DETECTION OF PSYCHROTROPHIC BACTERIA IN MILK BY GAS-LIQUID CHROMATOGRAPHY

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ABSTRACT

DETECTION OF PSYCHROTROPHIC BACTERIA IN MILK
BY GAS-LIQUID CHROMATOGRAPHY

By

Rena Maria Pierami

The objective of this study was to develop a rapid qualitative procedure for the detection of the growth of psychrotrophic bacteria in milk. Pyrolysis-gas-liquid chromatography (PGLC) and direct analysis of headspace vapors by gas-liquid chromatography (GLC) are two techniques that have been used for the identification and differentiation of microorganisms. Due to the ease and simplicity of these techniques, each one was evaluated for its applicability in analyzing the microbial quality of milk.

Samples of raw and pasteurized milk were inoculated with Alcaligenes viscolactis, Bacillus pumilus, Pseudomonas fragi, or Pseudomonas perolens; uninoculated milk samples were used as controls. All samples were incubated at 7±1 C, and the growth of bacteria was followed by plating the milk to determine the Standard Plate Count and the Psychrotrophic Bacteria Count. The concentration of bacteria in the control

was low initially and remained low and insignificant in comparison to the high concentrations of bacteria in the inoculated milk samples.

In preparation for pyrolysis aliquots of the milk samples, at various times during incubation, were lyophilized. The dried milk was then pyrolyzed at 800 C, and the resulting fragments were separated by GLC. The pyrograms obtained from analysis of the controls were visually compared with the pyrograms of the inoculated samples to determine if the growth of the psychrotrophs had caused any differences in the pyrolytic profiles of milk. No major differences were observed in any PGLC studies. Due to the inability to obtain consistent results and due to interferences in the pyrolytic reaction from the normal constituents of milk, under the conditions used in this investigation PGLC was found to be impractical for determining the presence of psychrotrophs in milk.

For headspace vapor analysis aliquots of the incubated milk samples were transferred into serum vials or Erlenmeyer flasks, sealed, and heated in a 60$\pm$1 C water bath for 15 minutes with constant shaking. A representative sample of the headspace vapors was then removed with a gas-tight syringe from the container, and the syringe was injected into the gas chromatograph. Varying volumes of milk and headspace samples were analyzed on a 10% Corbowax 20M on 80/100 mesh Chromosorb

W column and a 4% Apiezon L on 80/100 mesh Gas Chrom Z column to determine which sampling conditions yielded the best results. Two major peaks and at times three other peaks appeared on the chromatograms obtained from analysis of inoculated milk. Three peaks were presumptively identified as acetaldehyde, acetone, and ethanol by comparison of the retention times of the peaks with the retention times of pure standards. As the milk was incubated, changes occurred in the patterns of the peaks; these changes could be correlated to changes in the concentrations of the volatile compounds due to production and utilization of the compounds during bacterial metabolism.

The present standard microbiological method for the enumeration of psychrotrophs in milk requires incubation at 7±1 C for 10 days. Even though differences were not evident on the chromatograms until the milk contained relatively high concentrations of bacteria, the differences were observed before any organoleptic changes in the milk could be detected. The volatile by-products of microbial metabolism, therefore, could provide a measure of the activity of psychrotrophic bacteria in milk and make analysis of headspace vapors by GLC feasible as a quality control method for evaluating the microbial condition of milk.

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INTRODUCTION

Psychrotrophs are microorganisms capable of growing at 7 C or less, irrespective of their optimum growth temperature. Since psychrotrophic bacteria can grow at refrigeration temperatures, their presence in milk is primarily responsible for limiting the keeping quality and causing spoilage of the milk. The present standard microbiological method for the enumeration of psychrotrophic bacteria in milk involves incubation at 7 C for 10 days. This method is, therefore, not useful in quality control work because of the length of time required to obtain results.

In recent years several gas chromatographic methods have been developed for the identification of microorganisms or their metabolic by-products. Pyrolysis-gas-liquid chromatography was used in 1965 to identify members of the family Enterobactericeae (Reiner, 1965) and has since been used widely for identifying other microorganisms. In a pyrolytic reaction a pellet of dried bacteria is decomposed at a high temperature into a variety of chemical fragments. Each microorganism produces its own unique pyrolytic profile, thus allowing differentiation of organisms. Another technique has used GLC analysis of volatile compounds in foods as a means of identifying organisms in the food. This method

involves directly sampling the headspace vapors of foods and subjecting them to GLC.

This study was designed to develop a more rapid qualitative procedure for enumerating the presence of psychrotrophs in milk. Such a procedure would be able to detect potential spoilage problems earlier in the production of milk than now possible. Based on the ease and simplicity of PGLC and headspace vapor analysis by GLC, the feasibility of adapting these techniques for the microbiological analysis of milk was determined.

LITERATURE REVIEW

Psychrotrophic Bacteria

The potential shelf-life of milk depends primarily upon its microbiological quality. Since milk is normally stored under refrigeration temperatures, microorganisms growing in milk must be capable of functioning and surviving at low temperatures. Such bacteria have been termed psychrotrophs or psychrophiles (Hammer and Babel, 1957).

The definition of psychrophilic bacteria may be expressed in several different ways, depending upon the view of the investigating bacteriologist; however, the main point of any definition is that psychrophilic bacteria are capable of growing at low (i.e., refrigeration) temperatures. The 1968 International Dairy Federation (IDF) seminar on psychrotrophs in milk concluded that "in the dairy industry, psychrotrophs are defined as those microorganisms which can multiply at a temperature of 7 C or less, irrespective of their optimum growth tempterature." The same seminar also defined psychrophiles as being psychrotrophs with an optimum temperature of less than 20 C (IDF, 1969). These IDF definitions have been adopted for use in this study.

The IDF definition of psychrotrophs is based on the ability of microorganisms to grow at low temperatures.

According to Witter (1961), this definition is only one of four major ways of classifying microorganisms which grow at low temperatures. The other three methods include classification by the optimum growth temperature of the organism, by the method of enumeration, and by criteria independent of temperature. However, classification of an organism by optimum growth temperature does not indicate whether the optimum temperature refers to the temperature necessary to attain the fastest rate of cell multiplication, the temperature at which maximum cell population can be obtained, or a combination of both of these criteria (Witter, 1961). In addition, all microorganisms capable of growing at low temperatures do not have the same maximum growth temperature, so no upper temperature limit could be set for psychrotrophs, as is done with mesophiles. Characterizing psychrotrophs by criteria other than temperature does not define the bacteria but rather categorizes them according to certain abilities which they possess (Witter, 1961). For example, psychrotrophic organisms are mainly Gram negative rods that are catalase positive, asporogenous, motile, non-acid forming, and incapable of surviving pasteurization; however, other organisms which possess these characteristics are not psychrotrophs. As stated above, in this study organisms that can multiply at 7 C or less, irrespective of their optimum growth temperature and other characteristics, will be referred to as psychrotrophs.

Bacteria representing several genera can grow at

low temperatures; the most common ones include: Achromobacter, Alcaligenes, Enterobacter, Escherichia, Flavobacterium, and Pseudomonas species (Frazier, 1967; Parker et al., 1953, Thomas et al., 1959; Witter, 1961). Members of the genus Pseudomonas are the psychrotrophs most often encountered in dairy products, particularly Pseudomonas fragi and Pseudomonas fluorescens (Parker et al., 1953; Witter, 1961). Some Gram positive bacteria including Bacillus species, such as Bacillus cereus, Bacillus coagulans, and Bacillus licheniformis, have been isolated from pasteurized milk; due to their ability to form endospores, these bacilli can survive the pasteurization process (Grosskoph and Harper, 1969).

The correlation that exists between the shelf-life potential of milk and its psychrotrophic population is extremely close. Storage at low temperatures will reduce the growth rate and biochemical activity of bacteria and, therefore, increase the keeping quality of milk; but at low temperatures psychrotrophs can increase rapidly and cause the milk to spoil (Witter, 1961). In addition to the usual psychrotrophic flora, Kennedy and Weiser (1950) observed that certain mesophilic bacteria can adapt themselves to function at temperatures lower than normal. Storing milk at 10 C for 3 to 5 days provides enough adaptation time for such mesophiles; thus the presence of mesophilic bacteria can increase the cold-thriving population within milk and enhance spoilage conditions.

The presence of psychrotrophic bacteria tends to be

more common in raw milk than in pasteurized milk (Olson et al., 1955). Since most psychrotrophs are relatively heat sensitive, pasteurization decreases the number present and leaves only a few types of psychrotrophs to predominate (Kennedy and Weiser, 1950). The temperature at which milk is held before pasteurization greatly influences the types of microorganisms that survive, but has only a slight effect upon the percentage of survivors (Kennedy and Weiser, 1950).

According to Witter (1961) and Thomas (1969), psychrotrophic bacteria have become more important to the dairy industry due to longer holding times of both raw and pasteurized milk at refrigeration temperature and increased emphasis on the keeping quality of milk. Psychrotrophic bacteria can enter milk from many sources, including cows, water, and equipment used during processing operations (Hammer and Babel, 1957). The number of psychrotrophs present in a milk sample depends upon the level of contamination, both initially and after pasteurization, and upon the prevailing sanitary conditions during processing (APHA, 1972; IDF, 1969; Olson et al., 1955).

The growth and metabolism of psychrotrophic bacteria and their enzymes can cause several defects in milk between the time of production of the milk and consumption (IDF, 1969). Since psychrotrophs are usually non-pathogenic, most of the defects that they cause are organoleptic, including the creation of off-flavors, odors, discolorations, and changes

in texture (APHA, 1972; Hammer and Babel, 1957; Olson et al., 1955).

At present the most common way of assessing the microbiological quality of milk involves analysis to determine the Standard Plate Count (SPC) or the Psychrotrophic Bacteria Count (PBC), the latter is similar to the SPC, except the plates are incubated at a lower temperature for a longer period of time (APHA, 1972). The PSC and PBC, however, require a minimum of 2 and 10 days, respectively, before results are obtained; hence there is a great need for less time-consuming procedures for enumerating the presence of psychrotrophic bacteria in milk (1972).

The methods for enumerating the presence of psychrotrophs in milk are as diversified as the definitions. Every aspect of the plating technique has been varied, from the media used to the times and temperatures of incubation Thomas, 1969; Witter, 1961). The use of various combinations of time and temperature of incubation results in different counts and can cause a lack of standardization within the diary industry (Baumann and Reinbold, 1963; Witter, 1961). The 1968 IDF seminar saw the need for a standard reference plating method and recommended that one be developed (IDF, 1969).

The 13th edition of Standard Methods for the Examination of Dairy Products (APHA, 1972) contains a standard plating method for detecting psychrotrophs which uses Standard Methods Agar with incubation at 7±1 C for 10 days. This method is a compromise between previous methods that have used 5 or 10 C, and incubation at 7±1 C for 10 days has given the highest counts in several studies (Baumann and Reinbold, 1963).

Attempts have been made to use selective media for the enumeration of psychrotrophs (Thomas, 1969; Witter, 1961). Since the predominate psychrotrophs in milk are Gram negative rods, agents inhibitory to the growth of Gram positive bacteria, including violet red bile, crystal violet, and penicillin, have been used as selective agents with incubation at higher temperatures in order to obtain counts within a shorter period of time; however, this procedure does not allow differentiation between true psychrotrophs and mesophilic Gram negative rods which could grow under the same conditions (Thomas, 1969).

Chemical tests have been utilized for evaluating psychrotrophic organisms in milk, but these tests have not been successful. The lactate-ion concentration test can be useful for assessing the shelf-life of raw milk but not pasteurized milk (Witter, 1961). The dye reduction tests, methylene blue and resazurin, indirectly measure bacterial densities in milk in terms of the time

necessary for a blue dye-milk mixture to change color (APHA, 1972). These methods depend upon the ability of bacteria in milk to grow and utilize oxygen dissolved in the mixture and hence lower the oxidation-reduction potential of the mixture (APHA, 1972). The dye reduction tests are performed at 36±1 C, and at this temperature psychrotrophs do not reduce oxygen as actively as mesophiles present in the milk do (APHA, 1972); thus the tests correlate more closely to the SPC than the PBC and are poor estimates of the psychrotrophic bacterial population in refrigerated milk (IDF, 1969; Thomas, 1969; Witter, 1961). The dye reduction tests could be made more effective for enumerating psychrotrophs, by holding the milk at 12.8 to 18.5 C for 18 hours prior to conducting the test at the normal 36±1 C temperature (Thomas, 1969). Other reduction tests designed specifically for detecting psychrotrophs have not produced satisfactory results (Witter, 1961). For any chemical test to be accurate in detecting psychrotrophic bacteria in milk, the method must take into consideration the level of bacterial contamination, the rate of increase of bacteria with storage time, and the activity of bacteria in causing the spoilage of milk (Witter, 1961).

Pyrolysis-Gas-Liquid Chromatography

Introduction

Pyrolysis-gas-liquid chromatography (PGLC) is a controlled thermal fragmentation reaction in which a sample is decomposed into chemical fragments at high temperatures, followed by separation and detection of these fragments by gas-liquid chromatography (Leathard and Shurlock, 1970). Pyrolysis-gas-liquid chromatography is an extremely useful technique for analyzing involatile compounds that cannot be subjected to normal gas chromatographic operations (McKinney, 1969; Reiner, 1971). Reiner (1971) found that solid samples, heated in an inert atmosphere to a point of vaporization, will behave similarly to an ordinary mixture of gases injected into a gas chromatograph.

Pyrolyzed fragments can provide both qualitative and quantitative analytical data about the original solid sample, recorded on a chart called a pyrogram (McKinney, 1969; Reiner, 1971). Generally, it is not the individual peaks but rather the overall pattern of the pyrogram that is important in providing useful information (Leathard and Shurlock, 1970; Reiner, 1971).

Pyrolysis-gas-liquid chromatography is a rapid and easily performed technique that lends itself well to automated data processing (Mitz, 1969; Reiner, 1971). McKinney (1969) thought that computerizing PGLC results would permit the

development of a library of standard pyrograms or "finger prints," and that such a library could be used for identifying test samples, by comparing the unknown pyrograms with known standard pyrograms. Simmonds (1970) stated that mass spectrophotometry could be used in conjunction with PGLC for identifying specific individual peaks of pyrograms.

Identification of Microorganisms by PGLC

The techniques of PGLC have been used successfully in recent years for identifying pure cultures of microorganisms. When a small pellet of dried bacteria is subjected to a pyrolytic reaction, a pyrogram with a definite pattern of peaks is produced (Reiner and Kubica, 1969; Reiner and Hicks, 1971). This pyrogram is a measure of the major chemical components (carbohydrates, lipids, nucleic acids, and proteins) of the organism's parent structure and is always a constant factor (Mitz, 1969; Reiner and Ewing, 1968; Reiner and Kubica, 1969). Reiner and Kubica (1969) found that for precise reproducibility and accurate monitoring of the biochemical events of an organism's metabolism, the pyrolytic reactions should be performed within closely defined time and temperature ranges; but some peaks are specific for certain organisms and will even appear when different columns are used and under varied operating conditions. Reiner (1965) found that in some cases the only distinguishing features between two strains or species were

the ratios of peak heights. Microbial pyrograms are so reproducible and consistent that even after more than 2 years of storage certain mycobacteria produced pyrograms similar to the ones produced when the organisms were initially analyzed (Reiner et al., 1969). Using defined conditions, Reiner and Kubica (1969) were able to detect contaminated samples from the pyrograms produced by analysis of the bacterial cultures which they were studying.

Pyrolysis-gas-liquid chromatography has been used to classify and identify several organisms, including: Mycobacteria spp., Salmonella spp., Clostridium botulinum, types A, B, and E, strains of Vibrio cholerae, and Aspergillus flavus (Cone and Lechowich, 1969; Haddadin et al., 1973; Reiner and Kubica, 1969; Reiner et al., 1969; Reiner et al., 1971; Vincent and Kulik, 1970). Reiner et al. (1972) concluded that the pyrolytic peaks produced by certain Salmonella spp. were representative of the serotype and cell wall sugars of that species; and Haddadin et al. (1973) found PGLC to be no more difficult to perform than conventional microbiological methods for categorizing vibrios.

Preparation of Microorganisms for PGLC

In studies of microorganisms utilizing PGLC techniques, pure cultures of the test organism are grown in selective media, harvested, and lyophilized in preparation for pyrolysis. The dry weight of samples analyzed in various

studies has ranged from 0.1 to 1.1 mg (Cone and Lechowich, 1969; Reiner and Ewing, 1968). To avoid the possibility of instrument variation and/or nonhomogeneous cell populations, most samples have been pyrolyzed twice in succession on either the same or different gas chromatographs (Reiner and Kubica, 1969; Reiner et al., 1969).

Interpretation of Pyrograms

Most pyrolytic profiles of microorganisms have been analyzed visually for distinctive characterisitcs and have been compared to each other for notable similarities and differences by superimposing one pyrogram on top of another (Sekhon and Carmichael, 1972). Due to variations in sample size and in homogeneity between samples, the curves are not always superimposable; to compensate for this, investigators have shifted the pyrograms slightly, using a reference peak, so that homologous peaks could be compared (Sekhon and Carmichael, 1972). For easier evaluation the pyrograms are usually divided into complexes of individual peaks, identifiable by retention time (Haddadin et al., 1973; Reiner and Kubica, 1969; Vincent and Kulik, 1970), and the relative peak heights are computed, based either on the height of a certain peak (Oyama and Carle, 1967; Reiner and Kubica, 1969). Identification of an unknown organism is usually accomplished by superimposing its pyrogram on the pyrogram of a known organism and observing the "unknown"

pyrogram for the presence or absence of peaks at a given retention time and the existence of similarities or differences in the ratios of the major peaks (Oyama and Carle, 1967; Vincent and Kulik, 1970).

Several studies have indicated that the distinction between the pyrograms of different organisms is based on minute differences in protein matter, particularly in the organisms' relative amounts of amino acid residues (Oyama and Carle, 1967; Reiner and Ewing, 1968). Oyama and Carle (1967) in evaluating their results assumed that the largest peaks represented differences in protein content and were the most informative for describing major relationships among organisms. Cross-checks of an organism's identity could be made by examining pyrograms obtained from the organism, after it had been grown in different selective media. Huis In't Veld et al. (1973) found that pyrograms of some bacteria were similar even when the organism had been cultured in four different media. Reiner (1971) concluded that PGLC has been used successfully for identifying bacteria and has enormous future potential.

