3-1964

A study of origins of volatile sulfhydryls in milk

Andrew Wesley Gibbs

To the Graduate Council:

I am submitting herewith a thesis written by Andrew Wesley Gibbs entitled "A study of origins of volatile sulfhydryls in milk." I have examined the final electronic 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 Animal Husbandry.

B.J. Demott, Major Professor

We have read this thesis and recommend its acceptance:

J.T. Miles, Melvin R. Johnston

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

March 10, 1964

To the Graduate Council:

I am submitting herewith a thesis written by Andrew Wesley Gibbs entitled "A Study of Origins of Volatile Sulfhydryls in Milk." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairy Manufacturing.

B. L. Demott
Major Professor

We have read this thesis and recommend its acceptance:

Melvin Johnson

J. T. Miles

Accepted for the Council:

Hilton A. Smith
Dean of Graduate School

A STUDY OF ORIGINS OF VOLATILE SULFHYDRYLS IN MILK

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Andrew Wesley Gibbs

March 1964

Acknowledgements

The author feels and expresses his appreciation and indebtedness to Dr. B. J. Demott for his pleasant encouraging valuable guidance and help in planning and conducting this study. Appreciation is expressed also to Mr. Joel Collins for his assistance in statistical analysis of data. The author exceedingly feels obliged to one and all who contributed in the completion of this study.

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CHAPTER I

INTRODUCTION

The development of off flavors plays a very important part in the acceptance of dairy products by the consumer. The term "flavor" denotes a blend of complex chemical compounds. These chemical compounds are sometimes volatile under various processing procedures and on storage of product after it has been removed from its native state. It is the job of the research worker dealing with the flavor compounds to elucidate their exact chemical nature and to quantitatively express their approximate concentrations in various products. Also, the exact influence of processing techniques on the release of the volatile compounds must be determined so that steps can be taken to alter the release of the volatiles from the products as they are processed or stored.

Flavor research on volatile compounds has been hampered in the past by the inadequacy of available analytical procedures. Now modern instrumentation is opening the way to investigations which will unlock the causes of certain flavor developments and provide a sound basis for control.

Modern methods of processing sometimes contribute to a large extent to the development of undesirable flavor components. Perhaps in no industry is this more pronounced than in the processing of milk and milk products. Of great importance is the cooked flavor of milk which results when milk is heated to high temperatures. Some research workers believe that the cooked flavor occurs chiefly in the beta-lactoglobulin fraction of the

serum proteins. Upon the denaturation by heat this protein fraction is believed to be altered by the changing of cystine to cysteine which releases a free sulfhydryl group which is thought to be the chief constituent of the cooked flavor.

Research by Gould (20) and by Jackson (33) has shown, however, that the amino acids themselves can be denatured with the release of sulfhydryls. Four constituents in milk containing sulfur in their molecular structure are the amino acids, cystine, cysteine, methionine, and Vitamin B_1 (thiamine). Specific experiments dealing with the volatility of sulfur from these compounds in milk has not been found in the literature. Therefore, the present work was undertaken to study the volatility of sulfur from these compounds at the "critical" or threshold temperature and slightly above this temperature. The influence of various processing temperatures of milk in a dairy plant operation on the destruction of Vitamin B_1 has also been investigated in this study.

CHAPTER II

REVIEW OF LITERATURE

I. RESEARCH ON VOLATILE FLAVOR COMPOUNDS

Research in the field of volatile compounds in the food industry aims at prevention of the causes of off flavors or the preservation of normal flavors. By logic the chemical characterization of a volatile compound in flavor research is the first objective, followed by isolation, concentration, and purification. The chief difficulty is to find means that are operative on a microscale. Some volatile compounds in milk are effective at levels of only a few parts per million. Some recently developed research tools have done much to alleviate the need in this area. These are chromatographic methods, and vastly improved spectrophotometers and colorimeters.

Whitney, et al.(66) have used a modification of steam distillation to separate and identify the stale component in dried whole milk and butter oil. The distillation was carried out under a vacuum of 70 mm at 45° C. The distillate was trapped in H_2O at 0° C and was then extracted with petroleum ether. The ether was then evaporated to give the concentration stale component. When this fraction was then dissolved in fresh butter oil and blended with fresh products to the composition of original milk it yielded a detectable stale flavor in concentrations as low as 6.2 ppm.

By ether extraction Patton and Josephson (53) removed the volatile compounds from skim milk heated to 260° F for 60 minutes. The ether was then evaporated to give a concentrated product. A taste panel judged this

concentrate to have a strong caramelized odor. Fractionation of the flavor concentrate by selective solvent extraction and vacuum distillation yielded three fractions which had different solubility properties in water, petroleum, ether, and ethyl ether. The ether soluble fractions contained the caramelized odor and the water soluble had the sulfide taste and smell of the typical cooked flavor. This evidence may explain why the sulfhydryl groups in heated milk disappear as the development of caramelized flavor progresses. Repeated tests for nitrogen on both fractions gave negative results.

Flake, et al. (17) developed a method for isolating the volatile flavor and odor material thought to be responsible for the typical highly activated flavor of irradiated milk. The process consisted of steam distillation, purification of distillate by charcoal, and then extraction with ether. Sulfhydryls were shown to be present due to the formation of methylene blue. Casein treated by this method gave a strong burnt flavor to unirradiated milk. The production of sulfhydryls was measured by the formation of methylene blue from its precursor. Flake, et al. (18) also irradiated liquid systems of methionine, cystine, and cysteine with ultra-violet light. When a small amount of these irradiated samples were added to skim milk they gave the same effect of burnt flavor as irradiated casein. The flavor of milk containing irradiated cysteine was very strongly cooked and suggestive of flavor of whole milk irradiated with ultra-violet light. Steam distillation of irradiated milk produced a distillate that gave a positive nitroprusside test.

Miller and Zimmerman (52) devised a technique to study the volatile acidity in milk, comparing the methods of steam with vacuum distillation. The authors attempted to use the volatile acidity as a measure of the evaluation of sterilizing treatments of different processes. Steam distillation gave no formic acid and released volatile sulfides. Vacuum distillation at 24 mm Hg gave a good recovery of formic acid. Upon addition of acids to milk the steam distillation gave a higher recovery of added acids than did the vacuum distillation. However, the steam distillation tended to produce hydrolysis of triglycerides in solution and the vacuum distillation did not. Total volatile acidity varied directly with the degree of heat treatment.

Dill, et al. (13) used a steam injection and vacuum heating to study the heat activated compounds in skim milk over a temperature and time range of 190 to 300° F for 2 to 150 seconds. The heat activation of sulfhydryl groups as measured by silver nitrate titration followed a direct relationship to heating temperatures in those samples held for 2 seconds. The volatile sulfur compounds, liberated as a result of above heat treatments were collected by vacuum distillation and precipitated in mercuric chloride solution. Gas chromotography was used to resolve and identify the volatile materials. Volatile compounds were formed at heat treatments greater than a critical treatment concomitant with a loss in titratable sulfhydryl groups in the milk. The volatile fraction consisted primarily of hydrogen sulfide. The appearance of methyl sulfide in the volatile fraction appeared to correlate more closely to the presence of a cowy odor in the raw skim milk than to heat treatments.

Townley and Gould (62) used the process of heat distillation to study the release of volatile sulfhydryls when milk was heated to varying temperatures and held for long periods of time. The sulfhydryls were trapped in an alkaline zinc acetate solution which was then treated with the dye, P-amino-dimethylaniline, which when treated with hydrogen sulfide, reacts to form methylene blue. The intensity of methylene blue was then read colorimetrically and the concentration of sulfur calculated from colorimeter readings of known standards. Using this technique they found concentrations of 18 micrograms of volatile sulfur at 76° C of momentarily heating milk and on holding at 80° C for thirty minutes found the concentration of sulfur to be 110 micrograms per liter of milk. Further increases of both holding time and temperature led to an increase in the release of volatile sulfur from heated milk up to a point of 95° C. Beyond this temperature no further increase of volatile sulfur release was noted even at prolonged holding time.

