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Title Identification of Some Compounds Contributing to the Stale Flavor Defect of Sterilized Concentrated Milk

Abstract approved _____ (Major Professor)

Stale flavor development has been recognized as a defect of stored dry milk powders for several years. Recently, stale flavor development has been found to occur upon storage of sterilized concentrated milk, and is recognized as the principal limiting factor to commercial utilization of this process (Seibert, 1963).

Some attempts have been made to identify the volatile flavor components of sterilized concentrated milk (Patel et al., 1963; Bingham, 1964). The flavor components responsible for the stale flavor defect as it occurs in sterilized concentrated milk have not been identified, however.

The purpose of this work was to identify the compounds responsible for the stale flavor defect of sterilized concentrated milk. It was felt that this information was essential to an understanding of the staling phenomenon, which in turn might eventually lead to correction of the defect.

Commercial samples of sterilized concentrated milk were obtained. Stale flavor development was hastened in some of the samples by storing them at 21°C. Subjective flavor panel evaluation of stored and fresh samples revealed significant differences between the two.

Gas chromatographic analysis of the volatile head space components by the procedure described by Morgan and Day (1965) revealed only minor differences between the fresh and stale samples. It was reasoned, therefore, that the compounds responsible for the stale flavor defect were primarily of a less volatile nature.

A technique for isolating the higher-boiling flavor components was developed. This technique consisted of lyophilization of the sterile concentrated milk, uniform wetting of the lyophilized milk with water to 10% moisture, solvent extraction of the fat and flavor components from the moistened milk powder, and reduced-temperature, reduced-pressure steam distillation of the flavor components from the extracted fat. The resulting flavor extract was studied by gas chromatography in conjunction with mass spectrometry.

A base-treated pre-column was used in front of the regular gas chromatography column to remove fatty acid peaks from the chromatograms. A technique, which consisted of repeatedly trapping (from several successive chromatograms) particular regions of the effluent from a non-polar column onto a short section of packed column and re-chromatographing the trapped components on a polar column, was

developed to build up the concentration of flavor components and to improve the separation of components for mass spectral analysis.

The following compounds were positively identified in the flavor extract from stale sterile concentrated milk: 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, benzaldehyde, napthalene, a dichlorobenzene, δ-decalactone, benzothiazole, and o-aminoacetophenone. Acetophenone was tentatively identified. Of these compounds, 2-heptanone and the dichlorobenzene were positively identified in the extract from fresh sterile concentrated milk, and δ-decalactone was thought to be present.

The ketones and δ-decalactone undoubtedly make some contribution to the stale flavor defect (USDA, 1964). The identification of o-aminoacetophenone in stale sterilized concentrated milk supplements its identification in stale nonfat dry milk powder (Parks, Schwartz and Keeney, 1965), and further implicates it as an important compound in the stale flavor defect. This compound possesses a characteristic "grape-like" odor. Benzothiazole has not previously been identified in milk products. It possesses a characteristic "rubber-like" odor. Its possible significance in the stale flavor defect will require further study.

IDENTIFICATION OF SOME COMPOUNDS CONTRIBUTING TO THE STALE FLAVOR DEFECT OF STERILIZED CONCENTRATED MILK

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

IDENTIFICATION OF SOME COMPOUNDS CONTRIBUTING TO THE STALE FLAVOR DEFECT OF STERILIZED CONCENTRATED MILK

INTRODUCTION

Sterilized concentrated milk is a rather recent development in the processing of milk. It is one of a series of products developed by man in attempting to extend the storage life of milk, a highly nutritious but readily perishable food. Sterilized concentrated milk offers several advantages over other methods of preserving milk. Among these advantages are the following:

(1) Since it is a sterile product, sterilized concentrated milk is not subject to bacterial decomposition. It can thus be stored and transported without refrigeration.

(2) Being a concentrate, sterilized concentrated milk represents a more efficient medium for the distribution of milk nutrients than fluid whole milk. This applies not only to distribution in this country, but also in impoverished areas of the world where milk nutrients are badly needed and where refrigeration is often unavailable.

(3) Since the finished product exists in a liquid state, it is more readily reconstituted than are dry milk powders.

(4) Sterilized concentrated milk does not possess the strong caramel flavor that is associated with evaporated milk. A much

less intensive heat treatment is given sterilized concentrated milk than is given evaporated milk. Sterilized concentrated milk is sterilized by an ultra-high temperature, short time process and subsequently aseptically canned, whereas evaporated milk is sterilized in the can.

(5) Sterilized concentrated milk does not appear to be susceptible to lipid oxidation and the resulting development of oxidized flavors which often occur in dried milk products.

Commercial processes for the production of sterilized concentrated milk have been developed. Commercial utilization of the product has been limited by two problems: physical instability and flavor instability. The problem of physical instability, namely gelation and fat separation, has been solved by proper control of process parameters and the addition of polyphosphates to the product. The problem of flavor instability has not been solved, however.

Sterilized concentrated milk develops a definite stale flavor upon storage. This defect generally occurs within two to three months of storage at 70°F (21°C), but may be delayed to six months or longer if the product is refrigerated. Since one of the assets of the product is its ability to be stored without refrigeration, staling presents a serious problem to the general acceptance of the product. A similar staling effect has been noted for several years in dried milk products, where it is frequently accompanied by the

development of oxidized flavors.

The volatile compounds responsible for the stale flavor defect have not been identified. It was felt that the identification of the compounds responsible for the stale flavor defect would aid in determining the mechanism of the staling phenomenon. It was hoped that this information might eventually lead to correction of the stale flavor defect. Hence, this study of the compounds responsible for the stale flavor defect was undertaken.

REVIEW OF LITERATURE

Nature of the Stale Flavor Defect

The term, stale, as applied to a flavor defect of stored milk products is rather nebulous, and has been used to describe a multitude of off-flavors that develop upon storage of dry or concentrated milk products. The term has often been used when a definite off-flavor is detected, but the flavor criticism does not fit into a more specific category such as rancid, cooked, oxidized, etc. Bassette (1958) has expressed the opinion that the term, stale, may relate to a degree of off-flavor rather than to a description of a particular off-flavor.

Various authors have used the term, stale, to describe the off-flavor detected in stored milk products. Stale flavor was first described as a flavor defect of stored dry milk powders. Lea, Moran and Smith (1943), Tarassuk and Jack (1946, 1948), Jenness, Coulter and Larson (1946), Henry et al. (1948), Whitney and Tracy (1949), Shipstead and Tarassuk (1953), and Bassette (1958) have all referred to the stale flavor defect of stored milk powders. Other descriptive terms applied to the flavor defect include "burnt feathers" (Coulter, Jenness and Crowe, 1948), "cereal type" (Bassette, 1958; Bassette and Keeney, 1960) and "gluey" (Henry et al., 1948).

More recently, stale flavor development has been found to occur in sterilized concentrated milk. Siebert et al. (1963) and Bingham (1964) have pointed out that the limiting factor in the storage life of sterilized concentrated milk is flavor deterioration. Samples of sterilized concentrated milk used in the former study were criticized as being cooked initially and stale after three months storage at 75°F. Patel et al. (1962) indicated that a characteristic "old rubber flavor" develops upon storage of sterilized concentrated milk.

Isolation of Volatile Flavor Components from Milk Products

A number of techniques have been developed to study the volatile flavor components of milk products. These techniques can be classified into the following categories:

(1) Direct analysis of head space vapors

(2) Gas entrainment and trapping of volatiles

(3) Distillation

(4) Solvent extraction.

Direct Analysis of Head Space Vapors

Bassette, Ozeris and Whitnah (1962, 1963) developed a method for studying the volatile components of liquid milk products. In this technique, a small sample of milk (two ml) was placed in a glass serum vial containing 1.2 grams of anhydrous sodium sulfate and

capped with a rubber serum stopper. After agitation and heating to 60°C, a one ml sample of head space was withdrawn with a Hamilton gas tight syringe and injected into a gas chromatograph. Sodium sulfate reduces the vapor pressure of the water in the milk, while heating vaporizes the volatile components. The combined action of these two factors enriches the head space with vapors of the volatile flavor components. Jennings, Viljhalmason and Dunkley (1962) utilized a similar procedure using a larger sample, eliminating the sodium sulfate, and heating to 80° C instead of 60°C.

These techniques are useful for the more volatile flavor components, but not for the higher boiling compounds. Although of lower volatility, higher boiling compounds frequently have lower organoleptic threshold values and may therefore be of significance.

Gas Entrainment and Trapping of Volatiles

Sweeping of samples with nitrogen or other gas and trapping the volatile components in a cold trap has been applied to the study of food flavor volatiles. Nawar and Fagerson (1960) developed an enclosed system whereby nitrogen was continuously recycled through the sample and through a cold trap chilled with liquid air. The volatiles could then be swept from the trap onto a gas chromatography column by a valve arrangement. Hornstein and Crowe (1962 b) developed a system whereby the volatiles were trapped onto a short

section of packed gas chromatography column. This section of the column could then be attached by Swagelok fittings to the regular column in the gas chromatograph. Morgan and Day (1965) further modified this technique by trapping directly onto a U-shaped section of the regular gas chromatography column. Sodium sulfate and heat were again used to enrich the head space vapors. The general technique of gas sweeping and trapping of the volatile components has been used successfully in isolating the more volatile components from food systems. Again, the method is not well adapted to the isolation of higher boiling components.

Kurtz (1965) has used a technique similar to gas entrainment for recovering flavor volatiles from whole milk powder. A short path migration, at one micron pressure, from the powder directly to a liquid-nitrogen cooled condensor was used. While no sweep gas is used in this technique, it is similar in that the volatiles are transferred directly from the product to a cold trap. An interesting development of this work was the finding that powders possessing an oxidized flavor were improved by this treatment, whereas stale powders were not. This indicates that the compounds responsible for stale flavor are of low volatility, or are tightly bound to the powder due to structural properties. This would indicate that more rigorous isolation procedures must be used to recover stale flavor components.

Distillation

Various distillation procedures have been utilized to recover volatile flavor components from larger volumes of milk products. Day, Forss and Patton (1957 a) utilized a reduced pressure, reduced temperature nitrogen distillation to remove the volatile components from irradiated skim milk. Water was trapped in a wet ice trap and two ethanol-dry ice traps, while the more volatile components were trapped in subsequent liquid nitrogen cooled traps. The liquid nitrogen traps could be removed from the distillation system and attached directly to a gas chromatograph. Patel et al. (1962) used a similar system to isolate the volatile components of sterilized concentrated milk; the traps consisted of two liter round bottom flasks placed in ice water or ethanol-dry ice. Bassette (1958) utilized a similar system to recover the volatiles from reconstituted instant nonfat dry milk. A modified system in which the cold traps were replaced by traps containing 2, 4-dinitrophenylhydrazine reagent, to form derivatives of distilled carbonyl compounds, was also used by Bassette (1958).

Patton and Tharp (1959) described a reduced pressure, reduced temperature steam distillation apparatus which was used for the distillation of milk fat. A similar system was used by Day and Lillard (1960) to recover the volatiles from autoxidized milk fat. Whitney, Paulson and Tracy (1950) utilized a reduced pressure,

reduced temperature steam distillation to recover the volatiles from milk fat which had been solvent extracted from stale dry whole milk. The collected distillate was subsequently extracted with petroleum ether, and the petroleum ether evaporated to yield a concentrated stale flavor fraction.

Lindsay, Day and Sandine (1965) utilized an elaborate low pressure, low temperature steam distillation apparatus for the recovery of volatile components from large quantities of starter culture. The trapped distillate was subsequently extracted with ethyl ether in a liquid-liquid extractor.

Chang (1961) used countercurrent steam distillation in a 30 plate Oldershaw column to isolate the flavor components of reverted soybean oil. Dutra, Jennings and Tarassuk (1959) developed a continuous steam distillation apparatus to study the flavor volatiles of evaporated milk. In this system, the sample was continuously added to and removed from the distillation chamber.

Libbey, Bills and Day (1963) described a technique for the isolation of lipid soluble volatile components. In this technique milk fat or other lipid material is subjected to a low temperature, high vacuum distillation in a molecular still. The high vacuum and the formation of monomolecular layers of the lipid material make this an efficient distillation procedure.

The various distillation procedures have been criticized by

Bingham (1964) and others as possibly leading to the formation of artifacts. Although this might be a valid criticism, it can be circumvented in off-flavor studies by treating a control sample in an identical fashion and comparing the off-flavor volatiles with those of the control. One example of possible artifact formation in the distillation of milk fat would be the generation of methyl ketones. Patton and Tharp (1959) found that whereas high temperature (200°C) distillation of milk fat does generate a series of ketones, reduced pressure, reduced temperature distillation produces only acetone. Further, Langler (1964) has shown that maximum production of methyl ketones from milk fat requires 120-140°C for 30 minutes. Temperatures of 40°C are most commonly encountered in reduced pressure, reduced temperature distillations. Hence, the formation of artifacts by steam distillation procedures may not be as serious a criticism as some have suggested.

Solvent Extraction

Patton (1961) has described a solvent partitioning technique for recovering flavor components, with the exclusion of fat, from liquid processed milks. In this technique, an organic solvent such as ethyl ether or petroleum ether is layered onto the milk sample in a volumetric flask, which is then placed on a wrist action shaker for one hour. The organic solvent layer is subsequently decanted

and the solvent evaporated. The resulting fraction is suitable for injection into a gas chromatograph. This technique was used by Cobb and Patton (1962) to study the flavor components of evaporated milk. Muck, Tobias and Whitney (1963) used a similar technique in studying the flavor components of aged evaporated milk, and found that different types of flavors were extracted with different solvents. Petroleum ether and hexane appeared to extract more of the flavors described as stale, aged, or storage, whereas ethyl ether appeared to isolate more of the cooked or caramel flavors.

By necessity, the agitation of the sample-solvent system must be rather gentle to prevent extraction of the fat and to prevent the formation of unbreakable emulsions. It is questionable whether this gentle action has the ability to extract components which, due to their chemical structure or physical properties, might exist in close association with milk protein.

Whitney and Tracy (1950) showed that butter oil containing the stale flavor components could be extracted from stale dried whole milk by organic solvents. To free the fat for extraction, the powder was wetted with 95% ethyl alcohol. The alcohol treated powder was subsequently extracted in a soxhlet extractor with petroleum ether. Satisfactory yields of stale butter oil were recovered by this technique.

Wetting of the powder with ethyl alcohol was based on an

earlier finding by Lampitt and Bushill (1931) that only a small proportion of the fat could be extracted from untreated dried milk powder by organic solvents. Increasing the moisture content of the powder to 10% crystallized the lactose, freeing the fat for essentially complete recovery by organic solvents. Whitney and Tracy (1950) found that ethyl alcohol has an action similar to that of water in that it crystallizes the lactose, thereby freeing the fat for extraction. Kurtz (1965 b), in studying the recovery of added ketones from dried whole milk by direct short path migration to a cold finger, found that treatment of the powder with water to free the fat also freed added ketones, and presumably other volatile components.

Bassette (1958) used a solvent extraction technique to extract the fat from instant nonfat dry milk. Methanol was used to wet the powder instead of ethanol. Quantitative yields of fat were obtained. The flavor and odor of the extracted fat resembled that of the parent dry milk. It was concluded that the character of the milk powder was carried directly into the fat.

