CORRELATION OF SUBJECTIVE AND OBJECTIVE METHODS IN THE STUDY OF MILK FLAVORS

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I hereby recommend that this dissertation prepared under my direction by Salvador Retamoza Leyva

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be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy

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As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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SIGNED: Salvador Reta Mazer L.

Dedicated to my wife, Laura Elena, and to my children, Salvador, Jr. and Laura Belen.

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TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF TABLES

LIST OF TABLES--Continued

LIST OF ILLUSTRATIONS

ABSTRACT

Correction of subjective/objective analyses was applied to ten lots each of three commercial brands of market milk. The criteria used were as follows: a) oxidized flavor--copper concentration, peroxide levels, tocopherol content and aldehydes as determined by the thiobarbituric acid test; b) feed flavor--GLC analysis of milk sample steam distillates; and, c) rancid flavor--acid degree value of the milk lipids.

Statistical treatment of data included analysis of variance with simple and multiple correlation studies of the parameters involved. Sensory evaluation was performed by a panel of three judges and individual scores were given to oxidative, rancid and feed flavor. These scores were correlated with the results of objective measurements. A simplified version of the American Dairy Science Association scoring guide was employed.

Copper was determined colorimetrically, showing concentrations within the range reported in the literature. Its influence in promoting oxidation of milk was not totally correlated under the conditions of collection and flavor scores assigned by the panel. Some individual samples, however, indicated good relationship between levels of copper and oxidative scores.

totally correlated under the conditions of collection and flavor scores assigned by the panel. Some individual samples, however, indicated good relationship between levels of copper and oxidative scores.

Peroxide levels were also analyzed by colorimetric procedures. This test significantly correlated with the extent of oxidation present in the milk samples. The thiobarbituric acid test was employed to evaluate the extent of oxidation as a function of the amount of aldehydes present. This test was useful in determining the extent of oxidation in samples with a degree of oxidation sufficient to give an absorbance of 0.03 and above. Tocopherol was determined colorimetrically on column chromatographed milk lipids. Levels of this naturally occurring antioxidant were within the range previously reported. However, there was poor correlation between tocopherol levels and oxidized flavor intensity.

Acid degree values were determined by direct titration of the free fatty acids in milk fat previously isolated by detergent deemulsification techniques. The method was efficient in following the extent of rancidity and the correlation with the flavor scores was very good. Except for a few cases, rancidity seemed not to constitute a serious off-flavor problem in the samples studied.

high correlation coefficients. Tentative identification of the compounds present in the chromatograms revealed the presence of acetaldehyde, acetone, butanone, propanol, ethanol and isopropanol. Substractive chemical reaction of the steam distillates with proper reagents confirmed the carbonyl nature of the volatiles detected.

Interrelations among the parameters that influence flavor in milk was also investigated by statistical analysis.

CHAPTER 1

INTRODUCTION

The subjective/objective approach in studying a particular problem concerned with the quality, acceptance and shelf life of food products is a valuable methodology. In the present work, flavor characteristics of the beverage fluid milk were studied for possible correlation between objective determinations of several variables which are known individually or in combination to play important roles in the acceptance of the product in the market, and the sensory characterization of them. Excluding flavors of microbiological origin, the literature generally concedes that off-flavors in milk products can be traced either 1) to the feed consumed by the producing animal, or 2) to hydrolytic or oxidative deterioration of the milk lipids.

Since there is a lack of published information relating to the simultaneous evaluation of several milk off-flavor by exhaustive chemical and statistical analyses, this study was initiated. Furthermore, the sampling procedure permitted an approximate 85 percent surveillance of the conditions in a market milk supply being provided for a population of about 2,000,000 potential consumers. In this

work, packaged, pasteurized, homogenized milk samples representing products being locally marketed were collected at suitable intervals. Subjective flavor characteristics were evaluated by a trained panel using the American Dairy Science Association's official flavor scoring system. Each of these samples were analyzed for evidence of volatile feed flavor components by gas liquid chromatography. Hydrolytic rancidity was determined as a function of the total free fatty acids in the sample. Oxidative deterioration was related to four determinations: a) copper contamination, b) naturally occurring tocopherol, c) aldehyde compounds, and d) peroxides.

In order to establish valid relationships between objective/subjective flavor information, analysis of variance techniques were used in the statistical treatment of the data.

CHAPTER 2

LITERATURE REVIEW

Milk cannot be considered a highly flavored food, yet it does possess a delicate, pleasant flavor (61). The flavor of fresh fluid milk is difficult to describe because it is basically a bland product. From a gustatory standpoint, it can have either a sweet character as a result of its lactose content, or a slight salty taste due to the presence of chloride salts (61). Keeney (42), p. 183, observed that "Fresh milk imparts a pleasant smooth sensation to the mouth and there should be no evidence of astringency." A basic olfactory quality characteristic of all fresh milks exists, but the normal intensity of the flavor is such that for all practical purposes, milk has very little flavor. However, this does not mean that all milks are flat, since milk flavor is highly susceptible to change as a result of farm and processing practices, in addition to changes that can occur in the milk itself.

When the intensity of the normal flavors present in milk is altered by the presence of abnormal amounts of products due to feeding practices, contamination before and after processing, and spontaneous changes of flavor when in storage, consumer objection may be evidenced. Of equal

importance is the situation wherein the flavor intensity is below that which generates an observable consumer objection (subliminal level). This case results in possible decreased consumption levels without a complete awareness of a specific flavor abnormality. So, the dairy industry is confronted with the problem of marketing a product free of or with very slight amounts of objectionable flavors in order to gain a favorable consumer response for its products. This is one of the important reasons why the most advanced knowledge of the factors that can contribute to the formation of those off-flavors, their interrelations, and mechanisms of their development, are of great concern to the dairy industry.

Feed Flavor

Feed or weed flavor can be traced to the type of feed consumed by the producing animal, the surrounding conditions prevailing during the time of milking and the inhalation of strong odors derived from the feed. However, the role of the feed in producing flavor in milk does not always follow a standard pattern. Jenness and Patton (37) indicate that on occasion flavors are produced which could be directly related to the type of feed consumed, but in other cases this was not necessarily true, with flavor characteristics being noted in milk but not found in the feed (61). To a certain extent, the problem of feed flavor

can be controlled by improving feeding practices, such as removing animals from the feed a few hours prior to milking, feeding the highly flavored product immediately after milking and proper ventilation.

The mechanisms of transmission and incorporation of the compounds suspected as being responsible for this type of milk flavor have received a great deal of attention by various investigators. Dougherty et al. (15) conducted experiments which demonstrated the role of three pathways in the transfer of feed flavors to milk. Two animals were fitted with trachael and ruminal fistula that permitted the flavor substances under study to be added directly into the rumen or the lungs. Gases generated in the rumen could be allowed to pass directly into the lungs or interrupted by passing fresh air into the tracheal fistula. Table 1 summarizes the results. When the vapors of an onion slurry were introduced into the trachea, no detectable off-flavor was observed in the milk after 90 minutes. A low level was detected when the slurry was introduced into the rumen and eructated gases prevented from entering the lungs. A pronounced flavor occurred in the milk in a short period of time when the eructated gases were not excluded from the lungs. The role of the rumen was evident when onion slurry was incubated with rumen ingesta and the vapors then passed through the tracheal fistula into the lungs. The off-flavor which occurred in the milk was not characteristic of the onion. In another study by Petersen and Brerenton (64), forced

Table 1. Transmission of Flavor from Onions to Milk[a]

Pathway	Response
Onion slurry --→ Tracheal cannula --→ Lungs	No flavor up to 90 minutes
Fresh air --→ Tracheal cannula	Flavor intensity low up to 45 minutes
Onion slurry --→ Ruminal cannula	Flavor intensity low up to 45 minutes
Cow breathing normally	Flavor intensity low up to 45 minutes
Onion slurry --→ Ruminal cannula	Pronounced flavor in 15 minutes
Onion slurry incubated 30 minutes at 37 C with rumen ingesta --→ Lungs	Pronounced flavor in 15 minutes
Rumen ingesta --→ Lungs	No flavor up to 90 minutes

a. Ref. (61).

inhalation of odors from several pure substances and some natural materials showed that some of them were transmitted to the mammary gland via the respiratory system, producing off-flavors in milk. Feed flavor was induced in milk from cows which had been forced to inhale the odor of corn or alfalfa silage for two hours.

Morgan and Pereira (58) studied the volatiles entrained from several grass and corn silages by condensing in cold traps and separation by gas liquid chromatographic techniques. The volatiles from grass silages were found to be mixtures of low molecular weight alcohols, aldehydes, ketones and esters of short chain fatty acids. From corn silages, a similar composition was observed, except that some were not consistently detected and butyrates were positively absent. They postulated that rapid transmission of some of these compounds (or all) to the mammary gland via the respiratory system could be responsible in part for the development of feed flavor in milk cows fed silage.

Honkanen, Karvonen and Virtanen (33) carried out experiments using odorless, purified diets consisting of cellulose, starch, sucrose, urea, inorganic ammonium salts, minerals, vitamins A and D, and corn oil. The animals produced milk with its characteristic flavor and free of off-flavors.

The results of studies in which 1-2 gram portions of various compounds were added to the rumen recovered from the milk by vacuum distillation and quantitatively analyzed by gas

chromatography are listed in Table 2. One of the important results in this work is the fact that aliphatic alcohols with an odd number of carbon atoms were transferred to the milk in greater degree. Of the ethyl esters administered, only those with an even number of carbon atoms in the fatty acids enter the milk, while the methyl esters are not transferred in detectable amounts.

Shipe et al. (72) demonstrated that the low boiling, volatile compounds identified by Morgan and Pereira (58) could be transmitted to the milk via the cow's respiratory system. Methyl sulfide, acetone, butanone, and cis-3-hexanol imparted flavors that resembled some of the characteristics of feed flavor. Gordon and Morgan (23), using techniques such as vacuum distillation, gas chromatography combined with mass spectrometry, and selective pre-column qualitative reactions were able to identify 18 out of some 22 compounds regularly present in feed flavored milk. Six of these were reported to be always present, and mixtures with them simulated to a great extent the flavor associated with feed flavored milk. These compounds and their concentrations are listed in Table 3.

Numerous studies have reported the use of gas liquid chromatography (GLC) in an attempt to correlate sensory properties with objective measurements. The works of Bassette, Ozeris and Whitnah (3) and Jennings, Viljhalmsson and Dunkley (38) are among them. Simple correlation

Table 2. Transfer of Some Aliphatic Compounds to Milk via the Rumen

Compounds	Amount Fed (gm)	Maximum Concentration (µg/liter of milk)	Total Amount Transferred to Milk (µg)	% of Total Amount Fed
Alcohols				
n-Pentanol	2	380	250	0.013
n-Hexanol	2	30	20	0.001
n-Heptanol	2	120	100	0.005
n-Octanol	2	---	---	---
n-Nonanol	2	75	100	0.005
Aldehydes				
2-methyl propanal	2	1500	600	0.03
Hexanal	2	15	10	0.0005
Heptanal	2	20	10	0.0005
Octanal	2	30	10	0.0005
Ketones				
2-pentanone	2	1300	1000	0.05
2-hexanone	2	1100	800	0.04
2-heptanone	2	750	250	0.013
Esters				
Methyl esters of C_6-C_{10}	2	---	---	---
Ethyl butanoate	2	120	120	0.06
Ethyl hexanoate	2	60	60	0.003
Ethyl octanoate	2	120	100	0.005

Table 3. Concentration in ppb of Volatiles in Feed Flavored Milk[a]

Compound	Moderately[b] Flavored	Strongly[c] Flavored	Highest[d] Concentration Detected
Methyl sulfide	8-10	25-30	100
Acetone	2000-3000	3600-4800	18000
Butanone	250-350	500-1000	1400
Isopropanol	80-120	300-400	2500
Ethanol	1300-1500	10000-20000	75000
Propanol	100-130	1200-1500	4500

a. Source (23).

b, Feed flavored milk with a score of 38-39.

c. Flavor score range of 35-36.

d. In any sample analyzed during experimental period.

techniques are suitable for some food characteristics (feed flavor). By measuring the total peak area or change in height in one or more peaks, one estimates the intensity of the flavor present, especially if previous analyses have identified such a peak with a particular flavor characteristic.

Keller and Kleyn (44) devised a method by which total peak areas of headspace chromatograms of milk samples from cows fed haylage could be correlated to sensory properties. Analysis of variance techniques completed the study. It was reported that as the total chromatogram area increased, the flavor score decreased. No identification or quantification was attempted.

Feed flavor in low concentrations can be considered mildly unpleasant and could score as high as 39 (good flavor). Its presence or total elimination from the product must be weighed in conjunction with other factors relating to consumer acceptance, economics, type of feed involved, availability of the feeds, cleaning and manufacturing methods or other solutions tending to minimize the problem. Feed flavor in higher concentrations is considered to be unnaturally sweet and aromatic and may score as low as 36 (poor). Most feed flavored milk falls in that category Strong feed flavor is marked as low as 32-36, the same range used to score oxidized and rancid and considered unsuitable for fluid use (57).

Rancidity in Milk

All raw milks are susceptible to the development of rancid flavors as a result of liberation of fatty acids by lipases which are naturally present in milk. A rancid flavor in milk is characterized by a sharp, unclean, astringent taste that lingers in the mouth for some time after the sample has been tasted (57). It is accompanied by an obnoxious odor when the flavor is intense.

A lipase has been defined as an enzyme hydrolyzing the esters from emulsified glycerides at an oil-water interface (14). This situation gives rise to variables not ordinarily found in enzyme reactions. Factors such as total surface area, permeability of the emulsion, type of glyceride and degree of agitation of the reaction medium must be taken in account for the results to be meaningful (71). Other factors common to enzymes such as pH, temperature, inhibitors, activators, enzyme and substrate concentration, etc., will also affect activity.

Lipases in milk seem to work in "systems" or "groups", meaning by this that lipase is not a word used exclusively to characterize a sole enzyme, but rather a number of enzymes with a common property the release of free fatty acids (FFA). At least two different lipase systems have been reported in milk (81). One is irreversibly adsorbed on the fat globule membrane when freshly drawn milk is cooled, and the other is associated with the case in milk

plasma. Lipases are apparently inactive in the udder at the time of milking.

With regard to lipase activity, raw milks can be classified into either one of two groups: a) those which develop rancid flavor spontaneously without any treatment other than cooling of the freshly drawn milk, and b) those which develop rancid flavor after an activation treatment (71). Activation could include treatments such as: a) excessive agitation, b) homogenization, c) centrifugal separation or clarification, d) warming the milk to 27-30°C and recooling to lower temperature, e) freezing and thawing, and f) the addition of small amounts of raw milk to pasteurized, homogenized milk (61, 62).

It is generally accepted that high temperature short time (HTST) pasteurization of milk does not inactivate lipase completely (41). Added to this situation is the fact that current handling and pumping of raw milk from the milking machines to the farm tank through pipelines, and bulk hauling of milk, create varying degrees of agitation. In raw, normal or processed milk, the fat globules are covered by a membrane of phospolipid material which can be considered protective. Any disturbance or weakening of this membrane promotes the attack of natural lipases with increased rancidity. It must be noted that pasteurization does not eliminate or reduce off-flavors already present, but does stop further development to a certain degree. The

presence of FFA in fresh, unlipolyzed milk may be the result of an incomplete esterification in the mammary gland (37). Kintner and Day (50) found that the composition of the FFA in fresh milk was similar to that of the acids bound in the milk glycerides.

The literature seems to conflict regarding the type of fatty acids in what proportion they contribute to the development of the off-flavor known as rancidity. The composition of the FFA has been the subject of several studies. Milk fat is unique among fats in that it contains substantial amounts of low molecular weight fatty acids. It has been suggested by Jenness and Patton (37) that the release of those lower acids, especially butyric, imparts characteristic rancid flavor to milk. Harper (29) and Harper, Schwartz and El-Hagarawy (30) demonstrated that butyric acid is a major constituent liberated from milk fat by lipase specificity in liberating butyric acid from milk fat triglycerides. Kumar and Lalka (52) found that butyric acid is preponderant in the alpha position of the triglyceride molecule. Jensen et al. (39) found that pancreatic lipase shows no preference for the butyric acid linkage as such. Discrepancies in results have been in part due to different approaches to the problem. Factors such as the extension of the incubation period, migration of acyl groups, etc., can influence the results. Kintner and Day (50) using lyophilization, silicic acid adsorption and GLC

techniques, studied the major FFA in milk and their distribution in several milk fractions. The results of their work can be summarized as follows: a) pasteurization causes a slight decrease in total FFA and all acids except 18:0, and b) most of the FFA of milk were distributed in the fat and the fat globule membrane fraction.