Analysis of Headspace Vapors by Gas-Liquid Chromatography

Headspace Vapors of Foods

The volatile substances that a food contains are usually complex mixtures of organic compounds which vary in

concentration and volatility (Dimick and Corse, 1956). Volatiles impart distinctive characteristic aromas to foods and often can be used as indicators of food quality.

Direct gas chromatographic analysis of the headspace gases over a food has proven to be an efficient alternative to the existing classical methods for detecting volatile compounds (Fleming et al., 1969; Issenberg, 1971). Due to the fact that sample handling and preparation time are minimized with direct vapor techniques, the variability often encountered in multi-step procedures is eliminated, thus making direct sampling more applicable for quantitative analyses (Issenberg, 1971).

The volatiles of a food could include the metabolic products of various microorganisms growing within that food (Fleming et al., 1969); hence Guarino and Kramer (1969) speculated that analysis of headspace vapors of a food could be a rapid method for microbiologically examining the food. The chromatographic patterns obtained from direct sampling of microbially-produced volatiles in many food products have been used for the characterization and identification of several bacterial species (Bassette et al., 1967; Guarino and Kramer, 1969). Comparative studies of the headspace vapors of milk, which had been inoculated with pure cultures of organisms, have shown that bacteria having different properties produce dissimilar chromatograms; whereas closely related organisms produce similar chromatographic profiles. There was enough variation in these profiles, however, to allow

differentiation of the organisms (Bassette and Claydon, 1965; Bawdon and Bassette, 1966).

Preparation of Milk for Headspace Vapor Analysis

In previous studies of microorganisms in milk by analysis of the headspace vapors, investigators used commercial, homogenized, pasteurized milk, which had been autoclaved and vacuum-distilled (Bassette et al., 1967; Bawdon and Bassette, 1966; Toan et al., 1965). The purpose of autoclaving and distilling the milk was to eliminate any interfering substances that could be detected by a gas chromatograph and to destroy undesirable microorganisms. In addition, after such treatment the milk could be stored at refrigeration temperatures for 3 months without developing volatile materials caused by microbial contamination (Bassette et al., 1967; Bawdon and Bassette, 1966; Toan et al., 1965). Active cultures of test organisms used were maintained in various broths, prior to their inoculation into milk (Bassette et al., 1967; Guarino and Kramer, 1969; Toan et al., 1965). The concentrations of bacteria used to inoculate milk samples ranged from 2,000 cells per milliliter (cells/ml) in one study to 22,000 cells/ml in another (Bassette et al., 1967; Guarino and Kramer, 1969). During these studies the milk was incubated at specific temperatures and analyzed periodically by a direct vapor method (Bassette et al., 1967; Guarino and Kramer, 1969; Toan et al., 1965). Uninoculated milk served as a

control, and the growth of the bacteria in the milk was followed by using standard plating methods on Plate Count Agar (Bassette et al., 1967; Toan et al., 1965).

Use of Gas-Liquid Chromatography (GLC) for Headspace Vapor Analysis

A gas chromatograph that is to be used for examining headspace vapors of foods must be equipped with a detector that is insensitive to water, yet extremely sensitive to other compounds. Flame ionization detectors have been found to be the most suitable type of detection system and have been employed by a majority of the studies using direct vapor sampling (Bassette et al., 1962; Bassette et al., 1967; Buttery and Teranishi, 1961; Marshall, 1971; Palo and Ilkova, 1970).

Most techniques have been altered to improve the sensitivity of detecting volatile compounds and to eliminate conditions that could decrease detection. Too much water vapor can lower the sensitivity of response from volatile compounds (Sundararajan et al., 1968); thus hygroscopic salts such as sodium sulfate are frequently added to samples in order to reduce the amount of water vapor present in the headspace (Bassette et al., 1972; Guarino and Kramer, 1969; Marshall, 1971; Toan et al., 1965). In addition to the use of hygroscopic salts, samples can be heated in a water bath with intermittent shaking, prior to analysis, to help saturate the headspace with the volatiles and to increase their vapor pressure (Guarino and Kramer, 1969; Marshall, 1971).

Marshall (1971) and Kepner et al., (1964) found that alteration of headspace vapors by either of the above conditions changed the true ratio of volatiles present but made detection of volatiles easier.

Identification of Volatile Peaks

Some studies have included identification of the chromatographic peaks obtained by direct vapor analysis (Bassette et al., 1967; Collins, 1971; Loney et al., 1968; Palo, 1971); whereas, other studies have not attempted complete identification, since total identification is not considered necessary for headspace gas sampling to be useful in quality control work (Buttery and Teranishi, 1961). Loney et al., (1968) and Palo (1971) conducted prechromatographic selective reactions on milk samples to distinguish the functional groups of the volatile components. Qualitative reagents were added to the foods to cause a reaction that resulted in the elimination of a peak representative of a specific volatile compound. Bassette et al. (1962), Bassette et al. (1967), Collins (1971), Gordon and Morgan (1972), and Bassette et al. (1963), for example, added hydroxylamine hydrochloride to food samples to remove carbonyls from the headspace gases and to eliminate the carbonyl peak from the chromatogram.

Another means of identification has been the standardization and comparison of the retention times of peaks of unknown compounds with the retention times of known compounds

(Flemming et al., 1969; Palo and Ilkova, 1970; Toan et al., (1965). In order to make accurate positive correlations, the concentrations of the pure standards used for establishing references should be similar to the concentration of the standards within the test product themselves (Bassette and Ward, 1969; Palo and Ilkova, 1970; Toan et al., 1965); and for totally precise identification, standardization should be carried out on more than one type of column packing for example on polar and non-polar stationary phases (Oaks et al., 1964).

If chromatographic peak areas are integrated, the results of headspace sampling could be computer analyzed (Richard et al., 1971; Tassan and Russell, 1974). Richard et al. (1971) predicted that computer programs could be written, which could enter the stored data from integrated analyses and, under computer control, plot simulated chromatograms.

Use of Headspace Vapor Analysis for Quality Control

If constant operating conditions are used, direct gas chromatographic analysis of a food's headspace vapors will yield reproducible results and thus can be valuable in quality control work (Buttery and Teranishi, 1961; Sapers et al., 1970). Several studies comparing the direct injection of liquid solutions with headspace vapor sampling have shown the latter to yield superior results (Bassette et al.,

1962; Gasco, 1969). The direct injection of vapors offers few possibilities of artifact formation, and this technique can be used to measure concentrations below the parts per million range (Kepner et al., 1964).

One limitation of direct analysis headspace vapors is the difficulty of detecting compounds that have high boiling points ("high-boilers"), particularly those present in the headspace in trace quantities. To improve detection, a method of concentrating or enriching the presence of the "high-boilers" in the headspace is necessary (Nawar, 1966; Nawar and Fagerson, 1962).

Volatile Compounds Produced by Bacteria Growing in Milk

The volatile fraction of milk contains several compounds including acetaldehyde, acetone, ethyl alcohol, and methyl sulfide (Jennings et al., 1962; Loney et al., 1968; Palo and Ilkova, 1970). Bassette et al. (1963) found the amount of volatiles present in untreated raw milk would increase during refrigerated storage, probably because of deterioration. Bassette et al. (1967) examined the production of volatile materials in milk by several species of bacteria, including: Escherishia coli, Enterobacter aerogenes, Streptococcus lactis, Streptococcus diacetilactis, Lactobacillus acidophilus, Lactobacillus casei, Achromobacter lypolyticum, and Pseudomonas fragi. The results of the study

were that four principal volatiles (acetaldehyde, ethyl alcohol, diacetyl, and methyl sulfide) were observed in all of the inoculated samples. When members of the family Enterobacteriaceae were used as inocula, the same group of volatiles was detected, but differences in the relative concentrations of the volatiles allowed differentiation of the microorganisms (Guarino and Kramer, 1969). Toan et al., (1965) correlated the growth of E. aerogenes with the production of methyl sulfide odors in milk, but little change was visible in the chromatograms until the E. aerogenes had reached a concentration of at least 10^6 cells/ml. In the work of Bassette et al. (1967) on the production of volatiles by bacteria, it took 1 to 2 days, and at times 4 days, for some organisms to produce detectable quantities of volatile materials. When P. fragi was inoculated into milk, the organisms produced little volatile material detectable by GLC even though the milk had a fruity odor and a high bacterial concentration (Bassette et al., 1967). Guarino and Kramer (1969) concluded from their work using Alcaligenes faecalis and Pseudomonas aeruginosa that chromatograms representing the headspace vapors of non-dextrose fermenting bacteria show little volatile activity.

In summary, the characteristics of psychrotrophic bacteria and the effects that they have on milk have been reviewed, in addition to two gas chromatographic procedures that have been used successfully in recent years for the

identification of microorganisms. Since the present standard method for the microbiological enumeration of psychrotrophs in milk requires at least 10 days before results are obtained, there is a great need for a less time-consuming procedure (APHA, 1972). The ease and simplicity of both the pyrolytic and gas chromatographic techniques make these methods applicable as possible screening devices in a wide variety of areas. Direct headspace analysis by GLC, however, requires less time to obtain results and has been used for examining the volatile components of several food products, indicating its potential for routine monitoring of the microbiological quality of foods.

MATERIALS AND METHODS

Microorganisms

Four microorganisms were used in this investigation. Alcaligenes viscolactis, a psychrotrophic strain of Bacillus pumilus, and Pseudomonas fragi were obtained from the Department of Food Science and Technology, University of California at Davis. Pseudomonas perolens was obtained from the Meats Laboratory, Department of Food Science and Human Nutrition, Michigan State University. The cultures were maintained in Nutrient Broth (Difco Laboratores, Detroit, MI), incubated at 7±1 C, and transferred 2 days prior to their inoculation into milk.

Microbiological Analyses of Milk

All of the milk samples were microbiologically analyzed according to the methods described in the 13th edition of Standard Methods for the Examination of Dairy Products (APHA, 1972). Both the Standard Plate Count (SPC) and the Psychrotrophic Bacteria Count (PBC) were determined; however, for the SPC the plates were incubated at 25±1 C rather than 32±1 C for 2 days. The incubation temperature and time for the PBC was 7±1 C for 10 days. The medium used was Plate

Count Agar (PCA; Difco), and samples were diluted in phosphate buffer.

Preparation of Milk

Raw milk was obtained from the M.S.U. Dairy Plant and cold-sterilized with a hydrogen peroxide (H_2O_2)-catalase treatment (Walker and Harmon, 1965) in order to reduce the number of bacteria that were initially present in the milk. Prior to treatment a sample of the raw milk was removed for microbiological analysis.

According to Roundy (1958), the treatment of milk with H_2O_2 causes the selective destruction of many undesirable bacteria without adversely affecting the milk itself. The efficiency of the H_2O_2 process depends on the bacterial quality of the milk at the time of sterilization, the concentration of H_2O_2 used, the temperature of the milk, and the duration of the treatment (Luck, 1956; Roundy, 1958). Walker and Harmon (1965) found that a 0.05% concentration of H_2O_2 and a temperature of 120 F (48.9 C) were effective bactericidal conditions.

In this study the raw milk was heated in a water bath equipped with a Bronwill Model 20 constant temperature circulator (Bronwill Scientific Co., Rochester, N.Y.) to 48.9 C and treated with 0.05% H_2O_2 for a period of 30 minutes. At the end of the contact time , the milk was cooled to 75 to 100 F (23.9 to 36.7 C). In order to decompose any residual

H_2O_2 remaining in the raw milk, catalase (Nutritional Biochemical Corporation, Cleveland, Ohio) was added. Several studies have found that cooling the milk after the H_2O_2 treatment enables the catalase to function more effectively in breaking down H_2O_2 to water and oxygen (Luck, 1956; Underkofler, 1968; Walker and Harmon, 1965). According to Roundy (1958), the addition of excess catalase is not harmful; therefore, three to four times the theoretical amount of catalase needed to destroy the H_2O_2, diluted with five times its volume of cold water was used.

To ensure the catalase had decomposed all of the residual H_2O_2, a few drops of freshly-prepared 30% potassium iodide and 2% soluble starch solutions were added to samples of both treated and untreated milk, and the resulting colors were compared. Identical colors in the treated and untreated were an indication of complete H_2O_2 destruction (Roundy, 1968). At the end of the cold-sterilization treatment the SPC and PBC were determined, as above, to evaluate the effectiveness of the treatment in reducing the initial bacterial concentration of the milk. The raw milk was then dispensed into flasks and inoculated with the test organisms.

The pasteurized milk used in this study was also obtained from the M.S.U. Dairy Plant, but it was not treated with the H_2O_2-catalase system, since the initial bacterial counts were low. The pasteurized milk was directly dispensed into flasks and inoculated with bacteria.

Growth Studies

Two days prior to inoculation into milk, 48-hour cultures of the organisms in Nutrient Broth (Difco) were plated on PCA (Difco) to determine the approximate concentration of each culture. Using these plating results as a basis, appropriate dilutions of the cultures were made in phosphate buffer, and finally a 1:100 dilution was inoculated into milk to obtain the desired concentration of bacteria. An equal volume of uninoculated milk was used as a control sample, and all milk samples were incubated at 7±1 C after inoculation. During incubation the growth of bacteria in the control and the growth of organisms in the inoculated sample were followed by plating the milk to determine the SPC and PBC.

Gas Chromatograph

A Series 5750B Research Gas Chromatograph (Hewlett-Packard/F&M Scientific Division, Avondale, PA) equipped with a Moseley 7128A Dual-Pen Strip Chart Recorder (Hewlett-Packard/F&M Scientific Division) was used for all analyses. The detector was a flame ionization detector.

Analysis of Milk by Pyrolysis-Gas-Liquid Chromatography (PGLC)

Column

The column used was 10 feet long with a 1/8 inch outside diameter (OD), made of stainless steel, and prepacked with 100/110 mesh Anakrom ABS coated with 5% Carbowax 20M, treated with terephthalic acid (Supelco, Inc., Bellefonte, PA). The column was conditioned for 72 hours at 200 C prior to use and was reconditioned between samples for 1 hour and overnight at 200 C.

Operating Parameters

A Model 80 Pyrolysis Unit (Hewlett-Packard/F&M Scientific Division) was used for the analyses. The operating parameters of the pyrolysis unit and gas chromatograph were as follows:

Gases: Helium (carrier) -- 60 psi; 50ml/minute flow rate through the column
Hydrogen -- 10 psi; 30 ml/minute
Air -- 33 psi; 500 ml/minute

Temperatures:
Pyrolysis -- 800 C for 12 seconds
Column -- 80 to 200 C; held at 80 C for 3 minutes; programmed at 4 C/minute rise; held at 200 C for 4 minutes.
Injection port -- 250 C
Detector -- 275 C

Attenuation and Sensitivity -- minimum setting of 8×10^1
Chart Speed -- 0.25 inch per minute

Preparation and Pyrolysis of Milk

Only raw milk was used in the pyrolysis study, and the cultures which were inoculated into the milk were A. viscolactis, a psychrotrophic strain of B. pumilus, and P. fragi. Prior to inoculation into milk, the cultures were grown for 24 hours at 7±1 C in H_2O_2-treated milk. One-tenth milliter aliquots of appropriately diluted 24-hour cultures were used to inoculate 99.9 ml volumes of H_2O_2-treated milk. The uninoculated control and the inoculated milk were incubated at 7±1 C, and samples for analysis were taken at 0, 2, 4, 6, and 8 days. A portion of each sample was used to determine the SPC and PBC, and 5 ml aliquots were frozen at -22 C and later freeze-dried on a VirTis Lyophilozer (The VirTis Co., Inc., Gardiner, N.Y.). The lyophilized samples were stored at 4 C.

For PGLC analysis a 0.9 mg sample of lyophilized milk was carefully placed in the jaw end of a pyrolysis probe. The probe was fitted into the injection part of the gas chromatograph via an adaptor and connected to the Model 80 Pyrolysis Unit, and the sample was automatically pyrolyzed.

Analysis of the Headspace Vapors of Milk by Gas-Liquid Chromatography (GLC)

Columns

Two types of column packings were used. One column was 6 feet long with a 1/8 inch OD, made of stainless steel,

and prepacked by Supelco, Inc. with 80/100 mesh acid-washed Chromosorb W coated with 10% Carbowax 10M. The second column had the same dimensions but was packed with 80/100 mesh Gas Chrom Z coated with 4% Apiezon L. These columns will henceforth be referred to as Carbowax 20M and Apiezon L, respectively.

The columns were conditioned for 72 hours at 200 C prior to the first injection of samples and were reconditioned between injections for 30 minutes and overnight at 200 C.

Operating Parameters

The operating parameters of the gas chromatograph for headspace vapor analyses were as follows:

Gases: Helium (carrier) -- 60 psi; 40 ml/minute flow rate through each column

Hydrogen -- 10 psi; 30 ml/minute

Air -- 33 psi; 500 ml/minute

Temperatures:

Column -- 70 to 150 C; held at 50 C for 3 minutes; programmed at 8 C/minute rise

Injection port -- 185 C

Detector -- 190 C

Attenuation and Sensitivity -- minimum setting of 16x1

Chart speed -- 1 inch per minute for the first 3 minutes; then 1/2 inch per minute.

Preparation of Samples and Standards

Vial Method: Two milliliters of milk were removed from the incubated milk samples at the time of each analysis. The 2-ml sample was put into a screw-capped serum vial (7-ml volume) containing 1.2 g of anhydrous sodium sulfate. The vial was sealed and heated in a 60±1 C Metabolyte water bath (New Brunswick Scientific Co., New Brunswick, N.J.) for 15 minutes with constant shaking. The depth of water in the bath was slightly above the level of milk in the vial. At the end of the heating period, the screw-cap of the vial was replaced with a teflon-faced rubber septum.

A sample of the headspace vapors was obtained from the vial with a Hamilton 1002-LTN gas-tight syringe (Anspec Co., Ann Arbor, MI). The vapors were drawn into the barrel of the syringe, evacuated into the vial, and refilled three times before a 1-ml sample was removed. The syringe was immediately injected into the gas chromatograph.

The syringe was cleaned between injections by means of an air aspirator which pulled air through the syringe. This cleaning method eliminated the possibility of any compounds remaining in the needle and interfering with the next analysis (Bassette and Glendenning, 1968).

