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LILIANA URREA ISAZA for the degree of Master of Science in Food Science and Technology presented on June 3 , 1986

Title: RAPID METHODS FOR THE DETERMINATION OF POST-PASTEURIZATION CONTAMINATION OF FLUID MILK AND SHELF-LIFE PREDICTION

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Several methods for estimation of the potential shelf-life of pasteurized fluid milk products were evaluated for their efficacy in this investigation. These methods were evaluated and compared to sensory, biochemical and bacteriological indices through a series of experiments conducted on different brands of commercially pasteurized fluid milk. The methods evaluated included: Standard Plate Count (SPC), Psychrotrophic Bacteria Count (PBC), Modified Psychrotrophic Bacteria Count (MPBC), the Moseley keeping quality test (MKQT), Parmelee tube test (PTT), tetrazolium salt-resazurin test (TRT), modified Parmelee tube test (MPTT), and p-iodonitrotetrazolium violet-phenazine methosulfate test (INT-PMS).

Several different conditions of preliminary incubation (PI) were attempted in an effort to accelerate outgrowth of psychrotrophic bacteria and hence obtain sufficient numbers and metabolic activity to reduce the redox potential indicator dye.

Correlation coefficients (r) and chi-square (χ^2) values were obtained in an attempt to detect significant relationship between the parameters studied and the potential shelf-life of the product. Results suggested that the PTT, TRT and MPTT tests were not reliable predictors of the potential shelf-life of pasteurized milk (r values between -0.445 and 0.734, non-significant $P>0.05$). The INT-PMS Test at 21°C for 20 minutes following PI at 21°C for 25 hours provided the best estimate of the potential shelf-life of pasteurized whole milk ($r= -0.840$). This method shows some potential as a method for determining post-pasteurization contamination: it was accurate (92.3%), rapid (<26 hours), simple, inexpensive (4.54 to 9.64 cents/sample), and sensitive (it was able to detect less than 1 PBC/ml and less than 5.0×10^1 total CFU/ml in fresh milk if bacteria were able to reach 1 PBC/ml and 1.0×10^3 total CFU/ml during PI). However its accuracy could be significantly affected by the intensity of the pasteurization heat treatment given to the milk due to possible denaturation of the whey proteins and release of heat activated reducing substances (-SH groups).

RAPID METHODS FOR THE DETERMINATION OF POST-PASTEURIZATION CONTAMINATION OF FLUID MILK AND SHELF-LIFE PREDICTION

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Liliana Urrea Isaza

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To my husband, mother and father, for their stallworth support and steadfast love.

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RAPID METHODS FOR THE DETERMINATION OF POST-PASTEURIZATION CONTAMINATION OF FLUID MILK AND SHELF-LIFE PREDICTION

INTRODUCTION

The problem of maintaining an adequate shelf-life or achieving extended keeping quality has always been of great concern to milk processors. The presence of psychrotrophic bacteria which are capable of producing extracellullar proteolytic and lipolytic enzymes in refrigerated, pasteurized milk frequently leads to shortened shelf-life, loss of saleable products and consumer dissatisfaction. Consumers tend to lose confidence in specific brands of milk that spoil or lose their general palatability before the pull (sell-by) date or before reasonable time has elapsed after product purchase.

Economic necessity as well as standards of quality required by regulatory agencies and consumers have forced milk processors to produce higher quality products with extended shelf-life. The ability to accurately detect and enumerate milk spoilage bacteria (psychrotrophs) in recently processed milk could provide important information on the sanitary conditions and temperature control of processing operations; this might provide the necessary information for the processor to significantly extend product shelf-life.

Generally there is a direct relationship between contamination after pasteurization and the shelf-life of the final product. Pasteurized milk eventually spoils when held at refrigeration temperatures due to the action of psychrotrophic contaminants which come from improperly cleaned and sanitized equipment located between the pasteurizer and the final container. The result of such growth is definite flavor deterioration in finished products (as used by the consumer). The most common defects observed have been fruity and rancid (172,185,219). Putrid, potato-like, cheesy, bitter, unclean, soapy and fishy off-flavors have been associated with proteolysis and/or lipolysis by psychrotrophic bacteria (94,115, 159, 162, 219, 232).

The bacterial population requiered to cause detectable sensory changes in milk varies among genera, among species and within genus. Some investigators have reported population levels at which flavor changes occur (158,198). However, other researchers have found that the number of bacteria was not as important as the types of bacteria which were able to degrade milk components (77,157,174).

Within the last few years, the management of U.S. dairy processing plants has become more and more aware that procedures formerly used to measure plant sanitation are outdated and ineffective. Semmingly, bacterial plate

counts (either the SPC or psychrotrophic count) or coliform tests on freshly processed product provide no means to predict the shelf-life of pasteurized milk products. The Moseley test has been widely applied and generally accepted as a reliable indicator of the keeping quality of fluid milk (14,49,95,175). However, the 7 to 9 day period of time requiered to obtain results renders the Moseley test rather impractical for monitoring the quality of perishable products.

Many other tests have been recommended to determine the microbial quality and shelf-life of milk and dairy products. They include: incubation at elevated temperature, preliminary incubation, use of selective media with inhibitors or dyes, dye reduction tests, detection of metabolites, rapid screening,etc. However, at the present time, there is not single method that meets all the requierements of the ideal test for post-pasteurization contamination as Blankenagel (14) proposed: (1) accurate: it would indicate or reflect the number of organisms which gained entry to the product after pasteurization. Hopefully, such a test would also differentiate between thermoduric bacteria and post-pasteurization contaminants (2) rapid: the results would be available within at least 15 - 20 hours (3) simple to perform and (4) economical (less than $2.00/sample).

The objectives of this study were: (1) to develop a dye reduction test for determining the absence or presence of any level of psychrotrophic bacteria in commercially pasteurized milk (2) to develop a practical test method, adaptable by the dairy industry, that will readily predict the shelf-life potential of fluid milk products and (3) to develop a test method in which the results correlate closely with both sensory evaluation and microbiological analysis of milk samples.

The approach for the rapid detection of population levels of psychrotrophic bacteria in pasteurized milk employed the use of several dye reduction tests: (1) Parmelee tube test for psychrotrophs (2) tetrazolium salt-resazurin test (3) tetrazolium salt test (4) modified Parmelee tube test (5) p-iodonitrotetrazolium violet-phenazine methosulfate test. Trials with these methods were conducted in commercial milk samples to evaluate different sensory, biochemical and bacteriological parameters.

REVIEW OF LITERATURE

DEFINITION OF PSYCHROTROPHS

The definition as well as the terminology for microorganisms that are able to grow at temperatures as low as 0°C has confused microbiologists since the beginning of this century. In 1887 Forster (55) first observed bacterial growth at 0°C, but it was not until 1902 that Schmidt-Nielsen (165) defined psychrophiles as bacteria which are not only able to survive at refrigeration temperatures but also have the ability to multiply at 0°C.

The term psychrophile is derived from the Greek words "psychros", which means cold and "philos", which means loving. Therefore, this term implied that such microorganisms grow best at low temperatures and that they are cold-loving. However, several investigators objected the use of this term since it implies a preference for growth at low temperature. Actually, these microorganisms grow better at 20°C or higher (88,90,180,191,198).

Psychrophiles have been defined in many different ways based on: optimum growth temperature, growth at low temperature, methods of enumeration and other criteria unrelated to temperature. Olson et al. (146) described psychrophiles as those bacterial species

which are capable of relatively rapid growth at low temperatures, generally within the range of 1.7°C to 7.2°C. Morita (133) described psychrophilic bacteria as organisms having an optimal temperature for growth at 15°C, a maximum growth temperature of 20°C and a minimum growth temperature of 0°C or below. This is also the classical definition used in most microbiology textbooks. However, most microbiologists think of psychrophiles as microorganisms that can grow at low temperatures.

Since the term "psychrophiles" inappropriately labels microorganisms capable of growth at low temperatures, other names have been proposed (48,100, 180,185,196,198). Morita (133) suggested that those mesophilic microorganisms that could grow at 0°C are more correctly termed psychrotolerant or psychrotrophic than psychrophilic. Most of the literature published after the year 1960 seems to refer to psychrotrophs rather than psychrophiles. The term psychrophile should refer only to those microorganisms whose optimum temperature of growth or minimum temperature of generation is achieved by low temperatures (48,100).

In a 1976 meeting of the International Dairy Federation, psychrotrophs were defined as microorganisms that grow at 7°C or less, irrespective of optimal temperature (35,105,184). In application

to the dairy industry and for technological reasons, psychrotrophs are considered to be those microorganisms which can multiply relatively rapidly in milk and dairy products at refrigeration temperatures of 3°C to 7°C.

TEMPERATURE-GROWTH RELATIONSHIPS

Growth Temperatures

Psychrotrophs are considered to preferentially grow more readily at 20°C or higher(88,90,91,180,198). Lawton and Nelson (111) described the optimum growth temperature range as 21-32°C for most of the isolates they investigated. Elliott and Michener (50) reported that optima for growth of most microorganisms are 20 to 30°C with some organisms having optima of 30 to 45°C and only a few at 15°C or below.

The optimum growth temperature of psychrotrophs can be determined either by observing the generation times during the exponential growth phase of the culture or ascertaining the exact temperature at which the generation time is shortest. Many investigations have been made since 1903 about generation times for psychrotrophs and psychrophiles (11,58,64,71,90,133, 141,170). Yano et al. (234) studied generation times for psychrotropic bacteria in refrigerated raw milk

and found a range of 6.6 to 12.7 hours at 5°C and 12.2 to 26.1 hours at 0°C.

The maximum growth temperature for psychrotrophs usually is stated as 30°C, but some have maxima of 37 to 45°C (86,91,180). The minimum growth temperature for psychrotrophic bacteria in foods systems has been reported to be -10°C (90,184).

Response to Low Temperatures

The Arrhenius equation has been used to describe how chemical reactions are affected by temperature (6,85, 92,133). By using this procedure, Ingraham postulated that a psychrophile could be distinguished from a mesophile (89). The Arrhenius curve for true psychrophiles is linear to 0°C and below, but the curve for psychrotrophs deviates from linearity at 4-5°C and for mesophiles it deviates from linearity at even higher temperatures (85,92). On the other hand the μ values in the Arrhenius equation do not adequately describe the growth characteristics of psychrophiles, psychrotrophs and mesophiles according to several other researchers.

It has been postulated that many psychrophilic bacteria compensate for growth at low temperatures by synthesizing increased quantities of enzymes (85,133). Support for this hypothesis is indirect, but several workers (25,78,84) have shown

significantly higher RNA and protein levels of microorganisms grown at low temperature than at optimal growth temperatures. Some psychrotrophs exhibit a preferential release of proteolytic and lipolytic enzymes at lower temperatures (50,232).

If enzymes are to function at low temperatures then necessary growth substrates must be able to enter the cell. The ability of microorganisms to grow at low temperatures has been linked to cell permeability and substrate intake (85,91,92). Several researchers (89,134) have reported that the minimum temperature for growth of mesophiles is due to low temperature inhibition of substrate up-take. Consequently, considerable interest has been shown in the lipid composition of microorganisms in relation to their response to growth at low or sub-optimum growth temperatures, since lipids form the bulk of the cytoplasmic membrane. Alterations in the physical or chemical composition of the membrane lipids may alter membrane functions. Many microorganisms respond to growth at sub-optimum temperature by synthesizing increased proportion of unsaturated fatty acids, at the expense of saturated fatty acids (25, 51, 102). It has been argued that these changes decrease the melting point of the membrane lipids, thereby maintaining their integrity and function

(51,61). However, not all microorganisms apparently undergo changes in fatty acid composition with a decrease in growth temperature (5,58). The significance or reality of a change in unsaturation of cell lipids due to decreased temperatures has not been resolved and no known physiological basis exists for changes to occur in amounts or types of lipids with change in temperature.

Moderate temperature changes can alter physiological or permeability functions of microorganisms that grow at low temperature. The permeability barrier of the psychrophile bacteria cell can be damaged sufficiently to allow leakage of intracellular components: protein, DNA, RNA, amino acids or total cell lysis (91,92,191).

Studies with marine psychrophiles has shown that these microorganisms increased the amounts of respiratory enzymes at sub-optimum temperatures, which resulted in growth maintenance, but decreased cell yield (85,133). Minimum uptake of oxygen and maximum cell yield occurred at the optimum growth temperature for the microorganisms. However more research seems needed before definite correlations between cell yield, oxygen utilization and growth rate can be established.

Frank et al. (58) concluded that growth at low temperatures may be possible because the structure and enzymes of psychrotrophic Pseudomonas species were similar to those at higher temperatures.

