To the Graduate Council:

I am submitting herewith a thesis written by Mansour Tavangaran entitled "Development of Vitamin Fortified Buttermilk." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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DEVELOPMENT OF VITAMIN FORTIFIED BUTTERMILK

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Mansour Tavangaran

August 1980

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. H.O. Jaynes, his major professor, for his help, guidance and patience throughout the graduate program. The author wishes to thank Dr. Jaynes for his assistance, support and encouragement in the planning, conducting, and reporting of the present study. Appreciation is also extended to Dr. A. Draughon for her close guidance, assistance and encouragement throughout the study, and to Dr. B.J. Demott for his advice and assistance, as well as to Dr. P.M. Davidsom for serving on the thesis committee.

The author also wishes to thank the faculty and staff of the Department of Food Technology and Science, especially Dr. J.T. Miles, Department Head, for their help through his program. Thanks are also extended to Miss L.S. Post for her assistance in the statistical analysis and Mrs. Ola Sanders for her continued assistance in sensory evaluation.

Sincere appreciation is expressed to his family, especially his senior brother Nasser Tavangaran, for their unlimited support and encouragement. He is grateful to all his new friends in Knoxville for their role in making graduate studies delightful.

ABSTRACT

Cultured buttermilk was fortified with vitamin A palmitate and vitamin D_3 in order to make the product nutritionally equivalent to other fluid milk products. Changes in vitamin content, pH, and Titratable Acidity (TA) were monitored during storage over 21 days at 4° C and 10° C.

A high performance liquid chromatograph technique was developed to measure vitamin content. Fortified buttermilk samples were lyophilized and subjected to a hexane extraction procedure. The extract was cleaned on a Sep-pak silica cartridge and eluted with hexane-ethyl acetate (70:30). The samples were chromatographed through a reverse phase μ-bondapak C_{18} column with methanol-acetonitrile (50:50) as the mobile phase.

Fortified buttermilk and unfortified controls were stored 21 days at 4° and 10° C. An analysis of variance of vitamin stability indicated a significant decrease in concentration due to time. Temperature of storage had an inverse effect. An analysis of variance for pH and TA values showed a significant difference between fortified and control buttermilk samples held under the same conditions of storage. A decline in pH with an accompanying increase in TA was noted in all samples upon storage, particularly in the fortified buttermilk samples.

The results of organoleptic analysis indicated no significant difference between fortified and control buttermilk samples stored under the same conditions.

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CHAPTER I

INTRODUCTION

Cultured dairy foods constitute a very important part of the dairy industry. During the last several years, fluid milk and related items have faced a steady decline in sales while sales of non-fat cultured dairy products have assumed considerable importance in the American diet and are becoming increasingly important in supplying many of the nutrients that milk is known to contain.

The popularity of cultured dairy foods is due to several factors. They are nutritious, appetizing, convenient, and relatively inexpensive. Such milk, being in a state of temporary preservation, may be held for several days without any detrimental effects. The delicate and pleasing aroma of cultured dairy products cannot be found in other foods.

The three main cultured milk products manufactured in the United States are buttermilk, yogurt, and sour cream. While yogurt and sour cream are gaining in popularity, buttermilk has been a neglected member of the dairy food family for some time (31). The consumer attitude towards buttermilk has been changing in recent years. The United Dairy Industry Association reports (39) have indicated that there was an overall drop in beverage use of buttermilk as evidenced by a rise in the percentage of people who never drink it from 56 percent in 1973 to 62.3 percent in 1975. Of the population who drink buttermilk, older and lower income groups were reported to be substantially better consumers of the product (2,39).

The outlook for buttermilk sales is not bright unless some changes are made. Some industry people feel that the decline in sales is due to its poor quality while others propose that the decrease in buttermilk popularity is due to its lack of promotion and consumer education (39).

Recently, dairy companies have found that buttermilk lends itself well to promotion. Some experts feel that a market exists for a product with a higher nutritive value, a relatively distinct yet delicate flavor, low acidity, and low viscosity which may reduce the negative attitude of consumers and increase buttermilk usage as a beverage rather than as an ingredient (2,31,39).

Buttermilk, while a poor source of vitamin A and D, can lend itself to the addition of these vitamins. Buttermilk, fortified with vitamin A and D, could be considered a substitute for milk and soft drinks by people appreciating a cool and refreshing drink of the highest quality as well as by those older persons who believe buttermilk corrects certain types of digestive disorders.

This experiment was undertaken as an investigation into the improvement of the nutritional quality of buttermilk through preparation of a vitamin A and D fortified cultured buttermilk product, determining the extent of the correlation of vitamin addition to flavor quality, and developing a high performance liquid chromatographic method to study the stability of vitamins under the anticipated storage conditions.

CHAPTER II

LITERATURE REVIEW

I. GENERAL

For centuries, man has known that milk spoils easily, but when allowed to sour under favorable conditions, the resultant product can have a pleasant flavor and can be kept for several days. The manufacture of fermented milks is thought to have originated in the near East and then spread through central and eastern Europe (24). Historically, the first fermented milk was discovered when raw milk, soured in a bag made from a calf's stomach, was subsequently consumed. In early history different tribes of people treated milk differently, resulting in a variety of fermented milk products which were given different names. The most common fermented milk products today are buttermilk, yogurt, kefir, koumiss, bulgarian buttermilk, and acidophilus milk. The most characteristic flavor of fermented milk is lactic acid resulting from bacterial action. Some products undergo an alcoholic fermentation along with the lactic fermentation. Such products as kefir and koumiss thus contain ethyl alcohol and CO_2 in addition to lactic acid.

Fermented milk products are usually manufactured by using active commercial bacterial cultures of known organisms called "starter cultures." Therefore, the term "cultured" is frequently substituted for the term "fermented." The term "cultured dairy products" is widely accepted and includes such products as buttermilk, yogurt, sour cream, and acidophilus and bulgarian buttermilk (25). While all of these

products constitute an important part of the dairy industry, emphasis in this study will be limited to cultured buttermilk.

True buttermilk is the liquid remaining after the fat is removed from milk or cream by the process of churning butter (18,25). Buttermilk obtained from sweet cream or milk has a composition similar to that of ordinary skim milk (25,56), whereas buttermilk made from sour cream contains less lactose than skim milk, sweet cream, or buttermilk, as well as some lactic acid (6,56). The quality and shelflike of these buttermilks depends heavily on the quality of cream used for making butter. Cultured buttermilk evolved from the difficulty of consistency obtaining a fine flavored buttermilk as a by-product of buttermaking. It may be prepared by culturing true buttermilk or, as is more widely practicd, culturing skim milk with buttermilk starter cultures that produce lactic acid and a desirable flavor and aroma.

Very little true buttermilk is available for retail consumption. Nearly all of the buttermilk by-product from the manufacture of butter is condensed and/or dried for use in bakery goods, pancake flour, ice cream and animal feed (25).

Cultured Buttermilk, Definition and Standards

The United States Public Health Service (USPHS) Grade A pasteurized milk ordinance (52) defines cultured buttermilk as "a fluid product resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized skim milk or pasteurized low fat milk." Cultured milk or cultured whole milk buttermilk is defined as "a fluid product resulting from the souring by lactic acid producing bacteria or similar culture of pasteurized milk."

In recent years, direct acidification of milk with food grade acids has been used in the manufacture of such products as buttermilk, cottage cheese, sour cream, sour half-and-half, and dip bases (11,27,28). The USPHS defines acidified milk and milk products as "milk and milk products obtained by the addition of food grade acids to pasteurized cream, half-and-half, milk, low fat milk or skim milk, resulting in a product acidity of not less than 0.20 percent expressed as lactic acid" (52).

There were no federal standards before July 1, 1979 for cultured milk or cultured buttermilk, but these products were regulated by state state standards (11). The minimum concentration of milk solids not fat (MSNF) required in cultured buttermilk ranged from 8.0 to 9.0 percent, with a majority of states requiring 8.25 percent MSNF (49,51).

Recently, the Food and Drug Administration has proposed a standard of identity for cultured buttermilk and acidified milk (17). All cultured buttermilk shipped in interstate commerce after July 1, 1979 would have to be in compliance. This specification covers the requirements for pasteurized cultured buttermilk, cultured lowfat milk and cultured milk for use by all federal agencies.

> The products covered by this specification shall be of the following types as specified:
>
> Type I--cultured buttermilk (less than 0.50 percent milk fat).
>
> Type II--cultured lowfat milk (not less than 0.50 percent nor more than 2.0 percent milk fat).
>
> Type III--cultured milk (minimum 3.25 percent milk fat).
>
> Types I and II shall contain added vitamins A and D, when specified by the procuring agency. When vitamins A and D are specified or not specifically excluded by the procuring agency, they may be added. When vitamins A and D are specified, they shall meet the potency requirements. Vitamin A content may be increased by the addition of a concentrate of vitamin A dissolved in an appropriate carrier. Vitamin D content may be increased by the addition of a concentrate of

> vitamin D of food grade quality dissolved in a food oil, but if the oil is not milkfat, the quantity added shall be not more than 0.01 percent by weight of the finished product.

Compositional requirements for cultured buttermilk are presented in Table 1. The finished products should possess a pleasing and desirable characteristic aroma and acid flavor and should be free from undesirable flavors and odors. The products should be free from gas resulting from abnormal fermentaton, wheying off, and practically free from entrapped air.

Economic Aspects

Buttermilk sales represent 2.0 - 2.5 percent of total fluid milk sales. In 1976, 1,020 million pounds of buttermilk were sold nationwide (3). If the average retail price was 55 cents per quart, it would amount to over 262 million dollars.

Buttermilk sales vary in different areas of the United States. The term, "Buttermilk Belt" has been used to refer to those states where buttermilk sales are the highest (40). The "Buttermilk Belt" includes sections of the southern and southeastern United States where buttermilk sales exceed 3 percent of fluid milk sales. In Georgia, South Carolina, Alabama, Tennessee, Mississippi and in parts of Arkansas, Kentucky and Louisiana, buttermilk represents more than 6 percent of fluid product sales. Buttermilk sales exceed 8 percent of the sales volume in Alabama, South Carolina and Mississippi. In most areas of Texas and Oklahoma, buttermilk accounts for more than 4 percent of fluid milk sales. In all other federal market order areas, buttermilk sales are less than 3 percent of the total fluid milk sales (40).

