A GAS CHROMATOGRAPHIC STUDY OF THE FLAVOR DETERIORATION IN HIGH-TEMPERATURE-SHORT-TIME FLUID STERILE MILK

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ABSTRACT

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by James R. Kirk

A study has been made of the flavor deterioration that takes place in high-temperature-short-time (HTST) fluid sterile milk during storage at 4.4°, 22°, and 36°C. for three months. Fluid milk was sterilized in the commercial DeLaval Vacu-Therm-Instant-Sterilizer unit at a temperature of 140.5°C. ± 1°C. for approximately 4.0 seconds; the milk samples were then collected in previously sterilized glass containers in an aseptic atmosphere.

Body defects that developed in the fluid sterile milk during storage were cream layer formation, age-thickening, and sedimentation. All sterile milk samples showed the presence of sedimentation by one month of storage. Age-thickening, an incipient sign of gelation, was observed in the 22° and 36°C. storage samples by two months of storage, but was not evident in the 4.4°C. storage samples until three months. Cream layer formation was found to occur after only one month in the storage samples held at 22° and 36°C. This body defect was not evident in the 4.4°C. samples after three months storage.

Organoleptic evaluation, which was carried out during the storage of the HTST fluid sterile milk, indicated that stale flavor was present in the milk after two months of storage at 22°C. This sensory evaluation also indicated that the rate of product staling was a function of the temperature at which the product was stored, since fluid sterile milk was judged slightly stale after only one month of storage at 36°C. Separation of the flavor components, which had been isolated from the HTST fluid sterile milk by low-temperature, reduced-pressure distillation, was accomplished by gas chromatography using a 1/4 inch O.D. x 6 foot packed column and a dual hydrogen flame detector. The flavor components that were tentatively identified in the sterile milk control samples (zero time storage) by retention time and functional group analysis data were: ethanal, propanal, furfural, ethanol, butanol, acetone, and 2-butanone. Storage samples yielded in addition to the aforementioned: 2-pentanone, 2-heptanone, butanal, hexanal, and heptanal. Also, it was observed that the fluid sterile milk samples that had been judged stale by organoleptic evaluation exhibited a 30-50% increase in acetone concentration, as well as a definite increase in all of the tentatively identified carbonyl compounds.

Confirmation of some of the flavor compounds tentatively identified by the gas chromatography was made by thin-layer chromatographic separation of their 2,4 dinitrophenylhydrazones. These hydrazone derivatives were first separated on a Magnesia-Celite 545 column according to homologous classes prior to thin-layer separation. Methyl ketones, saturated alkanals, and 2,4 dienals were the three classes identified on the absorption column; however, confirmatory TLC could only be carried out on the methyl ketones and saturated alkanals. These data confirmed the presence of ethanal, acetone, and 2-butanone in the control samples, while ethanal, butanal, hexanal, acetone, 2-butanone, and 2-heptanone were isolated from the three months 22°C. storage samples.

Analysis of the data collected from both gas and thin-layer chromatography indicates that the stale flavor is probably due to the synergistic interaction of these tentatively identified compounds with those developing during product storage playing a more definite role in the stale flavor.

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INTRODUCTION

The importance of milk as an article of food in the human diet existed from the time of recorded history as can be attested by the ancient Sanskrit writings of India. Dairy conditions from these ancient times until 1850 showed little change either in the methods of production or manufacturing; thus each family depended upon privately owned cattle for its supply of milk and dairy products. The process of pasteurization was developed by Pasteur in 1864, and a few years later Jacobi and Soxhlet, realizing the value of pasteurization as a public health safeguard and a means of increasing the self-life of milk, advocated the pasteurization of milk. By the early 19th century pasteurization was common practice in America and Europe.

Although pasteurization is effective in destroying the pathogenic bacteria, and the bulk of the non-pathogens in milk, it does not preclude bacterial spoilage. Thus a new processing method, milk sterilization, was developed as a means of increasing the shelf life of dairy products by elimination of bacterial spoilage. Sterilization has proved effective against bacterial deterioration; however, the resultant sterile products have a flavor which is much less desirable than that of normally processed products. Storage of these sterile products for prolonged time periods results in further flavor deterioration characterized as "stale" or "stale-oxidized". The stale or stale-oxidized flavor defect has been shown to arise in part from the deterioration of the milk lipids.

Webb, in 1963, commented on the formidable task of obtaining an acceptable flavor in sterile dairy products. He stressed the fact that both the stale-oxidized, and the cooked-carmelized flavors, that now develop in commercially sterilized products must be eliminated before their markets can be fully exploited.

To accomplish the task of maintaining normal product flavor, researchers have concluded that the following three steps must be achieved: a) all compounds contributing to off-flavor must be detected, b) origins of off-flavor compounds must then be elucidated, and c) steps taken to prevent formation of these off-flavor compounds.

Flavor chemists feel that attainment of these goals ultimately lies in the elucidation of the compounds that contribute to the natural falvor of the fresh product. Utilizing this information as a common reference point, research can then be directed to the problem of off-flavor detection and analysis.

It is the intention of this research, with the aid of some of this previously secured information on the flavor chemistry of dairy products, to shed some light on the problem of stale-oxidized, or as it is more commonly known storage flavor, which is a common defect of heat-processed dairy products upon storage. The primary concern of this research is the study of the development of off-flavors in high-temperature, short-time (HTST) processed fluid sterile milk under storage conditions of refrigerated, ambient, and tropic temperatures.

REVIEW OF LITERATURE

Flavor Defects Associated with Sterile Dairy Products

The most common flavor defect associated with fluid sterile milk is the stale or stale-oxidized flavor that usually manifests itself between the second and third month of product storage at 70°F. (Patel et al., 1962). The stale or stale-oxidized flavor defect, although never fully defined, has been studied by Bassette and Keeney (1960), Nawar et al. (1963), Bingham (1964), Arnold et al. (1966), and all have indicated that both the Maillard browning reaction and lipid oxidation may be involved in its formation.

Other off-flavors which detract from the flavor of sterile dairy products are the cooked flavor and the sweet-carmel taste, which are present because of the stringent heating conditions needed for product sterilization (Harland and Ashworth, 1945; Gould and Lomoner, 1939; Hutton and Patton, 1952; and Hodge, 1953).

Thus, if we are to obtain a product that has any future market value, we must find a way to prevent these undesirable flavor deteriorations from occurring whether they be in processing or during storage.

Sample Collection Techniques

Sampling flavor compounds of foods has proven to be a somewhat difficult task because of the relative ease with which artifacts can be incorporated into the sample by the recovery procedure. This is especially true of dairy products, since they easily pick up off-flavors and only small concentrations of some compounds, in some cases parts per billion, are needed to cause these off-flavors. The isolation of flavor components from milk is even more difficult than most foods, since fresh milk normally has a delicate bland taste and aroma. Therefore, it was necessary to first develop recovery techniques capable of concentrating these flavor components to a point whereby they could be detected by present-day scientific instruments, and at the same time assure the original integrity of the compounds responsible for imparting these flavors.

Mabbitt and McKinnin (1963) removed the volatile components of milk by entrainment of the volatiles in a sweep gas and condensed them in a trap cooled by liquid nitrogen. They felt this method caused minimal change in composition of the volatiles. The success of this method depended only on having a pure gas supply in which to entrain the volatiles. Libbey et al. (1963) utilized the technique of steam stripping under reduced pressure for recovering the flavor volatiles associated with the lipid phase in dairy products of high fat content. This method also utilized a fractional collection technique for recovering the volatiles from the distillate by using various trap temperatures ranging from 0°C. to -195.8°C.

Winter et al. (1963) recovered the volatile components of butter by an oxygen-free water emulsification of the butter, followed by a reduced pressure distillation of the emulsion in a falling-film, thin-layer distillation column. This technique had value in that by performing these steps in a medium of nitrogen, atmospheric oxidation of the fat during volatile extraction was eliminated, thus eliminating artifacts due to oxidation. El-Negoumy et al. (1961) utilized a molecular still and Skellysolve B in isolating and concentrating butter-oil volatiles. Patton (1961) suggested a unique solvent extraction technique for recovering flavor compounds in processed milks. This was accomplished by allowing the protein adsorbed on the fat and that of the fat globule membrane to act an an interface and prevent contact between fat and solvent, thereby excluding unwanted fat from the extracting solvent. A reverse-phase, liquid-liquid extraction system for recovering the volatile organic components of citrus juices was developed by Senn (1963). Nawar and Fagerson (1960) employed gas-liquid extraction in a recycling system for recovery of food volatiles, which permitted recovery of high percentages of the flavor volatiles because of minimal losses to the atmosphere.

Bassette et al. (1962) developed a means of studying milk volatiles by sampling headspace gas. Here a 2 ml. sample of the milk to be analyzed was added to a serum vial containing

1.2 gm. of sodium sulfate. The vial was then capped with a rubber septum, shaken for 5 minutes, and heated at 60°C. for 3 minutes. Sampling of the headspace gas was accomplished by inserting the needle of a gas-tight syringe through the rubber septum and removing the desired volume of sample. This procedure enabled the recovery of volatiles at a concentration as low as 1 ppm. Recently, Morgan and Day (1965) have adapted headspace gas sampling to the on-column trapping system developed by Hornstein and Crowe (1962) using nitrogen as the entrainment gas and liquid nitrogen as the cooling medium for condensing the flavor volatiles on the packed column. This eliminated the need of gas transfer systems and the heating of volatile traps; extremely good reproduction is obtained with this method. Bradley and Stine (1962) devised a method for sampling headspace gas from sealed tin containers. The device forms an air-tight puncture in the lid of the container, from which the desired volume of gas is then removed by inserting the needle of a gas-tight syringe through the rubber septum of the sampling device. MacKay and Berdick (1961) also used a method of sampling headspace gas for analysis of flavor volatiles.

Reduced-pressure, low-temperature distillation technique was also developed by Day et al. (1957) for collecting flavor compounds from gamma-irradiated milk. This method utilized the technique of fractional collection of flavor volatiles in wet ice, dry ice-ethanol, and liquid nitrogen traps. Since this technique was adopted as the method of flavor volatile recovery from the HTST fluid sterile milk to be analyzed in this research program, it will be discussed more specifically in the procedure section.

As evidenced by this short review of the more noteworthy volatile collection techniques, numerous and imaginative system designs have been employed in flavor volatile extractions. Although each has attempted to eliminate the artifacts which may involuntarily arise during analysis, no one system to date has been developed which eliminates all of the shortcomings found in our present recovery systems.

Means of Identification of Isolated Components

Gas liquid chromatography provides a suitable means of separation and tentative identification of volatile flavor components by use of retention volume data. To the investigator who is working with an entirely unknown mixture of compounds, these data have limitations, since some components of the mixture, upon gas chromatographic separation, may exhibit the same, or nearly the same retention data, and yet possess an entirely different functional group (Walsh and Merritt, 1964). This problem has been resolved by collecting the gas chromatographic effluents or, as is more recently the case, splitting the sample stream to the gas chromatographic detector, and employing infra-red spectrometry (Billis and Slowinski, 1956; Wheaton and Wentworth, 1959; Chang et al., 1961) mass spectroscopy, or time-of-flight mass spectrometry (Ebert, 1961; Gohlke, 1959; Linderman and

Annis, 1960; McFadden et al,, 1963), for more conclusive identification. Because of the cost of such instrumentation, this type of confirmatory analysis is beyond the reach of most researchers and identification of volatile flavor components must be made by gas chromatographic retention data, coupled with functional group analysis. Thin-layer and paper chromatography have also proved to be invaluable tools in this work.

Functional group analysis is basically a rather uncomplicated technique which consists of reacting aliquots of an unknown chemical mixture with special reagents to confirm or negate the possible presence of aldehydes, ketones, alcohols, esters, mercaptans, amines, and sulfides. Aldehydes can be detected by Schiff's reagent, 2,4 dinitrophenylhydrazine (Bassette et al., 1962; Walsh and Merritt, 1960; Lynn et al., 1956; Badings and Wassink, 1963), ketones by 2,4 dinitrophenylhydrazine, and acid hydroxyl amine (Bassette et al., 1960; and Badings and Wassink, 1963), alcohols by 3,5 dinitrobenzoates, ceric acid (Cheronis and Entrikin, 1957; and Hoff and Feit, 1964), esters by ferric hydroxymate, basic hydroxyl amine, and sodium hydroxide (Walsh and Merritt, 1960; Bassette et al., 1962; Hoff and Feit, 1964), mercaptans by alcoholic silver nitrate, lead acetate, isatin, and sodium nitroprusside (Walsh and Merritt, 1960; and Shriner et al., 1956), and sulfide with mercuric chloride (Bassette et al., 1962).

Thin-layer chromatography has also proved to be an invaluable aid in the identification of the carbonyl components of flavor isolates as shown by Parks et al. (1964), and Badings and Wassink (1963) who have established an extremely refined procedure for the separation of the homologous classes of both aldehydes and ketones. This technique coupled with the method for class separation by absorption-chromatography developed by Schwartz et al. (1962), and Schwartz et al. (1963), has endowed the flavor chemist with a reliable tool for identification of these flavor components. More recently Soukup et al. (1964) accomplished the identification of 2,4 dinitrophenylhydrazones by gas chromatographic analyses. This latter technique is especially useful since derivatives need not be purified and trace amounts of these compounds can be detected.

Recently Merritt and Walsh (1962) developed a new method of functional group classification and subsequent qualitative identification of chromatographic peaks by the use of dual column gas chromatography in which each of the two columns has a different liquid phase column packing. This enables qualitative identification of the peak to be made solely by means of retention volume constants.

Flavor Components of Milk and Milk Products

In the past decade researchers in the field of dairy chemistry have enjoyed much success in isolating and determining natural and off-flavor components in dairy products. The reason for these many advances has been the development of new instrumental techniques.

The majority of the compounds isolated and identified have been carbonyl in nature, and many have been positively linked to certain natural desirable or undesirable flavors. The following is a chronological summary of the known compounds that have been isolated, the worker(s) who have isolated these compounds, and where known, the characteristic flavor with which each has been associated.

Table 1. Chronological review of flavor components isolated from stored dairy products.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
2 hydroxypropanal C_7 and C_9 unsaturated ketones a compound with the empirical formula $C_{12}H_{20-22}O$	Oxidized butteroil	oxidized	Keeney and Doan (1951a, 1951b, 1951c)
C_7-C_9n-alkanals	dry whole milk (old sample)		Shipstead and Tarassuk (1953)
C_4-C_{11} alk-2-enals C_6-C_9 alk 2,4 dienals acetone ethanal n-hexanal crotonaldehyde	oxidized skimmilk	oxidized	Forss et al. (1955a, 1955b)
methyl sulfide	fluid milk	cowy flavor	Patton, Day, and Forss (1956)
delta-decalactone delta-dodecalactone	evaporated and dry whole milk	stale and coconut	Keeney and Patton (1956a, 1956b)
ethanal acetone butanone formaldehyde n-hexanal methyl alcohol	gamma irradiated skimmilk		Day et al (1957)

Table 1. Continued.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
ethyl alcohol methyl mercaptan methyl sulfide			
furfural acetaldehyde	heated skimmilk	stale	Morgan et al. (1957)
acetaldehyde 2-pentanone 2-heptanone	evaporated milk	typical evaporated flavor	Dutra et al. (1959)
2-pentanone 2-heptanone acetone	evaporated milk		Wong et al. (1958)
C_3-C_{15} methyl ketones unheated milkfat- acetone only	heated milk fat	heated	Patton and Tharp (1959)
formaldehyde acetaldehyde acetone butanone methylpropanal 3-methylbutanal furfural diacetyl hexanal nonanal	nonfat dry milk	storage stale	Bassette and Keeney (1960)
C_5-C_{10}n-alkanals C_5-C_{10}alk 2-enals 2,4-heptadienal 2-heptanone	washed cream	oxidized	Forss et al. (1960b)
C_1-C_{10}n-alkanals C_5-C_{11}alk-2-enals acetone all odd number C_5-C_{15}n-alk-2-ones but - 2-enal	autoxidized milkfat	oxidized	Day and Lillard (1960)

Table 1. Continued.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
n-hexanal n-heptanal hex-2-enal 2-heptanone 2-pentanone 2-nonanone 2-undecanone acetone C_3C_5, C_8-C_{10} alkanals C_5, C_7, C_8, C_9 alk-2-enals C_3-C_9alk-2-ones hepta-2,4 dienal	milk fat	fishy flavor	Forss et al. (1960)
acetone C_1-C_{10}n-alkanals C_5-C_{11}alk-enals	milk fat		Day and Lillard (1960)
ethanal methyl sulfide acetone	fluid milk		Wynn et al. (1961)
C_1-C_3, C_5-C_{10}, C_{12}n alkanals C_3-C_9, C_{11}, C_{13}, C_{15} methyl ketones C_5-C_{11} alk-2-enals cis furfural trans furfural benzaldehyde	dry whole milk (badly deteriorated powder)		Parks and Patton (1961)
ethanal acetone 2-pentanone α-pentyl acetate dimethyl sulfide	sterile concentrated milk		Patel et al. (1962)
oct-1-en-3-one	butteroil	metallic	Forss, et al. (1962)
ethanal acetone butanone 3-methyl butanal 2-methyl propanal	raw milk	normal flavor	Wood and Aurand (1963)

Table 1. Continued.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
alk-2-enals alkanals alk-2,4-dienals	milk fat	oxidized	Keith and Day (1963)
C_5-C_{16}n-alkanals C_6-C_{11}alk-2-enals C_3-C_{12}alk-2,4 dienals	butteroil from spontaneously oxidized whole milk		Parks et al. (1963)
formaldehyde propanal ethanal acetone 2-butanone or 2-pentanone a compound similar to heptaldehyde an unsaturated dicarbonyl or hydroxycarbonyl	stale dry whole milk	stale	Nawar et al. (1963)
methyl sulfide	cultured cream or butter		Day and Lindsay (1963)
2-pentanone 2-heptanone 2-nonanone 2-undecanone caprylic acid 2 tridecanone delta-decalactone delta-dodecalactone gamma-dodecalactone	evaporated milk	storage	Muck et al.
methyl sulfide acetone butanone ethanol isovaleraldehyde ethyl alcohol chloroform	fresh cream	natural	Wong (1963)
3-methoxy-4-hydroxybenzaldehyde	heated milk	carmel flavor	Cobb et al. (1963)

Table 1. Continued.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
formaldehyde ethanal isobutyraldehyde isovaleraldehyde n-hexanal n-nonanal phenylacetaldehyde acetone 2-heptanone 2-nonanone diacetyl acetoin	fresh butter	normal flavor	Winter et al. (1963)
C_3-C_8 alkanones C_1-C_{12} alkanals C_4, C_5-C_{12} alk-2-enals	sunlight exposed milk		Wishner and Keeney (1963)
dimethyl sulfide	butter	normal	Day et al. (1964)
oct-1-en-3-ol	oxidized milk and butter	mushroom	Stark and Forss (1964)
n-hexanal n-heptanal 2-hexanal 2-heptanone	milk fat	fishy flavor	Forss (1964)
n-amyl vinyl ketone	oxidized fat	metallic flavor	Forss (1964)
ethanal propanal acetone butanone 2-pentanone	sterile		Bingham (1964)
methyl sulfide acetaldehyde acetone	milk		Toan et al. (1965)

Table 1. Continued.