Keeney and Patton (1956) used a soxhlet extraction with ethyl ether as the solvent to study the coconut-like flavor defect of dried cream and dried whole milk. The dried powder was rehydrated to 8% moisture before extraction. Parks, Schwartz, and Keeney (1964) used a soxhlet extraction to recover a "grape-like" fraction from nonfat dry milk. Hexane was used as the solvent, with 10% water

being added to the powder. The water was added to a slurry of the hexane and powder in a Waring blendor, thus providing for uniform distribution of the water. The wetted powder was subsequently placed in the soxhlet extraction thimble and extracted with the same hexane.

A disadvantage of these latter solvent extraction techniques is that the flavor components are not extracted directly, but are partitioned into the fat phase. They must then be further isolated from the fat. The reduced pressure, reduced temperature steam distillation and high vacuum molecular distillation techniques previously discussed offer convenient methods of isolating the flavor compounds from the extracted fat.

An advantage of these solvent extraction techniques is that the combined action of the water or other wetting agent and the solvent tends to remove compounds that exist in close association with milk protein. Such compounds might not be recovered by other techniques.

Identification of Flavor Volatiles

Several techniques have been developed for the identification of food flavor volatiles. Carbonyl compounds have been most commonly identified by the formation of 2, 4-dinitrophenylhydrazones (Morgan, Forss and Patton, 1957; Bassette, 1958; Dutra, Jennings and Tarassuk, 1959; Day and Lillard, 1960; Parks and Patton, 1961;

Muck, Tobias and Whitney, 1963; Parks, Schwartz and Keeney, 1964). These derivatives are separated and identified by paper and column chromatography, ultraviolet studies, and melting point data. Various other qualitative reagents can be used to form derivatives of carbonyls or other classes of compounds.

The development of gas chromatography techniques was an important breakthrough for the flavor chemist. The gas chromatograph is sensitive to very small concentrations of volatile components, which is generally the case in flavor studies. Although gas chromatography does not provide a positive identification of the compounds, it does provide a ready method for separating the various components of a flavor mixture. Clues as to the tentative identity of the various components eluting from the chromatographic column can be gained by comparing retention times with known compounds, and by smelling the effluent gas stream. Gas chromatography has been widely used to study the flavor volatiles of milk products (Day, Forss and Patton, 1957 b; Patton, 1961; Cobb and Patton, 1962; Jennings, Viljhalmason and Dunkley, 1962; Patel et al., 1962; Bassette, Ozeris and Whitnah, 1962, 1963; Libbey, Bills and Day, 1963; Muck, Tobias and Whitney, 1963; Parks, Schwartz and Keeney, 1964; Lindsay, Day and Sandine, 1965).

Chang, Ireland and Tai (1961) described an infrared gas cell for the collection of gas chromatographic fractions for infrared

analysis. This technique is useful for determination of the functional groups present in a particular component eluting from the gas chromatography column.

Day, Forss and Patton (1957 b) used mass spectrometry to identify volatile components of gamma-irradiated skim milk. McFadden et al. (1963) have developed a technique for using a time-of-flight mass spectrometer in conjunction with a capillary gas chromatograph to obtain fast scan mass spectra of compounds eluting from the column. Day and Anderson (1965) used this technique to analyze the neutral flavor volatiles of blue cheese, while Lindsay, Day and Sandine (1965) used it to analyze the volatile components of starter culture. These techniques offer considerable promise in the study of flavor components, and will undoubtedly see increased application in the future.

Identity of Stale Flavor Components

The initial attempt to isolate stale flavor components was made by Whitney and Tracy (1949, 1950). These workers found that the stale flavor components were partitioned into the fat phase when the reconstituted milk was fractionated into cream and skim milk fractions. They also found that this partitioning effect occurred when the milk fat was extracted from the powder by organic solvents. Whitney, Paulson and Tracy (1950) found that the stale flavor

components could be steam distilled from the extracted butter oil. This information was obtained by adding the isolated fat or flavor distillate back to fresh milk and subsequently evaluating the resulting product organoleptically. No data as to the identity of the compounds responsible for the stale flavor was presented, however.

Bassette (1958) studied the volatile components of stored instant nonfat dry milk by low pressure steam distillation and formation of the 2,4-dinitrophenylhydrazone derivatives of the distilled carbonyl compounds. Formaldehyde, acetaldehyde, acetone, 2-butanone, 2-pentanone, 2-methyl butanal, hexanal, nonanal, cis-2-furfuraldehyde, and trans-2-furfuraldehyde were positively identified, while heptanal, octanal, decanal, dodecanal, and tetradecanal were tentatively identified from powders that were criticized as having stale, hay-like, or cereal-type flavors. Parks and Patton (1961) studied the volatile carbonyl compounds of dry whole milk by a similar procedure. The following compounds were identified from an average powder: C_3-C_7, C_9, C_{11}, and C_{15} n-alk-2-ones, C_1, C_2 and C_4 alkanals, and cis- and trans-furfuraldehyde. In contrast, a badly deteriorated powder was found to contain C_3, C_4, C_9, C_{11}, and C_{15} n-alk-2-ones, C_1-C_3, C_5-C_7, C_9, C_{10}, and C_{12} n-alkanals, benzaldehyde, two mono- and two di-unsaturated carbonyls.

Nawar et al. (1963) used a solvent extraction technique similar to that used by Whitney and Tracy to extract stale milk fat from stale

dry whole milk. The extracted fat was subjected to Girard's-T extraction and carbon tetrachloride distillation techniques. Heptaldehyde, formaldehyde, acetaldehyde, propionaldehyde, acetone, 2-pentanone, and possibly 2-butanone were isolated and tentatively identified. Two other components were also isolated, one possessing spectra and chromatographic characteristics of a saturated monocarbonyl compound, and the other possessing characteristics of either an unsaturated dicarbonyl or hydroxycarbonyl compound.

Muck, Tobias and Whitney (1963) studied the flavor compounds present in aged evaporated milk after isolating the flavor components by the petroleum ether solvent partitioning technique. The flavor compounds identified were 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, δ-decalactone, γ-dodecalactone, δ-dodecalactone, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, and palmitic acid. Acetone and 2-butanone were tentatively identified.

Parks, Schwartz, and Keeney (1964) identified o-aminoacetophenone as the flavor compound responsible for the grape-like flavor of stale nonfat dry milk. The compound was obtained by soxhlet extraction of the powder with hexane, extraction of the hexane with an aqueous acid solution, treatment of the acid solution with sodium bicarbonate to make an alkaline solution, and back extraction with hexane. The o-aminoacetophenone was identified by gas chromatography,

mass spectrometry, and formation of the 2,4-dinitrophenylhydrazine derivative. Subjective organoleptic analysis of this compound in milk revealed that it did not cause stale flavor by itself, but that it enhanced the stale flavor of slightly stale products. These authors expressed the opinion that o-aminoacetophenone is an important component of a group of compounds which collectively are responsible for typical stale milk flavor.

The volatile flavor components of sterilized concentrated milk have not been as widely investigated as those of nonfat dry milk and dry whole milk. Patel et al. (1962) recovered the flavor volatiles by steam distillation and presented evidence that ethanal, dimethyl sulfide, acetone, 2-pentanone, and a pentyl acetate were present in sterilized concentrated milks. They concluded that these compounds were related to flavor changes during storage. The pentyl acetate was present in the largest amount of all of the volatiles detected.

Bingham (1964) used gas chromatography to study the volatiles of sterilized concentrated milk. Ethanal, propanal, acetone, butanone, 2-pentanone, pentanal, 2-hexanone, 2-heptanone, and dimethyl sulfide were tentatively identified. It was also reported that the pentyl acetate previously reported by Patel et al. (1962) probably came from the antifoam emulsion used in the steam distillation pot. It was concluded that the more volatile methyl ketones, such as acetone and 2-butanone, were probably not of significance in flavor

deterioration, because they were found in much higher concentrations in the raw and forewarmed milks than in the stored finished product.

A recent report by the USDA (1962) on dairy research programs has indicated that so far as is now known, methyl ketones and lactones are important in the stale flavor defect. It is also speculated that o-aminoacetophenone and other as yet unidentified compounds might also be of significance.

By and large, the stale flavor defect has not been characterized, particularly as it occurs in sterilized concentrated milk. Many of the volatile compounds that have been reported can be ruled out as causative agents of stale flavor due to their lack of characteristic odor properties. On the other hand, compounds possessing marked odor properties have been isolated but not identified. The identity of these compounds will undoubtedly aid in the characterization of the stale flavor defect.

Origin of Stale Flavor Components

The mode of formation of stale flavor components has for some time been the subject of considerable disagreement. This is especially true with regard to the stale flavor of dried milk products. Tarassuk and Jack (1946) reported that the stale flavor of dried milk powder is connected with materials associated with the fat,

and that oxygen is the chief contributing factor in the development of stale flavor. Tarassuk and Jack (1948) also reported that the usual deterioration of dry milk and dry ice cream mix powders in storage is independent of browning, and that under oxidative conditions, stale and oxidized flavors will develop with no browning at all. These findings implicate lipid oxidation as the principal route to stale flavor components.

On the other hand, Kunkel, Coulter and Combs (1946) reported that stale flavors may develop at oxygen levels as low as 0.5%. Jenness, Coulter and Larson (1946) indicated that fluorescing and brown-colored substances were formed in dried milk during storage under conditions that favored stale flavor deterioration. Coulter, Jenness and Crowe (1948) concluded that the characteristic stale flavor of stored dried milk was probably the result of both lactose-protein changes and oxidation of lipids. Coulter, Jenness and Geddes (1951, p. 98-99) in a review article on dry milk products stated that the typical flavor of stale dry whole milk was a composite flavor which arises from oxidation of lipids and the browning reaction. This viewpoint has since been reiterated by Bassette (1958) and Keeney (1963).

Part of the confusion surrounding the origin of the stale components might be attributed to the ambiguous use of the term, stale. It is highly possible that the stale flavor referred to by Tarassuk

and Jack (1946, 1948) and that referred to by Coulter and Jenness et al. (1946, 1948, 1951) were in actuality two different organoleptic flavor sensations. It has recently been shown by Tamsma, Mucha and Pallansch (1963) that in organoleptic evaluation of stored samples of foam dried whole milk by trained judges, the percent of stale judgements increased as the oxidized judgements decreased. This indicates that an oxidized flavor may mask stale flavor and further complicate the organoleptic detection of stale flavors.

Tamsma, Mucha and Pallansch (1963) also found that the addition of certain anti-oxidants was effective in inhibiting oxidized flavor development upon storage of foam dried powders, but that stale flavors still developed. This finding indicates that mechanisms other than lipid oxidation are responsible for the formation of stale flavor components.

The situation in sterilized concentrated milk is somewhat different from that of dried milk powders in that there is little likelihood that lipid oxidation occurs to any extent. Keeney and Patton (1956) have pointed out that in evaporated milk, reducing substances which inhibit lipid oxidation are formed during the heating process. A similar situation exists with sterilized concentrated milk, albeit to a lesser extent due to the less intense heat treatment. This factor, combined with the lack of free oxygen in the vacuum packed can, tend to rule out lipid oxidation as a significant flavor deterioration

mechanism in sterilized concentrated milk.

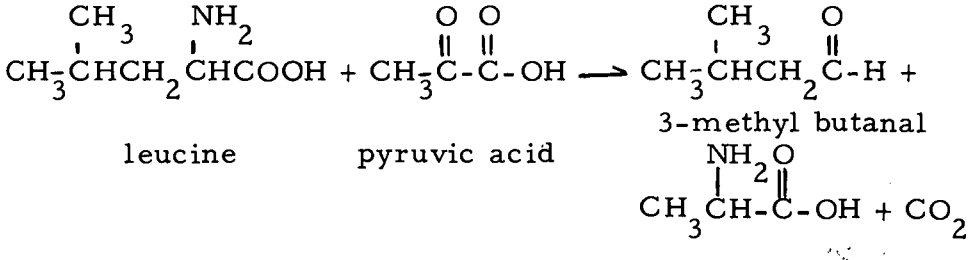
Sprecher, Strong and Swanson (1965) have recently studied the effect of staling of sterilized concentrated milk on the phospholipids. Phospholipids are highly susceptible to oxidative attack. Little difference was found between the phospholipids of fresh sterile concentrate and nine-month old concentrate exhibiting an intense stale flavor. It was concluded from this study that staling of concentrated milk does not involve oxidation of the phospholipids.

No attempt will be made here to discuss the mechanism of lipid oxidation or of browning. A good review of the former may be found in Chapters 2-4 of the symposium book edited by Schultz, Day and Sinnhuber (1962). The review by Hodge (1953) on browning is still the most complete review on this subject that is available.

The operation of these deteriorative mechanisms in milk product systems has been studied. Forss, Pont and Stark (1955) identified the volatile compounds associated with oxidized flavor in skim milk. Acetone, acetaldehyde, n-hexanal, crotonaldehyde, and C_5-C_{11} 2-unsaturated aldehydes were found. Day and Lillard (1960) identified the C_1-C_{10} n-alkanals, the C_5-C_{11} alk-2-enals and acetone from autoxidized milk fat. It was reported that 63% of the isolated monocarbonyl compounds isolated were n-alkanals and 34% alk-2-enals. The previously mentioned identification of several aldehydes in stale milk powders supports the theory that lipid oxidation does

occur in these powders. On the other hand, the absence of significant quantities of aldehydes in stale sterile concentrated milk tends to rule out lipid oxidation upon storage of this product.

The effect of browning on milk has been discussed in a review by Patton (1955). In this review, the Strecker degradation is implicated as a principal source of volatile flavor components in the form of low molecular weight aldehydes. The Strecker degradation involves an amino acid, a dicarbonyl compound, and heat. In the process of the reaction, the amino acid is degraded to an aldehyde of one less carbon atom. As an example, leucine may be degraded as follows:



leucine pyruvic acid 3-methyl butanal

The dicarbonyl may be of the form $-\overset{O}{\overset{\|}{C}}-(C=C)_n-\overset{O}{\overset{\|}{C}}-$, where n = O or an integer. Glucose and presumably lactose can be decomposed by heat to form the necessary dicarbonyls for the reaction to proceed. Trace quantities of free amino acids also exist in milk.

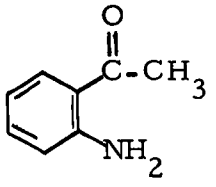
In addition to lipid oxidation and Strecker degradation mechanisms, various heat induced changes have been shown to occur in milk. The formation of methyl ketones as a result of heating milk

fat has been reported by Patton and Tharp (1959), and further studied by Langler (1964). The existence of acetone, 2-pentanone, and 2-heptanone in evaporated milk was reported by Wong, Patton and Forss (1958), while Parks and Patton (1961) found C_3-C_7, C_9, C_{11} and C_{15} methyl ketones in an average dry whole milk powder. Van der Ven, Bergemann and Schogt (1963) have shown that methyl ketones are formed from β-keto esters present in the milk fat which are hydrolyzed and decarboxylated by the action of heat and water.