Temperature Adaptability

Reports of the adaptation of microorganisms to growth at lower temperatures are few and inconclusive. Generally, the ability of microorganisms to grow at 0°C is a specific property of some microorganisms and cannot be acquired by culturing microorganisms at lower temperatures. Azuma et al. (5) reported unsuccessful attempts to adapt bacteria to low-temperature growth by serial transfers; microorganisms that are transferred to lower growth temperatures may require an induction period to facilitate biochemical changes necessary for substrate metabolism, enzyme synthesis and/or cell permeability.

There is genetic evidence for conversion of a mesophile to a psychrotroph by transduction (144), ultraviolet light (5) as well as mutation triggered by ultraviolet light from a psychrotroph to a mesophile (181). However Morita (133) concluded that a true psychrophile can not be produced by these methods since psychrophiles contain more than one thermolabile enzyme and several membrane differences when compared to mesophiles. Psychrophiles and psychrotrophs generally have higher levels of unsaturated fatty acids than do mesophiles, and solute transport may be affected by the degree of unsaturation of fatty acid chains in membrane lipids (85). Since

phage have been isolated from psychrotrophic bacteria (42,43,106) a transduction effect between cold-loving organisms is possible (133). Unfortunately, none of the host cells for phage are psychrophiles (113).

One hypothesis of microbial evolution is that thermophiles were the first organisms to evolve (23), followed by the mesophiles and finally the psychrophiles. The evolution of psychrophilic bacteria is probably the result of many genetic events, since psychrophiles generally contain more than one enzyme that is abnormally thermolabile, as well as marked membrane differences. Organisms that have survived a cold environment for generations may have experienced mutations that caused some of the key proteins to become relatively heat labile (1). There are probably many pathways that lead to psychrophily, since it has been found that a specific abnormally thermolabile enzyme in one psychrophile may not be abnormally thermolabile in another psychrophile (133).

TYPES OF PSYCHROTROPHIC BACTERIA

Psychrotrophs may include long or short bacilli, cocci or vibrios; Gram-positive or Gram-negative bacteria; spore-formers or nonspore-formers, and aerobic, facultative anaerobic or anaerobic microorganisms. Some researchers have described the morphological characteristics and Gram reaction; whereas, others used additional tests to identify

the genera (36). Characteristics that have been used to identify the isolates are cell shape, motility, Gram reaction, oxidative - fermentation of carbohydrates, production of ammonia from arginine, oxidase and catalase reaction , lipolysis, proteolysis, action on litmus milk, acid fastness and spore formation (36).

Gram-negative Rods

It has been observed that most psychrotrophs that spoil milk at temperatures slightly above freezing are predominantly Gram-negative, non-sporeforming, catalase-positive rods (88, 180). The relative frequencies of occurence of the predominant taxonomic groups of psychrotrophic bacteria isolated from milk products have been summarized by Thomas and Druce (191) and Cousin (36).

Pseudomonas sp. are the predominant microorganisms in refrigerated raw milk, with Achromobacter, Alcaligenes, Aeromonas, Flavobacterium and coli-aerogenes organisms and Enterobacteria sp. consistently present in less frequent proportion (44, 56,75,97,107,115,131,167,178,180,183,190,232). According to one study, Pseudomonas account for 20.4 - 69% of the total population of raw milk (87).

Pseudomonas sp. have also been shown to be the predominant psychrotroph in refrigerated commercially

pasteurized milk, followed by Achromobacter and Alcaligenes sp. with psychrotrophic strains of coli-aerogenes organisms occasionally constituting an appreciable proportion (44,154,168,216). Pseudomonas sp. were demonstrated to account for 62.1-70.1% of the total population of pasteurized milk (187) and 46.3 - 62.8% of pasteurized cream (187). It has been reported a much higher incidence of Pseudomonas and Achromobacter (44) and a lower incidence of coli-aerogenes organisms in pasteurized than in raw milk (44,132,183,191,195,204). Van Der Zant (220) found that certain species of Pseudomonas tend to inhibit the growth of Achromobacter, this may explain the frequent dominance of Pseudomonas sp. in milk products refrigerated for extended storage times.

Gyllemberg et al. (75) have studied the relative changes in the microflora population of pasteurized milk during refrigeration at 7°C. At the time of sampling psychrotrophic bacteria (Pseudomonas fluorescens, Pseudomonas fragi, psychrotrophic Pseudomonas aeruginosa, Alcaligenes viscolactis and Enterobacter)only constitued 6.1% of all microflora compared with 57.2% after 48 hours at 7°C. By comparison mesophilic and thermoduric bacteria (Alcaligenes tolerans, Corynebacteria and Micrococci) decreased from 93.8% of total flora to only 43.0% after holding at refrigerated storage.

Gram-positive Bacteria

Gram-positive bacteria are commonly isolated from raw milk; however these populations are generally less than the Gram-negative bacteria. The most frequently isolated Gram-positive microorganisms are species of Micrococcus,Bacillus and Arthrobacter (72, 88, 90,103,115,131,169,232).

The presence of Gram-positive bacteria in pasteurized milk and dairy products, has become more important since many bacteria of this type form spores which can survive typical heat treatments (pasteurization) applied to the products. Hence spore-formers have been linked to the spoilage of these products (38,71,127). Thomas (189) reported that the generation time of Bacillus species was about 45 hours at 5°C, compared to 7 hours for some Gram-negative rods. Hence, spoilage caused by these spore-formers is slower and requires prolonged storage at refrigeration temperatures for selective conditions. Of all microbial species isolated from milk and dairy products, Bacillus cereus has been one of major concern since it frequently develops a "bitty cream" defect, (bitter off-flavor and coagulation), and has been reported to cause food poisoning (34,37,40,127,153). Futhermore Bacillus cereus constituted 37% of the spore population in raw

milk in these studies.

The use of higher pasteurization temperatures and extended cold storage of pasteurized milk have led to greater concern about heat-resistant or thermoduric bacteria. The following variables: type present, cell numbers, total flora, product shelf-life, storage temperature, etc, are very important in ascertaining the relative significance of these microorganisms in milk; presently, their relative importance or significance have not been resolved. So-called "thermoduric psychrotrophs" are usually Gram-positive rods or cocci, most often from the following genera: Arthrobacter, Bacillus, Clostridium, Corynebacterium, Lactobacillus, Microbacterium, Micrococcus or Streptococcus (217,224).

In 1979, Collins emphasized the need for control of heat-resistant psychrotrophic bacteria, especially Bacillus species in milk and milk products and their potential impact on shelf-life. He stated that even if post-pasteurization contamination was controlled absolutely and all milk was stored at less than 1°C, from-cow-to-consumer, the typical shelf-life of milk could still be limited to three to four weeks, due to eventual spoilage caused by psychrotrophic heat-resistant, spore-forming bacteria (ie. Bacillus strains).

SOURCE OF PSYCHROTROPHS

Psychrotrophs are ubiquitous in nature; soil, water, plants and animals form the natural habitat for these organisms (55,90,135,180,183,185,196,232).

Most psychrotrophic microorganisms in milk and dairy products are usually derived originally from soil, water and vegetation (135,196). Soil, grass and hay can contain psychrotrophs that exceed 1×10^7/gram (198). Morse et al. (135) reported that the psychrotrophic count of water ranged from less than 10 to more than 100000 microorganisms/ml, with a median of 10 to 560/ml.

Few of the psychrotrophs found in milk have been isolated from the air in clean milking parlors or dairies (183,185,186) but dust collected from milking equipment surfaces and from cereal grains contained from 1 to 300 million psychrotrophs/gram (196). The main source of airborne microorganisms in dairy plants are worker activity, floor drains, fans and dust (194).

Occasionally, fecal contamination, due to poor milking conditions, may contribute psychrotrophs to milk, since both fresh and dry manure can contain millions of such bacteria per gram (185,196,198).

Poorly cleaned and sanitized dairy farm and processing plant equipment probably constitute major

sources of contamination for raw milk by psychrotrophs (183,185,191,196,198,208,214,232). The psychrotrophic microflora of milk contact surfaces has been determined by various research workers(28,188,198, 208,215). Psychrotrophic plate counts of rinses obtained from pipelines milking systems had a higher percentage of counts >1000/ft^2 than did milking machines (198,215). Standard plate counts at 30°C of pipeline milking plants revealed that Gram-negative rods were the predominant flora in "poorly-cleaned" systems, whereas streptococci predominated in "properly-cleaned" systems (188). Gram-negative rods were the primary microorganisms present on surfaces of farm bulk tanks, they constituted 38.6% of the flora for "well-cleaned" tanks and 76.7% of the flora of "poorly-cleaned" tanks (188). Cannon (27) found that pipeline gaskets were a major source of psychrotrophic spore-formers.

Post-Pasteurization Contamination

Since there is a conclusive evidence that typical psychrotrophs are destroyed during pasteurization, it is logical to assume that their presence in commercially pasteurized milk is a result of post-pasteurization contamination (116,192,232).

Many investigators have demonstrated in the recent years that psychrotrophs may be introduced at one or

or more points during handling of heat-treated milk (49,120,197). Post-pasteurization contamination frequently results from improperly cleaned and sanitized equipment, as well as from airborne contamination (191). Improperly sanitized bottles and containers have been implicated as additional sources of recontamination of pasteurized milk. Airborne contamination can add psychrotrophs to the milk and at 7.2°C, one psychrotroph in a half-gallon carton of milk could increase to 1 x 10^6 psychrotrophs/ml in 8 days or less (191). Manual valves and gaskets on take-down pipe lines are extremely critical areas (49). Paper and bottle filling machines have many vulnerable areas that can contaminate the product before or during filling (49) and probably represents the most common single source of post-pasteurization contamination.

Post-pasteurization contamination is an important source of psychrotrophs in milk. However, it is not the only source. Since 1971, numerous reports (16, 130,169,171,189) have appeared indicating that psychrotrophic spore-formers can survive pasteurization, outgrow during refrigerated storage and reduce the shelf-life of milk and other dairy foods.

INCIDENCE OF PSYCHROTROPHS IN MILK

Psychrotrophs in Raw Milk

The occurence and population levels of psychrotrophic bacteria in raw milk primarily depends on the type and number of microorganisms present, conditions under which the milk was produced and the temperature and length of storage time before it is processed.

Thomas and Thomas (198) reported that milk produced under sanitary conditions usually contains less than 10% of the total microbial flora as psychrotrophs, but milk produced under unsanitary conditions can contain more than 75% as psychrotrophs.

The age of milk affects psychrotrophic colony counts. Counts of 50 psychrotrophs/ml of milk have been reported immediately after milking (115). Psychrotroph counts ranging from 1700 to 49000/ml were observed for day-old milk. Two day old milk had from 4300 to 71000 psychrotrophs/ml. Psychrotrophic counts of 400000; 2.1 million and 11 million/ml of raw milk stored at 5°C for 1,2 and 3 days, respectively have been recorded (115). With hygienic production conditions and storage at 4.4°C or under, milk can be safely held for 72 hours before processing. With heavy initial contamination and storage at 7°C or

above, a relatively rapid build-up of psychrotrophs and the production of slightly "unclean" or even rancid off-flavors may occur within 48 hours of milking (119,127,137,149).

The increase in number of psychrotrophs is generally not the result of the initial number of psychrotrophs, but rather the presence of actively multiplying psychrotrophs (196). In a study reported by Thomas and Thomas (212), low-count milks had microflora composed of Micrococcus and Corynebacterium species. High-count milks had proteolytic, lipolytic and/or fluorescent pseudomonads present in large numbers (212).

Psychrotrophs in Pasteurized Milk

In several studies, psychrotrophic bacteria counts on freshly processed milk indicated that most of these organisms were destroyed by conventional pasteurization, ultra high temperature (UHT) treatments or laboratory pasteurization (53,129,225,232). In contrast, in studies conducted by Washam et al. (224), Standard Plate Count(SPC) determination of 500 freshly pasteurized milk samples obtained from 8 dairy plants showed counts of <100->10000/ml with 81.6% of the samples having counts of < 1000/ml. Even with UHT milks, SPC results ranged from 9-140/ml, according to Finley et al. (53).

With conventional pasteurized milk samples held at 4.4 or 7.2°C, Psychrotrophic Bacteria Count (PBC) increased from <1/ml to $>10^8$/ml after 10 days at 7.2°C (225). The same commercial pasteurized milk when subject to laboratory pasteurization had a PBC of <1/ml after 10 days at 7.2°C. Findings such as these indicate that post-pasteurization contamination is an important source of psychrotrophs in milk.

Post-pasteurization contamination contributes most microorganisms which eventually grow and spoil pasteurized milk during refrigerated storage. Thomas et al. (192) summarized the psychrotrophic bacterial content of bottled pasteurized milk recorded by different investigators in the U.S.A., Sweden, Denmark and Britain. Colony counts at 5-7°C did not often exceed 100/ml and counts of <10/ml were not uncommon with efficient HTST plant operation. Oliveria and Parmelee (143) found at least 20 psychrotrophs/ml in freshly pasteurized commercial and laboratory milk samples in the United States. One study, using 64 samples of pasteurized milk from nine dairies showed that after 72 hours at 7°C, 67% of the milks contained actively multiplying psychrotrophs (192). Jones and Langlois (97) observed that retail milks had the highest psychrotrophic counts in June and October and the lowest PBC's ocurred in December and March.

