To the Graduate Council:

I am submitting herewith a thesis written by Margaret Steele Rumbley entitled "A Procedure for Determination of Total Sulfur and Inorganic Sulfate Content of Food Stuffs." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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A PROCEDURE FOR DETERMINATION OF TOTAL SULFUR AND INORGANIC SULFATE CONTENT OF FOODSTUFFS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Margaret Steele Rumbley

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ABSTRACT

A procedure for determination of total sulfur and a procedure for determination of inorganic sulfate content of the edible portions of foodstuffs common to the American diet were investigated. The test food was New York State variety green cabbage. Total sulfur (as sulfate) was determined by the Parr sulfur bomb atomic absorption spectrophotometric method. The determination was made with and without added sodium sulfate standard. Good recovery and precision, 101.4 ±2.3%, were obtained by this method. An attempt was made to determine total sulfur (as sulfate) as the sum of the four sulfur components: inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur. The sum of these components was 11.2% higher than the total sulfur obtained by the Parr sulfur bomb atomic absorption spectrophotometric method. Therefore, replications and a recovery experiment were completed on the method for fractionation and determination of inorganic sulfate. The determinations were made with and without added sodium sulfate standard; recovery and precision were 101.2 ±6.9%. Replications and recovery experiments were not completed on the methods for fractionation and determination of organic sulfate, ethereal sulfur and amino acid sulfur.

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CHAPTER I

INTRODUCTION

It has been well documented that sulfur and the sulfur cycle are important to the nutrition and well-being of humans (1). Sulfur is emitted into the atmosphere by natural sources (primarily sea spray and decomposition of organic matter) and by people (primarily through industrial pollution) (2). Sulfur is taken up from the atmosphere by oceans, rivers, soils and vegetation. Robinson and Robbins (2) reported that industrial emission of sulfur dioxide doubled between 1940 and 1965. Data are unavailable to establish whether or not there has been a net accumulation of sulfur dioxide in the atmosphere as a result of industrial pollution. Nevertheless, in recent years strict measures have been taken to control sulfur pollution of the atmosphere and to restrict the use of sulfur-containing fertilizers. Allaway (3) argued that the demand for increased food production due to increased world population along with strict control of air pollution and use of purified fertilizers has created a threat to the sulfur cycle which eventually will result in reduced vegetational growth. Such a change in one aspect of the sulfur cycle without compensating in another part could also result in a reduction in human well-being.

A method for assessment of human sulfur status should be employed in order to insure optimum health and well-being. A dose response test based on urinary glucuronic acid salicylamide : sulfuric acid salicylamide

ratio has been developed for use in evaluating sulfur status of the rat.[1] Contingent upon further investigation using human subjects, this test is also a possible method for use in assessment of human sulfur status.

An assessment of nutritional status must include information on sulfur content of the diet as well as biochemical and clinical parameters. At the present time, available food composition tables reflect only the sulfur-containing amino acids (4,5) and total sulfur content of foodstuffs (6,7). More recently it has been shown that dietary inorganic sulfate (1) and organic sulfate,[2] as well as total sulfur and amino acid sulfur, influence sulfur metabolism in the rat. Methods are needed for fractionation and determination of these sulfur compounds in foods in order to apply the present knowledge of sulfur nutrition to an assessment of human sulfur status.

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[1] Kurzynske, J. S. (1975) Investigation of phenolic hydroxyl chemicals as model compounds for determining sulfur status. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

[2] Bowling, S. T. (1969) The utilization of the sulfur of sulfolipid by the albino rat. Unpublished MS thesis. The University of Tennessee, Knoxville.

CHAPTER II

REVIEW OF LITERATURE

Exogenous Sulfur Compounds

There are many sulfur-containing compounds in the foods ingested by humans. Organic sulfur is the dominant form while inorganic sulfate constitutes only a small percentage of the total sulfur in the diet (8). The two sulfur-containing vitamins, thiamin and biotin, and the sulfur-containing amino acids, methionine and cystine, have long been regarded as the only exogenous sulfur compounds needed to supply the total dietary sulfur requirement of humans.

Recently, inorganic sulfate has been shown to be useful in the synthesis of at least one metabolically important sulfur-containing compound, as well as being an important metabolic regulator (1). Ester sulfates have also been shown to be useful as a source of exogenous sulfur since the incorporation of $^{35}SO_4^{=}$ from ^{35}S-sulfolipids has been demonstrated.[3] Insulin, antidiuretic hormone, gastrin II, sulfhydryl enzymes, coenzyme A, acyl-carrier protein, mucopolysaccharides, heparin, bile salts, glutathione, mercapturic acids and lipoic acid are other metabolically important sulfur-containing compounds which are ingested in small amounts (8); whether or not these compounds are also important exogenous sources of sulfur has not been investigated.

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[3]Bowling, S. T. (1969) The utilization of the sulfur of sulfolipid by the albino rat. Unpublished MS thesis. The University of Tennessee, Knoxville.

Volatile sulfur-containing compounds produced during catabolism of common metabolites include hydrogen sulfide, dimethyl sulfite and methanethiol, among others (9). Masters and McCance (10) showed that these volatile compounds were lost from a variety of vegetables that were dried at 50° for 48 hours. These investigators reported that approximately 1 to 70% of the total sulfur was lost from the vegetables studied. One might assume that these compounds make varying contributions to the edible portions of certain processed and stored vegetables if loss of them is not controlled. Fruits, nuts, cereals, meats and beer were also investigated, and no loss was reported under the conditions of the study.