Table 1

Compositional Requirements for Cultured Buttermilk

	Type I	Type II	Type III
Milk fat (%)			
(not more than)	0.50	2.00	
(not less than)		0.50	3.25
Milk solds not fat (%)			
(not less than)	9.00	9.00	8.25
Phosphatase Activity			
(Schaner Rapid Method)	1 mcg	1 mcg	1 mcg
Titratable Acidity			
(as lactic acid)			
%	0.80 - 0.95	0.80 - 0.95	0.80 - 0.95
pH	4.40 - 4.50	4.40 - 4.50	4.40 - 4.50
Vitamin D			
(per quart)	400 IU	400 IU	----
Vitamin A			
(per quart)	2000 IU	2000 IU	----

Reference 17.

Total buttermilk sales declined from 1,149 million pounds in 1971 to 995 million pounds in 1978 (3). This decline in sales amounts to estimated losses of approximately 30.6 million dollars to the dairy industry.

Buttermilk acceptability seems to be related to the cultural background of consumers (40). For example, buttermilk sales are higher in Northern Louisiana than in Southern Louisiana where the population is primarily of Acadian ancestry. Buttermilk sales in Texas are relatively high in all areas except the San Antonio market which has many Spanish speaking people.

A study (2) of dairy sales in 10 metropolitan areas revealed that the heaviest buttermilk consuming area was Pittsburgh where 29.5 percent of the families indicated that they had served buttermilk within the seven day period preceding the receipt of the survey questionnaire. Los Angeles was second (22.5 percent) and Fort Wayne, Indiana was third (18.6 percent). The East Coast metropolitan areas indicated lower consumption of buttermilk (Baltimore, 10.9 percent; Philadelphia, 10.6 percent; and New York, 7.9 percent). The lowest consumption of buttermilk was in New England (Boston 5.3 percent).

Quality of Cultured Buttermilk

Unfortunately, the quality of buttermilk found in the marketplace today is not very good. Studies over the past few years showed this fact to be quite serious. A review of recent buttermilk scoring clinics reveal alarming facts about the quality of buttermilk sold today which is a logical explanation why sales are dropping. Before we can talk about increasing the future sales of buttermilk, we need to improve the

quality attributes of this product in every way to hold its present position (1,21,40).

The consistent production of high quality, good flavored cultured buttermilk constitutes a major challenge facing the dairy industry. There is little uniformity in buttermilk throughout the United States. The quality and composition of buttermilk is more variable than the quality and composition of many other dairy products (40). A survey of buttermilk from ten regional dairies in Oregon by Keenan (21), shows a wide variation of flavor scores, concentration of diacetyl, titratable acidities and pH values. Kosikowski (23) conducted a nationwide survey of methods for manufacturing buttermilk and reported variations in culture inoculum, pasteurization temperatures, incubation time, break-ng acidity and amounts of fat in the products.

Another problem in producing buttermilk of uniform quality is consumer preferences. Recently, Richter (40) pointed out that consumers in different areas of the United States prefer buttermilks with different levels of fat, acidity and viscosity. Therefore, it may not be feasible to try to establish uniform standards of identity throughout the United States. However, it is important to produce a buttermilk which has a distinct but pleasing, slightly sweet, lactic acid flavor. The flavor should be somewhat tart, yet mild, with no indication of excessive sourness. While buttermilk has been called "Lemonade of the Dairy Industry" it should not produce the flavor sensation which is too sour. The flavor of good buttermilk should be clean, fresh, and entirely free from any unpleasantness. The typical aroma of good buttermilk closely resembles that of high quality butter made from fresh cream of low acidity.

A good body is indicated by a smooth, rich, creamy consistency with entire freedom from lumps. It should impart the sensation of a "soft, velvety feel" to the palate. Viscosity refers to the consistency of the product as it is poured from the bottle. Excessive viscosity is often associated with lumpiness and lack of smoothness, whereas insufficient viscosity is closely related to a watery or flat flavor, dull color, and wheying-off. Each is severely criticized by the consumer. Good buttermilk pours from the bottle in a manner similar to rich cream or smooth, thick gravy.

Some of the most common defects of cultured buttermilk include: lack of fine flavor, unclean, putrid and bitter flavors, excess acid flavor, wheying-off, too little viscosity, gassy or foamy buttermilk (19,23). The lack of desirable flavor and aroma usually results from an insufficient level of activity of flavor producing bacteria or from the action of spoilage organisms. Contamination by coliforms and psychrotrophs may result in enzymatic changes such as proteolysis which may lead to unclean, putrid, or bitter flavors in cultured buttermilk. In addition, these organisms may produce an excess of CO_2. These defects can be controlled through proper cleaning and sanitation practices. Excessive acidity is due to overripening of the buttermilk and can be avoided by shortening the incubation period or by reducing the amount of starter. Some practices that lead to whey separation include improper acidity control, low pasteurization temperatures, violent agitation, and long storage at high temperature. Whey separation can be controlled by proper manufacturing practices and by addition of gelatin or other acceptable stabilizers where permitted

(18,19). Viscosity of buttermilk appears to be related to the acidity during ripening. Excess acidity causes higher viscosity and a thicker body. A thin body may be due to too low an acidity at breaking time, low SNF content of milk, excess agitation and high storage temperature (43). Viscosity defects can be controlled by proper culture selection and strict control of processing practices. In some instances, gelatin and fat may be added to improve the body of cultured buttermilk (1,18,19).

Nutritional Aspects

The nutrient composition of cultured buttermilk varies widely, partly due to variations in raw milk supply and the type of buttermilk manufactured. The fat content of all types of buttermilk has been reported to range from 0.5 to 3.5 percent (49). However, the fat content of cultured buttermilk made from skim milk or low-fat milk reportedly varied from 0.98 - 1.02 percent (49). Most of the buttermilk produced in Tennessee, Georgia, Alabama, and South Carolina contains approximately 0.5 percent fat. The fat content of buttermilk is generally higher in the western area of the Buttermilk Belt (40). However, no federal market order area in the United States reported an average fat content for buttermilk greater than 2.0 percent (50).

The protein contents of 47 commercial buttermilk samples varied from 2.8 - 3.6 percent (34). The mean protein content of buttermilk was 3.25 percent. In addition to fat and protein, cultured buttermilk contains many other nutrients. The nutritional composition of cultured buttermilk has been reported by the National Dairy Council (34,35). The nutrients provided by one cup (245 gm) of buttermilk are shown in Table 2.

Table 2

Nutritional Composition of Cultured Buttermilk
(Provided by One Cup)

Water	90%
Food Energy	90
Protein	9 gm
Carbohydrates	12 gm
Fat	Trace
Vitamin A	10 I.V.
Calcium	296 mg
Iron	0.1 mg
Thiamin	0.1 mg
Riboflavin	0.44 mg
Niacin	0.2 mg

Reference 35.

A highly controversial aspect of the nutritional qualities of cultured buttermilk is its acclaimed therapeutic value and special health-giving properties. Burke (10) cited the work of Metchnikoff who sought to prove that the prolongation of life might be closely associated with the daily consumption of fermented milk which certainly played no small part in its popularity. A similar book by Douglas, also noted by Burke (10), in which Douglas discussed the health benefits of lactic acid producing bacteria in the intestinal tract, served to stimulate public interest in the product as well. According to numerous researchers its value is to be found partly in the high acid content which exerts a detrimental effect on objectionable bacteria, and partly due to its superior digestibility. The casein of cultured buttermilk is in a finely divided state which approximates the first step in digestion when milk is taken into the stomach. This fact alone contributes much to its healthfulness and value, because the curd can no longer form hard, tough masses in the intestinal tract which is appreciated by older persons.

Vitamin Fortified Cultured Buttermilk

In recent years nutritionists have put great emphasis on high protein and played down high fat foods and as a result milk consumption has decreased during adult life (30,42,46). With recent life styles, the majority of people spend much of their life time working inside buildings and rely on chance for selection of their diet. Therefore, nutrients such as vitamin A and D which are not abundantly distributed in nature, have become important contributing factors to many diseases in adult life (16,26,42,46).

Vitamins A and D are required throughout life (7,26). Outside of the very specific role of these vitamins in the visual system and mineralization of bone (Table 3), they are necessary for growth, reproduction, and maintenance of good health. Diet surveys indicate that the majority of adolescents do not take vitamin supplements and vitamin A and D deficiencies (Table 4) such as keratomalacia, and degenerative bone diseases have found great importance in our senior population (12,16,37,42,46).

Since skim milk is often the principle product from which cultured buttermilk is prepared, it is apparent (Table 5) that fat and the accompanying fat soluble vitamins A and D are the only lacking constituents (24,48). However, cultured buttermilk can be supplemented with these vitamins in order to help make this product as nutritious as milk, which is almost a complete food. Fortification is the most rapid and the most economical method of promoting the intake of nutrients. It involves the least dietary changes and makes maximum use of the product (7).

Level of Vitamin Addition

The Food and Nutritional Board of the United States and the National Academy of Sciences state that a level of 2000 I.U. vitamin A should be obtained from fats, oils, and dairy products. The National Research Council recommended daily dietary allowances for vitamin A and D be set at 5,000 I.U. vitamin A and 400 I.U. of vitamin D (32,33).

The vitamin A requirements may be modified by abe, growth, calorie intake, physical activity and special needs. Although further

Table 3

Functions of Vitamin A and D

I. Vitamin A:

1. Maintenance of proper vision
2. Maintenance of spermatogenesis in the male
3. Maintenance of the placenta and prevention of resorption of the fetus in the female
4. Maintenance of bone development and growth
5. Maintenance of the mucous-secreting cells of epithelia, the biosynthesis of glycoproteins, and the prevention of keratinization
6. Interaction with vitamin E in regulating stability of biological membranes
7. Involved in production of corticosteriods and in glycogensis
8. Interrelated with thyroid hormone function
9. Influences synthesis of serum and muscle protein

II. Vitamin D:

1. Improving active transport of calcium and phosphorus in the small intestine by two independent mechanisms
2. Mobilization of calcium and phosphorous from the bone fluid
3. Improving renal reabsorption of calcium and phosphorous
4. Adjusting calcium absorption in intestine

Reference 42.

Table 4

Primary Vitamin A and D Deficiencies

I. Vitamin A:

General

- Loss of appetite
- Inhibited growth
- Increased susceptibility to infections
- Death

Skin

- Dry and scaly
- Poor hair production

Eyes

- Night blindness
- Keratomalacia
- Extensive keratinisation of lining cells

Respiratory tract

- Loss of ciliary epithelium
- Multiple infections
- Keratinisation of mucuous membrane

Digestive tract

- Loss of gland activities
- Poor absorption
- Infections

Urinary tract

- Metaplasia of epithelium and increased tendency to stone formation

II. Vitamin D:

Rickets in young children

Osteomalacia in adults

Reference 42.