However, post-pasteurization contamination is not the only source of psychrotrophs in milk. Research within the last decade has identified two groups of microorganisms which can survive pasteurization temperatures and limit or reduce the shelf-life of milk; namely, spore-forming and nonspore-forming Gram-positive bacteria. Washam (224) identified spore-formers as species of Bacillus and nonspore-formers as species of Corynebacterium and Arthrobacter which survived four exposures at 71.7°C for 16 seconds and then grew differently at 7.2°C as thermoduric psychrotrophs. Tinuoye and Harmon (217) identified thermoduric psychrotrophs as Bacillus subtillis, Lactobacillus casei, Micrococcus flavus and Streptococcus faecalis from laboratory, pasteurized milk. These microorganisms had generation times of 7 to 27 hours at 1.7 to 7.2°C and they could reach sufficient populations cause defects.

With the increased use of high temperature-short-time pasteurization (HTST) and extended refrigerated storage of milk, the spore-forming psychrotrophs may become more important to the dairy industry. HTST pasteurization probably promotes spore germination and under acceptable conditions, outgrowth of spores and vegetative cell multiplication proceed (34). Recent

research has also indicated that some Gram-negative bacteria may survive pasteurization temperatures (178,213,226). Weckbach and Langlois (226) concluded that as the concentration of a psychrotrophic Pseudomonas sp. was increased in raw milk, a greater number of the bacteria survived the heat treatments and that higher temperatures for shorter holding times were less effective than lower temperatures for longer times in destruction of this particular psychrotrophic organism.

BIOCHEMICAL CHANGES CAUSED BY PSYCHROTROPHS AND EFFECTS ON QUALITY OF PASTEURIZED MILK.

Growth of psychrotropic bacteria is primarily responsible for limiting the keeping quality of milk and dairy products held at temperatures below 7°C. They are capable of many biochemical reactions, activities involved in cell synthesis and maintenance, which cause milk spoilage. Slight biochemical changes occur in the early growth phase of psychrotrophs, which result in a "lack of freshness" or a "stale" off-flavor of milk. Upon subsequent cold storage a variety of defects become apparent. Development of these off-flavors and odors is usually a result of proteolysis and/or lipolysis by psychrotrophs. Many researchers have isolated psychrotrophs from milk that have shown lipolysis and/or proteolysis (103,115,126, 142,185,191,193,198,199,222). Shultze (166) reported

after working with 586 cultures of psychrotrophs isolated from pasteurized dairy products that about 90% of the cultures were either lipolytic or proteolytic, and 66% were both.

Psychrotrophic microorganisms may have an indirect, as well as, direct effect on the quality of finished milk products. Indirectly, psychrotrophs produce off-flavors and odors during growth in stored refrigerated raw milk which may carry over into the pasteurized milk even though the organisms fail to survive pasteurization. Directly, organisms that survive pasteurization or that result from post-pasteurization contamination can reduce the shelf-life and the quality of the finished product.

Lipolysis

Numerous psychrotropic microorganisms can produce the enzyme lipase which causes rancid off-flavors and odors in milk, which frequently makes products unacceptable to consumers (41,45,109,110).

Lipolyzed flavors, such as soapy, rancid and bitter, are caused by the enzymatic lipolysis of milkfat into free fatty acids, such as butyric, caproic, caprylic, capric and lauric, by lipase. The liberated short-chain fatty acids are assumed to serve as a substrate for subsequent esterification and

development of fruity off-flavors in milk (161).

Lipase activity was reported by Gelpi (62) for most psychrotrophs isolated from milk, cottage cheese and cream. The lipase activity correlated well with an increase in acid degree value (ADV) and off-flavors.

Lipase production by psychrotrophs varies with the specie, as does the optimum temperature, pH and enzyme specificity (21,22,122,125,138,139,140,154,199).

Phospholipase

Recent research has suggested that phospholipases may be important in milk spoilage. Fox et al. (57) isolated 58 microorganisms, which produced phospholipase C from fresh and spoiled homogenized milk. Most isolates were species of *Pseudomonas*, particularly *P. fluorescens*. These authors postulated that phospholipase degraded the milk fat globule membrane and thus increased the susceptibility of milkfat to the action of lipases.

Proteolysis

Release of various nitrogen components or degradation of individual protein fractions have been observed in proteolysis caused by psychrotrophs. Psychrotrophic bacteria produce proteases which are able to hydrolyze casein and whey proteins, which

generally leads to bitter off-flavor development and coagulation of milk (172,177). Also, fermented or stale off-flavors (with a proteolytic character) have frequently been encountered in aged milk during cold storage (3,173). Unclean and putrid off-flavors and related odors may be attributed to protein breakdown into bitter peptides and further decomposition of amino acids to "putrid end products" that have putrid-like or unclean (dirty) sensory characteristics.

Heat-Resistant Enzymes

Inactivation of lipase and proteases by heat has been important to dairy processors because enzymes that survive pasteurization can be detrimental to the keeping quality of products. Driessen and Stadhouders (46) found that some lipases were inactivated at 52.5 to 57.5°C, but others were heat-resistant (with a D-value of 16 minutes at 130°C). Lipases of Pseudomonas, Achromobacter, and Serratia species were heat-resistant, but those of Alcaligenes and Flavobacterium species were not; according to Stadhouders and Mulder and Thomas and Druce (179,192). Lipases from several psychrotrophs were evaluated for their ability to survive ordinary pasteurization of 72°C for 15 to 20 seconds (45). Results in studies conducted by Kishonti and Sjostrom (104) indicated that 0.3 to 170 minutes were required

before 90% inactivation of the lipases was achieved. Ultra-high temperature (UHT) treatment of about 150°C was necessary to destroy lipolytic and proteolytic enzymes of psychrotrophic bacteria (104).

West et al. (228) reported that the best method of inactivating heat-resistant proteases was to heat for 1 hour at 55°C, since this resulted in inactivation of 87 to 90% of the protease in milk. No enzyme reactivation was observed after this treatment. Barach et al. (7) investigated the possibility of using low temperatures to inactive proteases since these enzymes were stable to high temperatures. Heating purified protease from Pseudomonas species for 10 minutes at 55°C destroyed more than 90% of the proteolytic activity.

VARIOUS METHODS FOR ENUMERATION OF PSYCHROTROPHS AND PREDICTION OF MILK KEEPING QUALITY

The keeping quality of refrigerated pasteurized milk is largely determined by the number and activity of the psychrotrophic bacterial population of milk.

The present accepted method for determination of psychrotrophs requires incubation at 7°C for 5 to 10 days. (118). Bauman and Reinbold (10) examined differences in psychrotrophic bacteria results obtained by incubation at 5 or 7°C for 7 to 10 days. Incubation

at 7°C for 10 days apparently produced the highest counts, but this may not be the best enumeration procedure. One disadvantage of the "standard" method is that it requieres a relatively long time and the product may already have been consumed before the bacterial count was consumated. On the other hand, initial bacterial counts of various types have proven to be of limited value in predicting the keeping quality of milk (15,54,76,93,108, 121). The ideal shelf-life prediction test should take into account: (1) the type of psychrotrophic contaminants; (2) rate of increase of psychrotrophs during storage; and (3) the psychrotrophic bacterial "activity" in producing metabolites responsible for spoilage. Due to this limitations, other methods have been recommended for ascertaining psychrotrophic counts and/or the prediction of milk shelf-life.

Incubation at Elevated Temperatures

Recommendations have been made for incubation of psychrotrophic plates at temperatures higher than 7°C in an effort to reduce the time necessary to obtain results. (Table 1).

Table 1. Various recommended elevated temperatures for enumeration of psychrotrophic bacteria.

Investigators	Conditions
Waes (223)	16 hours at 17°C plus 3 days at 7°C
Lück (115)	2 days at 27 or 32°C
Juffs (99)	24 hours at 15°C plus 72 hours at 5-7°C
Luck and Hopkins (116)	3 days at 15°C or 2 days at 15°C plus 1 day at 7°C or 1 day at 17°C plus 3 days at 7°C
Oliveria and Parmelee(143)	25 hours at 21°C
Griffiths et al. (66,67)	25 hours at 21°C
McKellar and Oehlrich(123)	45 hours at 18°C

Preliminary Incubation

Various pre-incubation procedures have been closely studied in an effort to substantially reduce the time required for estimation of psychrotrophic bacteria in dairy products. The Moseley keeping quality test is widely used in the U.S. fluid milk industry to determine

shelf-life in lieu of detecting specific populations of microorganisms (14,118,136). Milk and cream samples are stored at 7.2°C for 5,7 or 10 days before plates are incubated at 32°C (136). The milk also may be flavored (taste and/or aroma assessment) after 5,7 or 10 days storage at 7.2°C. Bishop and White (12) recently concluded that the Modified Psychrotrophic Bacteria Count (MPBC) at 21°C for 25 hours (after the milk has undergone preliminary incubation of 14 hours at 21°C) was the best bacterial enumeration indicator of potential shelf-life ($r=-0.782$).

Other pre-incubation procedures for detection of psychrotrophic bacteria have been suggested (Table 2).

Table 2. Suggested pre-incubation procedures for detection of psychrotrophic bacteria

Investigators	Conditions
Johns (96)	18 hours at 13°C followed by microbiological testing
Cuperus et al. (39)	24 hours at 25°C followed by inoculation onto plate count agar containing benzalkon A-50%
Bishop and White (12)	14 hours at 21°C
Griffiths et al. (68)	25 hours at 21°C followed by plate counts on selective agar
Oliveria and Parmelee (143)	15 hours at 21°C followed by a dye reduction test.

Parmelee(143) and Bodyfelt (17) have suggested the use of media enrichment (trypticase soy broth) to expedite the maximun effectiveness of preliminary incubation procedures.

Selective Media with Inhibitors or Dyes

Antibiotics, dyes and other chemicals have been used by various investigators to inhibit Gram-positive

bacteria in milk samples in an effort to measure or monitor Gram-negative psychrotrophs. Freeman et al. (59) tested 32 different dyes and 26 chemicals for their ability to inhibit Gram-positive bacteria. Only five chemicals were considered to be effective: sodium desoxycholate, alkyl dimethyl benzyl ammonium chloride, methyl dodecyl trimethyl ammonium chloride, alpha-bromo-lauric acid and alpha-bromo-myristic acid.

Many investigators have experimented with media containing penicillin for the isolation or detection of spoilage microorganisms in milk and cream (112, 113,114,115,186). Waes (223) observed that Plate Count agar containing 1 I.U. penicillin, and incubated at 25°C for 3 days was an effective rapid method for enumeration of psychrotrophs in pasteurized milk.

Brant et al. (20) tried several media containing various Gram-positive bacteria inhibitors such as triphenyltetrazolium chloride, crystal violet, nacconol, wetting agent, cetrimide, diamide and other chemicals. These workers questioned the merits of employing a singular selective medium considering the nonhomogeneous psychrotrophic flora found in most milk samples.

Various methods for enumerating psychrotrophs have been based on use of media that incorporate dyes to inhibit Gram-positive bacteria (60,115,116,147,148, 176). Antopol et al. (2) found the ditetrazolium

salts: (1) neotetrazolium chloride (NTC), (2) blue tetrazolium (BT), and (3) nitro-blue tetrazolium (NBT) to be more effective as selective bacterial growth inhibitors than the monotetrazolium salts (triphenyltetrazolium chloride (TTC) and iodo-nitrotetrazolium chloride (INT). In the Antopol et al. study, Gram-negative organisms seemed more resistant to the inhibitory effects of all tetrazolium salts tested. These researchers also reported an inverse relationship between the inhibition of bacterial growth by all the tetrazolium salts tested and the reduction of these compounds to their respective formazan.

In Europe, violet-red bile agar has been used for estimating the psychrotrophic count of milk and cottage cheese (206). Foung and Miller (60) found that bromothymol blue, safranin O, janus green and methylene blue allowed Gram-negative bacteria to grow, but inhibited Gram-positive microorganisms. In this study, crystal violet was found to inhibit Pseudomonas and Alcaligenes species and E. coli, as well as Gram-positive bacteria.

Cuperus et al. (39) developed a method for limiting the growth of Gram-positive bacteria which correlated fairly well with shelf-life observations conducted at 7°C, after milk samples were pre-incubated at 25°C for 24 hours. In this study selective counts were obtained by addition of benzalkon A-50% to Plate Count agar.