Sulfur dioxide and sulfite salts are food additives which retard enzymatic and nonenzymatic discoloration of foods and inhibit bacterial growth in foods (11). These additives are considered to be nonnutritive, and, therefore, their role as an exogenous source of sulfur is assumed to be unimportant.[4]

Methods for Determining Sulfur Content of Foodstuffs

Much time and effort have been devoted to developing quantitative analytical procedures for determination of sulfate. Sulfate determination is important because sulfur in various other forms is frequently converted into sulfate ion and determined as such. Gravimetry, titrimetry, turbidimetry, colorimetry, nephelometry and atomic absorption spectrophotometry are used to measure sulfate ion.

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[4]Smith, J. T. (1978) Personal communication.

There are several combustion methods which oxidize sulfur to the sulfate form and have been used to determine total sulfur content of various materials. Dry ashing with magnesium nitrate, wet ashing with nitric and perchloric acids and combustion in a bomb or flask are frequently used (12). According to Johnson and Ulrich (13), wet ashing is superior to dry ashing for determining all elements except boron and chlorine. This is primarily because there is no danger of loss of the elements since the temperature of the digest cannot exceed the boiling point of perchloric acid. Either the oxygen bomb or oxygen flask, which are closed systems, must be used to obtain accurate absolute values for sulfur content. If relative values are acceptable, then nitric and perchloric acids may be used. The oxygen flask is useful for samples up to 150 mg. The oxygen bomb is useful for samples from 0.2 gm up to 1 gm. A disadvantage of the combustion flask and bomb is that the sample must be dry in order to burn.

Beswick and Johnson (7) investigated the effectiveness of three methods for determination of sulfur as sulfate obtained by oxygen flask combustion of foodstuffs. Colorimetric determination with barium chloroanilate showed interference by chloride, nitrate and phosphate. Interfering cations were removed by ion exchange. The investigators did not indicate the limit to detection of this method. Titration of 5 to 100 μEq of sulfate with barium perchlorate showed no systematic error (mean deviation, 0.2%). Phosphate showed interference when present in greater amounts than sulfate. The turbidimetric method lacked precision; the relative mean deviation was 5.6% at the 10 μEq level and 1.1% at the 30 μEq level. Magnesium, calcium, aluminum, ferric and ferrous iron,

phosphate and nitrate were shown to cause interference.

An indirect method for determination of sulfate was developed by Roe et al. (14). Barium may be measured by atomic absorption spectrophotometry since it is easily reduced to the atomic state. Sulfur is not easily reduced to the atomic state and thus had not been analyzed directly by atomic absorption until recently (15). Sulfur and barium are present in a one-to-one ratio in barium sulfate. In low concentrations, atomic absorption offers a sensitive procedure for determination of barium and thus of sulfate. The limit to detection of the Perkin-Elmer model 303 spectrophotometer is 0.4 ppm (16). Phosphate is known to interfere with atomic absorption spectrophotometric determination of barium. Roe et al. were able to reduce phosphate interference by using lanthanum chloride. Kirkbright and Wilson (15) investigated the use of a water-cooled demountable hollow cathode lamp for direct determination of sulfur using the Perkin-Elmer model 303 spectrophotometer. The limit to detection at 180.7 nanometers (nm) was reported to be 5.0 ppm, when sulfur was introduced into the nitrous oxide-acetylene flame as an aqueous solution of ammonium sulfate.

A photonephelometric method used in conjunction with the Parr sulfur bomb was developed by Toennies and Bakay (17). The method permits determination of sulfur in nucleoprotein with a precision of ±1% on samples containing 3 μg of sulfur. Ions including ammonium, sodium, potassium, calcium, ferric iron, chloride, nitrate and phosphate caused no interference even when present at high concentrations. The limit to detection by this method was 0.01 ppm. Evidence showed that the Parr sulfur bomb oxidation products interfered with nephelometric determination of sulfate.

Katz and Golden (18) used nitric and perchloric acids for wet combustion of organic matter. Complete recovery of cysteine and methionine were obtained after 2 hours of digestion, using a 50 to 1 ratio of combustion fluid to organic material. Using a gravimetric finishing method, the limit to detection was 680 ppm. Calcium and ferric iron have a strong tendency to co-precipitate with sulfate and thereby interfere with gravimetric determination of sulfur.

Masters (19) used a hydrogenation method for determination of total sulfur. The hydrogenation method depends on reduction of sulfur to hydrogen sulfide. The sample is heated in the presence of a flow of hydrogen. Hydrogen sulfide is collected in 2% $CdCl_2$, and the CdS precipitate is determined. The CdS is dissolved in a known excess of 0.01 N I_2 and acidified with concentrated HCl. The excess I_2 is titrated with 0.01 N $Na_2S_2O_3$, and the amount of sulfur is calculated. No information was given on the limit to detection of this method. Recovery of methionine was 100.5% and that of cysteine was 100.4%. The author reported that there was no interference from alkali metals. With this method, smaller samples can be analyzed more accurately than with the nitric-perchloric acid gravimetric method. The method was reported to be shorter than the oxidation process and to cause no risk of losing sulfur by volatilization. McCance and Widdowson (6) used the hydrogenation method to determine total sulfur content of 700 foods.