Table 5

Average Composition of Milk and Cultured Buttermilk

	Average Composition of milk	Average Composition of cultured buttermilk
	%	
Water	87.30	91.30
Protein	3.30	3.40
Fat	3.70	0.65
Lactose	5.00	3.40
Lactic Acid	0.16	0.60
Ash	0.70	0.65

Reference 24.

adjustments in the RDA levels are not being considered, higher doses of these vitamins are well tolerated and can be ingested with safety under normal conditions (32).

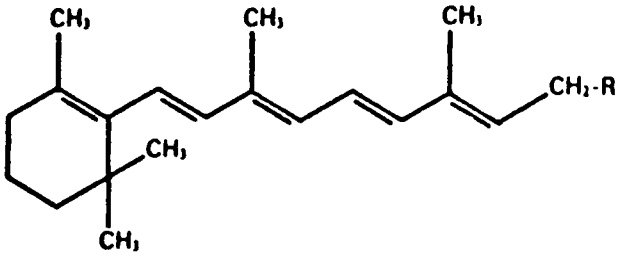
Hypervitaminosis from A and D can occur only if sufficiently high doses (over 10 x the RDA levels) are taken for long periods of time (months and years) (20,29). There has not been any reports of hypervitaminosis A or D resulting from the consumption of fortified foods (20,41).

Application Forms and Methods of Vitamin Addition

Various application forms of vitamin A and D have been developed to meet the demands of the food industries. Large quantities of synthetic vitamin A as palmitate and Vitamin D_3 (cholecalciferol) (Figure 1) are used to supplement processed foods. They possess the highest biological activity and are now the major sources of vitamin A and D in the diets of many people (7).

Vitamin A and D should be given with an adequate protein intake for proper and maximum vitamin utilization. But most of all, the ideal food carriers of added vitamins should be consumed by target population on the basis of preference, habit, and economics which makes buttermilk a logical food to be fortified with these vitamins (8,26).

Cultured buttermilk as an ideal carrier of vitamin A and D and can be fortified in several ways. A water-dispersible stabilized A palmitate and D_3 beadlet product of a vitamin A palmitate and D_3 emulsified concentrate can be dispersed in warm liquid skim milk and then added to the holding tank where skim milk is being made ready for

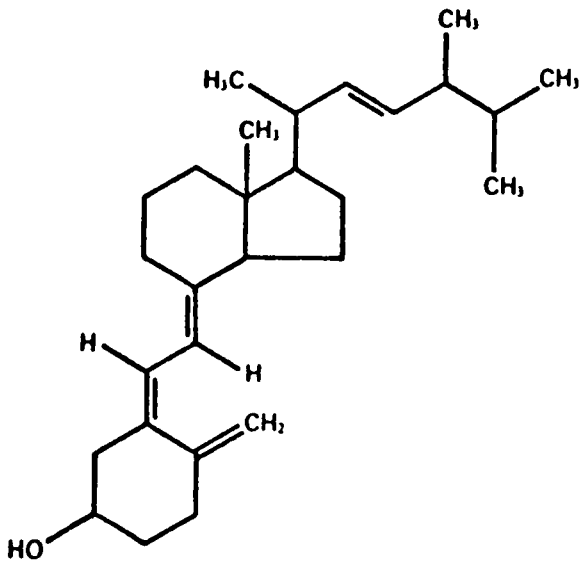


Vitamin A

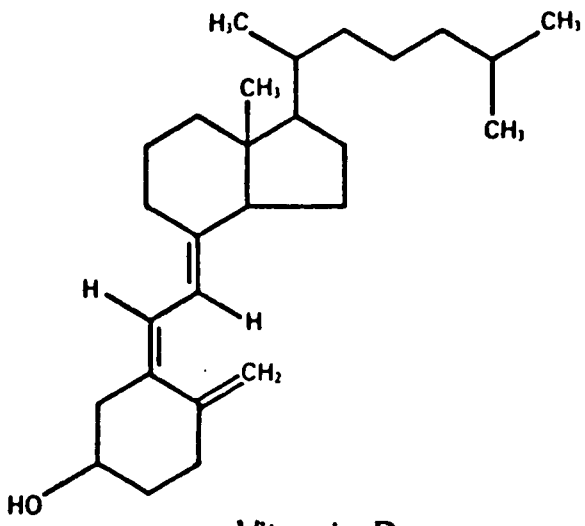
R = OH, Alcohol

= O_2CCH_3, Acetate

= $O_2CC_{15}H_{31}$, Palmitate



Vitamin D_2



Vitamin D_3

Figure 1. Structural formulas of vitamin A and D. The two most important forms of vitamin A + D, in terms of nutrition and fertification, are vitamin A palmitate and vitamin D_3.

inoculation. These are simple methods as it is easy to weigh out the required quantity of beadlets for a known volume of skim milk or to open a can, pour out, and flush out the contents of the emulsified concentrate into the tank (7,20).

Stability of Added Vitamins

Surprisingly, little data have been published on the stability of added vitamin A and D in fortified foods. Much of the information is recorded in the food industry's records. Available data are sketchy and at times difficult to extrapolate to buttermilk because insufficient details are usually available on pH, moisture content, and other significant variables. However, adequate vitamin A stability (Table 6) has been attained when fluid non-fat milk has been properly fortified with vitamin A palmitate in an emulsified base (13,22). It has also been reported that there is a direct correlation between moisture content and vitamin A and D losses during long storage (7). Cox and coworkers (15) reported that the presence of dissolved oxygen, mineral acids, and ultraviolet light are known to destroy vitamin A and D as well as their isomers. Better stability has been reported by other investigators under inert gas packaging and low temperature storage (15).

Methods of Vitamin Measurement

Within the last 15 years, several general reviews of analytical methodology for vitamin A and D have been published (4,5,38,45,53). Except for possibly gas-liquid chromatography and high performance liquid chromatography, all are based on three things: Vitamin A

Table 6

Stability of Added Vitamin A in Fluid Skim Milk

Storage time, 40° F	Vitamin A IU/qt	Retention %
Initial	6,400	100
1 day	6,120	96
4 days	5,740	90
7 days	5,300	83
11 days	4,400	70
14 days	4,260	67

[a]Palmitate 250-S dispersed in pasteurized warm skim milk and cooled to 40° F.

Reference 13.

absorbance at 320 nm and D absorbance at 265 nm, Vitamin A fluorescence, and both vitamins forming colored products with antimony trichloride.

The present method of the Association of Official Analytical Chemists (AOAC) for both vitamins in margarine is based on a spectrophotometric analysis (4). The official AOAC method for feeds, premixes, and foods is the Carr-Price antimony trichloride colorimetric procedure (4,5,45). Many analysts seem to have difficulties with the Carr-Price method, which has no doubt encouraged development of other methods.

In recent years, the principal centers for vitamin A and D determinations have shifted from research laboratories to processor, control, and regulatory laboratories. That shift has led to more practical approaches to methods. Many believe that, if necessary, we can forego some losses of accuracy and precision to achieve some of the desirable characteristics shown in Table 7 (36).

There have been several studies using gas-liquid chromatography for determining vitamin A and D, but in general the procedure is considered unsuitable for that purpose (36,44,54). Temperature and other conditions in the column tend to destroy vitamin A. Even with special treatments, results are not satisfactory. No reports were found on the use of GLC for determination of these vitamins in foods.

A number of absorbents has also been tried for separating these vitamins from other fat-soluble substances. Neutral alumina has been the absorbent most widely used. It required an exact adjustment of solvent composition and a more exacting elution. Failure to separate

Table 7

Desirable Characteristics of Methods for Routine Analysis of Vitamin A and D in Foods

General application to all foods (and feeds)
Simple, rapid - few steps and transfers
Simple, readily available equipment used
Adaptable to routine use, rugged
Minimum training and skills required
Minimum quantities solvents and reagents used
Minimum purification of reagents
Minimum of interfering reactants retained
Low cost
Safety
Low corrections for losses in analysis
Results reproducible, good accuracy and precision
Compatible with that for the provitamin A determination
Capable of automation

Reference 36.

and elute the vitamin from other substances may result from using alumina with too much or too little absorption activity (36,44,57).

A new and rapidly developing entry into the vitamin A and D field is high-performance liquid chromatography (HPLC). It offers more promise than gas chromatography. The column used in the separations are either absorption or partition types (57). Both normal and reverse-phase solvent systems are used, but the reverse-phase seems to be most often tried for fat soluble vitamin separations. Several HPLC manufacturers have brochures showing separations of fat-soluble vitamins from synthetic mistures of pure vitamins, or vitamin concentrates. Sharp peaks are shown with elapsed retention times of 4 to 20 minutes or more, depending on the system selected for the tests. Analytical results on determinations of vitamin A in vitamin mixtures by HPLC have been published (14,47,53,55,57). In this work, reproducibility was usually stressed over accuracy and sensitivity was enhanced by selective wavelength detectors.

Thompson and Maxwell in 1976 (47) developed an HPLC method to determine vitamin A in milk and reported that results agreed with those by both spectrophotometric and fluorometric assays. The recoveries with replicates were 99 percent and the coefficient of variation was 6.8 percent.

However, the competitive nature of the industry and increasing demands of the government and state regulations has constantly required improvements in vitamin separation technology. As it was mentioned, HPLC has been shown to be a reliable and time effective method for the analysis of fat soluble vitamins in milk and milk products. The

technique is believed to provide the user with the advantages of speed of analysis, sensitivity of detection, minimal sample preparation and excellent precision (47).

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF MATERIALS

Pasteurized milk and skim milk was obtained from the University of Tennessee creamery and the Vitex A-D was a generous gift from Vitex/American Vitamins (Diamond Shamrock Corporation, St. Louis, Missouri). Vitamin A palmitate and D_3 standards were obtained from Sigma Chemical Company (St. Louis, Missouri). All solvents were HPLC grade and were obtained from Fisher Scientific Company (Atlanta, Georgia).

II. PREPARATION OF THE SAMPLE

Skim milk (8 gal) standardized to 0.8 percent fat by adding whole milk was heated in 10-gallon stainless cans at 85° C for 30 minutes. Following the heating process, the milk was cooled to 21.5° C by circulating cold water. To one can 3.2 ml Vitex A-D was added to give buttermilk fortified to a level of 2000 I.U. of vitamin A and 400 I.U. of vitamin D per quart. An inoculum of 1.5 percent buttermilk culture (Hansens H-44) was added to both cans. Following addition of Vitex A-D and the starter, the mixture in both cans was agitated thoroughly with a hand mixer and incubated at 21.5° C to attain a titratable acidity of about 0.80 percent. The coagulum was then broken by agitation and 32 g salt was added to each can for desired flavor. After the complete mixing of the salt, the buttermilk was cooled to below 10 C and filled into qt. gable-top cartons, then held at temperatures of 4° C or 10° C.