Kristofferson, et al. (45) used an aerated distillation procedure to investigate the role of volatile sulfur in the flavor of cheddar cheese. They acidified the sample of cheese with sodium citrate and 1:1 hydrochloric acid and placed the slurry in a closed test tube. They then connected the tube to a flask containing zinc acetate and bubbled purified air through the cheese for thirty minutes. They then added to the flask containing the volatiles an organic dye which changes to methylene blue. Using the colorimetric determinations at a wave length of 660 mμ, they found 1.4 micromoles of hydrogen sulfide to be liberated per gram of cheddar cheese judged to be of good flavor. Kristofferson and Gould (44) later used this same technique to study the changes in hydrogen sulfide

during the controlled curing of cheddar cheese. The cheese made from raw milk, which had the highest flavor score, contained the most hydrogen sulfide, 1.8 micromoles, after two months and 3.4 micromoles per gram after twelve months of storage. Lawrence (48) later found higher concentration of hydrogen sulfide in cheese than did Kristofferson which he attributed to a loss of volatile sulfide in the distillation setup of Kristofferson and Gould (44). Lawrence stated that the system must be made as air tight as possible. Using a nitrogen atmosphere, Lawrence found a concentration of hydrogen sulfide at hooping of New Zealand cheddar cheese at 20 micrograms which increased on storage at 6 months to a concentration of 55 micrograms per gram and remained constant thereafter, while the intensity of typical cheddar flavors increased. There appeared to be no simple relationship between characteristic cheddar flavor and hydrogen sulfide concentration. Marbach and Doty (49) later used the dye reduction technique to measure the hydrogen sulfide released from meat subjected to varying doses of gamma irradiation. Heating at 65° C for two hours in a water bath produced 4 micrograms of hydrogen sulfide per gram of meat from a dosage of 2,000,000 roentgen equivalent physicals of gamma irradiation.

Thus, research workers have used a variety of tools to investigate volatile compounds contributing to both offensive and pleasant flavors in the food industry.

II. HEATED FLAVORS

The most fundamental preservation process for milk and dairy products is the use of heat, in its various forms. The most critical

problem in the use of high heat treatments is the development of cooked flavor. This off flavor develops at or near 74° C according to the study made by Josephson and Doan (38). Gibson (19) made a study of the flavor of milk pasteurized at 275° F for 3 seconds in a plate type heat exchanger. Milk pasteurized as such developed a scorched flavor which on aging, developed into a "cowy or barny" flavor. The flavor intensity increased during storage and was dependent on the storage temperatures used; the higher the temperature, the more rapid the change. Humbert and Gibson (31) later conducted a study using high temperatures and short periods of heating in an effort to arrive at a temperature where milk could be held at a time of 3 seconds at 285° F without development of a harsh cooked flavor. The cooked flavor was only slightly more pronounced than at 245° F. After a five-day storage period, milk processed at 245° F lost all trace of cooked flavor, whereas, the milk processed at 285° F had a faint cooked flavor.

Blankenagel and Humbert (5) investigated the effect of heating milk at 180 to 285° F for 3 seconds. As the processing temperature was increased a denaturation of serum protein occurred, followed by an increase in cooked flavor. At 265° F the SH content and cooked flavor were at a maximum; decreasing at higher temperatures. At this temperature, almost all of the beta-lactoglobulin was denatured. Similar results were also found by Josephson and Doan (38) and by Townley and Gould (62).

Amerdson and Swanson (3) studied the cooked flavor in dried, whole milk. They found that the spray drying process is not responsible for the

cooked flavor which is a common defect of spray dried whole milk. The cooked flavor comes from the heat treatment the milk goes through before concentration and drying. Various heat treatments were applied to fluid milk before concentrating. Samples were withdrawn before concentration and were compared with reconstituted milk made from spray dried powder. Taste panel members could find no difference in cooked flavor between the two milk samples. This substantiated their thinking that the cause of cooked flavor is in the heat treatment before the actual process of spray drying takes place.

Marquardt and Dahlberg (50) studied the influence of heat on development of cooked flavor in cream containing 40% fat. They processed cream at 143 and 150° F for thirty minutes in steel and glass lined tanks. Cooked flavor was noted in steel lined tanks. No cooked flavor was observed in glass lined tanks. The cooked flavor disappeared after 24 hours of storage at 40° F in all samples. Viscosity of cream was greater at 150° F than at 143° F. Whole milk, similarly treated, did not develop a cooked flavor. Results of this study show that a heated or cooked flavor is obscured in the complexities of protein denaturation and loss of Vitamin B_1. This invites one to accept the challenges of further elucidating and preventing cooked flavor development in milk.

III. SOURCE OF VOLATILE SULFHYDRYLS IN HEATED MILK

The serum proteins are thought to be the only heat denaturable source of sulfhydryls in milk. Belec and Jenness (4) studied the effects on heating on casein and found the only notable denaturation to be of a high degree of dephosphorization and a decrease in available calcium.

However, neither of these phenomena were shown to be associated with the release of sulfhydryls in milk as it was heated.

Hutton and Patton (32) using a silver nitrate titration procedure found heated casein to contain no free -SH groups and further fractionation by electrophoresis and pH alterations showed that the beta-lactoglobulin can account for practically all of free -SH groups. Hutton and Patton further stated that it is the conversion of -SH to hydrogen sulfide which causes cooked flavor.

Many of the research workers of cooked flavor are primarily interested in the fraction of beta-lactoglobulin which represents about 50% of the total serum proteins. The serum or whey proteins make up approximately 0.6% to 0.7% of the milk (37).

Apparently the milk serum proteins in their native state have a definite coiled configuration similiar to other proteins. Their stability is maintained by hydrogen bonding, hydration and salt balance. Heat denaturation of serum proteins has the following effect: evolution of hydrogen sulfide, formation of cooked flavor of milk, and a lowering of the oxidation reduction potential. The hydrogen sulfide is evolved from the free -SH groups which are thought to be oxidized to H_2S. The -SH groups are apparently held in the coiled protein in such a way as to make them unreactive. Jackson (33) could find no evidence of free -SH groups as measured by nitroprusside test on raw milk until he treated it with a strong oxidizing agent, sodium cyanide.

An important aspect of the heat denaturation of serum proteins is a decrease in solubility in acid solution. At pH 4.6 heat denatured serum

proteins are precipitated with casein on acid additon states Jenness (35). Harland and Ashworth (26) also precipitated the denatured serum proteins with saturated sodium chloride.

By the use of paper electrophoresis, Jenness (35) obtained and studied the difference in serum protein fractions to sensitivity of different heat treatments. The least degree of heating causes the immune globulins to be acid precipitable followed in order by the blood serum albumin, the beta-lactoglobulin, and finally the alpha-lactalbumin which is very resistant to heat denaturation. Similar results were obtained by Larson and Roleri (47) after they had removed the denatured serum protein with casein at pH 4.6 using a quantitative electrophoresis procedure.

Several investigators have studied the difference in nitrogen content of milk before and after heating to determine denaturation of serum proteins. Hetrick and Tracy (30) heated milk at 170° F for 12 seconds and found a 58% reduction in the amount of albumin and globulin. Denaturation of albumin and globulin together were determined by increase in nitrogen content of casein and a decrease in nitrogen content of albumin and globulin. When heat treatment was reduced to the minimum time-temperature relationship necessary to inactivate phosphatase, no denaturation of albumin and globulin occurred. Menefee, et al. (51) found no denaturation of proteins in skim milk heated to 145° F for thirty minutes as measured by the total nitrogen content during process of evaporation with a forewarming temperature of 203° F.

Rowland (55) found a reduction in creaming power of milk heated at 63° C for thirty minutes and attributed this to the denaturation of albumin and globulin. At temperatures of 60° C or above the decrease in creaming

power was closely related to the percentage of albumin and globulin denatured. At 80° C, 95% of the total albumin and globulin was denatured and at 65° C, 25% was denatured. Similar results were obtained by Jenness (35) upon precipitation of denatured serum proteins with saturated sodium chloride.

An interesting phenomenon of protein denaturation is evidence of a complex between serum protein and casein during heat treatment. Trautman and Swanson (63) produced a stable complex between alpha casein and beta-lactoglobulin during forewarming treatments used in manufacture of evaporated milk. Stability of evaporated milk was dependent on this complex as evidenced with use of sulfhydryl blocking agents. Confirmation of a complex between fractions of casein and serum proteins was done by Fitzpatrick and Sullivan (16). They showed a disappearance of beta-lactoglobulin fraction on the ascending pattern of electrophoresis of milk that had been heated at 242° F for 2 minutes. On descending, the casein and beta-lactoglobulin existed as an assymetric peak. Concentration of heated milk led to a stable complex which again could be disassociated at 280° F heating for 10 seconds. Similar results on complex formation in heated skim milk were obtained by Davies and White (11), Weinstein, et al. (65), and by Zittle, et al. (68).