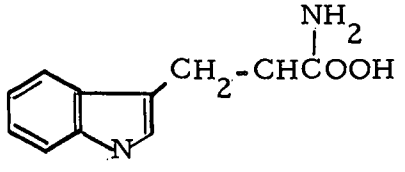
Gould (1945), and Gould and Frantz (1946) reported the formation of volatile acids, principally formic acid, in milk subjected to high heat treatments. Patton (1949, 1950 a, b) reported the formation of furfuryl alcohol, 5-hydroxy-methyl furfural, and maltol in skim milk subjected to high heat treatment. It was further reported by Patton (1950 c) that the formation of furan compounds, particularly furfuryl alcohol, appeared to be a consideration of carbohydrate chemistry alone. Keeney, Patton and Josephson (1950) reported the existence of acetol and acetic acid, and probably acetaldehyde in autoclaved condensed skim milk samples. These heat induced compounds which have been identified were produced by heat treatments greatly in excess of those given sterilized concentrated milk or dried milk powders. It is possible, however, that the reactions responsible for these heat induced changes might be initiated by heat treatment of the product and subsequently proceed at a slow

rate during storage of the product.

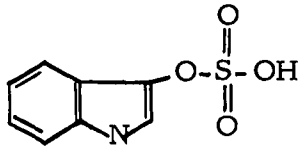
The identification of o-aminoacetophenone in stale nonfat dry milk by Parks, Schwartz and Keeney (1964) opens the door to as yet unexplained deteriorative mechanisms. The structure of this compound suggests that it might be derived from tryptophan, indican, or kynurenine.



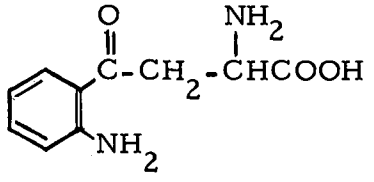
o-aminoacetophenone



tryptophan



indican (indoxyl sulfate)



kynurenine (3-anthraniloyl-alanine)

Kynurenine is a common metabolite of tryptophan, being found in the urine of numerous laboratory animals fed supplemental tryptophan. Tabone, Magis and Troestler (1947) have shown that kynurenine can be converted to o-aminoacetophenone by treatment with aqueous sodium hydroxide and heat. Tabone, Mamounas and Robert (1951) subsequently found that incubation of tryptophan at 37°C in a saturated sodium carbonate solution converted tryptophan to o-aminoacetophenone, with kynurenine appearing as an intermediate in the reaction. It can thus be seen that these compounds are all

closely interrelated, and may be involved in a common mechanistic pathway. Further mechanism studies will have to be undertaken before the occurrence of o-aminoacetophenone in milk can be explained. The possibility of other intermediates or further breakdown products of unknown reactions occurring in stale milk products must also be considered.

EXPERIMENTAL PROCEDURE

Sample Source

Samples of sterilized concentrated milk used in this study were provided by Nodaway Valley Foods, Inc., Corning, Iowa, and Carnation Research Laboratories, Van Nuys, California. These were commercially processed samples, the former being currently marketed under the tradename of Quilk. Both products were 3:1 concentrates. The Carnation samples were processed by forewarming to 265°F (129.5°C) for 15 seconds and sterilizing at 270°F (132.2°C) for 70 seconds or 295°F (146°C) for 3.5 seconds. The finished product contained 9.8% fat and 34.3% total solids, including 0.14% added Quadrafos (a polyphosphate, added to inhibit gelation). The Nodaway samples were sterilized at 273°F (134°C) for 30.5 seconds; the forewarming temperature was not indicated. The sterilized product was sterile homogenized at 4800 psi, and subsequently deaerated. The canned product contained 1.6 parts per million oxygen in the headspace of the can. The Nodaway product contained 9.5% fat and 34.0% total solids, again including added polyphosphate.

Stale flavor development was hastened by subjecting part of each batch to 21°C storage. Control samples were held at 1°C.

That there was a significant difference between the stale and

control samples was determined by organoleptic evaluation of the samples. A panel consisting of 10 members experienced in judging milk was presented with samples of fresh whole milk, fresh sterile concentrate (two months at 1°C), aged sterile concentrate (nine months at 21°C), and fresh sterile concentrate containing five parts per billion added o-aminoacetophenone. The latter sample was included to check the hypothesis of Parks, Schwartz and Keeney (1964) that this compound did not in itself cause stale flavor. All sterile concentrated milk samples were made up to whole milk concentration before presentation to the judges, and all samples were of a uniform temperature when presented. The same series of four samples was presented to the 10 judges on three different occasions. The proposed ADSA scorecard, as shown in Figure 1, was used.

The fresh and aged sterile concentrate samples were also included in a series of samples presented to a student preference panel consisting of 123 Oregon State University students. Calvin and Sather (1959) had previously shown that there was high correlation (0.89) between preferences of student panels and household consumer panels. It was therefore felt that the student preference panel might tend to give a more accurate reflection of consumer evaluation of the sterile concentrated milk samples. The fresh and aged sterile concentrated milks were included with fresh whole milk and a sample of Milkman (Foremost Dairies, Inc.) dry milk. The preferences

Name: _____ Date: _____

Astringent Sl. 9, 8: def. 7, 6: pron. 5, 4					
Caramelized Sl. 7, 6: def. 5, 4: pron. 3, 2					
Chalky Sl. 9, 8: def. 7, 6: pron. 5, 4					
Cooked Sl. 9, 8: def. 7, 6: pron. 5, 4					
Lactone Sl. 8, 7: def. 6, 5: pron. 4, 3					
Scorched Sl. 6, 5: def. 4, 3: pron. 2, 1					
Stale Sl. 6, 5: def. 4, 3: pron. 2, 1					
Other: Name and Intensity (See supplemental guide)					
Flavor Score - may not be higher than lowest score from above Score 10 for no criticism					

Supplementary Guide
Flavor Descriptions for Concentrated Milk

Criticisms	Slight	Definite	Pronounced
Acid	6, 5	4, 3	2, 1
Bitter	6, 5	4, 3	2, 1
Feed	9, 8	7, 6	5, 4
Flat	9, 8	7, 6	5, 4
Metallic	6, 5	4, 3	2, 1
Oxidized	6, 5	4, 3	2, 1
Rancid	6, 5	4, 3	2, 1
Salty	8, 7	6, 5	4, 3
Unclean	6, 5	4, 3	2, 1
Weedy	6, 5	4, 3	2, 1
Foreign	6, 5	4, 3	2, 1

Figure 1. Proposed ADSA Scorecard for Concentrated Milk for Beverage Purposes (Used for Trained Panel Evaluation of Milk Samples)

were indicated on a nine-point hedonic scale, ranging from 1, dislike extremely, to 9, like extremely.

Volatile Head Space Studies

The more volatile components were studied by the procedure described by Morgan and Day (1965). In this procedure, six ml of sample (sterile concentrated milk made up to whole milk concentration) was placed in a 20 ml screw capped vial containing 3.6 g of sodium sulfate. The plastic screw cap had two 1/8-inch diameter holes drilled in the top, 3/8-inch apart. A round rubber septum of the same diameter as the vial was placed on the vial, and the screw cap tightened to a snug fit. A small spatula was dipped into Dow Corning antifoam AF emulsion and then touched onto the inside of the rubber septum before the septum was placed onto the vial. This amount of antifoam was sufficient to prevent excessive foaming during the nitrogen purging step.

The sample vial was then placed on the apparatus as pictured in Figure 2. The inlet needle was a No. 22 B-D Yale needle, two inches long. The outlet needle was a No. 22, $\frac{1}{2}$-inch long. The gas chromatography column was attached to the outlet side of the apparatus and the U-shaped section of the column immersed in methyl cellosolve-dry ice. The nitrogen purge gas was then turned on at a flow rate of eight ml/minute. The sample vial was immersed in

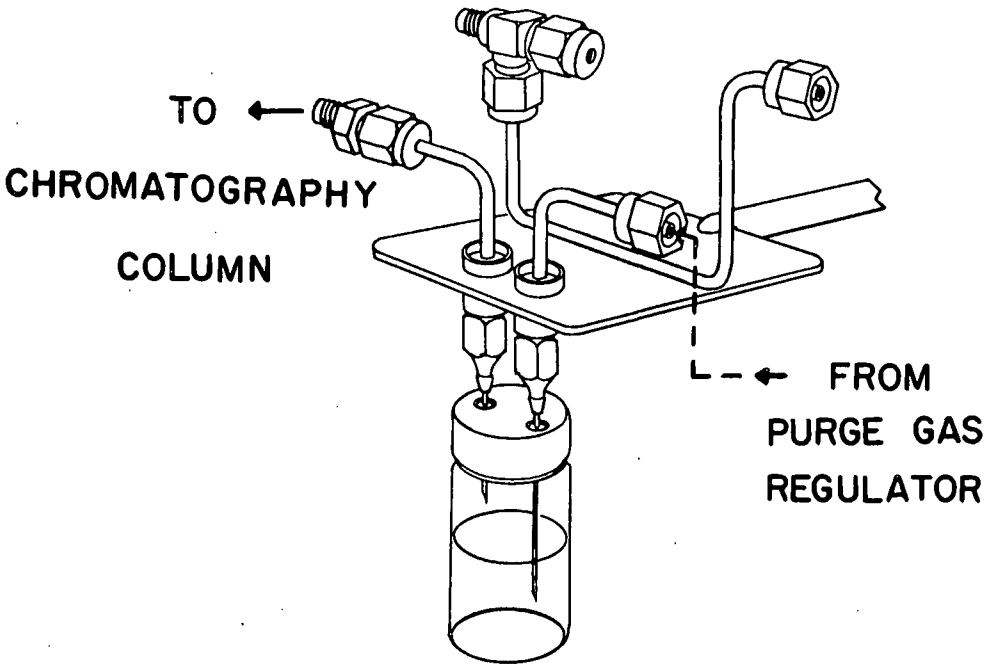


Figure 2. Apparatus Used for Volatile Head Space Studies (Morgan and Day, 1965)

an 80°C water bath during the collection period. The volatile components were collected for 10 minutes. The column was then reattached to the gas chromatograph injection port, and the carrier gas turned on.

The same apparatus was used for chromatographing known compounds for retention time data. In this case, the GLC column was connected to the Swagelok-T with the injection port. The nitrogen gas was connected to the inlet side of this line. Known compounds could then be injected through the injection port and collected in the U-shaped section of the column.

An F & M Model 810 gas chromatograph with hydrogen flame detectors was used for the volatile head space studies. This instrument was well adapted to this work due to its sensitivity and the rapid equilibration of the oven at the desired temperature. The operating parameters used were as follows:

Injection port temperature	200°C
Oven temperature	50°C and 80°C
Detector temperature	200°C
Range	10
Attenuation	x 1
Carrier gas flow rate	30 ml/minute
Hydrogen pressure	12 psi
Air pressure	26 psi

The column used for the head space analysis was a 12 ft x 1/8-inch OD stainless steel column packed with 4.0 g of 10% 1,2,3-tris-(2-cyanoethoxy) propane on 80-100 mesh Celite 545. The column

was conditioned for 48 hours at 125°C and 10 ml/minute nitrogen flow before use, and overnight between uses.

Solvent Extraction Studies

In light of the difficulties encountered in isolating higher boiling compounds by head space techniques, it was believed that other isolation techniques would be necessary. The success of Whitney and Tracy (1950) in recovering stale flavor components of dry whole milk by solvent extraction, and the success of Parks, Schwartz and Keeney (1964) in isolating o-aminoacetophenone, a highly polar, high boiling compound, by solvent extraction suggested that this would be the best technique to try.

Preparation of Solvents: The various solvents used in the preliminary solvents extraction experiments were prepared as follows:

Methanol: Reagent grade methanol was used as such.

Ethanol: Absolute ethanol, reagent grade, was used as such.

Chloroform: Reagent grade chloroform was rendered carbonyl free by the method of Schwartz and Parks (1961).

Ethyl ether: Reagent grade ethyl ether was purified by the procedure of Valseth (1953), followed by distillation.

Hexane: High purity grade hexane was treated for removal of carbonyls by the method of Hornstein and Crowe

(1962), after which it was redistilled.

Isopentane and Petroleum ether: High purity grade isopentane and reagent grade petroleum ether were treated in three liter quantities with 300 ml of concentrated sulfuric acid with stirring on a magnetic stirrer for six hours to destroy carbonyls. The acid treated solvent was redistilled over potassium hydroxide pellets.

Preliminary Experiments: Preliminary solvent extraction studies indicated that extraction of the fat from the liquid sterile concentrated milk by organic solvents was not feasible. The emulsions that formed upon agitation of the solvent-milk system were exceedingly stable.

The possibility that the sterile concentrated milk could be lyophilized before solvent extraction was considered. It was found that this could be done without loss of the stale flavor components. Samples of the lyophilized milk rehydrated to whole milk concentration with distilled water possessed the same characteristic stale flavor as samples of the original sterile concentrate diluted to whole milk concentration with distilled water. Hence, it was concluded that lyophilization could be employed in the extraction procedure.

A pilot-plant freeze drier was used to accomplish lyophilization. Sterile concentrated milk was placed in two-quart Pyrex baking pans to a depth of about $\frac{1}{2}$-inch (equivalent to approximately 750 ml of sample). Each pan was then covered with Saran wrap, and

placed in a -29°C blast freezer overnight. The frozen samples were lyophilized at four to five mm pressure for 12-14 hours, with the heating plates of the freeze-drier maintained at 32°C throughout the drying cycle. The lyophilized milk was scraped from the drying pans and coarsely ground in a large mortar and pestle in preparation for subsequent solvent extraction.

Experimental Solvent Extraction Systems: Various solvent extraction techniques were attempted before a successful method was developed. To be applicable to the problem of isolating the stale flavor components, the solvent system had to meet the requirements of recovering the characteristic stale flavor components in the fat in good yield while not interfering with subjective analysis of the isolated fat.

The first approach to solvent extraction of the stale flavor components was a soxhlet extraction of the lyophilized milk with ethyl ether. The typical odor associated with the stale sterile concentrate could not be detected in the fat extracted in this manner. After evaporation of ethyl ether from the powder, the powder was rehydrated with water. The typical stale odor was evident in the rehydrated milk, indicating that the stale flavor components were not recovered by this procedure.

Folch et al. (1951, 1957) reported the use of a 2:1 chloroform-methanol solvent system for quantitative extraction of lipid material

from animal tissues. Smith, Ambrose and Knobl (1964) had also used chloroform-methanol to extract the lipid material from fish meal. It was felt that this system might be applicable to the problem at hand. Hence, the former procedure was attempted. In this procedure, the lyophilized material was homogenized in a Waring blendor with 20 times its weight of 2:1 chloroform-methanol for four minutes. The homogenate was filtered through a Buchner funnel lined with Whatman No. 5 filter paper. The filtrate was placed in a separatory funnel, and distilled water in the amount of 20% of the volume of the filtrate was added. The funnel was shaken vigorously, and then placed in a 1°C room overnight to allow the chloroform-fat and methanol-water layers to separate. The chloroform-fat layer was collected, dried with sodium sulfate, and the chloroform evaporated on a rotary evaporator. Considerable difficulty was experienced with a residual chloroform odor which interfered with the judgement as to the success of the extraction procedure. This residual odor could not be eliminated by extensive evaporation at reduced pressure. The best method found for obtaining an odor judgement of the extracted fat was to dip strips of chromatography paper into the fat and allow the residual chloroform to evaporate from these strips. The odor of the fat remaining on the paper strip could then be determined. The characteristic odor of the stale concentrated milks was not detected in the fat extracted by the chloroform-methanol

procedure. Other modifications of this procedure using the same solvent system were attempted, but none appeared to be successful in extracting the stale flavor components in good yield.