III. PREPARATION OF VITAMIN STANDARDS

Vitamin A palmitate standard was prepared by dissolving 1 g of the vitamin in 20 ml acetone and then diluting to 100 ml with 95 percent ethanol (standard A). The working standard for HPCL was prepared by pipetting 1 ml of standard A and bringing it to a volume of 100 ml with hexane to give a concentration of 0.1 mg/ml (standard B).

Vitamin D_3 standard was prepared by dissolving 500 mg of the vitamin in 500 ml Hexane (Standard A) and the working standard was prepared by pipetting 0.5 ml of standard A and bringing it to a volume of 50 ml with hexane to give a concentration of 0.01 mg/ml (standard B).

IV. ASSAY PROCEDURE FOR VITAMINS A AND D

Prior to HPLC analysis, samples of buttermilk were lyophilized and 10 g of dried sample was mixed with 50 ml hexane, blended for 30 sec in a Waring blender, and filtered through Whatman #42 paper. Ten ml of hexane extract was chromatographed on a sep-pak Silica Cartridge and washed with 1 ml hexane. Elution of vitamins was with 2 ml hexane-ethyl-acetate (70:30). All the fat-soluble vitamins were contained in this fraction. The fraction was almost evaporated to dryness under nitrogen gas and then brought to a volume of 2 ml in hexane. Twenty µl of this eluate was injected into the HPLC to be analyzed.

The recovery studies were done by adding known amounts of Vitex A-D to the skim milk prior to extraction. Blanks were run in duplicate prior to spiking to correct samples for percent recovery.

V. COMPOSITIONAL ANALYSIS

Fat and total solids were determined by the Mojonnier method. Vitamins A and D were determined by HPLC. The pH was measured with a Fisher Accumet (Fisher Scientific, Atlanta, Georgia) pH meter and the standardization of the instrument was accomplished with pH 4.0 and pH 7.0 standard buffer solutions. For the titratable acidity, 9 ml aliquots of buttermilk sample were titrated with 0.1 N NaOH using phenolphthalein indicator.

VI. CHROMATOGRAPHIC CONDITIONS

Vitamin assays were conducted using a liquid chromatograph (Waters Associate Inc., Milford, Massachusetts) equipped with a M6000 A pump and a U6K septumless injector throughout the study. A model 440 UV detector with constant wavelength of 254 nm at a sensitivity of 0.05 absorbance units full scale was used as the detection system. The reverse phase μ-Bandapak C_{18} column was eluted with methanol-acetonitrile (50:50) mobile phase at a flow rate of 1 ml/min. Chromatograms were recorded on an Omniscribe (Houston Instrument) double pen recorder with a chart speed of 0.2 in/min.

VII. QUANTITATIVE DETERMINATION OF VITAMINS A AND D

Identification of the vitamins was made on the basis of retention time at maximum peak height. A procedure for quantitation was standardized by adding known concentrations of the Vitex A-D solution to skim milk and analyzing the samples. A standard curve of peak heights

vs. known concentrations of vitamins was prepared for Vitex A-D in skim milk and vitamin standards. The concentration of vitamins was calculated from average peak heights of triplicate injections of buttermilk samples by direct comparison with peak heights of vitamins in the standard curve. The peak height method of quantitation was chosen to minimize interferences by neighboring, overlapping peaks.

VIII. ORGANOLEPTIC EVALUATION

The effect of vitamins on the flavor and aroma of buttermilk was examined both initially and at 7 day intervals over a 3 week storage period. A consumer panel of 30 people were asked to evaluate the samples for difference and preference in a triangle test on the basis of flavor and aroma at each time period. For all the sensory tests the samples were coded with three-digit random number (Larmond, 1977) and presented in 9 oz clear plastic cups. The samples were presented cold under red light to enable better judgment. The consumer panel was conducted using the sensory evaluation booths in the sensory laboratory. Panelists were staff members and students of the Food Technology and Science Department of the University of Tennessee.

XI. STATISTICAL ANALYSIS OF DATA

The vitamin content of samples measured by HPLC, pH, and TA were analyzed by analysis of variance with a split design to show the effects of storage temperatures, time, replications and their interactions. The data from the sensory triangle tests were also statistically analyzed to determine whether there was a significant difference or overall performance among the samples when the buttermilk was fortified.

CHAPTER IV

RESULTS AND DISCUSSION

I. EVALUATION OF EXTRACTION PROCEDURE

The precision of the extraction procedure was tested by analyzing replicate subsamples of one buttermilk sample for vitamin A palmitate and vitamin D_3. Duplicate HPLC runs were made on each of six samples taken from the same quart of buttermilk stored 18 days. A mean value was calculated for each of the six extractions. An analysis of variance showed that the variation in recovery procedures was not significantly different at the 0.05 level among extractions (Table 8). The mean value of the six extractions showed a coefficient of variation of 2.73 percent for Vitamin D_3 and 0.88 percent for vitamin A palmitate. This suggested a method by which the correction factor due to extraction loss could be calculated, and it indicated that the extraction procedure, or recovery, was consistent within instrumental error.

An estimation of vitamin concentration was made prior to the addition of starter culture to the milk. The correction factor index was determined upon comparison of these concentrations to vitamin A palmitate and vitamin D_3 standards containing the same amount of vitamins as originally added to the milk and were injected daily to compensate for unpredictable variation in the HPLC.

II. PEAK IDENTIFICATION AND QUANTITATION

The chromatograms for vitamin A palmitate and vitamin D_3 standard solutions were obtained under the same conditions as the test sample.

Table 8

Analysis of Variance for Precision of the Single Extraction Procedure Used in the Recovery of Vitamin A Palmitate and Vitamin D_3 from Fortified Buttermilk Stored 18 days

		Vitamin A Palmitate		Vitamin D_3	
Source	DF	Mean Square	F-Ratio	Mean Square	F-Ratio
Extraction	5	1500.68	1.32^{ns}	409.93	2.04^{ns}
Duplicates	6	1133.59		200.67	
Total	11				
Mean			638.58±5.61		212.17±5.78
Coefficient of Variation			0.88%		2.73%

^{ns}Not significant at the 0.05 level.

Ten injections of the standard solutions were made and the retention times of vitamin A and D were recorded (Table 9). Vitamin A palmitate had a mean retention time of 2.84 minutes. The retention times ranged from 2.58 minutes to 3.01 minutes. Vitamin D_3 was eluted at a mean value of 7.46 minutes. The retention times ranged from 7.45 to 7.48 minutes. The coefficients of variation for retention times were 0.50 percent for vitamin A and 0.80 percent for vitamin D. From the results, the retention time of vitamin A palmitate and vitamin D_3 peaks proved to be reliable indicators for vitamin identification.

To illustrate the specificity of the method, the retention times for compounds which could interfere with vitamin identification were determined. Trans-retinol, which is similar in structure to vitamin A palmitate, was analyzed but produced a peak well separated from vitamin A palmitate. Of the solvents tested, only ethyl acetate was found to be positioned in close proximity to a vitamin. Ethyl acetate was used in the elution of the vitamins and interfered with the peak of vitamin A palmitate. Therefore, ethyl acetate was removed from the samples under nitrogen gas. If present, the concentration would be very low and a method was devised to correct for the effect on the vitamin A palmitate peak. An unfortified sample was subjected to the vitamin extraction procedure and evaporated. The sample was then analyzed for the solvent (ethyl acetate) peak height which gave a value to be subtracted from the vitamin A peak in fortified samples.

The procedure was standardized by adding different known concentrations of vitamin A and D standard solutions to skim milk. After analysis, the peak heights were plotted vs. known concentrations of

Table 9

Reproducibility of Retention Times of Vitamin A Palmitate and Vitamin D_3 Standards

Run No.	Vitamin A Palmitate Retention Time	Vitamin D_3 Retention Time
	min.	
1	3.01	7.45
2	3.00	7.48
3	2.58	7.45
4	3.01	7.45
5	2.58	7.45
6	2.59	7.48
7	3.01	7.45
8	3.02	7.45
9	3.01	7.48
10	2.58	7.45
Mean	2.84±0.22	7.46±0.017
Coefficient of variation	0.05%	0.80%

the vitamin solutions. The same concentrations of the vitamin standards were also made in hexane and injected directly. A linear relationship between peak heights and the known vitamins concentration was found over the range of 0.002 - 0.014 mg/g for vitamin D and 1.04 - 1.16 mg/g for vitamin A (Figure 2 and 3). The lower values obtained for the milk sample peak heights as compared to the directly injected standard peak heights with the same concentration were thought to be due to the extraction procedure as well as the instrumental change. After corrections, a mean value of peak height was indicated to be a reliable measure for determination of vitamins per injection of unknown sample. A chromatogram obtained for cultured buttermilk extract to which a known amount of vitamin A and D had been added is shown in Figure 4.

III. STABILITY STUDY

The effects of temperature and storage time on the stability of vitamin A palmitate and vitamin D_3 as determined by analysis of variance are presented in Table 10.

A large difference was noted between the amounts of vitamin A palmitate and vitamin D_3 added prior to storage and the amounts remaining upon completion of the storage period. The vitamin losses during storage of the buttermilk varied in magnitude and were significantly affected by time and temperature of storage.

The length of the storage time was found to be highly significant. The temperature of storage was found to be significant to the 0.01 level. These findings correlated well with research done previously by others (29).