According to these findings, most of the FFA of normal flavored milk are associated with the lipid phase and the fat globule membrane. In normal flavored milk, even though the total quantities of FFA may be large, the quantity available in the serum is small (7, 29). In case of lipolysis, which is supposed to occur at the interphase, the FFA are distributed in the aqueous phase where they eventually exceed the flavor threshold of rancidity. In their work, Kintner and Day (50) found that the short chain fatty acids (C_4 and C_6) partitioned more to the aqueous phase where their flavor is diminished due to salt formation. Larger chain acids partitioned more to the lipid phase. This would allow for the presence of relatively large amounts of unesterified fatty acids in normal milk without a noticeable rancid flavor. The flavor panel did not detect rancidity until the acid degree value (ADV) reached 2.74, which is well above the level normally considered rancid or in the process of becoming rancid.

Finally, Scanlan, Sather and Day (70) reported that only the even numbered fatty acids from C_4-C_{12} account for the contribution of fatty acids to the rancid flavor, but that no single acid exerted predominant influence.

Oxidized Flavor

The problem of lipid oxidation in fluid milk and a number of its products has been a matter of concern of the dairy industry for a long time. The importance given to its study is a direct expression of the need for better understanding of the mechanisms leading to its formation and ways of reducing its presence.

Due to the unique characteristics and complexity of milk, there are a large number of factors related directly or indirectly to lipid oxidation. These include the site of the reactions, catalytic agents and others. The development of new analytical techniques has increased the understanding of this reaction mechanism. Yet, the complete reaction parameters have not been totally solved or elucidated.

It is generally recognized that milk fat is mostly responsible for the development of the oxidized flavor. Lipid deterioration due to autoxidation of unsaturated fatty acids is a proven fact. The formation of peroxides, hydroperoxides, low molecular weight carbonyl compounds, such as aldehydes (saturated and unsaturated), ketones and others,

impart characteristic flavor and their presence in oxidized milk has been established (61, 62).

Kinsella, Patton and Dimick (49) used the term "Flavor Threshold Value" (FTV) to denote the minimum concentration of chemical compounds that could give a sensory response by imparting oxidative flavor from autoxidation of milk fat. The FTV values are dependent upon the dispersing media or the physical nature of the medium. Generally, flavor potential is stronger in an aqueous (lipophobic) than in an oil (lipophilic) medium. This also depends on the polarity of the particular flavor compound; substances of low polarity (long chain hydrocarbons) have low FTV in aqueous media, whereas more polar substances have lowest FTV in lipophilic media (49).

Most of the products of lipid autoxidation, at detectable concentrations, are undesirable because of their adverse effect on flavor although one characterized exception is cis-4-heptenal. This substance imparts a creamy flavor to some dairy products (butter, cream fudge) at an average level of 1.5 parts per billion and it is commercially used as a flavoring agent. It arises from the autoxidation of isolinoleic acid (61). Some of the FTV are listed in Table 4. Table 5 lists flavors and associated compounds with oxidized milk fat.

The concentration necessary to impart an off-flavor is very often so low that oxidation needs not to progress

Table 4. Flavor Threshold Values (FTV) of Autoxidation Products from Milk Fat[a]

Compounds	FTV in Milk (3.8% fat) (ppm)	
Ethanol	1.20	
Propanal	0.43	
Butanal	0.19	
Pentanal	0.13	
Hexanal	0.05	
Heptanal	0.12	
Octanal	0.46	
Nonanal	0.22	
Decanal	0.24	
n-2-hexenal	0.067	
n-2-heptenal	0.077	
n-hepta 2,4 dienal	0.049	
n-deca 2,4 dienal	0.0005	(water)
Oct-1-ene 3- ol	0.01	(skim)

a. From Kinsella (49).

Table 5. Some Descriptive Flavors and Associated Compounds Identified in Oxidized Milk Fat[a]

Flavor	Compounds
Oxidized	octanal, 2-heptenal,2,4-heptadienal
Cardboard, tallowy	n-octanol, n-alkanals (C_9,C_{11}) 2,4 dienals
Oily	n-alkanals, 2,4 dienals, 2-hexenal
Painty	n-alkanals (C_5-C_{10}), 2,4 dienal
Fishy	n-alkanals, 2-alkenals, 2,4 dienal, 2-alkanones (C_3-C_{11})
Grassy	2-alkenal, 2,6 dienal (C_9)
Metallic	oct-1-en-3-one (1,3, octenone)
Mushroom	oct-1-en-3-ol
Cucumber	2,6 dienal (C_9)
Nutmeg	octadienal; 2,4 dienals
Creamy	4-cis-heptenal
Fruity	n-alkanals (C_5,C_6,C_8,C_{10})

a. Ref. (49).

substantially before the off-flavors become evident. For example, Patton, Barnes and Evans (65) reported that 2.4-decadienal, which imparts a characteristic deep fried fat or oily flavor, is detectable in aqueous solutions at levels in the vicinity of 0.5 parts per billion.

Oxidized flavor is characterized by a cardboard or tallow-like taste, which is sharp when the sample is held in the mouth but clears up almost immediately when swallowed or expectorated (57). It is an off-flavor which develops upon storage and, with the exception of sunlight flavor, is more prevalent in pasteurized cream, skim milk and fluid cream products than in homogenized milk (61, 57). Although oxidized flavor may be found immediately when secreted, it is generally an off-flavor that requires time to develop. Some of the factors known to influence the extent of oxidation are copper, iron, rust, excessive chlorine, air incorporation, storage temperatures, heat treatment and exposure to light.

Oxidation in Fluid Milk

Fluid milks have been classified by Thurston, Brown and Dustman (84) into three categories with regard to their ability to undergo oxidative deterioration:

1. Spontaneous. Those milks that spontaneously develop off-flavor within 48 hours after milking.

2. Susceptible. Those milks that develop off-flavor within 48 hours after contamination with copper.
3. Resistant. Those milks that exhibit no oxidized flavor even after contamination with copper and storage for 48 hours.

With the introduction of equipment resistant to corrosion in the dairy industry, the incidence of oxidation problems in milk has been significantly reduced, although it has not been totally eliminated (37, 61). The resistance of some milks to oxidative deterioration even in the presence of added copper has been explained by Greenbank (24) on its resistance to change in the oxidation-reduction potential. That a correlation exists between the appearance of an oxidized flavor and conditions favoring a mild oxidation, as measured by the oxidation-reduction potential, was shown by Greenbank. The role of enzymes as catalytic agents promoting oxidative changes in milk was once considered to be valid. The role of xanthine oxidase in this regard was the subject of study by Smith and Dunkley which found no evidence of that role (74). The site of oxidation in fluid milk is the phospholipids associated with the fat globule membrane. Other factors implicated in the mechanism are the ratio of dehydroascorbic to ascorbic acid, the natural copper complexed with the proteins in the fat globule membrane, oxygen levels and phospholipids (48, 75). The literature has reported that some connection or correlation

could be established between the susceptibility to oxidation in milk or other dairy products, and the percentage and distribution of naturally occurring pro- and antioxidants.

Carbonyl Compounds in Oxidized Dairy Products

Considerable research has been devoted to the odorous compounds formed during the autoxidation of dairy products. Early identification studies on the subject were unable to differentiate between isomeric forms of various compounds. Data reporting concentrations in the range of ppb still merit careful attention. Table 6 summarizes the carbonyls that have been identified in several selected dairy products. The origin of these compounds could be traced to the autoxidation of the unsaturated fatty acids (oleic, linoleic, and linolenic) and dismutation products. The association of phospholipids in the promotion of the formation of several of these compounds is also reported. Triglycerides and cholesterol esters may be involved but to what extent is unknown (61).

Several problems exist when an attempt is made to correlate the off-flavor with specific compounds or groups of compounds, for example: a) the variety of compounds produced, b) analytical difficulties for quantitative evaluation of oxidized products, c) differences in threshold values of individual compounds, d) similarity of flavors imparted by individual compounds, e) a possible additive,

Table 6. Carbonyls Identified in Autoxidized Dairy Products[a]

Product	Alkanal	Alk-2-enal	alk-2,4-dienal
Skim milk, copper induced	C_2 , C_6	C_4-C_{11}	C_6-C_{11}
Whole milk, spontaneous oxidation	C_5-C_{10}	C_6-C_{11}	C_6-C_{12}
Whole milk, light induced	---	C_4,C_6-C_{11}	---
Dry whole milk air packed	C_1-C_3 C_5-C_{10}	C_5-C_{11}	traces
Butter oil, exposed to light	C_1-C_{10}	C_4-C_{11}	C_7 , C_9
Butter, cold storage defects	C_5-C_{12}	C_5-C_{12}	C_7,C_9,C_{10},C_{11}

a. From (62).

synergistic, or antagonistic effect, due to the interaction of components, and f) the possible existence of unidentified compounds (49).

Antioxidants

The use of antioxidants in dairy products is not permitted by United States' standards at the present time. Many compounds such as esters of gallic acid, butylated hydroxyanisole, norhydroguaiaretic acid (NDGA), hydroxyquinone and dihydroquercitin have been employed as antioxidants in studies of dairy products. These compounds apparently exert their influence by interrupting the chain reaction in autoxidation by capturing of the free radicals necessary for continuation of hydroperoxide formation (61, 85).

Tocopherols in Milk

Reports in the literature (25, 86) indicate that the use of green feeds tends to inhibit and that the use of dry feeds tends to promote the development of oxidized flavor in dairy products. Investigations in this regard had focused in the transfer to milk of natural antioxidants. Of these compounds, alpha tocopherol has received most of the attention.

Witting (86) stated that tocopherols are poor antioxidants and that very little, if any, increase in stability is attained by their addition to any food product containing linoleic acid or other more highly unsaturated fatty acids.

He based his report on studies of the kinetics of autoxidation in vitro. However, this is not to say that addition of tocopherol may not increase product stability slightly, but the beneficial effects are rather small as compared to the order of magnitude anticipated.

One point concerning the antioxidant capacity of naturally occurring tocopherol is related to its concentration in milk which is in the order of 12-30 µg/g of milk fat. These values vary depending on feeding practices and the season of the year (40).

A recent study by Goering et al. (22) concluded that direct addition of emulsified tocopherol in the range of 75 µg/g fat would serve to control both spontaneous and copper induced (0.1 ppm) oxidized flavor after 2 or 5 days of storage. In the same work, they observed that supplementing the cow's diet with alpha tocopheryl acetate (5 g/day) lowered the flavor score (0 = none, 1 = questionable, etc.) in milk, but only spontaneous oxidized flavor was controlled. They reported a 50 percent increase in tocopherol content of the milk as a result of this type of supplementation.

Vitamin E as Diet Additive

Natural foodstuffs contain vitamin E and polyunsaturated fatty acids (PUFA) in balanced quantities. In general, the tocopherol content of most fats and oils parallels

their PUFA content. Since fats and oils are our principal source of vitamin E, a diet low in PUFA tends to be low in vitamin E, and vice versa. The inverse relationship between dosage and absorption tends to ensure that human tissues will contain an optimum level of alpha tocopherol. The various proposals for massive additions of vitamin E to the diet are derived from considerations other than simple nutritional status. Vitamin E and other synthetic lipid antioxidants have beneficial effects in certain acute or chronic intoxications. The possible erroneous belief that increasing antioxidant concentration will decrease the yield of lipid peroxidation-free radical initiation has led to suggestions that vitamin E might ameliorate the deteriorative processes associated with the general phenomenon of aging (86).

Krukovsky, Whiting and Loosli (51) found a significant correlation between the tocopherol content of milk and its ability to resist autoxidation. Erickson, Dunkley and Smith (19) found that the tocopherol in the fat globule membrane is oxidized more rapidly than that inside the fat globules, hence tocopherol inside the globules would be ineffective in retarding oxidized flavor. Dunkley, Franke and Robb (16) stated, however, that the concentration of alpha tocopherol in milk should not be used as the sole criterion for predicting oxidative stability and that the concentration of copper must also be taken into account. Spontaneous milk oxidation was reported by Bruhn and Franke (10) to be

directly proportional to the copper content and inversely proportional to the alpha tocopherol content of milk.

In recent studies concerning the direct addition of tocopherol to milk for control of oxidized flavor, King (47) reported that alpha tocopheryl acetate in hexane or ethanol solutions were inefficient to prevent oxidation even at levels of 400 µg/g of milk fat. On the other hand, emulsified tocopherol (dispersed in a non-ionic agent commonly used to deemulsify milk fat) gave complete protection at a level of 25 µg/g of milk fat. These findings agree with the report of Witting (86) that increased ingestion of vitamin E (alpha tocopherol) results in decreased absorption. Since alpha tocopherol is present in tissues largely in subcellular membranes, it is not surprising that the incorporation and storage in such sites is severely restricted. Erickson, Dunkley and Smith (19) found that fat globule membrane contains the highest concentration of alpha tocoperol/g milk fat (44 µg/g lipid) but only accounts for 8 percent of the total content in the milk.

King (47) proposes the use of properly emulsified tocopherol be added to the milk directly at the farm. He also recommended concentrations in the range of the natural content.

Catalytic Effect of Copper

It has been demonstrated by many researchers that the role of copper in milk is an agent promoting lipid oxidation. Being a normal component of milk, the problem of controlling oxidative deterioration due to its catalytic presence is mainly a problem of avoiding contamination from external sources. Other metals such as iron are also present in milk, but copper has been shown by specific chelating agents to be the main catalytic agent in the development of oxidized flavor in milk (75). The potential ability of copper to promote oxidation should not be confined to the sole criterion of actual concentration in the milk, but also to the presence or absence of several other factors including tocopherols and ascorbic acid. Furthermore, the distribution of natural copper in milk and the content in several of the fractions permits a better understanding of the phenomenon. The range of copper concentration in milk, from reports of several authors, varies from 25-210 ppb. The only source of copper is the feed of the animal and it is transmitted to the milk by the blood stream (12, 61). King and Dunkley (48) reported that oxidation may occur regardless of the copper concentration in the milk; however, no oxidation problems could be traced to milks having a copper content less than 60 ppb.

King and Dunkley (48) studied the relation of natural copper in milk to the incidence of spontaneous oxidized

flavor. They observed no difference between pasture and dry feed in the natural copper concentration in the resulting milk. When cows on dry feed were drenched with large doses (10 g) of copper sulfate, there was an increase in the copper content of milk and the intensity of spontaneous oxidized flavor. Smith and Dunkley (75) demonstrated the role of copper as the only one involved in lipid oxidation by employing the sequestering agent neocuproine which effectively binds the metal, preventing the promoting of oxidized flavor. They also postulated that ascorbic acid interacts with the metal in promoting oxidative action when concentrations of copper are below 100 ppb.

The distribution of copper in milk proteins was determined by Aulakh and Stine (1). Using techniques of equilibrium dialysis they found that miscellar casein bound most of the copper present in the solutions. Fat globule membrane protein had the second greatest affinity for copper ions. Much less copper was bound by alpha casein, beta-lactoglobulin and beta-casein fractions. Chen and Tobias (12) observed the migration of copper between different fractions of milk and concluded that 35 percent of the naturally occurring copper in milk is associated with the fat fraction and 65 percent with the skim milk. On storage, added copper migrated into the non-fat fraction until 0-7 percent remained in the fat. King and Dunkley (48) observed that 10 to 35 percent of the naturally occurring

copper was concentrated at the surface of the fat globules, whereas most of the added copper was uniformly associated with the proteins in skim milk.

King and Dunkley (48) observed that milks which develop oxidized flavor spontaneously had a higher total copper concentration in the fat globule membrane than did milks classified as susceptible or resistant. Copper-protein complexes are known to catalyze lipid oxidation in fluid milk systems. It has been concluded that the close proximity of a copper-protein complex to the phospholipids, which are also associated with the fat globule membrane, is an important consideration in the development of an oxidized flavor in fluid milk (12).

Aulakh and Stine (1) suggested the possible special spatial configurations or orientation in the interaction metal-lipo-protein complex as a mechanism of oxidized flavor development catalyzed by copper.

Sunlight Flavor

It is well known that exposure of milk to light can cause flavor changes in the product. The oxidized flavor due to sunlight can originate with only 15 minutes of exposure producing flavor characterized as "cardboard", "cooked cabbage" and "burning hair" (57). Exposure of milk to bright fluorescent light in the dairy case for an extended period may cause an oxidized taste, particularly with

clear glass or polyurethane containers. Amber glass and plastic coated paper offer partial protection.

In spite of all the published information, the exact chemical nature and extent of the flavor changes due to sunlight have not been completely elucidated. The early literature refers to the flavor defect under the general term of sunlight, but actually two distinct flavors occur in exposed wbole milk: a) an oxidized flavor as a result of lipid oxidation, and b) an activated flavor which originates in the proteins of milk (37, 61, 62). The activated flavor has been shown to be dependent, although not entirely, in the presence of riboflavin (61, 62).