In view of the success of Whitney and Tracy (1949) in extracting stale milk fat from stale dry whole milk, it was decided that their method should be attempted. In this procedure, 100 g quantities of lyophilized milk were blended with 850 ml of 95% ethanol and 6.45 ml of distilled water for one hour in a Waring blendor. The resulting mixture was filtered through a Buchner funnel. The filtrate was placed in a separatory funnel, and ethyl ether and distilled water were added, each in a volume equivalent to the volume of filtrate. The funnel was shaken for two minutes, and the phases then allowed to separate. Some difficulty was experienced with emulsions. Saturation of the water with sodium chloride solved this problem, however. The ethyl ether layer was recovered and used in a soxhlet extraction of the residue from the Buchner funnel.

A similar experiment was attempted using methanol as the water-dispersion and wetting agent. The extracted fat from both of those experiments was found to possess some definite odor properties, but still was not characteristic of the stale flavor defect. Further criticisms of this procedure were the length of time involved to extract any appreciable amount of material, and the large volumes of solvent required.

As previously mentioned, Muck, Tobias and Whitney (1963) had found that different solvents seemed to extract different types of flavors, and that hexane in particular seemed to be effective in extracting stale flavors. It was reasoned that isopentane, being a hydrocarbon, would have solubility properties similar to hexane, making it an even better choice than hexane due to its lower boiling point ($28^{\circ}C$ vs $69^{\circ}C$) and its consequent ease of removal. A procedure similar to that used by Bassette (1958), who saturated nonfat dry milk with methanol before soxhlet extraction with hexane, was attempted, using isopentane as the solvent. Fifty grams of lyophilized milk was ground in a mortar and pestle with 50 mls of methanol. The resulting wet, coarse powder was placed in the extraction thimble and extracted with isopentane for 10 hours. The isopentane extract was washed with water to remove methanol, dried with sodium sulfate, and the isopentane evaporated. The extracted fat exhibited some of the odor properties of stale sterile concentrate, although not in very great concentration. The residual powder still possessed the characteristic stale odor, indicating that the stale flavor components had not been extracted completely.

The identification of o-aminoacetophenone as a flavor component of stale nonfat dry milk suggested that the compounds responsible for the stale flavor defect might be tightly bound to milk protein due to polar functional groups. It was reasoned that raising the

pH of the milk, in conjunction with the wetting procedure, might aid in releasing such flavor compounds for solvent extraction. Evidence supporting this hypothesis was obtained by adjusting the pH of sterile concentrated milk by adding solid potassium carbonate. As the pH was adjusted upward to pH 9.9, the stale odor became more intense. The increase in odor intensity was directly proportional to the amount of potassium carbonate added. A pH of 8.0 was selected as optimum, for at this pH the stale odor was enhanced but the sample was not altered physically. At a pH of 9.9, the sample became glue-like in consistency.

Following this hypothesis, the previous isopentane extraction procedure was repeated using methanol containing solid potassium carbonate such that the methanol-lyophilized milk mixture was pH 8.0. An alternative method was to add the potassium carbonate to the milk before lyophilization. The resulting extract possessed a very strong odor, although it did not appear to be typical of the stale odor. To determine what compounds were responsible for this odor, two 700 g batches of lyophilized milk were extracted by this procedure. The isopentane was evaporated from the extract, and the extracted fat was subjected to a low pressure molecular distillation. Because difficulty was experienced in keeping the pressure below 50 μ, the fat was subsequently subjected to a reduced temperature, reduced pressure steam distillation. The distillates from both distillations

were extracted with ethyl chloride, and the ethyl chloride was evaporated on the evaporator described by Bills, Khatri, and Day (1963). Approximately 30 ml of a highly odorous extract remained. Gas chromatographic analysis indicated that this extract was a series of methyl esters. It was then realized that the presence of methanol under alkaline conditions (established by the addition of sodium bicarbonate) in the organic solvent (isopentane) provided optimum conditions for ester exchange reactions with the fatty acids in the milk glycerides. With this fact in mind, the use of an alcohol as a wetting agent was abandoned, for it seemed feasible that the esterification process could possibly occur even in the absence of added base, catalyzed at localized sites by amino or other basic groups inherent in the dried milk. Water was thus selected as the sole wetting agent for the lyophilized milk.

The problem thus became one of getting approximately 10% water distributed uniformly throughout the lyophilized milk. The first attempt to solve this problem was to slurry the lyophilized milk in a solvent in which water was slightly soluble, such as ethyl ether, and adding the required amount of water dropwise. This procedure resulted in only a small proportion of the dried powder being wetted and sticking to the sides of the blendor. An alternative procedure was therefore attempted in which an organic solvent in which water is insoluble, such as hexane, was used. This procedure is

the same as that used by Parks, Schwartz, and Keeney (1964) to isolate o-aminoacetophenone from three year old dry milk powder. This procedure resulted in a very fine powdered product that was uniform in appearance. It was concluded that this method was the most acceptable for wetting the powder prior to extraction. Some of the fat was extracted into the organic solvent during the actual wetting process, and the remainder was subsequently removed by soxhlet extraction with the same solvent used in the wetting process. The resulting extracted fat possessed a definite stale odor, reminiscent of the original milk. Isopentane was also tried as a solvent in this same procedure (a shield with an aspirator connection was made for the blendor base to protect against explosion). It appeared that hexane did a more complete job of extracting the stale flavor components. Hence, hexane was selected as the extraction solvent.

Adopted Extraction Procedure: The procedure finally adopted for extraction of the fat and stale flavor components was as follows. One hundred gram quantities of lyophilized milk were slurried with 400 ml of hexane for five minutes. The hexane was then filtered into the round bottom flask used for the solvent extraction, and the resulting powder was placed in the extraction apparatus. A large soxhlet extractor was used for the extraction. The extraction thimble consisted of a cylinder of Whatman No. 3 chromatography paper plugged at one end with glass wool. The thimble provided adequate

drainage for the siphoning of the soxhlet apparatus. The extractor was of sufficient size to hold the powder from 800 g (eight 100 g batches) of lyophilized milk. By using two Waring blendors simultaneously it was possible to prepare 800 g of sample for extraction in approximately one hour. The powder was extracted until four trips of the siphon had occurred (three to four hours). The extraction thimble containing the sample was then removed and a new sample placed in the extractor. The same solvent was used to extract several batches of the same milk. A total of three 800 g batches of both stale and fresh lyophilized sterile concentrated milk were extracted by this procedure. The three extracts were pooled, and the solvent removed on a rotary evaporator. Only enough heat was applied in the water bath of the rotary evaporator to prevent the formation of frost on the outside of the evaporation flask.

Steam Distillation of Extracted Fat

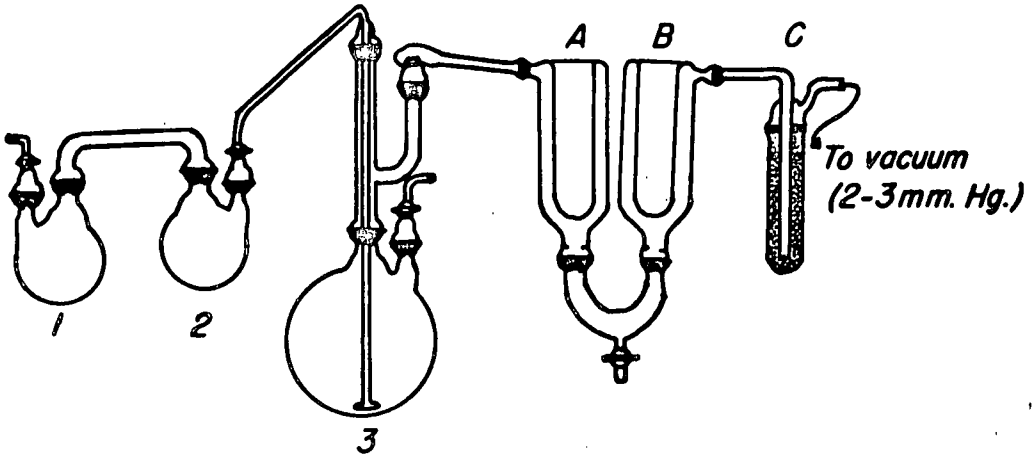
Isolation of the stale flavor components from the stale milk fat differed from the procedure used by Parks, Schwartz and Keeney (1964). Whereas they carried out an acid extraction of the hexane-fat mixture with the express purpose of isolating the one particular compound possessing a "grape-like" odor, a reduced temperature, reduced pressure distillation of the extracted fat was carried out in this work with the hope of isolating all of the compounds

responsible for the stale flavor defect. The distillation apparatus is shown in Figure 3.

Following the distillation, the two liquid nitrogen Dewar-type traps were thawed, and the distillate was collected and extracted three times with U. S. P. grade ethyl chloride in a cold room. The ethyl chloride extract was dried overnight with sodium sulfate, and the ethyl chloride evaporated on the apparatus described by Bills, Khatri and Day (1963). Trace quantities of hexane carried through from the fat to the stale flavor extract. Final concentration of the extract was thus achieved by passing a stream of nitrogen over the surface until approximately 0.3 ml of extract remained. The extract obtained from stale samples of sterile concentrated milk, possessed a strong odor described as stale, hay-like, or cereal-like. The odor of fatty acids, ketones, and lactones, and possibly of the grape-like component, could be detected in the extreme. On the other hand, the odor from the control samples was much less intense, and had a lactone-type quality. The strong hay-like quality was not evident in the control extract.

Gas Chromatography of Flavor Extract

Gas chromatography of the flavor extracts was performed initially on a Barber-Colman Model 20 gas chromatograph equipped with a β-ionization detector. Argon was used as the carrier gas.



1. Steam generator (500 ml.)
2. Steam trap (")
3. Steam-stripping flask (2 liter); warmed to 40°C.

A & B. Cold finger traps (liquid nitrogen)
C. Glass bead trap (")

Figure 3. Apparatus Used for Reduced-Temperature, Reduced-Pressure Steam Distillation of Extracted Fat.

A 12 foot x 1/8-inch OD stainless steel column packed with 3.8 grams of 20% Apiezon M on 100-120 mesh Celite 545 was used. Chromatograms were run isothermally at 130°C and 200°C. The carrier gas pressure was 20 psi, the cell voltage was 1250 volts, and the relative gain was set at 30. The effluent gas stream was split approximately in half by a Swagelok T-capillary tube arrangement, with half flow going to the detector and the other half passing through a heated port and vented to the atmosphere. The effluent gas stream could thus be smelled, and the odor significance of the various components eluting from the column could be determined.

It was determined by smelling the effluent gas stream that several components were being hidden under larger peaks of the chromatogram. These large peaks appeared to be fatty acids, which tend to tail considerably on an Apiezon column. Consequently, a two foot x 1/8-inch stainless steel column packed with 20% Apiezon M on 100 - 120 mesh Celite 545 containing 5% by weight sodium hydroxide was prepared. When placed in front of the regular Apiezon column, this base-treated column retained the fatty acids and allowed the resolution of components that were previously overshadowed by the fatty acid peaks.

Mass Spectrometry of Stale Flavor Components

Rapid scan mass spectra of the flavor components were obtained on an Atlas-MAT CH-4 Nier-type mass spectrometer (a nine inch, 60 degree sector, single focusing instrument). Spectra were recorded on a Honeywell 1508 Visicorder. A Barber-Colman Series 5000 temperature programming gas chromatograph with a flame ionization detector was used in conjunction with the mass spectrometer. One-half of the effluent stream from the gas chromatography column was directed through the EC-1 gas inlet valve of the mass spectrometer. When this valve was opened to the proper position, a portion of this effluent stream entered into the high vacuum system of the mass spectrometer, and rapid scan spectra could be taken at various points of the chromatogram as desired. The remainder of the effluent stream was vented to the atmosphere and could be smelled.

The operating parameters of the mass spectrometer were as follows:

Filament current	60 μ-amps
Electron energy	30 ev
Accelerating voltage	3000 v
Analyzer pressure	$\sim 2 \times 10^{-6}$ mm Hg
Multiplier voltage	1.85
Scanning speed	5 seconds to scan m/e 25 to m/e 250

For the initial mass spectrometry work, a 12 foot x 1/8-inch OD stainless steel column packed with 4.7 g of 20% Apiezon M on

100 - 120 mesh Celite 545 was used, in conjunction with the base-treated pre-column previously described, to obtain chromatographic separation of the flavor components. Apiezon M was chosen because of its bleed stability at higher temperatures. A column stationary phase with a low bleed rate at the desired temperatures is essential for mass spectrometry work in order to avoid interfering m/e peaks (m/e is the ratio of mass to charge) in the spectra.

Helium was used as the carrier gas for the gas chromatographic separation of the stale flavor components for the mass spectrometry work. The carrier gas pressure was set at 65 psi, which resulted in a flow rate of 40 ml/minute at room temperature. This higher flow rate helped to sharpen the peaks, thus increasing the ratio of sample to carrier gas at the peak maximum, and making the resulting spectra stronger. The hydrogen pressure was maintained at 13 psi, and the air pressure at 65 psi. The best temperature program for the stale flavor mixture was found to be an initial temperature of 100^{o}C for five minutes followed by a temperature increase of five degrees per minute to 200^{o}C, and holding at this temperature for 15 minutes. This program provided for optimum separation of the early eluting components without sacrificing sharpness of the later eluting peaks.

It should be noted that the time of maximum response of the chromatographic recorder did not indicate when the maximum amount

of a particular component was in the mass spectrometer. A lag period existed between these two maxima. The extent of this lag period at various points in the chromatogram was determined by monitoring a particular m/e peak, such as m/e 43, on the mass spectrometer, and timing the difference between maximum chromatograph detector response and maximum response in the mass spectrometer. Hence, spectra were taken on unknown peaks after the appropriate interval of time from maximum detector response of the gas chromatograph in order to obtain the strongest possible spectra. Whenever feasible, background spectra were taken just before a component eluted from the gas chromatography column so that m/e peaks resulting from that particular component could be determined by difference.

It was possible to obtain good spectra of some of the larger peaks of the Apiezon chromatogram by this procedure. Definite changes from one spectra to the next could be detected for some of the earlier peaks, and some of these were identified. The spectra of the latter peaks of the chromatogram were very similar, indicating the possible existence of an unresolved mixture. In taking spectra, particular attention was given those peaks having definite odor properties, as determined by smelling the effluent gas stream, that appeared to make them significant in the stale flavor defect. Significant odors were detected in the latter peaks of the chromatogram,

indicating that some of the components in this area were important in the stale flavor defect.