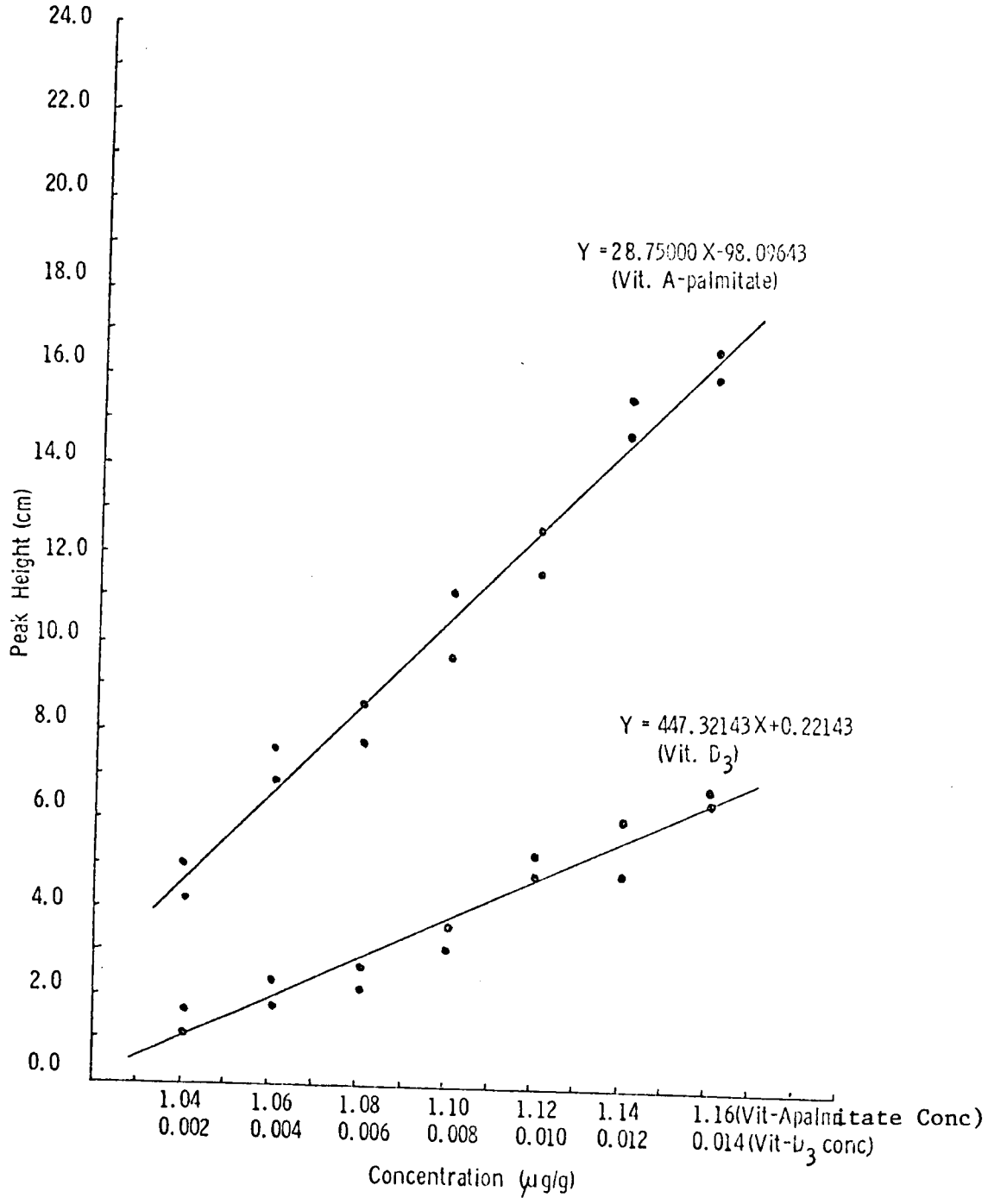


Figure 2. Linear dynamic range of vitamins A palmitate and D_3 extracted from skim milk.

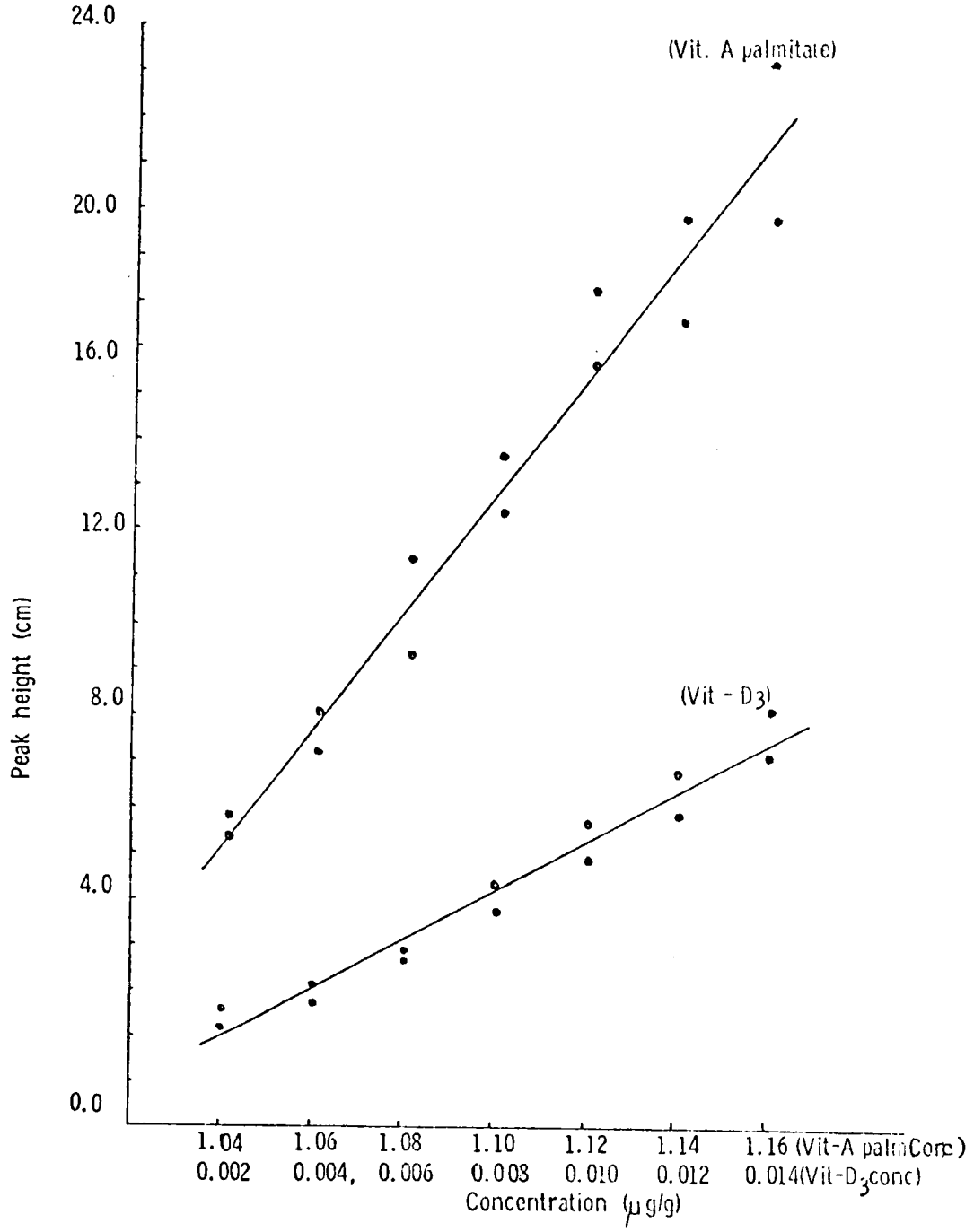


Figure 3. Linear dynamic range of vitamins A palmitate and D_3 standards.

Operating conditions:

Instrument:	Water Associate HPLC
Column:	μ Bondapak/C18
Mobile phase:	50% Acetonitrile/50% Methanol
Program:	Isochratic elution
Flow rate:	1.0 ml/min
Detector:	Model 440 UV abs (254nm) at 0.05 AVFS

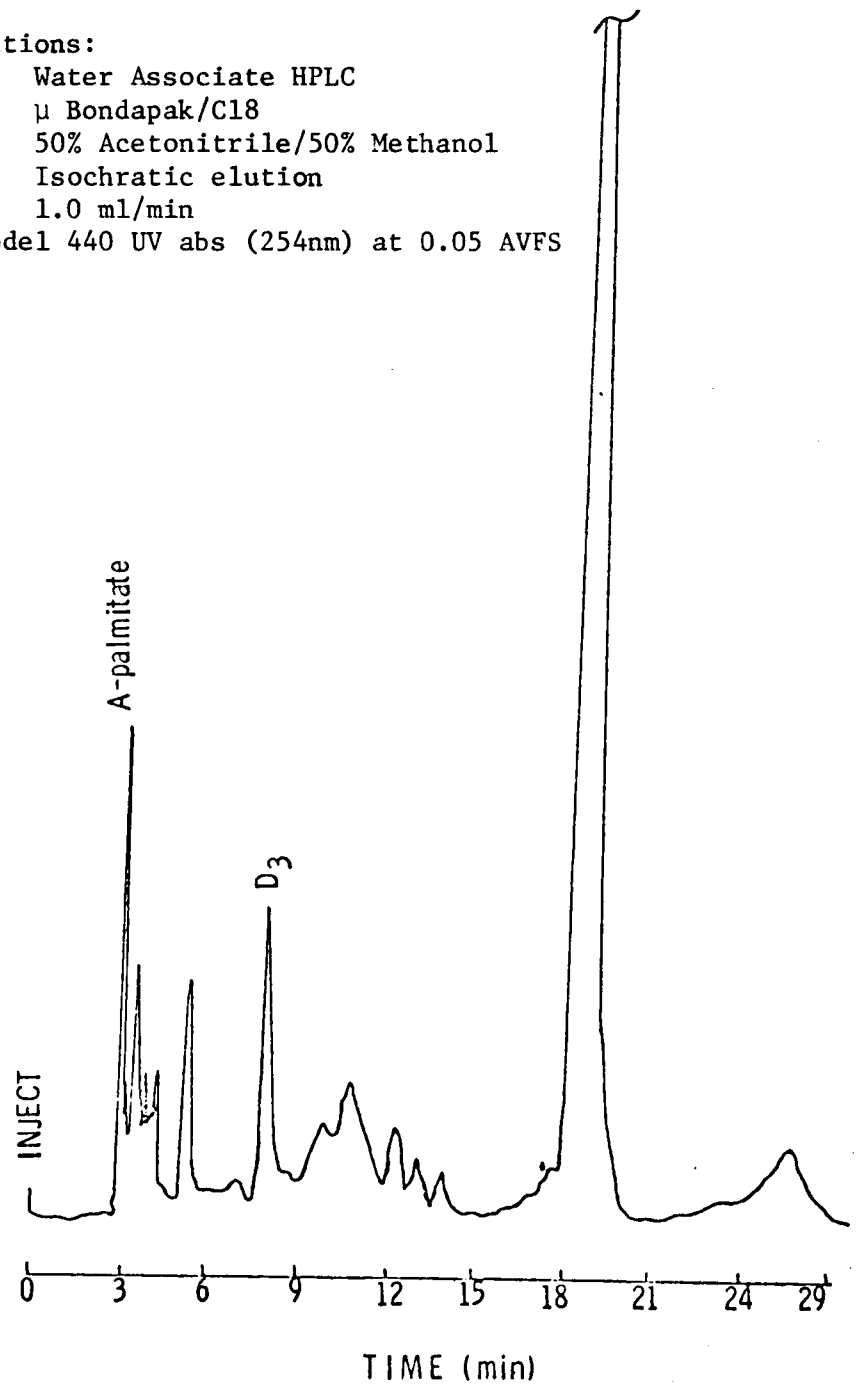


Figure 4. Liquid chromatogram of vitamins A palmitate and D_3.