Recent investigations by Timasheff and Townend (61) have shown a presence of two different fractions of beta-lactoglobulin called A and B. Jenness and Gough (36) found differences in rates of denaturation by heat between these two fractions. The A fraction was heat denatured faster than B in 1% solutions of the lactoglobulin and also in milk. Energies of activation were 65.6 and 77.1 K cal/mole for A and B, respectively. Another difference noted between A and B was a change from the native proteins to

a primary denatured form which is precipitable at pH 5.0 but has the same electrophoretic mobility as the native protein.

Jenness and Patton (37) state that hydrogen sulfide is perhaps most responsible for the cooked flavor in heated milk. The hydrogen sulfide is produced by the freeing of -SH groups from cysteine on heating. They are further oxidized to hydrogen sulfide. Jenness and Patton (37) state that this process might take place according to the following scheme:

$$HOOC\text{-}CHNH_2\text{-}CH_2\text{-}SH + H_2O \rightarrow HOOC\text{-}CHNH_2\text{-}CH_2OH + H_2S \quad \text{or}$$

$$2COOH\text{-}CHNH_2\text{-}CH_2\text{-}SH \rightarrow (COOH\text{-}CHNH_2\text{-}CH_2)_2\ S + H_2S$$

Jenness and Patton (37) state that the protein associated with the fat globule membrane could also be denatured by heat and was even denatured at a lower temperature than was the serum proteins. Larson and Jenness (46) also noted this on measuring the reducing power of different fractions of milk by the use of the O-iodobenzoate titration method. The reducing power of fat globule membrane was actually found to be higher than for the serum fractions.

Kiermeier and Hamed (40) point out that -SH groups in heated milk increase with the rise of the presence of certain fat globule membrane constituents and of beta-lactoglobulin.

IV. FACTORS AFFECTING THE RELEASE OF VOLATILE SULFHYDRYLS AND COOKED FLAVOR IN MILK

Modern processing methods makes use of high heat treatment and short heating periods. Among the benefits are continuous processing, greater keeping qualities and minimization of off flavors.

Townley and Gould (62) defined the critical temperature of heating milk to be the minimum temperature which releases free sulfhydryls in milk. Several experiments showed this temperature to be about 76° C at thirty minutes of holding time. They further found that an increase in holding time led to an increase in development of free sulfhydryls. Generally, the same results were found for an increase in temperature up to a certain point above which an increase in temperature did not bring about further production of sulfhydryls. This temperature ranged from 85 to 95° C. Burton (7) later conducted experiments showing the upper limit to be about 80 to 85° C for thirty minutes.

Dill, et al. (12) later showed that the maximum value of sulfhydryl production was reached at 260° F with a holding time of 150 seconds. Volatiles coming from a vacuum chamber were trapped in a lead acetate solution. At the critical temperature, the solution showed a black precipitate of lead sulfide indicating that free sulfur was being liberated from the milk.

Results on the critical temperature studies of sulfhydryl production vary chiefly due to the manner of heating. Zweig and Block (69), using holding times of thirty minutes, showed that the first appearance of free sulfhydryls occurs somewhere between 58 and 69° C.

Gould and Sommer (22) found 70° C to be the critical temperature for a thirty minute heat treatment. A lowering of the oxidation reduction potential occurred at a lower temperature than 70° C, but the presence of cooked flavor was questionable by the taste panel.

Some investigators prefer using the thiamine disulfide titration procedure to establish the critical point at which sulfhydryls first appear. As the free sulfhydryls are further lost, due to oxidation, the TDS values decreese. Boyd and Gould (6) heating milk to 90° C momentarily found the thiamine disulfide values to be about 21 meq/l as cysteine hydrochloride. Heating to 90° C for thirty minutes yielded TDS values of about 4 to 7 depending on the type of product.

Gould and Keenney (21) used the thiamine disulfide titration method to study the influence of various holding times on the quantity of free sulfhydryls liberated. The temperatures used were 160, 170, and 190° F at periods from 5 minutes to 30 minutes. Heating to 160° F for 30 minutes gave a TDS value of 4 and at 190° F for 30 minutes the TDS values were 3. Further decreases of TDS values occurred on storage at 40° F.

Coulter, et al. (8) used the TDS values to study the release of free sulfhydryls in an ultra-high temperature apparatus. The TDS values reached a maximum during heat treatment of milk, then decreased. A higher maximum is reached and decrease is slower in the absence of oxygen. A higher maximum is reached by high temperature short time heating of 95° C for 2 minutes than for a lower temperature and longer time.

Yoshino and Wilson (67) used the $AgNO_3$ amperiometric titration to estimate the sulfhydryl and disulfide groups in milk. Cysteine, beta-lactoglobulin, milk and whey were titrated at different pH levels in sodium acetate and ammonium nitrate buffers. The cysteine content of beta-lacto-globulin in sodium acetate at pH 10.2 was 96% of theoretical. The addition

of sodium lauryl sulphate increased the availability of -SH groups. A decrease in titratable -SH groups in heated milk coincided with the denaturation of serum proteins. Titratable -SH groups were decreased and -SS groups increased in concentrated milk by sterilization.

Some investigations have used steam injection and vacuum chambers to study the release of sulfhydryls in milk and to determine if these techniques could remove the cooked flavor from milk. Kleyn and Shipe (41) found the least amount of damage done to serum proteins at a temperature of 210° F for 10 seconds in comparison to longer holding times at higher temperatures. Heating at temperatures above 220° F caused a decrease in whey protein nitrogen from 0.942 to 0.603 mg/liter. Further evidence showed a low correlation between nitroprusside test and cooked flavor. At 240° F the nitroprusside test was negative but there was a distinct cooked flavor and the distillate contained 0.031 meq. cysteine-HCl per liter of milk. At 210° F only a slight cooked flavor and a negative nitroprusside test existed and the concentration of sulfur expressed as cysteine-HCl was 0.021 meq. per liter.

Dill, et al. (12) studied the volatile sulfhydryl groups in a steam injection vacuum system at temperatures of 160° F to 300° F using periods from 15 to 150 seconds. After heat treatment, some of the milk was processed through a vacuum chamber at 130° F and then cooled to 40° F. Some milk was heated in the apparatus and cooled without the vacuum treatment. The sulfhydryl titers increased with increasing heat treatments, for both vacuum and non-vacuum treatments up to a point, 210° F, beyond which the titers decreased in those samples subjected to vacuum treatment. Possible explanation was a volatilization of free sulfur compounds due to the increased

steam treatment. Remaining heat activated sulfhydryls decreased on storage, concomitant with decreases in cooked flavor as determined organoleptically.

Graves, et al. (23) point out that cooked flavor was obtained by heating milk to 175° F for 19 seconds in a regular high temperature short time pasteurizer. Because of the high initial temperature at the flow diversion valve, boiling occurred between a level of 7.5 and 10 inches of vacuum. An expert panel found a highly significant improvement in cooked flavor as vacuum was increased up to 10 inches. The consumer panel found maximum improvement to be at 7.5 inches of vacuum. The vacuum treatment also reduced the intensity of feed and other off flavors.

Other research in cooked flavor has involved the investigation of certain metallic ions, usually copper on the release of free sulfhydryls in milk. Gould and Sommer (22) found the addition of 5 ppm of added copper was sufficient to prevent cooked flavor at 90° C. However, bitter flavor developed upon addition of 1 ppm or above. A temperature of 60° C was necessary to develop cooked flavor. They also added 1% of a 0.5 M solution of sodium sulphite to raw milk and found this to produce the same degree of cooked flavor as highly heated milk. The only difference being that a bitter flavor developed on storage at 40° F.

Heated milk that had a high degree of cooked flavor was treated with 1 ppm of copper sulphate solution. At low temperature storage, this was enough to decrease 90% of free -SH groups at the end of three weeks. Similar results were obtained by Josephson and Doan (38).