Smith and Witter (176), in an evaluation of 17 inhibitory chemicals, concluded that only crystal violet and neotetrazolium chloride at 2mg/l proved reliable for the selective enumeration of psychrotropic bacteria. Griffiths et al. (68) reported two methods which could be employed with equal accuracy for the prediction of cream shelf-life. In the first method, samples of cream were pre-incubated at 21°C for 25 hours and selective counts were obtained by addition of the following Gram-positive inhibitors: crystal violet, penicillin G and nisin to the counting media. In the second procedure, the inhibitors were added directly to the milk sample prior to the pre-incubation step.

Dye Reduction

Various dye reduction methods have an extensive history of use as an indirect method of measuring microbial population of milk (115). Basically, these tests measure the metabolic activity of microorganisms and only serve as an index of microbial loads. Previously, the methylene blue test had been used for many years as an indicator of bacterial activity; as such it gives a misleading indication of milk

products keeping quality (24,115). This method is not considered applicable for monitoring psychrotrophic bacterial activity since many psychrotrophs do not reduce methylene blue. The resazurin test has been suggested as a way to predict keeping quality; however, it suffers from some of the same shortcomings as the methylene blue test (115).

Catchick and Gibson (31) developed a 16-hour test for detecting post-pasteurization contamination, which was based on resazurin dye and an overnight (16 hours) incubation with sodium desoxycholate at 0.5% concentration. Parmelee (155) modified this method by conducting the sample incubation at 32°C instead of 37°C. Parmelee's version of the resazurin test failed to detect 17.6% of milk samples, which were "moderately contaminated" after pasteurization. This test detected only 8.3% of milk samples which were known to have a slight degree of post-pasteurization contamination. Olson (145), proposed a rapid method for shelf-life prediction which included pre-incubation of milk sample at 21°C for 16 hours and use of resazurin. Zall et al. (235) reported that pre-incubation of milk samples for 5 hours at 30°C prior to adding reducing dyes eliminated the effect of somatic cells in reducing resazurin.

Bodyfelt (17) proposed a neotetrazolium chloride-resazurin method (NTC-R) for shelf-life prediction of pasteurized milk. Parmelee (156) advocated a modified resazurin test which was claimed to have an accuracy of about 80% in detecting samples (within 16 hours) that develop psychrotrophic counts in excess of 1 million/ml after 10 days storage at 7.2°C. Parmelee indicated an accuracy of about 95% in predicting high psychrotrophic counts when a 15 hour pre-incubation of the sample was employed prior to conducting a 16-hour dye reduction test.

Some studies have shown that a triphenyltetrazolium chloride medium is more sensitive than methylene blue or resazurin for detecting psychrotrophs in raw milk (2) and that triphenyltetrazolium chloride is also more sensitive than resazurin when a series of pure psychrotroph cultures were used as test organisms (182). Triphenyltetrazolium chloride has been used most frequently of the various dyes to measure metabolic activity of microorganisms since it appears to be less toxic than the other tetrazolium salts (227).However, in several studies, p-iodonitrotetrazolium violet (INT) has indicated that it is more rapidily reduced than other tetrazolium salts (101,4).

Bartlett et al. (8) found conditions which permitted a more rapid recognition of bacterial growth

than had been previously reported using various tetrazolium salts. Final concentrations of 1.0 mg of iodo-nitrotetrazolium chloride (INT) per ml of sample and 0.06 mg. per ml of phenazine methosulfate (PMS) (an intermediate electron carrier) potentiated the reduction of the tetrazolium salt.

Blankenagel (14) reported that pre-incubation for 18 hours at 25°C, served to coincidentally eliminate any heat-activated reducing substances (-SH groups), that could affect results of dye reduction tests.

Detection of Metabolites

Through metabolic activity, microorganisms that grow in milk produce either slight or marked changes in the carbohydrates, proteins and milkfat. Hence, some research efforts have been directed at detection of these metabolites or components that result from such metabolic activity. Analytical techniques are available which are capable of measuring trace amounts of metabolites or increases in their concentration within short time periods. Samples can be specially prepared, analyzed and evaluated with adequate sensitivity and reproducibility (83) and hence suffice as a possible method for detecting extremely low levels of psychrotrophs in milk.

Heeschen (82) and Heeschen et al. (83) concluded that changes in pyruvate concentration can be used to detect the presence of microorganisms in milk since pyruvate is a key metabolite produced during degradation of carbohydrates, proteins and milk fat. Pyruvate is present at a concentration of about 1.5 ppm in normal milk. Tolle et al. (218) reported that only levels of 1×10^5 to 1×10^6 psychrotrophs/ml could be detected when the pyruvate concentration exceeded 1.5 ppm. Harmon and Marshall (79) reported that use of a pyruvate difference test (pyruvate concentration after incubation minus pyruvate concentration before incubation) was required to predict shelf-life since some microorganisms can metabolize pyruvate. This study left many unanswered questions regarding the applicability of the automated pyruvate method to Grade A raw milk. These investigators also suggested that psychrotrophs are variable in their ability to produce pyruvate. Zandstra and deVries (236) questioned the use of the pyruvate test for refrigerated milk, since it does not provide an estimation of the number of contaminants in milk due to delayed metabolic activity at refrigeration temperatures.

Bassette et al. (9) characterized the bacteria in milk samples by measuring volatile compounds that resulted from microbial end products with a gas liquid

chromatograph (GLC). Pierani and Stevenson (158) noted that acetaldehyde may be the best indicator of the various chemicals detected by GLC as the consequence of large populations of psychrotrophs in milk. However, in this study, no differences were observed between controls and treatments until counts of 1×10^5 to 1×10^6 psychrotrophs/ml were attained. Vasavada and White (221) screened selected psychrotrophs for their ability to reduce diacelyl, but this method might be of limited value in detecting presence of psychrotrophs in milk products.

The B-D glucose absorption test was developed to further measure Gram-negative bacterial counts since it had been established that glucose is readily absorbed by many psychrotrophic bacteria (73,233). Clark (32) evaluated the procedure with some minor changes, described by Raabo and Terkildsen (160). The B-D glucose absorption test showed poor sensitivity for differentiation of serial dilutions of psychrotrophic bacteria.

Clark (32) also experimented with a carbon-14 labeled 2-deoxyglucose affixiate test in an attempt to measure Gram-negative bacteria within a short period of time. This method showed good sensitivity for differentiation of serial dilutions of bacteria but it was considered too expensive as a practical

procedures for industry to use in shelf-life monitoring.

The Limulus amoebocyte lysate test (LAL) has been demonstrated to be specific and highly sensitive to bacterial endotoxins that are associated with Gram-negative bacteria (29,98). Clark (32) found this to be a potential method for detection and estimation of psychrotrophic bacteria in fluid milk due to its apparent sensitivity, definitive endpoint determination, but it appeared to be too expensive method to be used by the milk industry. Mikolajcik and Brucker (128) reported that LAL was a rapid (1 hour), sensitive (1000-10000 psychrotrophic bacteria/ml) and accurate (r=0.9851) method to indicate the number of psychrotrophic bacteria present in milk samples. Bishop and White (12) found that endotoxin (lipopolysaccharide) level was a definite indicator of potential shelf-life (r=-0.896). The same investigators found that protease activity was related to potential shelf-life (r=-0.474) but it did not appear to be a reliable initial indicator. Proteolysis was certainly a cause of reduced shelf-life, but not a viable predictor.

In Europe, a highly specific enzyme that is associated with the cell surface of Gram=negative bacteria is being investigated to assess psychrotrophic load

in foods (151). This enzyme, amino peptidase, is not present in Gram-positive bacteria.

Rapid Screening

To eliminate the lengthy incubation periods for facilitating psychrotrophic bacteria counts, work has centered on rapid methods for obtaining counts. One method involves a membrane filter technique to enumerate Gram-negative bacteria. Filters are placed on yeast extract glucose medium and then incubated at 21°C initially for 12 hours, followed by incubation for 72 hours at 10°C. Colonies are stained with methylene blue and observed microscopically (10).

The direct epifluorescent filter technique (DEFT) allows enumeration of organisms with counts in the range of 5×10^3 to 1×10^8 bacteria/ml in milk within approximately 30 to 60 minutes. However, this method reflected a relative insensitivity having a lower limit of 5.75×10^3 per gram, for detection of bacteria in pasteurized cream (69). A significant correlation ($r=0.72$) was obtained between the ability of a cream to withstand storage at 6°C for 7 days and the counts obtained using the DEFT technique after pre-incubation of cream samples containing crystal violet-penicillin-nicin at 21°C for 25 hours (69). Rodriguez and Pettipher (163) found that a single DEFT

count made on a sample of pasteurized milk pre-incubated with inhibitors for 18 hours at 30°C can give a good indication of the probable keeping quality of the product at 5°C. Results also showed that a single DEFT count made on a sample of pasteurized milk (pre-incubated without inhibitors at 20°C) can provide a satisfactory indication of the probable keeping quality of the milk at 11°C.

Growth and metabolism of microorganisms causes unique and significant changes in the electrical impedance of the medium in which they are growing. This is due to changes in the chemical composition of the medium as ionized end products are produced through metabolic conversion of nutrients. Cady et al. (26) noted that data collected from automated impedance measurement could possible provide a 9 to 14 hour impedance-based test for keeping quality of milk. Bossuyt and Waes (19) investigated whether impedance measurements are suitable to detect post-pasteurization contamination of milk and showed impedance measurement to be useful for this objective.

Bishop et al. (13) proposed a rapid impedimetric method for determining the potential shelf-life with results available in 25-38 hours after processing (18 hours preliminary incubation plus 7 - 20 hours impedance detection). Griffiths, and Phillips (65)

developed a method to detect post-heat treatment contamination by monitoring impedance charges of cream containing inhibitors for the growth of Gram-positive organisms using Standard Plate Count agar at 21°C. Bishop and White (12) concluded that impedance detection with a modified crystal violet-triphenyltetrazolium chloride agar was a reliable indicator of potential shelf-life (r=0.906) and could be used to "categorize" milks as to their potential qualities. A simple prediction equation for shelf-life of pasteurized fluid-milk was also proposed as follows: Shelf-life (days) =0.560 + 1.400 (IDT) - 0.032 $(IDT)^2$, where IDT = impedance detection time.

It has been suggested that the ATP concentration of milk and other foods can be related to bacterial concentration. Bossuyt and Waes (18) developed a rapid method to detect post-contamination in pasteurized milk. Milk was incubated for 24 hours at 30°C with benzalkon A-50% and crystal violet as Gram-positive inhibitor and then the bacterial ATP content was determined. ATP can be easily measured, as light is emitted when an enzyme-substrate complex (luciferin-luciferase), which is obtained from fireflies, is reacted with ATP. Griffiths et al. (70) found a method which combines preliminary incubation (21°C for 25 hours) with ATP photometry allowing detection of

post-heat treatment contaminants in dairy products within 26 hours of manufacture.

Another screening method used catalase activity as an indicator of psychrotrophs, since many of these microorganisms are catalase positive (81). Two ml of a hydrogen peroxide solution were added to milk which was then incubated for 2 hours at 25°C, then 2ml of 70% TCA were added and finally the percentage of hydrogen peroxide that was degraded was determined. A microfilter count method was modified for use in enumerating mesophiles, psychrotrophs and coliforms in raw and pasteurized milk (30), this method required less time, media and space than did standard methods.

Comparison of Various Methods

Many tests have been developed in an effort to determine the microbial quality and shelf-life of milk and dairy products. Since most of these tests have specific advantages and disadvantages, dairy processors have doubts or are confused about the best method to use for their operations. Recent reviews have been published on the various methods that can be used (14,36,80,150, 200,204,229) and comparisons of some of these methods (33, 52,147,148,201,207,230,231). Blankenagel (14) proposed the following criteria for the ideal test to detect keeping quality of pasteurized milk. The

ideal test should be accurate and provide an indication of the exact number of pertinent microorganisms in the product(including a differentiation between thermoduric and contaminants), provide results rapidly, preferably within a day and be simple to perform and economically feasible.

Hartely et al (80), after reviewing various methods concluded that no single test could probably determine everything that is necessary to assess the microbiological quality of milk and other perishable dairy products.

The Moseley test has generally demonstrated a relatively high correlation to shelf-life, and is currently the industry method of choice, since its first introduction in the 1950's. Bishop et al. (13) obtained a correlation coefficient of -0.84 for the relationship between the Moseley test and actual product shelf-life. Felmingham and Juffs (52) found that the Moseley test correlated closely with initial psychrotrophic counts and organoleptic data after storage and was more sensitive and reliable than the methylene blue keeping quality test for determining the shelf-life of milk products. The Moseley test did not correlate well with the test of Oliveria and Parmelee (143), Blankenagel's inhibitory test (14) or coliform counts. The Moseley test compared favorably with a modified 4-day Moseley test, the inhibitory

test of Freeman et al. (59), and sensory evaluation.