Flask Method: An alternative method of preparing milk for headspace vapor analysis involved the addition of 36 ml of the incubated milk to a 50-ml Erlenmeyer flask containing 21±0.2 g of anhydrous sodium sulfate. The flask

was sealed with a #2 rubber stopper having a small hole in it. The barrel assembly of a Pressure-Lok series B gas syringe (Precision Sampling Corp., Baton Rouge, LA) was fitted into the hole of the rubber stopper. The flask and syringe were then heated in a water bath, using the same conditions as in the vial method.

After the heating period the vapors in the headspace of the flask were drawn into the syringe and evacuated into the flask three times prior to withdrawing the sample. A 2.5-ml sample of the vapors was locked into the barrel, and the syringe was removed from the flask and fitted with a 23-gauge x 2-inch luer-lok needle. The syringe needle was immediately injected into the gas chromatograph, and the syringe was unlocked to release the vapors.

External standardization of the gas chromatograph: To account for minor changes in instrument sensitivity, an external standard was examined under the same operating conditions used to examine the milk. On each day that samples were analyzed, the headspace vapors of a freshly prepared 1 part per million (ppm) acetone solution were analyzed before and after all the milk samples. A standard response for acetone was established, and the peak heights of the chromatograms were standardized by the following equation:

$$\frac{\text{standard acetone response}}{\text{actual daily response}_{\text{1 ppm acetone}}} \times \text{observed component peak height} = \text{corrected component peak height}$$

The peak heights obtained by analyzing standard compounds were also corrected with the above equation.

Interpretation of Results

In an attempt to tentatively identify some of the peaks that appeared on the chromatograms obtained by examining the headspace vapors of milk, various standard compounds were analyzed by a similar direct vapor method on both the Apiezon L and Carbowax 20M columns to establish their retention times. The standards and the concentration ranges tested were: acetaldehyde, 50 to 1,000 parts per billion (ppb); acetone, 50 to 1,000 ppb; ethanol, 1 to 50 ppm; acetic acid, 1 to 50 ppm; and lactic acid, 0.01 to 85%. Presumptive identification of an unknown volatile was made when the peak of the unknown had the same retention time as one of the standard solutions on both the Carbowax 20M and Apiezon L columns. To eliminate fluctuations in retention times due to experimental or instrumental variation, the retention time of each peak was adjusted to be the time each compound was retained by the stationary phase of the column after the appearance of the air peak (Macleod, 1973). In this study, all peaks will be described in terms of adjusted retention times (t'R).

Interpretation of the data was based on visual examinations; the chromatograms and pyrograms of the control milk sample were compared with those of the inoculated milk

samples by placing one curve on top of the other and illuminating them from behind, so that both curves could be seen at once. The retention times of the peaks were determined; and the chromatograms and pyrograms were examined for similarities in the patterns produced by the peaks, for differences in the heights of the peaks, and for differences due to the absence of existing peaks or the presence of additional peaks.

RESULTS

Growth of Bacteria in Milk Samples

Initial Standard Plate Counts (SPC) in the raw milk obtained from the M.S.U. Dairy Plant ranged from 7.0×10^3 to 3.0×10^4 cells/ml. After treatment of the milk with H_2O_2 and catalase, the bacterial populations ranged from 1.0×10^2 to 1.2×10^3 cells/ml. The Psychrotrophic Bacteria Counts (PBC) for the raw milk were approximately 10^2 cells/ml before H_2O_2-catalase treatment and 10^1 cells/ml after treatment. Table 1 shows representative counts (SPC and PBC) of raw milk both before and after treatment with H_2O_2 and catalase.

Table 1. Plate counts (cells/ml) of raw milk, before and after treatment with H_2O_2 and catalase.

Sample	Before	After
(a) SPC		
1	1.4×10^4	2.3×10^2 Est[a]
2	2.7×10^4	3.0×10^2 Est[a]
3	1.4×10^4	1.6×10^2 Est[a]
(b) PBC		
1	1.1×10^3	1.5×10^1 Est[a]
2	7.2×10^3	1.0×10^1 Est[a]
3	6.7×10^2	1.0×10^1 Est[a]

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

The plating records for each study done are presented in the Appendix (Tables 3-10). Serial dilutions of pure cultures were made into milk to obtain the desired levels of bacteria. In the various studies the initial PBC for each species ranged as follows: A. viscolactis, 45 to 20,000 cells/ml; B. pumilus, 75 to 3,000 cells/ml; P. fragi, 65 to 20,000 cells/ml; and P. perolens, 60 to 2,700 cells/ml. The PBC of the control milk samples ranged from 10 to 1,600 cells/ml.

Analysis of Milk by Pyrolysis-Gas-Liquid Chromatography

Variations in the Operating Parameters of PGLC

In preliminary experiments with raw milk, the operating parameters of PGLC were varied in an attempt to determine the conditions that would give the best results. Pyrolysis temperatures that have been used successfully for the identification of microorganisms have varied from 770 to 900 C (Huis In't Veld et al., 1973; Stack, 1967). The pyrograms obtained when samples of milk were pyrolyzed at both 800 and 1000 C were compared, and 800 C was chosen for normal use since this temperature consistently gave better results. The column temperature was programmed to rise from 80 to 200 C at 4 C/minute. Other rates ranging from 2 to 8 C/minute were tried; rates faster than 4 C/minute tended to miss or "mask" some of the pyrolytic peaks, while 2 C/minute

was impractical in terms of time required for analysis.

Sample sizes were restricted to 0.5 to 1.0 mg (dry weight) due to the requirements of the Model 80 Pyrolysis Unit, and a 0.9 mg sample was selected as the size for all analyses. In pyrolytic studies concerning identification of bacteria (Reiner and Ewing, 1968), the sample size was normally much smaller, e.g., 0.1 to 0.3 mg.

Analysis of Raw Milk by PGLC

Raw milk was used for all of the pyrolytic studies. Prior to inoculation with bacteria, the raw milk was treated with H_2O_2 and catalase to reduce the number of bacteria initially present. To determine the effects this treatment had on the pyrolytic profiles produced by milk, lyophilized samples of both untreated and treated milk were pyrolyzed. The similarity of the resulting two pyrograms was an indication that the peaks that appeared were fragments produced by the pyrolysis of the milk itself.

The general pyrolytic pattern of the controls varied considerably. In analyses using one "Carbowax 20M" column, as many as seven peaks appeared throughout the entire programmed temperature range from 80 to 200 C; in subsequent experiments using other Carbowax 20M columns, presumably containing the same packing, the pyrograms of the control had only three main peaks at 80, 175, and 195 C, respectively. The discrepancy of results obtained, when different columns

containing the same packing were used, will be explained in the discussion section of this text.

The pyrograms of raw milk inoculated with A. viscolactis, B. pumilus, or P. fragi contained the same number of peaks as the corresponding control, and except for slight differences in the heights of these peaks, the pyrograms showed little change from the control. Figure 1 shows the pyrograms obtained from the PGLC analysis of a control sample and a milk sample inoculated with P. fragi. Both samples had been incubated at 7±1 C for 48 hours, and the PBC of the inoculated sample was 1.4×10^7 cells/ml.

Analysis of Headspace Vapors by Gas-Liquid Chromatography (GLC)

Headspace Analysis of Cultures

Examination of the headspace vapors of Nutrient Broth cultures of A. viscolactis, B. pumilus, P. fragi, and P. perolens, by the same method used for milk samples, failed to produce chromatograms showing evidence of volatile compounds. This was an indication that any peaks appearing on the chromatograms of the milk samples could be attributed to the volatile components of milk or by-products produced in the milk by microbial metabolism.

Figure 1. Pyrolytic profiles of raw milk incubated for 48 hours at 7±1 C and analyzed by PGLC on Carbowax 20M column: (a) Control, (b) *P. fragi*.

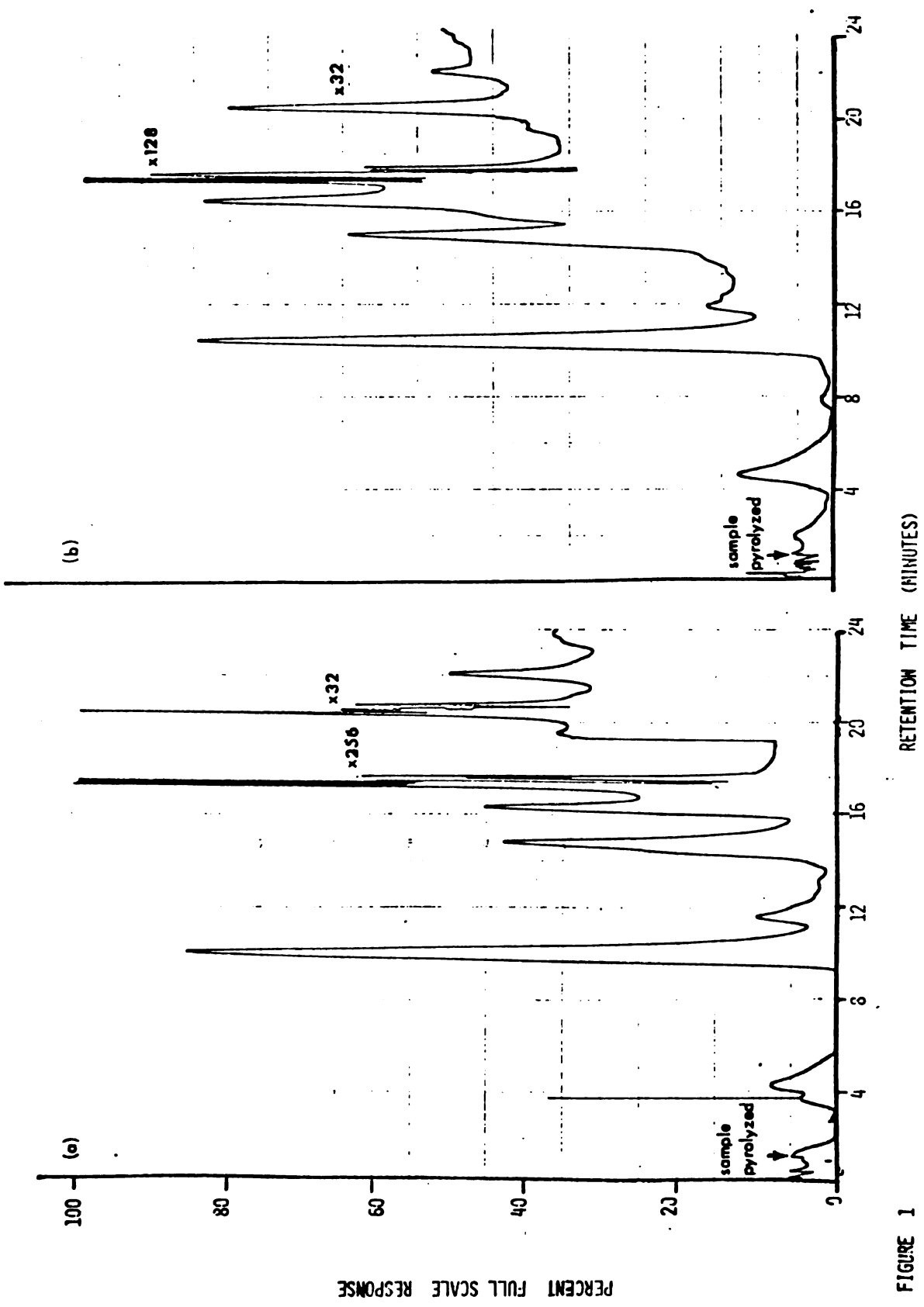
(a)
(b)
x256
x32
x128
x32
sample pyrolyzed
sample pyrolyzed
PERCENT FULL SCALE RESPONSE
RETENTION TIME (MINUTES)

FIGURE 1

Headspace Analysis of Standard Solutions

The adjusted retention times of various standard compounds analyzed on the Carbowax 20M and Apiezon L columns are presented in Table 2.

Table 2. Retention times of standard compounds analyzed by headspace vapor analysis on Carbowax 20M and Apiezon L columns.

Standard Compound	Retention Time (seconds)	
	Apiezon L	Carbowax 20M
Acetaldehyde	10[a]	15[a]
Acetone	14	30
Acetic Acid	26	70
Ethanol	30	40
Lactic Acid	20	15

[a]Time retained by stationary phase after appearance of air peak.

Analysis of the Headspace Vapors of Milk

Samples of the headspace vapors of uninoculated milk and milk that had been inoculated with psychrotrophic bacteria were analyzed by GLC at various intervals on both the Apiezon L and Carbowax 20M columns. The chromatogram of each inoculated sample was then visually compared with the corresponding control chromatogram.

Five peaks were generally produced by the growth of A. viscolactis, B. pumilus, P. fragi, and P. perolens in

milk. Peaks II, III, and IV were identified as being acetaldehyde, acetone, and ethanol. Peak I was not identified, and peak V was believed to be acetic acid. The retention time for peak V tended to fluctuate, so that this peak did not always emerge at the same time in different analyses.

Analysis of Pasteurized Milk: Figures 2, 3, and 4 compare the chromatograms of the control and of milk inoculated with A. viscolactis obtained by analysis on the Carbowax 20M column, and the chromatograms of the control and of milk inoculated with P. fragi obtained by analysis on the Apeizon L column. Figure 2 shows the chromatograms after inoculation, and Figures 3 and 4 show the chromatograms after incubation of the milk for varying lengths of time.

1. Control: Initially, only one peak was produced by the control sample on both columns. This peak was presumptively identified as acetone, since its t'R was identical to the t'R of a standard acetone solution. The acetone peak was labeled in the chromatograms as peak III. On the Apiezon L column acetone was the only volatile to appear throughout each study, and on the Carbowax 20M column the acetone peak was not the only peak present but it was always the tallest peak. In the control sample the acetone peak was generally higher than the corresponding peak in the inoculated milk samples. After 1 week, another peak having a t'R of 15 seconds began to appear on the chromatograms obtained from analysis on the Carbowax 20M column. This peak was identified as acetaldehyde and labeled II.

Figure 2. Gas chromatograms of the headspace vapors of pasteurized milk analyzed by GLC, initial analysis (0 day): on Carbowax 20M column (a) control, (b) A. viscolactis; and on Apiezon L column (c) Control, (d) P. fragi.

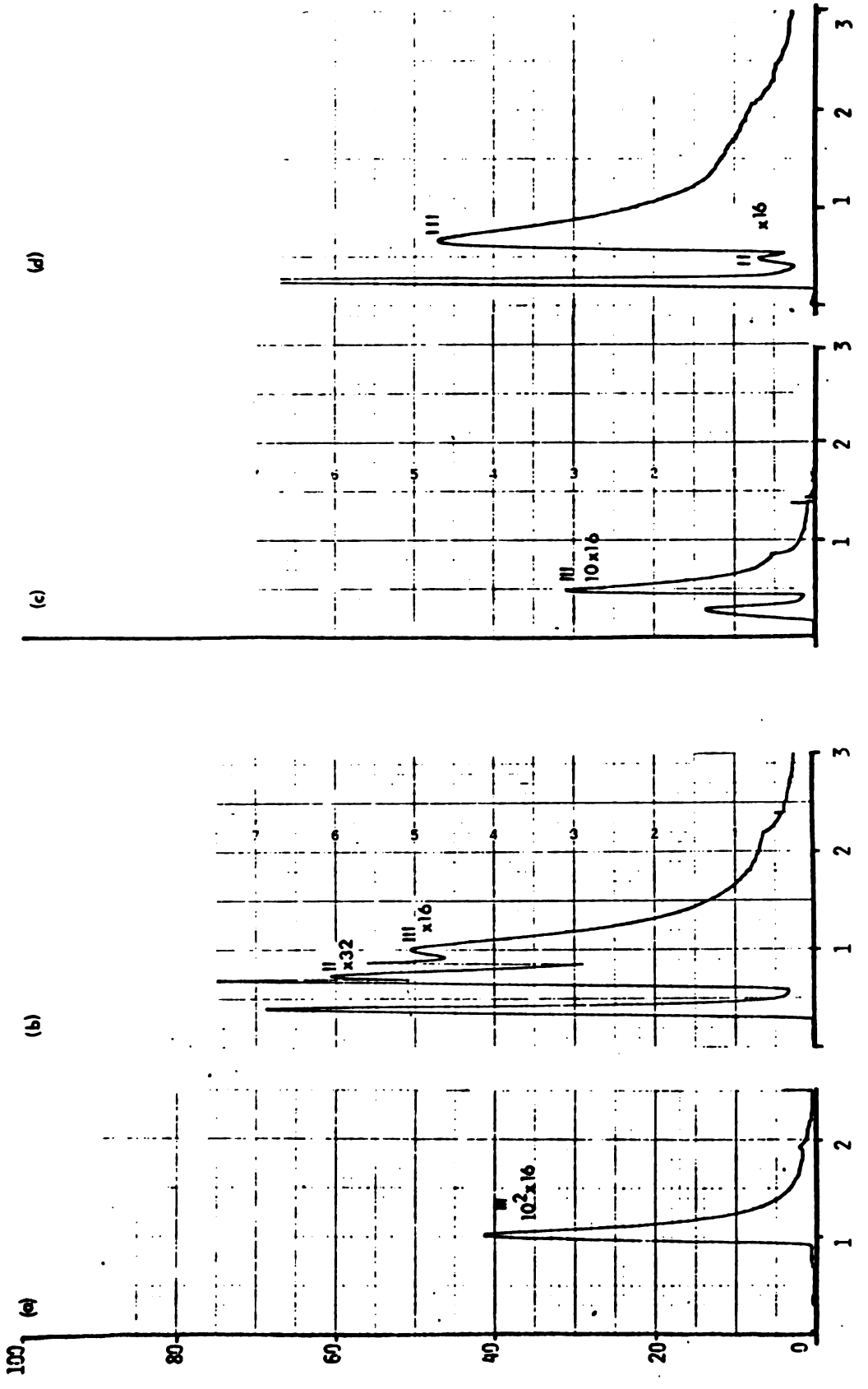
PERCENT FULL SCALE RESPONSE
100
80
60
40
20
0
(a)
(b)
(c)
(d)
10^2x16
II
III
x32
x16
10x16
x16
RETENTION TIME (MINUTES)

FIGURE 2

Figure 3. Gas chromatograms of the headspace vapors of pasteurized milk analyzed by GLC, analysis at 4 days: on Carbowax 20M column (a) Control, (b) A. viscolactis; and on Apiezon L column (c) Control, (d) P. fragi.