Gould and Sommer (22) investigated the change in pH and its effects on sulfhydryl production. They changed the pH to range from 5.8 to 7.4 by addition of either lactic acid or 6.2 M sodium hydroxide. Results showed that as the pH is decreased -SH production is slightly retarded, whereas,

an increase in pH had the opposite effect. A shift of 1.6 unit pH varied the temperature at which cooked flavor appears by 6 to 8° F.

The cooked flavor, according to the work of Josephson and Doan (38), disappears upon storage. Kristofferson, et al. (43) later verified this fact and stated that the decrease in cooked flavor was attributed to two things; time of holding in pasteurization process and temperature of storage conditions. Preheating of milk followed by pasteurization produced less -SH groups than pasteurization at 63° C alone. Total concentration of H_2S in milk pasteurized at 90° C for thirty minutes was 245 micrograms per liter of milk. Fluctuations in loss of sulfhydryl groups from heated milk was greatest during the first 48 hours of storage at 4° C.

Zweig and Block (69), however, did not find a loss of -SH groups from heated milk on storage. Hydrogen peroxide was used as an oxidizing agent and had little effect on the loss of sulfhydryls during storage. Milk was heated at 76° C, slightly above the critical temperature. No decrease after heating or in storage was found using a silver nitrate amperiometric titration procedure.

Despite the objectionable cooked flavor, the production of free sulfhydryls and cooked flavor of milk has some beneficial effects. Jenness and Patton (37) state that raw milk contains a deleterious factor or factors that depresses loaf volume when incorporated into dough for bread making. This effect can be overcome by heat treatment and this improvement in baking quality produced by heat treatment has been found to parallel the denaturation of serum protein. He states that denaturation occurs in the immune globulin phase when milk serum proteins are heated then fractionated.

Greenback and Wright (24) found that high heat treatments reduced the oxidation-reduction potential of milk. This was found to be attributed to the release of free sulfhydryls in the milk. High heat treatments used in the manufacture of dried milk solids produced a high quantity of -SH groups which Greenback and Wright found to be suitable anti-oxidants. Thus, on storage, dried milk with a high heat treatment was found to have superior keeping qualities as compared to the low heat methods of conventional means of drying milk. Similar results were obtained by Smith and Macleod (59) on their investigation of pasteurization effects on stability of homogenized milk. The degree of oxidized flavor was less in homogenized milk as the temperature of pasteurization was increased. Milk was stored in ultra-violet light at 40° F in an attempt to induce oxidized flavor development in milk. Increases of pasteurization temperatures brought about a high production of sulfhydryls, which the authors thought to have a protective effect on milk against oxidized flavor. Shipe (58) later found that aero-vacuum treatment, in conjunction with high temperature short-time pasteurization, reduced the development of oxidized flavor.

Jackson, et al. (34) found that temperatures used to sterilize canned cream led to a pitting of containers with a purplish pigment. They identified this substance as being iron sulfide and attributed this to the denaturation of cysteine and methionine which then reacted with iron of tinplate to produce iron sulfide. Dilute solutions of these amino acids were sealed and heated at temperatures used in processing canned cream. A typical purple pitting of the can occurred and again the substance was found to be iron sulfide.

VI. USE OF AMINO ACIDS IN VOLATILE SULFHYDRYL RESEARCH

Gould (20), using the tripeptide glutathione, studied the release of sulfhydryls from heated milk, containing varying amounts of glutathione. He reported that glutathione losses proceed at a rapid rate from heated samples at 40° C up to 4 hours. Losses were from 75% to complete destruction. Heating of milk to high temperatures before addition of glutathione stabilized the disappearance of glutathione. Reduced glutathione was destroyed and Gould advanced the theory that some of it is converted to the oxidized form. Complete loss of the reduced form was noticed in some heated samples. Harper and Brown (27) used sulfur - 35 labeled cystine and cysteine obtained from dosing a cow with S-35 labeled cystine infusion into the blood stream. Ninety-nine percent of the S-35 was found in the milk protein fraction and only as cystine or cysteine. Labeled milk and a simple buffer solution containing S-35 cystine and riboflavin were exposed for 4 hours to cool white fluorescent light and the volatile degradation compounds were trapped differentially. Based on specific activity of S-35 the concentrations of products released from the milk and buffer systems, respectively, were estimated as 0.0003 and 0.0002 ppM for H_2S, 0.0007 for RSH, 0.0019 for R-S-R.

Thiamine Concentrations and Losses in Heated Milk. Raw milk contains about 0.4 mg per liter of the sulfur containing Vitamin B_1 (thiamine). Thiamine is very heat labile occurring more so in conditions approaching neutrality. Weckel (64) investigated the destruction of thiamine in commercially pasteurized and sterilized samples of milk. Critical losses of methionine and cystine were also noted in the sterilized samples.

Daniels and Giddings (9) investigated various methods of pasteurization and their influence on the destruction of thiamine in milk. Milk was pasteurized in a holding vat at 144° F for thirty minutes. Samples were taken before and after pasteurization. Thiamine content of the raw milk varied from 0.29 mg per liter to 0.40 mg per liter. After pasteurization the thiamine content varied from 0.21 mg to 0.34 mg. Nine percent of the thiamine was lost by the holding method.

Dutcher and Guerrant (14) also investigated the various methods of pasteurization on thiamine content of milk. Three different temperature levels and treatments were used; 62° C for thirty minutes in reduced pressure, pasteurization with aeration and boiling thirty minutes. Maximum destruction of thiamine was 33%. The greatest losses occurred under diminished pressure. No explanation was given for this by the authors.

Elvejehm (45) investigated samples of milk obtained before and after pasteurization in four dairies. All four dairies used vat pasteurization of 145° C for thirty minutes. Average destruction of thiamine was found to be about 25%. Similar results were obtained by Kraus and Erb (42) in feeding experiments with rats using vat pasteurized milk.

Sherman and Spahn (57) found no measurable destruction of thiamine in milk powder when dried in free access to air at periods up to 48 hours at 100° C. They also found the extent of destruction to be dependent on the hydrogen ion concentration.

Halliday (25) investigated the influence of various lengths of heating time and pH levels on the thiamine concentration in protein free milk heated at 70° C for periods of one and four hours. pH levels used

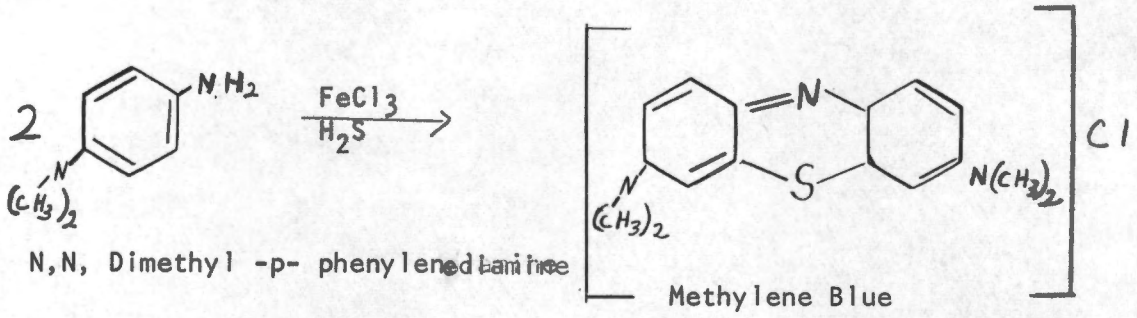
were 4.3, 7, and 10. Solutions at pH 10 showed no vitamin potency even in unheated samples. Thirty per cent loss of thiamine occurred at pH 7. Heating for four hours at pH 7 caused a loss of 58 per cent.

Davidov and Bekhova (10) studied the storage losses of thiamine in evaporated milk. Pasteurization alone had little effect on the vitamins except ascorbic acid in the milk. As a result of pasteurization and evaporation, however, the milk lost about 14% thiamine. Further decreases occurred when the product was stored at 8° to 12° C for a year. Decreases totaled 47% in comparison to thiamine content of fresh milk.