It became apparent from the spectra of the components eluting from the Apiezon column that some method of building up the concentration of certain of the components and of obtaining better separation of components was necessary. The logical method of increasing the concentration of particular components was to successively trap these components as they eluted from the Apiezon column. The use of a polar stationary phase, instead of the non-polar Apiezon, appeared to be the best method of obtaining better separation of components. Hence, it was decided that these two steps could be combined by repeatedly trapping the regions of interest from the Apiezon column onto a short packed column, and re-chromatographing the trapped components on a polar column.

The traps for the trapping procedure consisted of 1.5 foot x 1/8-inch OD stainless steel tubing packed with 20% Apiezon M on 100 - 120 mesh Celite 545. The traps were conditioned prior to use at 225^{o}C and at a helium flow rate of five ml/minute for 24 hours. For collection, these traps were immersed in methyl cellosolve-dry ice, and attached to the split from the gas chromatograph. A 20:1 collection port to detector split ratio was used for collection purposes. The majority of the sample thus went to the collection traps, but just enough went to the detector to provide a chromatographic record

of the injected sample. The traps were attached to the effluent port when the appropriate region of the chromatogram was reached. The collection traps were sealed with Swagelok caps between each collection. Two regions of the effluent from the Apiezon column were designated as important and were collected in this manner. The first of these regions was from approximately 22 minutes after the start of the temperature program to 28 minutes, and the second region was from 30 minutes to 45 minutes. These two regions were trapped from six consecutive temperature programmed chromatograms (approximately six μl of flavor extract injected for each chromatogram) on the Apiezon column. A certain amount of solvent was necessary in the flavor extract to enable the syringing of the extract. Since the first collection trap was attached after the solvent had been vented to the atmosphere, the trapping procedure provided a means of isolating the flavor components in the absence of any solvent.

It was determined that Carbowax 20M was a polar stationary phase which was stable enough at high temperatures to be used in conjunction with the mass spectrometer. The column bleed rate, when temperature programmed from 100°C to 200°C, was slightly greater than that of Apiezon M. Rapid scan mass spectra of the column bleed material at temperatures as high as 225°C indicated that only the 58, 73, and 88 m/e peaks were present in detectable

amounts. No higher mass numbers were evident. With this in mind, Carbowax 20M was selected as the polar stationary phase. A 12 foot x 1/8-inch OD stainless steel column containing 3.8 g of 20% Carbowax 20 M on 80 - 100 mesh Celite 545 was used.

The trapped components were re-chromatographed by installing the collection trap in front of the Carbowax 20M column, turning the carrier gas on, and setting the oven temperature to the desired level. The carrier gas pressure was set at 50 psi (to give a flow rate of approximately 30 ml/minute through the combined trap and column). The components contained in the first trap were separated by temperature programmed gas chromatography, using the same program as was used for the Apiezon column. The components collected in the second trap were separated by operating the gas chromatograph isothermally at 200°C. The latter parameters were selected because o-aminoacetophenone was suspected to be one of the components in the second trap, and it required approximately 45 minutes to elute from the polar Carbowax column at 200°C. If this component was indeed one of the compounds present in the trap, lower operating temperatures would cause it to be so far out in the chromatogram and the peak so diffuse that a good spectrum could not be obtained.

Identification of Stale Flavor Components

The various components were identified by a combination of

mass spectral fragmentation patterns and relative retention times on the gas chromatography columns. If a particular spectrum indicated a particular compound structure the compound was obtained and its retention time was checked on the particular column and under the conditions used when that spectrum was obtained. If the retention time of the suspected compound agreed with that of the unknown peak on which the spectrum was obtained, identification was considered positive. In general, all of the data available in the way of mass spectra and retention times on various columns was used to identify the components.

RESULTS AND DISCUSSION

Subjective Evaluation of Samples

The results of the trained panel evaluation of fresh whole milk, fresh sterile concentrated milk, aged sterile concentrated milk, and fresh sterile concentrated milk containing five parts per billion added o-aminoacetophenone are presented in Table 1. The average of the scores given each sample under each criticism, as well as average total scores, are presented in this Table. An analysis of variance of the total scores was performed (Li, 1957, p. 205-207), as indicated in Table 2. The analysis of variance revealed a significant difference among the samples at the 0.01 level of significance. Calculation of the Least Significant Difference (LSD) revealed that the total score of each sample was significantly different from the total score of each of the other samples at the 0.01 level of significance. Similarly, an analysis of variance was performed on the average score given each sample under the stale criticism, with a significant difference among the samples again detected. These calculations are indicated in Table 3. Calculation of the LSD indicated that for the stale criticism, fresh whole milk was significantly different from each of the other samples at the 0.01 level of significance, and that fresh sterile concentrated milk was significantly different from aged sterile concentrated milk at the 0.01 level of significance and from

TABLE 1

Average Scores for Each Criticism and Average Total Scores of Milk Samples Evaluated by Trained Panel

	Fresh Whole Milk	Fresh Sterile Concentrate	Aged Sterile Concentrate	Fresh Sterile Concentrate + o-amino-acetophenone
Criticism				
Astringent	10.0	9.70	8.87	9.53
Caramelized	10.0	9.73	7.90	9.50
Chalky	10.0	9.63	9.40	9.43
Cooked	9.20	8.33	8.33	8.67
Lactone	9.67	9.27	8.67	9.13
Scorched	9.80	9.30	7.70	9.20
Stale	9.84	6.97	5.36	5.75
Total Score	8.24	5.86	3.20	4.63

TABLE 2

Analysis of Variance of Total Scores, Trained Panel

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Judges	29	101	3.49	2.68
Treatments	3	400	133.3	102.3**
Error	87	113	1.3	
Total	119	614		

F-value necessary for significance at 0.01 level: 4.04

$LSD_{.05}$: 0.587

$LSD_{.01}$: 0.774

TABLE 3

Analysis of Variance of Stale Criticism Scores, Trained Panel

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Judges	29	365.0	12.6	3.40
Treatments	3	366.5	122.17	31.9**
Error	87	322.5	3.71	
Total	119	1054.0		

F-value necessary for significance at 0.01 level: 4.04

$LSD_{.05}$: 0.987

$LSD_{.01}$: 1.30

the fresh sterile concentrated milk containing five parts per billion added o-aminoacetophenone at the 0.05 level of significance. No significant difference was detected between the aged sterile concentrated milk and the fresh concentrated milk containing five parts per billion added o-aminoacetophenone.

It is interesting to note the significant difference between fresh whole milk and fresh sterile concentrated milk. When the fresh sterile concentrate was tasted by itself by some of these same panel members, the response was highly favorable. The response appears to be less favorable when the fresh sterile concentrated milk is compared directly with fresh whole milk.

The significant difference between the stale criticism scores

of fresh whole milk and fresh stale concentrated milk might be attributable to the nature of the scorecard used. A slight stale flavor must receive a mandatory score of six or five, as compared to a score of 10 if no stale flavor is detected. Thus the magnitude of the difference in numerical score between no stale flavor and slight stale flavor may not indicate as marked a flavor difference as between a slight stale and a pronounced stale flavor, where consecutive decreasing numbers are assigned to increasing intensity of stale flavor.

The lack of a significant difference between the stale criticism scores of aged sterile concentrate and fresh sterile concentrate containing five parts per billion added o-aminoacetophenone, combined with the significant difference between each of these samples and fresh sterile concentrated milk without any added o-aminoacetophenone, would indicate that this compound is indeed important in the stale flavor defect. The average total score of each of these samples was significantly different from each of the other two at the 0.01 level of significance, with the total score of the fresh sterile concentrate containing added o-aminoacetophenone falling about midway between the other two. This would seem to indicate that other compounds also contribute to the flavor differences between the fresh and aged sterile concentrated milks.

The results of the student preference panel evaluations are

shown in Table 4. The computer analysis of variance of these data is shown in Table 5. Significant differences were detected at the 0.01 level. From the LSD values, it can be seen that the preferences for the fresh and aged sterile concentrated milks were significantly different from each other at the 0.01 level of significance, and that the preference for each of these milks was significantly different from that of fresh whole milk.

From these results, it was concluded that the fresh and aged sterile concentrated milks were indeed different, not only in total score and consumer preference, but also in degree of stale flavor. Comparison of the flavor components of these samples thus seemed to be a logical approach to the identification of the compounds responsible for the stale flavor defect.

Volatile Head Space Studies

Examples of the gas chromatograms of the volatile components of fresh sterile concentrated milk (two months at 1°C) and aged sterile concentrated milk (nine months at 21°C) are shown in Figure 4. These chromatograms were run on a 1, 2, 3-tris-(2-cyanoethoxy) propane column at a column temperature of 80°C, using the technique developed by Morgan and Day (1965). Chromatograms were also run at 50°C; the 50°C chromatograms showed fewer peaks than the 80°C chromatograms.

TABLE 4

Frequency Distribution of Preference Scores Given Milk Samples by Student Panel

Score	Fresh Sterile Concentrate	Aged Sterile Concentrate	Dry Milk	Fresh Whole Milk
1 (dislike)	3	25	3	0
2	11	25	13	1
3	22	24	16	4
4	33	18	23	7
5	11	7	20	18
6	18	11	10	19
7	15	9	29	26
8	10	4	9	36
9 (like)	0	0	0	12
Total	123	123	123	123
Mean Preference Score	4.64	3.37	4.91	6.69

TABLE 5

Analysis of Variance of Preference Scores, Student Panel

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Judges	122	863.7	7.08	3.13
Treatments	3	692.8	230.92	102.16**
Error	366	827.2	2.26	
Total	491	2383.7		

F-value necessary for significance at 0.01 level: 3.78

$LSD_{.05}$: 0.376

$LSD_{.01}$: 0.494

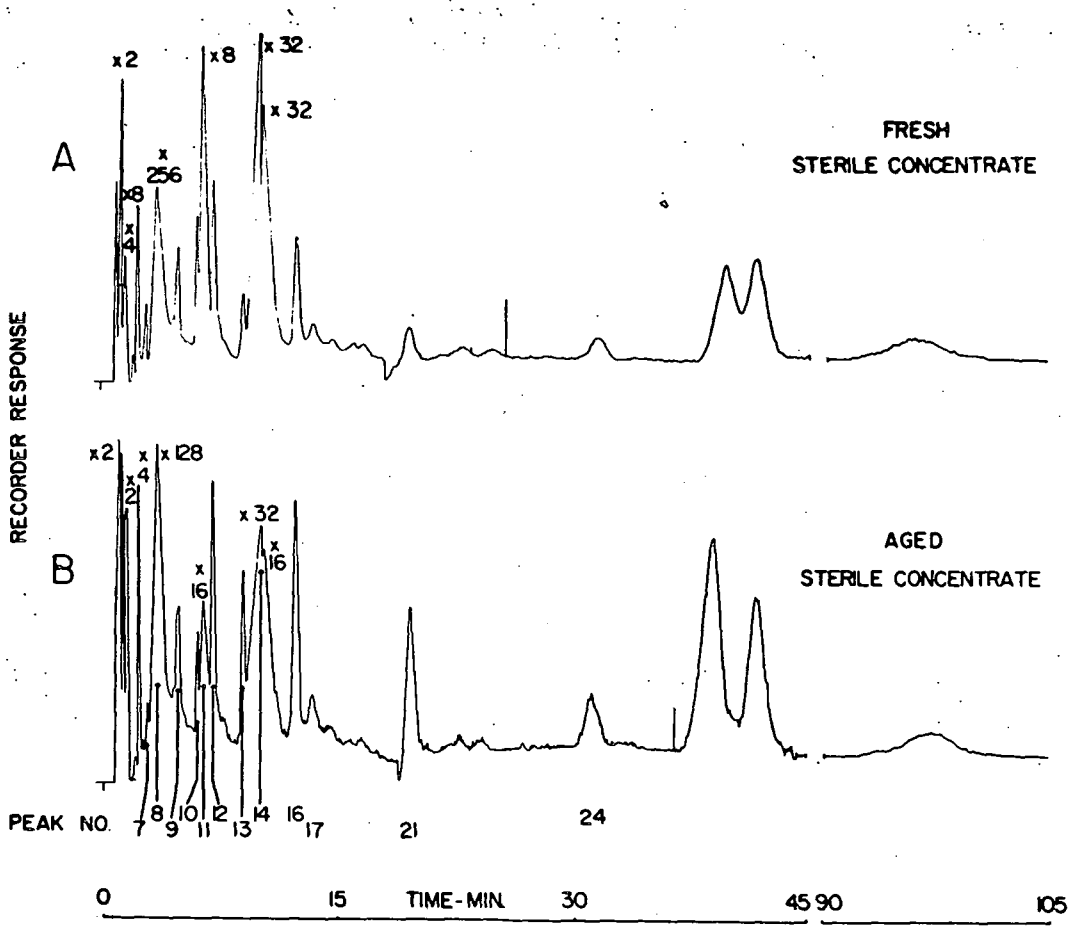


Figure 4. Chromatograms of Volatile Head Space Components of (A) Fresh and (B) Aged Sterile Concentrated Milks; 1, 2, 3-tris- (2-cyanoethoxy) Propane Column, 80°C.

The tentative identity of some of the peaks of the chromatograms shown in Figure 4 was determined by running known compounds and checking for agreement of relative retention times (relative to acetone) at both temperatures. The peaks tentatively identified are listed in Table 6. The largest peak of the chromatograms, peak 8, did not come from the milk; this peak was found in approximately the same quantity when six ml of distilled water containing 3.6 g of sodium sulfate and a spatula tip of Dowex antifoam AF emulsion was subjected to the same procedure. It was concluded that this compound originated from the antifoam. Similarly, peak 21 of the chromatograms was also found to be present in a blank run.

With the exception of isobutyraldehyde, isovaleraldehyde, and ethyl acetate, all of the compounds tentatively identified had been previously tentatively identified in sterile concentrated milk by Bingham (1964). The presence of the two aldehydes, isobutyraldehyde and isovaleraldehyde, can be explained by Strecker degradation of valine and leucine, respectively.

The fact that the identity of all of the peaks of the volatile head space chromatograms was not established was not of great concern, as this was not the major reason for performing the head space studies. The principal purpose of these chromatograms was to compare fresh and stale sterile concentrated milks in order to check the hypothesis that the compounds responsible for stale flavor

TABLE 6

Tentative Identity of Peaks Shown in Figure 4
(Chromatogram of Volatile Head Space, 80°C
on 1,2,3-tris (2-cyanoethoxy) Propane Column)

Peak Number	Relative Retention Time (to acetone)	Tentative Identity	Relative Retention Time of Known	Agreement in 50°C Chromatograms
7	0.438	acetaldehyde	0.443	Yes
8	0.549	(from antifoam)	0.552	(Yes)
9	0.755	isobutyraldehyde	0.748	Yes
		or		
		ethyl formate	0.770	No
10	0.950	ethyl acetate	0.947	Yes
11	1.00	acetone	1.00	Yes
12	1.10	ethanol	1.12	?
13	1.41	2-butanone	1.43	Yes
14	1.59	methyl butyrate	1.57	Yes
16	1.93	2-pentanone	1.92	Yes
17	2.10	dimethyl disulfide	2.10	Yes
21	3.36	(from antifoam)	3.43	(Yes)
24	4.92	2-heptanone	4.94	Yes

were mainly higher boiling compounds. Comparison of the chromatograms of fresh and aged sterile concentrate as shown in Figure 4 reveal no qualitative differences. No new peaks have appeared in the chromatogram of the stale milk that were not present in the fresh milk. Although quantitative evaluation of the chromatograms is difficult, it can be seen that the relative sizes of each of the peaks of the chromatogram of the stale concentrate is not markedly

different from the relative sizes of each of the peaks of the chromatogram of fresh concentrate. These results thus suggest that the significant difference detected between the flavor of the fresh and stale sterile concentrates are not due to differences in the more volatile components. The hypothesis that stale flavor is caused primarily by higher boiling components is thus supported by the volatile head space studies.