Compound	Source	Flavor Defect	Worker(s) and Reference No.
2-heptanone 2-nonanone 2-undecanone 2-tridecanone benzaldehyde napthalene alpha-dichlorobenzene benzothiazole ortho-aminoacetophenone delta-decalactone	sterile concentrated milk	stale	Arnold et al. (1966)

A recent review by Day (1965) also carries a complete list of the carbonyl compounds from flavor isolate of dairy products along with the melting points and ultraviolet absorption maxima of their 2,4 dinitrophenylhydrazone derivatives.

Origin of the Flavor Compounds

In dealing with milk that has been heated to a temperature of 75°C. or above, even momentarily, the first unnatural off-flavor that one encounters is the so-called cooked flavor as described by Harland and Ashworth (1945).

The intensity of this cooked flavor, as shown by Harland and Ashworth (1945) is a function of two parameters: degree of heating and duration of the heating interval. This flavor arises from compounds liberated by the heat denaturation of the milk serum proteins as shown by Harland and Ashworth (1945), Harland et al. (1955), Hutton and Patton (1952), Meneffe et al. (1941), Rowland (1937), and Rowland (1938). The particular protein involved has been shown by Larson and Jenness (1950) to be the β-lactoglobulin fraction of raw milk serum proteins. Hutton and Patton (1952) later confirmed β-lactoglobulin as the primary source of sulfhydryl groups while the fat globule membrane material was also shown to be a minor contributor.

Concomitant with the appearance of the reactive sulfhydryl groups in heated milk there also appears a reduction in the oxidation-reduction potential of the milk. The inhibitory effect of heat treatment of milk on oxidative deterioration has been reported by the following workers; Hutton and Patton (1952), Gould and Sommer (1939), Josephson and Doan (1939), Gould and Keeney (1957), and Patton (1955), and has been attributed to the preferential oxidation of the activated sulfhydryl group rather than the unsaturated fatty acids. The exact mechanism of sulfhydryl oxidation and the reaction products of sulfhydryl oxidation are at this time still unknown.

The origin of the sulfides and mercaptans which are present in milk is also not completely understood, but according to Patton (1955) and Dutra et al. (1959) sulfhydryl groups can be converted to volatile sulfides, mainly hydrogen sulfide. The presence of methyl sulfide in milk has been attributed by Patton et al. (1956) to methyl sulfone which is present either as a normal constituent of cow's blood or by bacterial decomposition of algae in the rumen of the cow. Recently Toan et al. (1965) have shown that a high Aerobacter aerogenes population can also cause an increase in the methyl sulfide content of milk.

Day et al. (1964) have suggested that dimethyl sulfide can be produced from three precursors: a) normal metabolic breakdown of methionine, which has been proven to be the production route from normal dimethyl sulfide levels, b) dimethyl-beta propiothetin, c) and methylmethionine-sulphonium salt. The two latter precursors are believed to account for excessive dimethyl sulfide levels. These precursors are of plant origin and would have to be transmitted via the rumen to the milk. This pathway for transmission of such volatiles has been demonstrated by Dougherty et al. (1962).

Lipid deterioration in milk can be caused by lipolysis and/or autoxidation, both of which can cause development of off-flavors. In this work, however, our concern will be with oxidative deterioration of the lipid phase and the secondary products of this oxidative mechanism, aliphatic aldehydes and ketones both saturated and unsaturated, and alcohols. Saturated and unsaturated aldehydes have been shown to impart characteristic off-flavors to milk at very low concentrations

(Boldingh and Taylor, 1962; Wong and Patton, 1962; Patton and Thorp, 1959; Van der Ven et al., 1963; Wishner and Keeney, 1963; Parks et al., 1963; Day et al., 1957; Forss, 1964; Forss et al., 1960). Day et al., (1963) have presented evidence concerning the additive interaction exhibited by these carbonyl compounds to give a detectable flavor even though all of the carbonyls were present at subthreshold concentrations.

The origin of these aldehydes has been the subject of research for many years, and one of the most popular and substantiated theories for aldehyde formation is that of autoxidation of the lipid fraction of milk (Stull, 1953; Badings, 1960; and Schultz et al., 1962). Although little is known concerning the initiation step in the autoxidation of the unsaturated fatty acids to form free radicals, Farmer and Sutton (1943) have elucidated the resulting propagation reactions, which yield hydroperoxides, and free radicals capable of propagating the reaction. In addition to hydroperoxides, peroxides are also known to arise from this mechanism (Parks et al., 1963; Lewis and Quackenbush, 1949; and Willites, et al., 1953).

Hydroperoxides in themselves are bland and odorless compounds causing no off-flavor; however, these hydroperoxides are extremely unstable compounds and their dismutation leads to the formation of carbonyls, alcohols, and epoxides, as secondary reaction products. A good review of this topic has been written by Badings (1960).

Winter et al. (1963) postulated that some aldehydes present in butter may originate from amino acids by enzyme-catalyzed oxidative decarboxylation. If this hypothesis is correct, then formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, phenylaldehyde might originate from glycine, alanine, valine, leucine, and phenylalanine respectively, which occur in the proteins of milk. Depending upon the previous history of the milk some of these amino acids may also exist as free amino acids.

Dutra et al. (1959) in support of this theory have shown that acetaldehyde can in fact originate from alanine. Wong (1963) has demonstrated that isovaleraldehyde, which gives rise to a malty flavor, may arise from the metabolic breakdown of leucine by <u>S</u>. <u>lactis</u> var. <u>maltigenes</u>.

Many theories exist as to the identity or the contribution of various classes of aldehydes in causing oxidized, and stale-oxidized flavor in processed dairy products. Parks et al. (1963), for example, in studying carbonyl compounds in butteroil from nonoxidized and spontaneously oxidized milk, indicated that alk 2,4 dienals (especially 2,4 decadienal) play an important role in off-flavor development, whereas the saturated aldehydes have slight, if any, significance in this role.

Forss et al. (1960) and Bassette and Keeney (1960) on the other hand, have shown the presence and flavor significance of the lower saturated aldehydes in oxidized dairy products. These findings have been collaborated with the research of Nawar et al. (1962), who identified the C_1 to C_9

saturated alkanals in oxidized milk fat, and postulated their contribution, using the additive interaction theory of Day et al. (1963), as a basis for their suppositions. There is uncertainty as to the part played by the C_1 to C_{12} saturated aldehydes in oxidized flavor. All workers seem to be in general agreement concerning the involvement of the mono- and di-unsaturated alkenals along with the branched chain aldehydes in the oxidized flavor of dairy products, as shown by the research of Bassette and Keeney (1960), Day and Lillard (1960), Parks et al. (1961), and Forss (1955a, 1955b).

Origins of the saturated, unsaturated, and branched chain aldehydes have been determined with a fair degree of certainty. Kawahara (1952), Farmer and Sutton (1943), Badings (1960), Day and Lillard (1960) and Gaddis et al. (1961) have been unable to show oxidative reaction schemes for C_2, C_3, C_4, C_6, C_8, C_9, C_{11} alkanals; C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11} alk-2-enals; 2,4 C_7, 2,5 C_8, 2,4 C_9, 2,4 C_{10}, 2,5 C_{11}, 3,6 C_{12} alkdienals; 2,4,7 C_{10}, 2,4,7 C_{13}, 2,5,8 C_{14} alktrienals; and finally 2,4,7,10 C_{16} alketetraenal, from oleic, linoleic, linolenic, and arachidonic acid. Parks et al. (1963) have shown that the C_{11}-C_{16} saturated aldehydes present in milk could arise from two possible sources: a) synthesis of milk fat and/or b) hydrolysis of plasmalogens or neutral plasmalogens during the process of pasteurization. Investigations by Van Duin (1958) and Parks et al. (1961) seem to support this theory by showing that the C_{11} to C_{18} saturated aldehydes are bound to glycerol of the plasmalogen fraction in butteroil.

The branch chained aldehydes that have been found to exist in milk systems have not been identified with any type of lipid degradation. The suggestion has been made that they are products of the Strecker degradation of their respective amino acids (Bassette and Keeney, 1960). Jackson and Morgan (1954) have shown conclusive evidence for 3 methylbutanal formation by Strecker degradation of leucine by S. lactis var. multigenes.

Dutra et al. (1958), using radioactive tracers, have elucidated two additional pathways for acetaldehyde formation: a) lactose breakdown, and b) deamination-decarboxylation of alanine.

The other group of carbonyl compounds that is responsible for off-flavor development in milk and milk products is the ketones. Ketones with both saturated and unsaturated structures have been shown to impart characteristic off-flavors, even at very dilute concentrations (Bingham, 1964; Wong et al., 1958; Nawar et al., 1962; Wong and Patton, 1962; Patton and Tharp, 1959; Langler and Day, 1964; Parks and Patton, 1961; and Schultz et al. 1962). Langler and Day (1964) also pointed out that ketone mixtures exhibited the same synergistic interaction as was shown for aldehydes.

Methyl ketones, one class of the volatile ketonic flavor compounds that have been isolated from pasteurized, concentrated, and sterile concentrated milks (Bingham, 1964; Boldingh and Taylor, 1962; Wong et al., 1958; Muck et al., 1963; Wong, 1963; Dutra et al., 1959; and Winter et al., 1963) have very controversial origins. The fact that methyl ketones have been isolated from milks that have had no heat treatment seems to give evidence that their formation is easily initiated. Acetone is the only methyl ketone that has been consistently isolated from unheated dairy products, and it is thought to be present from the time of secretion as shown by Patton and Tharp (1959). Boldingh and Taylor (1962) postulated that the methyl ketones have as their origin the beta-keto acids bound to the glycerides. Wong and Patton (1962) presented evidence to support the Boldingh-Taylor theory, when they showed that methyl ketones could be easily produced by spontaneous decarboxylation of beta-keto acids, which are intermediates in the beta oxidation of fatty acids. Recently, Van der Ven et al. (1963) have presented evidence in support of this theory by demonstrating that there are beta-keto esters in milk and that methyl ketones are formed in the presence of heat and moisture. He suggests that the C_5-C_{15} methyl ketones are derived from the C_6-C_{16} beta-keto acid glycerides. This work was accomplished by reacting the milk fat with Girard-T-reagent and subsequently identifying the pyrazoles. These data have been substantiated by Parks et al. (1964), who have isolated and identified beta-keto esters as the precursors of methyl ketones in milk fat. It has also been shown by Wong et al. (1958), Patton and Tharp (1959), Parks et al. (1964), Langler and Day (1964), and Schwartz et al. (1965), that prior to formation of methyl ketones in milk fat, hydrolysis must be effected whether by heating in the presence of water or by saponification. Evidence presented by Patton and Tharp (1959), showing that fresh unheated milk fat is devoid of such ketones, and the latter findings of Langler and Day (1964), concerning the inhibition of methyl ketone formation when milk fat was dried over calcium hydride for 18 hours, also seems to lend support to this hydrolysis theory. Nawar et al. (1962), have studied heat-induced changes in milk fat and did not show the necessity for the presence of moisture in formation of either even or odd numbered methyl ketones in freshly prepared butteroil; however, no mention was made of completely drying the oil before heating was initiated, so that moisture present in the oil could have been sufficient for hydrolysis. These researchers also feel that methyl ketone formation could not occur as a result of atmospheric oxygen attacking the lipid phase because of the abundance of reducing substances present in commercially processed dairy products.

α,β unsaturated ketones have been shown by Ellis (1950) to arise as the first reaction product in metal catalyzed lipid oxidation rather than the α,β unsaturated hydroperoxide proposed by Farmer et al. (1943). Ellis also postulated that these unsaturated ketones complex with molecular oxygen to form a compound having properties of peroxides. The traditionally accepted theory is that the α,β unsaturated ketones arise from the dismutation of the hydroperoxides formed by the mechanism described by Farmer et al. (1942).

Staling, a deteriorative reaction which takes place in dry and fluid milk products, has never fully been defined. Supplee (1926) as long ago as 1923, recognized the stale, musty, or gluey flavor in stored, high-moisture milk powders. Lea et al. (1943) have termed the stale flavor that developed in dry whole milk as heated, burnt, scorched, or cooked, and in 1962 Patel et al. (1962) described this condition as "old-rubber" in sterile concentrated milk.

Lea et al. (1943) believed that stale flavor consisted of two compounds: a) the burnt, or carmel taste, which they associated with the protein or carbohydrates; and b) a "butter-toffee" flavor which they felt was associated with the fat phase of dry whole milk powders. Whitney and Tracy (1949, 1950) fractionated stale dry whole milk into cream, skimmilk, butter, buttermilk, butteroil and butter serum, and concluded that stale flavor is concentrated in the fat phase. They were careful to point out that this was not evidence as to the origin of the flavor. Whitney and Tracy (1949) also determined that there was a greater concentration of stale flavor per unit weight in the products that were high in phospholipids.

Bassette and Keeney (1960), in studying the storage defect associated with non-fat dry milk, indicated that both Maillard browning and lipid oxidation were involved in this flavor deterioration. Evidence was presented for the presence of both odd and even chained aldehydes and ketones, and for the presence of branched chain aldehydes. Furfural and diacetyl were also identified as reaction products of the Maillard browning reaction. These data also agree with the later research findings of Nawar et al. (1963) who felt that there were at best two different components capable of inducing the stale-flavor sensation, one component was extractable as a Girard-T-derivative and was shown by infrared absorption spectroscopy to contain two functional groups; a) one in the lactone-ester region, and b) one in the ketone-aldehyde region. The second flavor component capable of eliciting a stale-flavor response was found in the carbon tetrachloride vapor from the volatile extraction technique and appeared to contain both saturated and unsaturated carbonyl compounds. The data from these latter two flavor fractions agree with the findings of Parks and Patton (1961) who suggested that staleness may be caused bya combination of compounds, being either aldehydes or ketones, and their concentrations determining which flavor was elicited; stale or oxidized, the former being caused by a lower carbonyl concentration than that necessary for oxidized flavor. The origin of these carbonyls has been shown earlier as secondary reaction products of the oxidized triglycerides and phospholipids in milk fat.

The presence of the lactone-ester region in the infrared spectrophotometric analysis of stale flavor isolate has given support to the postulation that the coconut and fruity flavors exhibited in early storage of dairy products originate from lactone formation. Keeney and Patton (1956a, 1956b) identified the origin of the coconut flavor as delta-decalactone. Tharp and Patton (1960) identified the compound contributing to the fruity flavor to be delta-dodecalactone. In both cases these flavor compounds are believed to arise from action of heat on the milk fat, since no lactones were found in unheated milk. This theory was later affirmed when Mattick et al. (1959) showed the reaction scheme for delta-decalactone formation from 5-hydroxydecanoic acid, which exists in native butterfat as a simple ester. This same research also demonstrated that both the degree of heat treatment in the processing technique and temperature of storage are of primary importance in the development of the delta-decalactone from 5-hydroxydecanoic acid. Muck et al. (1963) and Arnold et al. (1966) have also showed the involvement of lactones in stale flavor development by isolating them from stale evaporated milks.

The last cause of off-flavor development to be considered is that of non-enzymatic, or Maillard browning, which develops during the storage of dairy products. Tarassuk and Jack (1948) also pointed out that the naturally occurring phenomenon of non-enzymatic browning could be related to the development of stale and oxidized flavors in processed milk products. The mechanism postulated for this browning reaction by the French chemist Maillard, and discussed by Lea (1950) is concerned with the interaction of carbohydrates with amines and free amino acids. Harland et al. (1947), Kass and Palmer (1940), Patton (1952), and Patton and Josephson (1949a, 1949b) showed that in milk systems the reactants were lactose and casein. Evidence from Patton (1952), and Patton and Flipse (1953) pointed to the epsilon-amino group of lysine as being the key reactant group of casein in the browning mechanism. The mechanism of browning in the sugar-amine systems is extremely complex. The literature concerning these reactions in model systems reveals seven different types of reactions that have been shown to take place; however, the extent of which each is present in the natural browning of products is not known. The initial stage of the reaction involves sugar-amine interaction yielding an N-substituted glycosylamine. It is postulated by Lea and Hannan (1950) that these N-substituted glycosylamines isomerize to 1-amino-1-deoxy-2-ketoses, which is known as the Amadori rearrangement. The intermediate stage in the sugar-amine browning systems follows the Amadori rearrangement and as discussed by Hodge (1953) can lead to sugar dehydration, sugar fragmentation, formation of furfurals, reductone-like reducing substance, fluorescent substance, and the degradation of amino acids by the Strecker reaction. The final stage of browning is a polymerization reaction to form unsaturated, fluorescent, colored polymers. The main reactions of this polymerization reaction as discussed by Hodge (1953) are thought to be aldol condensations, aldehyde-amine polymerization, and the formation of heterocyclic nitrogen compounds, called melanoidins. Hodge (1953), Hodge and Rist (1953) along with Lea and Hannan (1950a, 1950b) have discussed at length these reactions and how they fit into the findings concerning the casein-lactose complexes.

The most important manifestation of the non-enzymatic browning system is the formation of the browning pigments, melanoidins, which according to Kass and Palmer (1940) and Patton (1952) are chemically bound by milk proteins and are responsible for the discoloration and flavor of milk systems. Patton (1955) has also proposed that when milk reaches this stage of discoloration there are at least four flavor components associated with it: 1) carmel or malty, resulting from sugar decomposition and Strecker degradation of amino acids, 2) "stewed meat", arising from methionine decomposition, and the presence of hydrogen sulfide which arises from the presence of reactive sulfhydryls, 3) hydrolytic rancidity, arising from fat hydrolysis, and 4) coconut, resulting from the formation of lactones in the milk fat phase as previously discussed.