Methional, formed by degradation of the free methionine in the milk serum, has been reported to be one of the principal contributors to the presence of activated flavor (21). Singleton, Aurand and Lancaster (73) did not believe that lipid oxidation contributed to sunlight flavor since lipid oxidation was slow compared to protein oxidation. At the same time, they demonstrated a relationship between riboflavin destruction, tryptophan destruction, and the intensity of the sunlight in milk. Stull (79) stated that light induced oxidation of the lipids in milk could occur simultaneously with, or subsequent to, the activated flavor. Witting (86) observed that alpha-tocopherol was ineffective as an inhibitor of light induced oxidation.

Finley and Shipe (21) reported the isolation of a flavor producing compound from light exposed milk. They showed that a low density lipoprotein fraction is a principal source of light induced flavors in milk, with the protein portion undergoing a partial degradation resulting on losses of thryptophan, tyrosine, lysine, cysteine, and methionine. The lipid portion was found to be partially oxidized. They concluded that the light induced changes in the lipid protein were different from those induced by addition of copper ions to milk.

Recently, Bassette (2) directed his attention to the problem of a minor change in concentration of volatile materials in milk exposed to light. Using gas chromatographic analysis of head space vapors of steam distillates from milk, the changes in the concentration of acetaldehyde, propanal, methyl sulfide, acetone, butanone, n-pentanal and n-hexanal were evaluated before and after exposure to sunlight and fluorescent light. He concluded that an increase in the content of acetaldehyde in sunlight irradiated skim milk could explain its formation in non-fat fractions of the milk, and increases in all other carbonyl compounds could be traced to milk fat deterioration. The overall effect in the concentration of volatiles derived from sunlight exposure was considerably greater than milk under fluorescent light.

Fat Oxidation Detection Techniques

Due to the importance of an effective quality control for the detection of oxidized flavor in dairy products, several tests have been designed for that purpose, with different characteristics and responses to the extent of the off-flavor present. The degree of oxidative deterioration expressed as the amount of hydroperoxides per unit weight of fat can be estimated by several methods. The modified Stamn method, which is considered to be the most sensitive, is based in the reaction of oxidized fat and 1.5 diphenylcarbohydrazyde to yield a red color (62). The Loftus-Hill and Thiel approach involves conversion of the ferrous ion to the ferric state in the presence of ammonium thiocyanate, presumably by hydroperoxides, to yield the red color pigment ferric thiocyanate. Parks (61, 62) stated that these methods based on the direct or indirect determinations of hydroperoxides, but which do not consider previous dismutations of these primary reaction products, are not necessarily indicative of the extent of the reaction, nor do they tend to correlate well with the degree of off-flavor in the product.

The thiobarbituric acid method (TBA), with two variations, is widely used to determine the degree of lipid oxidation in milk products. Both are of approximately equal sensitivity and based on the condensation of two molecules of TBA with one of malonaldehyde, with the formation of a

red color complex (63). The TBA method of King (46) has been used in relation to oxidative processes in fluid milk. The TBA variation of Dunkley and Jennings (17) has been reported to be more applicable than the method of King in determining the extent of autoxidation in butter. Both methods have been used extensively in studies of autoxidation of extracted milk components and model lipid systems (62).

Singleton et al. (73) expressed the idea that the TBA test is totally appropriate for estimating the extent of copper induced oxidized flavor but is unsuitable for the detection of light activated off-flavor in milk. The methods based on the carbonlyl content of oxidized lipids are reported to correlate significantly with the degree of off-flavor in butter oil; however, they lack practicability and are considered not really suited for routine work. These methods determine the secondary products of autoxidation (62).

CHAPTER 3

MATERIALS AND METHODS

Source of Materials

Three commercial brands of market milk were periodically collected from retail food stores for the study. Packaged, pasteurized, homogenized fluid milk was used for the observations. Samples were coded for pull date and collection day. All samples were kept under refrigeration at 4°C until analyses were performed.

In order to provide a uniform replication of conditions as a function of time, various observations and analyses were arranged in order or priority, speed of reactivity and ease of execution in the following manner:

1. Sensory evaluation,
2. Acid degree value (ADV),
3. Peroxide value (PV),
4. Thiobarbituric acid test (TBA),
5. Gas liquid chromatographic (GLC) analyses of feed flavors.
6. Tocopherol determination, and
7. Copper content.

Tables 7, 8 and 9 summarize code numbers for each lot together with collection and pull dates. A total of

Table 7. Collection and Pull Dates for Experimental Milk Samples (Brand X).

Code	Collection Date	Pull Date	Difference (days)
X-1	09-23-75	09-28-75	5
X-2	10-07-75	10-15-76	8
X-3	02-05-76	02-12-76	7
X-4	02-12-76	02-21-76	9
X-5	02-20-76	02-27-76	7
X-6	02-26-76	03-03-76	6
X-7	03-04-76	03-12-76	8
X-8	03-10-76	03-19-76	9
X-9	03-24-76	04-01-76	8
X-10	04-06-76	04-16-76	10

Table 8. Collection and Pull Dates for Experimental Milk Samples (Brand Y)

Code	Collection Date	Pull Date	Difference (days)
Y-1	09-23-75	09-30-75	7
Y-2	10-07-75	10-14-75	7
Y-3	02-05-76	02-12-76	7
Y-4	02-12-76	02-21-76	9
Y-5	02-20-76	02-28-76	8
Y-6	02-26-76	03-06-76	9
Y-7	03-04-76	03-12-76	8
Y-8	03-10-76	02-17-76	7
Y-9	03-24-76	03-31-76	7
Y-10	04-06-76	04-14-76	8

Table 9. Collection and Pull Dates for Experimental Milk Samples (Brand Z).

Code	Collection Date	Pull Date	Difference (days)
Z-1	09-23-75	09-28-75	5
Z-2	10-07-75	10-14-75	7
Z-3	02-05-76	02-13-76	8
Z-4	02-12-76	02-21-76	9
Z-5	02-20-76	02-28-76	8
Z-6	02-26-76	03-03-76	6
Z-7	03-04-76	03-13-76	9
Z-8	03-10-76	03-16-76	6
Z-9	03-24-76	03-31-76	7
Z-10	04-06-76	04-13-76	9

ten lots from each brand were examined during the study, which included the period October 1975 to May 1976.

Collection dates of the samples, along with pull dates, were recorded for the possibility of relating chemical analyses to the relative freshness of the market milk at the time of collection. No sample 72 hours or more in storage was studied.

The ADV, PV and TBA test were carried out in most cases on the day of collection. Tocopherol and copper determinations each required one day. GLC determinations usually required additional time due to preparative techniques and conditioning the instrument for proper response.

Sensory Evaluation

Flavor evaluations of the milk samples were made within 24 hours after collection. All samples were scored using the American Dairy Science Association scoring guide. The evaluations were made by an experienced panel of three judges and the results recorded on individual cards. Prior to evaluation all samples were conditioned to room temperature.

Approximately 150 ml of each sample was transferred to a 250 ml Erlenmeyer flask previously cleaned, dried and steam deodorized. Samples were allowed to equilibrate from five to ten minutes before inspection. This provided enough head space in the flask to allow the volatile odors from

milk to collect in the space. Following gentle agitation by rotation of the flask, vapors in this space were sniffed as an initial criteria for evaluation of feed volatiles. About 50 ml of milk were transferred each time to clean glasses for subsequent evaluation of rancidity and oxidation and final observation for feed flavor.

The identity of each sample was kept on a master card by a non-judging assistant. The panel had no idea of the identity of the milk being analyzed in order to control any subconscious bias or preference.

Figures 1 and 2 present the format of the master card and scoring card used in the investigation.

Table 10 describes the scoring guide according to the American Dairy Science Association.

It should be noted that older publications (1971 and before) showed preference for a scoring guide with a 40 point basis for a "no criticism" score and the degree of sensory problems scored in a deductive point manner (example: definite feed-38). A simplified version of this system was employed in the present work.

Date:	Master Card	
Brand	Identity	Code

Figure 1. Master Card for Coding Samples to be Scored by Judging Panel

Name : Date :			
Sample			
Off-flavor	Score	Score	Score
Feed			
Oxidation			
Rancidity			

Figure 2. Individual Recording Form for Sensory Evaluations

Table 10. Scoring Guide According to the American Dairy Science Association.

Flavor	None	Slight	Definite	Pronounced
Feed	10	9.5	8	5
Oxidized	10	5.0	3	1
Rancid	10	4.0	1	0

In the simplified tri-state flavor program (57), milk is described as good, fair or poor. These flavor categories can be interpreted as follows:

40-39	Good
38-37	Fair
36-32	Poor.

Acid Degree Value (ADV)

Market milk and certain products manufactured from milk may, at times, possess a flavor described as rancid. The term is used in the dairy industry to denote the accumulation of free fatty acids (FFA) released hydrolytically from milk fat under the catalytic influence of endogenous lipases.

Although pasteurization inactivates all lipases in milk, the phenomenon of reactivation of these agents after treatment (flash pasteurization, 148°C for 1-2 seconds) has been suggested for some workers (41).

The methods used for quantitative determination of the extent of lipolysis can be grouped into two classes: a) those which require total fat extraction and subsequent direct titration, and b) those which use colorimetric procedures using comparative standards.

The method outlined by Thomas, Nielsen and Olson (83) was the choice for measuring total FFA as a possible indication of the degree of rancidity in the samples analyzed.

The technique involves the use of a nonionic surface active agent (BDI reagent) for fat isolation and titration with alcoholic KOH of suitable normality.

Other methods of milk fat isolation were tested (TeSa, Babcock) and compared with the BDI reagent. Although

the fat release was complete with such methods, ADV values were inconsistent, probably due to differences in cleanliness of the fat isolated, together with influence of the reagents.

The BDI reagent provided the cleanest, well-separated milk fat of the methods used for deemulsification.

It is recognized that the recovery of the fatty acids may not be entirely quantitative. Constant proportionality is assumed under the conditions of isolation.

Experimental Method

Equipment:

1. A speed controlled centrifuge that will hold standard 18 gm, 8% Babcock test bottles.
2. Water bath for maintaining test bottles at boiling temperatures, equipped with rack or tray to hold the bottles.
3. Water bath to hold test bottles at 54-60°C for tempering the fat column.
4. Standard 17.6 ml milk pipette.
5. 1.0 ml tuberculin type syringe with number 19 needle (10.16 cm).
6. 50 and 125 ml Erlenmeyer flasks.
7. 5 ml microburette or 25 ml burette.

Reagents:

1. BDI Reagent. Thirty gm of Triton X-100 (a non-ionic surface active agent manufactured by Rohm and Hass, Philadelphia, Pennsylvania) and 70 gm of sodium tetraphosphate are made up to 1 liter with distilled water.
2. Alcoholic KOH. Absolute ethanol is used in the preparation of standard KOH. This solution should be standardized frequently against standard potassium acid phtalate or other suitable standard. Preliminary tests indicated that 0.0095N KOH gave the best results.
3. Indicator Solution. This is prepared by dissolving 1 gm of phenolphtalein in 100 ml of absolute alcohol.
4. Absolute alcohol was prepared by refluxing one liter of commercial ethanol over 12 gm of zinc dust and 5 gm of KOH for 4 hours followed by distillation.
5. Petroleum ether (B.P. range 30-60°C).
6. Aqueous Methyl Alcohol. This consists of equal volumes of chemically pure methanol and distilled water.
7. Fat Solvent. Two parts of petroleum ether and one part of absolute ethanol (V/V).

Procedure

Recovery of Fat:

1. Transfer 35 ml of milk to a 18 gm, 8% milk test bottle using a 17.6 ml milk pipette.
2. Add 10 ml of the BDI reagent.
3. Place bottle in gently boiling water, agitate after 5 and 10 minutes.
4. After a total boiling time of 15 minutes, centrifuge for one minute
5. Add sufficient aqueous methyl alcohol to bring the top of the fat column to the 6% graduation. Centrifuge for an additional one minute period.
6. Place the bottle in the tempering bath for five minutes. The water level must be at or above the top of the fat column.

Titration:

1. Transfer one ml[1] of the tempered milk fat from the test bottle to a 125 ml Erlenmeyer flask using 1 ml syringe.
2. Dissolve the fat in 15 ml of the fat solvent mixture. Add 10 drops of the indicator solution.

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1. The yield of 2-3 milk test bottles.

3. Titrate to the first definite color with standardized alcoholic KOH solution. Occasionally, turbidity in the fat solvent mixture will be observed during titration. If this occurs, the addition of 2 or 3 ml of additional solvents will usually clear the mixture.
4. Express results in terms of ADV (ml of 1N base required to titrate 100 gm of fat).

Calculations of ADV;

1. Calculate the grams of the milk fat in the sample titrated by multiplying the ml of milk fat by its density (0.90 gm/ml)

   gm of milk fat = ml of milk fat by 0.90.

2. $$\text{ADV} = \frac{(\text{ml KOH used in titration} - \text{blank})}{\text{weight of fat}} \text{xN x } 100.$$

3. Blank is obtained as the titration value of the fat solvent in the absence of fat using 5 drops of indicator. Blank determinations should be run on each new batch of solvent and then re-titrated at frequent intervals thereafter.

Interpretations

It is generally accepted that current high temperature short time (HTST) pasteurization of milk does not completely inactivate lipases (41, 71). There is a tendency for rancidity in pasteurized milk to intensify during storage in household refrigerators after purchasing from food markets. Lipases are active at low temperatures and, therefore, low storage temperatures are no assurance of lipolysis prevention (71).

Kason et al. (41) reported that the ADV in commercial pasteurized milk increased by 0.3 to 0.6 during refrigerated storage for several days. This may be a 50 percent increase from the initial value. The work of Jansen (35) showed an inverse relationship between flavor score and ADV. The criterion established by Thomas et al. (83) in their relationship patterns for ADV and extent of rancidity was the one employed in this work.

A panel of trained and experienced judges detected unequivocally the presence of rancidity when ADV reached values of 1.3 and 1.6. A slight rancidity was detected by one of the judges when the ADV was 0.9.

The following scoring guide for rancidity by the use of the ADV values:

ADV	Description
0 - 0.90	No rancid flavor (-)
1.0 - 1.30	Very slightly rancid (+)
1.30 - 1.60	Slightly rancid (++)
1.60 - 2.50	Definitely rancid (+++)
2.50 - and above	Pronounced rancid (++++)

The American Public Health Association recommends the following criterion (76):

ADV	Status
0.4	Normal milk
0.7 - 1.10	Borderline milk
1.2 - 1.5	Many people can taste rancidity
1.5 - and above	Unsatisfactory

Kason et al. (41), in their report of lipolysis changes during storage of pasteurized market milk, seemed to favor the above notation, considering 1.1 ADV as the acceptable limit for rancidity.

Thiobarbituric Acid Test (TBA)

Malonaldehyde occurs in foods and biological preparation only as a product of lipid oxidation. It can be assayed rather simply in aqueous extracts or distillates without the necessity of extracting the lipids. It is considered to be the only compound now known that meets the criteria of a good test substance for lipid oxidation (53). Its colored reaction product with 2-thiobarbituric acid (TBA) has been used widely to follow the oxidation of lipid systems. Tarladgis, Pearson and Dugan (82) concluded that the metabolite was the oxidation product of unsaturated fatty acids. Malonaldehyde is also reported to react with several food constituents including amino acids and proteins (62).

The application of the reaction to evaluate oxidation in dairy products was proposed by Dunkley and Jennings (17) who demonstrated that the TBA test correlates closely with numerical flavor scores of milk samples having oxidized flavor of varied intensity. The TBA test is based upon a reaction involving the formation of a red color when oxidized milk is acidified and heated with 2-thiobarbituric acid.

The method used in this work is that outlined by King (46) which is essentially a simplified version of the Dunkley method which included boiling for maximum color

development and extracting the color with a mixture of iso-amyl alcohol and pyridine 2:1.

King demonstrated that the use of high temperatures when the test is applied to fluid milk produced lactose degradation with possible interference in the TBA reaction and values. A satisfactory modification of milk uses trichloroacetic acid (TCA) to remove fat and protein and ethanolic TBA to increase the rate of color formation at 60°C, a temperature at which lactose degradation is minimized.

Experimental Method

Equipment:

1. Standard 17.6 ml milk pipettes.
2. Water bath at 60°C.
3. Filter paper, Whatman No. 42.
4. Spectronic 20 or suitable colorimeter.

Reagents:

1. Trichloroacetic acid solution containing 1 gm/ml.
2. 95% ethanol.
3. TBA solution (0.1M) prepared by dissolving 1.4 g of TBA in 100 ml of ethanol. The preparation of the TBA reagent is facilitated by heating to 60 C in a water bath. The reagent undergoes deterioration and should not be stored longer than three days (46).