CHAPTER III

I. MATERIALS AND METHODS

The distillation of volatile sulfhydryls was accomplished by using the procedure of Townley and Gould (62) as shown in Figure 1. The volatile sulfur produced was measured by the conversion of N,N, dimethyl -p- phenylenediamine reagent to methylene blue. The methylene blue is thought to be formed by the following reaction (39):



N,N, Dimethyl -p- phenylenediamine — Methylene Blue

One thousand milliliters of raw milk obtained from the University Creamery was heated as a single sample. The samples were heated at temperatures of 75° or 80° C for thirty minutes in a thermostatically controlled water bath accurate to ± 1° C. Ten trials were conducted at each temperature using added cystine, methionine, or glutathione in separate experiments. The heating and subsequent distillation of the volatile sulfhydryls was accomplished under conditions of a steady flow of nitrogen at a rate of approximately five bubbles per second into milk in order to pass the vapors into the receivers containing the zinc acetate. The purification of the nitrogen gas was accomplished by passing it through distilled water and alkaline pyrogallol to remove impurities and/or oxygen. Oxygen could cause an oxidation of sulfhydryls to H_2S which would cause some to be lost before it reached the receivers. The flow of nitrogen was started when

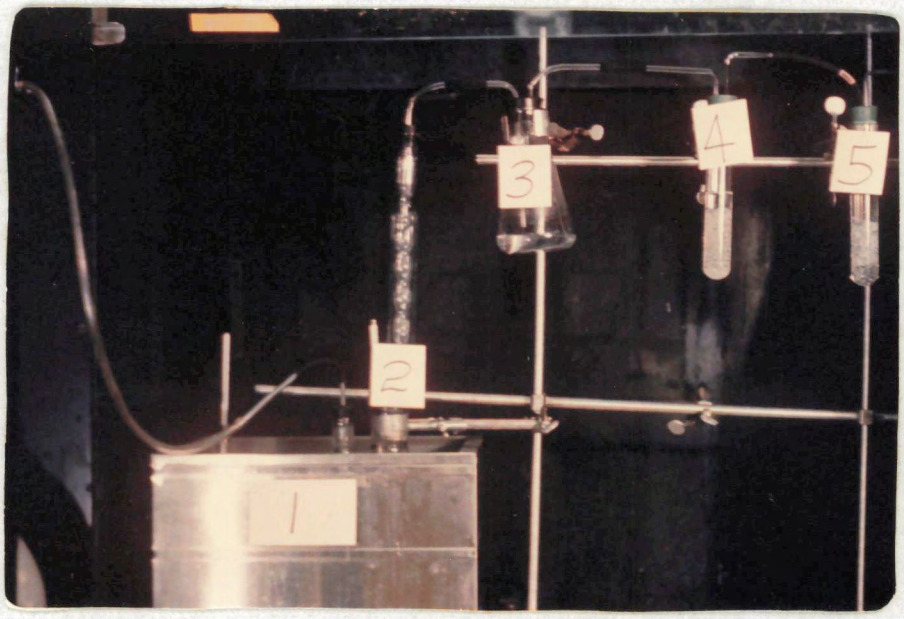


Figure 1. Photograph of apparatus used for heating milk and trapping the volatile -SH groups. 1. water heater; 2. flask and condenser; 3. foam trap; 4.& 5. receiving tubes containing alkaline zinc acetate.

the temperature of the milk reached 75° or 80° C and was continued for an aspiration period of thirty minutes after the heating period. The alkaline pyrogallol was prepared by the method of Townley and Gould (62) whereby thirty grams of pyrogallic acid was dissolved into 100 ml of distilled water and 2.5 volumes of 50% sodium hydroxide added. The volatile sulfide receivers, about 1/4 filled with 4 mm glass beads, contained 50 ml of the alkaline zinc acetate. The stock alkaline zinc acetate was prepared by mixing 25 ml of 20% zinc acetate and 40 ml of 10% NaOH diluted to one liter by adding distilled water. Nearly all of the volatile sulfhydryls coming from the heated milk were trapped in the first receiver.

Approximately 10 minutes were required for the water bath to reach the desired temperature. At the end of the heating period the hot water was removed and the flask cooled with ice water and the nitrogen flow continued for thirty minutes. At the end of the aspiration period the solution and glass beads were removed from the receivers and the receivers rinsed with distilled water, then with 20 ml of 1:4 hydrochloric acid. The acid rinse was withheld from the alkaline receiving solution to avoid the possibility of an error due to the liberation of hydrogen sulfide before the indicators were added.

Twenty-five milliliters of N,N, -p- phenylenediamine reagent prepared in stock by adding 500 ml of distilled H_2O and 1 gram of N,N, dimethylaniline, was added to alkaline zinc acetate mixture. Five milliliters of 0.02 molar solution of ferric chloride in 4% hydrochloric acid was added as well as the acid rinsings from the receiver. The flask was stoppered and set in the dark for about three hours to allow for color development.

The solution was then placed in a 300 ml Erlenmeyer flask. The beads and receiving flasks were rinsed several times with double distilled water;

the rinsings added to the Erlenmeyer and the mixture made up to 300 ml with distilled water.

The solution was then placed in a dark cabinet to retard any denaturation of dye before intensity was determined. The intensity of color was determined with a Bausch and Lomb colorimeter using a 620 millimicron wave length and the concentration of methylene blue read from a previously constructed graph. An example of these is shown in Figure 2.

Six hundred and twenty millimicron wave length was chosen after preparing a curve of transmissions at different wave lengths from 35.0 to 650 mμ with 620 mμ being at the top of the curve.

The colorimeter concentration curve (Table I) of methylene blue was prepared at wave length 620 which was then used to determine the concentration of methylene blue developed from the hydrogen sulfide collected in alkaline zinc acetate solution evolved in the vapors of different samples of heated milk.

The quantity of methylene blue was then used to calculate the quantity of sulfur evolved from various samples of milk on the basis that one molecule of hydrogen sulfide caused the formation of one molecule of methylene blue. The molecular weight of methylene blue is 319.85 and sulfur 32.066, therefore, $\frac{32.06}{319.85}$ X mg of methylene blue = mg sulfur evolved.

Due to formation of sulfhydryls the milk develops cooked flavor which can be detected by taste and the sodium nitroprusside test as described by Josephson and Doan (38). This test consists of measuring five milliliters of milk into a test tube, saturating with ammonium sulfate by adding an excess of the solid and shaking the tube. Five drops of 4.5% solution of sodium nitroprusside $Na_2(NO)Fe(CN)_5 \cdot 2H_2O$ are added and the mixture shaken. Five drops of 28% ammonium hydroxide are added and the

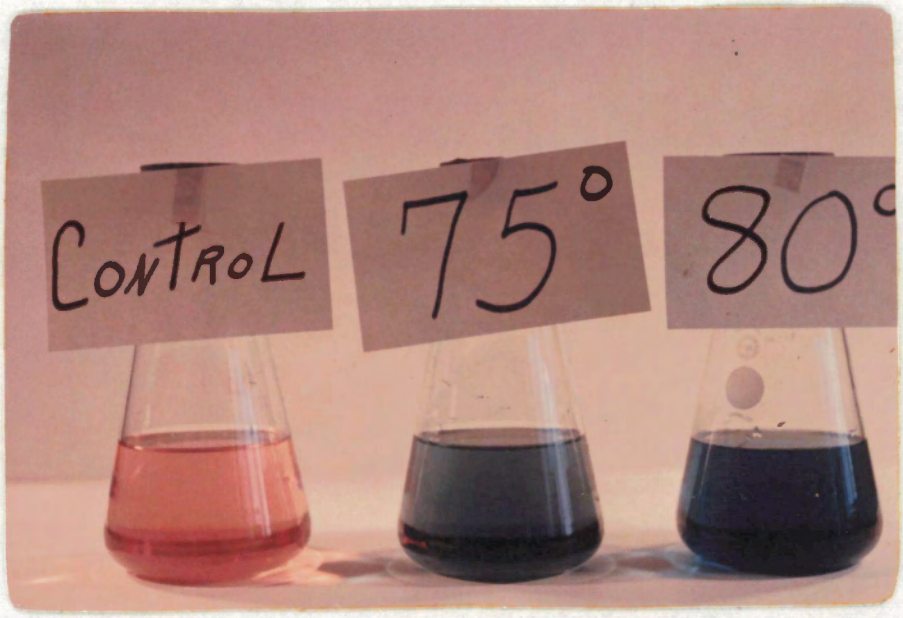


Figure 2. Example of color changes occurring due to the production of volatile sulfhydryl groups.
Control - methylene blue precursor, N,N, dimethyl -p-phenylenediamine
75° C - precursor + sulfhydryls from milk heated at 75° C for thirty minutes
80° C - precursor + sulfhydryls from milk heated at 80° C for thirty minutes

TABLE I

RELATIONSHIP BETWEEN CONCENTRATION OF METHYLENE BLUE AND LIGHT TRANSMISSION

Per Cent Transmittance	Concentration of Methylene Blue (mg/l)
95	0.20
90	0.25
87	0.50
80	0.75
75	1.00
70	1.45
65	1.60
60	1.75
55	1.95
50	2.25
45	2.45

mixture agitated and the developed color compared to standards. The test must be conducted at a temperature below 20° C and the results should be read within 30 minutes after additions of the reagents. The presence of a free sulfhydryl group will cause the sample to be pink in color. Substances such as creatinine can also cause a positive nitroprusside but these are not ordinarily found in milk.