Dodgson and Price (20) liberated polysaccharide sulfate by acid hydrolysis. A turbidimetric method was used for quantitative analysis of the liberated sulfate. Conditions for elimination of ultra-violet absorbing products were investigated. Complete (total) recovery was

obtained after hydrolysis at 105° to 110° for 3 hours using 1.0 N HCl. The limit to detection of this method was 450 ppm.

Johnson and Ulrich (13) determined inorganic sulfate and organic sulfur concentrations in dry plant materials. Inorganic sulfate was reduced to hydrogen sulfide which was swept from the reaction vessel with a stream of nitrogen gas and trapped in zinc acetate solution. The sulfide was reacted with p-amino dimethylanaline to form reduced methylene blue. The leuco-methylene blue was oxidized by ferric iron to methylene blue which was measured using a colorimeter. This method distinguished between inorganic sulfate and organic sulfur when the plant sample was treated directly with the reagents. Organic sulfur was obtained by difference after determination of total sulfur by wet digestion of the material.

Hydrolysis followed by chromatographic analysis (21,22) is a common procedure for isolation and quantitation of amino acids present in plant and animal materials. To avoid loss of cysteine and methionine during acid hydrolysis in the presence of carbohydrate, performic acid oxidation of cystine to cysteic acid and methionine to methionine sulfone should first be carried out as described by Lewis and Shanley (23).

In summary, methods for determination of total sulfur (7,19) and amino acid sulfur (21,22) content of foodstuffs are available in the literature. One method was found for fractionation of inorganic sulfate followed by estimation of organic sulfur based on the difference between total sulfur and inorganic sulfate (13). No method was found for fractionation and determination of organic sulfate and ethereal sulfur in foodstuffs. A method for fractionation and determination of each of

the aforementioned sulfur components of foodstuffs is needed in order to apply the present knowledge of sulfur nutrition to an assessment of human sulfur status. The purposes of the present study were to investigate a possible method for determination of total sulfur content of foodstuffs and to investigate a possible method for fractionation and determination of inorganic sulfate content of foodstuffs. In addition, an attempt was made to fractionate and determine organic sulfate, ethereal sulfur and amino acid sulfur in the test food.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Plan

The foodstuff used as a test food was New York State variety green cabbage which was purchased by the Department of FSNFSA from M&S Produce Company, Knoxville, Tennessee. Each cabbage head weighed approximately 1600 g. Ten 100 g (edible portion) samples from one head of cabbage were cooked and analyzed for total sulfur content using a Parr sulfur bomb-atomic absorption spectrophotometric (AAS) procedure. Five of the samples were analyzed without addition of Na_2SO_4 standard, and five were analyzed with added Na_2SO_4 standard. A single 100 g sample from the same head of cabbage was analyzed for inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur. The total of these four components was expected to represent the total sulfur content of the sample. Ten 100 g samples taken from a second head of cabbage were cooked and analyzed for inorganic sulfate. Five of these samples were analyzed without addition of Na_2SO_4 standard, and five were analyzed with added Na_2SO_4 standard. Addition of the sulfate standard was used to measure recovery by the procedures followed. Cooking loss of volatile sulfur-containing compounds was determined using two (100 g) samples taken from a third head of cabbage. One of these samples was cooked in a sodium hydroxide solution, and the other sample was cooked in demineralized water. All reagents and methods are described in the sections which follow. All glassware and other equipment were rinsed with demineralized

water. The results were averaged, and the standard deviation from the mean was calculated as an indication of the precision of the methods used. All data calculations were performed using an Olivetti-Underwood Programma 101. The scheme for determination of total sulfur and for fractionation and determination of inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur is presented in Figure 1.

Sampling and Cooking Procedure

Each of the two heads of cabbage was cut into two halves beginning at the core end using a Universal French knife with a 17.5 cm stainless steel blade. A wedge, weighing approximately 105 to 125 g including core, was cut from one of the cabbage halves. All subsequent samples were cut in the same manner. The sample was weighed on a tared 15 cm diameter watch glass using a Mettler P1200 balance to verify that the sample weight exceeded 100 g. Forty ml of demineralized water were placed in a Pyrex saucepan (1500 ml capacity). The water was brought to a boil on a 14.5 cm diameter burner of an electric range. The heat control was set on medium high to bring the water to a boil. Immediately after boiling commenced, the cabbage wedge was placed in the saucepan, and the heat control was reduced to the low setting. The cabbage was cooked with the lid on for exactly 12 minutes from the time that the heat setting was reduced. After cooking, the liquor from the sample was poured into a 250 ml beaker, and the sample was placed on a 15 cm diameter Pyrex watch glass where it was allowed to cool for 5 minutes. The cabbage core was then cut away and discarded because it was assumed that the core is not commonly ingested by Americans. Cabbage (100 g) was