More detailed discussions concerning the origins of these flavor compounds can be found in the publication of Hodge (1953), Hodge and Rist (1953) and Patton (1955).

EXPERIMENTAL PROCEDURE

The sterile milk used in this research project was produced by a commercial process using the DeLaval Vacu-Therm Instant Sterilizer, which is a high-temperature, short-time steam injection sterilization unit.

In this unit the raw milk was preheated to 77°C. and then instantaneously heated by steam injection to 140.5°C ± 1°C. Following an approximately 4.0 second hold at this temperature the sterile product was then cooled to 77°C. in a reduced-pressure vessel. The desired post-sterilization temperature was achieved by controlling the vacuum on this vessel by a hand operated release valve. The purpose of this vacuum cooling procedure was to remove the water added by the steam injection process and thus assure a proper total solids/water ratio in the sterile product. At this point the milk was homogenized in an aseptic Manton Gaulin homogenizer at a pressure of 4000 psig. (3000 psig. on the first stage and 1000 psig. on the second stage). Proper control of temperature and pressure are essential to assure satisfactory homogenization, and minimizes the development of cream layer and sediment in the sterile product during storage.

Following homogenization the milk was cooled to a temperature of 12.5°C. in an aseptic plate heat exchanger and then piped directly to an aseptic balance tank to await sampling, or further processing.

The procedure for sterilization of the DeLaval VTIS system was modified from that programmed in the automatic sterilizing cycle. This modification resulted from a lack of steam pressure on the final aseptic balance tank, which was observed to be 7 psig. Since the sterilizing program was based on an F_0 of 15 minutes, it was necessary to calculate a new F_{234} with a z of 18°F., which resulted in a sterilizing period of 3.9 hours.

Sample Collection Techniques

One gallon glass bottles were used as the storage containers for the sterile fluid milk samples. The procedure for cleaning and sterilizing these bottles was as follows: the bottles were washed twice in detergent solution, thoroughly rinsed in tap water, rerinsed with distilled water, and loosely capped. The bottles were then steam sterilized for 20 minutes at 15 psig., after which the bottles were cooled and the caps securely sealed. Thirty bottles were used for each set of samples taken, and there were three sets of samples collected.

Thirty presterilized bottles were then placed in a 48" x 24" x 30" Plexiglass dry-box for further processing. This dry-box was constructed with a gas inlet at each end, two arm holes fitted with rubber gloves in the front panel, a one-half inch hole in the back panel through which a sterile sampling hose entered the chamber and a sterile air filter fitted to the top of the box. The purpose of this dry-box was to provide a sterile atmosphere in which sampling could be carried out and thus reduce the possibility of air-borne contamination.

Sterilization of the atmosphere in the chamber was accomplished with the use of the sterilizing gas, ethylene oxide, by the following procedure: a hose leading from the pressure reducing valve of the ethylene oxide-carbon dioxide gas cylinder was connected to one of the gas inlets, both inlets were then opened, and the pressure on the reducing valve adjusted to approximately 3 psig. The system was permitted to remain under these conditions for five minutes to assure complete purging of the atmosphere in the dry-box. At various intervals during this purging operation the gas jet used as an exhaust vent was closed for 15 to 20 seconds and then reopened, thereby accelerating the exchange of atmospheres in the sampling chamber.

At the completion of the five minute purge interval the inlet of the sterile air filter was capped, the gas outlet closed, and the pressure of the ethylene oxide-carbon dioxide gas mixture in the chamber adjusted to 3 psig. At this point the gas inlet was closed and the chamber and its contents underwent sterilization for eight hours. Ethylene oxide is both explosive and toxic, and, since a concentration of 50,000 ppm. is not tolerable for more than a few minutes, a well-ventilated room should be used for such research.

Upon completion of the sterilization period a vacuum pump was connected to one of the gas jets. The jet was opened, the pump started, and the cap was then immediately removed from the inlet of the sterile air filter. The system was evacuated for two hours in order to replace the ethylene oxide-carbon dioxide gas in the sterile chamber with sterile air. This was done to preclude the presence of ethylene oxide in the headspace gas of the samples which could react with the milk fat during storage. Completion of this evacuation phase completed the sterilization procedure, and with the attachment of a presterilized hose to the sampling valve of the aseptic balance tank, the system was ready for sampling. During sampling the sterile bottles were uncapped a few seconds before they were to be filled and were immediately recapped after filling.

After all of the samples had been taken the sampling valve was closed, the hose disconnected and the bottles removed from the sampling chamber. They were then placed in storage cabinets maintained at 4.4°C., 22.0°C., and 36°C. to await analysis at predetermined intervals of zero, one, two and three months.

Volatile Recovery

The recovery of flavor components from fluid sterile milk was accomplished with a low-temperature, reduced-pressure distillation technique described by Day et al. (1957) for recovering the volatiles from gamma-irradiated skimmilk.

The standardized procedure for recovery of flavor components from both fresh and storage fluid sterile milk was as follows: six liters of the sterile product at a temperature of $10^{\circ}C.$ were placed in a 12-liter distillation flask. 186 mg. of Dow-Corning Antifoam AF Emulsion was added and the pressure in the system was reduced to 20 to 30 mm. mercury. Nitrogen gas was used to control the pressure and afforded three benefits: a) the bubbling of the nitrogen into the system provided good agitation, b) a nitrogen atmosphere prevented oxidation during distillation, and c) the addition of nitrogen acted as a sweep gas to carry off both the water vapor and flavor volatiles from the distillation flask. No heat was applied to the distillation system until the operating pressure of 20 to 30 mm. mercury was reached, at which time the temperature was immediately raised to $40^{\circ}C. \pm 1^{\circ}C.$ The sample was then allowed to distill for a period of four hours and the distillate from the sterile milk was fractionally collected in various traps: wet ice, dry ice and ethanol and liquid nitrogen. Upon completion of the distillation period the distillation flask was removed from the system and the volatiles from the traps were distilled over to the final liquid nitrogen trap. This was accomplished by salting out each distillate trap with anhydrous sodium sulfate and then varying the temperature and pressure on the trap until the volatiles were moved from the aqueous distillate to the next trap.

This procedure of transferring all volatiles to the final liquid nitrogen trap was deemed standard procedure after previous gas chromatographic analyses showed that no volatiles were in the wet ice or dry ice traps that were not present in the liquid nitrogen trap.

Following the distillation and concentration techniques in the volatile recovery procedure the volatiles were kept in liquid nitrogen until gas chromatographic analyses were performed.

Gas Chromatography

Gas chromatographic analyses were performed on an F & M model 810 research gas chromatograph equipped with a dual flame ionization detection system, a model 50 automatic attenuator, and a column oven designed for automatic temperature programming from ambient to 400°C.

The columns used were prepared in a manner outlined by Dal Nogare and Juvet (1962). A six foot length of 1/4 inch O. D. copper tubing was packed with 25% w/w carbowax 20 M on acid-base washed 80/100 mesh Chromosorb W. Packing was accomplished with the aid of a Vibra-graver tool and both columns were closely matched to insure minimal baseline drift during the temperature programming sequence. Following the packing of both columns they were installed in the model 810 chromatograph and conditioned for one week by continually programming the oven temperature from ambient to 210°C. at a rate of 1°C. per minute for two days, and then holding the temperature at the upper limit for the remaining five days. At this time the helium flow rate in both columns was set at 50 ml/min. The instrument was then checked for drift during a programmed run of 4°C/min., and the helium flow on the reference column, B column, adjusted so as to balance the background signal emitted by the analytical column at the upper limit of the temperature program. At this point the instrument was used for analysis of the flavor distillates.

The following conditions were established for the gas-liquid chromatographic analysis of the extracted flavor distillates:

range setting-----------------1x
attenuation-------------------lower limit 4
recorder sensitivity----------1 mv.
temperature-------------------12 minutes isothermally at 70°C., then 4°C. per minute to an upper limit of 210°C.
detector temperature----------285°C.
injection port temperature----250°C.
detector:---------------------hydrogen flame
flow rates:
    carrier gas--------helium 50 ml/min.
    hydrogen-----------63 ml/min.
    compressed air-----500 ml/min.
recorder speed----------------0.25 inch/min.
column data-------------------length 6 feet 1/4 inch O. D. 25% carbowax 20 M on acid-base washed 80/100 mesh chromosorb W.

Sampling of the Flavor Isolate for Analysis

The glass stopper was removed from the cold finger, while it was still submerged in liquid nitrogen, and a rubber stopper, fitted with a rubber septum and covered with a double layer of parafilm, was inserted in its place. The cold finger was then removed from the liquid nitrogen and warmed to a temperature of 70°C. It was important to warm the cold trap slowly and remove some of the pressure from the trap by periodically inserting a hypodermic needle through the septum.

Preliminary analyses demonstrated that no volatiles were lost through this technique. After the temperature of the trap reached equilibrium with the temperature of the water bath a gas tight syringe was used to remove a 6 ml. gas aliquot from the trap. The sample of gas was then injected into the chromatograph. Relative retention times were obtained for the isolated components from the flavor distillate, and these were always run in duplicate. Often the individual flavor constituents are similar, or are structural isomers, or have the same relative retention time as another compound. Thus, in order to make some tentative identification as to the components present, it was necessary to perform functional group analyses. The technique of Hoff and Feit (1964) in which these reactions were carried out in the vapor sampling syringe was adapted because of its ease and efficiency. The only modification that was found necessary was the use of 1 ml. of reagent for proper reaction. Reactions for aldehyde, ketone, ester, alcohol, and various sulfur compounds were performed on the distillate vapor from each extraction. These chromatograms were then compared with those of the untreated vapor to aid in establishing the functional groups and the identity of the compounds present.

Once the information had been collected from these chromatograms these data were compared to the relative retention times of known compounds that had been chromatographed under similar conditions. Using all of this information an effort was made to tentatively identify some of the components that were responsible for the stale-oxidized flavor that developed during storage.

Thin Layer Chromatography

Another technique that was utilized to determine the identity of these off-flavor compounds was thin-layer chromatographic identification of the 2,4 dinitrophenylhydrazone derivatives of the carbonyls present in the flavor isolate. The hydrazone derivatives were formed using a modified procedure of Lawrence (1965), in which a 2:1 ratio of 2,4 dinitrophenylhydrazine reagent to aqueous flavor distillate was mixed in a glass stoppered bottle and allowed to remain quiescent for 12 hours. The 2,4 dinitrophenylhydrazones were then extracted from the mixture with 30 ml. of carbonyl-free hexane, prepared by the method of Schwartz and Parks (1961). The aqueous layer and upper hexane layer, containing the 2,4 DNP derivatives, were then permitted to separate in a separatory funnel. The hexane extraction was then repeated using 20 ml. aliquots of carbonyl-free hexane until no color was apparent in the hexane. All hexane extractions were then collected and evaporated to dryness using a rotary evaporator. Because of the small quantities of 2,4 DNP hydrazones present, melting or mixed melting points could not be done. Identification of the 2,4 DNP hydrazones was then made by thin layer chromatography of the homologous classes of carbonyl derivatives. The class separation performed on the mixture of 2,4 DNP hydrazones was that described by Schwartz et al. (1962) using a Magnesia-Celite 545 column. This gives the elution sequence of methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals. These various classes, which could easily be identified on the column because of the colored bands they exhibited, were then analyzed spectophotometrically to determine their absorption maximum, which assists in identification of the eluting class.

These homologous classes were then separated and identified using the thin-layer chromatographic techniques reported by Badings and Wassink (1963) for the separation of 2,4 DNP hydrazones on Kieselguhr G-Carbowax 400 impregnated plates. All known 2,4 DNP hydrazone derivatives of the carbonyls used for identification of unknown were prepared and doubly recrystallized according to the procedure discussed by Shriner and Fuson (1948).

Bacterial and Organoleptic Analyses of Storage Sterile Milk Samples

All HTST fluid sterile milk samples, prior to the recovery of the flavor components by low-temperature, reduced-pressure distillation were analyzed for the presence of bacteria by the Standard Plate Count Method. Standard plate counts were made utilizing the technique described in Laboratory Manual-Methods of Analysis of Milk and Its Products. A one milliliter sample of undiluted milk was plated since these analyses were made merely to ascertain whether or not the milk was sterile at the time of flavor extraction, and thus assure that none of the flavor compounds isolated were caused by bacterial decomposition of the milk during storage.

The HTST fluid sterile milk was also subjected to organoleptic evaluation prior to the flavor distillation of the volatiles. The fluid sterile milk was graded on a numerical basis using the grading system set forth by the American Dairy Science Association and the Dairy Industry Science Association for scoring milk and milk products. The normal range in grading market milk according to this system is from 31 to 40 points with 31 showing a product of near unsalable quality and 40 denoting an excellent product devoid of criticism. A breakdown of the scoring range for the broad classification of excellent, good, fair, and poor is as follows: excellent, 40; good, 38-39.5; fair, 36-37.5; poor, 35.5 or less. In the organoleptic evaluation of the HTST fluid sterile milk samples, the particular defect or defects that were incurred were recorded as well as the numerical score.

RESULTS AND DISCUSSION

Organoleptic Flavor Evaluation

Organoleptic flavor evaluation of the HTST fluid sterile milk samples was employed throughout the storage study, and provided sensory evaluation of deteriorative changes as the flavor of the fluid sterile milk progressed from fresh to stale or stale-oxidized. As can be observed from Tables 2, 3, and 4 all samples exhibited a highly cooked flavor immediately after heat processing; however, this cooked flavor disappeared, or was masked by the appearance of other off-flavors during subsequent storage. A slight sweet or carmel flavor was also present in the freshly processed milk, as was a distinct coconut flavor. These cooked, carmel, and coconut flavors are attributable to the stringent heating conditions imposed upon the milk to effect sterilization. Harland and Ashworth (1945), Harland et al. (1955), Hutton and Patton (1952), Rowland (1937), and Larson and Jenness (1950) have shown the cooked flavor to arise from the interaction of sulfhydryl groups, freed by heat denaturation of the serum protein β-lactoglobulin, and to a minor extent, from the fat globule membrane protein. The interaction of these reactive SH groups forms volatile sulfides, mercaptans, and hydrogen sulfide, all of which are related to cooked flavor in milk. The coconut flavor present in the milk has been demonstrated by Keeney and Patton (1956b), Mattick et al. (1959), Tharp and Patton (1960), and Parliment et al. (1966) to arise from delta-lactones, which according to the Boldingh and Taylor theory originate from heat degradation of monohydroxy-acyl-triglycerides. The monohydroxy-acyl-triglycerides responsible for delta-lactones are those containing a 5-hydroxy fatty acid and two nonhydroxylated fatty acids. Parliment et al. (1966) have shown that application of heat to these monohydroxylated-acyl-triglycerides results in the hydrolysis of some 5-hydroxy fatty acids, which readily lactonize to form delta-lactones.

The sweet, or carmel flavor that also accompanies heated milk products, arises from the heat degradation of lactose, which Kass and Palmer (1940) describe as the carmelization of lactose by the casein, and the absorption of the lactocarmel by the colloidal caseinates.

Concomitant with the carmel flavor in the HTST fluid sterile milk is the off-white to tan color of the freshly processed milk. This discoloration became more apparent with storage of the milk at 22° and 36°C., while the samples at 4.4°C. exhibited no additional color change during storage. The slight discoloration observed with the control (zero time storage) samples is most likely the result of Maillard browning. Patton (1952) observed that browning was readily evident in milk samples heated to 120°C. for 7.5 minutes, and with the heating conditions imposed upon the HTST fluid sterile milk (140.5°C ± 1°C. for approximately 4 seconds) it is postulated that Maillard browning results with the consequent formation of melanoidins. The discoloration observed in all control samples as well as the carmelized flavor in one of the control samples, appears to lend support to the theory that sulfhydryl groups are very labile, and disappear readily either as volatile sulfides, or by disulfide interchange with kappa casein (Hartman and Swanson, 1965). The basis for this reasoning lies in the sulfhydryl inhibition mechanism, observed by Tarassuk and Jack (1948) and Patton and Josephson (1949b), for heat-induced browning. Hodge (1953) speculated that the inhibition mechanism concerned the addition of sulfhydryl compounds at the double bond of the Amadori rearrangement product (in the enol form), effectively blocking the heat-induced browning reaction in some systems. Thus if sufficient reactive sulfhydryls had been present to inhibit Maillard browning, discoloration should not have occurred.

Stale flavor development in the HTST fluid sterile milk is the major obstacle associated with flavor deterioration in storage. Until stale flavor was detected organoleptically in the stored fluid sterile milk samples a minimum score of 36 on the A.D.S.A.-D.S.I.A. scoring system was recorded for the storage samples. This would be a marketable product. Appearance of the stale flavor lowered the sterile milk score to 34, which is indicative of a product of near unsalable quality. The staling of HTST fluid sterile milk manifests itself between the second and third months of storage at 22°C. This same time lapse was recorded by Patel et al. (1962) for staling of sterile concentrated milk held at 21.1°C. It is also evident from Tables 2, 3, and 4 that the storage temperature to which the product is subjected plays an important role in the staling of storage milk products. Here we observe that two out of three samples of HTST fluid sterile milk held at 36°C. were judged slightly stale in their first month of storage. However, pronounced stale flavor was exhibited by the HTST fluid sterile milk somewhere between the first and second months of storage at 36°C. These specific data and the general aging of all sterile milk samples agreed well with the gas chromatographic survey data (Figures 1-10). Here the appearance and enhancement of stale flavor can be correlated with the increased number of carbonyls present as staling develops, as well as the increased concentration of those previously detected in the control samples. Such gas chromatographic data are relevant to stale flavor in stored dairy products, since Bassette and Keeney (1960) have reported the association of autoxidation and Maillard browning to stale flavor, and have corroborated the catalytic effects of high storage temperatures on both autoxidation and Maillard browning reactions.

The beneficial effect of refrigerated storage as an inhibitor on staling of HTST fluid sterile milk cannot be concluded from these storage data, although research has presented evidence that lower storage temperatures reduced the intensity of the off-flavors in the sterile milk. Patton (1955), in a review of Maillard browning in dry milk powders, ranks storage temperature second only to moisture content in factors contributing to Maillard browning. Badings (1960) likewise, feels storage temperatures are critical in autoxidation of milk lipids. Although the products are in a different physical state, it is apparent that elevated storage temperatures are an important factor in contributing to the stale flavor of HTST fluid sterile milk. This view is supported by Patton (1955), who suggests that the lowest feasible storage temperature should be employed in order that flavor, as well as color changes, will be inhibited.