II. THE DETERMINATION OF THIAMINE

The thiochrome assay method as used by Harris and Wang (28) and modified by The Association of Vitamin Chemists (2) was used to measure the thiamine destruction and thus relate this as a possible source of the volatile sulfhydryls in milk undergoing heat treatments. The thiochrome method is based on the conversion of thiamine to thiochrome, a substance which fluoresences under ultra-violet light. The conversion is made by oxidation with alkaline $K_4Fe(CN)_6$ solution. The resultant fluoresence is determined by the use of a photofluorometer, the amount of fluoresence being directly related to the thiamine concentration in original sample. Determinations of the thiamine content are made by oxidation of a known quantity of thiamine.

The thiamine was extracted from a 20 milliliter sample of milk by adjusting the pH to 4.0 with 15% trichloroacetic acid. To the resulting slurry was added 0.1 gram of takadiastase and 0.1 gram of papain. The samples were then incubated overnight at 40-45° C. They were then centrifuged and the supernatant liquid made up to a volume of 25 milliliters with distilled water. The insoluble residue was discarded. Five milliliters of the supernatant were then placed in a centrifuge and the impurities.

extracted by shaking for five minutes with an equal volume of isobutanol. The tube was then centrifuged and the isobutanol layer discarded. One and five-tenths milliliters of the washed sample was then added to a glass stoppered centrifuge tube. Two milliliters of methanol and three milliliters of alkaline potassium ferricyanide solution were added and the contents shaken for two minutes and then 10 milliliters of isobutanol were added. The resultant mixture was centrifuged and the lower aqueous layer removed with a narrow pipette equipped with a rubber teat. Two milliliters of ethanol were added to clarify and then the fluoresence determined on a Coleman Model 12 photofluorometer. A stock solution prepared according to Hennessey (29) containing 1 microgram of thiamine hydrochloride per 5 milliliters was oxidized to obtain a standard to calculate the concentration of thiamine in the unknown sample.

Sampling for Thiamine Determinations. All samples were analyzed for thiamine before and after heat treatments. The skim milk was pasteurized in the University Creamery as was the ultra-high temperature samples of whole milk. The heat treatments were 143° F for thirty minutes, UHT (194° F for 3 seconds) and 75 or 80° C for thirty minutes in a thermostatically controlled water bath accurate to ± 1° C with nitrogen agitation. All samples were stored at 40° F until the assays were performed.

Calculations of thiamine concentration per unit volume were made according to the following formula:

$$\frac{x - y}{x_1 - y_1} \cdot \frac{25}{5} \cdot \frac{1}{20} = \text{micrograms thiamine chloride per milliliter milk}$$

Where x = Standard 1 microgram in 5 milliliters oxidized thiamine

y = Standard 1 microgram in 5 milliliers not oxidized

x_1 = Unknown oxidized

y_1 = Unknown blank

25 = volume of extract

5 = milliliter of extract used

20 = milliliter of original sample

Radioactivity Measurements: Self-Absorption Determinations. Self-absorption is the phenomenon whereby radioactive particles are absorbed in their suspension media before reaching the surface where they can be detected and measured by counting techniques. This phenomenon is particularly serious in measuring the activity of materials emitting low energy beta rays. Sulfur - 35 emits a beta ray of 0.167 Mev which is a very low energy beta particle. Thus, to obtain an accurate measurement of the radioactivity in the $BaS^{35}O_4$ precipitates a self-absorption curve must be constructed and the resulting radioactive counts on the unknowns corrected for self-absorption.

The determination of the self-absorption factors was carried out as described by Schweitzer and Stein (56). The factors are determined by adding, to a given amount of the particular radioisotope, various amounts of carrier material. In the present investigation the carrier material used was Na_2SO_4 which was converted to $BaSO_4$. This procedure results in a series of $BaSO_4$ precipitates of different weights, but having the same amount of radioactivity. Using semi-logarithmic paper and plotting the counts per minute against the weights of $BaSO_4$ results in a straight line which can then be extrapolated to zero self-absorption.

In this investigation 2.0, 2.5, 3.0, 4.0, or 5.0 milliliters of a 12.5% solution of Na_2SO_4 were added to 10 milliliters of zinc acetate solution containing one microcurie of sulfur - 35. After precipitation of

the SO_4 as $BaSO_4$ the precipitates were dried, weighed and counted on a gas flow counter using a thin window detector. The self-absorption factor for a given weight of $BaSO_4$ was determined by dividing the zero self-absorption count by the observed count. For example, the zero self-absorption count extrapolated from the various weights of $BaSO_4$ was found to be 1733 counts per minute per gram of $BaSO_4$. The observed count on a sample which weighed 620 milligrams was 1118, thus the ratio $\frac{1733}{1118} = 1.549$ is the self-absorption factor for all $BaSO_4$ precipitates weighing 620 milligrams counted by this technique.

Preparation of S-35 labeled amino acids for dosing in the milk were made according to the following dilution scheme: 1 millicurie each of S-35 labeled cystine and methionine and 500 microcuries of reduced glutathione were received from the Schwarz Bio-Research, Inc. of Orangeburg, New York. The samples were placed in 1000 milliliters of distilled water and were further diluted to a concentration of one microcurie per 5 milliliter solution. Ten milliliters of solutions were then used to dose 1000 milliliters of raw whole milk which was then given various heat treatments, giving a final calculated activity of 0.0002 microcuries per milliliter of milk.

The specific activity of the cystine, methionine, and glutathione as received was 54.5, 157, and 32 microcuries per milligram, respectively. The quantities added to a liter of milk was thus 0.073, 0.025, and 0.06 milligrams of cystine, methionine, and glutathione, respectively. The calculated quantities of cystine and methionine in milk is about 285 and 175 milligrams per liter, respectively. The amount of glutathione in milk was not found in the literature, but the amount of all the proteose-peptone nitrogen in milk is about 5.0% of the total nitrogen. Thus, in the case of

cystine the dose amounted to about 0.026% of the cystine normally present in milk, and in the case of methionine, about 0.014%. These amino acids normally in milk, of course, are mostly in the form of a larger protein molecule and not free amino acids as were the dose which was added in these experiments.

Preparation of Volatiles for Counting. After a 30 minute heat treatment and just before the receivers were prepared for color development, ten milliliters of the zinc acetate solution were removed for determining the activity of the volatiles trapped in the solution. The samples were prepared for counting by using the $BaSO_4$ precipitation technique as described (1) and (54). This consisted of dissolving 2-3 grams of Na_2O_2 in 50 milliliters cold water in a 250 milliliter beaker. The zinc acetate solution was then added. The beaker was covered with a watch glass and heated on a steam bath until all the sulfur was oxidized to sulfate (indicated by a disappearance of yellow color). The sides of the beaker were then washed with 100 milliliters of HCl of dilution 1:4. Five milliliters of sodium sulfate carrier (125 gram Na_2SO_4 per liter) were then added. The mixture was then heated to boiling with constant stirring and 13 milliliters of 5% $BaCl_2$ solution were added. After allowing for settling, the completeness of precipitation was checked by adding a few drops of barium chloride. The barium chloride must be in excess to get complete precipitation. The beaker was then heated for an hour covered by a watch glass. After heating the supernatant liquid was poured off and the precipitate was washed into a tared stainless steel planchet fitted with a holder for the liquid portion. After settling out, the liquid was removed

and the precipitate washed with de-ionized water. After two washings the holder and liquid were removed and the planchet and contents dried under a heat lamp. The radioactivity (S-35) was then measured by a gas flow counter. All samples were counted on the same day, but heatings were alternated as 75^{o} C one day and 80^{o} the next day to eliminate any discrepancies in sampling preparation.