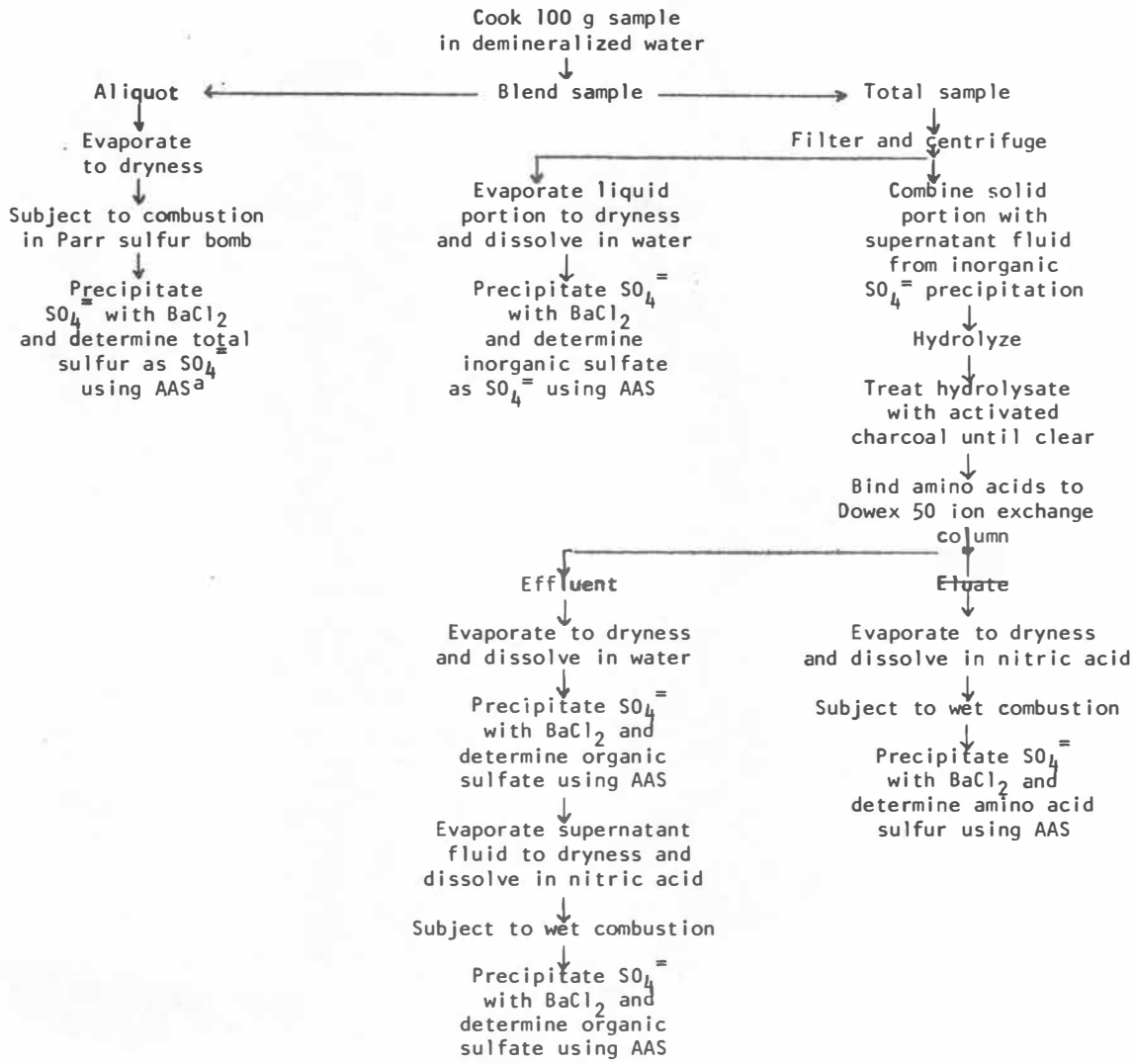

Figure 1. Scheme for determination of total sulfur and for fractionation and determination of inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur.

[a]Atomic absorption spectrophotometry (AAS)

Source: Smith, J. T. (1978) Personal communication.

weighed directly into a stainless steel blender jar. Any necessary trimming was done equally from all parts of the wedge, to the extent possible. To the cabbage in the blender were added 200 ml liquid, which included the liquor and demineralized water used to rinse the saucepan, the watch glass and the beaker that had contained the cabbage liquor. The sample was blended for 30 seconds at the low speed followed by 120 seconds at the high speed of a Waring Blendor model PB-5A. The sample was then prepared either for combustion in the Parr sulfur bomb or for inorganic sulfur determination.

Parr Sulfur Bomb Combustion

To prepare for combustion in the Parr sulfur bomb, the blended sample was transferred, nonquantitatively, to a 400 ml beaker. The contents of the beaker were then mixed on a Sargent S-76490 magnetic stirrer using a 3 cm long teflon coated stir bar. With a transfer pipette, two aliquot samples were transferred to two (250 ml) tared beakers and were weighed on the Mettler balance. Each aliquot weighed 5% (1/20) of the total weight of the sample. The weight of each aliquot was calculated as follows: weight of the blender jar and lid plus weight of the sample (100 g) plus weight of 200 ml water minus weight of the blender jar and lid divided by 20. Each sample was dried for 3 hours in a Precision Scientific Company 1052 convection oven at 55°. The sample was then allowed to cool in air before being stored uncovered in a desiccator overnight. A metal spatula was used to scrape the sample from the beaker. The sample, consisting of a pale yellow powder, was weighed in a tared 15 ml beaker on the Mettler P1200 balance. Each sample weighed approximately 0.5 g and was made into a pellet in a Parr