Body Defects Associated with HTST Fluid Sterile Milk During Storage at 4.4°, 22°, and 36°C.

The physical defects that were observed in HTST fluid sterile milk during the storage conditions of refrigerated, ambient, and tropic temperatures are those that have been commonly associated with sterile concentrated and evaporated milks during storage. Cream layer formation was the first body defect that was detected during the storage of HTST fluid sterile milk. This physical separation of the milk fat from the serum phase is explained by the inverse of Stoke's equation for falling bodies, which states that fat globules rise in milk systems for two reasons: a) a density differential exists between milk fat and milk serum, and b) the size of the fat globules. It was observed that even with homogenization pressure of 4000 psig. (3000 psig. 1st stage, and 1000 psig. 2nd stage) the fluid sterile milk still developed a cream layer after one month of storage at

Table 2. Organoleptic evaluation data for HTST fluid sterile milk under storage conditions of 4.4, 22 and 36°C. for a three months time interval (processing date 3/2/66).

Storage Time	4.4°C.		22°C		36°C.	
Control (zero time)	highly cooked coconut	35				
One Month	sl. cooked sl. coconut	37	cooked sl. coconut	37	sl. coconut sulfide flavor	36
Two Months	lacks fresh. cooked	36	lacks fresh. sl. cooked	36	stale	34
Three Months	astringent stale	34	stale sl. astringent	34	no sample	

Table 3. Organoleptic evaluation data for HTST fluid sterile milk under storage conditions of 4.4, 22 and 36°C. for a three months time interval (processing data 5/20/66).

Storage Time	4.4°C.		22°C.		36°C.	
Control (zero time)	highly cooked	37				
One Month	sl. cooked sl. carmel.	37	sl. cooked sl. carmel.	37	lacks fresh. sl. stale	34.5
Two Months	sl. cooked sl. coconut lacks fresh.	36	lacks fresh. sl. carmel	36	stale sl. carmel sl. coconut	34
Three Months	sl. carmel stale	34	stale	34	stale	34

*Organoleptic evaluations were made using the A.D.S.A.-D.S.I.A. point scoring system.

Table 4. Organoleptic evaluation data for HTST fluid sterile milk under storage conditions of 4.4, 22 and 36°C. for a three months time interval (processing date 6/9/66).

Storage Time	4.4°C.		22°C.		36°C.	
Control (zero time)	highly cooked sl. carmel.	36.5				
One Month	cooked lacks fresh.	36	sl. cooked lacks fresh.	36	lacks fresh. sl. stale	34.5
Two Months	lacks fresh. sl. carmel sl. cooked	36	sl. carmel. sl. cooked lacks fresh.	36	sl. carmel sl. stale	34
Three Months	lacks fresh. sl. cooked sl. carmel	36	lacks fresh. sl. stale sl. cooked	34	stale	34

*Organoleptic evaluations were made using the A.D.S.A.-D.S.I.A. point scoring system.

temperatures of 22°C. and 36°C.; however, sterile fluid milk held at 4.4°C. did not show any noticeable cream layer after three months of storage. Notation should be made that these samples were not subjected to any type of stock rotation or inversion during storage.

The question of what causes the clustering of these rising fat globules, and subsequent formation of the cream layer has never fully been determined. Dunkley and Sommer (1944) established that a protein is involved in this phenomenon, and have classified it as a euglobulin; however, they were not able to establish whether this protein was identical with the euglobulin of immune proteins. The exact effect that the euglobulin has on milk fat is not known. Presumably the protein alters the surface properties of the fat globules in a way which allows them to adhere to one another. It is also known that heating above minimal pasteurization temperature reduces the cream layer volume due to heat denaturation of the euglobulin. Homogenization may also have a similar effect.

Explanation of this cream layer or cream plug formation in the storage fluid sterile milk may be attributed to the high homogenization pressure. This has been shown as the cause of cream plug formation in half and half and other homogenized cream products. The euglobulin is not believed to be responsible since it would supposedly be denatured by both sterilization and homogenization (Patton and Jenness, 1959). The observation that creaming occurs in samples stored at 22° and 36°C., but not at 4.4°C. is also intriguing. This seems inconsistent with the early literature which reported that optimal conditions of temperature for creaming of fat in pasteurized products was 1.7° to 4.4°C. This temperature range would also show a greater density differential between the serum and fat phases of the milk system, than at any of the other storage temperatures.

Age-thickening, an incipient sign of gelation, was first tactually observed in the samples stored for two months at 22° and 36°C., and in the sample held at 4.4°C. for three months. The body of these fluid sterile milk samples could be compared to that of half and half, a dairy product containing at least 10% milk fat. This increase in viscosity is believed due to gelation, since no apparent increase in viscosity was detected by organoleptic observations of the sterilized milk samples, and would seem to discount heat coagulation during sterilization as the main cause of increased viscosity.

Gelation of sterilized milk appears to be a very complex phenomenon associated with the formation of a fat/casein complex, which has been shown to be influenced by the following variables: a) heat treatment given the milk, b) composition of the milk, c) concentration of total solids, and d) storage temperatures. The gelation phenomenon is directly related to the temperature of storage. Ellertson and Pearce (1964) have shown that at 4.4°C. concentrated sterile milk of 26% total solids had a gelation time of 50 weeks, while gelation occurred in less than ten weeks at 36°C.

Gelation seems to originate with heat processing, which causes the formation of large fat protein particles. The actual appearance of the gel structure does not begin until these large particles dissociated and rejoin to form short chains. Researchers have suggested that the bonding sites involved in gel structure are not available until the large protein particles dissociate. The gelation sequence has been studied by following the viscosity of milk from a time interval prior to processing until gel formation occurred in the concentrated product. Reduced viscosity was associated with the early stage of dissociation of large particles; these large particles, then dissociated to form smaller particles and short chains, which increased viscosity, and ultimately, a gel structure formed. Further studies on gelled milks by Sasago et al. (1963) seem to indicate the involvement of disulfide bonds in the binding of the fat globules with the protein, and the importance of calcium in the formation of the large particles.

Many researchers have shown that increased processing time and temperature reduces gelation by protein stabilization; however, with this increased heat treatment the problem of sedimentation during storage arises. Phosphates have also been utilized as a means of controlling gelation by complexing the casein micelles with polyvalent cations, which prevents fat-casein complexes from forming. The polyphosphate method of retarding gelation has proven quite effective, as shown by Leviton and Pallansch (1962), and Leviton et al. (1963), and has increased storage life of concentrated milk from 45 days (control) to 441 days (polyphosphates added). The exact mechanism involved in the retardation of gelation by polyphosphates is not known. Polyphosphates have a stabilizing effect on proteins and this may be involved in some way with the reactive sites of the fat globules. Ellertson and Pearce (1964) have shown that homogenization at elevated temperatures increases the viscosity of concentrated sterile milk and this increases the rate of gelation but it is not known whether or not this is due to the effect of homogenization on the casein micelles.

Sedimentation was also observed in the samples held for one month. The amount of sediment seemed to be constant in all samples and did not appear to be affected by storage temperature. This body defect is viewed as resulting from the effect of the ultra-high temperature of processing on the milk proteins.

In summary, it is felt that after three months of storage at 4.4°, 22°, and 36°C. only the cream layer formation in samples held at 22° and 36°C. was of significance, since normal agitation could not produce sufficient distribution of the two phases. The quantity of sediment could be considered insignificant and the age-thickening could be controlled by the addition of polyphosphates.

Parameters Incurred in Flavor Recovery Distillation, Sampling, and Gas Chromatography

The low-temperature, reduced-pressure distillation technique employed in the flavor volatile recovery system utilized in this research had two important parameters: a) pressure in the distillation flask, and, b) temperature of the distillation. Accurate control over this temperature-pressure combination was extremely important in the standardization of the flavor volatile recovery. This enables the researcher to compare data from different extractions with assurance that either an increase, or decrease in volatiles recovered from the fluid sterile milk was the result of flavor improvement or deterioration, rather than an inconsistency in the recovery technique.

When gas chromatographic analyses of the flavor isolates were initiated they were performed only on the liquid nitrogen cold trap. The reason for this procedure is that prior analyses of each individual cold trap showed no additional flavor compounds that were not detected in the final liquid nitrogen trap. Therefore, to minimize the number of volatile samples that would have to be chromatographed only the flavor isolate from the liquid nitrogen trap was analyzed.

As an enrichment procedure the volatile from each of the other cold traps was salted out and the volatiles transferred to the liquid nitrogen trap by manipulating the pressure and temperature on the cold traps. This was done in sequential order from the wet ice to the final dry ice ethanol trap. Following this standardized volatile enrichment procedure the flavor isolate was held in a cold trap in liquid nitrogen (-195.8°C.) until gas chromatographic analyses were performed.

When the flavor isolate held in the liquid nitrogen cold trap was to be prepared for gas chromatographic analysis, the standard taper glass stopper was removed and a rubber stopper, covered with a double layer of parafilm, and fitted with a rubber septum, was inserted in its place. The cold trap was then taken out of the liquid nitrogen bath and slowly warmed to 70°C. When the trap containing the isolate reached equilibrium with the 70°C. bath a 6 ml. sample of isolate volatiles was removed with a gas-tight syringe and injected into the gas chromatograph.

During the preliminary trials concerning the gas chromatographic analyses of the flavor isolate vapor an interesting observation was made concerning the sampling of the vapor. It was found that if the vapor sample was removed from the cold finger without releasing the existing pressure from the system only peaks 1 to 8 were chromatographed. On the other hand if this pressure was released and the trap permitted to remain at equilibrium temperature (70°C.) for a few minutes the normal flavor volatile spectrum was obtained. The reason postulated for this behavior concerns the vapor pressures of the low boiling volatiles present in the flavor isolate. It is believed that the low boiling volatiles build up such pressure in the system that the vapor pressure of the higher boiling volatiles is suppressed, and consequently cannot reach the vapor state. In substantiating this theory it was observed that when systems which showed only peaks 1 to 8 had the excess pressure released and were rechromatographed, the normal volatile spectrum was obtained. Another experiment that was performed to show this effect was the addition of a wide boiling point range of carbonyls to a cold finger containing distilled water. The trap was then heated to 70°C and a 6 ml. vapor sample was removed and gas chromatographically analyzed. Here again it was observed that until the pressure on the system was released only the low boiling volatiles were being sampled. Because of these findings it was adopted as standard procedure to release the excess pressure on the isolate trap before vapor samples were taken. Previous research also indicated that no volatile components were lost during this procedure.

Another observation made concerning the gas chromatographic analyses of the flavor isolate volatiles concerns functional group analysis. It was apparent that the functional group reagents that were employed did not always react quantitatively with the flavor components. This meant that in some cases the peaks involved were greatly reduced or eliminated while at other times the diminution was only slight. Possibly only the vapor that came into immediate contact with the functional group reagent was chemically treated, which limits the reliability of such a functional group classification.

Identification of Flavor Compounds Detected In HTST Fluid Sterile Milk by Gas Chromatography

The compilation of data interpreted from over 240 gas chromatographic analyses of sterile fluid milk is shown in Table 5. These data relate to the off-flavor development in fluid sterile milk during storage at 4.4°, 22°, and 36°C. In order that the absolute retention time data for each peak could be used as an identification constant, the average retention time of each peak was calculated and is recorded in Table 5. Some compounds isolated in the flavor distillate were found to have identical or nearly identical retention times with one another. This necessitated functional group analyses on the vapor of flavor isolate in conjunction with gas chromatographic analysis, in order to determine in which functional group the unknown compound belonged. Retention times of the proper class of standards could then be compared to the unknown peak for the purpose of tentative identification. The functional groups that were of interest in this research were aldehyde, ketone, alcohol, ester, and sulfide. The technique used in this functional group analysis procedure was that of Hoff and Feit (1964), which has been previously discussed in the procedure. Table 5 represents the compilation of the data found in Figures 1-10, which was a gas chromatographic survey of fluid sterile milk through a three month storage period at temperatures of 4.4°, 22°, and 36°C. The data in the four columns on the right in Table 4 enable the reader to determine the peaks that were present in the control and storage samples, and those that appeared or disappeared during the storage life of the HTST fluid sterile milk. This information also indicates if any particular peak was present at all storage temperatures, or at only one or two of the storage temperatures.

Peak number 1 through 5 were not conclusively identified; however, peaks 1 and 3 appeared to be aldehydes because of their marked response to both of the carbonyl reagents. The functional groups of the components in peaks 3', 5 and 5' could not be determined with sufficient consistency to give any useful information for peak identification. Peak 6 exhibited a retention time of 2.0 minutes, which corresponded to that of ethanal (1.9 min.). The retention time coupled with the information gained through functional group analyses suggested the identification of peak 6 as the C_2 alkanal. The presence of ethanal in the flavor isolate of fluid sterile milk has also been supported by thin-layer identification of its 2,4 dinitrophenylhydrazone, (Fig. 13). Peak 6', which had an average retention time of 2.8 minutes, has been tentatively identified as propanal. Peak number 7 has not been identified, but functional group analyses classified this component as an aldehyde. Since this peak did not correspond in retention time data to that of any low saturated aldehydes, it is possible that it belongs to the class of unsaturated alkanals. Peak 8 has been tentatively identified as acetone. The presence of acetone in the flavor isolate has also been shown by thin layer separation of its

2,4 dinitrophenylhydrazone (Figs. 16, 17). Peak 8' with a retention time of 4.2 minutes was confirmed as an aldehyde, but confirmation as to the carbon number was not obtained. It is interesting that butanal, whose presence in the flavor isolate was also shown by thin-layer separation of its hydrazone derivative, exhibits a retention time of 4.6 minutes. Because of these data it is believed that peak 8' is the C_4n-alkanal. Peak 9 was found to contain two components by functional group analyses. Both ethanol and butanone with identical retention times of 5.6 minutes on the Carbowax 20 M column were shown to contribute to the response of peak 9. This conclusion was drawn after functional group reactions with nitrite and sodium hydroxide showed ethanol present, but failed sufficiently to diminish the peak. This seemed to indicate the presence of another component. Further investigation with functional group reagents showed another component present, which was identified as 2-butanone. Peak 10 was not identified and could not be classified by functional group analyses. Peak 10' was functionally classified as an aldehyde, but did not correspond in retention time to any of the alkanal standards. Peak 11 was not identified, but was believed to contain both an alkanal and an alkanone. The reasoning behind this postulation is that during the course of the three storage studies, this peak would sometimes react with potassium permanganate, sometimes with acid hydroxyl amine, and sometimes with both reagents. Peak 11' was identified as 2-pentanone. It was not present in the control samples, but appeared to emanate from peak 11 during the first month of storage at 4.4°, and 22°C. This could account for the positive alkanone reaction that was recorded for peak 11. Peak 12 was not tentatively identified, but was functionally classified as a ketone, while peak 12', which was also unidentifiable, was classified as an aldehyde. Peak 13' was believed to contain either or both classes of carbonyls, as indicated by the group reagents. Functional group analyses and the retention time data of the known standards 2-hexanone and n-hexanal, served as the basis of this tentative identification. Hexanal was also identified as a 2,4 DNP hydrazone derivative in the flavor isolate of the 3 months 22°C. storage samples by TLC (Fig. 15). Peak 14 was shown as a possible C_3 alcohol by correlation with retention data for alcohol standards, and peak diminution when reacted with the nitrite reagent. Peak 14' was tentatively identified as n-butanol, not only because of its comparative retention time with a standard, and decreased peak height when treated with sodium nitrite; but also because of the increased peak height when the vapor sample was treated for the identification of esters with sodium hydroxide. Peaks 14, 15, and 16 were not identified, nor were any functional group analyses data obtained. Peak 16' with a retention time of 23.4 minutes was tentatively identified as heptanal. Peak 17 was identified as 2-heptanone; the C_7 alkanone has also been identified by thin-layer chromatography of its 2,4 dinitrophenylhydrazone derivative in the three months

22°C. storage samples. Peaks 18, 18', 18", 19 and 20 were not able to be identified. Peak 21 was shown to exhibit the same retention time as n-hexanol but tentative identification was not conferred upon this peak, since it did not consistently react with the functional group reagent employed for alcohol confirmation. Peak 22 was not tentatively identified, but it was functionally classified as an aldehyde. Peak 23 which exhibited the characteristic retention-time and functional group data of 2-nonanone was unable to be confirmed. The only other peak which was tentatively identified gas chromatographically was peak 24, which was believed to be furfural. Of the remaining peaks 25 to 30 the only information that could be deduced from the chromatograms was that peaks 27 and 29-30 were most probably aldehydes and peak 26 a ketone.

In the following section the components that have been gas chromatographically identified from the flavor isolate, and are believed to be of flavor significance, are discussed.

Flavor Volatiles Tentatively Identified in HTST Fluid Sterile Milk by Gas Chromatography and their Flavor Significance

Ethanal

Peak number 6, which has a retention time value of 2.0 minutes, has been tentatively identified as ethanal. Ethanal was found in the control and storage samples and as can be observed from the chromatograms (Figs. 1-10) the ethanal concentration remained quite stable from the control

Table 5. Retention times of flavor components detected in HTST fluid sterile milk during a three month storage survey by gas chromatography.