Procedure:

1. Pipet 17.6 ml of milk into a 125 ml Erlenmayer flask fitted with glass stopper, warm to 30°C.
2. Add one ml of TCA solution, followed by 2.0 ml of 95% ethanol.
3. Shake vigorously for 10 seconds and then leave it undisturbed for five minutes.
4. Filter through No. 42 Whatman paper.
5. Collect 4 ml of the filtrate and add one ml of the TBA reagent. Stopper the container (screw cap, 15 ml test tube), mix, and place in a 60°C water bath for 60 minutes.
6. Cool, determine optical density at 532 m.u. using distilled water as a reference. Should turbidity appear at this point, filtering again will usually remedy the situation.

Interpretation

In order to have some meaningful interpretation of the validity of the test, previously correlated objective-subjective values should be taken as a basis. The following table indicates the relation derived from the work of King (46) and was employed in our study (see Table 11).

Table 11. Relationship Between TBA Values and Sensory Evaluation.

Flavor Score	Description	Range of Optical Density at 532 m.u.
0	No oxidized flavor	0.01-0.023
1	Questionable to very slight	0.024-0.029
2	Slight but consistently detectable	0.030-0.040
3	Strong	0.041-0.056
4	Very strong	0.056 and above

Standard Curve

Although the method per se does not use a standard curve for the estimation of TBA values, a curve (Figure 3) was prepared in preliminary work to show the linearity of the TBA-malonaldehyde color reaction.

Malonaldehyde[1] (tetra ethyl acetal, Matheson Coleman) was used to prepare a stock solution (0.001M). This was further diluted (0.000001M) for appropriate range of color

1. Malonaldehyde does not exist in crystalline form due to its high hygroscopic and volatile character. It is customarily obtained from acid hydrolysis of its tetraethyl acetal form.

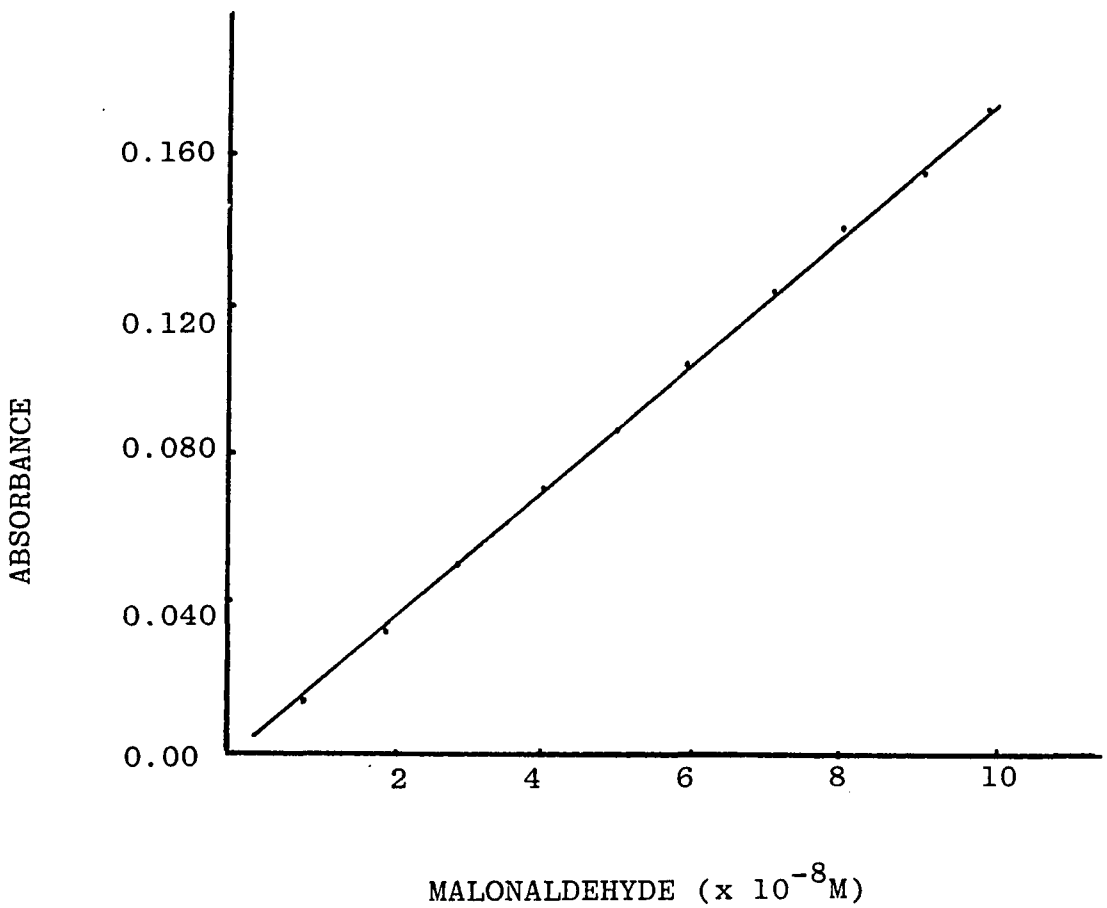

Figure 3. TBA--Malonaldehyde Standard Curve

intensities formed in the reaction with TBA. The color obtained was measured at the same wavelength and the conditions for the reaction were similar to those normally employed for the TBA test.

Peroxides (PV)

The estimation of the fat of PV in fluid milk is of major importance to the dairy industry. In butter, milk and dried milk, flavor deterioration is accompanied by a slight oxidation of the milk fat. Several chemical tests have been employed in order to monitor those changes in a simple, routine basis to gain a quantitative appreciation of the extent of such condition.

One of the early works (54) included the iodometric-peroxide method widely used in fats and oils, but considered not sensitive enough for the threshold values of dairy chemistry. The oxidation of ferrous ion to ferric ion and its estimation by the thiocyanate reaction has been the basis for a number of tests for determining peroxides in fats and oils (55). The work of Chapman and McFarlane (11) was directed to the determination of oxidation before flavor changes could be detected by sensory observation. Difficulties with the reagents employed prompted investigations by Hills and Thiel (31) who modified the solvents and established the most appropriate wavelength range for its colorimetric estimation. Stine et al. (77) directed their

attention to a better form of milk fat isolation. With the advent of new surface active agents, they tested various products with the successful development of the BDI reagent.

The method used in our work was a recent modification by Holloway (32) on the paper of Stine, using chloroform-methanol as a solvent for the milk fat instead of benzene-methanol proposed by Stine.

Experimental Method

Isolation of the Milk Fat. The same technique utilized for the recovery of fat by the method of Thomas et al. (83) in the determination of ADV is suitable for isolation of milk fat to be used in the analysis of peroxides.

Apparatus. The equipment needed is the same as used for ADV (p. 44). Added to the list is a colorimeter (Spectronic 20) as the only other instrument required. All glassware was cleaned to be free of iron and oxidized fat by detergent washing and soaking for 2 hours in 1:3 HNO_3 followed by distilled water rinsing.

Reagents. All chemicals should be Analytical Reagent Grade.

1. Fat Solvent. Chloroform-methanol 70:30 (v/v).

2. Ferrous Chloride Solution (0.014M). Barium chloride (0.4 g) dissolved in 50 ml glass distilled water is added slowly, with stirring, to 0.5 g of ferrous sulphate dissolved in 50 ml of distilled water. To this mixture is added 2.0 ml of 10N HCl. The barium sulphate precipitate is allowed to settle by gravity or by centrifuging and the clear solution is then decanted into a bottle protected from light. The solution remains stable for about a week.
3. Ammonium Thiocyanate Solution. 30 g of the salt is dissolved in distilled water and the volume made up to 100 ml.
4. Standard Ferric Iron Solution. Iron wire (0.250 g) is dissolved in 25 ml of 10 N Hydrochloric acid and oxidized with 1-2 ml of hydrogen peroxide. The excess peroxide is removed by boiling and the solution is diluted to 250 ml. This solution will contain 1 mg/ml.

Preparation of the Standard Curve

1. Dilute one ml of the standard ferric solution to 100 ml with chloroform-methanol 70:30 (v/v) obtaining a working standard solution of 10 µg/ml.

2. With this solution together with appropriate amounts of the cholorform-methanol solvent, prepare four tubes containing 5, 10, 15 and 20 µg of ferric ion in 9.9 ml of solution.
3. Add one drop of ammonium thiocyanate solution and one drop of water containing 2 ml of 10 N NCl per 100 ml.
4. Shake and hold the tubes for 5 minutes in subdued light and read at 505 m.u. in a suitable colorimeter against a blank of chloroform-methanol solvent. Plot optical density versus concentration of ferric ion. A straight line is obtained indicating close adherence to Beer's law for the concentration used.

Procedure

1. A volume of chloroform-methanol[1] equal to 9.9 ml less the volume of milk fat to be taken is pipetted into a screw cap test tube (15 cm x 1.9 cm). The volume of the oil depends on the peroxide value of the fat. For PV below 0.5, 0.2 to 0.5 ml of fat is convenient. PV's between 0.5 to 5.0 require 0.1 ml or less.

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1. Usually 9.6 ml since 0.2-0.3 ml of milk fat were the volumes used in the test.

2. One drop of ammonium thiocyanate and one drop of ferrous chloride solution are added in that order, the test tube is then shaken to dissolve the fat and reagents.
3. The color is developed under subdued light conditions and after 5 minutes the color is determined at 505 m.u. as indicated for the preparation of the standard curve.
4. A fat blank is necessary to compensate for some of the red components in the color of the milk fat. This blank contains fat and thiocyanate but no ferrous salt.
5. A reagent blank is also prepared with each batch of tests and subjected to the same treatment. This reagent blank contains no fat.

Calculations

The peroxide value is expressed in terms of milliequivalents of oxygen per kilogram of fat. In making these calculations, the percent transmittance for the blanks must first be converted to micrograms of iron per 10 ml of solvent by means of the standard curve (Figure 4). The net value of the unknown in terms of micrograms of iron /10 ml

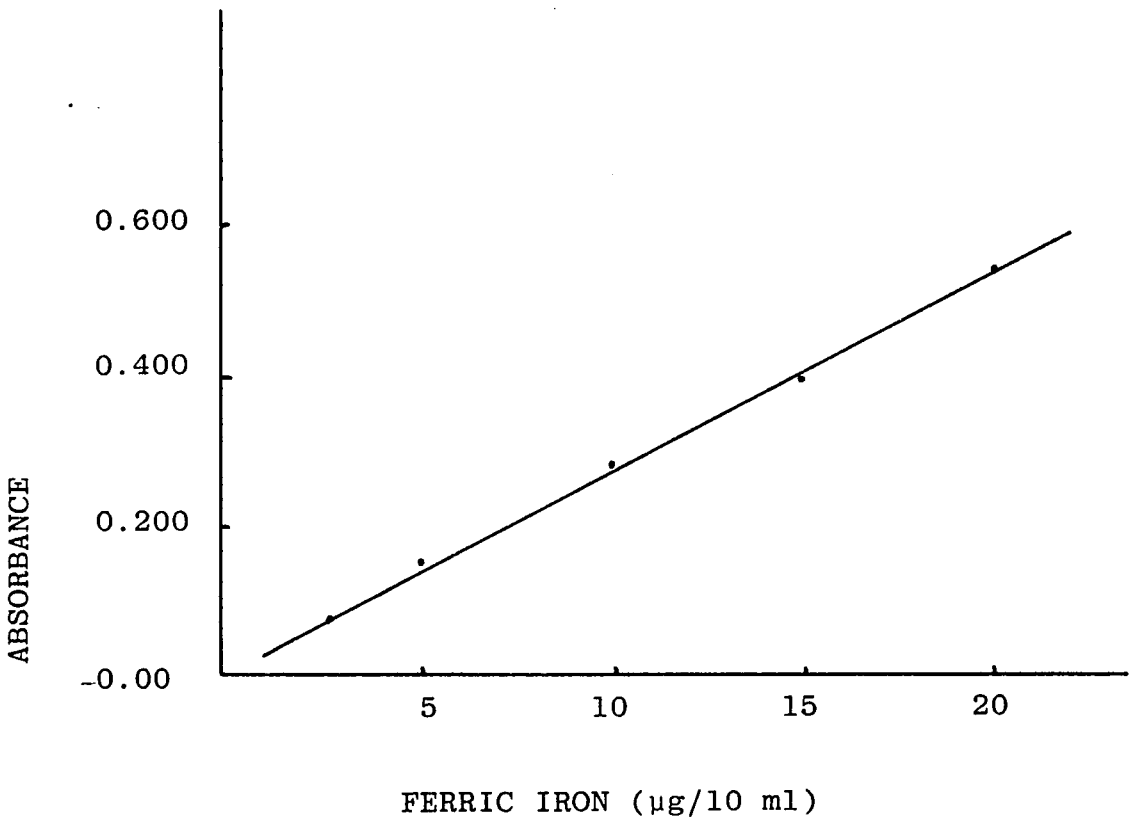

Figure 4. Peroxide Test Standard Curve.

of solvent is then calculated by deducting the sum of the fat and reagent blanks.

$$PV = \frac{\text{Net } \mu g \text{ iron } / 10 \text{ ml}}{\text{Weight of fat in grams} \times 55.85}$$

(as m.eq O_2/Kg fat).

Applications of the Test

One of the advantages of the ferric thiocyanate test is the small amount of sample required. The deemulsification techniques described by Stine permits the application of the test to milk samples as small as 30 ml and to homogenized milk and other products (77).

Color Reaction Development

The color of the reaction seems to originate through the following mechanism:

1. The oxidation of the ferrous ion to the ferric state by the peroxides or related compounds present in solution.

$$ROOH + FE^{++} \longrightarrow Fe^{+++} + OH^{-} + RO.$$

2. The radical can then undergo various subsequent reactions with the excess ferrous ions, the solvent or oxygen. High results are generally obtained in the presence of air and low results in its absence.

The color is formed by the presence of the ferric thiocyanate.

$$Fe^{+++} + 3\ SCN^{-} \longrightarrow Fe(SCN)_3.$$

Tocopherols

The literature appears to be in general agreement that the use of green feeds tends to inhibit and that of dry feeds to promote the development of oxidized flavors in milk. Investigations concerned with variations in the oxidative stability of milk as a result of feeding practices have centered on the transfer of natural antioxidants to milk (62). One group of these compounds is the tocopherols which, in milk, have been reported to exist in two forms: alpha tocopherol and gamma tocopherol--in very minute amounts (40). Alpha tocopherol is considered to be the only naturally occurring known antioxidant of consequence in milk.

Vitamin E consists of eight naturally occurring tocopherols. Each has different biological activities. They are readily oxidized, especially under alkaline conditions or when certain metal ions, most commonly iron, are present. Daylight accelerates the oxidation in the presence of iron. Fluorescent light has been found to be much less catalytic (2).

The group of compounds comprises four tocols and four analogous toco-trienols. Of these compounds, alpha tocopherol (5, 7, 8 trimethyltocol) is the form usually found in foods, although in some oils, such as corn oil, the beta or gamma form predominate (13). Table 12 describes the nomenclature of naturally occurring tocopherols.

The tocopherols are fat soluble compounds and hence are obtained from plant and animal materials in intimate association with other lipid constituents. Their primary chemical property from the analytical viewpoint is the ease of oxidation of the hydroxyl group. The pure compounds, when dissolved in analytical grade or purified solvents such as ethanol and stored in the dark and cold, are stable for many months (6, 43).

General Considerations

Due to the fact that tocopherols in milk products seem to be present and associated with fat globule membranes (19), the procedure devised for tocopherol estimation must extract most of them without losses due to oxidation or long manipulations. The problem is also relatively complex because of low tocopherol concentration in milk.

The procedure for determination of tocopherols in foods requires, in most cases, an initial purification step such as saponification of the sample with the following

Table 12. Nomenclature of Naturally Occurring Tocopherols.*

Tocols	Tocotrienols
1. 5,7,8-Trimethyltocol (α-tocopherol)	5. 5,7,8-Trimethiltocotrienol (α-tocotrienol)
2. 5,8-Diemthyltocol (β-tocopherol)	6. 5,8-Dimethyltocotrienol (β-tocotrienol)
3. 7,8-Dimethyltocol (γ-tocopherol	7. 7,8-Dimethyltocotrienol (γ-tocotrienol)
4. 8-Methyltocol (δ-tocopherol	8. 8-Methyltocotrienol (δ-tocotrienol)

* Taken from Bieri (6).

precautions being observed: a) air should be excluded, b) alkali should be very concentrated, c) the time should be kept to a minimum; and d) an antioxidant should be added (13). An additional precautionary measure during saponification is the use of a chelating agent such as etylenediaminetetracetic acid disodium salt (EDTA) in the amount of 0.1-0.2 g to bind traces of metals (6). The next step involves the extraction of the saponification mixture with peroxide free diethyl ether, petroleum ether or hexane. In order to separate the tocopherols from the rest of the compounds present in the mixture (sterols, carotenes, etc.), column chromatographic methods using alumina, celite-digitonin or silicic acid are frequently used. Paper and thin layer chromatography can also be used as a preliminary purification, although column separation is the method of choice (13).