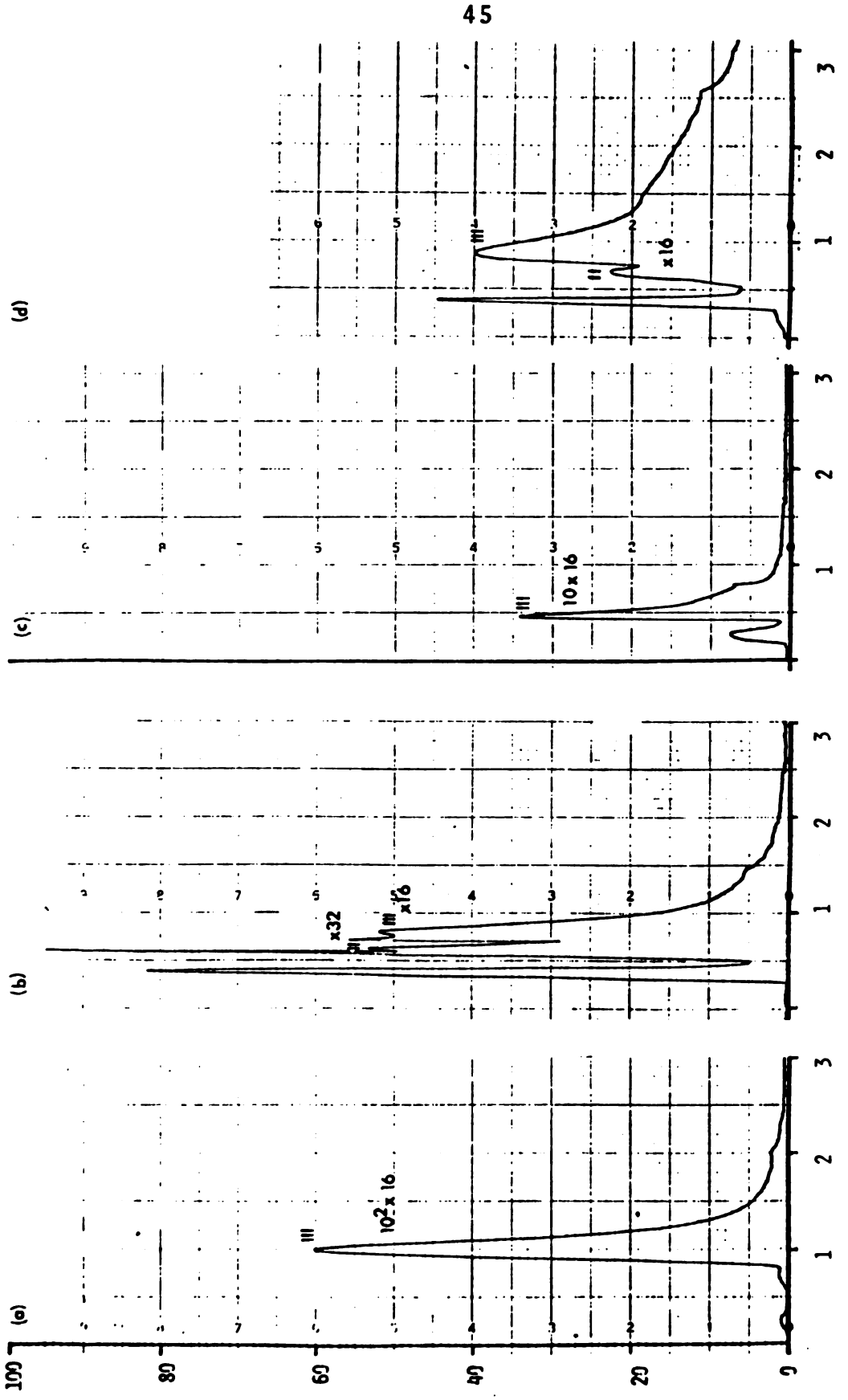
(a)
(b)
(c)
(d)
100
80
60
40
20
0
PERCENT FULL SCALE RESPONSE
1
2
3
RETENTION TIME (MINUTES)
III
II
I
10² x 16
x32
x16
10 x 16
x16

FIGURE 3

Figure 4. Gas chromatograms of the headspace vapors of pasteurized milk analyzed by GLC, analysis at: 8 days on Carbowax 20M column (a) Control, (b) A. viscolactis; and 6 days on Apiezon L column (c) Control, (d) P. fragi.

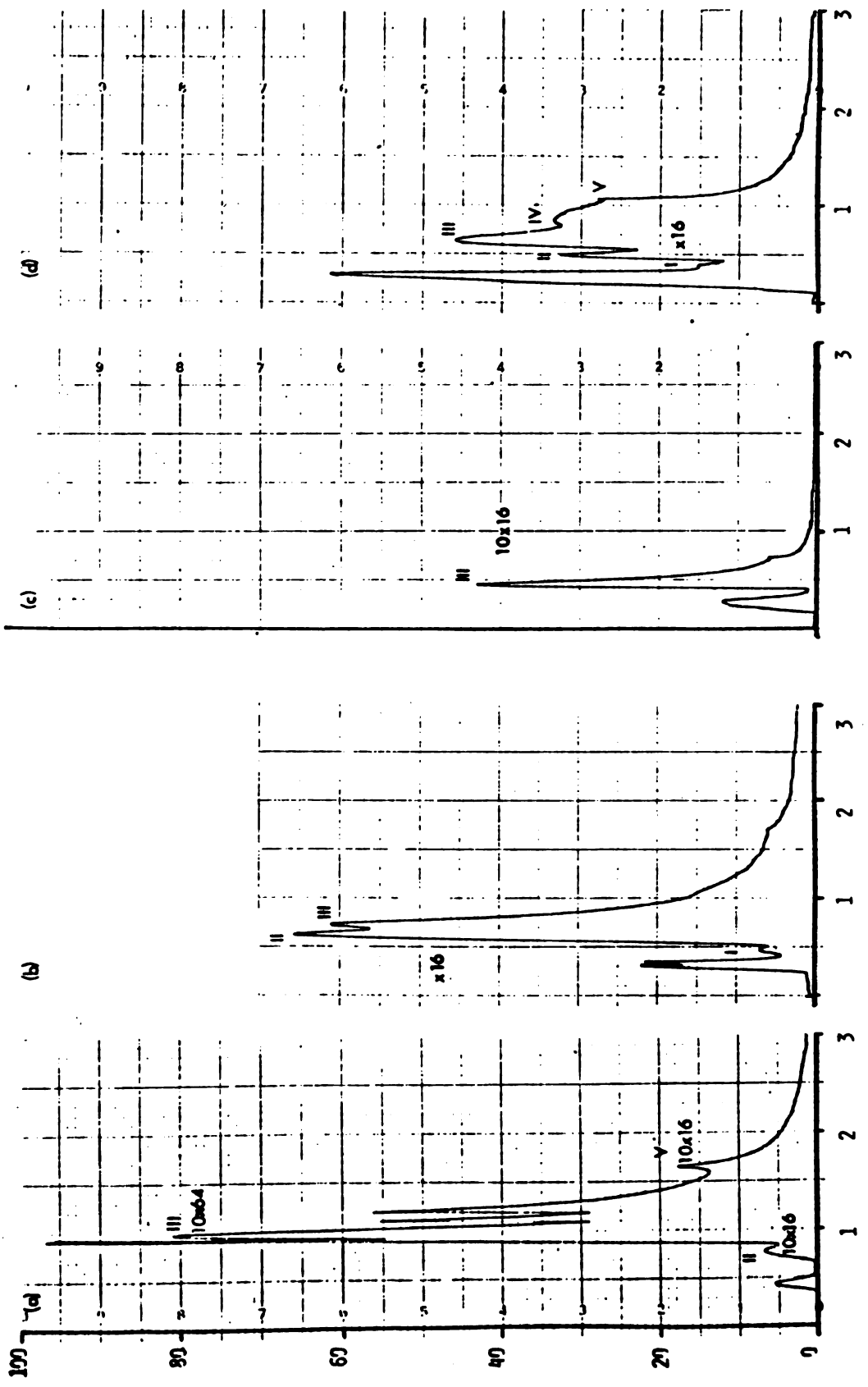
PERCENT FULL SCALE RESPONSE
RETENTION TIME (MINUTES)
(a)
(b)
(c)
(d)
II
III
IV
V
10x16
x16

FIGURE 4

2. Pasteurized milk inoculated with P. fragi: The headspace vapors of milk that had just been inoculated with P. fragi only produced one peak when analyzed on the Carbowax 20M column. This peak had the same t'R as the acetone peak (III) of the control milk and increased in height until the second day; thereafter, it declined (Figure 5). The pattern of the inoculated milk remained similar to that of the control until 3 days after inoculation, at which time the acetone peak began to form a doublet peak. By the fourth day the pasteurized milk contained over 10^7 cells/ml of P. fragi. The first apex of the doublet peak was presumptively identified as acetaldehyde (peak II) due to its t'R of 15 to 18 seconds. The second apex of the doublet represented acetone, and acetaldehyde and acetone were equal in height. After 5 to 6 days, two new small peaks appeared on the chromatograms; the first peak (peak I), which increased with time, was not identified. The second new peak was labeled V and, by comparison of retention time and the shape of the peak, seemed similar to peaks produced by the standard acetic acid solutions. Peak V had a distinct narrow shape. In some instances a small peak appeared in addition to peaks I, II, III, and V, after the milk was incubated approximately one week. This peak (IV) had a t'R similar to ethanol.

On the chromatograms produced by analysis on the Apiezon L column, a difference from the control was noticed on the second day due to the addition of peaks II (acetaldehyde)

Figure 5. Headspace volatiles in pasteurized milk inoculated with P. fragi and incubated at 7±1 C for 0, 2, 5, and 8 days as determined by GLC analysis on Carbowax 20M and Apiezon L columns. Legend: I, unidentified; II, acetaldehyde; III, acetone; IV, ethanol; V, unidentified. ▭, Carbowax 20M; ▨, Apiezon L; ▩, both columns.

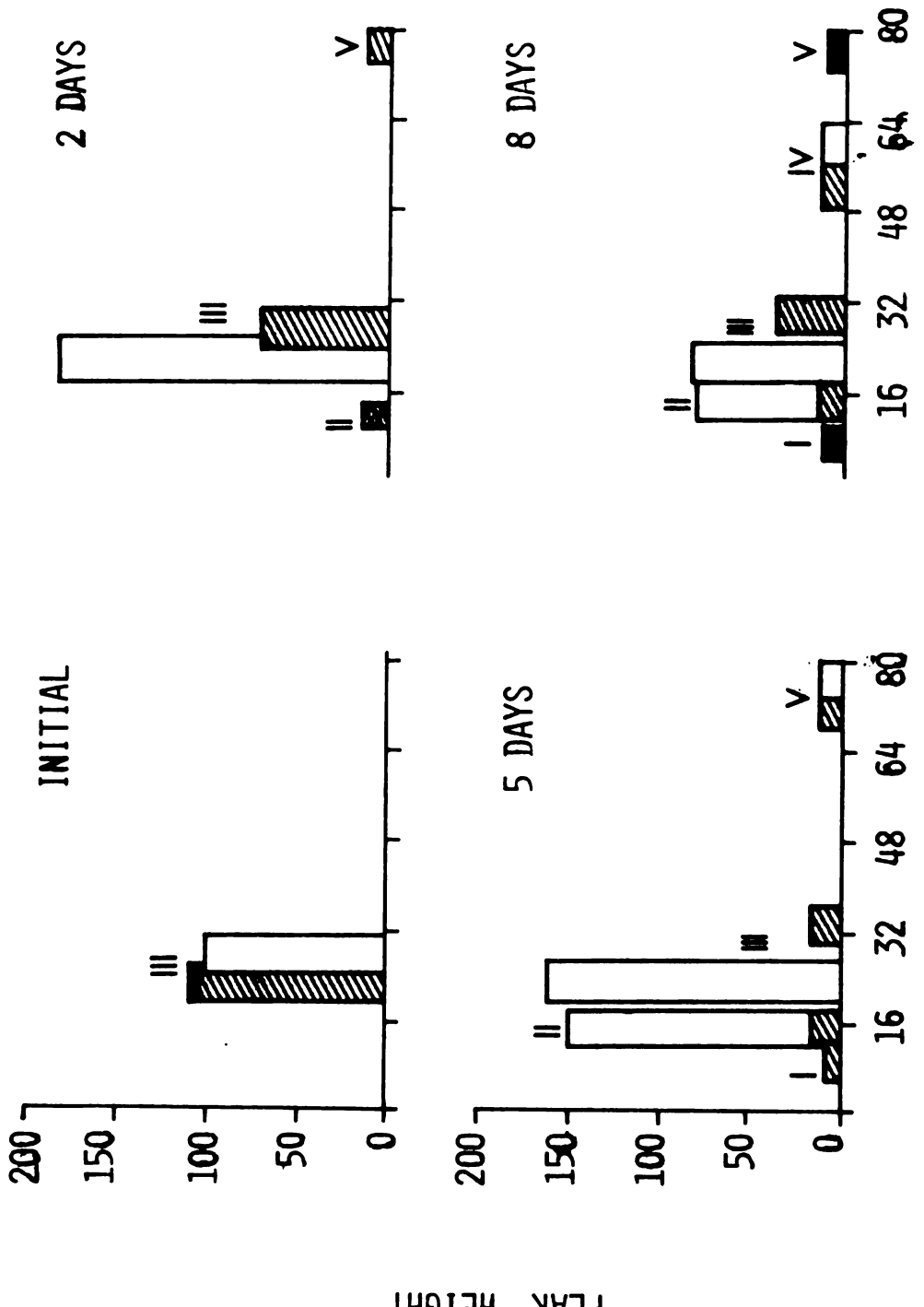
INITIAL
2 DAYS
5 DAYS
8 DAYS
PEAK HEIGHT
200
150
100
50
0
I
II
III
IV
V
16
32
48
64
80
ADJUSTED RETENTION TIME (SECONDS)

FIGURE 5

and V. Acetone, present in the milk initially, decreased with time. The appearance of additional peaks and the pattern that all of the peaks produced was similar to that discussed for the Carbowax 20M column.

3. Pasteurized milk inoculated with P. perolens: Initial analyses of the headspace vapors of milk inoculated with P. perolens on the Carbowax 20M column produced the acetaldehyde (II) and acetone peaks (III); whereas, the control produced only the acetone peak. The concentration of acetone increased through the second day and then began to decrease (Figure 6). On the second day peak V appeared, and on the fourth day peak I appeared; both peaks increased as the milk was incubated. After 5 days, there was a large decrease in acetaldehyde concurrent with the appearance of ethanol (peak IV) on day 6. By 15 days all of the peaks increased substantially, and in some studies acetaldehyde and acetone emerged as one peak.

On the chromatograms representing analysis on the Apiezon L column, differences between the control and inoculated milk were not detected until the second day, when peak I, the acetaldehyde peak, appeared on the chromatograms of the inoculated milk (Figure 6). By this time the milk contained over 10^5 cells/ml of P. perolens. Other changes that occurred were similar to the changes and patterns observed on the Carbowax 20M column.

Figure 6. Headspace volatiles in pasteurized milk inoculated with P. perolens and incubated at 7±1 C for 0, 4, 6, and 8 days as determined by GLC analysis on Carbowax 20M and Apiezon L columns. Legend: I, unidentified; II, acetaldehyde; III, acetone; IV, ethanol; V. unidentified. ▭, Carbowax 20M; ▨, Apiezon L; ▩, both columns.

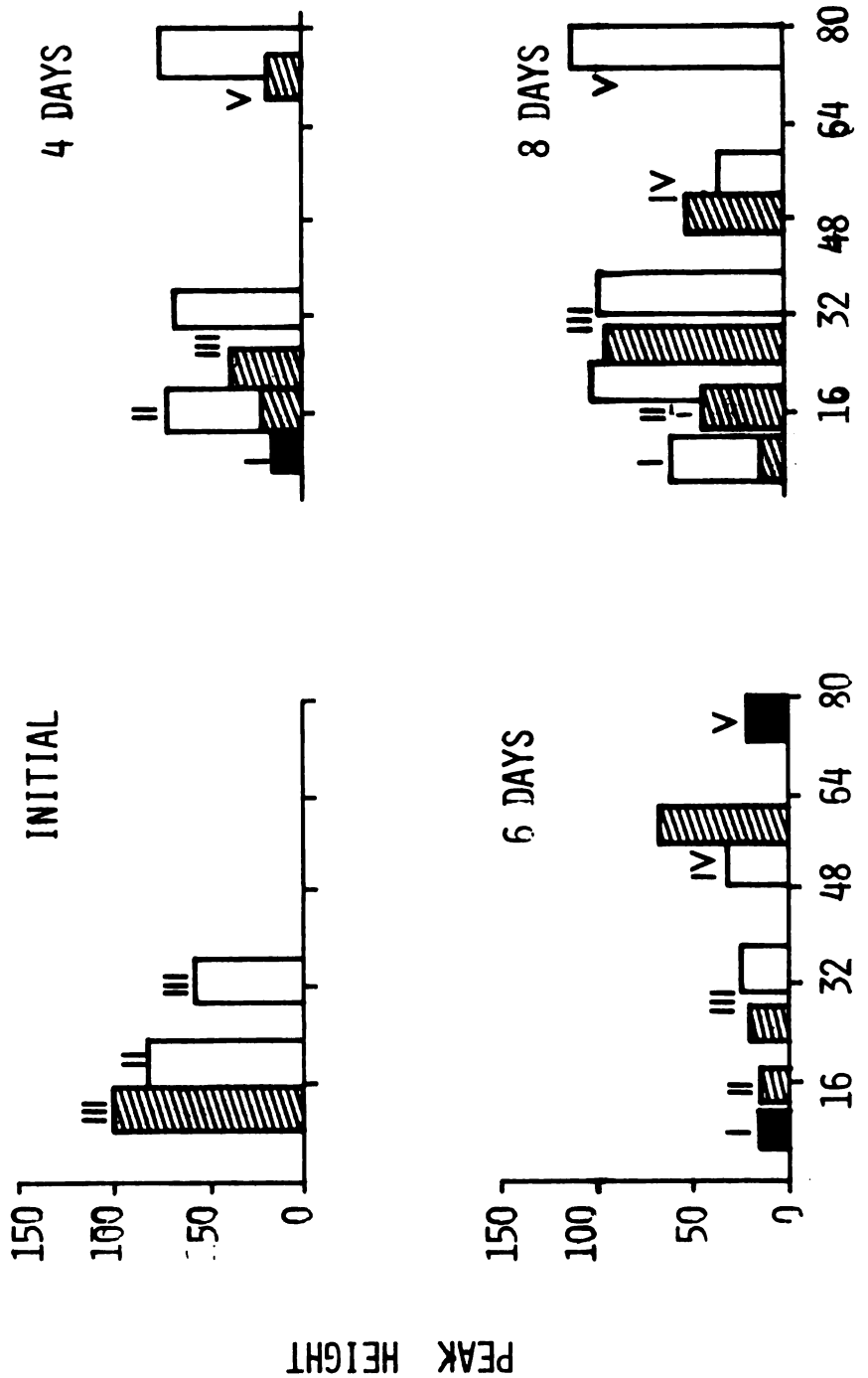
INITIAL
4 DAYS
6 DAYS
8 DAYS
PEAK HEIGHT
150
100
50
0
I
II
III
IV
V
16
32
48
64
80
ADJUSTED RETENTION TIME (SECONDS)

FIGURE 6

4. Pasteurized milk inoculated with B. pumilus: Initially, the headspace gases of pasteurized milk inoculated with B. pumilus produced a chromatographic pattern on the Carbowax 20M column similar to the uninoculated control, i.e., only acetone appeared. The growth of B. pumilus in milk caused the concentration of acetone to increase through the first few days of incubation. After 2 days, acetaldehyde appeared in the headspace profile of the milk as part of a doublet peak in combination with acetone; both the acetaldehyde and acetone decreased until the milk had been incubated for at least 1 week. Peak V, assumed to be acetic acid, also appeared after 2 days and increased slightly. Peak I was first visible after 5 days, and peak IV, ethanol, was apparent after 6 to 7 days. The appearance of ethanol occurred during the time that acetaldehyde was decreasing in concentration (Figure 7).