press model 2811. The sample was subjected to combustion using the Parr series 1900 oxygen bomb sulfur apparatus (24). The pellet was placed in the metal combustion capsule and combusted in the bomb with 10 ml demineralized water and oxygen at 30 atmospheres pressure. The bomb was allowed to stand in the water bath at least 15 minutes after firing. The residual gases were released slowly to atmospheric pressure over a period of not less than 1 minute. With a transfer pipette, the aqueous ash was transferred to a 50 ml centrifuge tube. The interior of the bomb and its parts were rinsed twice, and each rinse was added to the 50 ml centrifuge tube. Approximately 10 to 20 ml demineralized water were used for these rinsings.

Sulfate Determination by Atomic Absorption

The sample was analyzed for total sulfur as sulfate by the method of Roe et al. (14). Four ml of 15% (w/v) barium chloride solution were pipetted into the sample tube to precipitate the sulfate as barium sulfate. Using a Super-Mixer 12-812, Lab-Line Instruments Incorporated, the sample was mixed thoroughly and then centrifuged at 2600 x g for 5 minutes in an International model SBV centrifuge. The supernatant fluid was discarded. Five ml demineralized water were added to the precipitate which was again mixed thoroughly, centrifuged as before, and the supernatant fluid discarded. The washing of the precipitate was repeated to remove excess barium which had not reacted with the sulfate in the samples. After the supernatant from the third centrifugation had been discarded, the tube was left inverted to drain 2 to 3 minutes. It was then placed in the convection oven at 55° for 5 minutes to evaporate

all liquid from the sample. The precipitate was then dissolved in 10 ml of an alkaline disodium ethylenediamine tetraacetate (EDTA) solution which had been prepared by dissolving 10 g EDTA in 500 ml demineralized water, adding 20 g of sodium hydroxide and diluting to 2000 ml. After the precipitate was dissolved, it was necessary to dilute the total sulfur sample to 100 ml with demineralized water followed by a 1:10 dilution in order to measure percent absorption using the atomic absorption spectrophotometer.

Working standards were prepared from a stock sulfate solution made by dissolving 1.479 g of sodium sulfate in 500 ml demineralized water, to yield a concentration of 2000 ppm sulfate. The standards contained 0.10, 0.25, 0.40, and 0.50 ml of the sulfate solution, respectively. Demineralized water was added to each one to bring the volume up to 5 ml. Barium sulfate was precipitated and dissolved in 10 ml EDTA solution as described above for treatment of the samples. The standards contained 20, 50, 80, and 100 ppm of sulfate and were used to form a standard curve. A blank of demineralized water and a 20 ppm standard sample were run with each set of samples.

Samples and standards were aspirated into the flame of the Perkin-Elmer model 303 atomic absorption spectrophotometer which was prepared for determination of barium in the following manner. A barium hollow cathode tube was operated at 20 milliamperes. The instrument settings were: monochromator, 276 nanometers (nm) visible; gain 5-6; slit 3; flow rate of nitrous oxide (the oxidizer), 8.5; flow rate of acetylene gas (the fuel) slightly above 15. The nitrous oxide burner was ignited and the fuel flow adjusted to give a characteristic pink cone.

Percent absorption, an exponential function, was recorded and later converted to absorbance, a linear function, so that the μg sulfate per sample could be calculated using the following equation:

$\frac{\text{absorbance of unknown}}{\text{absorbance of standard}}$ X concentration of the standard (μg/ml) X volume of standard (ml) X dilution factor X factor representing the whole.

Fractionation and Determination of Inorganic Sulfate

If the blended sample was to be analyzed for inorganic sulfate, the liquid portion was separated from the solid portion using 18.5 cm diameter Whatman #4 filter paper in a 19 cm (inside) diameter Buchner funnel connected to a Little Giant model 13152 pressure/vacuum pump, Gelman Instrument Company. Approximately 15 minutes were required to separate the two portions. The filtrate was collected in a 2 liter Erlenmeyer flask. The residue was transferred to a blender jar using a teflon spatula, 200 ml demineralized water were added and the material was blended for 30 seconds at the high speed followed by 120 seconds at the low speed of the blender. This material was filtered through the Buchner funnel, and the filtrate was collected in the Erlenmeyer flask. The filtrate was believed to contain all inorganic sulfate of the cabbage sample since sulfate is known to be water soluble. If the sample was to be analyzed for organic sulfate, the residue was transferred quantitatively to a 1000 ml beaker and stored in the refrigerator without a preservative until further analysis. The residue was otherwise discarded. To remove solid material from the filtrate which was not removed by