Effect of Base-treated Pre-column

A chromatogram of the flavor extract obtained from stale sterile concentrated milk by the solvent extraction technique is shown in Figure 5A. This chromatogram was run at 200°C on a 20% Apiezon column. Figure 5B shows a chromatogram of the same extract upon insertion of the base-treated pre-column in front of the regular Apiezon M column. Although the extra two feet of column has increased the retention times of the various components, the general pattern of the chromatograms can be followed and comparisons of the two can be made. The retention times of various known compounds was determined both with and without the base treated pre-column to establish the exact relationship between the two chromatograms. By so doing, it was determined that the peaks designated as numbers 7, 9, 16 and 21 in Figure 5A were removed by the base-treated pre-column. As shown in Table 7, the retention

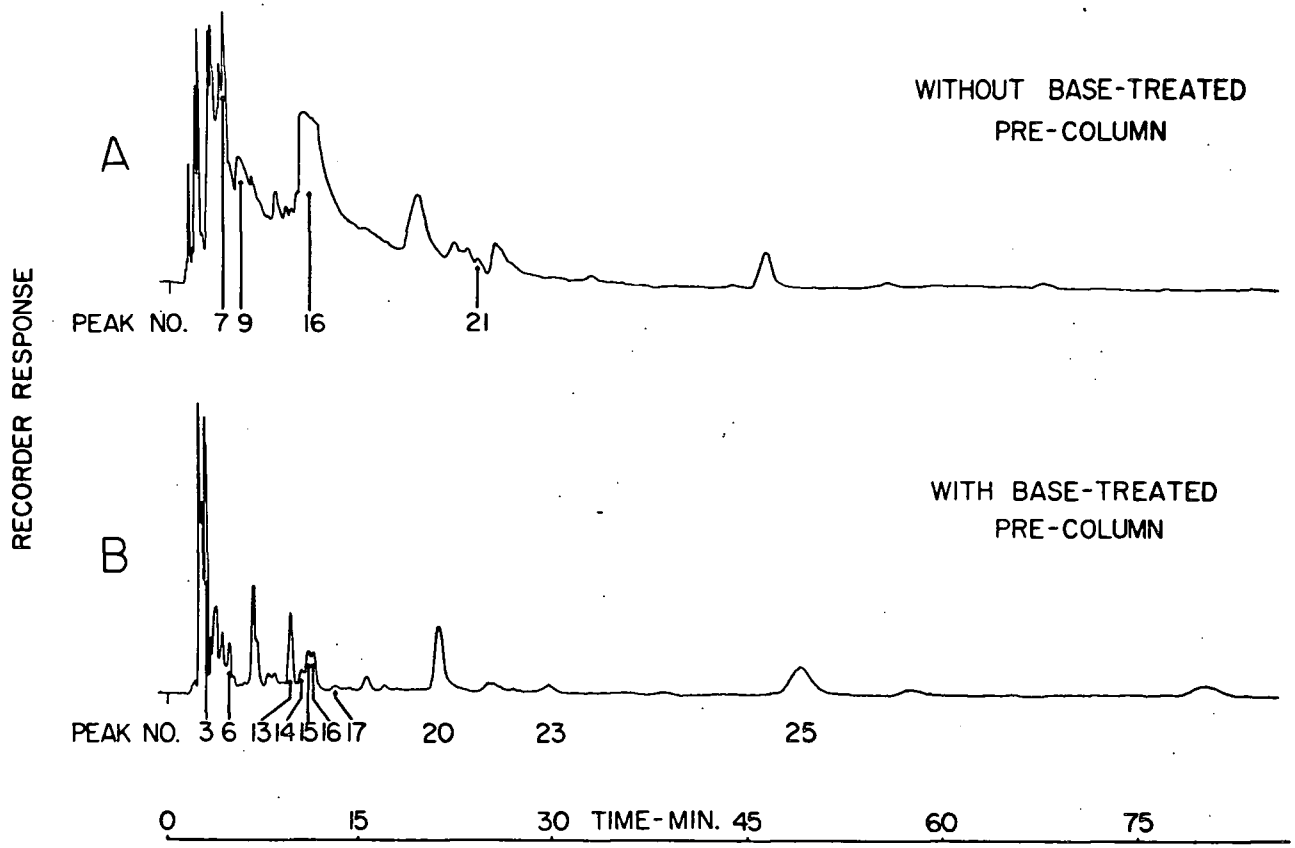


Figure 5. Chromatograms of Flavor Extract from Stale Sterile Concentrated Milk: (A) Without Base-treated Pre-column, (B) With Base-treated Pre-column; Apiezon Column, 200°C.

times of these four peaks agree with the retention times of butyric, caproic, caprylic, and capric acids, respectively. The area under peak 16 of Figure 5A was resolved into three other components when caprylic acid was removed by the base column. These three new components are indicated as peaks 15, 16, and 17 of Figure 5B.

The 130°C chromatograms show similar results, although fewer components elute from the column at this temperature. Those components that do elute from the column at 130°C are better separated than in the 200°C chromatogram. Tentative identification of some of the components appearing in the 200°C Apiezon chromatogram with the base-treated pre-column (Figure 5B), based on relative

TABLE 7

Identity of Peaks of Chromatogram Shown in Figure 5A Which are Removed by Base-treated Pre-column (Stale Flavor Extract, Apiezon Column, 200°C)

Peak Number	Retention Time (mm)	Identity	Retention Time of Known Compound (mm)	Agreement at 130°C
7	35	butyric acid	34	Yes
9	46	caproic acid	45	Yes
16	89	caprylic acid	87	Yes
21	202	capric acid	199	---

retention times, is presented in Table 8. As indicated in the Table, the odor of the effluent was generally in agreement with that of the suspected compounds. Other odors believed to be of significance were detected in the region of peaks 14, 15, and 16 of this chromatogram, but no tentative identification was established for these compounds.

TABLE 8

Tentative Identity of Certain Peaks of the Chromatogram Shown in Figure 5B (Stale Flavor Extract, Apiezon Column with Base-treated Pre-column, 200°C)

Peak Number	Relative Retention Time (to 2-heptanone)	Tentative Identity	Relative Retention Time of Known	Agreement at 130°C	Agreement of Odor
3	0.60	2-pentanone	0.59	Yes	?
6	1.00	2-heptanone	1.00	Yes	Yes
13	2.02	2-nonanone	1.98	Yes	Yes
20	4.47	2-undecanone	4.35	---	Yes
23	6.28	o-aminoacetophenone	6.17	---	Yes
25	10.40	δ-decalactone	---	---	Yes

Comparison of Control and Stale Extracts

Chromatograms of the fresh and stale concentrated milk flavor extracts are shown in Figure 6. These chromatograms were run on a 20% Apiezon M column with the base-treated pre-column. The chromatograms were temperature programmed from an initial temperature of 100°C, held for five minutes, to 200°C at the rate of 5°/minute.

A quantitative comparison of the two chromatograms is difficult due to variations in the amount of solvent present and the amount of sample injected. However, the comparison of unknown peaks with a known peak in each chromatogram, and comparison of the size of this known peak between the two chromatograms should give some indication of the relative amounts of the various components in each sample. Peak 5 of the chromatogram of the flavor extract from fresh sterile concentrated milk (Figure 6A) and peak 7 of the chromatogram of the flavor extract from stale sterile concentrated milk (Figure 6B) were identified as 2-heptanone by retention time and mass spectral data. Whereas these two peaks are of comparable size in each of the two chromatograms, several of the subsequent peaks are considerably larger in the stale milk chromatogram than in that of the fresh milk. Certain of the peaks in the stale milk are not present in the fresh milk. In comparing the two chromatograms,

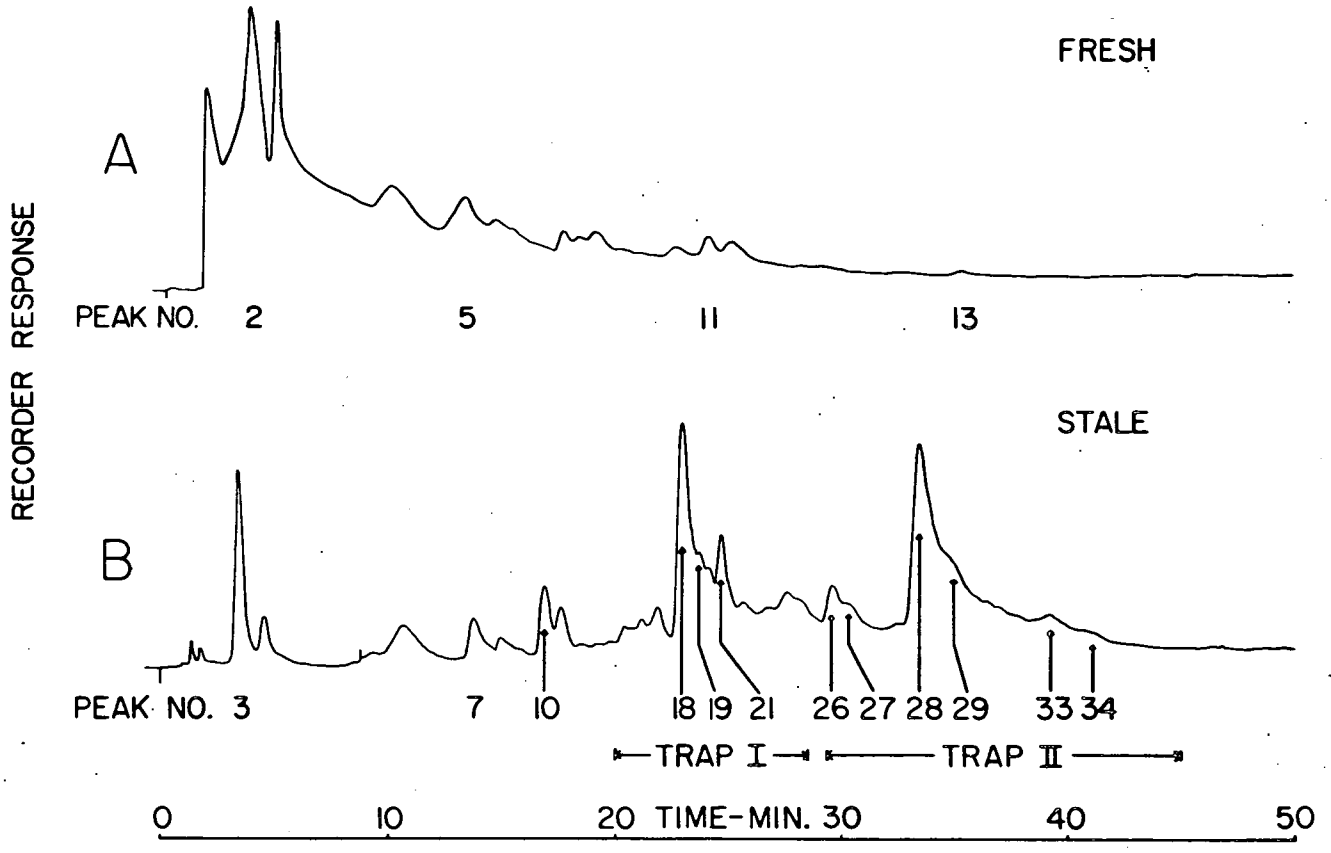


Figure 6. Chromatograms of Flavor Extracts from (A) Fresh and (B) Stale Sterile Concentrated Milks; Apiezon Column, Temperature Programmed at 5°C/minute from 100°C (5-minute hold) to 200°C.

it would appear that one or more of peaks 18, 21, and 24-34 of the stale sterile concentrated milk might be of significance in the stale flavor defect. Both peak 18 and peak 21 are significantly increased in the stale milk, and peaks 24-34 of the stale milk chromatogram do not appear to be present in the fresh milk extract.

Odors thought to be significant in the stale flavor defect were detected in the region beginning with peak 18 of the stale milk chromatogram and continuing to the end of the chromatogram. Although the nature of these odors changed, there was considerable residual odor from one component to the next making differentiation of the compounds rather difficult. A characteristic "horse barn" odor was detected in the region of peak 18, along with a strong "ketone-like" odor. An odor reminiscent of ground corn was detected in the region of peak 21. A persistent odor resembling the odor of rubber was detected in the region of peaks 26 and 27, and extending into peak 28. Although a very large peak, peak 28 did not appear to have an odor intense enough to overcome the background "rubber" odor. Peak 29, a shoulder of peak 28, had a "grape-like" odor, and peak 33 had a "coconut-like" odor. None of these odors, with the possible exception of the "coconut-like" odor, were detected in the fresh milk chromatogram. A trace of the "coconut-like" odor was detected in the region of peak 13 of the chromatogram of the fresh sterile concentrated milk.

Only peaks 3, 7, 10, 18 and 19 of the chromatogram of the stale milk extract (Figure 6B) were identified conclusively by mass spectral data that were obtained as these components eluted from the Apiezon column. These peaks were identified as hexane (solvent), 2-heptanone, benzaldehyde, 2-nonanone and a dichlorobenzene, respectively. Peaks 2, 5 and 11 of the chromatogram from fresh sterile concentrated milk extract (Figure 6A) were identified as hexane (solvent), 2-heptanone and a dichlorobenzene.

Gas Chromatography and Mass Spectral Identification of Trapped Components

The regions of the Apiezon chromatogram selected to be trapped for subsequent re-chromatography on the Carbowax 20M column are designated as Trap I and Trap II in Figure 6B. These two regions were selected because of the significant odors detected within these regions, and because the mass spectra of the components in these regions were very inconclusive due to poor separation of components.