Analysis with the Apiezon L column yielded no discernible differences between the control and inoculated milk in the number of peaks until the second day of incubation, when there were approximately 6.0×10^5 cells/ml of B. pumilus. Like the acetone peak, the acetaldehyde peak, which appeared on the second day, slowly decreased; whereas, peak V, also visible after the second day, did not change significantly. No ethanol was detected using the Apiezon L column, even though it was detected using the Carbowax 20 M column.

Figure 7. Headspace volatiles in pasteurized milk inoculated with B. pumilus and incubated at 7+1 C for 0, 2, 5, and 7 days as determined by GLC analysis in Carbowax 20M and Apiezon L columns. Legend: I, unidentified; II, acetaldehyde; III, acetone; IV, ethanol; V, unidentified. ▭ , Carbowax 20M; ▨, Apiezon L.

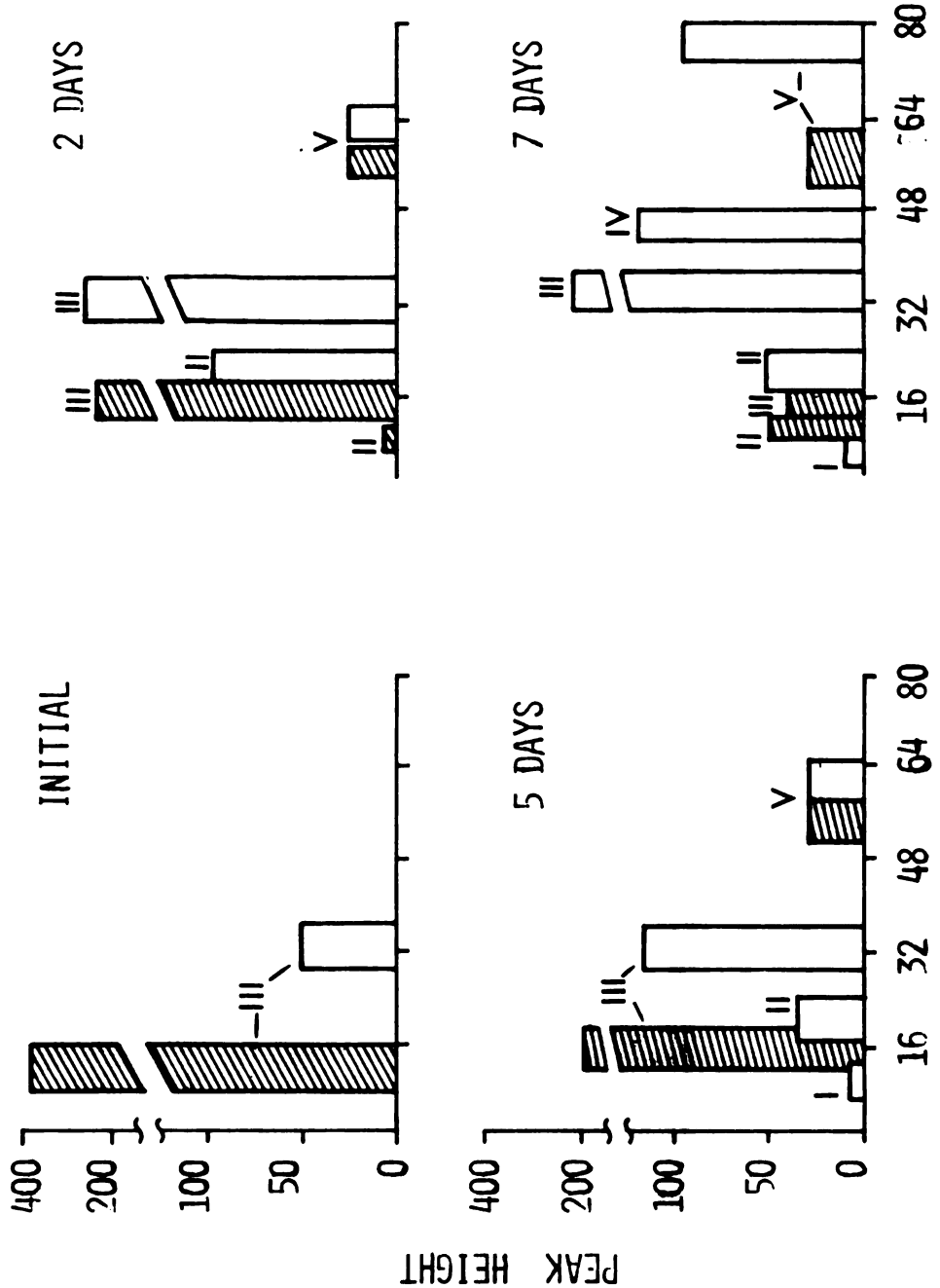
INITIAL
2 DAYS
5 DAYS
7 DAYS
PEAK HEIGHT
400
200
100
50
0
I
II
III
IV
V
16
32
48
64
80
ADJUSTED RETENTION TIME (SECONDS)

FIGURE 7

5. Pasteurized milk inoculated with A. viscolactis: The initial chromatogram produced by analysis of milk inoculated with A. viscolactis on the Carbowax 20M column was similar to the chromatogram of the control, since only the acetone peak was present. In one study, however, acetaldehyde was also present initially and in comparison to acetone, the acetaldehyde peak was higher, indicating the presence of a substantial amount of acetaldehyde. In the other studies that were done with pasteurized milk that had been inoculated with A. viscolactis, acetaldehyde did not appear until at least 3 days after inoculation (Figure 8). After the milk was incubated for approximately 5 days, peak III began to have 2 apexes. Comparison of retention times with standard solutions indicated that the first apex represented acetaldehyde, and the second apex, acetone. The formation of this doublet peak was more distinct with milk inoculated with A. viscolactis than with any other culture used in this study. Prior to the formation of the doublet peak and during the first few days of incubation, acetone was beginning to show a decrease. After the milk had been incubated for 1 week, both acetaldehyde and acetone showed an increase in peak height. Peak V was detected after the second or third day but changed only slightly in peak height.

Direct vapor analysis using the Apiezon L column showed no difference between the control sample and the pasteurized milk inoculated with A. viscolactis, until the

Figure 8. Headspace volatiles in pasteurized milk inoculated with A. viscolactis and incubated at 7±1 C for 0, 2, 5, and 8 days as determined by GLC analysis on Carbowax 20M and Apiezon L columns. Legend: I, unidentified; II, acetaldehyde; III, acetone; V, unidentified. ▭, Carbowax 20M; ▨, Apiezon L.

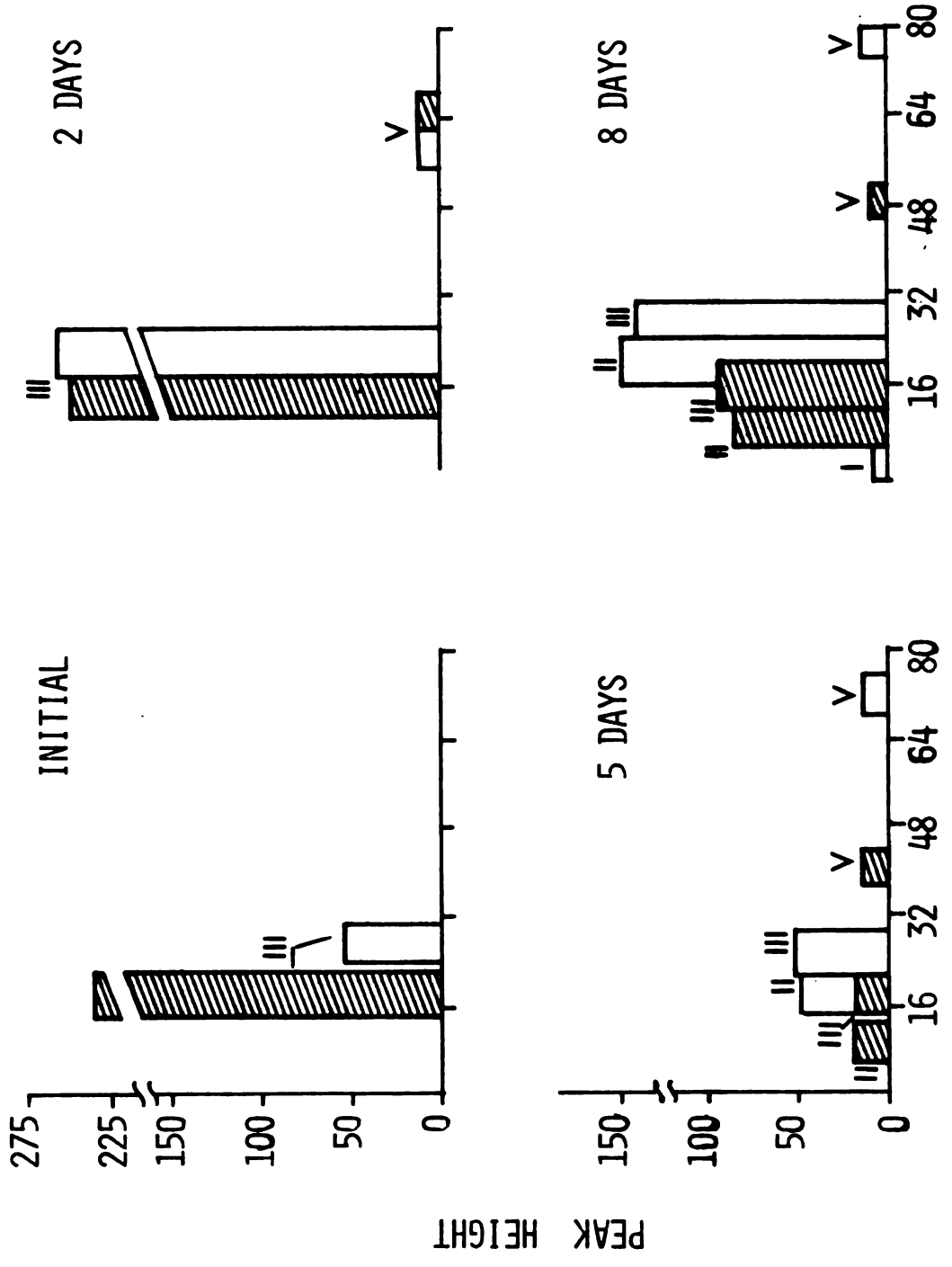
INITIAL
2 DAYS
5 DAYS
8 DAYS
PEAK HEIGHT
275
225
150
100
50
0
16
32
48
64
80
ADJUSTED RETENTION TIME (SECONDS)

FIGURE 8

milk had been incubated for 2 days and had over 10^6 cells/ml. Acetaldehyde and peak V appeared on the chromatograms in addition to the acetone peak which was present initially. Upon analysis by both the Apiezon L and Carbowax 20M columns, acetaldehyde and acetone similarly formed a distinct doublet peak. Peak I was not detected using the Carbowax 20M column, but it did appear on the chromatograms produced using the Apiezon L column. Peak V remained at approximately the same height in analyses on the Apiezon L column, and the shape of the peak was not as distinct as that produced by analysis on the Carbowax 20M column.

Analysis of raw milk: The chromatograms produced by analyzing the headspace vapors of raw milk inoculated with psychrotrophic bacteria were very similar to the results obtained with the raw milk control. The chromatograms in Figures 9 and 10 are, therefore, representative of all raw milk samples obtained on the Carbowax 20M and Apiezon L columns.

1. Control: The headspace vapors of the uninoculated raw milk control, analyzed on the Carbowax 20M column, always contained three peaks. The peaks were labeled on the chromatograms as II, III, and V. Peaks II and III were identified by comparison with standard solutions as being acetaldehyde and acetone, respectively. Comparison of peak V by retention time and by the shape of the peak, with standard solutions indicated that it possibly was acetic acid. The acetone peak

Figure 9. Gas chromatograms of the headspace vapors of raw milk analyzed by GLC, initial analysis (0 day): on Carbowax 20M column (a) Control, (b) P. perolens; and on Apiezon L column (c) Control, (d) B. pumilus

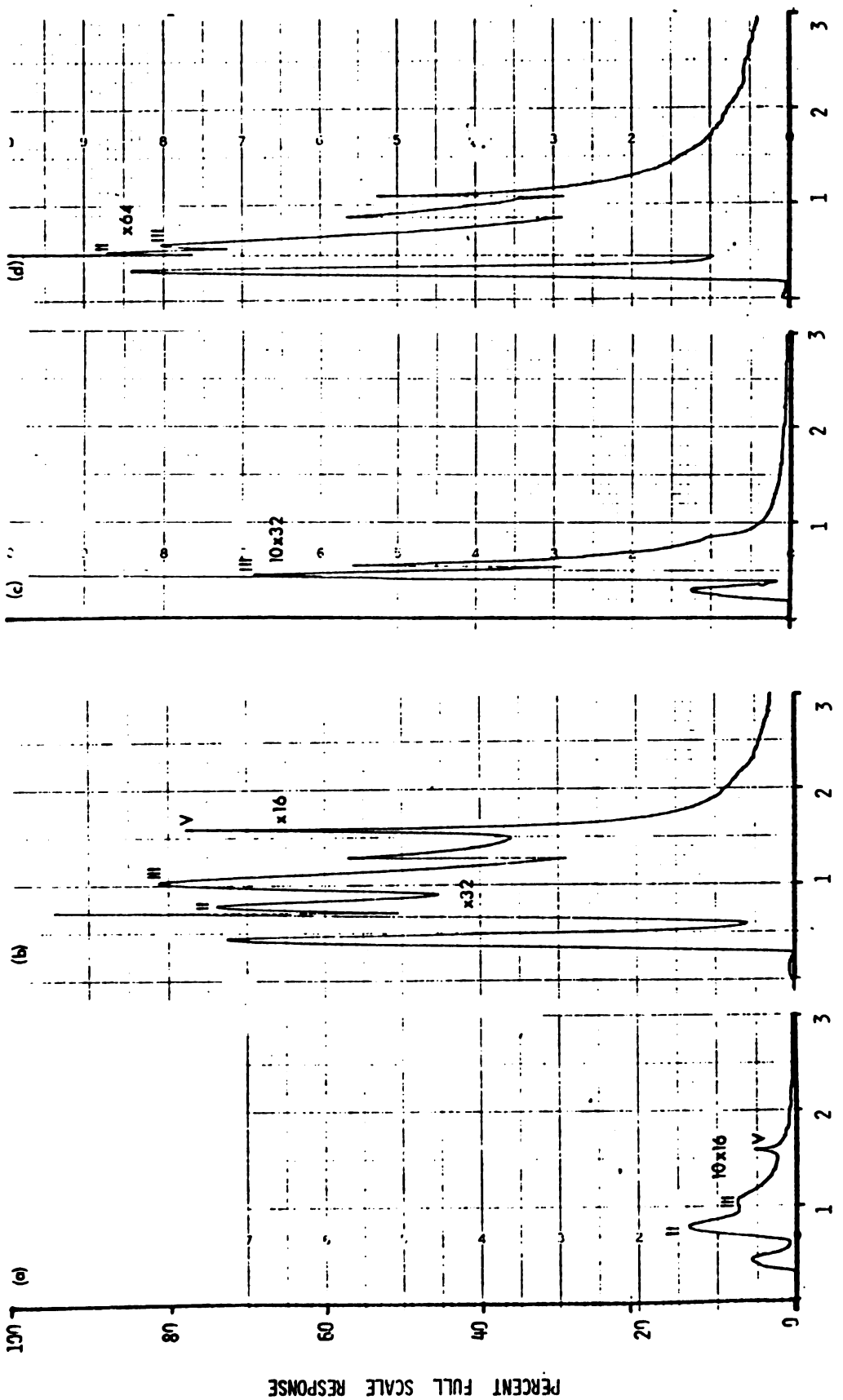
PERCENT FULL SCALE RESPONSE
100
80
60
40
20
0
(a)
II
III
10x16
V
(b)
II
x32
III
V
x16
(c)
III'
10x32
(d)
II
x64
III
RETENTION TIME (MINUTES)
1
2
3

FIGURE 9

Figure 10. Gas chromatograms of the headspace vapors of raw milk analyzed by GLC, analysis at: 2 days on Carbowax 20M column (a) Control, (b) P. perolens; and 5 days on Apiezon L column (c) Control, (d) B. pumilus.