Griffiths et al. (67) found an excellent correlation (r=0.97) between psychrotrophic counts conducted at 6°C for 14 days and at 21°C for 25 hours and Oliveria and Parmelee (143) obtained a correlation coefficient of 0.996 between counts obtained by the Standard Psychrotrophic Bacteria Count (7°C - 10 days) and counts obtained by the 21°C - 25 hours. Bishop and White (12) found that none of the direct counts, Standard Plate Count (SPC), Psychrotrophic Plate Count (PBC) and Modified Psychrotrophic Bacteria Count (MPBC) (21°C - 25 hours after preliminary incubation 21°C - 14 hours) correlated well enough with actual shelf-life determination to allow keeping quality prediction. Impedance detection times at 21°C and 18°C proved to have the most significant relationship to shelf-life, with correlation coefficient of 0.88 and 0.87, respectively. Therefore, the impedance method was found to have three distinct advantages over the Moseley test: (1) it was a better predictor of shelf-life; (2) it was less labor intensive; and (3) it required only 1-2 days to complete as opposed to 7-9 days for the Moseley procedures.

Griffiths et al. (69) found an acceptable correlation (r=0.91) when the numbers of bacteria in pre-incubated cream are enumerated by plate count (21°C - 25 hours)

and by the DEFT. A correlation of r=0.72 was obtained between the ability of cream to withstand storage at 6°C for 7 days and the counts obtained using the DEFT after pre-incubation of cream sample containing crystal violet, penicillin and nisin at 21°C for 25 hours.

Griffiths et al.(70) found that the ATP photometry method had better repeatibility than the Direct Epifluorescent Technique (DEFT) and plate count methods and had better reproducibility than the DEFT count.

EXPERIMENTAL

SAMPLE COLLECTION AND STORAGE

Samples of pasteurized milk products were collected from various stores and dairy processing plants in the vicinity of Oregon State University. One-half gallon and quart sized paperboard containers were most frequently obtained, but in a few instances, one gallon, pint and 1/2-pint paperboard containers were sampled. Also gallon and 1/2-gallon plastic containers were sampled. Although the majority of products were whole milk products; skim milks, low-fat milks and half-and-half products were also tested.

Milk samples were packed in ice and transported to the laboratory where they were stored at 1-3°C until analysis commenced.

To ensure a wide range of potential shelf-life for the milk samples obtained, several procedures were followed: (1) Milk samples were collected that indicated different ages at the time of purchase (1-15 days). (2) Samples were stored at 7.2°C for 0 to 7 days, then subjected to analysis at day "zero". Day "zero" corresponding to the actual date a particular carton was opened, and evaluation initiated after storage. (3) Spoiled milks which were held several days at 7.2°C were used to inoculate fresh samples to obtain

various degrees of psychrotrophic contamination. (4) Appropriate milk samples were pasteurized in the laboratory by heating, in a sterile 200 x 25mm screw cap test tube at 62.8°C (145°F) for 30 minutes or 71.6°C (161°F) for 15 seconds (water baths with continuous shaking).

Milk samples were refrigerated in combined water-ice baths during laboratory work.

MICROBIOLOGICAL PROCEDURES

Standard Plate Counts (SPC) and Psychrotrophic Bacteria Counts (PBC) were determined by the methods recommended by the American Public Health Association (118). Modified Psychrotrophic Bacteria Counts (MPBC) were determined by the procedure described by Oliveria and Parmelee (143); plates were prepared by the same methods used for the SPC, but plate incubation was at 21°C for 25 hours.

SPC agar (DIFCO Laboratories) was used to enumerate bacterial populations as appropriate. Phosphate buffered distilled water (118) was used in preparing the dilution blanks for preparing the 10^2 to 10^6 - fold dilutions.

PRELIMINARY INCUBATION PROCEDURES

Various pre-incubation (PI) procedures were evaluated exhaustively in an effort to reduce the time required for estimation of psychrotrophic bacteria.

Additionally, these time periods served to help eliminate some heat-induced reducing substances(-SH groups) that could interfere with the results of dye reduction tests (14). The various PI periods tried were: (1) incubation at 30°C for 5 hours (235), (2) incubation at 21°C for 16 hours (12), (3) incubation at 21°C for 25 hours (68), (4) incubation at 21°C for 21 hours , and (5) incubation at 30°C for 16 hours. (17).

In as much as PI of those milk samples that demonstrated extended shelf-lives could permit any Gram-positive organisms to grow rapidly and hence provide a false indication of Gram-negative bacterial contamination, chemical inhibitors were incorporated to prevent unwanted outgrowth of Gram-positive bacteria. The Gram-positive bacterial inhibitors evaluated here were: (1) sodium desoxycholate, (0.5%)(31,59,155,156), (2) neotetrazolium chloride, NTC, (0.0002%) (17,176), (3) tetrazolium violet, TV, (0.0002%), (4) tetrazolium blue chloride, TBC, (0.0002%), triphenyltetrazolium chloride, TTC, (0.0002%), and (6) a combination of two different tetrazolium salts (0.0002-0.0004%).

Microbial growth enhancers for Gram-negative bacteria were also used: (1) trypticase soy broth, (0.4%, 0.5%,0.6%,1.0%), (2) brain heart infusion (0.5%), and (3) yeast extract (0.5%).

Since most of the psychrotrophic bacteria present in milk are aerobic, several conditions for the PI test

were analyzed: (1) shaking a 20 ml milk sample in a screw cap tube, (2) shaking 20 ml milk in a test tube sealed with 0.45 μm filter paper, and (3) quiescently held milk samples(20 ml). All samples, were placed in 200 x 25 mm test tubes.

METHODS USED TO COMPARE THE EFFICIENCY OF DYE REDUCTION TEST

Moseley Keeping Quality Test

This commonly used test (45,136) consists of storing containers of milk products at 7.2°C for 5, 7 or 10 days and subsequently subjecting samples to a SPC at 30°C ± 2°C for 48 hours. Milk samples may also be tasted after 5, 7 or 10 days at 7.2°C.

Shelf-life Determination

The shelf-life of each milk sample was determined by sensory evaluation conducted daily by three experienced judges. When a given sample was considered unpalatable by taste or odor, the "previous day" was considered to be the termination point of the shelf-life of the sample. Numerical scores were assigned to indicate the intensity of psychrotrophic flavor. The numerical values were assigned as follows to reflect the degree of milk spoilage (psychrotrophic off-flavor): 0 = none; 1 = questionable; 2 = slight; 3 = definite or distinct;

4 = strong or intense. This scheme of assigned numerical values was taken from the scoring system used by Dunkley and Franke (47) for determining the extent of oxidized off-flavor in milk samples. Numbered samples were presented to the judges in random order at 7.2°C in portions of about 10 ml served in 50 ml beakers. Daily assessment of flavor characteristics of each sample provided a reliable method to determine the actual point of shelf-life termination.

DYE REDUCTION TESTS

Parmelee Tube Test for Psychrotrophs

The Parmelee tube test (PTT) (156) was conducted by the following procedure: 9 mls of each milk sample were added to a 200 x 25 mm screw cap test tube that contained 1 ml of 5.5% sodium desoxycholate solution and 1 ml of 6% trypticase soy broth. To this mixture 1 ml of 0.005% resazurin was added and mixed by inversion. The sample and additional materials were incubated at 30°C ± 2°C for 16 hours.

Observation of color changes provided an interpretation as to possible relative psychrotrophic bacterial populations as follows:

Table 3. Interpretation of the color endpoints of the Parmelee tube test.

Numerical Value	Color Change	Indication of Keeping Quality (KQ)
0	None (Blue)	Good potential KQ Low numbers or no psychrotrophs
1	Violet	Possible KQ problems Low to moderate psychrotrophs
2	Dark Pink	Fair to poor KQ Moderate psychrotrophs
3	Light Pink	Poor KQ High numbers of psychrotrophs
4	White	Poor KQ Extremely high number of psychrotrophs

The key method employed to evaluate the effectiveness (or reliability) of the Parmelee tube test and other shelf-life prediction test was the 7-day Moseley keeping quality test. Negative control milks were pasteurized in the laboratory at 71.6°C for 15 seconds; positive control milks were held at 7.2°C for several days. When this method was used as a control, 20 ml and 50 ml size milk samples were used for the dye

reduction tests; added reagents were increased in quantity to maintain the original proportions of milk and added reagents.

Tetrazolium Salt - Resazurin Test

The neotetrazolium chloride-resazurin (NTC-R) method for shelf-life prediction of pasteurized milk as proposed by Bodyfelt (17) was also evaluated. Two ml of sterile 10% trypticase soy broth (TSB) were added to 200 x 25 mm screw cap test tubes. One ml of 0.01% neotetrazolium chloride and 50 ml of each milk sample were added to each test tube. Two ml of 0.005% resazurin solution were then added and mixed by inversion. Samples were incubated at 30°C ± 2°C for 16 hours, tubes were inverted once, and the color endpoints recorded. In some analyses 20 ml of milk sample were used instead of the 50 ml quantity.

Observations for color changes provided interpretation of the possible relative population of psychrotrophic bacteria, as summarized in Table 4.

Table 4. Interpretation of the color endpoints of the neotetrazolium chloride-resazurin test.

Numerical Value	Color Change	Indication of Keeping Quality (KQ)
0	Purple	Good potential KQ Low numbers or no psychrotrophs
1	Violet	Possible KQ problems Low to moderate psychrotrophs
2	Dark Pink	Fair to poor KQ Moderate psychrotrophs
3	Light Pink	Poor KQ High numbers of psychrotrophs
4	White	Poor KQ Extremely high number of psychrotrophs

Other tetrazolium salts were examined following the same procedure employed for the NTC-R method: (1) tetrazolium blue chloride (TBC), (2) tetrazolium violet (TV), (3) triphenyltetrazolium chloride (TTC), and (4) a combination of two different tetrazolium salts.

Different time frames for determining the optimum color endpoints were also studied. Color development

within the sample tubes was observed over a time period of 0 to 16 hours, starting with addition of the dye, in an effort to find the optimum endpoint for the best prediction of shelf-life. The NTC-R method, as well as all tetrazolium salts and combination were tested for determination of the optimum color endpoint (5).

The 7-day Moseley keeping quality test was used as the standard procedure for testing comparative numbers and/or outgrowth of psychrotrophic bacteria in pasteurized commercial milk samples.

Tetrazolium Salt Test

The employed procedure was based on that reported by Bodyfelt (17), but the addition of resazurin was eliminated. The added tetrozolium salt(s) acted as both an inhibitor of Gram-positive bacteria and as well as a redox potential indicator. The test objective was to eliminate the effect of two added dyes (resazurin and tetrazolium salt) changing color in opposite directions. Resazurin changes from blue to colorless; by contrast several of the tetrazolium salts change from colorless to either red, violet or blue, depending on the given tetrazolium salt.

Two ml of sterile 10% TSB were added to a 200 x 25 mm screw cap test tube. One ml of 0.01% tetrazolium salt and 50 ml of milk sample were added to each test tube. It was capped, mixed by inversion and

incubated at 30°C for 16 hours. Following incubation, tubes were inverted once and the observed color was recorded.

Observations of color changes in the tubes provided an indication of the relative population of psychrotrophic bacteria (summarized in Table 5).

Table 5. Interpretation of the color endpoints of the tetrazolium salt test.

Numerical Value	Color Change	Indication of Keeping Quality (KQ)
4	Dark(Purple, pink,violet, or blue)	Poor KQ. Extremely high numbers of psychrotrophs
3	Medium (Purple,pink, violet or blue)	Poor KQ.High numbers of psychrotrophs
2	Light(Purple, pink,violet or blue)	Fair to poor KQ. Moderate psychrotrophs
1	Very light (Purple,pink, violet or blue)	Possible KQ.problems. Low to moderate psychrotrophs
0	White	Good potential KQ. Low numbers or no psychrotrophs

Modified Parmelee Tube Test (MPTT)

The procedure was essentially that reported by

Parmelee (156) but a tetrazolium salt is used as the redox potential indicator to assess the relative populations of psychrotrophic bacteria in milk samples. Fifty ml milk samples were used. The interpretation of color changes was based on the scheme shown in Table 5.

p - Iodonitrotetrazolium Violet - Phenazine Methosulfate Test (INT-PMS Test)

Bartlett et al. (8) and Sandine (164a) have reported methods or conditions which permit a more rapid indication of bacterial growth than has been previously reported using the tetrazolium salts. They used p-iodonitrotetrazolium violet (2-(4-iodophenyl) - 3 (4-nitrophenyl)-5-phenyltetrazolium chloride; INT)as a redox potential indicator of microbial growth. Phenazine methosulfate was added to accelerate the reduction of the tetrazolium salt.