filtration, the filtrate was transferred quantitatively to 40 ml plastic centrifuge tubes and centrifuged at 12,000 x g for 10 minutes using a refrigerated centrifuge, Lourdes "Beta-fuge" model-A. The centrifugate, 600 to 800 ml, was decanted into three (600 ml) beakers with approximately 200 to 270 ml of centrifugate in each beaker. If further analysis of the sample was planned, the residue was transferred quantitatively to the 1000 ml beaker containing the residue obtained during filtration. Otherwise, the residue was discarded. The centrifugate was then evaporated to dryness in the convection oven, at 55°, a process which required 16 to 20 hours. The residue which remained after evaporation was light caramel in color and dissolved only partially in the 20 to 30 ml demineralized water used to quantitatively transfer the residue from each beaker to a 50 ml centrifuge tube. The more complete the removal of solids during filtration and centrifugation, the more soluble was this residue. The total liquid used to dissolve the residue could not exceed 100 ml in order to allow precipitation of sulfate with 15% barium chloride. The sample was then analyzed for sulfate content using the method of Roe et al. (14), which was described previously. The sample was diluted to 50 ml with demineralized water after addition of the 10 ml EDTA. If the sample was to be further analyzed for organic sulfate, the supernatants from the washings of the barium sulfate precipitate above were added to the 1000 ml beaker containing the solid residue of the sample. Residue that did not dissolve was collected from the three washings of the precipitate. It was not always possible to collect all of this residue quantitatively, and it was eventually discarded.

Fractionation and Determination of Organic Sulfate, Ethereal Sulfur and Amino Acid Sulfur

To fractionate the organic sulfate, ethereal sulfur and amino acid sulfur, the sample was first hydrolyzed according to the method of Dodgson and Price (20). The volume of the residue and supernatant fluids which had been collected and saved in the 1000 ml beaker was estimated and an equal volume of 12 N HCl was added. A 15 cm diameter Pyrex watch glass was used to cover the beaker. The sample was hydrolyzed for 3 hours at 110° in a Fisher Isotemp oven. The sample was cooled to room temperature, and approximately 65 g activated charcoal was added to remove the humin which had accumulated during hydrolysis. The charcoal was separated from the hydrolysate using 18.5 cm diameter Whatman #4 filter paper in a 19 cm (inside) diameter Buchner funnel connected to the vacuum pump described previously. The filtrate was collected in a 2 liter Erlenmeyer flask. The charcoal residue was rinsed with 6 N HCl to remove all of the hydrolysate, which was canary yellow in color after cleaning. Following hydrolysis, the organic sulfate was assumed to be liberated. A method was then employed to separate the organic sulfate from the ethereal sulfur and amino acid sulfur. Sulfur in the latter two forms was assumed to have remained bound within these compounds after hydrolysis. The hydrolysate was passed through a Dowex 50 column, and the amino acids were bound to and then eluted from the column. The column was prepared as follows: Dowex 50W-X8, 200 to 400 mesh, hydrogen form sulfonated polystyrene resin was mixed with enough water to make a thick slurry in a 1000 ml beaker. The resin was poured into a 40 cm x 4 cm (inside diameter) glass column which was bedded with

glass wool covered with a thin layer of sand. The resin bed measured 19.5 cm x 4 cm. Two normal hydrochloric acid was passed through the column until the eluate reached pH 2, as measured with pHydrion paper with a pH range of 1 to 11, Micro Essential Laboratory. Then 2 N NaOH was passed through the column until the eluate reached pH 10. Demineralized water was passed through the column until pH 7 was reached by the eluate. The hydrolysate was then passed through the column. The effluent, approximately 600 to 800 ml, was collected in 3 (600 ml) beakers. The effluent which contained organic sulfate and ethereal sulfur was canary yellow in color. Approximately 200 ml 2 N HCl was passed through the column to quantitatively elute the amino acids from the column. All eluant was collected in a 400 ml beaker until the eluant color changed from canary yellow to colorless. Each of the samples was evaporated to dryness, approximately 16 to 20 hours, in a convection oven at 55°. There was an accumulation of a white crystalline material in all of the samples beginning within an hour or two after placing them in the convection oven. When dry, the residue of each sample was canary yellow in color, and the crystalline material was also covered with yellow residue. The three portions of residue containing the ester sulfates and ethereal sulfur compounds were transferred quantitatively with demineralized water to 3 (50 ml) centrifuge tubes. The crystalline material was insoluble and was rinsed until free of the yellow deposit. The total volume of the three samples could not exceed 100 ml in order to allow precipitation of sulfate with barium chloride. A 0.5 g portion of the white crystalline material was subjected to combustion in the Parr sulfur bomb as described previously and was found

to be noncombustible. The three samples containing ester sulfate and ethereal sulfur were then treated for determination of sulfate according to the method of Roe et al. (14). Each sample was diluted to 50 ml with demineralized water after the barium sulfate precipitate was dissolved in 10 ml EDTA. The supernatant fluids from above were decanted and saved in a 400 ml beaker after each washing of the precipitate. The supernatant fluid was evaporated to dryness, approximately 16 to 20 hours, in the convection oven at 55°. The sample containing the ethereal sulfur compounds was quantitatively transferred to three (50 ml) Erlenmeyer flasks with a solution of concentrated nitric acid : demineralized water (1:1). The sample containing the amino acids was quantitatively transferred to a single 50 ml Erlenmeyer flask with a solution of concentrated nitric acid : demineralized water (1:1). Sulfur in the three flasks containing ethereal sulfur and in the single flask containing amino acids was oxidized to sulfate by the nitric-perchloric acid wet combustion method of Katz and Golden (18). The modified combustion mixture consisted of: two volumes of concentrated nitric acid to one volume of 70% perchloric acid, containing 10 mg copper nitrate, $Cu(NO_3)_2 \cdot 3H_2O$, per ml. Five ml of the combustion fluid were pipetted into each Erlenmeyer flask. The flasks were heated on a Type 1900 Thermolyne hot plate in a well ventilated hood. The heating was slow during the first 30 minutes during which time a very violent reaction occurred. The samples were then heated at a high temperature for 2 or more hours. After this time, most of the perchloric acid was evaporated, and a wet, greenish-yellow to orange residue was obtained.