The separation of the individual tocopherols is usually accomplished by a thin layer chromatography (TLC). Techniques for visualization and identification of spots require a combination of fluorescent viewing under ultraviolet light (2540 A) and of spraying with ferric chloride--bathophenantroline reagent (56).

Areas are scraped from TLC plates, eluted by ethanol, centrifuged and then aliquots of the supernatant are taken for quantitative analysis by colorimetric reaction.

The use of GLC after purification offers good possibilities for the separation and determination of individual tocopherols in a single process with sensitivity of about 0.01 µg. However, the main difficulty is in achieving a good preliminary purification so that interfering substances, primarily cholesterol, do not mask the smaller amounts of tocopherol (6).

Other techniques such as absorption spectrophotometry are of little value for application to food due to the low intensity of tocopherols absorption in ultraviolet light. Fluorometry provides a very sensitive means of detecting tocopherols but rigorous purification of solvents is essential, otherwise high background values are obtained that offset the advantage of the technique (6, 13).

Determination of Tocopherols in Milk

The steps usually involved in tocopherol determination in biological systems include: a) extraction of the lipid material containing tocopherol, b) separation of tocopherol from interfering compounds, and c) the determination of the separated tocopherol. Lipid isolation from milk by churning or detergent action releases the triglyceride fraction but not the fat globule membrane which contains the phospholipids and lipoproteins (18). Data from

Erickson et al. (19) concluded that the concentration of tocopherol in the membrane lipid is more than three times higher than inside the fat globules.

Erickson and Dunkley (18) stated that the use of ethanol to break the lipoprotein complex and hexane for extraction of the lipid material is a more satisfactory technique.

In the same work (18), the separation of tocopherol from interfering substances was accomplished by use of a silicic acid column; however, a correction factor was necessary for carotenoids which were not eliminated.

The basis of the final step in most methods is the determination of ferrous ions resulting from reduction of ferric ions by tocopherol and the color formed by interaction with the complexing agent 2,2' bipyridine. The procedure has been improved by Tsen (85) increasing the sensitivity of the reaction by using bathophenantroline (4,7-diphenyl-1,10-phenantroline) as the complexing reagent.

The method of Low and Dunkley (56) eliminates this factor by using an alumina column with elution of tocopherol alone in a single fraction.[1] The rest of the method is essentially that of Erickson and Dunkley (18).

---

1. Worker (87) brings out the fact that in animal tissues, blood, milk fat, etc., chromatography on alumina to remove carotene, leaves tocopherol and vitamin A alcohol together in solution. However, the presence of vitamin A is being ignored since, at least over a short time interval, it has been shown to have no significant effect on the reaction.

Experimental Method

Reagents:

1. Absolute ethanol.
2. Hexane, technical grade. Pass twice through a column of activated silica gel.
3. Bathophenantroline solution. 194 mg in 100 ml of absolute ethanol (J. T. Baker). Store in dark bottles under refrigeration.
4. Stock solution of dl-alpha tocopherol (Roche). 0.5% w/v in absolute ethanol. Keep refrigerated in amber bottle. Prepare appropriate dilutions with absolute ethanol (1000, 100, 20, 5 µg/ml).
5. Ferric chloride solution. 27 mg of the hexahydrate salt in 100 ml of absolute ethanol. Store in dark bottle at 4°C.
6. Orthophosphoric acid solution, 0.1.M. 0.69 ml of 86% reagent acid in 100 ml of absolute ethanol.
7. Activated alumina (M. Woelm). Use from freshly opened bottle or activate for three hours at 110°C before use.
8. 2% absolute ethanol in hexane (v/v).
9. 2% acetone in hexane (v/v).
10. 20% absolute ethanol in hexane (v/v).

11. Anhydrous sodium sulfate.

12. 1 N HCl.

Isolation of Lipids

Isolate lipids by adding 15 ml of absolute ethanol and 1 ml of 1N HCl to a 10 ml sample in a 50 ml centrifuge tube. Heat in a water bath at 60°C for ten minutes with intermittent shaking. Add 10 ml of hexane, shake mixture for 20 minutes and centrifuge five minutes at 2000 x g. Transfer about 9 ml of the clear hexane extract to a 50 ml centrifuge tube and wash the hexane solution once by shaking 30 seconds with 20 ml of deionized water and centrifuge three minutes at 2000 x g. Remove hexane layer and dry it with 0.5 g of anhydrous sodium sulfate. Determine lipids gravimetrically in the extract with a 1 ml aliquot.

Chromatography

By vibration pack 2.5 g of alumina into a chromatographic tube (9-10 mm i.d. x 200 mm long) plugged with glass wool. Condition the column with 3 ml of 2 percent absolute ethanol in hexane followed by 5 ml of hexane. Add 5 ml of the hexane extract. Rinse the column's walls with 1 ml of two percent acetone in hexane. Elute carotenoids with 10 ml of the same eluant and discard the eluate. For elution of tocopherol add 2 ml of the 20 percent absolute ethanol in hexane and discard the eluate. Place a 10 ml volumetric flask under the column, add 8.5 ml of 20 percent absolute

ethanol in hexane, and collect eluate. Allow each separate addition of solvent to percolate completely through into column before a new addition.

Colorimetric Analysis

Develop color in the 10 ml volumetric flask by adding stepwise, with thorough mixing after each addition, 0.5 ml of bathophenantroline solution and 0.5 ml of ferric chloride solution. After two minutes of color development in subdued light, add 0.5 ml of phosphoric acid. Adjust sample to volume (10 ml) with 20 percent absolute ethanol in hexane (v/v) and read absorbance (O.D.) at 534 m.u. against a blank prepared by adding the color developing reagents to 8.5 ml of twenty percent absolute ethanol in hexane in a 10 ml volumetric flask. Read absorbance 20 to 30 minutes after the addition of phosphoric acid.

Calculate tocopherol:

μg tocopherol/g of lipid

$= KxA_{534}$/g of lipid in

one ml of hexane extract.

The constant K (20.3) was obtained from a standard curve prepared with alpha-tocopherol as the reference. The method measures the total free tocopherol in milk but not sterified forms (19). An average K value was determined from the

three lower values of the curve which represented the range in concentrations found (Figure 5).

Copper in Milk

Much attention has been given to the copper content of milk because of its catalytic effect on the development of oxidized flavor. Copper is a normal constituent of milk in amounts ranging from 20 to 200 µg/liter [ppb; see Murthy, Rhea and Peeler (59)]. The content varies widely depending upon individuality of the cow, stage of lactation, feeding practices, treatment and storage milk after processing, etc. (59, 62).

Some of the high values reported for Cu probably include an amount dissolved from metal equipment as contamination. Recent studies have shown, however, that milk can vary considerably in its copper content without involving contamination (28, 62). King reported a relatively high concentration of copper (200 ppb) in early lactation milk and a level of about 20-50 ppb during the remainder of the lactation period.

King and Dunkley (48) observed no difference between pasture and dry feed in the natural copper concentration in the resulting milk. However, when cows on dry feed were drenched with a 1 percent solution of copper sulfate they were able to raise the Cu content of milk by a few µg/liter.

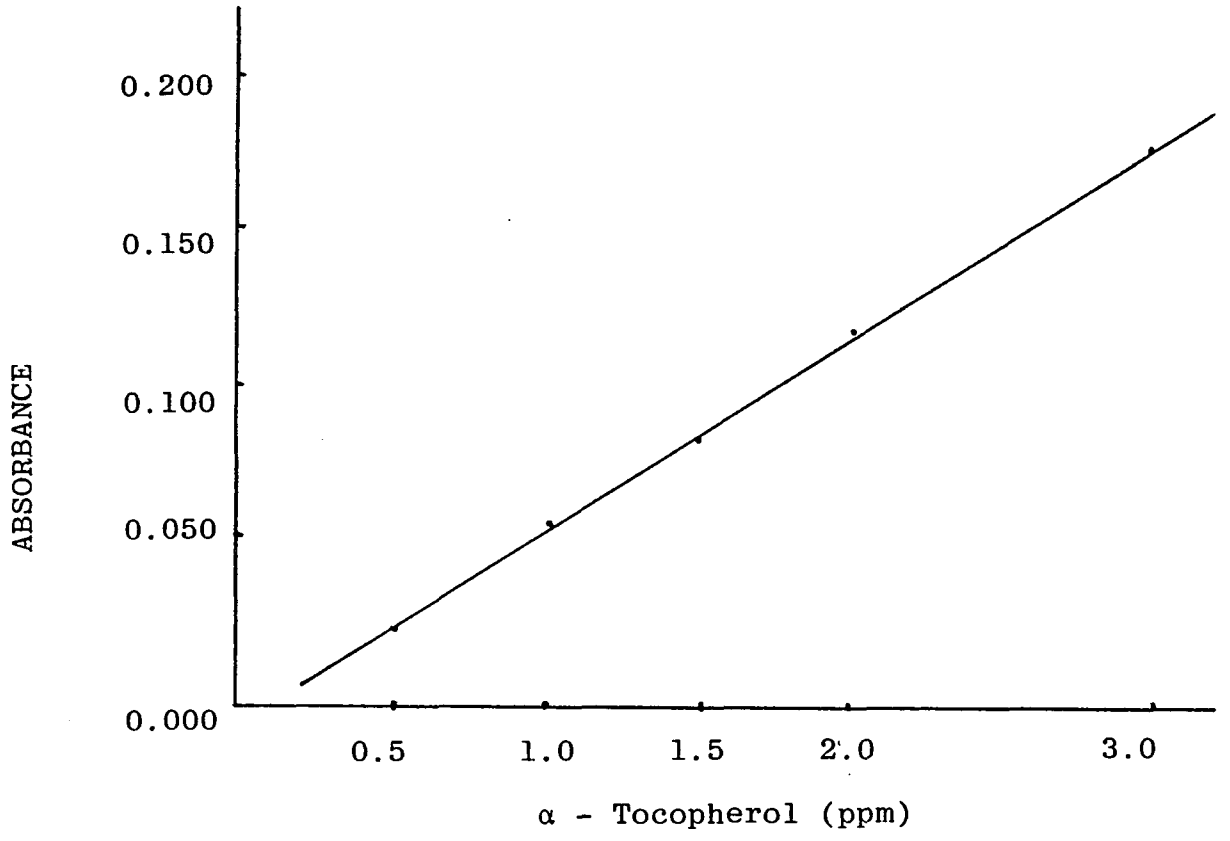

Figure 5. α-Tocopherol Standard Curve.

Copper is essential in the formation of hemoglobin. A deficiency of Cu produces anemias and other physiological disorder in animals (62).

Copper Analysis

Post milking contamination of milk with copper is a primary source of oxidized flavor and copper naturally occurring in milk has been correlated with spontaneous oxidized flavor (75). It is important, therefore, to control the copper content during processing of fluid milk and to minimize its content in the finished product in order to increase its stability.

Extraction Procedures

About 35 percent of the naturally occurring copper in milk is associated with the fat fraction (12). The total extraction of the element may be carried out by several methods which include dry and wet ashing, acidification for clarification, etc. Dry ashing, although considered accurate, is somewhat limited because of the time required and equipment not normally present in dairy plant laboratories (74). Wet ashing procedures are considered to be more rapid, but require larger amounts of acid that may not be entirely copper free, thereby increasing the chances of contamination. Extraction by acid precipitation of protein and fat and subsequent filtration is a quick and

simpler technique but having less sensitivity due to the large volume of filtrate and the use of filter papers that may not be copper free.

Determination

After extraction, the copper is usually analyzed by colorimetric methods or by atomic absorption spectrophotometry (AAS). The colorimetric assay involves the use of sequestering agents such as zinc dibenzyl dithiocarbamate, sodium diethyl dithiocarbamate or ammonium pyrrolidine dithiocarbamate (68). These salts are usually dissolved in organic compounds such as toluene, carbon tetrachloride, methyl isobutyl ketone and others. The color formed is extracted and read in a suitable colorimeter such as Spectronic-20.

Copper analysis by AAS requires the use of an instrument sensitive enough to detect the low concentrations normally found in milk. (In regard to accuracy, both colorimetric and AAS seem to provide enough reliability and the preference is dictated by instrumental facilities or volume of work to be performed.)

AAS also requires preparative techniques such as dry or wet ashing, elimination of interfering substances, and organic extraction with methylisobutyl ketone before aspiration (68). In the present work, several of these techniques or combinations of them were tested with a

certain degree of success. One of the problems of long manipulations consists of increasing chances for contamination working in the range of copper content in milk.

Colorimetric Determination of Copper in Milk

The use of trichloroacetic acid (TCA) as an agent for fat and protein precipitation and total copper release was reported by Rowland (69). Price (67) stated that most of the metal-protein bonds such as copper, zinc and iron are broken by TCA and transferred to the supernatant. He also suggested that organic tissues such as blood could be prepared by analysis (AAS) by mixing the serum with TCA, centrifuging and aspirating the supernatant.

After a preliminary survey of the colorimetric and other procedures, it was decided that a slight modified version of the method outlined by Smith (74) offered the best alternative for its application to copper determination in milk.

TCA precipitation of fat and protein followed by chelating extraction of the copper in the serum by zinc dibenzyl dithiocarbamate (arazate) offers the advantage of requiring no pH adjustment. Arazate is highly specific for copper in acid solutions (pH below 2). This reduces the possibility of contamination.

Experimental Method

Reagents:

1. Arazate in toluene (0.05%). A 0.05% solution of reagent grade zinc dibenzyl dithiocarbamate in redistilled toluene. Store in dark bottle.
2. Trichloroacetic acid (50%). Dissolve 500 g of reagent grade TCA with an equal amount of distilled water and extract in a 2 liter separatory funnel with 50 ml amounts of 0.05 arazate in toluene until the solvent layer becomes free of the color of the copper complex.
3. Nitric acid. 69-71% reagent grade nitric acid.
4. Standard solutions of copper containing 1 and 2 µg/ml of Cu^{++}.
5. Glassware. Soak the clean glassware in 1:3 nitric acid overnight and follow with threee thorough deionized water rinsings.

Procedure (Method of Additions)

Take three aliquots of 17.6 ml each of the milk sample and place them in Babcock cream test bottles. Add 1 and 2 µg of copper to two of the bottles. Then add slowly, with constant shaking, 5 ml of 50 percent TCA solution. Shake vigorously and place the bottles in a boiling water bath for 15 minutes with intermittent shaking every five minutes. Cool in ice water to approximately 10°C. Add

5 ml of 0.05% arazate in toluene and shake in a Babcock test bottle shaker for ten minutes. Centrifuge for five minutes, add sufficient deionized water to bring the toluene layer within the neck of the bottle and again centrifuge for five minutes. Carefully decant the toluene layer into test tubes, cool to room temperature, add a pinch of anhydrous sodium sulfate and clear by shaking in vortex mixer. Re-centrifuge for five minutes, transfer to clear cuvettes and read optical density in a Spectronic-20 colorimeter at 435 m.u. against a reagent blank prepared by substituting deionized water for milk in the above analysis. Simultaneously with the milk samples and reagent blank, run a milk blank by substituting 50 percent of TCA not freed of copper for copper-free 50 percent TCA and toluene for 0.05% arazate in toluene.

Plot the optical densities obtained for the three milk samples against the concentration of copper added (Figure 6). Extrapolate the resulting line corresponding to the absorbance value for the milk blank, draw a perpendicular to the X-axis. The concentration in µg of copper at this intersection of the X-axis represents the µg of copper present in the milk sample. This value is multiplied by 0.057 (1/17.6) to obtain micro g/ml of copper.