Table 10

Analysis of Variance of the Effect of Time and Temperature on Stability of Vitamin A Palmitate and Vitamin D_3

		Vitamin A Palmitate		Vitamin D_3	
Source	DF	MS	F[1]	MS	F[1]
A. Time	7	268277.39	80.94***	5180.27	35.56***
B. Temperature	1	135850.78	40.99**	1225.13	8.41*
C. Replication	1	40826.53	12.32**	2211.13	15.18**
C x B	1	1023.78	0.31ns	561.13	3.85*
C x A	7	32566.25	9.83**	762.70	5.23*
B x A	7	3643.64	1.10ns	163.55	1.12ns
Residual	7	3314.50	29.03	145.70	13.37
Total	31				

[1]* Significant at the 0.05 level.

** Significant at the 0.01 level.

*** Significant at the 0.001 level.

ns Not significant at the 0.05 level.

It was determined, as had been in this study, that as storage time and temperature increased, vitamin D_3 concentration and to a greater extent vitamin A palmitate concentration were markedly reduced. The higher storage temperature gave the highest percentage of loss in both vitamins.

The mean values for concentration and the percent retention of vitamin A palmitate and vitamin D_3 stored at 21 days at 4 and 10° C are presented in Table 11. The mean values for concentration of vitamin A palmitate in samples stored at 4° C ranged from 1440.5 IU/quart (Day 0) to 793.5 IU/quart (Day 21). The concentrations for vitamin A palmitate stored at 10° C ranged from 1434.0 IU/quart (Day 0) to 585.0 IU/quart (Day 21). The change in vitamin concentration from day to day over the storage period was found to be significant to the 0.01 level for samples hold at both 4 and 10° C.

The mean values for concentration of vitamin D_3 (Table 11) in samples stored at 4° C ranged from 359.0 IU/quart (Day 0) to 304.0 IU/quart (Day 21). The concentration in samples stored at 10° C ranged from 362.5 IU/quart (Day 0) to 283.0 IU/quart (Day 21). The change in vitamin concentration over the period of storage at both 4 and 10° C was found to be significant to the 0.05 level.

When the sum of squares for this effect was partitioned to determine the type of variation, the linear effect was found to be predominant for both vitamin A palmitate and vitamin D_3 stored at 4 and 10° C.

The vitamin A palmitate concentration after 21 days of storage at 4° C was determined to be two to three times lower than the initial

Table 11

Stability of Vitamin A Palmitate and Vitamin D_3 in Buttermilk as a Function of Storage Time and Temperature

Storage Days	Vitamin A Palmitate				Vitamin D_3			
	Concentration[1]		Retention[2]		Concentration		Retention[2]	
	4° C	10° C	4° C	10° C	4° C	10° C	4° C	10° C
	----IU/qt-------		------% -------		---IU/qt------		--- % ------	
0	1440.5[a]	1434.0[a]	72.0	71.7	359.0[a]	362.5[a]	89.8	90.6
3	1356.5[a]	1176.0[b]	67.8	58.8	319.0[abc]	305.5[b]	79.8	76.4
6	1284.5[a]	1170.5[b]	64.2	58.5	329.5[ab]	316.5[b]	82.4	79.1
9	989.0[bc]	883.0[c]	49.5	44.2	284.5[bcd]	284.5[bc]	71.1	71.1
12	1023.0[b]	889.5[c]	51.2	44.5	253.5[b]	246.5[cd]	63.4	61.6
15	986.0[bc]	831.0[c]	49.3	41.6	294.5[bcd]	284.0[bc]	73.6	71.0
18	846.5[bc]	708.0[cd]	42.3	35.4	275.0[cd]	237.5[d]	68.8	59.4
21	793.5[c]	585.0[d]	39.7	29.3	304.0[bc]	283.0[bc]	76.0	70.8
Means	1089.9	959.6	54.5	48.0	302.4	290.0	75.6	72.5

[1]Mean value of duplicate determinations.

[2]Buttermilk initially fortified with 2000 IU/qt vitamin A Palmitate and 400 IU/qt vitamin D_3.

[abcde]Mean values within the same column followed by the same letter are not different at the 0.01 level.

concentration. When stored at 10° C, the vitamin concentration was reduced to a level three to four times lower than the initial concentration. The reduction in vitamin A levels occurred suddenly after the fermentation of the milk started. The concentration of vitamin D_3 stored 21 days at 4° C showed a decrease only one to one and one-half times lower than the initial concentration. The reduction during storage at 10° C indicated about the same. These results agree with other investigators who reported that vitamin D_3 generally is more stable than vitamin A (29).

IV. pH AND TITRATABLE ACIDITY

The analysis of variance for pH and titratable acidity values (Table 12) indicated a significant difference between the pH and titratable acidity of fortified and unfortified buttermilk samples held under the same conditions of storage. Storage time and storage temperature individually had significant effects on the titratable acidity and pH of both fortified and unfortified samples. The interaction of storage time and temperature had a significant effect on pH at the 0.05 level while having no significant effect on titratable acidity. The interaction of time and sample treatments were found to be significant to the 0.05 level for pH but not for titratable acidity. This would indicate that the sample pH and conditions whicch influence pH are perhaps the important factors in the stability of vitamin A palmitate and vitamin D_3. The pH decline was also detected by taste panelists in the sensory evaluation of the product.

The mean values of pH and titratable acidity (TA) for buttermilk stored over 21 days at 4° and 10° C are presented in Table 13. Fortified samples stored at 4° C varied in pH from 4.35 to 4.59.

Table 12

Analysis of Variance of pH and Titratable Acidity in Vitamin Fortified and Unfortified Buttermilk as a Function of Storage Time and Temperature

Source	DF	pH		Titratable Acidity[2]	
		MS	F[1]	MS	F[1]
A. Time	7	$33642X10^{-6}$	28.12^{***}	$18803X10^{-6}$	176.25^{***}
B. Temperature	1	$12100X10^{-6}$	10.11^{**}	$2500X10^{-6}$	23.43^{***}
C. Treatment	1	$21756X10^{-6}$	18.18^{***}	$6006X10^{-6}$	56.30^{***}
D. Replication	1	$5625X10^{-6}$	4.70^{*}	$1225X10^{-6}$	11.48^{**}
A x B	7	$3093X10^{-6}$	2.59^{*}	$125X10^{-6}$	1.17^{ns}
A x C	7	$921X10^{-6}$	0.77^{ns}	$188X10^{-6}$	1.77^{ns}
A x D	7	$1497X10^{-6}$	1.25^{ns}	$436X10^{-6}$	4.08^{**}
B x C	1	$7225X10^{-6}$	6.04^{*}	$225X10^{-6}$	2.11^{ns}
B x D	1	$7656X10^{-6}$	6.40^{*}	$156X10^{-6}$	1.46^{ns}
C x D	1	$3025X10^{-6}$	2.53^{ns}	$25X10^{-6}$	0.23^{ns}
Residual	29	$1196X10^{-6}$	8.15	$107X10^{-6}$	40.53
Total	63				

[1]* Significant at the 0.05 level.

** Significant at the 0.01 level.

*** Significant at the 0.001 level.

ns Not significant at the 0.05 level.

[2] Expressed as percent lactic Acid.

Table 13

Mean Values for pH and Titratable Acidity for Fortified and Unfortified Buttermilk as a Function of Storage Time and Temperature

Storage Day	Fortified Samples pH[1] 4° C	pH[1] 10° C	TA[1] 4° C	TA[1] 10° C	Unfortified Samples pH[1] 4° C	pH[1] 10° C	TA[1] 4° C	TA[1] 10° C
0	4.50	4.59	0.76	0.76	4.63	4.63	0.73	0.74
3	4.55	4.49	0.81	0.83	4.61	4.54	0.77	0.81
6	4.53	4.46	0.83	0.84	4.57	4.51	0.81	0.82
9	4.50	4.44	0.85	0.87	4.56	4.46	0.84	0.86
12	4.45	4.43	0.87	0.88	4.54	4.46	0.85	0.87
15	4.48	4.46	0.88	0.88	4.51	4.47	0.87	0.88
18	4.44	4.46	0.88	0.90	4.47	4.43	0.87	0.88
21	4.35	4.42	0.90	0.91	4.43	4.42	0.87	0.88

[1]Values are means of duplicate samples.

Fortified samples stored at 10° C varied in pH from 4.42 to 4.59. The pH and TA data were analyzed and regression coefficients estimated (Tables A1 and A2, Appendix). These were used to generate prediction curves which are shown in Figures 5, 6 and 7. A steady decline in pH was noted, particularly in the fortified samples held in 10° C, until day 12 of storate whereupon this trend became inconsistent. Unfortified samples stored 21 days at 4° C varied in pH from 4.43 to 4.63. Unfortified samples stored at 10° C varied in pH from 4.42 to 4.63. Unfortified samples held at 4° C showed a consistent decline in pH over the storage period. Unfortified samples held at 10° C, however, showed a decline in pH to day 12 after which the pH levels no longer followed this trend. This same phenomenon was noted in fortified samples held at 10° C.

The inconsistency of pH readings for fortified and unfortified samples held at 10° C after 12 days of storage could be due to bacteriological action.

TA for fortified samples at 4° C ranged from 0.76 to 0.90 percent lactic acid. TA for fortified samples at 10° C ranged from 0.76 to 0.91 percent lactic acid. Unfortified samples held at 4° C showed a TA ranging from 0.73 to 0.87 percent lactic acid. When held at 10° C the range was 0.74 to 0.88 percent lactic acid. The TA of all samples increased during storage. The decrease in pH and the increase in TA during storage of both fortified and unfortified samples was more rapid at 10° C than 4° C.