The flasks were returned to the plate to dissolve the residue. The liquid was transferred quantitatively to a 50 ml centrifuge tube and treated for determination of sulfate as described by Roe et al. (14). The volume of liquid used to dissolve and transfer the residue could not exceed 100 ml in order to precipitate sulfate as barium sulfate. Each sample was diluted with demineralized water to 100 ml after the barium sulfate precipitate was dissolved in 10 ml EDTA.

Determination of Cooking Loss of Volatile Sulfur Compounds

Cooking loss of volatile sulfur-containing compounds was determined using two (100 g) samples. One sample was cooked in 40 ml demineralized water, and the other sample was cooked in 40 ml 0.5 N NaOH. These two samples were cooked, subjected to combustion in the Parr sulfur bomb and the sulfate was determined as described in the previous sections. The percent loss of volatile sulfur-containing compounds was calculated on the basis of the difference between the total sulfur (as sulfate) content of the two samples.

CHAPTER IV

RESULTS AND DISCUSSION

The present study was originally planned to develop a procedure for determination of inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur which could be used to determine these sulfur components in all foodstuffs common to the American diet. The results of a single determination of each of these components in cabbage are reported in Table 1. It was not certain that the sulfur in each component and the total sulfur in the test food were properly represented by the values shown in Table 1. Therefore, the investigator completed recovery experiments on total sulfur and inorganic sulfate. Replications and recovery experiments are needed to evaluate the methods for fractionation and determination of organic sulfate, ethereal sulfur and amino acid sulfur before a workable procedure for determination of inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur can be recommended.

Total sulfur represented by the sum of the four components was 11.2% higher than total sulfur obtained by the Parr sulfur bomb atomic absorption spectrophotometric (AAS) method reported in Table 2. Further investigation of each component is needed to resolve this discrepancy. The average recovery of total sulfur using the Parr sulfur bomb AAS method was 101.4% with a precision of ±2.3%. This recovery is slightly better, and the precision is approximately 50% better than the 95.5 ±4.1% reported by Roe et al. (14) for diet analysis of methionine using the Schoniger oxygen flask AAS method. The Parr sulfur bomb AAS method

Table 1. Total Sulfur in 100 g Cabbage Sample Based on Sum of Four Sulfur Components

Component	S as $SO_4^{=}$
	----mg---
Inorganic sulfate	115.4[a]
Organic sulfate	24.0
Ethereal sulfur	158.3
Amino acid sulfur	71.9
Total sulfur as $SO_4^{=}$	369.6

[a]Values are based on one determination.

Table 2. Recovery of Total Sulfur in 100 g Cabbage Samples Determined by Parr Sulfur Bomb AAS Method

Sample	Total S as $SO_4^=$ Without Added Na_2SO_4	Total S as $SO_4^=$ With Added Na_2SO_4		% Recovery
		Calculated	Found	
	mg			
1	331.8[a]	381.8	400.0[a]	104.8
2	331.8	381.8	383.4	100.4
3	331.8	381.8	391.8	102.6
4	331.8	381.8	383.6	100.5
5	335.1	385.1	380.3	98.8
Average	332.5			101.4±2.3

[a]Average of two determinations

for determining total sulfur was considered to be very reliable because a closed system was used for combustion of the foodstuffs (13), there were few steps and little handling of the sample, the limits to detection of the atomic absorption spectrophotometer are very low (16) and good recovery and precision were achieved with this procedure. Since the recovery and precision of the Parr sulfur bomb AAS method were good, it was assumed that there was no interference from other ions. Lanthanum chloride was not used to prevent phosphate interference described by Roe et al. (14) because it resulted in formation of an insoluble precipitate, and further analysis was not possible. It is highly unlikely that phosphate would be present in the material aspirated into the flame.

It is not possible to compare the results of the present study with those obtained by the turbidimetric method since Beswick and Johnson (7) did not determine total sulfur content of cabbage. The turbidimetric method lacked precision and resulted in interference by various ions when other foodstuffs were analyzed. Masters (19) found the total sulfur content of cooked winter cabbage to be 21.0 mg per 100 g cabbage. The total sulfur content of the winter cabbage analyzed in the present investigation was found to be 109.7 mg per 100 g of cabbage. The hydrogenation titration method (19) is believed to yield lower results than the Parr sulfur bomb AAS method because it requires more steps and more handling of the sample. Also, combustion of the sample was probably less complete and the limits to detection were probably greater than with the Parr sulfur bomb AAS method. Since no better methods are reported in the literature, the investigator recommends that the Parr sulfur bomb AAS method be used for determination of the total sulfur

content of foodstuffs as a check of the total sulfur content obtained by summing values of the four sulfur-containing components. One analysis, from weighing the sample to calculating the results, may be completed in a 14-hour period. Storage of the sample in a desiccator was only a matter of convenience and could be eliminated, reducing the time required to analyze one sample. Five samples could then be completely analyzed in an 8-hour period.