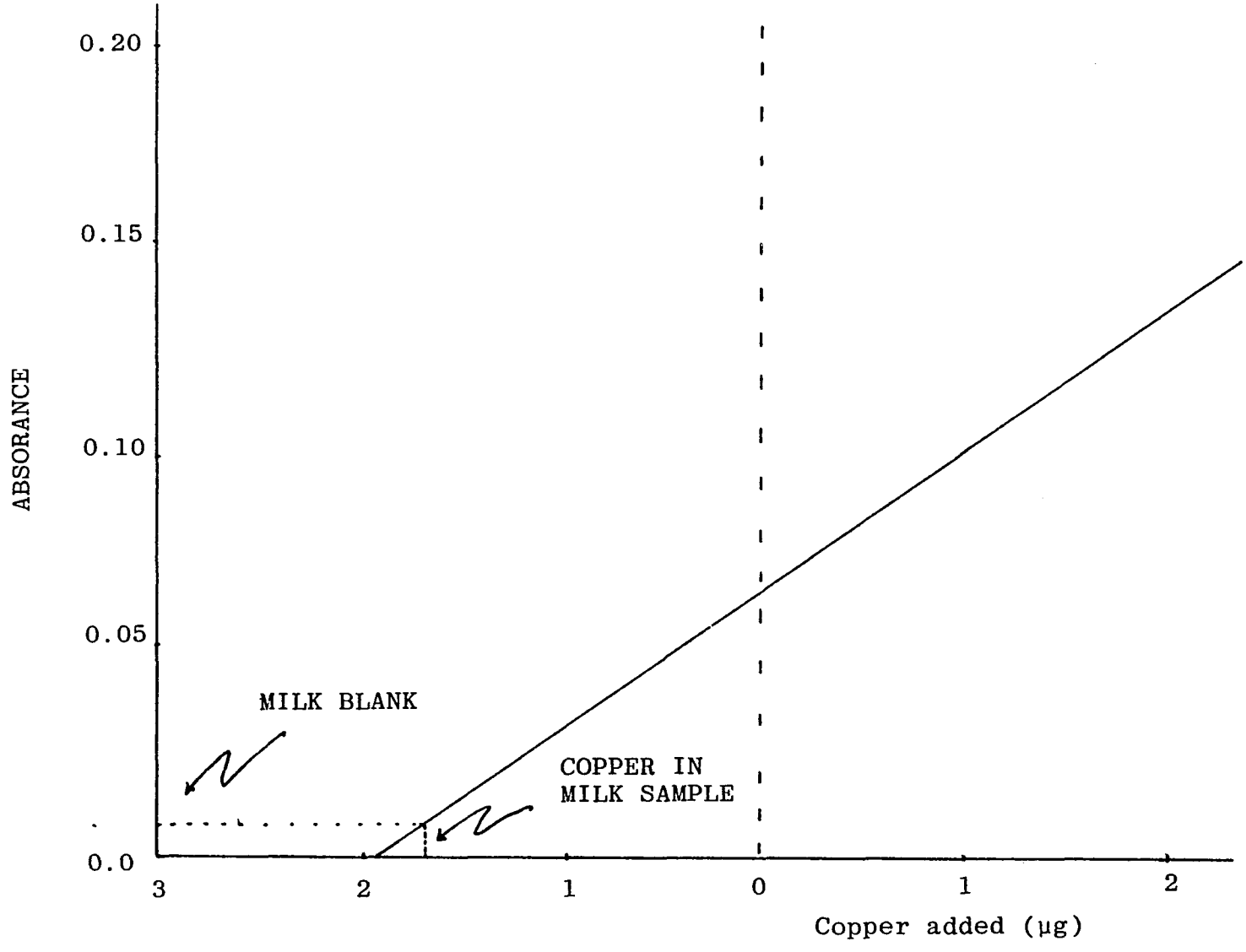

Figure 6. Typical Working Curve for Copper Determination

Gas Liquid Chromatography Analysis of Feed Flavor

Flavor is an integrated sensation having dimensions of taste, odor, texture, pain, temperature, color and even sound (34). Odor is the most important single contribution to the characteristic flavor of most foods. The coding systems by which we identify odor are extremely complex. To date no satisfactory correlation between chemical structure and odor quality have been developed. For example, compounds with very different structures may exhibit similar odors and very similar structures may have quite different odors. Present day odor theories place emphasis on size and shape of molecules rather than in functionality (27, 34). The following is a short review of the present day techniques used to study flavor characteristics correlated with chemical analysis in food products such as milk and other dairy products.

Isolation and Concentration

Numerous methods have been used to trap or isolate the compounds which are responsible for flavor. The main objective is the clear separation from the food and differentiation of the flavorful compounds. Most foods contain large quantities of volatile materials such as water, carbon dioxide, ethanol, etc., which contribute very little to flavor and must usually be removed during preparation of flavor isolates (34).

One of the early attempts to study milk vapors using gas liquid chromatographic techniques was reported by Jennings et al. (38). They employed headspace methodology with direct injection into the GLC. Storry and Millard (78) used steam distillation of milk fat to analyse volatile fatty acids by gas liquid chromatography. Patton (63) reported a simple solvent extraction technique for recovering flavor compounds, with the exclusion of fat, from processed milks. The basis of the method is a petroleum ether extraction of milk, evaporation of the solvent and the use of the residue for GLC analysis. In the distillation system of Bingham (8), nitrogen is passed through a milk sample to which anhydrous sodium sulfate has been added and carries volatiles through a foam trap and into a column immersed in liquid nitrogen. High vacuum, steam distillation was the method used by Tamsma et al. (80) to study chromatographic patterns from fresh and stale milks. The work of Gordon and Morgan (23), in which feed flavored volatiles from milk were analyzed, used heating and flash cooling combined with vacuum to collect volatiles from milk. Further concentration included steam distillation and headspace vapor analysis.

Bassette et al. (3, 4) devised a simple method of studying volatile components of milk. A 2 ml sample of milk is placed in a glass serum vial containing 1.2 g of anhydrous sodium sulfate and capped immediately with a

rubber serum stopper. After five minutes of agitation, the sample is heated at 60 C for three minutes and 1 cc sample of headspace vapor withdrawn and injected into the gas chromatograph.

In addition to liquid-liquid extraction, fractional distillation, high vacuum distillation, and other techniques such as adsorption of flavor volatiles in activated charcoal have been also tested (34). The application of this method has been more in the field of concentration of organic compounds from aqueous solution, and not widely used in flavor research (34).

Role of Gas Chromatography

The function of gas chromatography in flavor investigations can be divided into two categories: a) it can be used for separation and isolation of constituents of flavor concentrates isolated from food products, b) it can be used for separation, detection and quantitative determination of volatile compounds in vapor samples over food directly without prior concentration. In most flavor investigations, characterization of volatile compounds is necessary before direct vapor (headspace) analysis can be interpreted with any degree of confidence (22, 34, 60).

Identification Methods

The use of chemical functional analysis as a tool in helping to establish the identify of volatiles from milk

is illustrated in the work of Morgan and Pereira (58). The procedure involves reacting portions of the milk distillates with proper chemical compounds and rechromatography. The comparison between treated and untreated chromatograms reveals the functional groups present. Among the reagents currently in use for that purpose are: a) mercuric chloride to remove sulfides, (b) acidic hydroxylamine to remove aldehydes and ketones, c) potassium permanganate to oxidize primary and secondary alcohols, d) sodium bisulfite to reduce aldehyde and methyl ketones, and e) sodium borohydride to chemically reduce aldehydes and ketones to the corresponding alcohols.

Physical instrumental methods can be coupled to GLC to further improve the positive identification of volatiles derived from foods. The use of infrared spectroscopy provides one of the best ways of identifying functional groups present in a molecule. Nuclear magnetic resonance (NMR) has also been reported (34), but the combination of GLC and mass spectrometry is the instrument of choice since the amounts of material required for mass spectrum compares favorably with the amounts detected by gas chromatography (45). It is stated that a μg or less can give a useful spectrum [obtained by analysis of the effluent from the GLC column; see Khatri, Libbey and Day (45)].

Correlation with Composition

The complexities of sensory evaluation and uncertainties in interpretation of results of these evaluations usually result in researchers choosing either a chemical or a sensory approach to specific flavor problems. In spite of the problems involved, the two approaches when carefully applied to the same problem, result in far more knowledge than could be obtained from either one alone (34). With the development of increasingly sophisticated instrumental methods and with the application of advanced statistical analysis techniques, many meaningful and sensitive instrumental evaluations of the sensory properties of the food can be made (60).

Surveys of the literature indicate a number of articles which report correlations between some objective measurements and sensory evaluations (26, 27). However, empirical correlations may not reflect a valid relation between the measured sensory property and the objective technique (60). One of the problems is the number of closely related compounds that can be identified in volatiles from foods and to establish the number and combinations of them, and in what proportion they correlate positively with sensory methods. Furthermore, it is necessary to determine under what conditions these relationships are valid. In the study of the development of hexanal and

off-flavor in air packed potato granules (9), the accumulation of hexanal is an indication of increasing off-flavors during storage, but it is not directly responsible for the off-flavor.

Experimental Method

Headspace gas chromatography of atmospheric steam distillates from milk was the method used in the present study The method, with slight modifications, was originated in work published by Bassette and Ward (5). Total peak areas of the chromatograms obtained were further correlated with sensory evaluations. Retention times of purified standards together with functional chemical tests were used to help in component identification.

Preparative Technique
(Flavor Concentrates Isolation)

Reagents:

1. Anhydrous sodium sulfate.
2. Dow-Corning antifoam A (spray).

Apparatus:

1. A Kemmerer-Hallet type micro-Kjeldahl distillation unit, with 150 ml digestion-distillation flasks.
2. Serum vials, 15 mm x 42 mm, 5 ml capacity with self-sealing rubber caps.
3. Conical graduated test tubes (15 ml).

Steam Distillation of Milk

Fifty ml of milk (room temperature) sprayed with Dow-Corning antifoam A are transferred to the distillation flask of a Kemmerer-Hallet type micro-Kjeldahl distillation unit. The steam generator is operated to collect 5 ml of distillate in ten minutes while cold tap water is circulated through the condenser. A 12 cm long capillary pipette is connected to the outlet tube of the condenser so its end will fit within 3 mm of the bottom of a 15 ml conical graduation mark on the test tube. The distillation flask is connected and distillation started. After 5 ml $\mp$ 0.1 ml of distillate is collected, the conical tube is removed and quickly stoppered. Two 2 ml aliquots from the conical tube are pipetted rapidly into 5 ml serum vials each containing 1.2 g of anhydrous sodium sulfate. They are immediately stoppered with rubber serum caps and frozen until analyzed.[1] Exchanging the milk distillation flask with one containing water and continuing the distillation for about five minutes cleans the equipment and prepares it for the next sample distillation.

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1. The other variation was to transfer the distillates to a freezer until GLC analysis with the advantage of minimizing losses of volatile compounds due to gas leaking during storage.

GLC Analysis

1. Equipment. A Micro Tek Model DSS 170 gas chromatograph equipped with a dual flame ionization detector, coupled to a digital readout system (Infotronics Model CRS-108) and a printing integrator (Victor, Digital Matic) were used during the study of the feed flavor volatiles from milk.

   Two glass columns (0.4 cm x 145 cm) were packed in the laboratory by suction and vibration. The packing material used was 7.5% polyethylene glycol mono stearate + 0.75% phosphoric acid.

2. Operating conditions.

Column temperature	70°C
Argon outflow	60 ml/minute
Argon input	20 PSI
Hydrogen input	40 PSI
Temperature of the injection and dector points	285°C

3. Procedure. The vials containing the distillates were placed in a 60°C water bath for five minutes and then shaken for five minutes and again warmed in the water bath for an additional six minute period. A 1 ml sample of the head space gas was obtained by inserting the needle of the gas tight

syringe (1 ml, Hamilton No. 1001) through the rubber serum cap and drawing the vapors into the syringe. The syringe was evacuated and refilled twice. After the syringe was removed from the vial, volume was adjusted to 1.0 ml and the gas sample quickly injected into the chromatograph. The total time elapsed from removal to injection averaged eight seconds.

3. Treatment with chemical agents. A single test treatment was carried out with the steam distillates from milk in order to help establish identification of the peaks detected in the chromatograms. A 5 ml portion of distillates were substractively reacted and rechromatographed. Listed reagents were adeed directly to the vials containing 5 ml of milk distillate and 1.2 g of sodium sulfate, the vials sealed, and the reaction proceeded at room temperature unless otherwise indicated (23).
   a. Mercuric chloride. 0.4 g was added and reacted for 90 minutes to remove sulfides (23).
   b. Acidic hydroxylamine. 0.5 ml of a solution containing 0.4 g of hydroxylamine dissolved in 10 ml of 1N NaOH, added and reacted for two hours to remove both carbonyls and esters (23).

c. Potassium permanganate. 1 ml of a 1% aqueous solution added and reacted at 100°C for 20 minutes to oxidize primary and secondary alcohols (23).

d. Sodium bisulfite. 0.5 g added and reacted for four hours with continuous shaking to remove or substantially reduce both aldehydes and methyl ketones (23).

CHAPTER 4

RESULTS AND DISCUSSION

Sensory (Subjective) Evaluation

Feed, oxidized and rancid flavor scores for each individual brand are listed in Tables 13, 14 and 15. Feed flavor scores averaged 8.85, 9.08 and 7.31 percent for brands X, Y and Z, respectively. Scores from individual panel members showed very good agreement in feed flavor evaluation. Brand Z consistently had a feed flavor intensity which was well correlated with objective measurements (GLC). The average feed flavor scores for brands X and Y corresponded to samples with low feed flavor intensity. Refer to scoring guide on page 42.

Oxidative flavor scores for brands X, Y and Z averaged 9.13, 8.76 and 8.56 percent, respectively, which can be interpreted as either a nonexistent or very slight oxidative intensity. However, among individual samples, lots 2 and 4 of brand Y showed slight to definite oxidation and of brand Z, lot 1 scored slightly oxidized.

Except for very few cases (brand Y, lot 4 and brand Z, lot 3), rancidity (hydrolytic) was not detected to an extent of being considered a consistent off-flavor.

Table 13. Individual Panel Member Flavor Scores for Brand X Samples.

Lot	Feed			Oxidative			Rancidity		
	*I	II	III	I	II	III	I	II	III
1	9.0	9.5	9.0	9.0	9.0	9.0	10.0	9.0	10.0
2	8.5	9.0	10.0	9.0	8.0	5.0	8.0	10.0	10.0
3	9.0	9.5	8.0	10.0	9.5	10.0	10.0	9.0	10.0
4	9.5	8.5	8.0	9.0	9.5	10.0	10.0	9.5	10.0
5	10.0	9.5	8.0	9.0	8.5	9.0	10.0	10.0	10.0
6	8.0	8.0	5.0	10.0	9.0	8.0	10.0	9.5	10.0
7	8.5	8.0	10.0	10.0	10.0	10.0	10.0	9.0	10.0
8	10.0	8.5	7.0	10.0	9.0	8.0	10.0	9.5	10.0
9	9.0	10.5	9.5	10.0	9.5	10.0	10.0	10.0	10.0
10	10.0	9.5	8.4	10.0	9.0	8.0	10.0	10.0	9.0
$\bar{x}$	9.15	9.0	8.4	9.6	9.1	8.7	9.8	9.55	9.9
	$\bar{\bar{x}} = 8.85$			$\bar{\bar{x}} = 9.13$			$\bar{\bar{x}} = 9.75$		

* Panel members.

Table 14. Individual Panel Member Flavor Scores for Brand Y Samples.

Lot	Feed			Oxidative			Rancidity		
	*I	II	III	I	II	III	I	II	III
1	9.5	9.5	9.0	9.0	9.5	10.0	10.0	10.0	10.0
2	9.5	9.5	10.0	4.0	9.0	10.0	10.0	9.5	10.0
3	10.0	8.5	9.5	10.0	9.0	7.0	8.0	9.5	10.0
4	8.0	9.5	9.5	10.0	9.0	7.0	7.0	10.0	10.0
5	9.0	9.5	9.5	4.0	9.5	10.0	10.0	9.0	10.0
6	9.0	9.5	9.5	10.0	10.0	10.0	10.0	9.0	10.0
7	8.5	9.0	10.0	8.0	10.0	5.0	10.0	9.0	10.0
8	8.5	9.0	6.0	8.0	10.0	10.0	10.0	10.0	10.0
9	10.0	9.5	9.0	9.0	10.0	8.0	10.0	9.5	10.0
10	8.5	8.5	8.0	8.0	10.0	10.0	10.0	9.5	10.0
$\bar{x}$	9.05	9.2	9.0	8.0	9.6	8.7	9.5	9.5	10.0
	$\bar{\bar{x}}$ = 9.08			$\bar{\bar{x}}$ = 8.76			$\bar{\bar{x}}$ = 9.66		

* Panel members.

Table 15. Individual Panel Member Flavor Scores for Brand Z Samples.