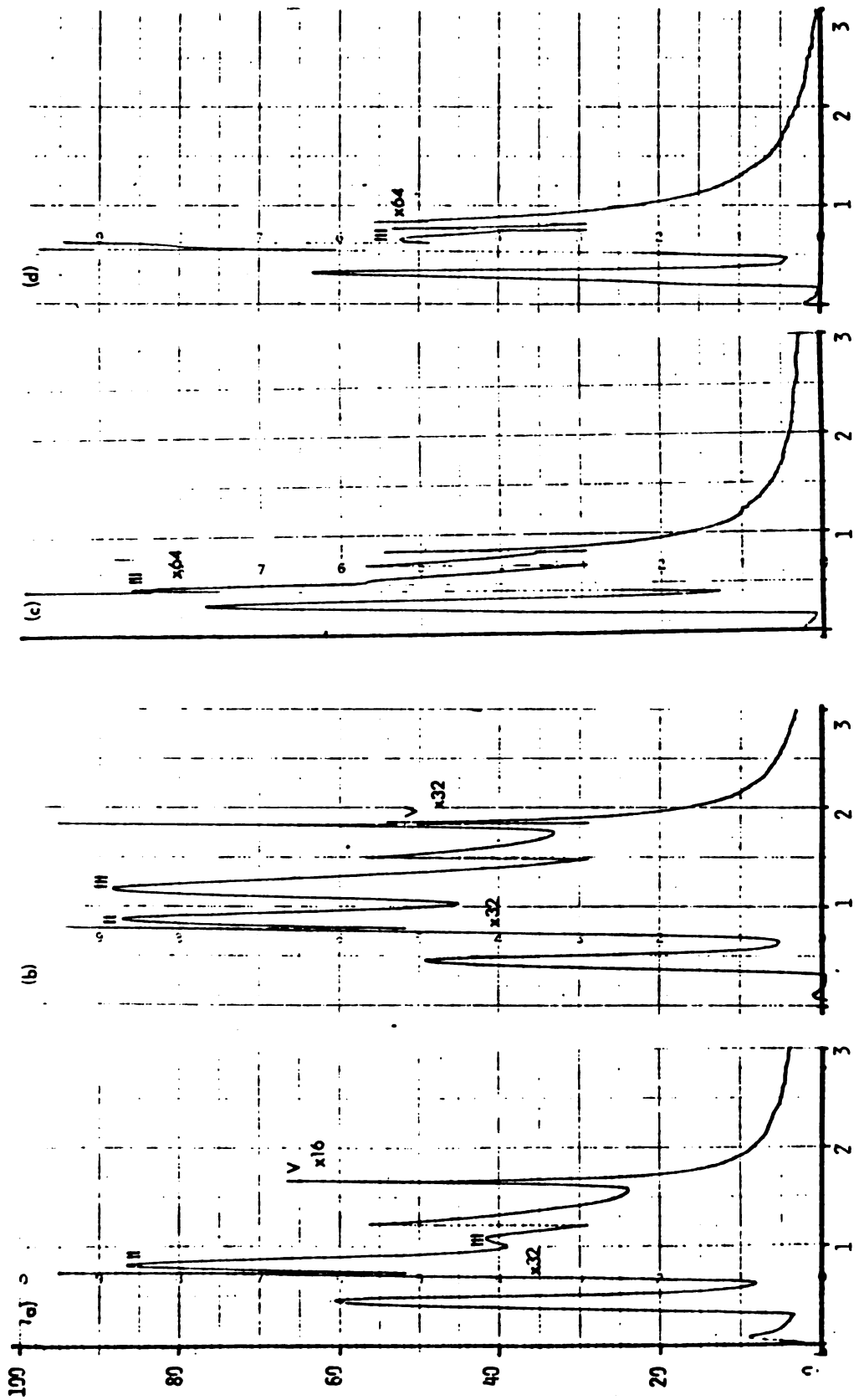
PERCENT FULL SCALE RESPONSE
RETENTION TIME (MINUTES)
(a)
(b)
(c)
(d)
x16
x32
x64

FIGURE 10

increased until the second day and then began to decline; whereas, the acetaldehyde peak generally showed slight increases in height. Peak V remained at the same height for 2 days, showed a large increase on the third day, and then gradually decreased.

Only one large peak was produced by analysis of the headspace vapors of the control milk on the Apiezon L column throughout the duration of each study. Due to the proximity of retention times of standard acetaldehyde and acetone solutions on Apiezon L columns, it was speculated that acetaldehyde and acetone were being detected simultaneously by the gas chromatograph, thus accounting for the appearance of one large peak.

2. Raw milk inoculated with psychrotrophic bacteria: Little change or difference was detected between the chromatograms of the inoculated milk samples and the control. Figures 11 and 12 are bar graphs showing the chromatographic patterns of milk inoculated with B. pumilus and P. fragi on the day of inoculation and at two other times during the incubation of the milk. Since the four organisms examined had only slight variations in volatiles produced in raw milk, the results for each species will be discussed as a group.

Analysis using the Carbowax 20M column yielded the same group of volatiles in both the control and inoculated milk, but there was a difference in the height of the peaks. When the plate counts of the control milk were analagous to

Figure 11. Headspace volatiles in raw milk inoculated with B. pumilus and incubated at 7±1 C for 0, 1, and 5 days as determined by GLC analysis on Carbowax 20M and Apiezon L columns. Legend: I, unidentified; II, acetaldehyde; III, acetone; V, unidentified. ▭ , Carbowax 20M; ▨ , Apiezon L.

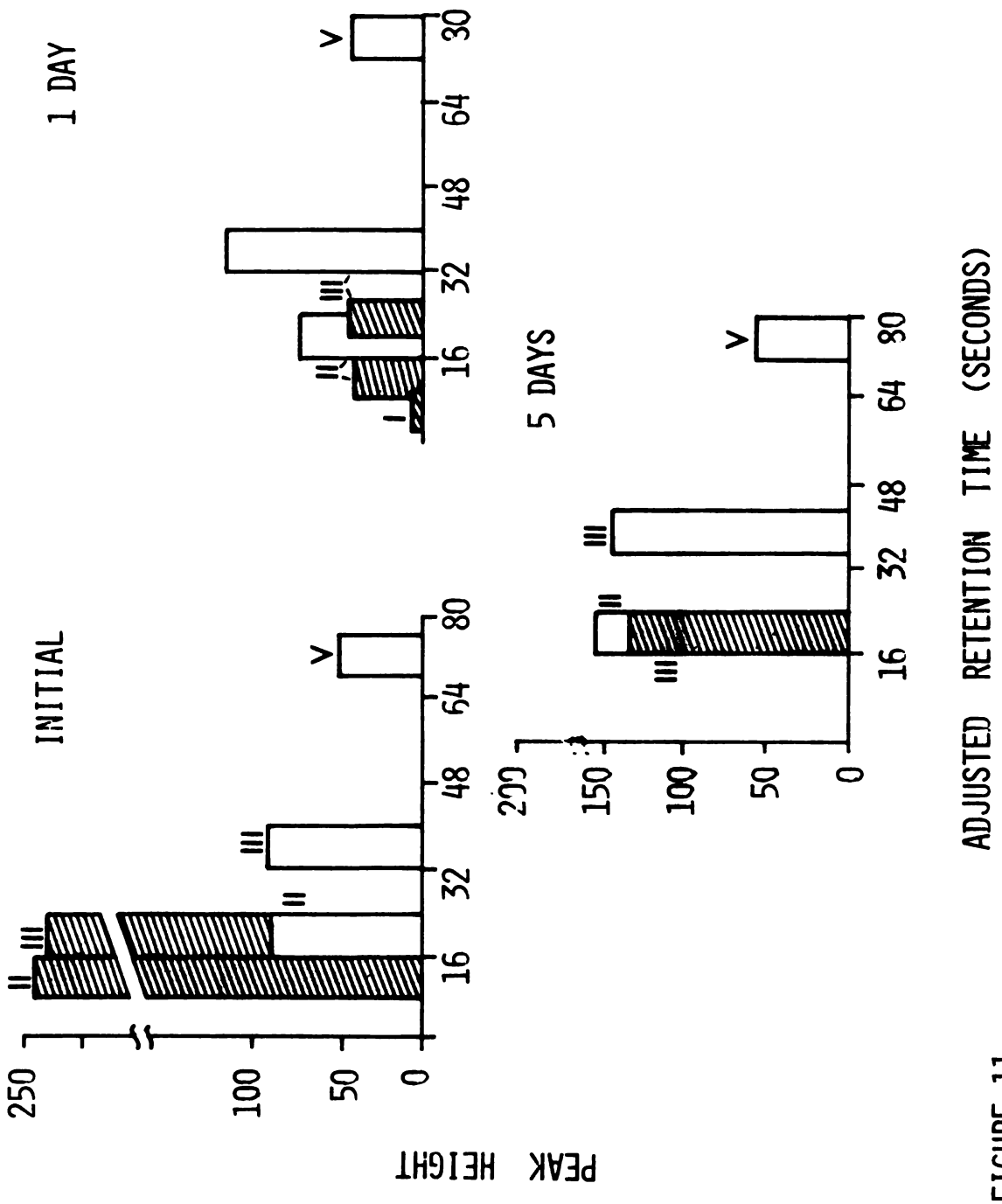
INITIAL
1 DAY
5 DAYS
PEAK HEIGHT
ADJUSTED RETENTION TIME (SECONDS)
250
100
50
0
200
150
100
50
0
16
32
48
64
80
I
II
III
V

FIGURE 11

Figure 12. Headspace volatiles in raw milk inoculated with P. fragi and incubated at 7+1 C for 0, 1, and 5 days as determined by GLC analysis on Carbowax 20M and Apiezon L columns. Legend: II, acetaldehyde; III, acetone; V, unidentified.
, Carbowax 20M; , Apiezon L.

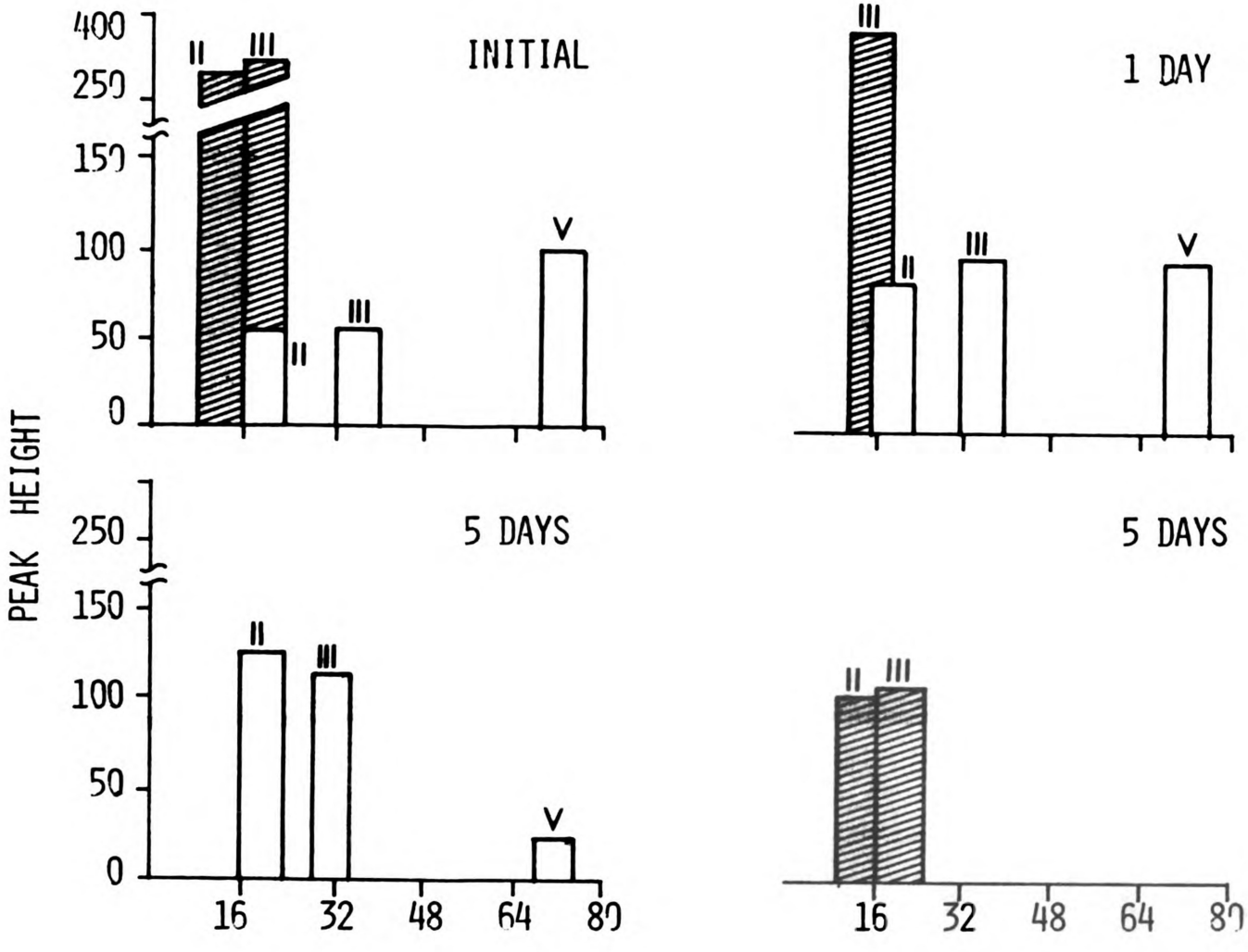
400
250
150
100
50
0
II
III
V
INITIAL
1 DAY
5 DAYS
5 DAYS
PEAK HEIGHT
16
32
48
64
80
ADJUSTED RETENTION TIME (SECONDS)

FIGURE 12

the plate counts of the inoculated milk, the heights of the peaks were also similar. In one study for example, when the PBC of the control was 10 cells/ml and the PBC of milk inoculated with A. viscolactis was 45 cells/ml, the heights of the acetaldehyde and acetone peaks on the chromatograms of each sample were equal. The concentrations of the volatiles changed at approximately the same time in all inoculated raw milk samples; for example, acetone always increased during the first and second days. After 1 week of incubation of the milk, the concentration of acetone in the control decreased; whereas, the concentrations of acetone in the inoculated milk samples showed large increases. By this time, visual spoilage of the milk was more evident in the inoculated samples than in the control.

The acetaldehyde peak (II) of the control tended at times to be two to three times higher than the acetone peak III), but in all of the inoculated samples, except for milk containing A. viscolactis, acetaldehyde and acetone were similar or equal in height. The ratio of peak II to peak III in milk inoculated with A. viscolactis was the same as the ratio in the control.

Analysis of the headspace vapors of raw milk on the Apiezon L column did produce a difference between the control and inoculated samples. Buth acetaldehyde and acetone (peaks II and III) appeared together as a doublet peak in the chromatograms of the inoculated milk; only acetone was present in

the control. The two peaks in the chromatograms of milk inoculated with A. viscolactis and B. pumilus decreased with time, similar to the acetone peak of the control; with P. fragi and P. perolens the two peaks showed an increase during the first 2 days. In some analyses of both raw and pasteurized milk on the Apiezon L column, one peak would occasionally be produced on a chromatogram where two peaks had been produced before; e.g., for raw milk inoculated with P. fragi and analyzed on the Apiezon L column (Figure 12), initially and after 5 days peaks II and III were visible on the chromatogram but on the second day only peak III appeared.

DISCUSSION

Growth of Bacteria in Milk Samples

All milk samples were plated to determine both the SPC and PBC. In this study the incubation temperature for the SPC test was 25±1 C (APHA, 1972). According to Witter (1961), in order to obtain maximum bacterial counts after storage of milk at refrigeration temperatures, the temperature of incumation should be lowered because of the growth of psychrotrophic bacteria. As the data in Table 1 and in the Appendix (Tables 3-10) show, in most samples analyzed in this study, the SPC was slightly higher than the PBC.

The SPC and PBC of the controls were low initially and remained low and insignificant throughout each study in comparison to the inoculated samples (Appendix, Tables 3 to 10). Generally, 1 week was required for the control sample to attain counts comparable to those of the inoculated samples. In pasteurized milk that had been inoculated with psychrotrophic bacteria, the fastest growth occurred within the first 48 hours, during which time there was as much as a 100-fold increase in bacterial concentration.