Peak No.	Ret. Time Min.	Tentative Identity of Peaks	Retention Time Knowns	Control	1 Month Storage	2 Months Storage	3 Months Storage
1	.5	Unknown		+	+	+	+
1'	.7	Unknown		-	-	-	-
3	.8	Unknown		+	+	+	+
3'	1.0	Unknown		-	+	-	-
5	1.4	Unknown		+	+	+	+
5'	1.8	Unknown		+	+	+(22°C)	+(36°C)
6	2.0	Ethanal*	1.9	+	+	+	+
6'	2.8	Propanal	2.9	+	+	+	+(4.4°C)
7	3.2	Unknown		+	+	+	+
8	3.6	Acetone*	3.4	+	+	+	+
8'	4.2	Butanal*	4.6	-	+(36°C)	+(36°C)	+(22°-36°)
9	5.8	Ethanol-Butanone*	5.6-(5.6)	+	+	+	+
10	6.8	Unknown		+	+	+(22°-36°C)	+
10'	7.2	Unknown		+	+(4.4°-36°)	+(4.4°-22°C)	+(4.4°-22°C)
11	8.6	Unknown		+	+	+	+(4.4°-22°C)
11'	8.9	Pentanone*	8.4	-	+	+(22°C)	+(36°C)
12	9.6	Unknown	+	+	+	+	
12'	11.0	Unknown		+	+	+(22°-36°C)	+
13	12.6	Unknown		+	+	+	+
13'	16.2	Hexanone-Hexanal*	16.4	+	+	+	+
14	17.4	Propanol	17.0	+	+	+(22°-36°C)	+
14'	19.2	Butanol	19.2	+	+	+(22°-36°C)	+
14"	20.4	Unknown		-	-	-	+(4.4°C)
15	20.6	Unknown		+	+(4.4°-36°C)	+(22°-36°C)	+
16	21.6	Unknown		+	+	+	+(4.4°-36°C)

16'	23.4	Heptanal	23.8	-	+	+(22°-36°C)	+(4.4°-36°C)
17	24.2	Heptanone*	23.6	-	+	+(22°-36°C)	+(22°-36°C)
18	26.0	Unknown		+	+	+(22°C)	+(4.4°-36°C)
18'	26.9	Unknown		+	+(36°C)	+(22°C)	-
18"	28.2	Unknown		+	-	-	-
19	28.8	Unknown		+	+(4.4°C)	+(22°C)	+(4.4°C)
20	30.2	Unknown		+	+	-	+(36°C)
21	30.6	Unknown		-	+(22°C)	+(22°C)	-
22	32.2	Unknown		-	+(36°C)	+	+(4.4°C)
23	33.4	Unknown		+	+	+	+
23'	34.0	Unknown		-	+(36°C)	-	-
24	34.8	Furfural	35.2	+	+	+(36°C)	-
25	36.4	Unknown		+	+(22°-36°C)	+	+(4.4°C)
25'	39.0	Unknown		+	+(36°C)	-	+(4.4°C)
26	40.4	Unknown		+	+(36°C)	+(22°-36°C)	-
27	45.2	Unknown		-	+	-	+(36°C)
29) 30)	48.4	Unknown		-	-	-	+

*Compounds identified form the flavor isolate of HTST fluid sterile milk by thin-layer chromatography of their 2,4 dinitrophenylhydrazone derivatives.

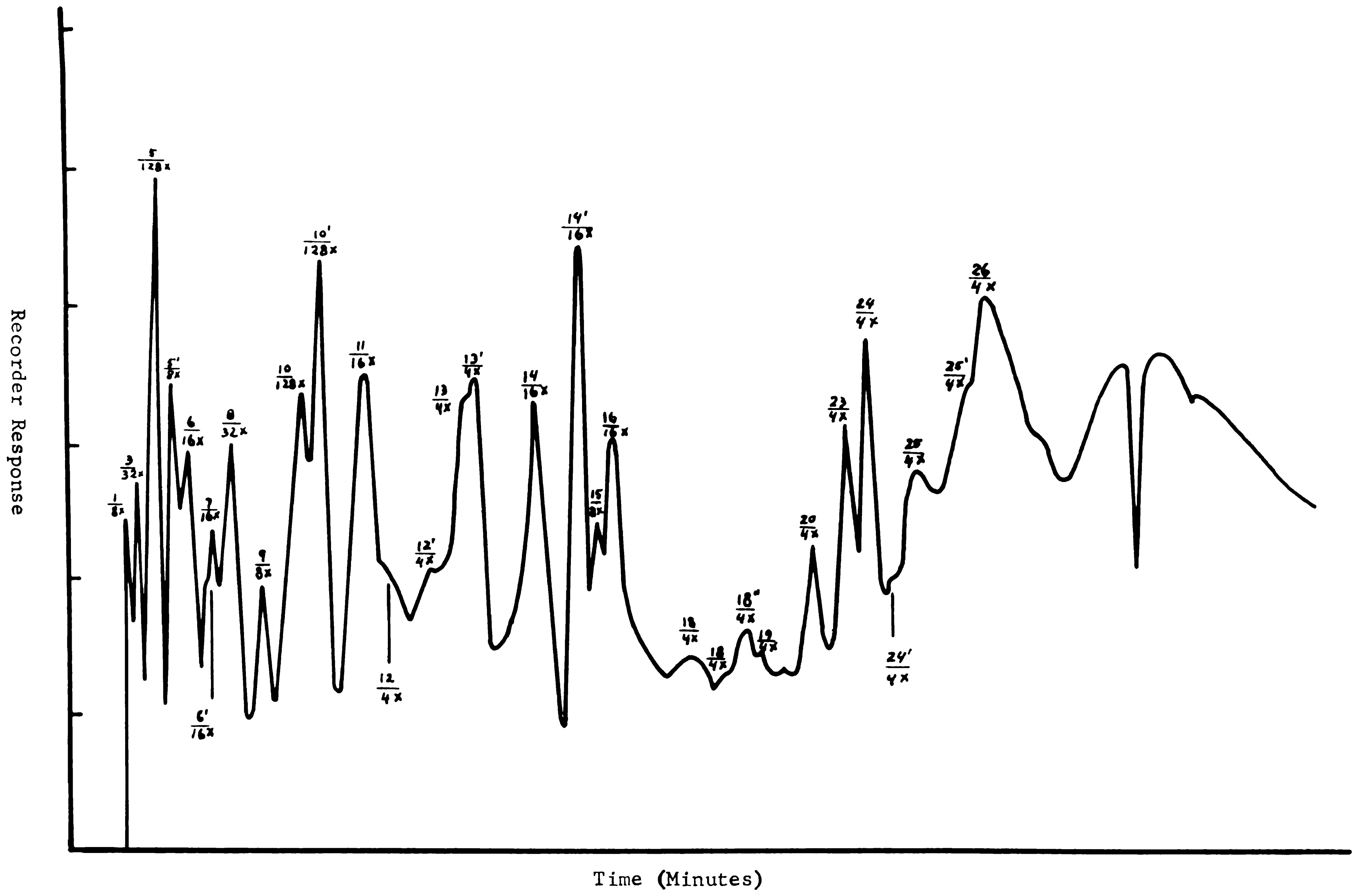

Figure 1. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk control sample.

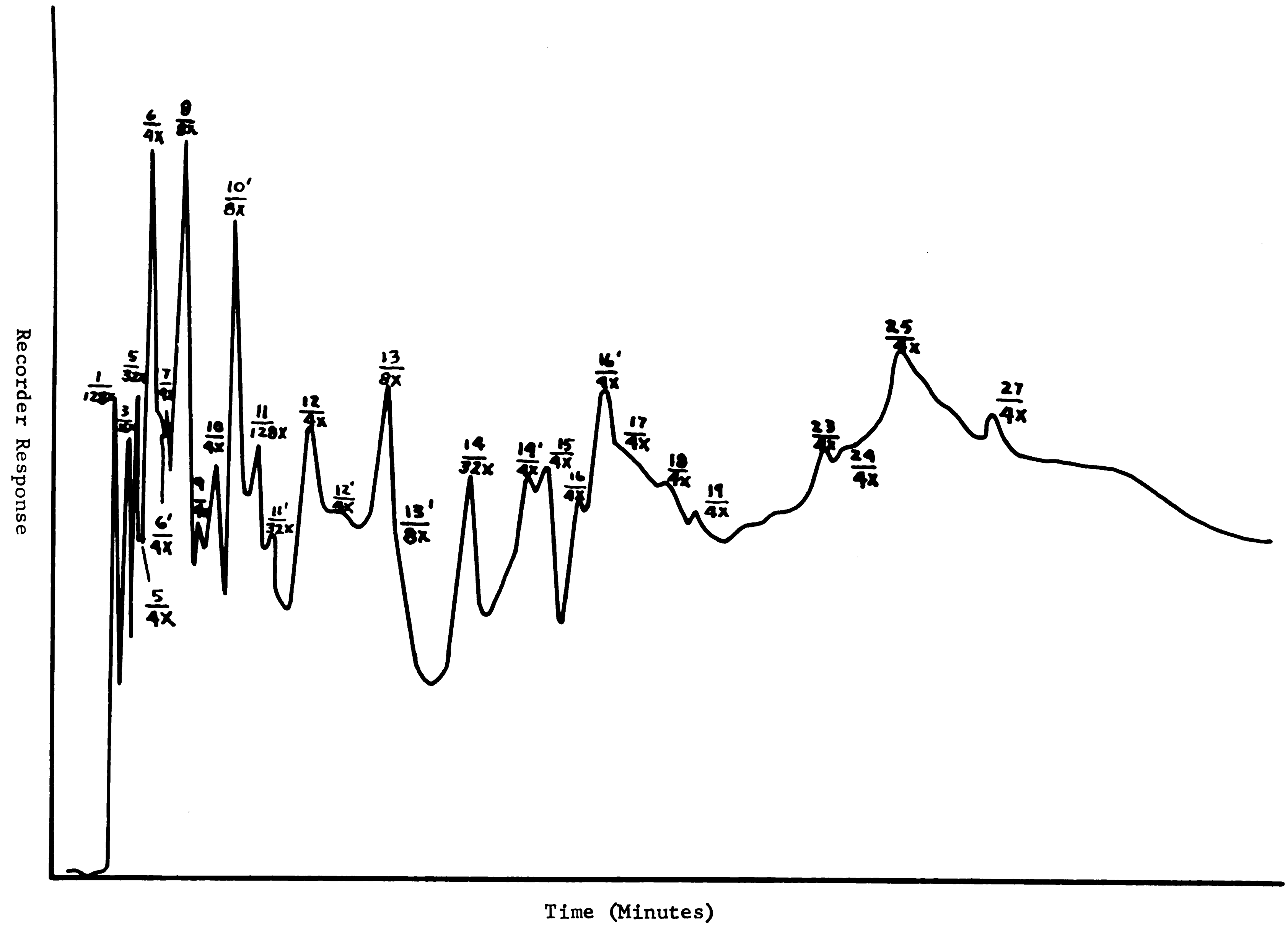

Figure 2. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after one month storage at 4.4°C.

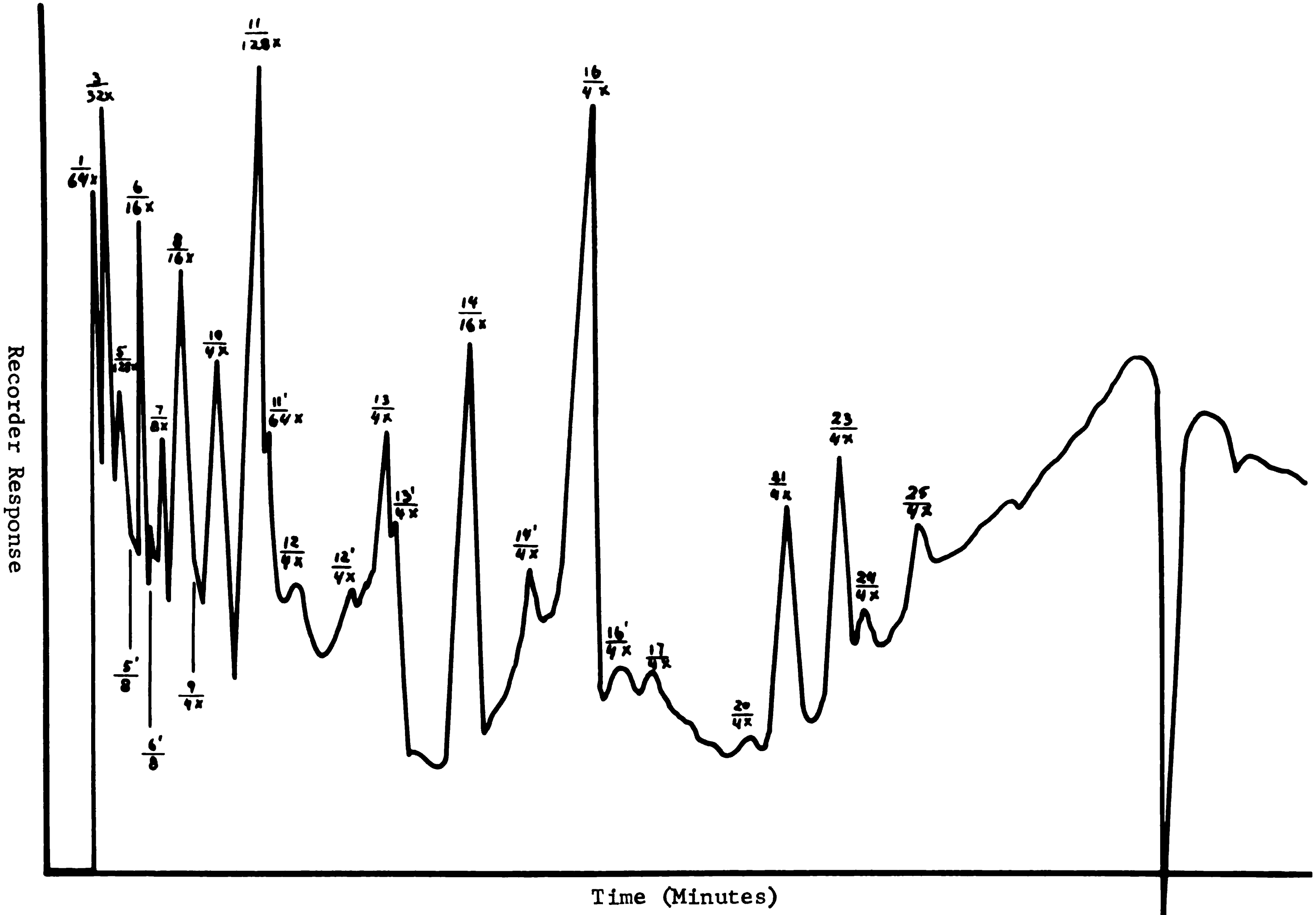

Figure 3. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after one month storage at 22°C.

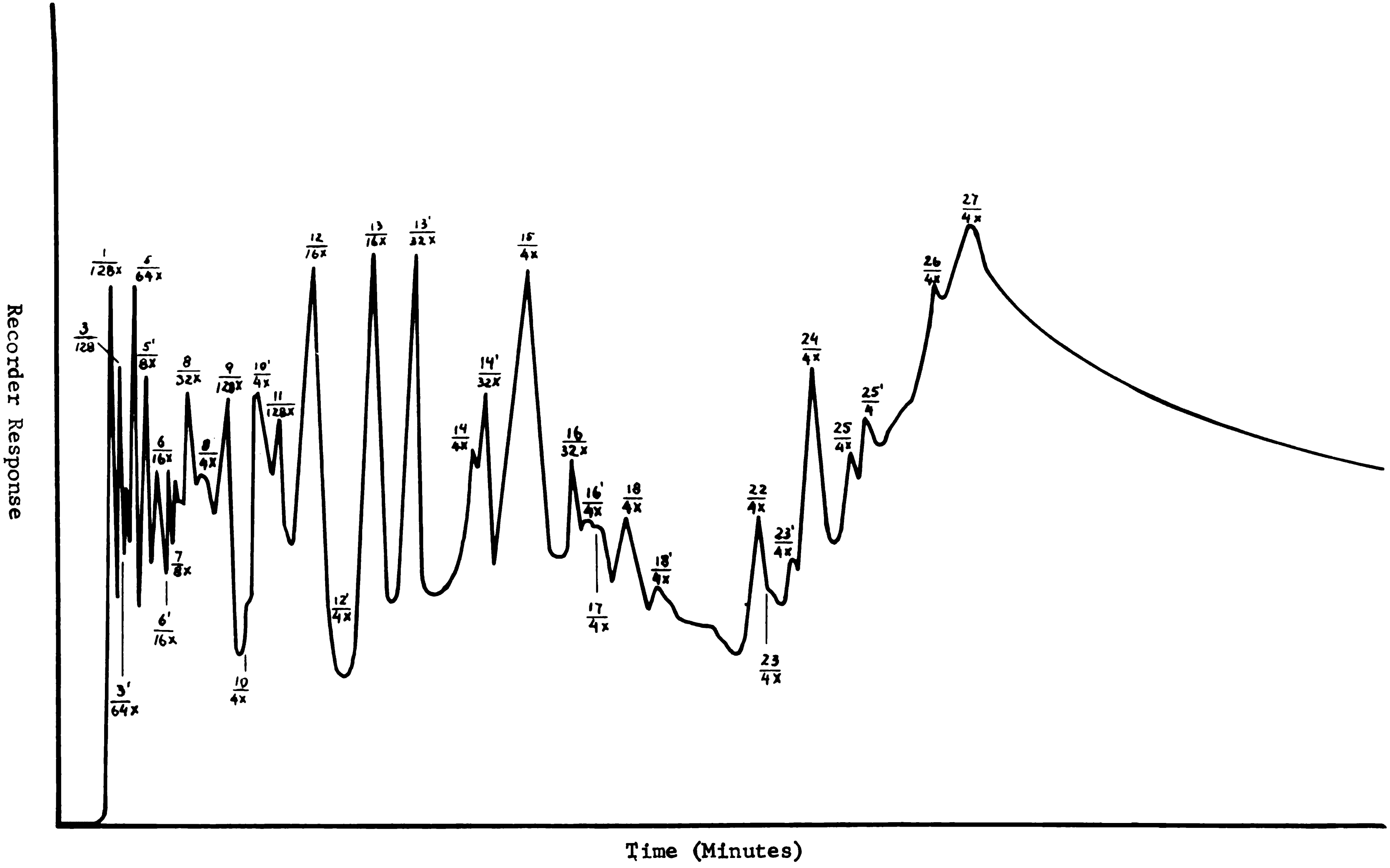

Figure 4. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after one month storage at 36°C.