V. ORGANOLEPTIC EVALUATION

Samples of buttermilk were presented to sensory panels in a triangle test to assess differences between fortified and control samples

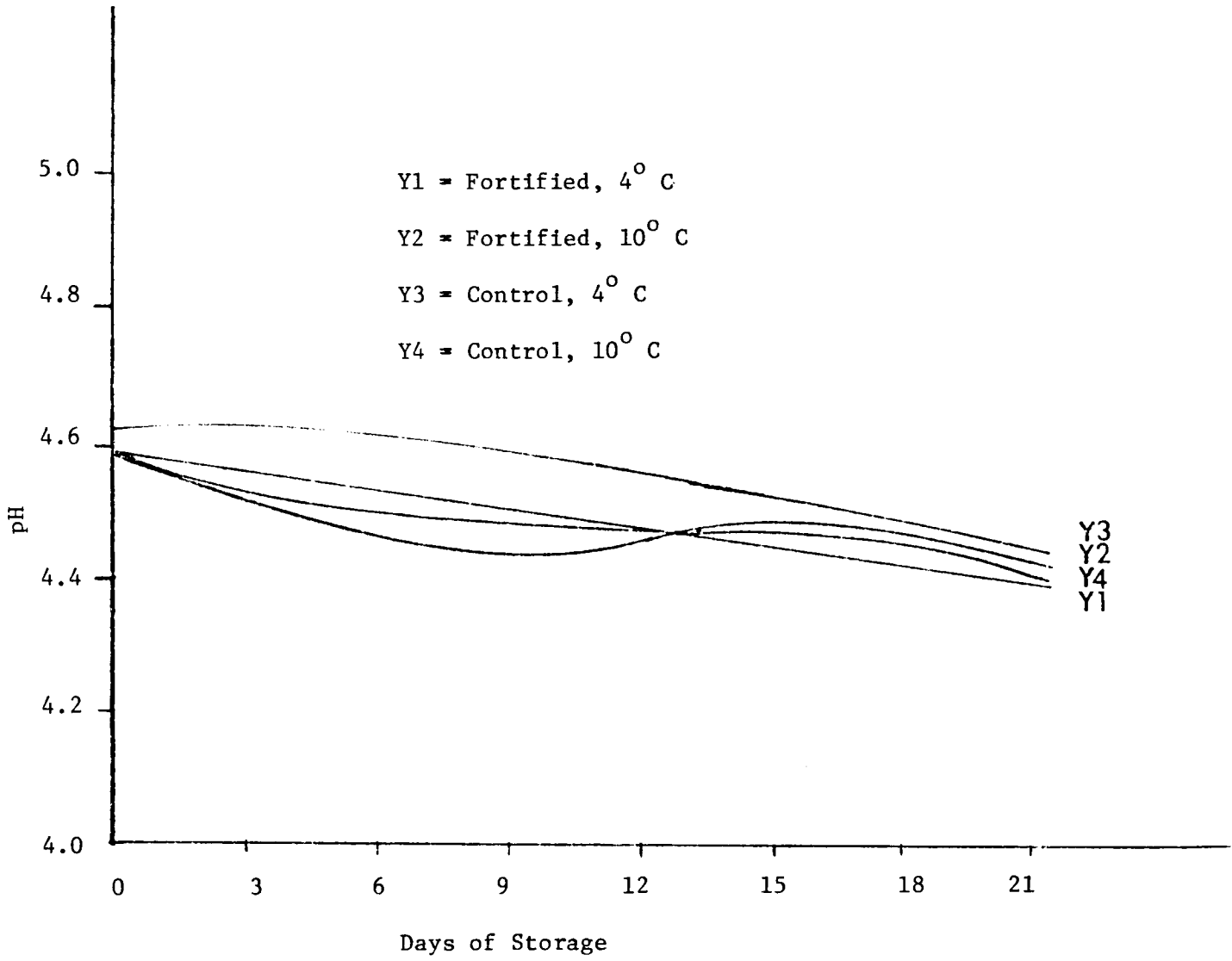


Figure 5. Computer predicted values of pH in buttermilk developed upon storage.

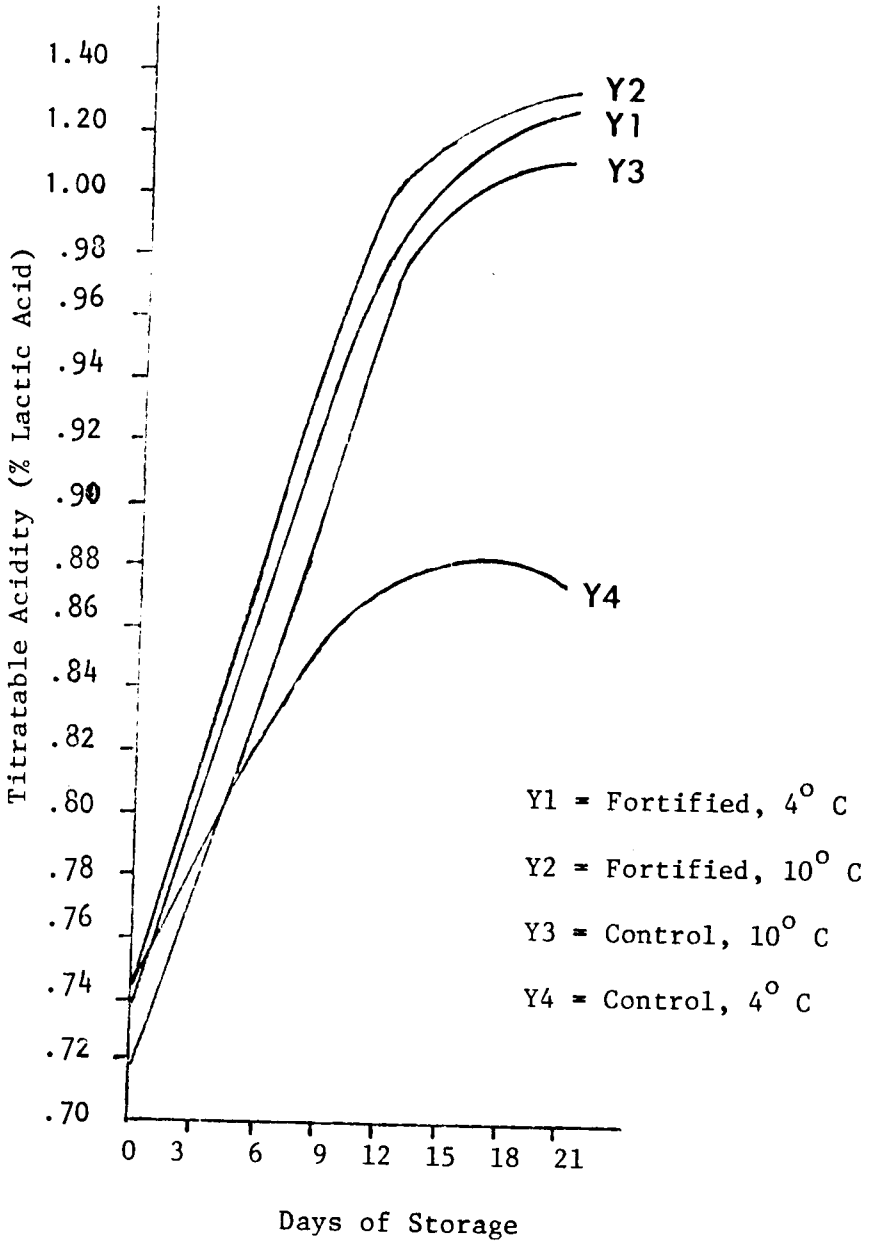


Figure 6. Computer predicted values of titratable acidity in buttermilk developed upon storage. Replicate 1.

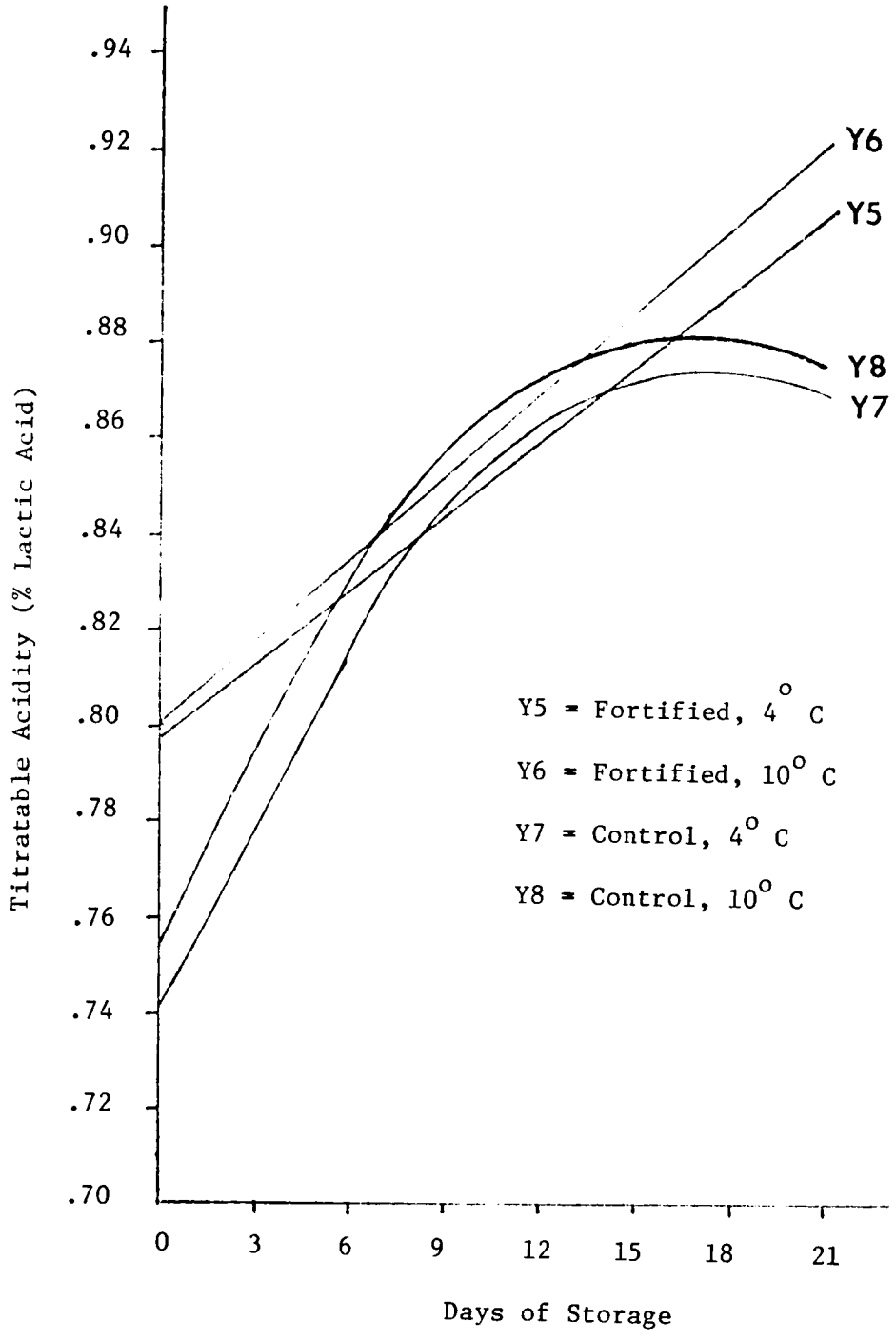


Figure 7. Computer predicted values of titratable acidity in buttermilk developed upon storage. Replicate 2.

over the storage period. No comparisons were made between temperature of storage.