Trap I

The chromatogram of the components of Trap I, chromatographed on a Carbowax 20M column and temperature programmed as with the Apiezon column, is shown in Figure 7. Of the various peaks in this chromatogram, good spectra were obtained only on peaks 8, 11, 12, and 17. Peak 8 appeared to be an aromatic

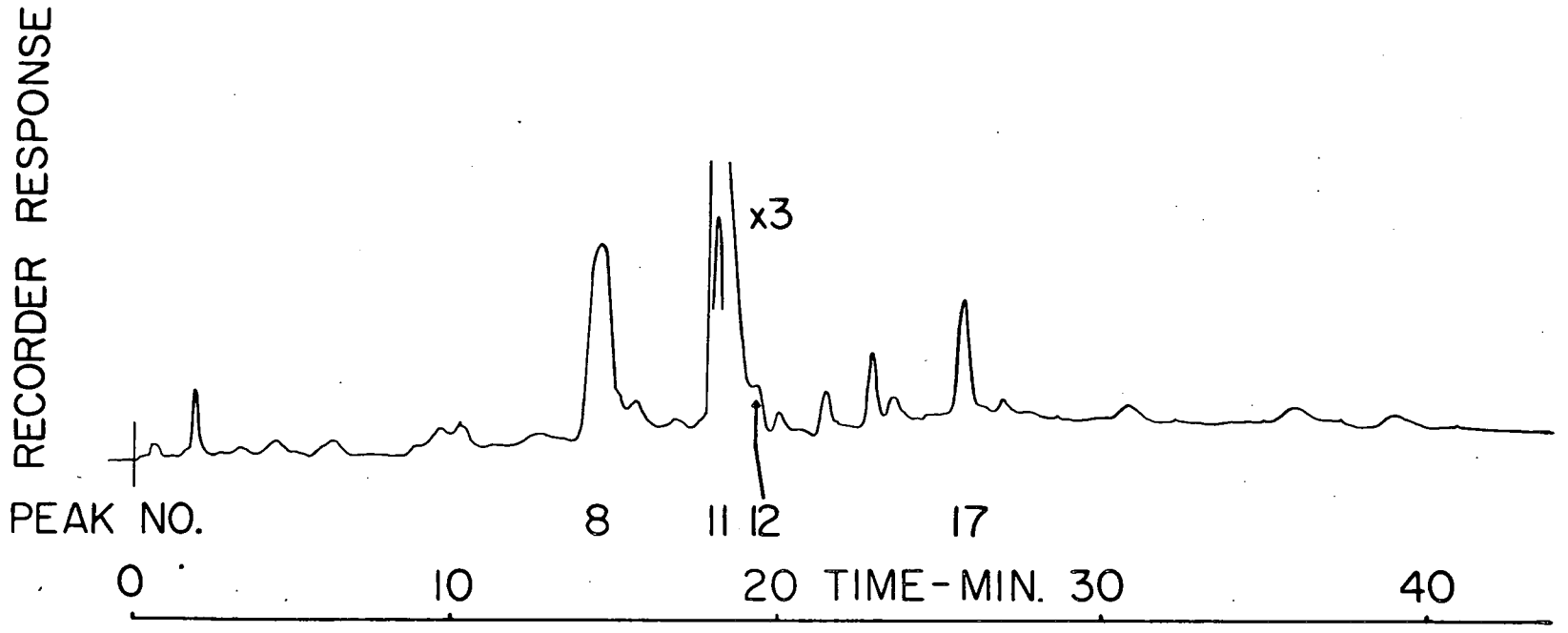


Figure 7. Chromatogram of Components Trapped in Trap I; Carbowax 20M Column, Temperature Programmed at 5°C/minute from 100°C (5-minute hold) to 200°C.

hydrocarbon with three carbons attached to the aromatic ring; definite identity could not be established.

As an example, the spectrum obtained from peak 11 of Figure 7 is shown in Figure 8; (note: m/e 28, 32, 40, and 44 are inherently present in all of the mass spectra and may be attributed to the following sources: 28, carbon monoxide and nitrogen; 32, oxygen; 40, argon; 44, carbon dioxide). The intensity of m/e 43 and 58 suggest that this compound is a methyl ketone; this conclusion is supported by the intensity of m/e 57, 71, 59, and 41. (Sharkey, Schultz, and Friedel, 1965). The parent ion (p) of the compound appears to be m/e 142, which was not present in the background spectra. The peak at p-15 (m/e 127), indicating loss of a methyl group from the parent molecule, further establishes that m/e 142 is the parent ion. Hence, the spectrum is interpreted to be that of 2-nonanone. Comparison of the spectrum with the spectrum of 2-nonanone in the API tables (American Petroleum Institute, 1948 to date) shows excellent agreement, and further substantiates the identity of the component. Finally, coincidence of the relative retention times of known 2-nonanone and the unknown compound, under identical gas chromatography conditions, is sufficient evidence for confirming the identification.

Peak 12 of Figure 7, appearing on the shoulder of peak 11 (2-nonanone), was identified as a dichlorobenzene on the basis of

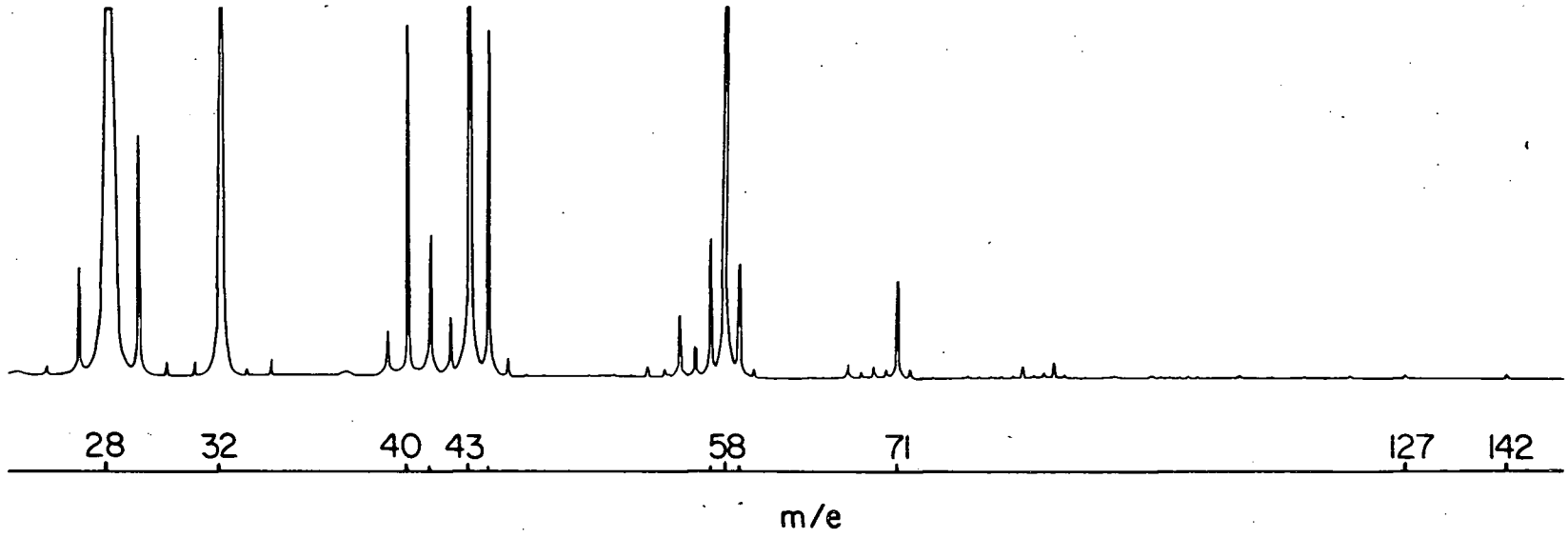


Figure 8. Mass Spectrum of Peak 11 of the Components of Trap I (Figure 7).

its mass spectrum. (American Society for Testing and Materials, 1963). The retention time of peak 12 was in fair agreement with that of o-dichlorobenzene, supporting the identification of this component as a dichlorobenzene. The spectrum of peak 17 tentatively indicated that his component might be acetophenone. The retention times were in agreement, and hence peak 17 was tentatively identified as acetophenone.

Trap II

The chromatogram of the components of Trap II, chromatographed isothermally on the Carbowax 20M column at 200°C, is shown in Figure 9. Although this temperature enabled o-aminoacetophenone to be eluted from the column as a peak sufficiently sharp to obtain a good mass spectrum, it did not provide for separation of the earlier components. Hence, these components could not be identified. To obtain an identification of these components, the process will have to be repeated and the components of Trap II temperature programmed to obtain better separation of the earlier eluting components. The number of components eluting from Trap II, in comparison to the apparent number of components in the region of the Apiezon column trapped in Trap II indicates the utility of this procedure in the separation of previously unresolved components.

Peak 1 of the chromatogram of Trap II was thought to be

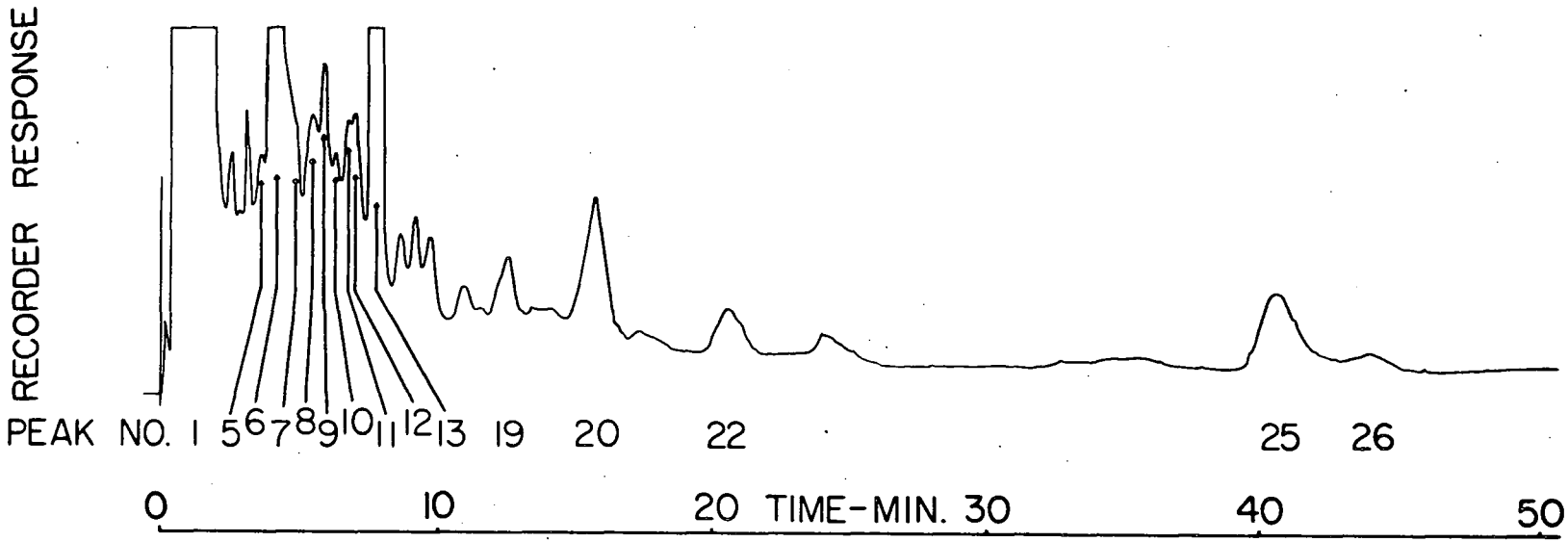


Figure 9. Chromatogram of Components Trapped in Trap II; Carbowax 20M Column, 200°C.

methyl cellosolve on the basis of the odor eluting from the column. Peaks 5 - 12 were not identified; spectra taken within this region indicated an aromatic character for some of the components. As previously mentioned, this region of the chromatogram will have to be temperature programmed to achieve optimum separation of components.

Peak 13 and peak 20 were positively identified as 2-undecanone and 2-tridecanone, respectively. Peak 19 was identified as naphthalene.

Peak 22 illustrates an interesting example of the technique of identifying compounds by GLC and mass spectra. The compound eluting at this point possessed an odor reminiscent of the "rubber" odor previously mentioned. No other evidence as to the possible identity of the compound was available. The mass spectrum of this compound, as well as the background spectrum taken just before this compound eluted from the column, are shown in Figure 10. It can be seen that m/e 135 and 108 are significantly increased in the mass spectrum of this compound. The peaks at m/e 58, 73, and 88 are the result of column bleed from the Carbowax 20M column, and remain constant in both spectra. Other m/e peaks that appear to be increased to some extent in the spectrum of the unknown compound are 37, 38, 39, 45, 63, 69, and 82. The doubly charged ion 135^{++} (appearing as m/e 67.5) which appears in the mass spectrum

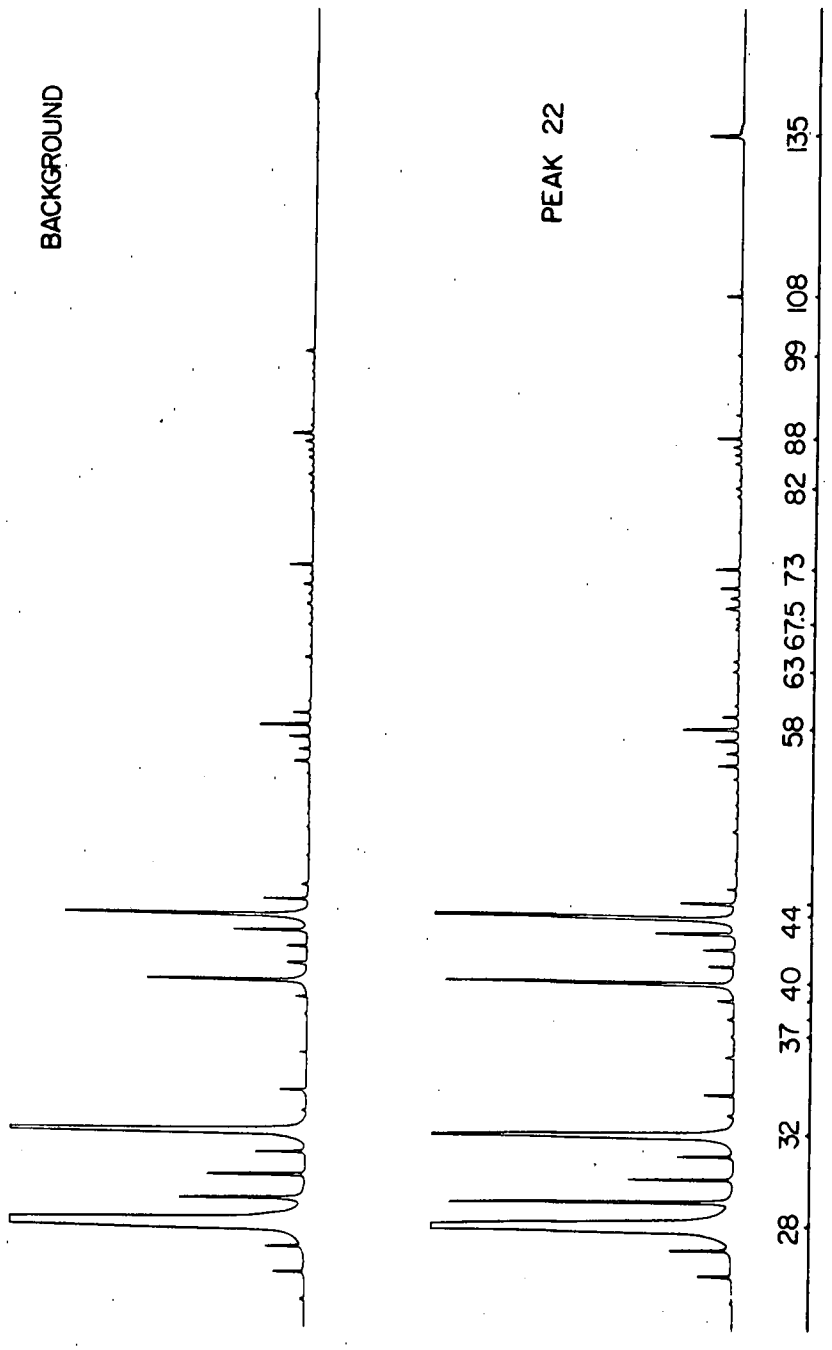


Figure 10. Background Mass Spectrum and Mass Spectrum of Peak 22 of the Components of Trap II (Figure 9).

of the compound is not present in the background spectrum. It appears that m/e 135 is both the base peak (largest) and the parent peak (molecular weight) of the unknown compound and that m/e 108 is the second largest peak. With this information, the ASTM index (American Society for Testing and Materials, 1963) of mass spectral data was consulted. The most probable identity of the unknown compound appeared to be benzothiazole, which has a base and parent peak of m/e 135, with m/e 108 equal to 35% of the size of the base peak. Further comparison of the spectrum of benzothiazole and the unknown compound shows good agreement, the known spectrum having significant peaks at 37, 38, 39, 45, 63, 67.5, and 69.