Lot	Feed			Oxidative			Rancidity		
	*I	II	III	I	II	III	I	II	III
1	8.0	8.5	8.0	5.0	9.0	9.0	10.0	10.0	9.0
2	7.0	9.0	9.5	8.0	9.0	10.0	10.0	9.5	10.0
3	7.0	7.5	5.0	10.0	8.0	10.0	10.0	6.0	10.0
4	5.0	8.0	9.0	10.0	9.5	10.0	9.0	9.5	8.0
5	7.0	7.0	5.0	9.5	9.5	8.0	10.0	9.5	10.0
6	7.0	7.0	8.0	10.0	8.0	8.0	10.0	9.5	10.0
7	7.0	7.0	5.0	7.0	9.5	10.0	10.0	9.5	10.0
8	9.5	7.5	8.0	8.5	8.0	8.0	10.0	9.0	10.0
9	8.0	8.0	8.0	8.0	9.5	7.0	10.0	9.0	10.0
10	5.0	7.0	7.0	10.0	9.5	9.0	10.0	10.0	10.0
$\bar{x}$	7.05	7.65	7.25	7.9	8.9	8.9	9.9	9.15	9.7
	$\bar{\bar{x}}$ 7.31			$\bar{\bar{x}}$ = 8.56			$\bar{\bar{x}}$ = 9.58		

* Panel members.

Average scores were 9.75, 9.66 and 9.58 percent for brands X, Y and Z, respectively.

ADV

The determination of ADV by the method of Thomas and its correlation with sensory techniques has been reported by Jansen (35). He found an inverse relation between flavor scores and ADV. The ADV for brands X, Y and Z averaged 0.97, 1.03 and 0.94 percent, respectively. According to the scoring guide from the work of Thomas (83), these values represent milk with no perceptible rancid flavor. This result is in good agreement with the flavor scores assigned by the panel. Table 16 presents average rancidity scores and their corresponding ADV. None of the average ADV's exceeded the acceptable limit reported by Kason (41) and expressed by the American Dairy Science Association as 1.1. No attempt was made to follow ADV variations on a day-to-day basis or to investigate milk fat percentage in relation with flavor scores and ADV. Calculations were performed by assigning milk fat a density of 0.90 g/cc.

Treatment of Data From Oxidized and Feed Flavor Observations

Data collected in the experiments involving oxidative rancidity and feed flavor were subjected to statistical treatment for analysis of variance and multiple regression analysis under the program titled "Special Package for the

Table 16. ADV and Flavor Scores for the Three Brands of Milk.

Lot	Brand "A"		Brand "B"		Brand "C"	
	A.D.V.	F.E. Rancid	A.D.V.	F.E. Rancid	A.D.V.	F.E. Rancid
1	1.05	10	1.17	10	1.05	10
2	1.45	8	1.05	10	1.05	10
3	0.95	10	1.05	8.5	1.05	10
4	0.81	10	1.00	9.0	0.65	9
5	0.93	10	0.93	10	0.76	10
6	0.54	10	0.72	10	0.65	10
7	0.84	10	0.90	10	0.95	10
8	0.74	10	1.16	10	1.05	10
9	1.05	10	0.84	10	1.16	10
10	1.37	10	1.50	10	1.05	10
$\bar{X}$ =	0.973	9.8	1.032	9.75	0.942	9.9

Social Sciences," directed by Dr. David B. Marks, Assistant Professor of Statistics, College of Agriculture, The University of Arizona, Tucson, Arizona. This was done to determine the possible influence of several parameters either in single or combined form on the flavor scores. All statistical analyses used 95 percent confidence intervals.

TBA Test

TBA test results and their corresponding flavor scores are listed in Tables 17, 18 and 19. Examination of the data reveals that the test was especially well correlated to oxidative flavor such as in lot 2 of brand Y and lots 1 and 2 of brand Z. No case of oxidation was detected in brand X; this being confirmed by the TBA test. The criterion used by King (46) and outlined on page 53 was utilized in our observations. Brands Y and Z had some slightly oxidized samples which were detected subjectively and gave higher TBA values. The correlation coefficient (r) of TBA values and oxidized flavor scores for the combined X, Y and Z brands was -0.34. However, samples with slight or definite oxidation were the most well correlated with TBA values (OD = 0.030-0.040), r =-0.70. The reason for the low correlation of TBA values with flavor scores, especially the high ones, was in agreement with King, who stated that "Ranking by a flavor panel was good for samples yielding optical density values above 0.04; however,

correlation fell off among the samples having low flavor intensity" (46). King also reported that contamination with copper up to 0.5 ppm does not alter the results. In the present study, the combined levels of TBA and copper had a low correlation (r = 0.16) with flavor scores.

Since the TBA test is performed under conditions that may expose lipid material to heat, light, oxygen and possibly trace metals, it is reasonable to assume that the test conditions themselves will contribute in varying degrees to the results obtained. Patton (64) suggested that copper or air can induce enals or dienals to generate absorbance at 532 m.μ. It has been demonstrated that malonaldehyde, a three-carbon compound, gives the characteristic absorbance at 532 m.μ. and Patton demonstrated that oxidized milk fat contains this compound.

Peroxides (PV)

PV levels correlated very well with sensory evaluations either by individual brand or in the three brands combined as one group. The highest PV detected was 0.420 and the lowest 0.052. These values are listed in Tables 17, 18 and 19. PV averaged 0.195 for the three brands. Brand X, with the best average flavor score (9.6), also had the least content of peroxides (0.155). Similar relationships were also observed in the rest of the samples analyzed.

Table 17. Oxidative Rancidity Related Determinations in Market Milk (Brand X).

Lot	TBA (OD)	PV (meq O_2/Kg fat)	Tocopherol (µg/lipid)	Copper (ppb)	Flavor Score
1	0.022	0.259	13.60	80	9.0
2	0.013	0.167	16.18	104	9.0
2	0.013	0.157	18.46	108	10.0
4	0.022	0.164	13.50	106	9.0
5	0.022	0.196	16.46	82	9.0
6	0.004	0.096	21.60	91	10.0
7	0.009	0.094	18.46	94	10.0
8	0.004	0.098	16.18	100	10.0
9	0.004	0.157	19.19	88	10.0
10	0.007	0.164	16.35	114	10.0
$\bar{X}$ =	0.012	0.155	16.90	97	9.6

Table 18. Oxidative Rancidity Related Determinations in Market Milk (Brand Y).

Lot	TBA (OD)	PV (meq O_2/Kg fat)	Tocopherol (µg/lipid)	Copper (ppb)	Flavor Score
1	0.013	0.178	13.92	103	9.0
2	0.022	0.260	11.40	100	4.0
3	0.013	0.256	13.92	98	10.0
4	0.018	0.174	17.40	104	10.0
5	0.018	0.222	21.50	115	4.0
6	0.004	0.052	19.00	91	10.0
7	0.009	0.155	19.06	100	8.0
8	0.013	0.246	21.36	81	8.0
9	0.004	0.192	20.26	90	9.0
10	0.022	0.420	17.40	108	8.0
$\bar{X}$ =	0.0149	0.215	17.52	99	8.0

Table 19. Oxidative Rancidity Related Determinations in Market Milk (Brand Z).

Lot	TBA (OD)	PV (meq O_2/Kg fat)	Tocopherol (µg/lipid)	Copper (ppb)	Flavor Score
1	0.036	0.312	11.40	140	5.0
2	0.027	0.259	13.50	110	8.0
3	0.009	0.165	16.08	108	10.0
4	0.018	0.164	13.50	136	10.0
5	0.018	0.170	18.28	140	9.5
6	0.009	0.255	11.72	128	10.0
7	0.009	0.170	11.40	131	7.0
8	0.013	0.269	19.06	142	8.5
9	0.013	0.222	13.50	128	8.0
10	0.009	0.182	18.28	155	10.0
$\bar{X}$ =	0.016	0.216	14.67	136	8.6

A relationship between PV and oxidized flavor scores, especially in the form of charts or tables, were not found in the literature. However, in considering the significance of the PV as a chemical test for lipid oxidation in milk fat, Hills and Thiel (31) reported a PV range from 0.06 to 0.30 for various samples obtained from fresh market milk. Fresh raw milk had a PV level of 0.02 to 0.03. In our study, a PV above 0.30 always corresponded to samples with some degree of oxidation as determined by sensory evaluation.

The PV test, correlated with oxidized flavor scores, had the highest correlation coefficient of the parameters observed ($r = -0.70$). This could be interpreted as being the most influential of the tests performed and a clear manifestation of the presence of oxidation. Statistical analysis considered the PV as the most significant of the variables involved in the study. The combined levels of TBA and PV also showed a relatively good degree of correlation with sensory evaluations ($r = -0.630$). The combined PV and tocopherols levels showed little relation with flavor scores and no valid interdependence was observed under the conditions of this study.

Jarvi (36) studied oxidative rancidity of soybean oil using a subjective-objective approach. He found a

valid relation between PV levels, GLC analysis of direct injection of heated oil samples and sensory evaluation scores. The application of a similar approach to oxidized milk fat has not been reported.

Tocopherols

In computing results for tocopherols using the method of Low and Dunkley (56), K values obtained from our prepared standard curve showed a slightly lower value than than reported by those authors (20.3 vs 20.6). Lipid gravimetric analysis ranged from 0.032 to 0.036 mg lipid/ml of hexane extract. The average value (0.033 mg lipid/ml) was used in the calculations. Earlier analyses of tocopherol in milk ($_{51}$) had higher figures than recent reports. The tocopherol content of market milk averaged 16.9, 17.5 and 14.7 µg/g liquid for brands X, Y and Z, respectively (see Tables 17, 18 and 19). These values are in agreement with reports by other recent investigators. Low and Dunkley (56) found values ranging from 14.7 to 22.1 µg/g lipid. Erickson and Dunkley (18) reported values from 11.6 to 30.1 µg/g lipid. Christie et al. (13) gave 7 µg/g lipid for freeze dried milk as α-tocopherol and the same value obtained by GLC.

According to Tsen (85), a possible disadvantage of the colorimetric procedure for tocopherol determination is that the various analogs do not give the same absorbance on

either a mass or a molar basis when using α-tocopherol as the standard. An error would be introduced in those samples which contain tocopherols other than the α-form. In milk, however, α-tocopherol is the only form present to any significant degree.

Relations between flavor scores and tocopherol content produced a low correlation coefficient ($r = 0.15$) for the number of samples studied. This indicates a low degree of influence of tocopherol levels on flavor scores. However, it is difficult to exclude the antioxidant property of tocopherols on the basis of these results since most of the samples tested gave high flavor scores showing little or no oxidation.

This confirms the findings of Dunkley et al. (16) who reported that, "Differences in oxidative stability of the milk were not significant, possibly because the cow produced milk with relatively high oxidative stability when they were fed the control ration (without a tocopherol supplement." They also reported a low correlation coefficient ($r = -0.17$) between flavor scores and tocopherol present in milk (daily supplemented rations of 100 mg α-tocopherol/cow).

Copper

Tables 17, 18 and 19 present the results of copper analyses. Brands X and Y had the lower values, 96.7 and 99 parts per billion (ppb), respectively. Brand Z with 131.7 ppb averaged the highest concentration. In comparison, Hankinson (28) reported values ranging from 72-136 ppb (μg/liter). Murthy et al. (59) analyzed copper content of market milk in several geographical areas of the United States. They found concentrations of 77-120 ppb for the Phoenix, Arizona, area with an average of 90 ppb. Our findings agree very closely with these results.

Deleterious effects of small amounts of copper (less than 100 ppb) in catalyzing undesirable flavor changes have been reported by Smith and Dunkley (74). In the present work, copper concentrations above 115 ppb accompanied flavor scores considered indicative of oxidation (brand Y, lot 5). Brand Z gave similar results when copper levels reached 130 ppb. No oxidation was found in brand X wherein the copper concentration did not exceed 115 ppb in any case. In relating copper and tocopherol content, brand Z, with the highest copper concentration, showed the lowest average tocopherol level. However, only two cases correlated well with flavor scores, tocopherol and copper tocopherol and copper concentration (brand Z, lots 1 and 7). In general, copper concentration below 100 ppb showed no

appreciable influence on the oxidized flavor scores given by the panel. This could mean that copper influenced the flavor scores in a negative manner as was demonstrated by the correlation coefficient ($r = -0.14$) and that this relation could not be considered of high significance in predicting any flavor scores based only on copper content.

In explaining the highest value of copper present in brand Z, diverse factors such as type of feed, production, location, or contamination from either of feed or milk during processing, could account for the differences in concentration in brands X and Y.

Although no apparent relation can be established, milk samples having the highest copper content also had the lowest feed flavor scores. The oxidation of fluid dairy products such as milk involves a complex biochemical system and may include many more parameters than those discussed here.

Feed Flavor

Brand Z had the most pronounced feed flavor of the those subjectively tested. The average scores were 8.85, 9.08 and 7.31 for brands X, Y and Z, respectively. The flavor scores are listed in Tables 13, 14 and 15.

Data obtained from the GLC analysis of milk sample steam distillates were correlated with the feed flavor scores in order to establish a possible subjective/objective

approach to the feed flavor problem. Statistical treatment of the data (simple linear regression) was carried out individually for each brand and for the combined group of the three brands. Flavor scores and their respective GLC peak areas in cm^2 are listed in Table 20.

Brand Z, which exhibited the most intense feed flavor, also had the greatest range of total peak areas. The correlation coefficient between flavor score and total peak area was high ($r = -0.79$). Brands X and Y, which had few cases of noticeable feed flavor, had higher "r" values; -0.934 and -0.920, respectively, This could mean that milk samples with high feed flavor scores (low intensity) produced consistently low total peak areas, whereas highly feed flavored milk, although producing larger chromatogram areas, presented more variations for a given flavor score. Further, it could mean that milk with high intensity feed flavor was not so well correlated with their respective peak areas as was the case for brands X and Y. From these correlation coefficients, an average combining the three brands produced -0.88 and an equation was developed to predict mean flavor scores from total GLC areas of the steam distillates of milk. These results are listed in Table 21.

Some tentative identification compounds present in the chromatograms was attempted by comparison with retention times of pure compounds which have been reported as feed flavor constituents. These are reported in Table 22. The

Table 20. Relation Between Total GLC Peak Areas (cm^2) and Their Corresponding Feed Flavor Scores.

Flavor Score	GLC Peak Areas (cm^2)	Range
10.0	3.4, 4.2, 4.8, 5.3	3.4-5.3
9.5	3.3, 3.5, 4.7, 6.8, 8.2	3.3-8.2
9.0	4.1, 4.6, 6.2, 6.3, 7.2	4.1-7.2
8.5	6.2, 6.7, 7.2, 8.1, 8.8	6.2-8.8
8.0	7.2, 7.9, 19, 11.8	7.2-11.8
7.0	9.8, 10.2, 11.6, 12.1, 13.0	9.8-13.0
5.0	11.60, 17.60	11.6-17.6

Table 21. Regression Analysis of Total Peak Area of Milk Samples as Affected by Feed Flavor Scores

Brand	Regression Equation [c]	Slope (m)	Intercept (y)	Correlation Coefficient (r)
X	[a]FS=11.3-41 GC[b]	-2.41	27.22	-0.934
Y	FS=11.8-0.43 GC	-2.31	27.16	-0.920
Z	FS=15-0.67 GC	-1.47	21.87	-0.790
Mean	FS=12.34-0.48 GC	-2.06	25.42	-0.880

a. FS = Flavor score.

b. CG = Area in cm^2.

c. $x = \frac{y-b}{m}$

Table 22. Relative Retention Times (acetone=1) of Various Pure Chemical Compounds Used as Standards of Comparison.

Compound	Relative Retention Time
Alcohols	
Methanol	1.09
Ethanol	1.35
Isopropanol	1.48
Butanol	3.85
Ketones	
Acetone	1.00 (54 sec)
Butanone	1.51
Pentanone	2.35
Aldehydes	
Acetaldehyde	0.96
Propionaldehyde	1.48
Butyraldehyde	2.08
Miscellaneous	
Ethyl acetate	1.38
Methyl sulfide	1.24
Chloroform	2.07

The number of peaks present in the chromatograms ranged from 5 to 12 but only peaks with an area larger than 0.2 cm^2 were considered for identification. The number of "responsible peaks" or those that were considered to be clearly a part of the total area and frequently present was from 5 to 7. Peaks numbered 1, 2, 3, 4 and 5 were tentatively identified as acetone, ethanol, isopropanol, butanone and propanol, respectively. However, acetaldehyde, methanol and acetone had close retention times to certain of these. (See Figures 7 and 8.) Methyl sulfide, which has also been reported present in feed flavored milk, had a retention time of 1.24. Chemical treatment of steam distillates with mercuric chloride and subsequent GLC analysis showed little or no effect in the number of peaks present or in the total peak area, meaning that methyl sulfide is present in very small amounts. Chromatograms from feed flavored milk, obtained in this work, showed great similarity to those previously published by Jennings et al. (38). Peaks 2, 3 and 4 seemed to account for most of the total peak area of definite feed flavored milk. This was also the case in the work of Jennings who showed that "alfalfa feed flavor milk" had increased peak height corresponding to 2, 3 and 4 in our work. Acetone, acetaldehyde and methyl sulfide were reported to be present.