CHAPTER IV

RESULTS AND DISCUSSION

Part I.

Listed in Table II are the results for ten trials at 75° C for thirty minutes using sulfur - 35 labeled cystine, glutathione, and methionine. The data shows that the ratio of S-35 from cystine to the grams of $BaSO_4$ produced from the volatiles is greater when milk is heated at 75° C for thirty minutes than at 80° C for thirty minutes. This indicates that cystine will be broken down at a lower temperature than the average of the other components in milk which contribute to volatile sulfur. Average sulfur liberation for each sample is also listed in Table II. The average concentration for ten trials was shown to be 53, 47, and 60 micrograms per liter for cystine, methionine, and glutathione, respectively. This was shown not to be significantly different from each other at the 99% level of probability. As shown in Table II the sample which contained glutathione gave the highest concentration of both sulfur - 35 and total sulfur liberation. However, taken on a ratio basis, cystine and glutathione show similar results of ratio between micrograms of sulfur and counts per minute of sulfur - 35, indicating little difference in resistance to heat at 75° C.

At 75° C methionine-dosed samples showed the lowest volatility of both total sulfur and sulfur - 35. The ratio of volatile sulfur - 35 to total sulfur was 1.12 compared to about 2.9 for the other two samples. This would tend to substantiate the work of Flake, et al.(17) in that sterilization temperatures higher than 75° C must be reached in order to significantly destroy methionine by heat treatments.

TABLE II

VOLATILITY OF SULFUR-35 LABELED CYSTINE, METHIONINE, AND GLUTATHIONE AT 75° C.

	Trials											
	1	2	3	4	5	6	7	8	9	10	Av.	SD*
Cystine												
cpm/g $BaSO_4$	163	175	160	150	156	125	113	155	170	160	153	±19.6
Sulfur	54	67	57	47	67	36	55	45	45	53	53	±12.0
Nitroprusside	2	3	2.5	2	3.5	2	2		2.5	3	2.3	
Methionine												
cpm/g $BaSO_4$	52	43	48	53	52	56	49	51	51	49	53.4	± 6.4
Sulfur	50	45	40	42.5	50	50	60	55	50	42.5	47.5	±12.2
Nitroprusside	3	2	2	2	2	3	3	3	2	2	2.4	
Glutathione												
cpm/g $BaSO_4$	185	125	150	185	190	196	185	180	180	175	175	±18.5
Sulfur	66	71	66	61	56	49	56	56	66	49	60	± 7.5
Nitroprusside	3	3	3	3	2	2	2.5	2.5	3	2	2.6	

$$\frac{\text{cpm per gram } BaSO_4}{\text{micrograms sulfur evolved}}$$

Cystine: 2.89
Methionine: 1.12
Glutathione: 2.91

$$*\text{Standard Deviation} = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N - 1}}$$

The nitroprusside tests on all samples were lower at 75° C than at 80° C as shown in Tables II and III. This seems to follow the recognized fact that as temperatures are increased the total volatile -SH groups are also increased which react with nitroprusside to give a pink color more intense as temperature is raised and also time of holding increased. As shown in Table II the average nitroprusside content was 2.3, 2.4, and 2.6 for the three samples containing cystine, methionine, and glutathione, respectively. The nitroprusside values were obtained by visual inspection and not by the use of a colorimeter. The nitroprusside values normally range from 0 to 6 (38), and the author observed that it was possible to detect changes which were judged to be 0.5 unit different than a similar sample. A reading in the final analysis of 2.3 or 2.4 merely represents the numerical average of values of all ten samples. Some workers have shown that there is a low correlation between nitroprusside test and cooked flavor, while others have shown that there is a good correlation. Perhaps the nitroprusside test is a better qualitative test than a quantitative one, but it seems to be a good measure of the heat activated -SH groups in milk.

Listed in Table III are the results of volatility of cystine, methionine, and glutathione heated at 80° C for thirty minute periods. Cystine showed a lower ratio of cpm/g $BaSO_4$/ microgram sulfur at 80° C than at 75° C indicating greater denaturation of other proteins in the milk at 80° C. The above ratio in those samples containing both glutathione and methionine was higher at 80° C than at 75° C. The total micrograms of sulfur evolved as measured by the dye reduction method was considerably higher at 80° C than at 75° C. This substantiates other work (38) that has

TABLE III

VOLATILITY OF SULFUR-35 LABELED CYSTINE, METHIONINE, AND GLUTATHIONE AT 80° C.

	Trials											
	1	2	3	4	5	6	7	8	9	10	Avg.	SD*
Cystine												
cpm/g $BaSO_4$	93	99	96	80	106	103	88	79	95	102	92	±9.3
Sulfur	134	113	87	103	103	92	103	92	108	103	108	±13.2
Nitroprusside	5	5	4	4	4.5	4	4.5	4	5	5	4.5	
Methionine												
cpm/g $BaSO_4$	72	65	67	63	64	48	62	60	64	61	63.0	±6.4
Sulfur	80.2	70.0	80.2	120	80.2	145	145	155	95	95	106.5	±19.7
Nitroprusside	5	3.5	5	5	4	5	5	5	4	4	4.55	
Glutathione												
cpm/g $BaSO_4$	225	230	235	215	210	210	215	220	220	215	229	± 9.5
Sulfur	154	123	103	103	111	123	123	115	118	123	119	±14.4
Nitroprusside	5	5	4.5	4	4.5	5	5	4.5	5	5	4.75	

$$\frac{\text{cpm per gram } BaSO_4}{\text{micrograms sulfur evolved}}$$

Cystine: 0.85
Methionine: 0.59
Glutathione: 1.99

* Standard Deviation = $\sqrt{\dfrac{\Sigma X^2 - \dfrac{(\Sigma X)^2}{N}}{N-1}}$

shown that as temperatures are increased up to a point (90° C.) an increase of sulfhydryl production takes place. Nitroprusside tests were also higher at 80° C, indicating a further release of free -SH groups into milk as it is heated.

Statistical analysis on all data procured was determined using the t test as described by Snedecor (60) for variance between group comparisons of equal allotment within each group. The formula is as follows:

$$t = (\bar{X}_1 - \bar{X}_2) \sqrt{\frac{n(n-1)}{\Sigma X^2}}$$

Where $\bar{X}_1$ = concentration in first group

$\bar{X}_2$ = concentration in second group

n = total number of experiments in both groups combined

Tables IV and V contain a summary of data analyzed statistically by the t test using group comparisons between 75° and 80° C and within samples of 75° and 80° C. Samples analyzed for differences between 75° and 80° C with both counts per minute and total sulfur evolved showed a significant difference at the 1% level of probability in all experiments except the sulfur - 35 labeled methionine. Likewise, sulfur liberation between treatments within 75° and 80° C were not significant at the 1% level. As shown in Table IV there was not a significant difference in the volatility of cystine and glutathione at 75° C or between methionine and cystine at 80° C.

When compared to these samples containing added cystine, those samples containing S-35 glutathione showed a greater ratio of cpm/g $BaSO_4$/ micrograms sulfur in all aspects except for counts per minute at 75° C and here it was higher than cystine, but not significantly. Of interest in

TABLE IV

STATISTICAL ANALYSIS ON SULFUR - 35
FROM 75° AND 80° C HEATED MILK

Treatment	Difference of Mean
	(cpm/g $BaSO_4$/ micrograms sulfur)
Between 75° and 80° C	
Cystine	60**
Methionine	13
Glutathione	54**
Within 75° C	
Glutathione and Cystine	23
Glutathione and Methionine	124**
Methionine and Cystine	101**
Within 80° C	
Glutathione and Cystine	137**
Glutathione and Methionine	166**
Methionine and Cystine	29

TABLE V

STATISTICAL ANALYSIS ON SULFUR LIBERATION AT 75° AND 80° C

Treatment	Difference (Micrograms)
Between 75° and 80° C	
Cystine	45**
Methionine	58**
Glutathione	60**
Within 75° C	
Cystine and Methionine	9
Cystine and Glutathione	2
Methionine and Glutathione	11
Within 80° C	
Cystine and Methionine	3
Glutathione and Cystine	16
Methionine and Glutathione	13

**Significant at $P=<0.01$

this study is the volatility of cysteine, not as such, but as glutathione. Glutathione is a tri-peptide made up of cysteine, glutamic acid, and glycine. Jackson, et al. (34) have shown that cysteine contributes more to sulfur "blackening" of canned cream than does cystine or methionine and thus the results obtained here seem to substantiate the work of Jackson, et al. (34) and by Gould (20). The low volatility of methionine also substantiates the work of Flake, et al. (18) who also showed a high temperature to be necessary for the destruction of methionine. In the present investigation the difference in the destruction of sulfur - 35 methionine between 75° and 80° C was not significant. The higher ratio of cystine at 75° than at 80° C perhaps might give greater significance to the chemical equation for the production of sulfhydryls as outlined by Jenness and Patton (37). Also higher temperatures could very well bring about a conversion of cystine to a less volatile form if such existed.