The basic principles involved in the INT-PMS test are summarized in Figures 1 and 2. Four different types of oxidation-reduction enzymes or electron transfering proteins participate in the electron transport from organic substrates to molecular oxygen. These substances are: (1) pyridine linked dehydrogenases: NAD or NADP as coenzymes; (2) flavin linked dehydrogenases: FAD or FMN as prosthetic groups; (3) iron-sulfate containing proteins and (4) cytochromes.

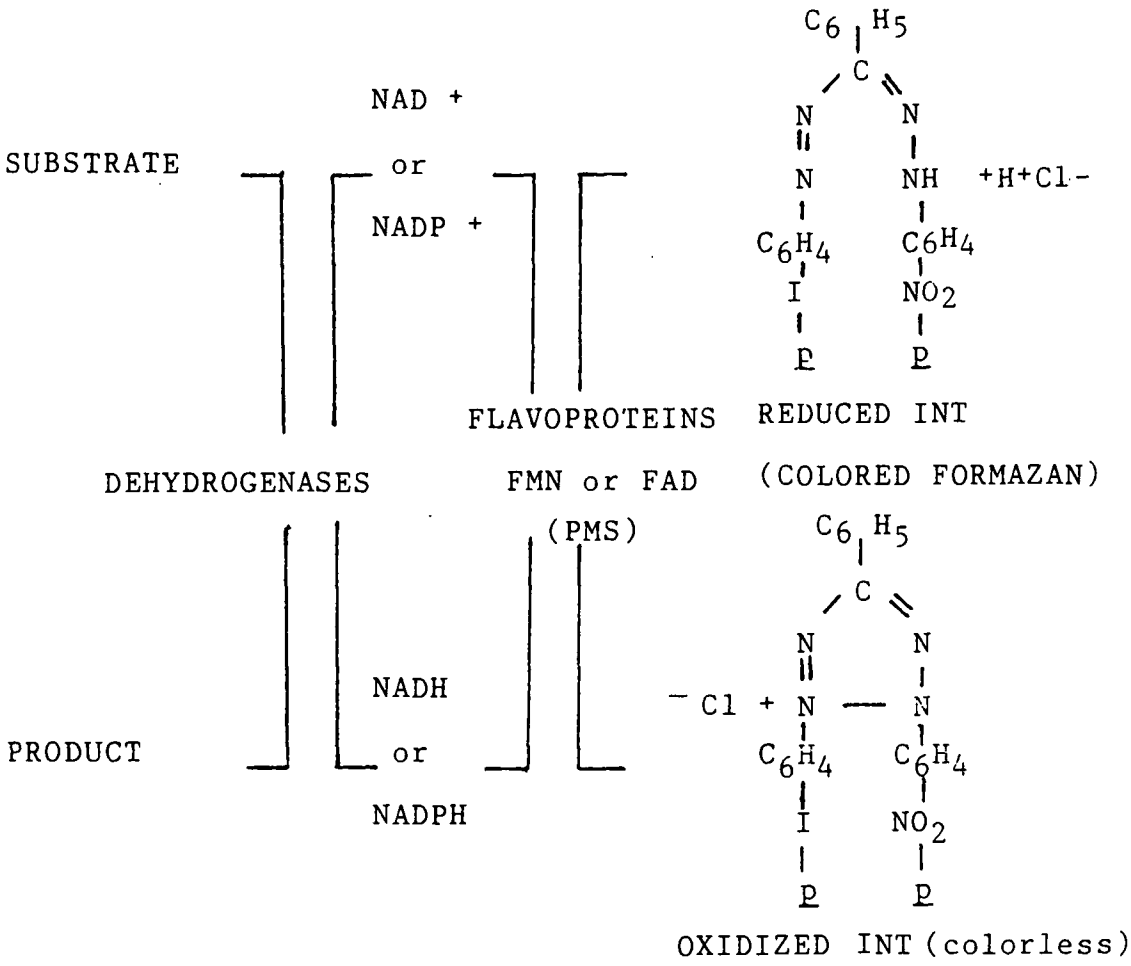

Figure 1. Basic principles involved in the INT-PMS test.

Phenazine methosulfate is an artificial electron carrier-acceptor from flavin dehydrogenases according to the equation $E\text{-}FADH_2$ + PMS oxidized = E-FAD+ PMS reduced.

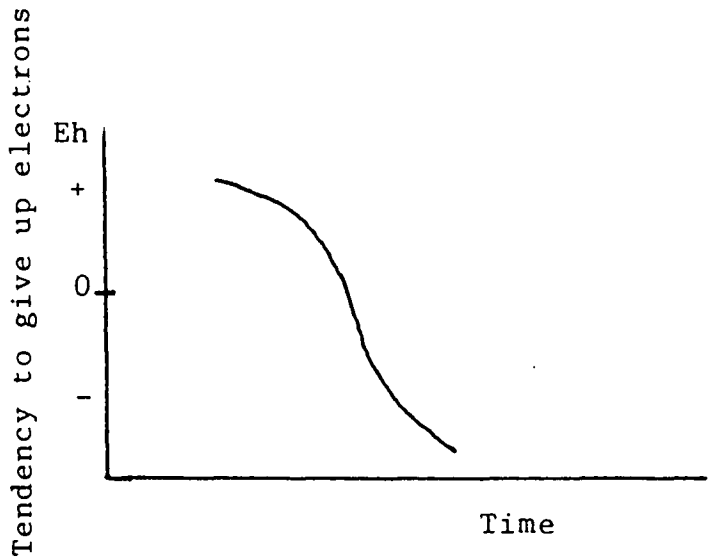

Figure 2. Electrons flow in the reduction of INT.

Electrons flow in the direction of the more positive Eh to the more negative Eh. Initial milk sample has a high positive Eh; when microbial growth occurs there is a reduction of the oxidation-reduction potential and the tetrazolium salts are reduced; signified by a color change.

In this study INT-PMS test was conducted according to the following procedures:

Sample Pre-incubation: Samples were subjected to PI before the dye reduction tests were conducted. Preliminary incubation conditions that were studied include: 21°C for 16 hours, 21°C for 21 hours and 21°C for 25 hours. Twenty mls of each milk sample were added to sterile screw cap culture tubes (200 x 25 mm) and placed in a "wrist action" shaker, set at position 1, of a 1-10 scale.

Milk Clarification: Forty mls of heat sterilized 0.6% sodium citrate (6 g dihydrate crystals in 1000 ml distilled water) were aseptically added to each test milk sample for the purpose of reducing the opacity of milk and providing a more transparent-like solution. Each tube and contents were agitated on a vortex mixer for 30 seconds and allowed to rest quiestently for 2 minutes. The sodium citrate was maintained at a temperature comparable to the atmosphere in which the dye reduction test was conducted (either 21°C or 30°C).

Dye Reduction Test: After the milk samples were pre-incubated and clarified, the dye reduction test was conducted. Filter sterilized phenazine methosulfate (1.2 ml of 0.05%) solution (0.05 g PMS in 100 ml distilled water) and 1.2 ml filter sterilized 0.2% p-iodonitrotetrazolium violet (0.2 g INT in 100 ml distilled water) were added to citrate clarified milk samples. Immediately, after addition of the dye, all samples were protected from any sources of light by using a black heavy cloth to cover the reaction tubes; this minimized the occurence of any light initiated reducing action on the dye in the milk samples. The protected samples were placed in a water bath at 21°C (or 30°C for some trials) for either 10, 20, 30 or 40 minutes. The samples were mixed by inversion and the observed color that developed recorded and categorized

according to the Dictionary of Color (117).

Observation of color changes provided an interpretation of the relative population of psychrotrophic bacteria as follows:

Table 6. Interpretation of the color endpoints of the INT-PMS test.

Numerical Value	Color Change	Indication of Keeping Quality
0	PL2A1 (White)	
1	PL2B1	
2	PL2C1	Variable depending on
3	PL2D1	preliminary incubation
4	PL2E1	conditions, temperature
5	PL2F1	of the dye reduction
6	PL2G1	test and time to read
7	PL2H1	the color endpoint
8	PL3I2	(Figures 3,4,5,6,7
9	PL2I1	and 8)
10	PL2J1	
11	PL3K8	
12	PL4L8	

Table 7. Interpretation of the INT-PMS test. Classification of milk samples to their relative level of post-pasteurization contamination.

Milk Category	Probable Level of Post-pasteurization Contamination
I	Low or no post-pasteurization contamination. Expected shelf-life greater than 14 days.
II	Minor to intermediate post-pasteurization contamination. Expected shelf-life between 8 and 14 days.
III	Serious post-pasteurization contamination. Expected shelf-life 7 days or less.

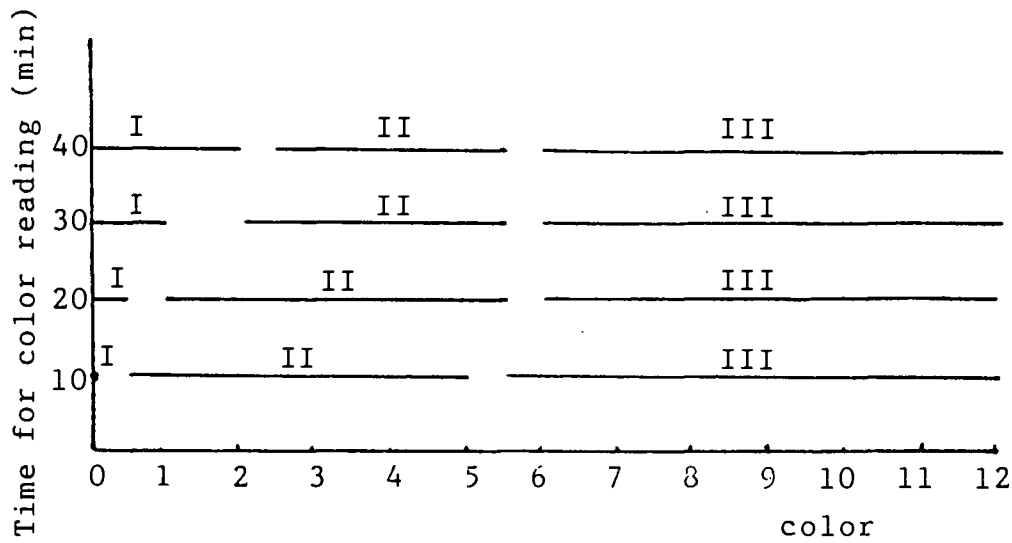

Figure 3. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 16 hours. Dye reduction test at 21°C.

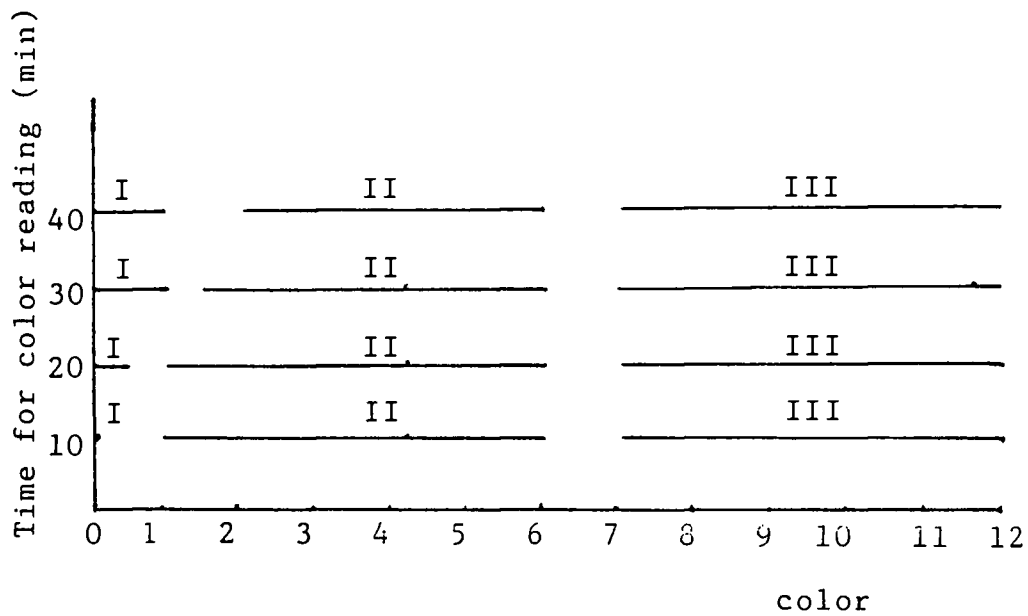

Figure 4. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 21 hours. Dye reduction test at 21°C.