Figure 9 shows the result of additional peak identification by chemical treatment of milk distillates with

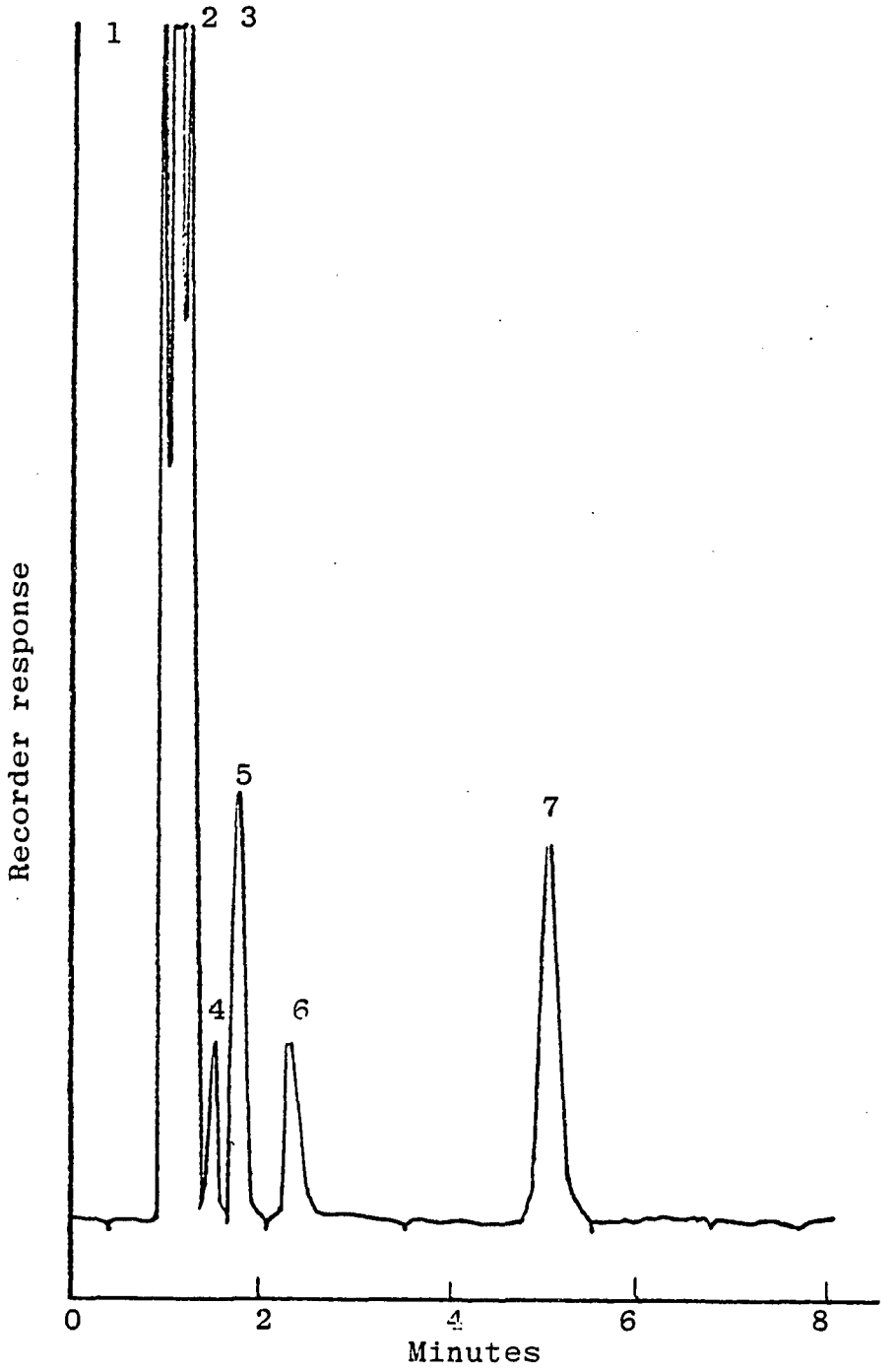

Figure 7. Typical Chromatogram of Milk Steam Distillate with High Intensity Feed Flavor (Peak area 11.80 cm^2)

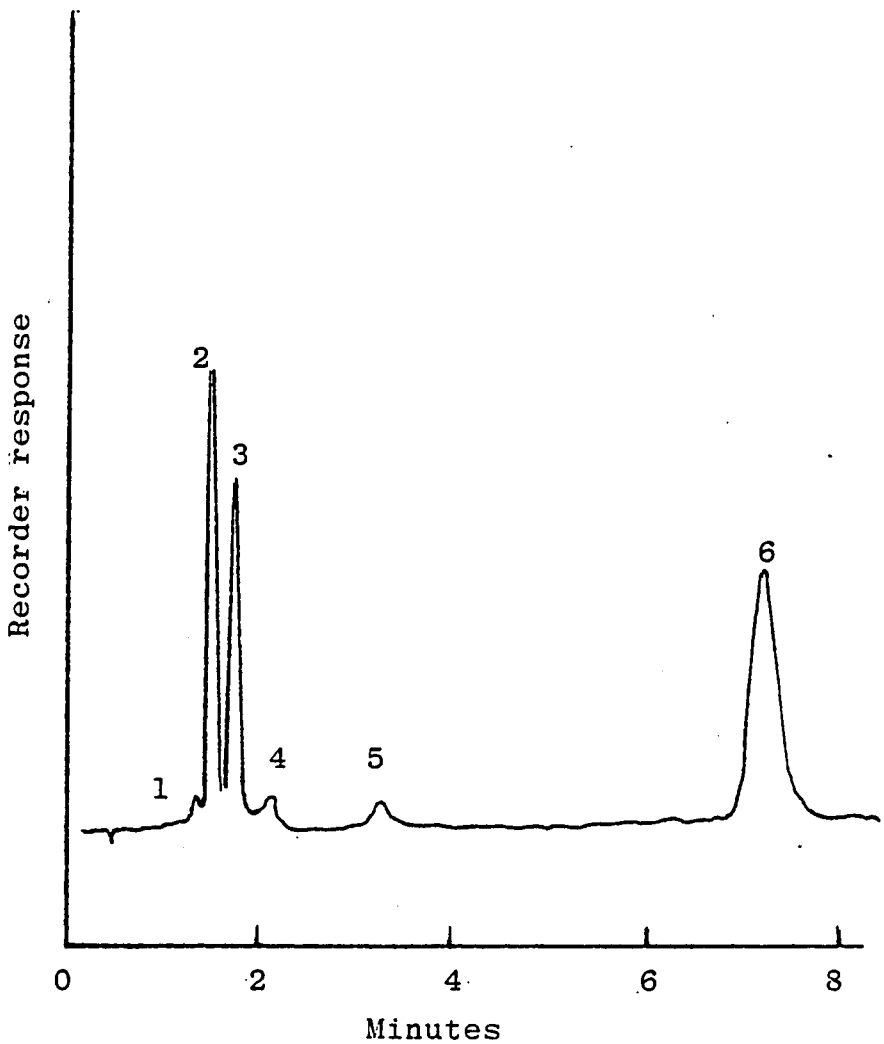

Figure 8. Typical Chromatogram of Milk Steam Distillate With Low Intensity Feed Flavor (Peak area 4.60 cm^2)

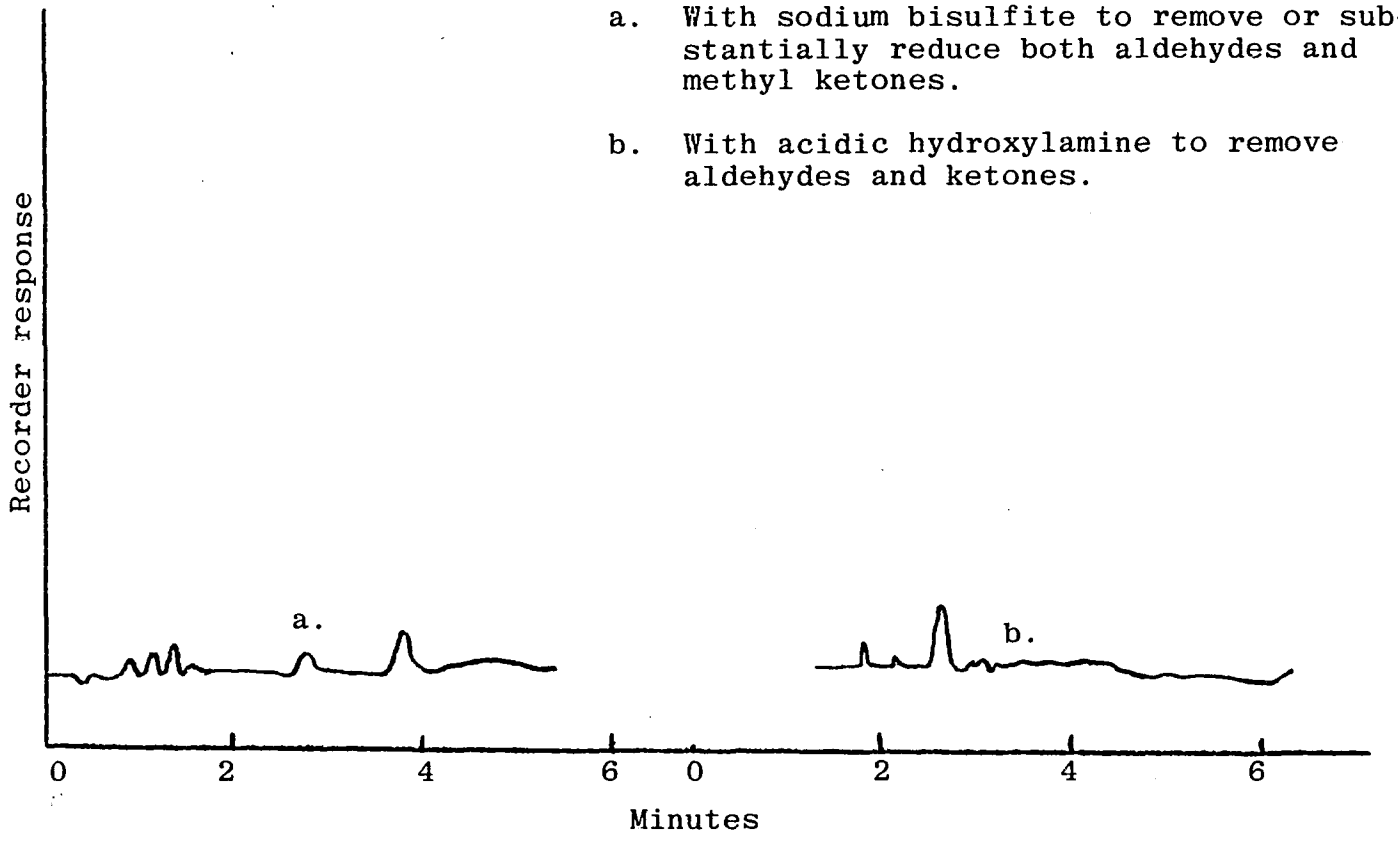

Figure 9. Chromatograms of Milk Steam Distillates After Substractive Reaction.

sodium bisulfite and acidic hydroxylamine to remove aldehydes and ketones. The drastic reduction in total peak area confirms that a large portion of the compounds present are of carbonyl nature. Feed flavor reduction in milk was recently reported (20) to be effective by simple withholding of feed two to four hours prior to milking. This finding was in agreement with recommendations previously outlined in this work. Figure 10 reports the relation between feed flavor scores and their areas by means of regression analysis.

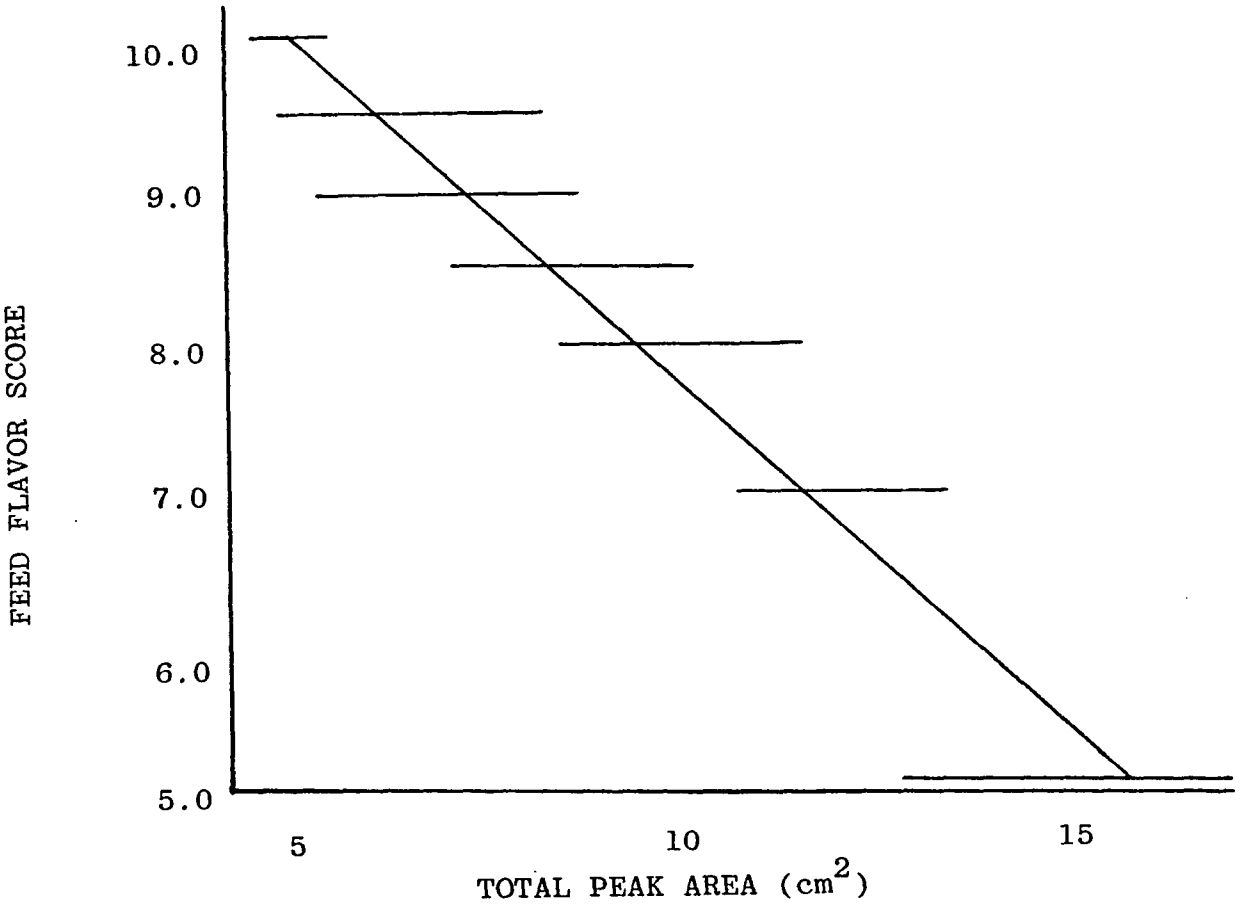

Figure 10. Relationship Between Gas Chromatogram Total Peak Areas and Feed Flavor Scores of Milk Samples

CHAPTER 5

SUMMARY

In this work, a steam distillation technique for the removal and collection of feed volatiles from market milks was used. Gas chromatographic analyses of these volatiles were successfully correlated with sensory evaluations for all samples. A regression equation was developed to predict flavor scores from GLC analysis as a function of total peak area.

At least 12 compounds were present in the chromatograms and 5 to 7 were regularly detected and accounted for most of the total area. Acetone, ethanol, acetaldehyde, butanone, isopropanol and propanol were tentatively identified using retention time comparison with pure compounds.

Peroxide values were highly correlated with oxidized flavor scores and proved to be a reliable test for detecting early oxidation in fluid milk lipids. TBA values had little correlation with oxidized flavor scores. Tocopherol levels of the milk analyzed were within the range reported in the literature. However, one of the brands exhibited relatively low average content. Tocopherol levels and flavor scores seemed to have a little correlation under the conditions of this sampling.

Copper concentrations were also within expected levels. However, one of the brands had a consistently higher copper content, suggesting the possibility of some contamination. Copper levels and flavor scores also had a low level of correlation, although samples with above average copper concentration tended to be evaluated as having more oxidized flavor.

Hydrolytic rancidity was not detected to any significant degree in the three brands analyzed. The ADV and their corresponding flavor scores showed very good agreement. In general, consistent feed flavor in one brand isolated cases of flavor oxidation and little or no hydrolytic rancidity was the pattern in this study.

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