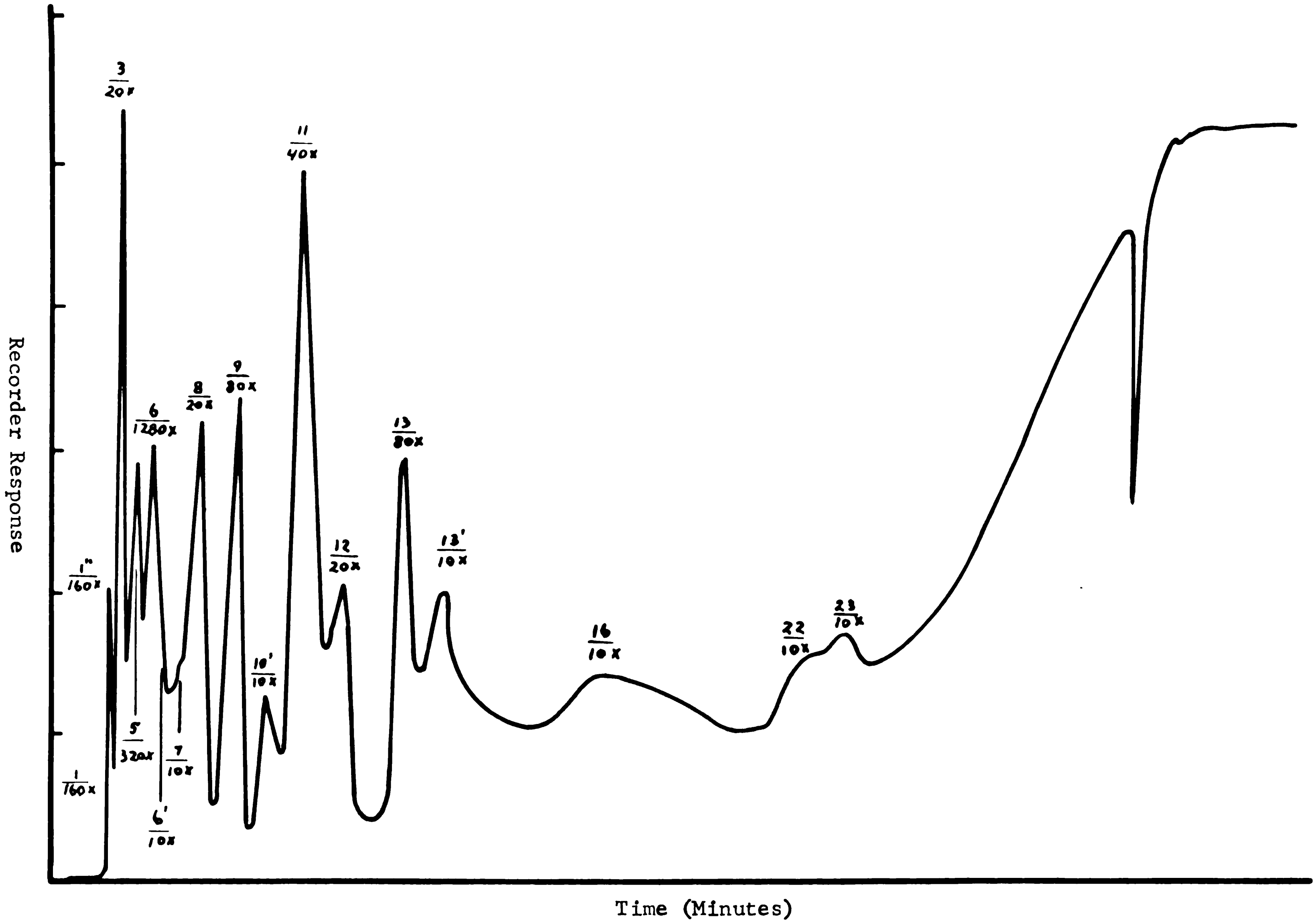

Figure 5. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after two months storage at 4.4°C.

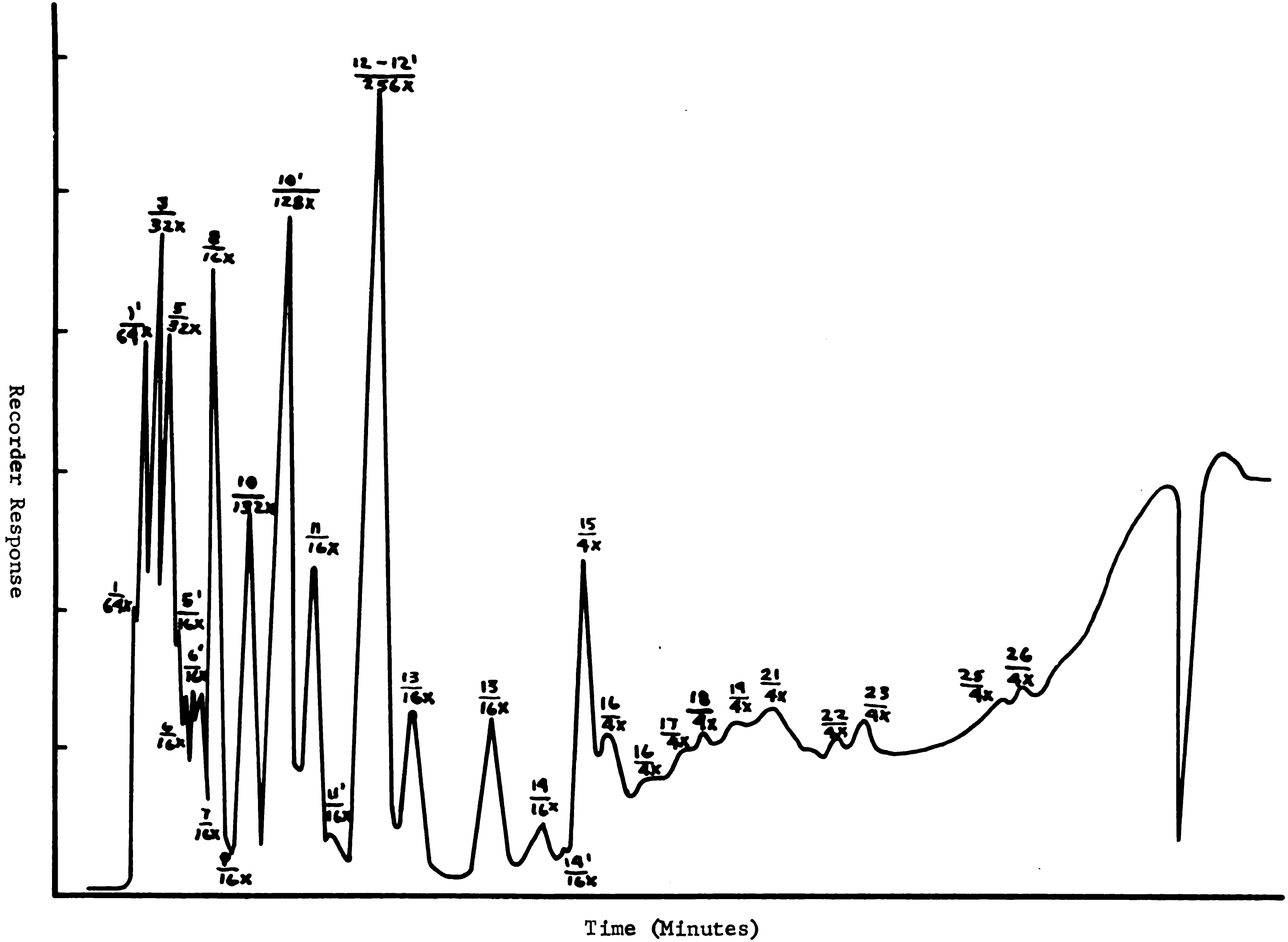

Figure 6. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after two months storage at 22°C.

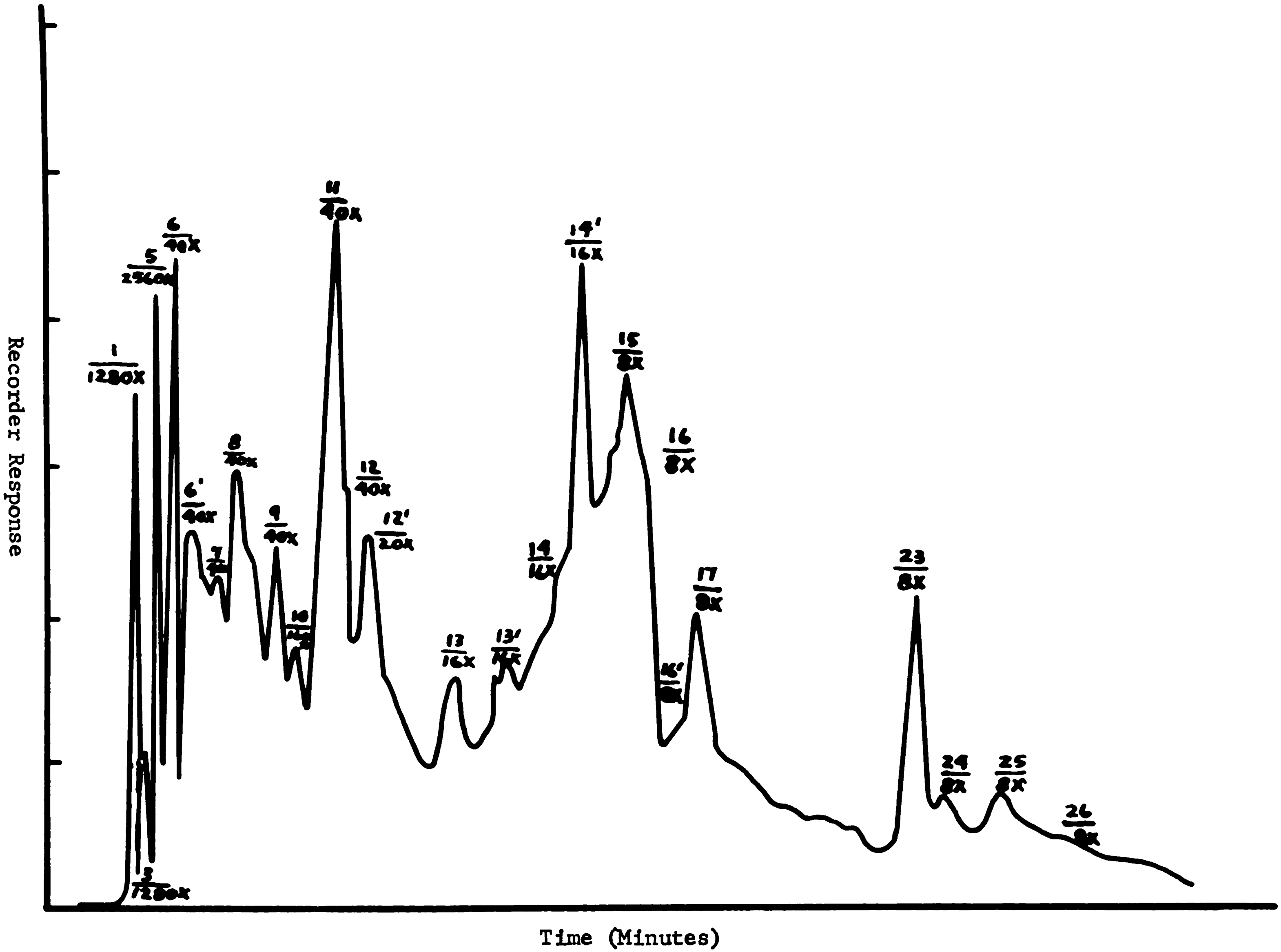

Figure 7. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after two months storage at 36°C.

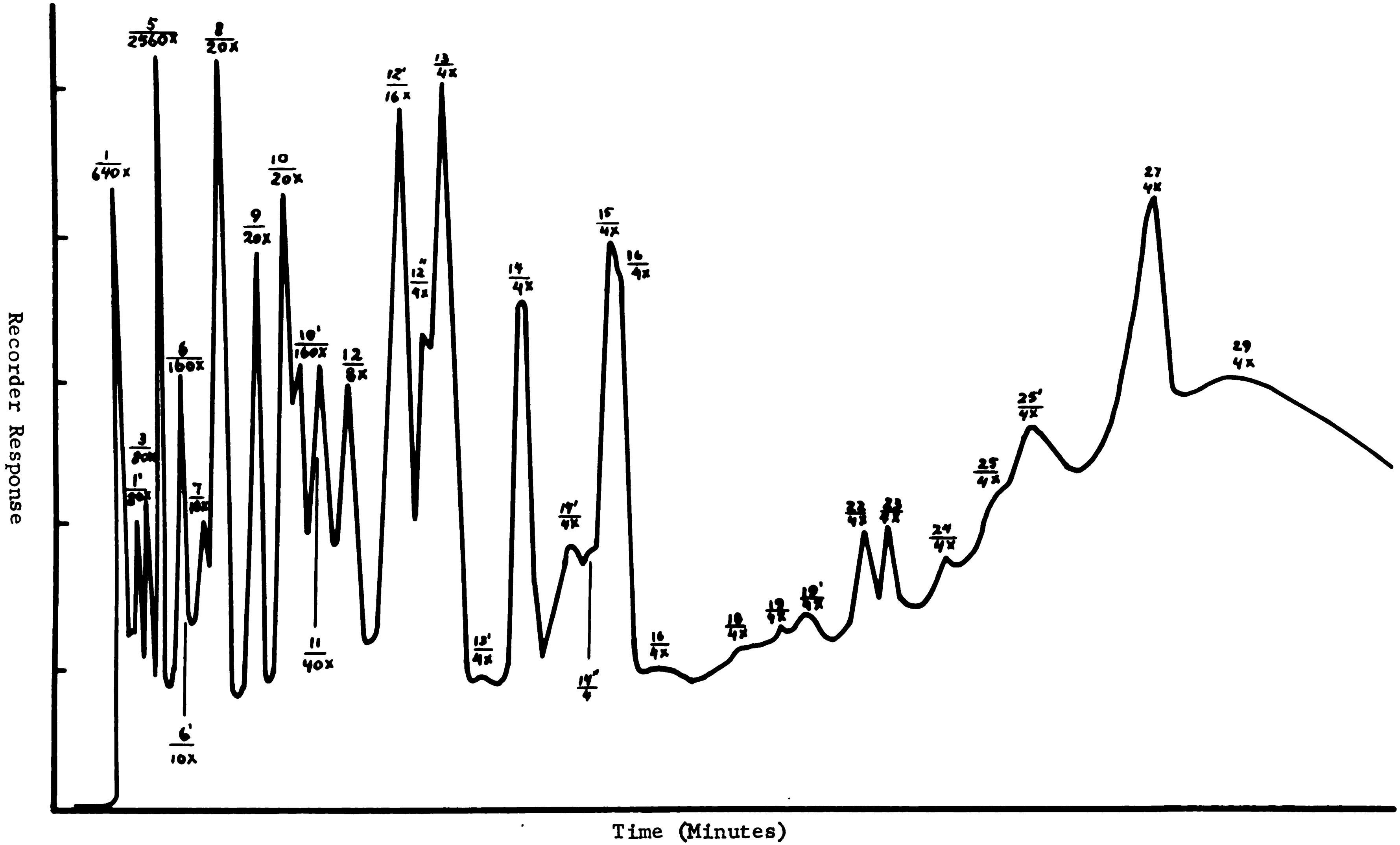

Figure 8. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after three months storage at 4.4°C.

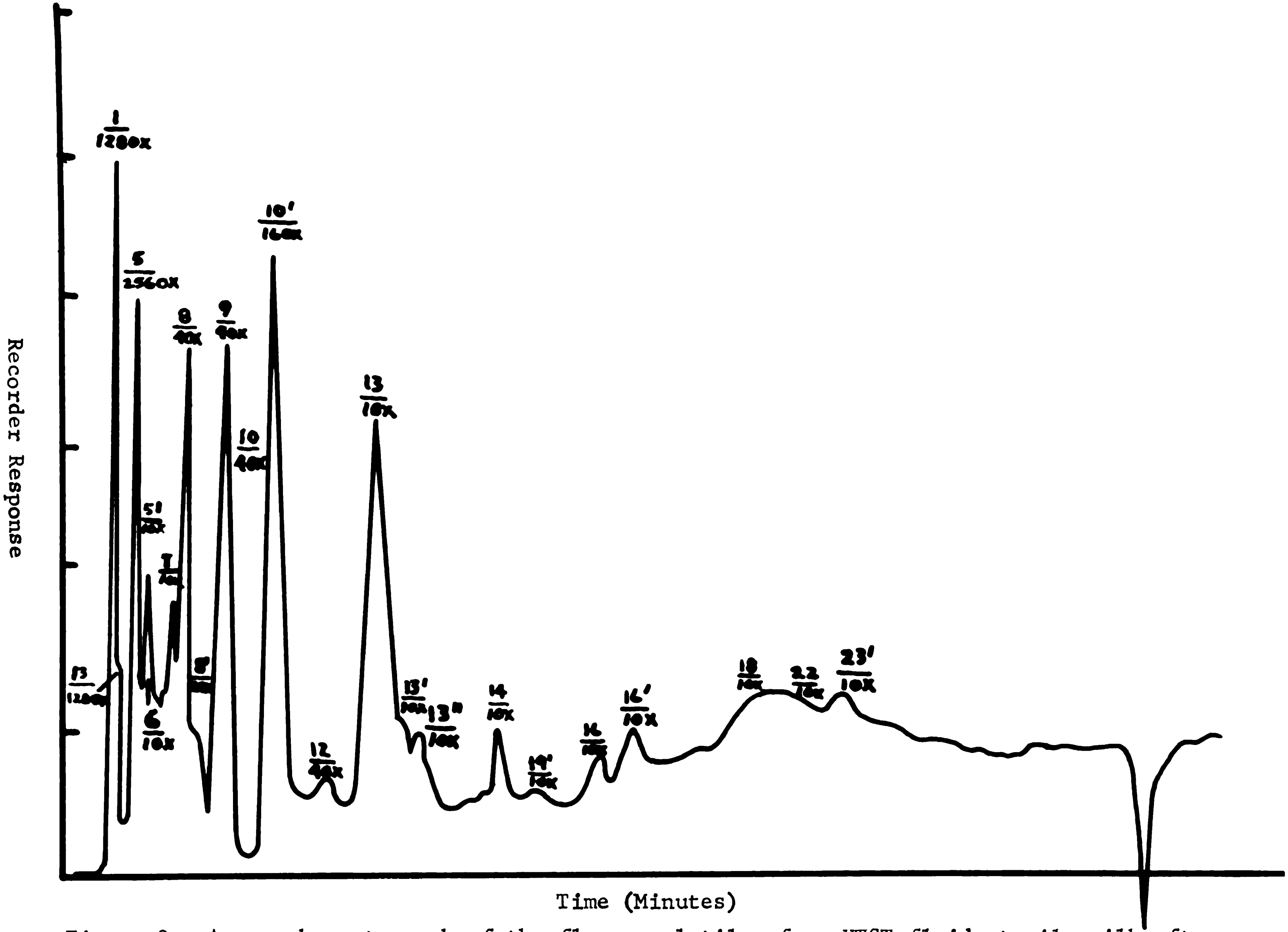

Figure 9. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after three months storage at 22°C.