Part II.

Data on the destruction by heat of thiamine in milk are reported in Table VI. The ultra-high pasteurized milk showed the greatest destruction of thiamine of any heat treatment applied. This is similar to the data by Halliday (25) who obtained a 58% destruction of thiamine in milk heated at 183° F for 3 minutes. The 54% loss found in this investigation is lower than that reported by Halliday but higher than that obtained by Davidov and Bekhova (10).

There is approximately 20% greater thiamine destruction by ultra-high temperature processing than by vat pasteurization. Similar results have been obtained by Daniels and Giddings (9) and by Kraus and Erb (42). The

TABLE VI

THIAMINE DESTRUCTION IN HEATED MILK
(micrograms per liter)

Treatment	Trial											
	1	2	3	4	5	6	7	8	9	10	Av.	SD*
Raw	450	500	500	485	490	525	500	500	515	525	499	
UHT	250	200	225	200	250	250	250	250	225	175		
Amt. Loss	200	300	275	285	240	275	250	250	290	350	271	±41.8
% Loss	44.4	60.0	55.0	58.8	49.0	52.4	50.0	50.0	56.3	66.7	54.26	± 6.5
75° C	375	375	360	360	350	450	375	350	350	300		
Amt. Loss	75	125	140	125	140	75	125	150	165	225	134	±43.12
% Loss	16.7	25.0	28.0	25.8	28.6	14.3	25.0	30.0	32.0	42.8	26.82	± 7.9
80° C	300	258	250	250	300	375	325	300	300	265		
Amt. Loss	150	142	250	235	190	150	175	200	215	260	196	±40.19
% Loss	33.3	48.4	50.0	48.5	38.8	28.6	3.50	40.0	41.7	49.5	41.38	± 7.6
											39	
Raw Skim	468	487	500	485	475	500	500	525	525	525	499	
Vat Past.	325	350	350	350	300	375	325	300	375	375		
Amt. Loss	143	137	150	135	175	125	175	225	150	150	156	±23.8
% Loss	30.6	28.1	30.0	27.8	36.8	25.0	35.0	42.9	28.6	28.6	31.34	± 5.4

*Standard Deviation = $\sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N - 1}}$

vat pasteurized skim showed a greater loss of thiamine than did the 75° C heating but this difference was shown not to be significant at the 95% level. However, the 80° C heating had the second highest rate of destruction of thiamine of any sample. Evidently the thiamine is destroyed quite rapidly on heating above a certain point.

Listed in Table VII is the summary of all treatments grouped together for the purpose of comparison. As noted, the greatest difference in amount of thiamine destroyed occurred in the comparison of 75° C for thirty minutes and ultra-high processed milk. The difference of 139 micrograms destroyed was significant at the P=<0.01% level of probability. The next greatest difference occurred between UHT and vat pasteurized skim. The 115 micrograms destroyed here is also significant at P=<0.01%. Highly significant differences were also noted in comparing 75 and 80° C and for 80° C and ultra-high processed milk. Non-significant differences were found between 75° C and vat pasteurized skim and also between 80° C and vat pasteurized skim. The values obtained on raw milk and vat pasteurized milk are similar to those reported by Weckel (64) on earlier findings.

As a source of volatile sulfhydryls in heated milk, thiamine undoubtedly would be of importance even though thiamine contains only 12% sulfur in its molecular structure. Based on this fact the ultra-high processed milk looses approximately 30 micrograms of sulfur from the destruction of thiamine. How this would influence degree of cooked flavor as evidenced by taste was not found in the literature or in these experiments. At vat pasteurization temperatures thiamine might contribute about 16 micrograms of volatile sulfur, where at 75° C, 14 micrograms might be

TABLE VII

STATISTICAL ANALYSIS ON THIAMINE DESTRUCTION IN HEATED MILK

Comparison of Treatment	Difference of Mean
UHT (194° F, (90° C) 3 sec) and vat pasteurized skim (145-150° F, (62-66° C) 30 min.)	115**
80° C - thirty minutes and 75° C - thirty minutes	72**
75° C - thirty minutes and vat pasteurized skim	22
75° C - thirty minutes and UHT	139**
80° C - thirty minutes and UHT	75**
80° C - thirty minutes and vat pasteurized skim	40

**Significant at P=<0.01

evolved and at 80° C, 30 micrograms. This might be compared to about 55 micrograms from cystine, methionine, or glutathione at 75° C, and slightly over 100 micrograms from each of these three latter sources at 80° C.

CHAPTER V

SUMMARY AND CONCLUSIONS

Milk containing sulfur - 35 labeled cystine, methionine and glutathione has been heated at 75° and 80° C for thirty minutes to study heat denaturation and subsequent loss of sulfhydryls from these sources. Using ratios of cpm/g of $BaSO_4$/microgram of sulfur evolved, very little difference existed between cystine and glutathione at 75° C, but significant differences were shown at 80° C with glutathione showing a higher ratio. Significant differences existed between ratios of cystine and glutathione as compared to methionine except at 80° C for cystine and methionine. Differences in ratio between temperature levels for methionine were not significant indicating that temperatures above 80° C must be reached in order to destroy methionine by heat treatments.

Significant differences in total sulfur were found between 75° and 80° C indicating that as temperatures are raised in this range greater release of free sulfhydryls occurs. Differences of sulfur liberation within each temperature level for all treatments were not significant indicating the same degree of accuracy in trapping the volatile sulfhydryls as they are evolved during heat treatments.

A study has been completed using pasteurization temperature of 145-150° F (62-66° C) for 30 minutes, 194° F (90° C) for 3 seconds, and 75° and 80° C for 30 minutes to determine destruction of thiamine by heat. Destruction of thiamine of 54, 26, 41, and 31% occurred at 194° F (90° C), 75° C, 80° C, and 145-150° F (62-66° C), respectively.

Liberation of 30 micrograms of sulfur occurred at the 194° F (90° C) level of heating, 16 micrograms at 145-150° F (62-66° C), 14 micrograms at 75° and 30 micrograms at 80° C. This might be compared to about 55 micrograms from cystine, glutathione, or methionine at 75° C and slightly over 100 micrograms for each of these three latter sources at 80° C.

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APPENDIX

TABLE VIII

SELF-ABSORPTION FACTORS FOR SULFUR - 35 IN $BaSO_4$
PRECIPITATES IN STAINLESS STEEL PLANCHETS

Milligrams of Sample	0	10	20	30	40	50	60	70	80	90	100
500	1.428	1.434	1.444	1.454	1.463	1.474	1.485	1.495	1.506	1.516	1.528
600	1.528	1.538	1.549	1.560	1.572	1.582	1.593	1.604	1.617	1.626	1.640
700	1.640	1.649	1.663	1.673	1.685	1.698	1.710	1.721	1.730	1.747	1.758
800	1.758	1.771	1.783	1.795	1.808	1.823	1.833	1.847	1.860	1.837	1.888
900	1.888	1.899	1.912	1.926	1.939	1.953	1.967	1.982	1.996	2.009	2.026
1000	2.026	2.029	2.052	2.067	2.080	2.096	2.113	2.126	2.140	2.157	2.172
1100	2.172	2.186	2.204	2.210	2.234	2.249	2.265	2.281	2.297	2.313	2.330
1200	2.330	2.346	2.363	2.380	2.398	2.412	2.430	2.448	2.467	2.482	2.501
1300	2.501	2.516	2.536	2.552	2.572	2.588	2.609	2.625	2.647	2.664	2.681
1400	2.681	2.704	2.772	2.740	2.758	2.777	2.801	2.820	2.840	2.860	2.880