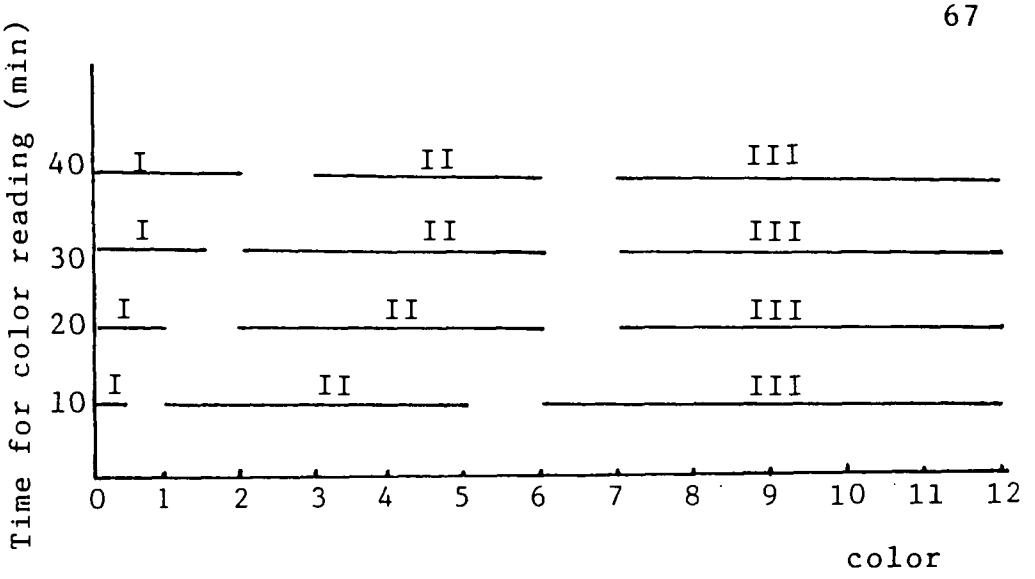

Figure 5. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 25 hours. Dye reduction test at 21°C.

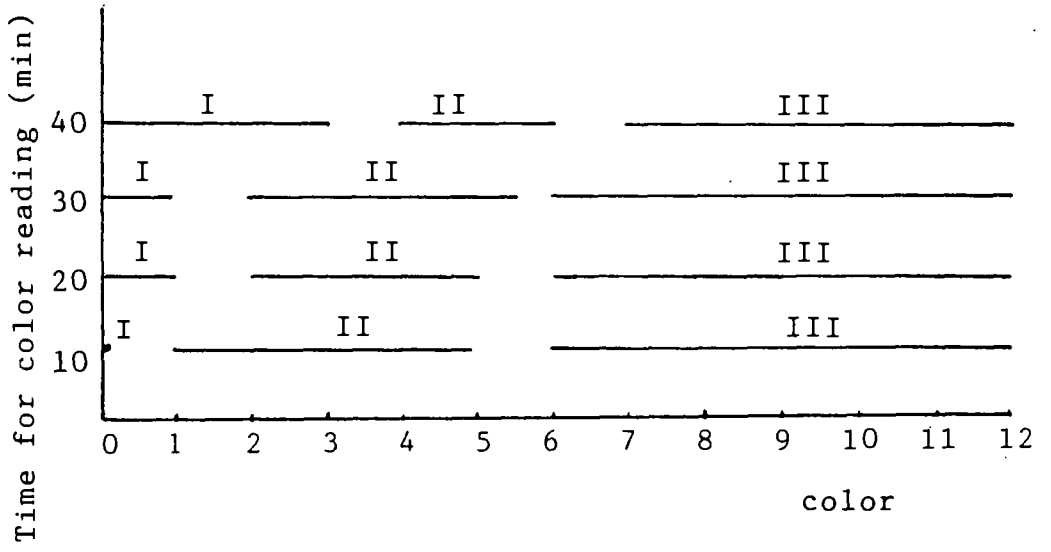

Figure 6. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 16 hours. Dye reduction test at 30°C.

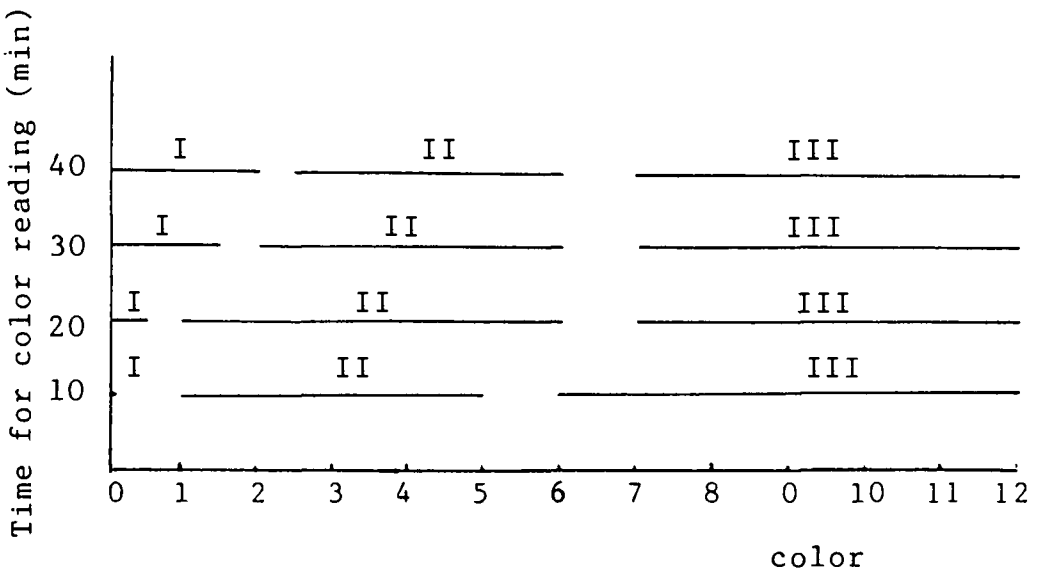

Figure 7. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 21 hours. Dye reduction test at 30°C.

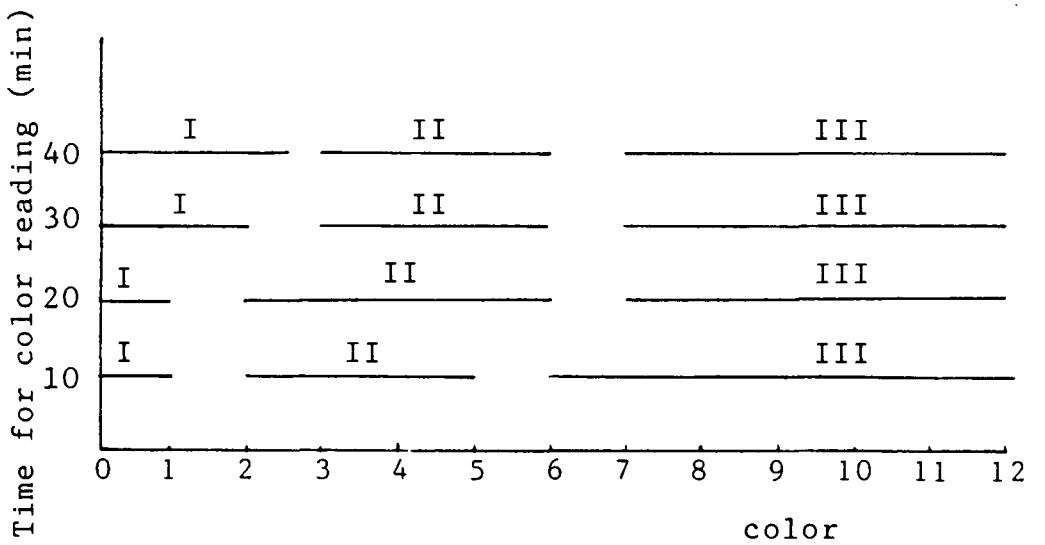

Figure 8. Interpretation of the color endpoints of the INT-PMS test. PI at 21°C for 25 hours. Dye reduction test at 30°C.

Negative controls were prepared by pasteurizing milk in the laboratory at 62.8°C for 30 minutes. Positive controls were held at 7.2°C for several days (Pasteurized milk). The method used to evaluate its efficiency was the actual shelf-life detection by sensory evaluation of the milk conducted on a daily basis.

RESULTS AND DISCUSSION

The word "potential" was used in all these shelf-life studies. It is a term necessary in any shelf-life study as the experiment progresses under ideal conditions, which would exclude any activities such as temperature abuse, unsanitary handling, etc., as could possibly occur in the home and the market place.

THE MOSELEY KEEPING QUALITY TEST

In this study, sample results from the 5 day-Moseley keeping quality test (SPC) appeared to correlate quite closely with the potential shelf-life of pasteurized whole milk (correlation coefficient, r = -0.820) (Figure 9). By contrast, flavor evaluation scores after the milk had undergone an incubation at 7.2°C for 5 days was a relatively poor indicator of potential shelf-life (correlation coefficient, r = 0.670).

Results of the 7 day-Moseley keeping quality test (SPC) appeared to correlate reasonably with the potential shelf-life of pasteurized whole milk samples (correlation coefficient, r = -0.750) (Figure 10). Flavor evaluation scores conducted after 7 days storage at 7.2°C showed good correlation (r = 0.820) with the potential shelf-life of pasteurized whole milk.

An analysis of results obtained during the course of this study and studies by several other investigators in the

Moseley keeping quality test (12,49,175) suggest an opportunity for classifying fluid milks into three categories with respect to their characteristics for potential shelf-life. This system of classification should only be considered as a general guideline to indicate the level of post-pasteurization contamination that occurred (Table 8, Figures 11,12,13 and 14).

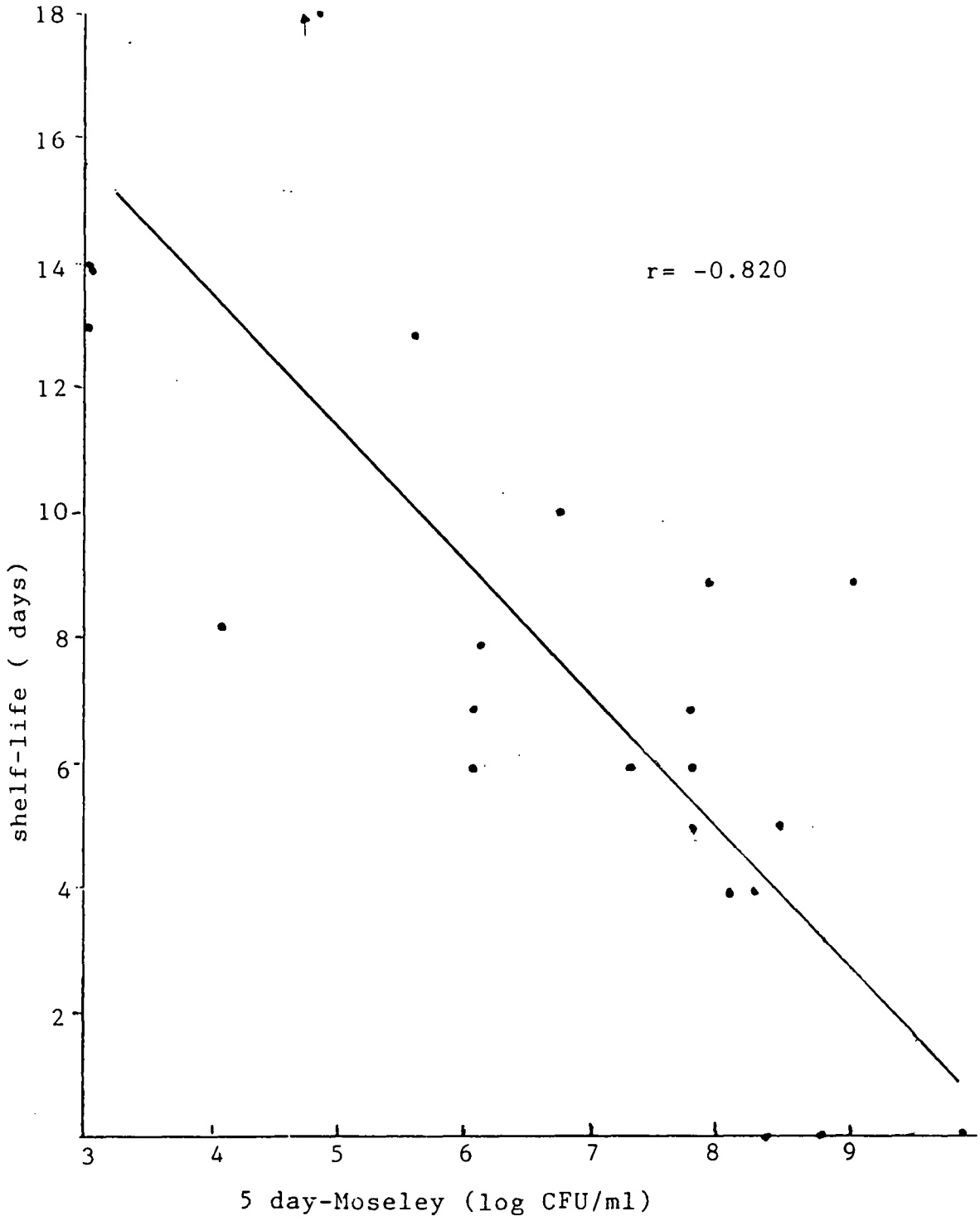

Figure 9. Plot of the log of counts by the 5 day-Moseley keeping quality test against potential shelf-life of milk at 7.2°C