The lag phase of bacteria inoculated into pasteurized milk was very short in comparison to the lag phase in raw milk. Figure 13 which represents a characteristic growth curve for

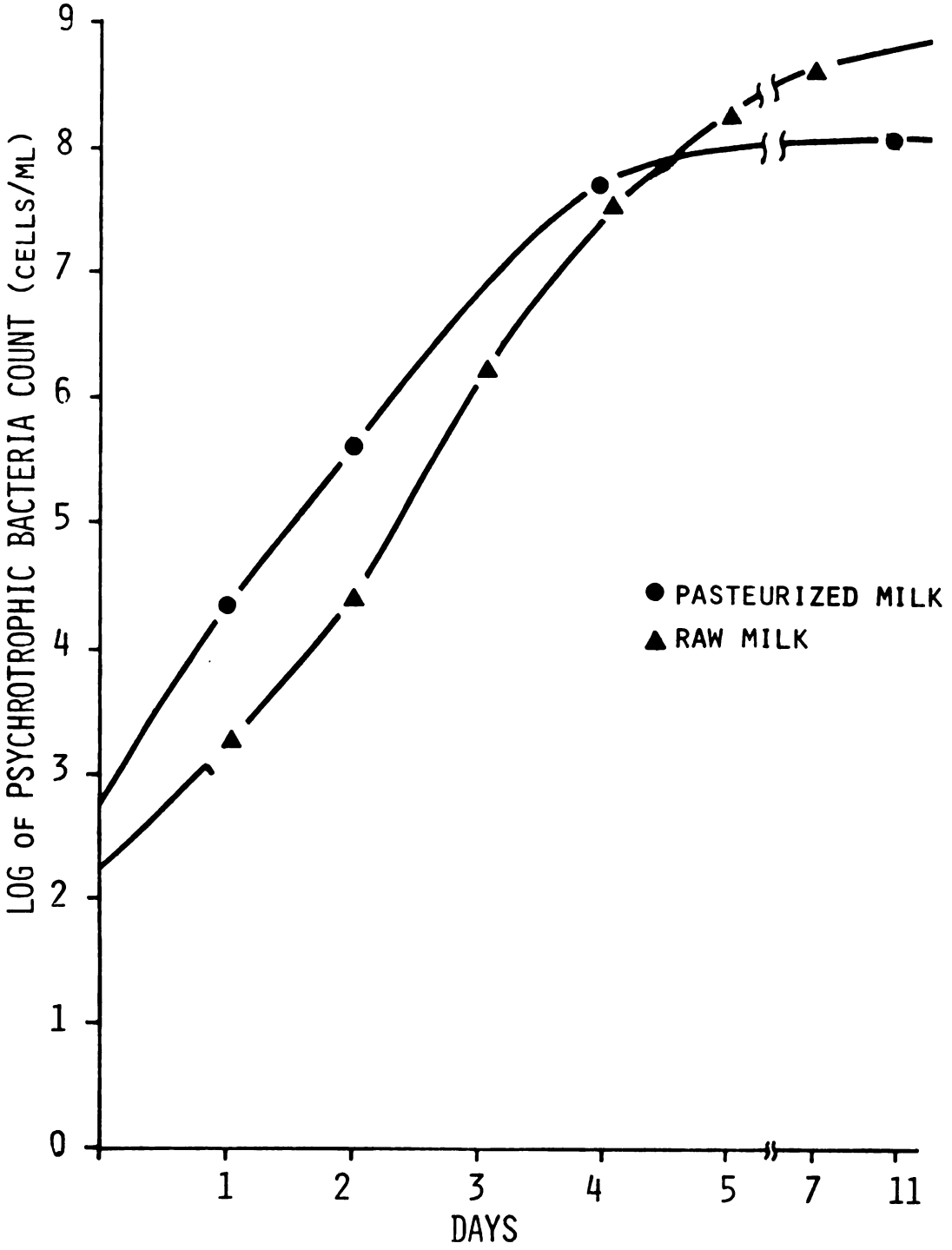

FIGURE 13 - GROWTH OF PSYCHROTROPHIC BACTERIA IN RAW MILK AND PASTEURIZED MILK INCUBATED AT 7±1 C

inoculated milk samples, shows the difference in growth rates between inoculated raw and pasteurized milk samples. Psychrotrophic bacteria grow slowly when fresh raw milk is first placed at low temperatures, but later the increase in growth is rapid (Hammer and Babel, 1957). In addition, psychrotrophs that have been heat-treated are more fastidious in their nutrient requirements for initiating growth and have a more pronounced lag phase (Witter, 1961). The raw milk used in this study was heated, just prior to inoculation, during the H_2O_2 treatment.

From day 2 to day 4 there was approximately a 10-fold increase per day in the number of bacteria in pasteurized milk. After 4 days the rate of all growth declined markedly, and by 13 days the populations of many samples began to decrease. The fastest growth rate for the cultures inoculated into raw milk was between 2 and 4 days, and after 5 days the bacteria entered the stationary phase of their growth cycle. Figure 13 shows that after 5 days the increase in cell numbers in both raw and pasteurized milk was small. According to Stanier *et al.* (1970), if an accumulation of toxic materials is the factor limiting growth, slight increases in total count can still occur in the stationary phase due to differences in the susceptibility of the bacterial cells. This situation is especially common when bacteria are grown in a rich medium.

Analysis of Milk by Pyrolysis-Gas-Liquid Chromatography (PGLC)

The main disadvantage of using PGLC to detect the presence of psychrotrophic bacteria in milk was the inability to reproduce results. The lack of reproducibility, in this study, was caused in part by variations of the column packing. Due to the buildup of sample residue on the column packing, column performance was acceptable for only a short period of time. In one study, after approximately 3 weeks of use the retention times of the peaks were observed to increase significantly. In addition, the baseline of the recorder began to "spike" and could not be stabilized at the conditions used. When new columns of identical packing were used, the pyrograms of the control and inoculated samples of milk were markedly different than those obtained in the previous experiments. The pyrograms obtained using the new columns contained fewer peaks. The cause(s) of this difference could not be determined by the analyst or the manufacturer of the prepacked column.

While general trends could sometimes be established (e.g., pyrograms of milk inoculated with high concentrations of bacteria, at times, tended to show additional peaks), the lack of reproducibility invalidated reasonable use of the data. Previous studies that have used PGLC for identification and differentiation of microorganisms have cited reproducibility as one of the major advantages of the method (Reiner and

Kubica, 1969). In this study even replicate analyses of the same sample, in succession, gave very different pyrolytic profiles.

Another disadvantage of using PGLC to detect the growth of psychrotrophs in milk was the high concentration of bacteria needed before any consistent differences could be noted. Only when the concentration of bacteria exceeded 10^7 cells/ml were differences in the pyrograms evident. Table 2 in the Appendix contains data on the growth studies of representative milk samples. According to Reiner (1971), PGLC gives qualitative and quantitative analytical data that can be used as screening devices in a wide variety of areas, particularly in quality control operations. While pyrolytic techniques can be employed as reproducible methods in microbial studies when pure samples of bacteria are used (Cone and Lechowich, 1969; Haddadin et al., 1973; Reiner and Kubica, 1969; Reiner et al., 1969; Reiner et al., 1971; Vincent and Kulik, 1970), the concentrations of interfering constituents from milk itself apparently make PGLC impractical for the analysis of microorganisms in milk. For each sample, the large concentration of milk constituents that are pyrolyzed seems to mask any detection of pyrolytic fragments of bacteria or bacterial products.

Reiner stated (in a letter dated July 18, 1973; Department of HEW, Center for Disease Control, Atlanta, GA) that PGLC has been used successfully with pure cultures of

organisms, and that it would most likely be diffcult to use PGLC to obtain useful information on mixed cultures, unless the method was being used to detect a specific compound. All psychrotrophs do not undergo the same metabolic processes in milk; therefore, it would be difficult to use pyrolytic tehniques to detect a single by-product, produced in sufficient concentrations by all organisms, that could serve as an indication of the growth of psychrotrophic bacteria in milk.

Analysis of Headspace Vapors by Gas-Liquid Chromatography (GLC)

Gas chromatographic analysis of the headspace vapors of milk has been used to identify microorganisms in milk (Bawdon and Bassette, 1966; Guarino and Kramer, 1969; Toan et al., 1965). The methods used in the studies cited above were relatively simple and could be adapted for routine quality control analyses of milk. Based on the work of Bawdon and Bassette (1966), Guarino and Kramer (1969), and Toan *et al*. (1965), the headspace vapors of milk were analyzed, in this investigation, in an attempt to detect the growth of psychrotrophic bacteria in milk.

Operating Parameters

A flame ionization detector was used because of its sensitivity to a wide range of organic compounds and its

insensitivity to inorganic gases, water vapor, and pressure surges from sample injections of large volumes (Nawar, 1966). Two types of column packings were used. Chromosorb W coated with Carbowax 20M was chosen as one column because of the interactions of Carbowax 20M with polar compounds; however, such polar stationary phases impose certain restrictions upon the operating parameters due to the tendency of these phases to give high recorder noise levels from substrate "bleed," which can affect detector sensitivity at column temperatures above 100 C. The second column packing used was Gas Chrom Z coated with Apiezon L. Apiezon L is a non-polar stationary phase, which separates compounds on the basis of boiling points, has a low vapor pressure, and is thermally stable; however, Apiezon L tends to allow peak tailing with carbonylic and alcoholic compounds. In addition to being acid washed, the Gas Chrom Z support was treated with dimethyldichlorosilane (DMCS) to make the support surface more inert and thus minimize its adsorption of volatiles.

Headspace Analysis of Standard Solutions

Under the operating conditions used in this investigation, several of the reference compounds had similar retention times, as Table 2 illustrates. Decreasing the flow rate of the carrier gas through the columns from 50 to 40 ml per minute resulted in a slight increase in retention times. Due to the proximity of these retention times, some

chromatographic peaks of either mixtures of the standard reference compounds or of the milk probably represented two or more of the volatile components. Likewise, due to differences in concentration, some of the more concentrated compounds probably masked the appearance of volatiles present in only trace amounts (Bassette and Ward, 1969). The detection of compounds was also dependent upon the sensitivity of the flame ionization detector to detect trace quantities of various compounds; for example, the conditions used in this study allowed detection of low concentrations of acetaldehyde and acetone but did not allow detection of low concentrations of lactic acid. This combination of detector insensitivity to lactic acid and the proximity of the retention times of acetaldehyde and lactic acid resulted in a failure to detect lactic acid in the milk samples analyzed in this investigation.

Analysis of the Headspace Vapors of Milk

The volatiles of foods can include the metabolic products of various microorganisms growing within the food (Fleming et al., 1969). Since the chromatograms produced by uninoculated control milk samples in this study showed little volatile activity, the peaks that were produced were presumably volatile compounds being formed in the milk as a result of bacterial metabolism. The respiration of organic compounds by microorganisms under ideal conditions causes the total conversion of substrate carbon to assimilatory

products and carbon dioxide (CO_2); however, at times the oxidation is incomplete, and only partially oxidized organic end-products are formed (Stanier et al., 1970). Acetaldehyde and acetone are intermediary products in several microbial fermentations (Doelle, 1969; Stanier et al., 1970) and, therefore, could be produced, if the oxidation of organic compounds in the milk was incomplete. Since A. viscolactis, B. pumilus, P. fragi, and P. perolens will have the same metabolic processes in raw and pasteurized milk, this discussion will apply to the results obtained for both.

Disaccharides are attacked by bacteria only after hydrolysis to monosaccharides; however, in the oxidation of disaccharides to bionic acids, hydrolysis is not necessary (Thimann, 1963). According to Doelle (1969) and Thimann (1963), lactose can be oxidized to lactobionic acid by several pseudomonads. The lactobionic acid is then slowly hydrolyzed to produce a hexose and a hexonic acid which can be further oxidized (Thimann, 1963). If a disaccaride is not hydrolyzed, it usually cannot be fermented.

According to Bergey's Manual (Breed et al., 1957), P. fragi and P. perolens cannot produce acid from lactose, but according to Thimann (1963), several pseudomonads can attack lactose to produce acetoin (acetylmethylcarbinol). Members of the genus Pseudomonas are capable of using many different organic compounds as the carbon source for their metabolism (Stanier et al., 1970). Unlike many other

bacteria, however, pseudomonads use the Entner-Doudoroff pathway rather than the Embden-Meyerhoff pathway to breakdown carbohydrates into pyruvic acid (Doelle, 1969; Rose, 1968). There are three possible metabolic pathways that lead from pyruvic acid to the formation of acetoin by microorganisms, and in one pathway diacetyl is a precursor of acetoin (Doelle, 1969). Bassette et al. (1967) studied the production of volatiles in milk by several bacteria, including P. fragi, and found that diacetyl was one of the principal volatiles produced, but P. fragi was not one of the species that produced diacetyl.

During the metabolism of a variety of compounds, part of the substrate disappearing is assimilated by microorganisms, while the rest of the substrate is broken down into various products (DeMoss and Bard, 1957); hence the assimilation of volatiles could be a reason why certain compounds produced in milk increased initially and then began to decrease. The bacteria could have been utilizing these volatile compounds for growth. It is known, that several pseudomonads can produce alcohol from sugars, and acetaldehyde is an intermediate product in an alcoholic fermentation (Thimann, 1963); thus the appearance of ethanol in the analyses of milk inoculated with pseudomonads could account for the decrease in acetaldehyde in the headspace vapors and would be a good example of the formation of one volatile compound during catabolism of another compound.

Members of the genus Bacillus are among the many groups of bacteria that utilize the Embden-Meyerhoff pathway to obtain pyruvic acid and then metabolize the pyruvic acid in a mixed-acid fermentation to produce various end-products (Stanier et al., 1970). The products produced in the fermentation of glucose by Bacillus species include carbon dioxide, 2,3-butanediol, acetoin, lactic acid, glycerol, ethanol, and acetic acid (Thimann, 1963). Acetoin is an intermediary product of the butanediol fermentation, and large amounts of acetoin are formed from sugars by several Bacillus species, including B. pumilus. The amount of acetoin formed reaches a maximum and then begins to decrease, even to a point where it has been completely utilized and is no longer present (Gorden et al., 1973). Due to the high yields of acetoin and 2,3-butanediol in a mixed-acid fermentation, the total amount of acid produced is decreased; thus, correlating the formation of these compounds with the production of acid could presumably be the reason peak V, suspected to be acetic acid, did not increase in height in the headspace vapor analyses during the growth of B. pumilus in milk.

Many psychrotrophs, including Alcaligenes spp. and Pseudomonas spp. are strongly lipolytic. Alcaligenes viscolactis readily hydrolyzes fats through the production of lipases. Certain lipolytic organisms can assimilate the products of fat hydrolysis into new compounds. Unlike many pseudomonads and bacilli, A. viscolactis cannot produce acetoin and also produces little or no acid from carbyhydrates.

The growth of certain bacteria in milk, such as A. viscolactis, causes the formation of viscous masses of milk. This type of spoilage, called ropy fermentation, is especially common when milk is held at low temperatures, since the organisms responsible for the spoilage grow well at refrigeration temperatures (Hammer and Babel, 1957). The formation of ropy masses in only the top cream layer of milk is due to the oxygen requirements of the organisms (Hammer and Babel, 1957). In the spoilage that occurred in milk inoculated with A. viscolactis and P. fragi, viscous masses of milk did form on the surface of the cream layer and along the top side walls of the flasks containing the milk.

Correlation of Bacterial Growth and Direct Headspace Vapor Analysis

Milk that had been inoculated with psychrotrophic bacteria was observed to contain two principal volatile components and at times as many as three other volatile compounds. The time of appearance and the rate of development of the volatiles was significant. The chromatograms of the milk inoculated with specific bacteria did not show any distinct differences from the chromatograms of the control, until there were at least 10^5 cells/ml, and most differences were not noted until the PBC was greater than 10^6 cells/ml. In similar work on the production of volatile materials in

milk by several species of bacteria, Bassette et al. (1967) found that it took 1 to 2 days, and sometimes 4 days, of incubation of the milk, before any volatile materials developed; and Toan et al. (1965) found that until there were greater than 10^6 cells/ml, the presence of E. aerogenes in milk produced few detectable changes in the volatile peaks.

The main trend observed in the chromatographic patterns produced by the volatiles, in this study, was the increase in the acetaldehyde and acetone (peaks II and III) during the first few days of incubation. These peaks reached a maximum between the second and fourth day and they began to decline. Toan et al. (1965) reported that the growth of E. aerogenes in milk caused methyl sulfide to produce a pattern similar to the one observed in this study; the concentration of methyl sulfide reached its maximum point at 4 days and then began to decrease. The bacteria in the pasteurized milk entered into the exponential phase of growth within 2 days, and the concentration of bacteria in the raw milk did not increase exponentially until 2 to 4 days after inoculation. The increase in the concentration of acetaldehyde and acetone, therefore, corresponded to the exponential growth period of the bacteria. When the growth rate began to level off at approximately 4 days, the concentration of volatiles began to decrease. As stated previously, if bacteria cannot derive assimilable carbon from substrates, they will grow at the expense of other organic nutrients furnished in the medium

(Stanier et al., 1970); hence when the bacteria reached a high population, they could utilize the volatiles, which had been previously produced in the milk, causing a decrease in concentration of those volatiles.

After approximately 7 days, the concentrations of volatile compounds in the milk began to increase, and by 14 days large increases in concentration were generally noticed. These increases were observed in all milk samples at the same time that visual spoilage became evident. The spoilage was predominant in the inoculated milk samples and included the production of off-odors, coagulation, and the formation of viscous masses of milk that adhered to the sides of the container.

Even when the chromatograms of the control and inoculated milk had the same number of peaks, there were differences between the two samples in the heights of the individual peaks. In several of the analyses of this study, the peaks of the control were higher than their corresponding peaks in the inoculated samples. Since the control milk normally contained fewer bacteria, a probable explanation would be that there was less utilization of certain volatile components by bacteria in the control.

The relative amounts of specific volatiles produced during growth of each organism in milk remained consistent when the headspace vapors of different samples of milk were analyzed in several studies over varying periods of time.

There were differences in the magnitudes of the peaks, but the similarity of the patterns produced by the peaks was an indication that reproducible results could be attained from analysis of the headspace vapors of milk. The ability to reproduce the chromatographic patterns was evident, for example, in samples of milk inoculated with P. fragi; in the examination of the headspace vapors by GLC at 1 month intervals for a period of 3 months a doublet peak appeared on the chromatograms after each milk sample had been incubated for 3 days.

Effects of Variation on Headspace Vapor Analysis of Milk

As was stated in the discussion of results of the pasteurized milk control, a distinct difference was noted in the results obtained using varying volumes of milk and headspace samples. The Materials and Methods Section has an explanation of the two methods (i.e., flask versus vial) used in preparing milk for headspace vapor analysis. Use of the flask method and a larger sample of the headspace gases yielded better results. This was especially evident with peak V which appeared as a definite peak with the flask method in comparison to a minute peak with the vial method. The reason for obtaining better results with the flask method was the ratio of headspace to milk volume. In the flask the ratio of headspace to milk was less than one; hence the

volatiles were concentrated into a smaller area, enhancing their detection. In the vial the conditions were opposite, since the ratio was greater than one, allowing dilution of the volatile materials. Nawar and Fagerson (1962) found that sample size relative to container size and the temperature of sampling were important factors in achieving reproducible results in direct vapor sampling.

A 60±1 C water bath was used in this investigation to heat the milk samples prior to analysis to increase the concentration of volatiles in the headspace. In an attempt to obtain better results, an 85 C water bath was also used, but the results were similar to those obtained at 60 C.

Comparison of the results obtained by analysis with the Apiezon L and Carbowax 20M stationary phases indicated that the Carbowax 20M phase gave better separation of the volatile components. A major disadvantage of the Apiezon L column was that sometimes acetaldehyde and acetone emerged together as one peak and at other times as two separate peaks. When standard solutions of acetaldehyde and acetone were analyzed individually on the Apiezon L column, acetone had a higher response than acetaldehyde, and its retention time was slightly longer; when the headspace vapors of a mixture of the two standards were analyzed, acetaldehyde and acetone merged together, and the peak produced had both a retention time and height intermediate to the retention times and heights of the peaks produced by the standards alone. This phenomenon of one peak representing a mixture of

volatiles seemed to occur primarily in the control milk sample.