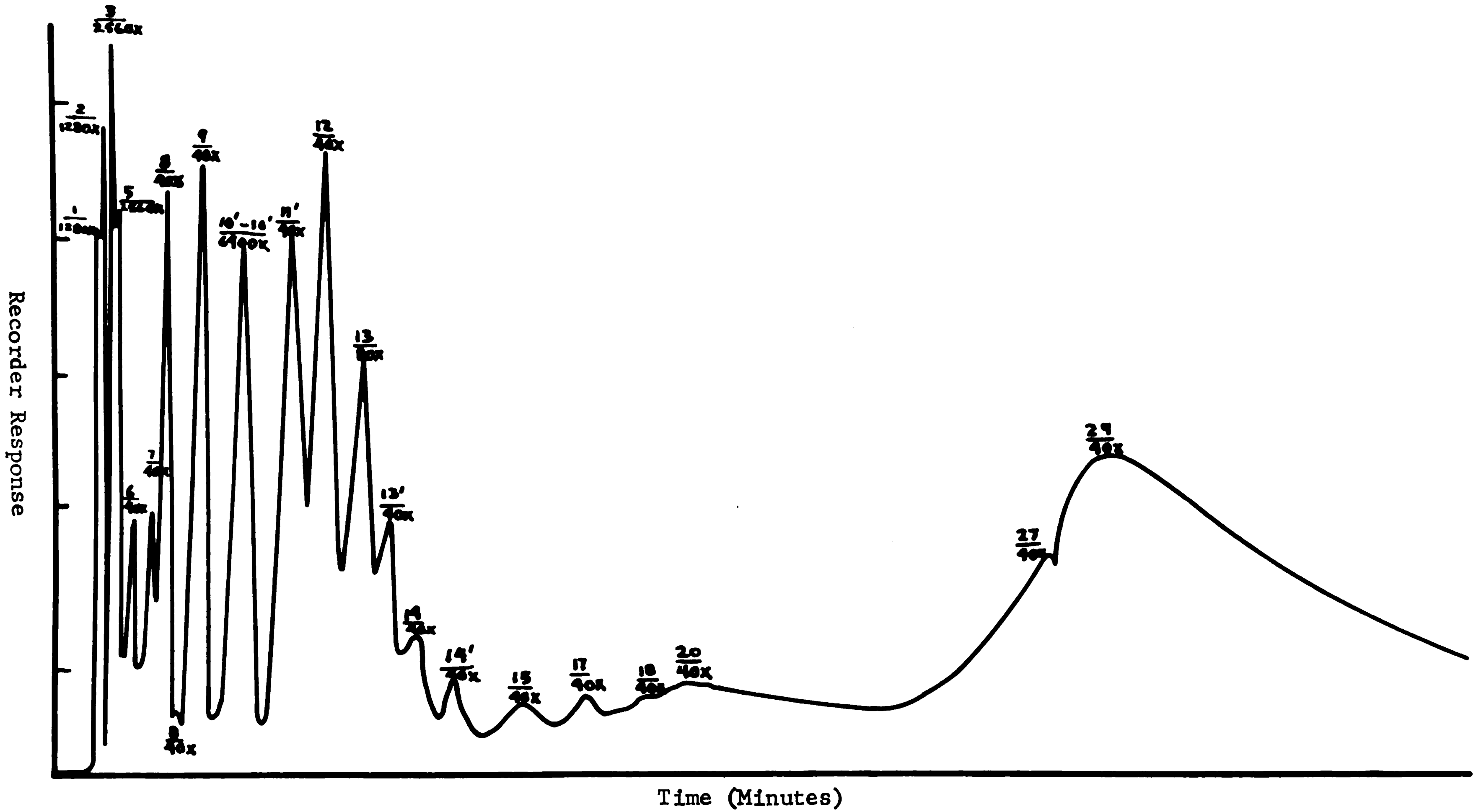

Figure 10. A gas chromatograph of the flavor volatiles from HTST fluid sterile milk after three months storage at 36°C.

samples through the first month of storage at all three storage temperatures. Between the first and second month of storage the concentration of ethanal increased. This increase was detected in both the second and third month storage samples with the largest concentrations being present at 4.4°C. The reason for the higher alkanal concentration being found in the refrigerated samples could be accounted for in two ways: a) that higher storage temperatures may favor aldolization of this active carbonyl, and/or b) since ethanal is only slightly less reactive than normal sugar fragentation products in forming carbonyl-amine complexes either or both of these mechanisms could account for the increased browning in the storage samples held at 22° and 36°C. Research has shown that amino compounds, including peptones and albumins do catalyze aldol condensation (Hodge, 1953), such higher alkanals could then more readily react with free amino groups present to form the carbonyl-amino complexes.

The contribution of ethanal to the stale flavor that develops in the storage fluid sterile milk is uncertain, since ethanal has also been identified as a flavor component of raw milk.

Propanal

Propanal has been tentatively identified as peak 6' in the gas chromatographic analyses of the fluid sterile milk. Propanal was observed in the sterile milk throughout its storage life, and its concentration appeared to be relatively constant until the third month at 22°and 36°C. At this time peak 6' disappeared from the chromatograms. The relatively constant concentration of propanal in the sterile fluid milk would seem to indicate that it may have resulted from the following two sources: a) hydrolysis of lower aldehydes from plasmalogens by sterilization and/or b) it may have been present in the milk before processing. Propanal has also been shown to arise from the autoxidation of linolenic acid.

Acetone

Acetone, a well known flavor component of dairy products, was tentatively identified by functional group analyses and retention time data. Acetone was also identified in both the storage and control samples by thin-layer chromatography of its 2,4 dinitrophenylhydrazone (Figs. 16, 17). The concentration of acetone in the control sample was quite high, due possibly to the hydrolysis of the β-keto ester or the decarboxylation of β-keto acids during sterilization of the milk. Subsequent storage of the fluid sterile milk for one month at 4.4°C. showed a decrease in acetone concentration; this loss was also noted in the one month 22°C. and the two month 4.4°C. and 22°C. storage samples. Increases in the acetone concentration were observed in the two months 36°C. and three months 22° and 36°C. fluid sterile milk samples, which had been judged stale. An increase of 30-50% in acetone concentration in the stale sterile milk samples was noted, depending upon the storage temperature. These increases were most probably caused by storage at elevated temperatures, which previous research has shown to cause spontaneous decarboxylation of β-keto acids resulting from the β-oxidation of fatty acids. Both Wong and Patton (1962) and Boldingh and Taylor (1962) have presented evidence concerning the ease with which this decarboxylation reaction occurs. If this supposition is true it appears as though the limiting reaction in the formation of acetone could be the β-oxidation of the fatty acids.

Butanal

The tentative identification of butanal by gas chromatography was supported by thin-layer chromatographic separation of its 2,4 dinitrophenylhydrazone (Fig. 15). Butanal was detected from the following storage samples: one month at 36°C., two months at 36°C., and three months at 22° and 36°C. The concentration of butanal detected from the storage samples increased quite noticeably between the first and second months of storage at 36°C., and then leveled off between the second and third months. The fact that butanal was not isolated from the control samples, as a hydrazone derivative, appears to indicate that it is not a normal flavor component of fluid milk before heat processing, or that it arose as a direct result of heat processing. This evidence suggest that the butanal probably originated as a result of lipid autoxidation. This could explain why the butanal was initially found at low concentrations. Then in later storage, as the autoxidative chain reactions progressed, the concentration of butanal increased.

Ethanol

Ethanol was the first alcohol in the homologous series that was tentatively identified in the gas chromatographic effluent. This identification was difficult, since functional group analyses indicated that peak 9 was a composite of two flavor compounds; ethanol and 2-butanone. Identification of ethanol was made on the basis of retention time, and its reaction with two functional group reagents. Peak 9 exhibited the normal decrease in recorder response when the flavor isolate vapor was treated with nitrite solution, which is used for alcohol confirmation. Another interesting change in the chromatogram occurred when the effluent coinciding with this peak was treated with sodium hydroxide. Under these alkaline conditions an increase in recorder response was noted. This was believed to indicate not only the presence of ethanol, but an increase in ethanol due to the hydrolysis of an ethyl ester or esters present in the flavor isolate. Peak 9, which contains both ethanol and 2-butanone, was present in all control and storage samples and increased significantly in concentration in the 36°C. storage samples at one and two months, and in the 22° and 36°C. three month storage samples. Because of the binary nature of this peak it was not possible to positively attribute the increase in concentration to either of the components; however, after observing that the peak increased only under elevated storage temperatures it seems likely that the increase was probably due to the methyl ketone present. The exact role played by ethanol in the flavor of dairy products is not fully understood, but Day et al. (1957) and Cobb et al. (1963) and others have shown its presence in connection with oxidized and carmel flavors in milk products. Both of these flavors have been implicated with the stale flavor exhibited in stored dairy products.

Butanone

The second component of peak 9 has been tentatively identified as 2-butanone, a C_4 alkanone, whose presence was also confirmed by thin-layer separation of its 2,4 dinitrophenylhydrazone (Fig. 16). Because of the ease with which methyl ketones can be formed in heated milk systems, it is believed responsible for the increase in peak 9 at elevated storage temperatures.

Hexanal and Hexanone

Peak 13' was tentatively identified and shown to contain two components, both carbonyl in nature. Storage increased the concentration of peak 13' with more definite increases being observed in the 36°C. storage samples. The initial presence of peak 13' was probably due to the presence of 2-hexanone, formed as a result of hydrolysis of the milk fat at the high processing temperature. The author suggests that hexanal does not appear until later in the storage life of the sterile fluid milk, when autoxidation of linoleic acid has been initiated. This may account for the large increase in concentration of peak 13' in the three months 22° and 36°C. storage samples. The presence of hexanal has also been shown by TLC analyses of its 2,4 dinitrophenylhydrazone (Fig. 15) which was isolated from the three months 22°C. storage samples, but not found in the control samples.

Butanol

Tentative identification of peak 14' as butanol was made from retention time data comparisons with standards, and functional group analyses. It was also apparent that the butanol peak increased when the flavor isolate was treated with sodium hydroxide. This increase was believed due to the presence of a butyl ester or ester in the flavor isolate; however, no confirmational data could be obtained to support this supposition. It is also interesting that the butanol concentration increased during product storage at temperatures of 22° and 36°C. These increases could have resulted from lipid autoxidation, by the dismutation of hydroperoxides, which has been shown to yield alcohols.

Heptanal

Heptanal has been tentatively identified as the flavor component present in peak 16'. Its presence was not detected by gas chromatographic analyses in the control samples, but was observed in all of the storage samples. The concentration of this flavor component was observed to increase during product storage with the most significant increases being in the two months 36°C. and three months 22°C. samples. Absence of this flavor volatile in the initial control samples would seem to eliminate processing as the factor causing its development, and suggests that lipid oxidation is the most probable factor accounting for its presence.

Heptanone

The identification of 2-heptanone as a flavor component, which appeared during storage of the fluid sterile milk was based on retention time and data from functional group analyses. 2-Heptanone was also identified in the thin-layer separation of the methyl ketones (Fig. 17) isolated from HTST fluid sterile milk held for three months at 22°C. Increases in the concentration of 2-heptanone during storage can be seen from inspection of Figures 1-10 and were more predominant in the storage samples held at elevated temperatures. This could indicate that 2-heptanone was relevant to the storage flavor of sterile fluid milk.

Furfural

Peak 24 has been tentatively identified as furfural. This component was present in both the control and storage samples up to two months. In the three months storage analyses this peak was absent at all temperatures. It should be noted that the supposed furfural concentration was shown to increase during the first two months of storage. Furfural is a common product of Maillard browning resulting from sugar fragmentation and dehydration as well as from hydroxymethylfurfural conversion, which can result from heat degradation of sugars. Because of the boiling point differential between the two compounds, this peak was believed to be furfural and not hydroxymethylfurfural. Hydroxymethylfurfural has a much lower boiling point (furfural--161.7°C., hydroxymethylfurfural--110°C.) than furfural and this would have permitted hydroxymethylfurfural to elute from the column prior to furfural. The interesting point concerning this tentatively identified compound is that it is not present in the three months storage samples, which undoubtedly contains more browning products than the previous storage samples. This loss could be explained by the aldol condensation of furfural, leading to the formation of the carbonyl-amine complex, and eventual melanoidin formation (Rice et al., 1947). Furfural, diacetyl, and hydroxymethylfurfural on the other hand are usually considered to be rather unreactive, and because of this their concentrations are sometimes used as an index to the degree of Maillard browning that has taken place. In view of these conflicting observations the exact fate of the furfural in the three months storage samples is not known. It may also be possible that the high sterilization temperature could have effected the milk system in such a way as to permit the furfural to react in the carbonyl-amine condensation. This condensation reaction has been shown by researchers to take place with lower carbon chain aldehydes, and keto acids, but at a rate slower than the reactions with normal fragmentation products (Hodge, 1953).

The participation of these tentatively identified flavor compounds in the stale, or stale-oxidized flavor which has been associated with stored dairy products both in the fluid and dry state can best be explained in the following manner. Whitney and Tracy (1949), who determined that stale flavor was of greater concentration in the fat phase of dairy products, directed the activities of future researchers to this starting point in determining off-flavor compounds responsible for causing the storage products to elicit stale flavor response. Bassette and Keeney (1960) in studying defects in non-fat dry milk powder indicated that both Maillard browning and lipid oxidation were involved. They presented evidence for both odd and even chained aldehydes, as well as for furfural and diacetyl. Forss et al. (1960b) have shown the significance of the lower saturated aldehydes C_1-C_9 in the oxidized flavor of dairy products. More recently Nawar et al. (1963) in working with stale flavor in dry whole milk powder identified formaldehyde, ethanal, and propanal from two separate flavor extractions, which were designed specifically to extract stale flavor components. Bingham (1964) in working with stale flavor in 3:1 concentrate tentatively identified ethanal, propanal, and n-pentanal as the n-alkanals present in the stale sterile concentrate.

The presence of methyl ketones has been shown both for oxidized and stale flavors. Arnold et al. (1966) have identified 2-heptanone, 2-nonanone, 2-undecanone and 2-tridecanone, by means of mass spectroscopy, as ketones present in the stale flavor of sterile concentrated milk. Nawar et al. (1963) identified acetone, 2-butanone, and 2-pentanone as the ketones present in stale dry whole milk powder. These data also agree with the findings of Parks and Patton (1961) who suggested that staleness may be caused by a combination of carbonyl compounds with their respective concentrations determining which flavor will be prominant: stale or oxidized. The former flavor apparently results at a lower carbonyl concentration than the latter. Thus, from what can be determined from previous research, and from the finding of this research, it appears that lower aldehydes and methyl ketones do contribute to the stale flavor. Those compounds which appear during product storage may have a greater contribution to this flavor defect.

Analysis of 2,4 Dinitrophenylhydrazone Derivatives

Tentative identification of carbonyls present in HTST fluid sterile milk flavor distillate was made by gas chromatographic analyses utilizing the techniques of volatile retention time, and functional group analyses. Confirmation of these data was accomplished by thin-layer chromatographic identification of their 2,4 dinitrophenylhydrazone derivatives. Following the formation of the 2,4 dinitrophenylhydrazones by the method outlined in the procedure, the carbonyl derivatives were separated according to class utilizing the procedure of Schwartz et al.(1962) (Figs. 14, 15, 16, 17). Examination of the column during the separation of carbonyl derivatives from the control samples showed the presence of a gray methyl ketone band, and a tan saturated aldehyde band. The presence of these two groups was also spectrophotometrically confirmed by observing their absorption maxima. Fraction 1 (Fig. 11) from the class separation of hydrazone derivatives was observed to have an absorption maximum at 365 millimicrons, and fraction 2 and 3 (Figs. 12, 13) absorption maxima at 355 millimicrons. These correspond to the absorption maxima of methyl ketones and saturated aldehydes as published by Schwartz et al. (1962). Class separation of the 2,4 dinitrophenylhydrazone derivatives from the three months 22°C. storage sample, which had been judged stale, showed the presence of 2,4 dienals (lavender band on the Celite 545-Magnesia column) along with the methyl ketone and saturated aldehyde bands. Here again the presence of methyl ketones and saturated aldehydes was confirmed spectrophotometrically using the Beckman DBG recording spectrophotometer (Figs. 11, 12, 13). It was also observed that in the storage samples the methyl ketones band was a charcoal color rather than gray, signifying increased methyl ketone concentration in the storage samples. Spectrophotometric confirmation of the presence of 2,4 dienals was not obtained from any of the three class separations of hydrazone derivatives from the storage samples because they could not be eluted from the column. Even after two liters of 100% chloroform had been passed over the absorption column the 2,4 dienals were not eluted. Working on the assumption that chloroform possessed insufficient polarity to strip the 2,4 dienals from the absorption column a mixture of 10%, 15%, and 30% v/v methanol to chloroform mixtures were tried, but these likewise failed to elute these compounds.

It was interesting that no monounsaturated aldehydes were observed in the class separation of the 2,4 dinitrophenylhydrazones of either the control or storage samples.

Thin-layer chromatographic separations of the methyl ketones in the control samples by the procedure described by Badings and Wassink (1963) confirmed the presence of acetone and 2-butanone (Fig. 16). Acetone, 2-butanone, and 2-heptanone were identified in the three months storage samples held at 22°C. (Fig. 17).

The absolute importance of acetone and butanone in contributing to stale flavor deterioration in fluid sterile milk is uncertain, since their presence has been detected in fresh unheated, as well as heated milks. Wong et al. (1958), however, have shown that the acetone concentration in heated milk is markedly increased over that of the unheated product. Increases in the acetone and 2-butanone concentrations were also gas chromatographically observed during the storage of the fluid sterile milk (Figs. 1-10).

2-Heptanone which, is not normally found in unheated milk, has been shown to arise from the decarboxylation of β-keto acids. β-keto acids have two possible precursors in milk fat: a) as intermediates in the β oxidation of fatty acids liberated from triglycerides, and/or b) from the hydrolysis of β-keto esters, which have been shown in milk fat by Van der Ven (1963), and Parks et al. (1964), both of whom have isolated and identified methyl ketone precursors in butterfat as β-keto esters.

The important condition required in the formation of methyl ketones in processed dairy products, as shown by Langler and Day (1964), is heating of the milk fat in the presence of moisture. In a process such as milk sterilization, hydrolysis of both fatty acids and β-keto esters has been shown to occur. The importance of water in this reaction cannot be overstressed, since inhibition of methyl ketone formation was observed when anhydrous milk fat was heated. In view of this evidence, it seems possible that the presence of 2-butanone in the controls and in the storage samples could also be attributed to heat sterilization of the fluid milk.