A sample of benzothiazole was then ordered from a chemical supplier. Upon arrival, this compound was chromatographed on the Carbowax 20M column with Trap II in place, under conditions identical to those used for chromatographing the components of Trap II. The retention time of this compound was in agreement (less than 3% deviation) with that of the unknown peak. A rapid-scan mass spectrum of the known benzothiazole was also taken as it eluted from the column. This spectrum agreed with that of the unknown compound. On the basis of this evidence, peak 22 of the components of Trap II was positively identified as benzothiazole.

The mass spectrum of peak 25 of the components of Trap II (Figure 9) and the background spectra are shown in Figure 11. The

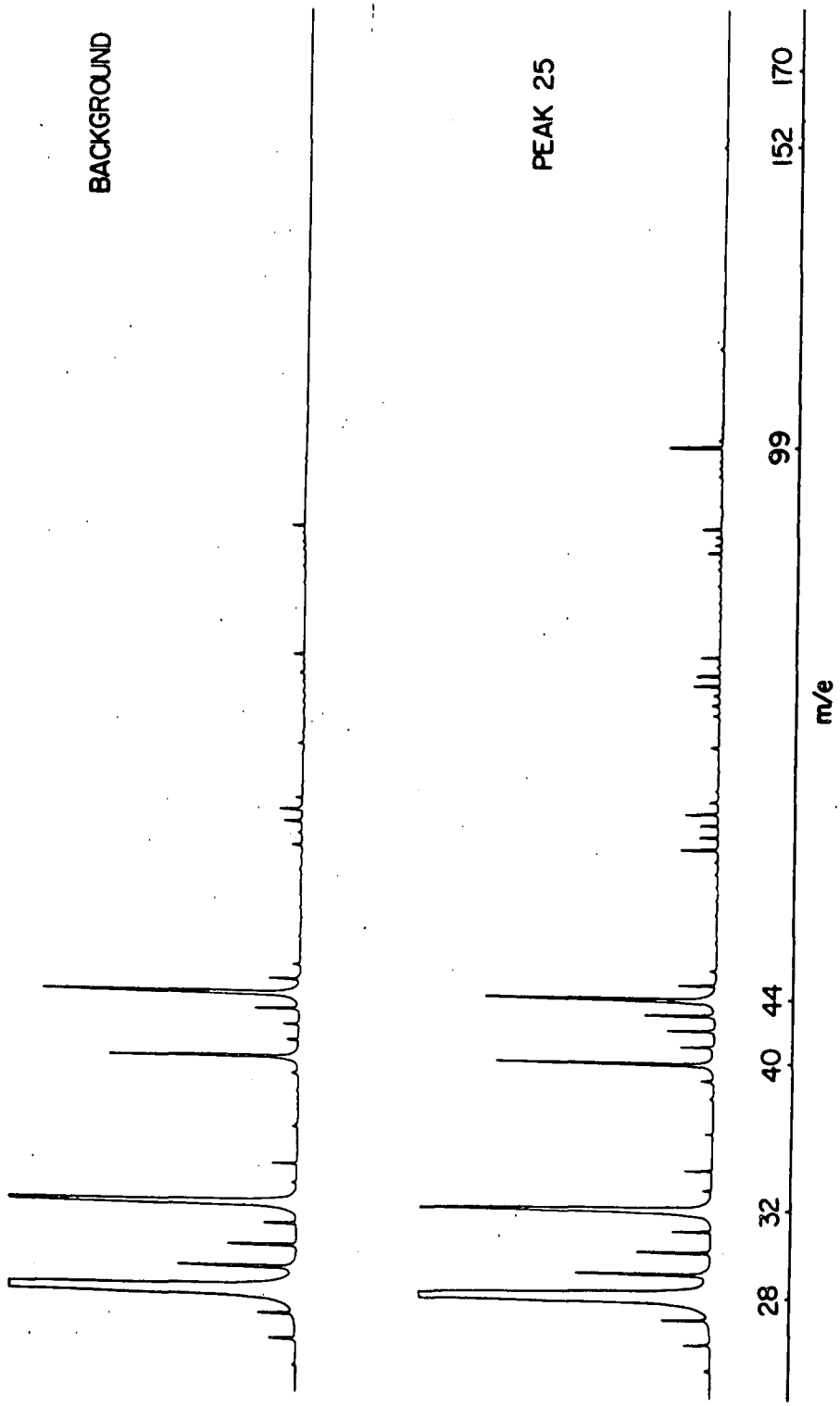


Figure 11. Background Mass Spectrum and Mass Spectrum of Peak 25 of the Components of Trap II (Figure 9).

large 99 peak immediately suggests that this component is a δ-lactone. (McFadden, Day and Diamond, 1965). The parent ion is rarely seen with lactones, but p-18 (loss of water) is a common occurrence. The peak at m/e 152 in this spectrum is likely p-18, and consequently the parent ion must be 170. This would suggest that the compound is δ-decalactone. The characteristic coconut-like odor of this peak further supports its identity as δ-decalactone. Finally, the coincidence of the retention time of this peak with known δ-decalactone under identical gas chromatography conditions positively identifies it as δ-decalactone.

Peak 26 of the components of Trap II was found to have a parent peak and base peak at m/e 135, with m/e peaks also appearing at 120 and 92. This information, along with the coincidence of retention times on both the Apiezon and Carbowax columns, and the characteristic grape-like odor of this component positively identified it as o-aminoacetophenone. Although the amount of this component appears to be small (as judged by GLC peak size), it was possible to determine its parent ion because of the nature of compound. The compound is quite stable to mass spectral fragmentation, thus making the parent peak the largest peak of the spectrum. This enabled the mass spectral determination of the molecular weight of the compound even though it is present only in very small concentration. The 120 (loss of $-CH_3$), and 92 (loss of $-\overset{O}{\overset{\|}{C}}-CH_3$) m/e peaks

were also helpful in identifying the compound.

Summary of Compounds Identified

A summary of the compounds identified by gas chromatography and mass spectral data are indicated in Table 9. The basis of identification of each compound is also included in this Table.

Of the compounds identified in the extract from stale sterile concentrated milk, only 2-heptanone and the dichlorobenzene were positively identified in the extract from fresh sterile concentrated milk. These identifications were also made by gas chromatography and mass spectrometry.

Significance of Compounds Identified

It is of interest to consider the significance of the compounds identified in this study. The methyl ketones are undoubtedly of significance from the flavor standpoint. Methyl ketones, and in particular 2-heptanone, have been found to possess low flavor thresholds (Langler and Day, 1964). It must be kept in mind that 2-heptanone was also identified in the extract from fresh sterile concentrated milk. The fact that the longer chain ketones were found in the stale sterile concentrate but not in the fresh would suggest that the reaction leading to the formation of methyl ketones proceeds slowly upon storage of the product. The reaction might well be initiated by the heat treatment given the product, and subsequently proceed at a rate

TABLE 9

Summary of Compounds Identified in the Flavor Extract from Stale Sterile Concentrated Milk

Identified	Retention Time Data		Mass Spectral Identification	Agreement of Odor
	Apiezon	Carbowax		
2-heptanone	+		positive	+
2-nonanone	+	+	positive	+
2-undecanone	+	+	positive	+
2-tridecanone	+	+	positive	?
benzaldehyde	+		positive	+
acetophenone	+	+	tentative	?
napthalene		+	positive	?
a dichlorobenzene	+	+	positive	?
δ-decalactone	+	+	positive	+
benzothiazole		+	positive	+
o-aminoacetophenone	+	+	positive	+
butyric acid	+			+
caproic acid	+			+
caprylic acid	+			+
capric acid	+			+

determined by storage temperature.

Benzaldehyde possesses a characteristic odor which might be of significance in the stale flavor defect. This compound has previously been identified by Parks and Patton (1961) from badly deteriorated dry whole milk powder, but has not previously been

reported in stale sterile concentrated milk. Its origin is unknown.

Acetophenone, although only tentatively identified, can be explained as a breakdown product of o-aminoacetophenone (loss of $-NH_2$ group). It does possess an odor reminiscent of the "ground corn" odor noted in the Apiezon chromatogram, and thus might be of significance in the stale flavor defect.

Napthalene possesses an odor of moth-balls, which could be of some significance. The origin of this compound is unknown.

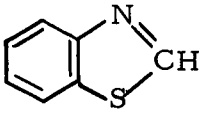
The dichlorobenzene was found both in the fresh and stale sterile concentrated milk. It is not considered to be of significance in the stale flavor defect. Its origin is unknown, although it is speculated that it might be a contaminant from diffusion pump oil (a chlorinated diphenyl) which could have carried into the product from the vacuum system used in the steam distillation procedure. Another possibility is that it might be a breakdown product of pesticide contamination in the milk.

The occurrence of δ-decalactone is not surprising, as it has been identified in a number of heated milk products. It was not positively identified in the fresh sterile concentrate but was thought to be present on the basis of gas chromatography retention time and odor. It appears that the amount of δ-decalactone in the stale milk is considerably more than in the fresh, suggesting that the heat-initiated reaction leading to lactone formation also occurs at

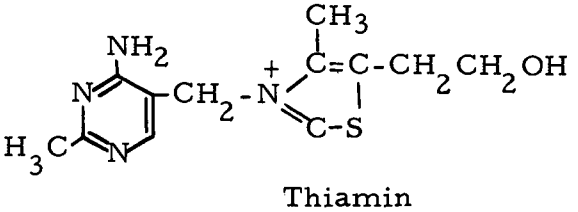
a slow rate during storage of the product. This compound, due to its very characteristic "coconut-like" odor, is likely very significant in the stale flavor defect.

The existence of benzothiazole in milk products has not been reported in the literature. This is understandable in that it is a high boiling compound (boiling point of 234°C), and does not readily yield to derivative formation. Mass spectrometry has not been applied in a sufficient number of cases to have identified this compound. Benzothiazole possesses a characteristic "rubber" odor, and might be responsible for the "old rubber" flavor of stored sterilized concentrated milk reported by Patel et al. (1962). Its effect on the flavor of fresh sterile concentrated milk is currently being studied to further determine its significance.

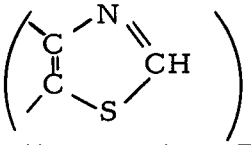
The structure of benzothiazole is as follows:



Benzothiazole



Thiamin

The thiazole ring  is found in thiamin (vitamin B_1), which occurs naturally in milk. The formation of benzothiazole needs to be further investigated before a mechanism for its formation can be postulated.

The identification of o-aminoacetophenone from stale sterile

concentrated milk further implicates this compound as a causative agent of stale flavor. This compound had previously been identified in stale nonfat dry milk, but not in sterile concentrated milk. It is speculated that this compound is one of the more important in the stale flavor defect. The possible origin of this compound has previously been discussed, and needs to be studied more extensively.

The significance of the fatty acids in the stale flavor defect is unknown. The same fatty acids were found in the flavor extracts from fresh and stale sterile concentrated milks when chromatographed without the base-treated pre-column. The free fatty acids of fresh and stale sterile concentrated milk were not quantitatively determined, however.

SUMMARY AND CONCLUSIONS

This investigation was concerned with the qualitative identification of the compounds responsible for the formation of stale flavor upon storage of sterilized concentrated milk.

Commercial samples of sterilized concentrated milk were obtained and stored at 21 ^{o}C to accelerate the development of stale flavors. Stored and fresh samples were compared organoleptically, and found to be significantly different.

The more volatile components of the fresh and stored liquid milk samples were compared by a nitrogen sweep on-column trapping procedure.

The higher boiling components were recovered by lyophilization of the liquid sterile concentrated milk samples, solvent extraction of the fat and flavor components from the lyophilized milk, and reduced temperature, reduced pressure steam distillation of the solvent extracted fat. The flavor components recovered in this manner were studied by gas chromatography in conjunction with rapid-scan mass spectrometry.

A gas chromatography trapping technique was utilized to build up the concentration of flavor components, and to obtain better separation of components. In this technique, certain regions of the effluent from a non-polar column (Apiezon M) were trapped

successively from several chromatograms, and the trapped components re-chromatographed on a polar column (Carbowax 20M). Rapid-scan mass spectra were then taken of the components as they eluted from the polar column. The findings of this investigation were as follows:

1. There is little difference in the more volatile components of fresh and stale sterile concentrated milk, suggesting that the compounds responsible for stale flavor are of a higher boiling nature.

2. Stale sterile concentrated milk can be lyophilized without loss of the compounds responsible for the characteristic stale flavor.

3. A solvent extraction technique, consisting of slurrying the lyophilized milk with hexane in a Waring blendor, adding 10% water by weight, and subsequent soxhlet extraction of the wetted powder with the same hexane was an effective method for the extraction of the compounds responsible for the stale flavor from the lyophilized milk.

4. The compounds responsible for the stale flavor defect can be isolated from the fat by a reduced temperature, reduced pressure steam distillation technique.

5. The technique of trapping the effluent from a particular region of a gas chromatogram several times in succession, and then re-chromatographing the trapped components on a different type of column is a good technique for increasing the concentration

of components and improving the separation of components for mass spectral analysis.

6. Of the compounds isolated by these techniques, the following were positively identified: 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, benzaldehyde, napthalene, a dichlorobenzene, δ-decalactone, benzothiazole, o-aminoacetophenone, butyric acid, caproic acid, caprylic acid and capric acid. Acetophenone was tentatively identified. The dichlorobenzene was present in both fresh and stale sterile concentrate and was believed to be an artifact.

7. Of the compounds identified, the methyl ketones, benzaldehyde, δ-decalactone, and o-aminoacetophenone have been identified in badly deteriorated dry milk powder. Their presence in stale sterile concentrated milk further implicates them as significant compounds in the development of stale flavor.

8. Benzothiazole has not previously been reported in milk products. It has a characteristic odor reminiscent of the odor of rubber, which might well make it of significance in stale flavor.

9. Several other compounds were isolated but not identified. Further application of these same general techniques is necessary to completely characterize the compounds responsible for stale flavor development.

10. Studies on the mechanism of formation of such compounds as o-aminoacetophenone and benzothiazole are also necessary to

completely understand the factors favoring the formation of stale flavor.

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