It was expected that in the test food, ethereal sulfur and amino acid sulfur would be the largest organic sulfur components since they are precursors to hydrogen sulfide, dimethyl sulfide and methanethiol, which are responsible for cabbage flavor and odor (8). The organic sulfate component was expected to be small since there are probably few mucopolysaccharides and sulfolipids in cabbage. Replications and recovery experiments are needed to evaluate the methods for fractionation and determination of organic sulfate, ethereal sulfur and amino acid sulfur. A second hydrolysis at a higher temperature and for a longer time may be needed to completely extract and ionize each component, since it was not certain that hydrolysis was complete after 3 hours at 110°. The white crystalline material which developed during drying of the effluent and eluate was not identified. Since the material was insoluble in water and noncombustible in the sulfur bomb, it was assumed to contain no sulfur. It was difficult to separate the crystalline material from the yellow residue, and some of the crystalline material was always transferred to the wet combustion flasks. Due to the presence of this material, sample loss occurred if the temperature was not carefully monitored during the entire digestion period. The procedure

for fractionation and determination of the four sulfur components required approximately 2-1/2 days from cooking the sample through calculation of the results. Once the entire procedure is developed for use on edible portions of foodstuffs, a smaller sample may be used in order to reduce the time required for analysis and to reduce the chance of error incurred from working with large volumes of sample material.

The value for inorganic sulfate reported in Table 1, page 23, was verified by additional findings reported in Table 3. The procedure for fractionation and determination of inorganic sulfate yielded an average recovery of 101.2% with a precision of ±6.9%. Although the precision was not as good as desired, better precision is not likely when analyzing biological materials. Greater than 100% recovery is possibly due to co-precipitation of calcium and ferric iron with sulfate. Error was incurred when handling the large sample volume (600 to 800 ml), which could result in variable loss of sample. Variable amounts of residual solid material which was not removed during filtration and centrifugation could possibly interfere with barium sulfate precipitation. It is desirable that the sample material be a clear liquid when the barium chloride is added for precipitation of barium sulfate. Although the volume of barium chloride was constant, the volume of the samples was variable (60 to 90 ml), which could have resulted in variable precipitation of barium sulfate. The latter source of error could be easily eliminated in future analyses of foodstuffs. Johnson and Ulrich (13) did not determine the inorganic sulfate content of foodstuffs, and there are no values with which to compare the results of the present

Table 3. Recovery of Inorganic Sulfate from 100 g Cabbage Samples

Sample	Total S as $SO_4^=$ Without Added Na_2SO_4	Total S as $SO_4^=$ With Added Na_2SO_4		% Recovery
		Calculated	Found	
		----------mg---------		
1	99.3[a]	149.3	161.6[a]	107.9
2	99.7	149.7	117.7[b]	78.6
3	113.6	163.6	173.2	105.9
4	119.6	169.6	165.8	97.8
5	115.4	165.4	154.4	93.3
Average				101.2±6.9[c]

[a]Average of two determinations

[b]Loss occurred during handling of sample

[c]Calculated without recovery from sample 2

investigation. Since there are no better methods reported in the literature, the method investigated in the present study is deemed acceptable for determination of inorganic sulfate.

Results of an attempt to determine cooking loss of volatile sulfur compounds are reported in Table 4. By cooking a sample of the test food in sodium hydroxide solution, sodium sulfide is formed from hydrogen or organic sulfides, and thereby loss of sulfur is prevented. Based on the difference between the total sulfur (as sulfate) content of samples cooked in sodium hydroxide solution and those cooked in demineralized water, the loss of sulfur by volatilization was found to be approximately 4% in the test food. Loss of volatile sulfur compounds would vary depending on the kind of vegetable (9).

Table 4. Cooking Loss of Volatile Sulfur Compounds in 100 g Cabbage Samples

Sample	Total S as $SO_4^=$ Cooked in Demineralized Water	Total S as $SO_4^=$ Cooked in 0.5 N NaOH
	mg	
1	88.98[a]	-----
2	-----	92.81[a]
	% Retention = 95.87	
	% Loss = 4.13	

[a]Average of two determinations

CHAPTER V

SUMMARY

Total sulfur (as sulfate) content of edible portions of the test food was determined by the Parr sulfur bomb atomic absorption spectrophotometric method. Recovery and precision of this method were 101.4 ±2.3%, which was better than results reported in the literature. The investigator recommends that this method be used as a means of checking the total sulfur content of foodstuffs obtained as the sum of inorganic sulfate, organic sulfate, ethereal sulfur and amino acid sulfur.

Total sulfur (as sulfate) obtained by the sum of the four components was 11.2% higher than total sulfur obtained by the Parr sulfur bomb atomic absorption spectrophotometric method. Further investigation of each component is needed to resolve this discrepancy. A recovery experiment on the procedure for inorganic sulfate determination yielded a recovery of 101.2% and a precision of 6.9%. Although the precision of the method was not as good as desired, better precision is not likely to be achieved in analysis of biological materials. The investigator recommends that the method herein described be used for fractionation and determination of inorganic sulfate since no better method was found in the literature.

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VITA

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