The appearance of 2-heptanone in the storage samples (Fig. 17) is believed due to the decarboxylation of β-keto acids during the prolonged storage at elevated temperatures.

The importance of methyl ketones in contributing to flavor deterioration in heat processed milk products held in storage, and possibly to the stale flavor has not been completely determined. However, it has been shown that, commercially feasible, inhibition of autoxidation, and associated oxidized flavor does not necessarily solve the stale flavor problem. Nawar et al. (1963) isolated stale flavor components from dry whole milk powder by Girard-T-reagent, and carbon-tetrachloride distillations, showing the association of acetone, 2-butanone, and 2-pentanone with stale flavor, Arnold et al. (1966) have confirmed the presence of 2-heptanone in stale sterile concentrated milk by mass spectroscopy. Day and Lillard (1960) have also identified acetone as a component in oxidized flavor of milk fat, and at the same time have found presumptive evidence for the involvement of odd-numbered C_5-C_{15} n-alkan-2-ones. Parks and Patton (1961) postulated that methyl ketones in a medium concentration elicit a stale flavor response, which they believe is due to their synergistic interaction.

Spectrophotometric analyses of the second and third fractions (Figs. 12, 13) collected from the column separation of the 2,4 dinitrophenylhydrazones from the control and the three months storage samples at 22°C. showed them to be saturated aldehydes. Acetaldehyde was identified in the control samples as well as the storage samples (Figs. 14, 15) and because of this was believed to originate from a source, or sources, other than lipid autoxidation. Dutra et al. (1959) have shown acetaldehyde formation form the enzyme-catalyzed oxidative decarboxylation (Strecker degradation) of alanine, and the breakdown of lactose by the action of heat during the processing of evaporated milks. It was also shown that the ascorbic acid, which is present in milk, appears to have a catalytic effect on the Strecker degradation of alanine, since the reaction was accelerated by addition of ascorbic acid. They also postulated that other amino acids could undergo this degradation, and yield carbonyls of considerably more flavor significance. It can be seen from the gas chromatographic data presented in Figs. 1-10 that the acetaldehyde level did increase during storage at all temperatures although the increases were noticeably less in the samples held at 4.4°C. These increases in acetaldehyde levels were believed due to lipid autoxidation as well as Strecker degradation of amino acids. Acetaldehyde has been related to stale flavor by the work of Nawar et al. (1963), who reported acetaldehyde and propanal present in two separate extractions, which were specifically designed to extract stale flavor components.

Two other saturated aldehydes were isolated, and tentatively identified by thin-layer chromatography from the three months storage samples: n-butanal and n-hexanal (Fig. 15). These compounds were also present in the gas chromatographs discussed earlier. Since these two compounds were not isolated as hydrazone derivatives in the control samples, it is believed that they arose as dismutation products of hydroperoxides resulting from lipid autoxidation, rather than the Strecker degradation of amino acids. Hexanal has been shown as a secondary reaction product from the oxidation of linoleic and arachidonic acids, which are both present in milk fat. Butanal has also been isolated from autoxidized milk fat by Day and Lillard (1960), and Nawar et al. (1962); however, as yet no reaction scheme has been postulated to account for its presence. The flavor significance of these lower saturated aldehydes in oxidized and stale flavors has been proposed by Nawar et al. (1962), who postulated that the C_1-C_9 alkanals contribute to the oxidized flavor. They are generally present in small concentrations, and function in synergistic interactions to cause perceptible flavor deterioration. Parks and Patton (1961) in following this line of reasoning found that low aldehyde concentrations appeared to elicit a stale flavor response, and as the concentration was increased the flavor observed was recorded as oxidized.

The 2,4 dienals whose presence was shown during the class separation of 2,4 dinitrophenylhydrazones from the storage samples, were believed to arise as the result of autoxidation of linoleic, linolenic and arachidonic fatty acids according to the mechanism of Farmer and Sutton (1943). It was unfortunate that these diunsaturated aldehydes could not be recovered from the column for confirmatory thin-layer chromatography, since there is general agreement among all workers as to the importance of diunsaturated alkenals in both oxidized and stale flavors. It was also observed during these class separations that no monounsaturated alkenals were present in either the control or storage samples.

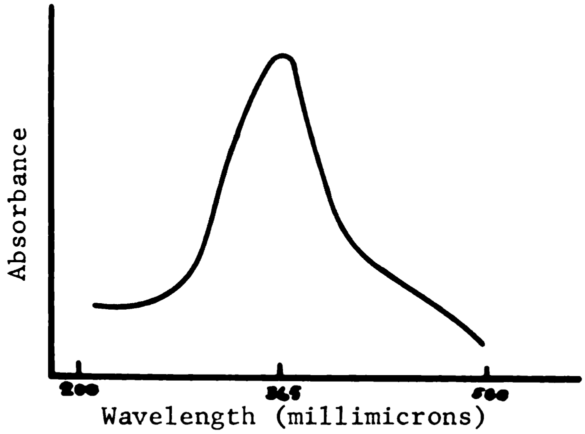

Figure 11. Visible absorption spectra of 2,4 DNP hydrazones in chloroform recovered from fraction 1 during column separation of 2,4 DNP hydrazones from sterile milk at 36°C. for three months, and control samples.

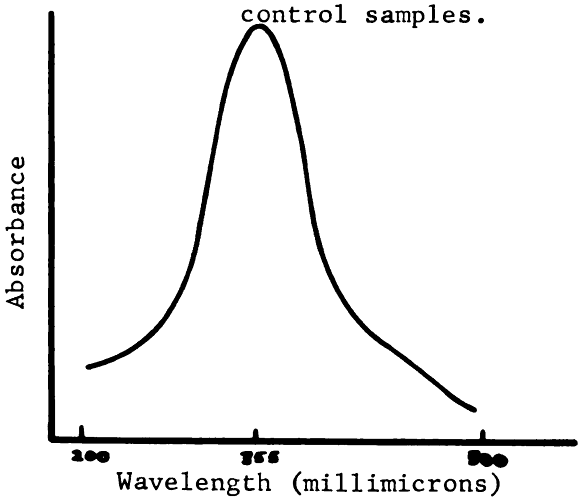

Figure 12. Visible absorption spectra of 2,4 DNP hydrazones in chloroform recovered from fraction 2 during column separation of 2,4 DNP hydrazones from sterile milk at 36°C. for three months, and control samples.

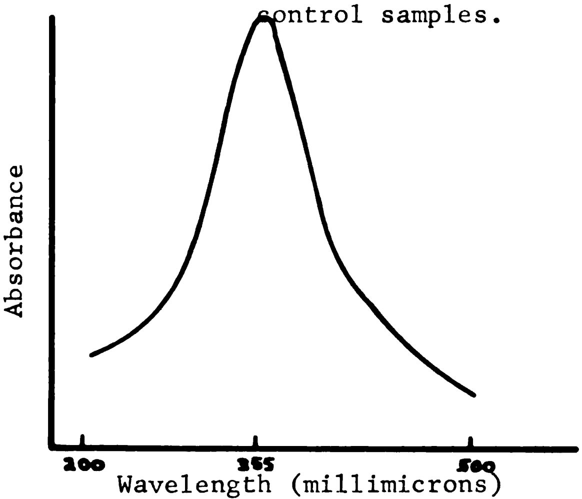

Figure 13. Visible absorption spectra of 2,4 DNP hydrazones in chloroform recovered from fraction 3 during column separation of 2,4 DNP hydrazones from sterile milk at 36°C. for three months, and control samples.

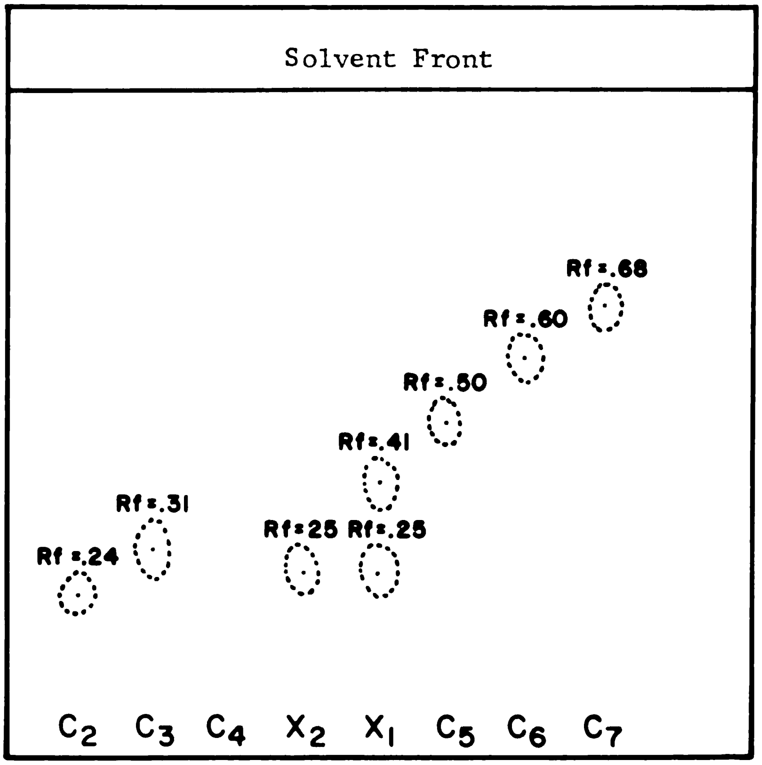

Figure 14. Separation of 2,4 DNP hydrazone derivatives of saturated aldehydes from HTST fluid sterile milk control samples by thin-layer chromatography.

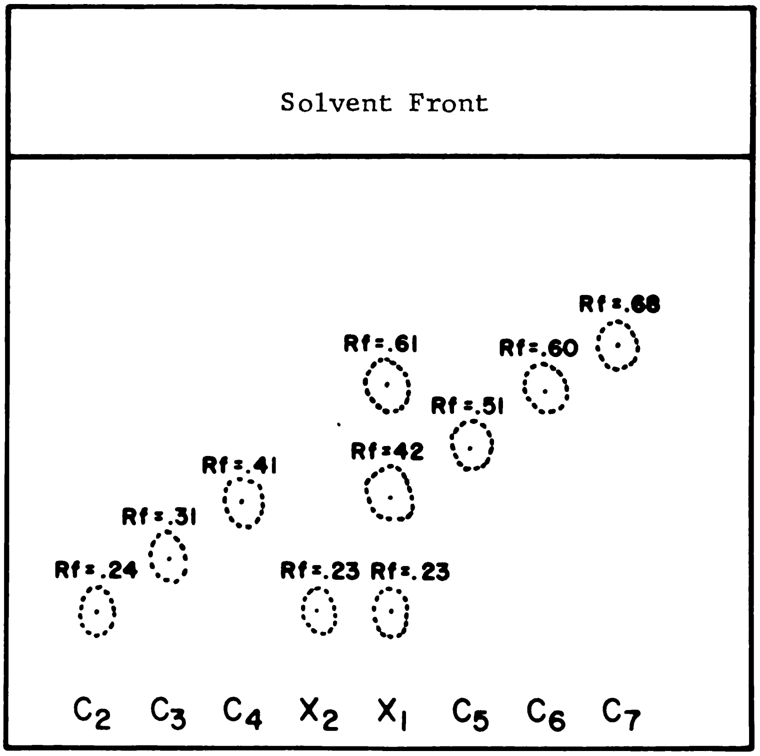

Figure 15. Separation of 2,4 DNP hydrazone derivatives of saturated aldehydes from HTST fluid sterile milk, held at 22°C. for three months, by thin-layer chromatography.

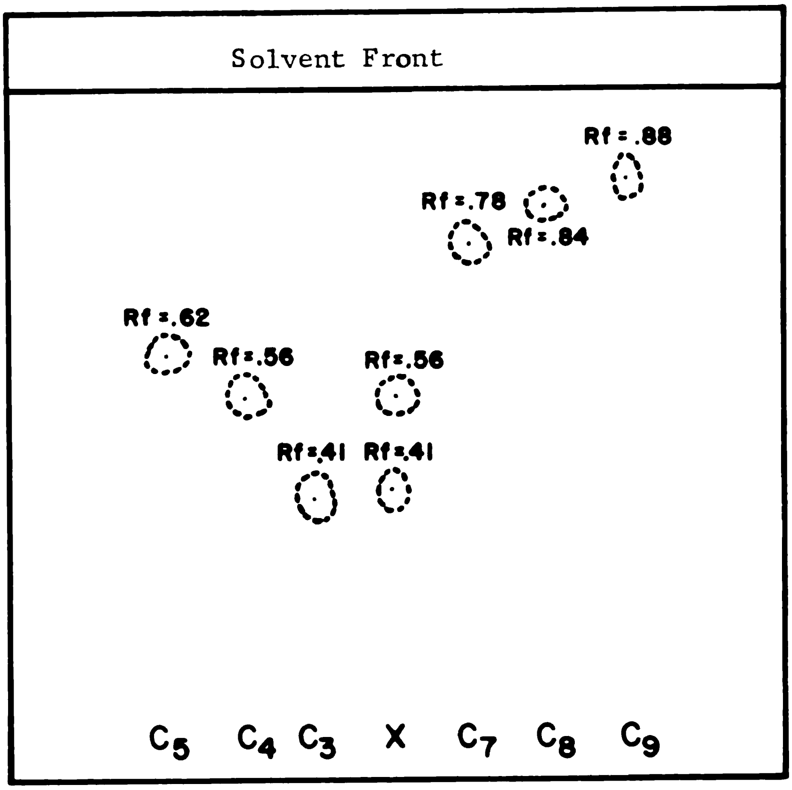

Figure 16. Separation of 2,4 DNP hydrazone derivatives of methyl ketones from HTST fluid sterile milk control samples by thin-layer chromatography.

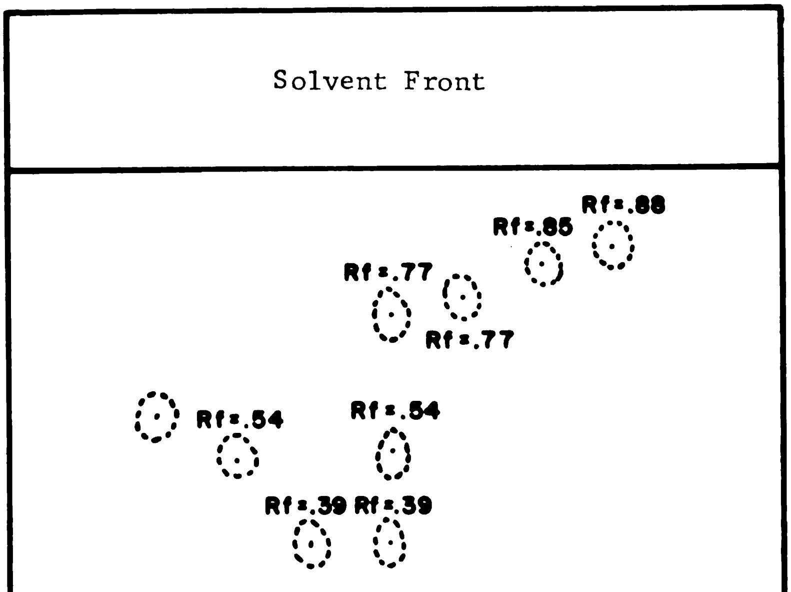

Figure 17. Separation of 2,4 DNP hydrazone derivatives of methyl ketones from HTST fluid sterile milk, held at 22°C. for three months, by thin-layer chromatography.

SUMMARY AND CONCLUSIONS

1. Fluid whole milk, which was sterilized at 140.5°C. ± 1.°C. for approximately four seconds in a DeLaval Vacu-Therm-Instant-Sterilizer, exhibited a highly cooked and slight sweet taste by organoleptic evaluation of the control samples. The color of these control samples was an off-white to tan, signifying the occurrence of Maillard browning during the sterilization process.

2. Fluid sterile milk stored at temperatures of 4.4° and 22°C. for two months still had an acceptable flavor. Stale flavor was organoleptically detected in the product after one month of storage at 36°C., and two months at 22°C.

3. Age-thickening and sedimentation were observed in the fluid sterile milk during storage. Sedimentation occurred in all sterile milk samples after one month of storage. Age-thickening, an incipient sign of gelation, was visually and tactually detected in the two months 22° and 36°C. samples, and by the third month in the 4.4°C. samples.

4. Severe creaming occurred in the fluid sterile milk after one month of storage at 22° and 36°C. After two months of storage at these temperatures a firm cream-plug had formed in the neck of the bottle. This defect was not observed in the sterile milk samples held at 4.4°C. for three months. All samples were held under static storage conditions.

5. Standard plate counts were made on the storage samples of the fluid sterile milk before flavor distillation to confirm the sterility of the milk samples and to insure that any flavors present in the sterile milk were not the direct result of bacterial contamination of the milk during storage. No organisms were detected in any of the sterile fluid milk storage samples used for extraction of flavor volatiles.

6. Flavor components were recovered from the fluid sterile milk by a low-temperature, reduced-pressure distillation technique. Separation of the flavor components in the flavor distillate was accomplished by gas chromatography using a packed column and a dual flame ionization detector.

7. Tentative identification was made of the flavor components separated gas chromatographically, using the retention time and functional group analysis data.

8. Flavor components that were tentatively identified in the fluid sterile milk control samples were: ethanal, propanal, acetone, 2-butanone, 2-hexanone, butanol, ethanol, and furfural.

9. 2-Pentanone, hexanal, butanal, heptanal, and 2-heptanone were tentatively identified from the fluid sterile milk during storage as well as those previously mentioned as present in the control samples. An increase of 30-50% in acetone concentration was also observed in the samples, which had been organoleptically judged stale. A general increase in volatile concentration was apparent in all of the sterile milk samples held in storage.

10. The presence of ethanal, butanal, hexanal, acetone, 2-butanone, and 2-heptanone in the samples of sterile milk was confirmed by thin-layer chromatography. Butanal, hexanal, and 2-heptanone were found to be present only in the storage fluid sterile milk samples. The presence of 2,4 dienals was observed in the storage samples during the class separation of the hydrazone derivatives, but these compounds were not present in the controls. No monounsaturated alkenals appeared to be present either in the control or storage samples. These organic compounds, whose presence has been shown in the fluid sterile milk, are believed to contribute to the stale flavor by their synergistic interactions, especially those which arose during the storage of the sterile milk samples.

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