A disadvantage to the use of either column is that with age, column performance decreases. Jackson and Kempton (1972) found that the average life for a 5% Carbowax 20M on Chromosorb G AW, 80/100 mesh column used for routine analyses of food preparations was 4 weeks. With increasing age, there are differences in retention times of the standards, and for continued accuracy in identification of unknown peaks, the retention times of the standards must be reachecked periodically. The column performance for the columns used in this study was good, and the retention times only varied slightly after 3 months of use.

Advantages and Disadvantages of Headspace Vapor Analysis of Milk

The main advantage of anlayzing the headspace vapors of milk by GLC was the ease and speed with which the test could be performed. Bawdon and Bassette (1966) stated that for the differentiation of coliform organisms in milk, direct vapor sampling gave reproducible results and required no special preparation; Guarino and Kramer (1969) concluded that the method could easily be applied to routine microbiological examination of foods. In this study, the method of directly sampling the headspace vapors of milk was successful in detecting the effects of the growth of psychrotrophic bacteria in milk. The volatile by-products of microbial metabolism

provided a measure of the activity of the psychrotrophic bacteria. Since most of the volatile compounds were absent in fresh milk, their appearance as the milk became older provided an effective means of estimating the microbiological quality of milk (Fields et al., 1968).

The main disadvantage of the method was that at low concentrations of bacteria, when only incipient activity had occurred, the amount of volatile compounds present in the milk was below the level of sensitivity of the flame ionization detector. Another disadvantage in detecting the volatile by-products of the bacteria was the insensitivity of the detector to certain volatiles, notably lactic acid and carbon dioxide.

Sterken and Kempton (1973) developed a gas chromatographic method for detecting the microbial metabolites in milk, in which alcohols and volatile acids were directly extracted from the milk with ether, and non-volatile fatty acids were methylated with BF_3-methanol. The spoilage process of refrigerated milk could then be followed by the appearance of various compounds on chromatograms; however, even using this extraction procedure, a few days of storage were still required before any differences were seen. In the study of Sterken and Kempton (1973), there was no correlation between the bacterial populations of the milk and the results of the gas chromatographic analysis.

In this study, even though the gas chromatograph did

not detect differences in the headspace vapors of milk until relatively high bacteria levels were reached, the differences were observed before any visual changes and off-odors had been produced. Bassette et al. (1967) and Toan et al. (1965) were unable to detect volatile production in milk by bacteria until the populations had exceeded 10^6 cells/ml; thus if the analysis of the headspace vapors of milk by GLC is to be used to estimate low concentrations of psychrotrophic bacteria, an improved method of concentrating the volatile materials will have to be developed.

The appearance of acetaldehyde and ethanol (peaks II and IV) on the chromatograms produced in any milk sample would indicate the presence of relatively high concentrations of psychrotrophic bacteria in the milk; however, the acetaldehyde peak would be a more positive indicator, since it was present in all of the milk samples analyzed in this investigation. Ethanol was not produced by all of the organisms studied and was normally not detected by the gas chromatograph until the milk had been incubated at 7±1 C for 6 days. Acetaldehyde was not detected by GLC analysis immediately after inoculation of the milk with bacteria but was usually detected within two days after inoculation; hence its presence in the volatile fraction of milk seemed to be due to bacterial metabolism and could be correlated to the presence of relatively high concentrations of bacteria in the milk. To improve the use of acetaldehyde as an index of the presence of

psychrotrophs in milk investigations should be done to improve the sensitivity of detecting this compound by the use of different methods and/or detection systems. In addition, acetaldehyde, in conjunction with other compounds such as pyruvic acid (Tolle et al., 1972), could serve as a sensitive index of the microbial quality of milk.

SUMMARY AND CONCLUSIONS

This study was undertaken to develop a less time-consuming procedure for detecting the presence of psychrotrophic bacteria in milk. Individual samples of raw and pasteurized milk were inoculated with four psychrotrophic bacteria and incubated at 7±1 C. Uninoculated milk samples were used as controls. The growth of organisms in the inoculated samples was followed by plating the milk to determine the SPC and PBC.

Two gas chromatographic methods were used for analysis of the milk. One method was pyrolysis-gas-liquid chromatography (PGLC), and the other was analysis of the headspace vapors by gas-liquid chromatography (GLC). No consistent differences were detected between the pyrograms of the control and inoculated milk samples in any of the PGLC studies done. Due to the inability to reproduce results and the large concentrations of interfering constituents from milk itself, PGLC was found to be impractical for determining the presence of psychrotrophs in milk.

For headspace vapor analysis by GLC, representative samples of the headspace vapors of milk were injected into a gas chromatograph and analyzed on Apiezon L and Carbowax 20M columns. Two major peaks, and at times three other peaks,

appeared on the chromatograms obtained from analysis of milk inoculated with A. viscolactis, B. pumilus, P. fragi, and P. perolens. Three of the peaks were presumptively identified as acetaldehyde, acetone, and ethanol by comparison of their retention times with retention times of known pure standard solutions. No distinct differences were noted between the chromatograms of the control and inoculated milk until the latter contained over 10^5 cells/ml. As the milk was incubated, changes were noted in the concentrations of the volatiles due to the utilization of the compounds for microbial metabolism.

Even though differences were not detected by the gas chromatograph until a relatively high bacterial population was reached, differences were observed well before any organoleptic changes could be detected or before the 10 days required to obtain plating results; hence the volatile by-products of microbial metabolism provided a measure of the activity of the psychrotrophic bacteria in milk. In addition, headspace vapor analysis required less sample preparation and analysis time than PGLC. These results indicate that analysis of headspace vapors by GLC has the potential to be a quality control method for evaluating the microbial condition of milk.

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APPENDIX

Table 3. Plate counts (cells/ml) of raw milk*

Sample	Days 0	2	4	6	8
(a) STANDARD PLATE COUNT**					
Control	$7.0x10^2$	$1.1x10^3$	$1.5x10^3$	$1.3x10^4$	$1.7x10^5$
Alcaligenes viscolactis	$3.0x10^4$	$2.6x10^6$	$4.1x10^7$	$1.5x10^8$	$5.1x10^7$
Bacillus pumilus	$2.7x10^4$	$3.0x10^6$	$3.1x10^7$	$7.1x10^7$	$7.1x10^7$
Pseudomonas fragi	$4.8x10^4$	$2.9x10^5$	$1.8x10^7$	$6.6x10^7$	$7.5x10^7$
**Incubated at 25 C for 48 hours.					
(b) PSYCHROTROPHIC BACTERIA COUNT**					
Control	$1.0x10^2$Est[a]	$1.0x10^2$Est[a]	$1.5x10^1$Est[a]	$6.2x10^2$	$8.7x10^4$
Alcaligenes viscolactis	$2.0x10^4$	$3.4x10^5$	$4.1x10^7$	$1.4x10^8$	$2.8x10^7$
Bacillus pumilus	$1.5x10^3$	$3.2x10^5$	$2.9x10^7$	$1.9x10^7$	$1.6x10^8$
Pseudomonas fragi	$2.0x10^4$	$4.7x10^5$	$1.2x10^7$	$2.4x10^7$	$7.5x10^7$
**Incubated at 7 C for 10 days.					

*Estimated count since all plates contained less than 30 colonies (APHA, 1972).
[a]Milk was analyzed by PGLC.

Table 4. Plate counts (cells/ml) of pasteurized milk.

Sample	Days 0	2	3	5	13
(a) STANDARD PLATE COUNT*					
Control	$4.1x10^3$	$3.2x10^3$	$5.9x10^3$	$3.4x10^3$	$2.5x10^5$
Bacillus pumilus	$4.4x10^3$	$2.8x10^6$	$3.6x10^7$	$7.9x10^8$	$2.0x10^8$ Est[a]
Pseudomonas fragi	$4.9x10^3$	$3.1x10^6$	$4.3x10^7$	$6.2x10^5$	$1.0x10^8$ Est[a]
Pseudomonas perolens	$3.2x10^3$	$3.9x10^6$	$3.9x10^7$	$7.8x10^7$	$2.5x10^8$ Est[a]
*Incubated at 25 C for 48 hours.					
(b) PSYCHROTROPHIC BACTERIA COUNT*					
Control	$1.6x10^3$	$2.8x10^3$	$5.3x10^3$	$4.6x10^3$	$2.0x10^5$
Bacillus pumilus	$2.2x10^3$	$2.0x10^6$	$2.2x10^7$	$6.8x10^8$	$1.5x10^8$ Est[a]
Pseudomonas fragi	$5.0x10^3$	$2.4x10^6$	$4.1x10^7$	$5.8x10^8$	$1.0x10^8$ Est[a]
Pseudomonas perolens	$2.7x10^3$	$1.0x10^6$	$1.5x10^7$	-	$3.5x10^8$ Est[a]
*Incubated at 7 C for 10 days.					

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 5. Plate counts (cells/ml) of pasteurized milk.

Sample	Days 0	2	4	6	14
(a) STANDARD PLATE COUNT*					
Control	1.1×10^{2} Est[a]	1.0×10^{2} Est[a]	1.1×10^{2} Est[a]	1.3×10^{2} Est[a]	$>3.0 \times 10^{4}$ Est[b]
Alcaligenes viscolactis	2.3×10^{3}	3.4×10^{6}	1.2×10^{8}	3.1×10^{8}	1.4×10^{8}
Pseudomonas fragi	2.6×10^{3}	4.0×10^{6}	2.2×10^{7}	3.5×10^{8}	5.8×10^{8}
*Incubated at 25 C for 48 hours.					
(b) PSYCHROTROPHIC BACTERIA COUNT*					
Control	1.0×10^{1} Est[a]	1.0×10^{1} Est[a]	3.5×10^{1} Est[a]	4.0×10^{1} Est[a]	5.0×10^{4}
Alcaligenes viscolactis	3.5×10^{3}	2.8×10^{6}	1.1×10^{8}	2.1×10^{8}	1.3×10^{8}
Pseudomonas fragi	2.6×10^{3}	3.1×10^{6}	2.5×10^{7}	3.3×10^{7}	4.3×10^{8}
*Incubated at 7 C for 10 days.					

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

[b]Estimated count since all plates contained greater than 300 colonies (APHA, 1972),

Table 6. Plate counts (cells/ml) of pasteurized milk.

Sample	Days 0	2	4	6	8	14
(a) STANDARD PLATE COUNT*						
Control	5.4×10^2	1.6×10^3	6.1×10^3	$>3.0 \times 10^5$ Est[a]	7.0×10^6 Est[a]	8.5×10^7 Est[a]
Alcaligenes viscolactis	2.1×10^3	1.6×10^6	5.8×10^7	1.9×10^8	5.3×10^8	4.7×10^8
Bacillus pumilus	8.0×10^3	7.7×10^5	4.7×10^7	1.3×10^8	2.2×10^8	5.5×10^8
Pseudomonas perolens	4.3×10^3	6.0×10^5	6.3×10^7	1.4×10^8	4.6×10^8	2.1×10^8
*Incubated at 25 C for 48 hours.						
(b) PSYCHROTROPHIC BACTERIA COUNT*						
Control	7.0×10^1 Est[b]	5.5×10^2	6.0×10^3	8.3×10^5	$>3.0 \times 10^6$ Est[a]	$>3.0 \times 10^7$ Est[a]
Alcaligenes viscolactis	1.7×10^3	1.4×10^6	2.4×10^7	1.1×10^8	1.6×10^8	2.8×10^8
Pseudomonas perolens	1.8×10^3	2.6×10^5	6.7×10^7	1.5×10^8	3.8×10^8	2.6×10^8
Pseudomonas perolens	1.8×10^3	2.6×10^5	6.7×10^7	1.5×10^8	3.8×10^8	2.6×10^8
*Incubated at 7 C for 10 days.						

[a]Estimated count since all plates contained greater than 300 colonies (APHA, 1972).

[b]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 7. Plate counts (cells/ml) of pasteurized milk.

Sample	Days 0	1	2	4	11
(a) STANDARD PLATE COUNT*					
Control	-	2.5×10^2 Est[a]	1.0×10^2 Est[a]	1.7×10^2 Est[a]	6.5×10^3
Bacillus pumilus	-	4.9×10^4	7.3×10^5	8.8×10^7	1.9×10^8
Pseudomonas fragi	1.6×10^3	1.7×10^5	2.5×10^6	8.3×10^7	1.4×10^8
Pseudomonas perolens	1.2×10^3	9.0×10^4	1.8×10^6	5.3×10^7	3.1×10^8
*Incubated at 25 C for 48 hours.					
(b) PSYCHROTROPHIC BACTERIA COUNT*					
Control	3.1×10^2	1.0×10^2 Est[a]	1.0×10^2 Est[a]	1.0×10^1 Est[a]	3.2×10^3
Bacillus pumilus	5.8×10^2	2.8×10^4	3.9×10^5	7.4×10^7	1.1×10^8
Pseudomonas fragi	9.2×10^2	1.2×10^5	1.2×10^6	4.3×10^7	7.0×10^8
Pseudomonas perolens	1.2×10^3	6.5×10^4	1.8×10^6	5.5×10^7	5.3×10^8
*Incubated at 7 C for 10 days.					

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 8. Plate counts (cells/ml) of raw milk.

Sample	Days 0	1	2	5
(a) STANDARD PLATE COUNT*				
Control	4.2×10^3	1.6×10^4	5.7×10^4	9.0×10^4 Est[a]
Alcaligenes viscolactis	8.8×10^2	5.9×10^4	6.1×10^4	1.1×10^8
Bacillus pumilus	2.2×10^2 Est[a]	4.5×10^4	4.6×10^4	1.3×10^8
Pseudomonas fragi	3.4×10^2	5.4×10^4	5.6×10^4	1.3×10^8
Pseudomonas perolens	3.6×10^2	5.3×10^4	5.8×10^4	4.1×10^7

*Incubated at 25 C for 48 hours.

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 8. (continued)

Sample	Days 0	1	2	5
(b) PSYCHROTROPHIC BACTERIA COUNT*				
Control	$1.0x10^{1}$ Est[a]	5.0 Est[a]	$5.0x10^{1}$ Est[a]	$1.0x10^{4}$ Est[a]
Alcaligenes viscolactis	$4.5x10^{1}$ Est[a]	$9.0x10^{2}$ Est[a]	$5.0x10^{3}$ Est[a]	$7.0x10^{7}$
Bacillus pumilus	$7.5x10^{1}$ Est[a]	$8.5x10^{2}$ Est[a]	$3.0x10^{3}$ Est[a]	$7.6x10^{7}$
Pseudomonas fragi	$6.5x10^{1}$ Est[a]	$1.9x10^{3}$ Est[a]	$6.0x10^{3}$ Est[a]	$1.1x10^{8}$
Pseudomonas perolens	$2.0x10^{1}$	$5.0x10^{2}$ Est[a]	$1.0x10^{3}$ Est[a]	$2.0x10^{7}$

*Incubated at 7 C for 10 days.

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 9. Plate counts (cells/ml) of raw milk.

Sample	Days 0	1	2	3	5	7
(a) STANDARD PLATE COUNT*						
Control	$1.6x10^2$Est[a]	$2.1x10^2$Est[a]	$1.8x10^2$Est[a]	$2.0x10^2$Est[a]	$1.7x10^2$Est[a]	$1.8x10^2$Est[a]
Alcaligenes viscolactis	$5.9x10^2$	$9.0x10^3$	$8.5x10^4$	$6.3x10^6$	$3.0x10^8$	$1.2x10^9$
Bacillus pumilus	$6.4x.0^3$	$8.0x.0^3$	$4.0x.0^4$	$1.9x.0^6$	$2.7x10^8$	$3.2x.0^8$
Pseudomonas fragi	$1.1x10^3$	$1.3x10^4$	$4.0x10^4$	$3.3x10^6$	$1.7x10^8$	$8.1x10^8$
*Incubated at 25 C for 48 hours.						
(b) PSYCHROTROPHIC BACTERIA COUNT*						
Control	$1.0x10^1$Est[a]	$1.0x10^1$Est[a]	$1.0x10^1$Est[a]	$1.0x10^1$Est[a]	$1.0x10^1$Est[a]	$1.0x10^1$Est[a]
Alcaligenes viscolactis	$2.5x10^2$Est[a]	$9.0x10^3$	$9.5x10^4$	$7.0x10^6$	$1.7x10^8$	$8.8x10^8$
Bacillus pumilus	$3.3x10^2$	$2.0x10^3$	$2.0x10^4$	$2.4x10^6$	$1.6x10^8$	$4.1x10^8$
Pseudomonas fragi	$6.0x10^2$	$1.4x10^4$	$7.0x10^4$	$2.0x10^6$	$1.3x10^8$	$5.0x10^8$
*Incubated at 7 C for 10 days.						

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).

Table 10. Plate counts (cells/ml) of raw milk.

Sample	Days 0	2	4	8	11
(a) STANDARD PLATE COUNT *					
Control	-	$1.1x10^4$	$2.3x10^4$	$3.0x10^4$	$1.7x10^4$
Bacillus pumilus	$1.8x10^4$	$2.2x10^5$	$4.0x10^7$	$2.7x10^8$	$5.1x10^8$
Pseudomonas perolens	$2.0x10^4$	$1.7x10^5$	$4.0x10^7$	$1.5x10^8$	$1.9x10^8$
*Incubated at 25 C for 48 hours.					
(b) PSYCHROTROPHIC BACTERIA COUNT*					
Control	$1.0x10^1$Est[a]	$4.0x10^2$	$1.0x10^3$Est[a]	$1.0x10^4$Est[a]	$2.0x10^2$
Bacillus pumilus	$9.5x10^2$	$6.6x10^4$	$1.9x10^7$	$1.8x10^8$	$9.6x10^8$
Pseudomonas perolens	$4.5x10^2$	$1.0x10^5$	$3.8x10^7$	$8.0x10^7$	$1.1x10^8$
*Incubated at 7 C for 10 days.					

[a]Estimated count since all plates contained less than 30 colonies (APHA, 1972).