The results of organoleptic analyses on the seventh day of storage at both 4 and 10° C indicated that there was no significant difference between vitamin fortified and unfortified buttermilk samples to 0.05 level. The overall acceptability was judged to be "fair" for all the samples.

After 14 days of storage, the flavor panel responses revealed that panelists detected off-flavors such as higher acidity, bitterness, and rancidity when they were asked to evaluate the samples stored at 10° C. This was believed to be due to psychortrophic bacteria which are known to grow at 10° C sufficiently to affect flavor during storage. These bacteria produce bitter, fruity, stale, and putrid types of off-flavors. Such contaminating microflora may have been responsible for the unfavorable flavor which developed. The samples stored at 10° C were not presented to panelists after the 14th day.

Sensory data for samples stored at 4° C over 21 days revealed no significant difference in flavor due to addition of vitamins (Table 14). Therefore it can be concluded that fortification would be very unlikely to introduce flavor variation in buttermilk. The summary of the overall quality of samples is given in Table 15.

VI. DISCUSSION

In spite of the economic and nutritional significance of buttermilk, it has long been a neglected product by the dairy industry as a

Table 14

Sensory Evaluation of Buttermilk by Triangle Test for Effects of Vitamin Addition, Temperature, and Storage Time

Days Storage	Replicate	Panelists	Correct Choices	Difference[1]	Preference
		No			
0	1	33	6	no	none
	2	29	9	no	none
7	1	25	11	no	none
	2	27	7	no	none
14	1	28	10	no	none
	2	31	13	no	none
21	1	22	7	no	none
	2	24	11	no	none

[1]Determined at 5 percent level of significance.

Table 15

Flavor Description of Fortified and Unfortified Samples as Perceived by Consumer Panel After 0, 7, 14, and 21 Days of Storage at 4° C and 10° C

Days of Storage	Fortified Samples 4° C	Fortified Samples 10° C	Unfortified Samples 4° C	Unfortified Samples 10° C
0	Mild Pleasant Thin	Acid Sharp Weak	Mild Natural Buttery	Acid Flat
7	Acid Salt Sour	Acid Chalky Lumpy	Acid Strong Salt	Acid Flat Other
14	Acid Pungent Thin Weak	Acid Rancid Putrid Bitter	Acid Flat	Cooked Acid After taste Lumpy
21	Acid Thin Flat	Putrid Acid Rancid Oxidized Bitter Cooked Lumpy Other	Acid Flat	Acid Cooked Lumpy Pungent Unpleasant

result, by the consumer in fact, buttermilk has received the lowest priority in the product support plans of the United States Dairy Industry Association (1). Consumers seem to view buttermilk as a dairy industry by-product, lacking in quality and wholesomeness (2). The segment of the population to whom buttermilk would be the most advantageous, older and low income groups, are often those who are deficient in the nutrients buttermilk would supply (2,3). Buttermilk is more easily digestable, by older, milk intolerant individuals and since skim milk forms the basis of the product, it contains low fat and cholesterol. Buttermilk is also less expensive than milk.

The attention of this study was directed toward developing a vitamin A and D fortified cultured buttermilk to compensate for the deficiency of these important fat soluble vitamins in the buttermilk skim milk base. The changes in the stability of vitamin A and D corresponding to the changes in pH and TA during 21 days of storage at 4 and 10° C were investigated to determine the feasibility of such a fortification procedures.

The first step in this investigation was to select a rapid, convenient method for the determination of vitamin A and D. Present chemical and biological methods require tedious and time consuming extractions and purification prior to chromatography.

Recent advances in HPLC instrumentation and column packing have enabled separation and determination of fat-soluble vitamins. Williams et al. (1972) resolved these vitamins by using reverse phase partition chromatography and a permaphase ODS Column. Vitamins A and D were also resolved at Dupont by using the Zorbas ODS Column at 50° C (53).

The VYdac 201 TP reverse phase Column and acetonitrile-methanol (50:50) mobile phase have also been successfully employed. No significant interference has been reported between vitamin A and the separation of vitamin D by this methods.

In order to develop a chromatographic system for this study, several solvent systems of differing proportions were compared during preliminary trials. Acetonitrile-methanol (50:50) mobile phase was selected for better resolution and retention time. A variety of HPLC columns can be used to separate vitamin A and D. Some provide more resolution than others. After comparison of several available types, the reverse phase μ-bandapak C18 Column was chosen.

The application of a reverse phase liquid chromatographic system had several advantages over normal phase separation. The major advantage is the decreased effect of water on the surface active sites of chemically bonded supports. It has been suggested that water is omnipresent in each eluent and influences more or less the retentions on polar stationary phases like silica (57). Commercially available "pure solvents" contain significant but variable quantities of water. The use of a reverse phase separation system minimizes the influences of water in two ways: (1) the surface activity of the reverse phase HPLC Column is much less than that of a normal phase silica column and since (2) hydrocarbon eluents are normally not used in this type of system, equilibration of water in the eluent and water absorbed onto the stationary phase is rapid. For all of these reasons one would, therefore, expect more reproducible separations.

Finally, highly polar lipids present in milk can be retained on a silica column and damage the column. Since polar compounds are not readily retained on reverse phase columns, minimum deterioration of the HPLC should be expected. However, the condition of the column is a critical factor for repeatable and consistent results. To prolong the life of the reverse phase HPLC column, it is suggested that a precolumn filled with a larger particle size packing be used and that the column be routinely purged with appropriate solvents. This can be done either by solvent programing, using a gradient from 1 percent acetonitrile to 99 percent acetonitrile, or by separate isocratic runs, using pure acetonitrile and pure methanol.

One of the most important steps in vitamin A and D measurement was the extraction of these vitamins from other components. This was particularly true when a non-specific method was used for vitamin extraction from buttermilk. Methods have been published for extracting tablets and concentrates containing gelatin stabilized vitamin A and D without alkaline digestion (57). Dimethyl sulfoxide (DMSO) has been used to solubilize vitamin A in some concentrates but there are no published reports on trials using DMSO with foods containing vitamins A and D.

When sampling, if the vitamin A and D emulsion is uniformly distributed in the milk during the mixing procedure, the process becomes simplified and only 10 g samples of the well mixed material are required for the analysis. Uniformity can be checked by comparing results on a number of replicates from the same blended sample. If results are erratic, larger samples may correct the problem.

All stages of the analysis were required to be carried out rapidly to minimize vitamin A and D exposure to air, bright light, and heat, especially when not in solution. During analysis small quantities of vitamin A and D may be lost due to oxidation, unextracted residues, transfer, and the chromatographic procedure. Most importantly, vitamin stability is dependent on the interaction of temperature and time. The extraction loss may be corrected. However, large corrections cannot be made with confidence. It has been suggested that the corrections for losses in the analysis of vitamins A and D in food should be less than 7 percent as determined by collaborative tests (54). The results obtained by the method adopted should be reproducible with good accuracy and precision. In analyzing vitamin A in foods a coefficient of variation of less than 8 percent as an index of precision is suggested by collaborative tests (54,57). In this study, during the development of an extraction procedure for vitamin A and D in buttermilk, these suggested limits were not consistently achieved. One reason for this was low vitamin A and D content of samples as compared to levels in pharmaceuticals and concentrates where better analytical accuracy and precision are often reported. However, such limits are present goals to work toward.

An important consideration in HPLC is the number of vitamin A and D samples to be analyzed per day or week. How long can the production-line operators wait on analytical results? The considerable cost for HPLC and related hardware can be justified only if it is used frequently.

If the HPLC must be programmed for different types of analysis, changeover and preparation time as well as column clean-up time between series of samples must be considered.

HPLC offers several advantages over the vitamins A and D assays currently in use but at this time, the application of HPLC to analysis of vitamin A and D in foods is in the early stages of development. With further trials and experience, it may become a satisfactory method for use by those who wish to utilize it as an alternative to the present official method.

CHAPTER V

SUMMARY AND CONCLUSIONS

This study was undertaken to investigate the feasability of fortifying cultured buttermilk with vitamins A and D. Toward this end, an analytical procedure was developed in order to study the effects of changes in such a fortified product. This included a method of vitamin A and D extraction from buttermilk and an HPLC procedure for analysis of the added vitamins.

Stability of vitamin A palmitate and vitamin D_3 in buttermilk was monitored through the development of pH and TA and the changes in vitamin concentration over a 21 day storage period at 4 and 10° C. The means, standard deviation, and analysis of variance of vitamin concentration were computed to determine the effect of storage time and temperature. The effect of fortification, storage time, and temperature on buttermilk pH, and TA was also statistically evaluated. Fortification was found to have a significant effect on pH and TA. Time, temperature and replication were found to have significant effects on vitamin concentration as well as pH and TA.

The results of the vitamin stability determination indicated more than 54 percent of the vitamin A palmitate added to the buttermilk was recovered after 21 days of storage at 4° C and 48 percent was recovered upon storage at 10° C. Approximately 75 percent of the vitamin D_3 added to the buttermilk was recovered after 21 days of storage at 4° C and about 72 percent was recovered upon storage at 10° C.

Sensory evaluation of fortified buttermilk samples indicated no significant difference between fortified and unfortified samples stored under the same conditions.

Further research is necessary in order to standardize a method for extraction and determination of vitamins A and D in buttermilk. Work in the area of vitamin stability during storage of the product would appear to be necessary. This would include development of protective packaging, addition of stabilizers, and uniform manufacturing practices.

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APPENDIX

Table A1

Regression Equation for pH

$Y1 = 4.5875 - 0.00964T$	$r = 0.90826$
$Y2 = 4.58969 - 0.04137T + 0.00357T^2 - 9,44531X10^{-5} T^3$	$r = 0.97780$
$Y3 = 4.62583 - 0.00579T - 0.00015 T^2$	$r = 0.98854$
$Y4 = 4.62863 - 0.03414T + 0.00240 T^2 - 5.98578X10^{-5} T^3$	$r = 0.97688$

Table A2

Regression Equation for TA

$Y1 = 0.7425 + 0.0166667T - 0.00047619T^2$	$r = 0.95835$
$Y2 = 0.749583 + 0.0175198T - 0.00052249T^2$	$r = 0.96282$
$Y3 = 0.720833 + 0.014960T - 0.0003836T^2$	$r = 0.99374$
$Y4 = 0.7416667 + 0.016905T - 0.000503T^2$	$r = 0.93381$
$Y5 = 0.7975 + 0.00524T$	$r = 0.96478$
$Y6 = 0.8000 + 0.00583T$	$r = 0.95366$
$Y7 = 0.78875 + 0.0158532T - 0.0004696T^2$	$r = 0.99312$
$Y8 = 0.7529167 + 0.0154564T - 0.0004696T^2$	$r = 0.99644$

VITA

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