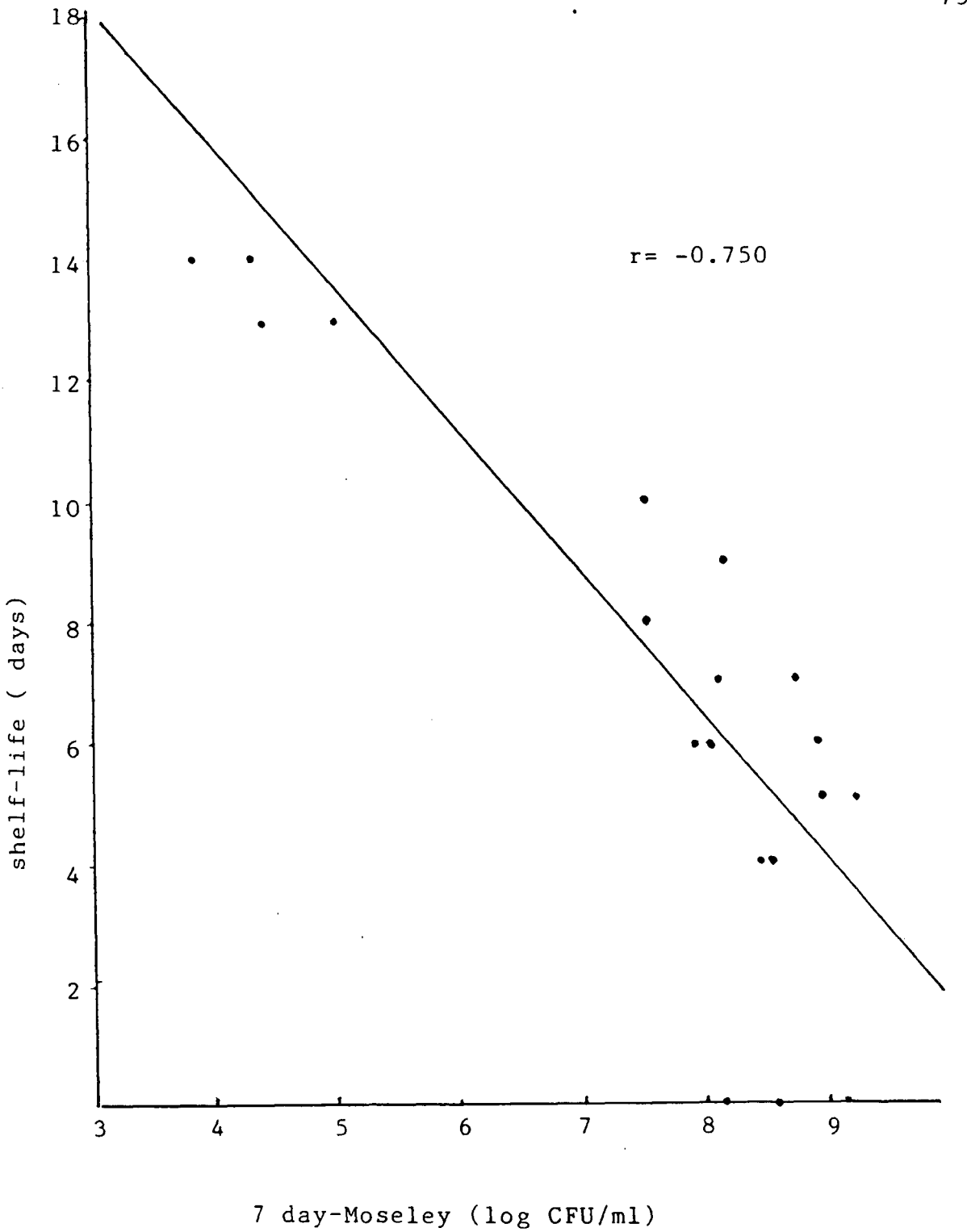

Figure 10. Plot of the log of counts by the 7 day-Moseley keeping quality test against potential shelf-life of milk at 7.2°C

Table 8. Classification of pasteurized whole milk by categories based on results of the Moseley keeping quality test.

Milk Category	5-day Moseley Count	7-day Moseley Count	Level of Post-pasteurization Contamination
I	$<1.0x10^3$	$<1.0x10^4$	Low or no post-pasteurization contamination. Expected shelf-life greater than 14 days.
II	$1.0x10^3$-$5.0x10^5$	$1.0x10^4$-$5.0x10^7$	Minor to intermediate post-pasteurization contamination. Expected shelf-life between 8 and 14 days.
III	$>1.0x10^5$	$>1.0x10^7$	Serious post-pasteurization contamination. Expected shelf-life less than 7 days.

The effectiveness of the Moseley keeping quality tests to appropriately classify pasteurized whole milk by categories of potential shelf-life was evaluated as part of this study (Table 9). Significant chi-squares (χ^2) ($P < 0.01$) indicated a lack of independ nce between the log of the 5 day-Moseley counts or the log of the 7 day-Moseley counts and expected shelf-life. The 7 day-Moseley keeping quality test appeared to be the better

method for prediction of potential shelf-life, according to the categories summarized in Table 8, having a higher degree of dependence between variables and a greater accuracy for predicting shelf-life potential.

Table 9. Effectiveness of the Moseley keeping quality test in predicting potential shelf-life of pasteurized whole milk by categories.

Test	χ^2	Measure of Dependence $\chi^2/n(t-2)$	% Accuracy
5 day-Moseley KQ	14.87*	0.354	71.4%
7 day-Moseley KQ	17.68*	0.421	85.7%

* Significant ($P<0.01$). Fisher's exact probability test: significant ($P<0.01$).
n:# of samples

The 7 day-Moseley keeping quality test was the comparative test used to evaluate the efficiency of several dye reduction methods evaluated during the course of this study.

PRELIMINARY INCUBATION STUDIES (PI)

Pre-incubation Trials with Growth Enhancers

The use of microbial growth enhancers such as trypticase soy broth (0.5%), yeast extract (0.5%), and brain heart infusion (0.5%) during sample pre-incubation at 30°C for 5 hours and at 21°C for 16 hours did not significantly increase the cell numbers of psychrotrophic bacteria within the incubation period. Application of the Dunnett's procedure to judge the significance of observed differences between each treatment and the control showed all differences to be non-significant (P>0.05) (Table 10).

Table 10. Log means of the increase in PBC during preliminary incubation(PI) with microbial growth enhancer.

PI	Control (no growth enhancer added)	0.5% Yeast Extract	0.5% Trypticase Soy Broth	0.5% Brain Heart Infusion	d'Dunnett Critical Value	
					P =0.99	P =0.95
5h-30°C	2.62	2.42	2.78	2.48	1.00	0.79
16h-21°C	3.89	4.41	4.17	4.10	0.98	0.74

PBC: Psychrotrophic Bacteria Count

Pre-incubation with Growth Enhancers and Inhibitors

The use of 0.5% sodium desoxycholate as inhibitor of Gram-positive bacteria without greatly affecting Gram-negative bacteria did not appear as effective in this study as was found to be the case by Freeman et al. (59) in their studies. Addition of sodium desoxycholate at 0.5% was an effective inhibitor of Gram-positive bacteria, but this compound also demonstrated inhibitory activity against psychrotrophic bacteria (Tables 11,12). Trypticase soy broth (1%) was used to enhance psychrotrophic bacteria growth during these studies and milk was pre-incubated at 30°C for 5 hours and 21°C for 16 hours. Application of the 7 day-Moseley keeping quality test demonstrated how milks with serious post-pasteurization contamination problems could be predicted to have minor or no post-pasteurization contamination when conducting the dye reduction test.

Table 11. Effect of using 0.5% sodium desoxycholate for inhibition of Gram-positive bacteria during Preliminary Incubation (PI) at 30°C for 5 hours.

Sample	PBC Fresh	PBC after PI	7 day-Moseley Keeping Quality Counts
1	$3.1x10^8$(EST)	$3.9x10^5$	$5.2x10^8$
2	$4.4x10^8$(EST)	$1.5x10^7$	$1.6x10^5$
3	$3.5x10^5$	$3.0x10^2$	$5.0x10^8$
4	$2.1x10^1$	$<1.0x10^0$	$1.6x10^7$

PBC: Psychrotrophic Bacteria Count
EST: Estimated

Table 12. Effect of using 0.5% sodium desoxycholate for inhibition of Gram-positive bacteria during Preliminary Incubation (PI) at 21°C for 16 hours.

Sample	PBC Fresh	PBC after PI	7 day-Moseley Keeping Quality Counts
1	4.8×10^{8}(EST)	$<1.0 \times 10^{2}$(EST)	5.7×10^{8}
2	1.9×10^{8}(EST)	$<1.0 \times 10^{2}$(EST)	7.8×10^{9}(EST)
3	1.0×10^{4}	$<1.0 \times 10^{1}$(EST)	2.2×10^{9}(EST)
4	1.5×10^{7}	$<1.0 \times 10^{0}$(EST)	2.0×10^{7}
5	1.0×10^{3}	$<1.0 \times 10^{1}$(EST)	1.6×10^{8}

PBC: Psychrotrophic Bacteria Count
EST: Estimated

The use of 0.0002% neotetrazolium chloride for inhibition of Gram-positive bacteria as proposed by Smith and Witter (176) apparently allowed too many Gram-positive microorganisms to grow as evidenced by coagulation of some milk samples when incubated at 30°C for 16 hours.

Basically, similar results were obtained when 0.0002% tetrazolium violet (TV), 0.0002% tetrazolium blue chloride (TBC), 0.0002% triphenyltetrazolium chloride (TTC) and total of 0.0002% and 0.0004% combinations of two of these tetrazolium salts were used. Quite possibly, the selected media employed to colonize psychrotrophic bacteria could cause differences in optimal inhibitor concentration. Smith and Witter (176) had used Trytic Soy agar.

Of various tetrazolium salts evaluated in this study, NTC and TBC appeared to be the most lethal to Gram-positive bacteria, causing the least coagulation problems during the incubation period.

Pre-incubation Studies Without Growth Enhancers or Bacterial Inhibitors

Twenty one degrees (°C) appeared to be an adequate preliminary incubation temperature to support growth of psychrotrophic bacteria. During pre-incubation at 21°C (16h).

significant increments in psychrotrophic bacteria counts occurred (Table 13), and only small increments in non-psychrotrophs bacterial counts seemed to occur (Table 14).

Table 13. Increase in Psychrotrophic Bacterial Count (PBC) during preliminary incubation (PI) at 21°C for 16 hours without addition of bacterial inhibitors or enhancers.

Initial PBC	Fold increase in PBC
$<1.0 \times 10^0$	4.4×10^3
$1.0 \times 10^0 - 1.0 \times 10^2$	1.0×10^4
$1.1 \times 10^2 - 1.0 \times 10^3$	1.0×10^4
$1.1 \times 10^3 - 1.0 \times 10^4$	2.2×10^3
$1.1 \times 10^4 - 1.0 \times 10^5$	3.2×10^3
$1.1 \times 10^5 - 1.0 \times 10^6$	8.5×10^2
$1.1 \times 10^6 - 1.0 \times 10^7$	1.1×10^2
$>1.0 \times 10^7$	8.0×10^0

n = # of samples = 40

Table 14. Increase in bacterial counts different than psychrotrophs during preliminary incubation (PI) at 21°C for 16 hours without inhibitors or enhancers.

Initial Bacteria Counts (Range)	Fold Increase in Bacteria Counts
$1.0 \times 10^1 - 1.0 \times 10^2$	Between 2 and 57 with mean, 26 and standard deviation, 20.

n = # of samples = 5

An initial pre-incubation of milk samples for 16 hours at 21°C, and subsequently 4 hours at 30°C did not provide encouraging results. The final 4 hour segment of incubation at that elevated temperature seemed to favor growth of non-psychrotrophic bacteria along with a non-significant increase in psychrotrophic count ($P>0.05$) during this additional incubation time. Milk samples that contained less than 1 psychrotroph per ml exhibited up to 1.1×10^5 total colony forming units per ml.

Since all the results from previous experiments during this work demonstrated various merits for use of 21°C as the optimum temperature to grow psychrotrophic

bacteria without bacterial growth inhibitors or enhancers, a complete study for evaluation of various periods of times for preliminary incubation at 21°C was conducted. Selected pre-incubation periods in this study were 16, 21 and 25 hours, based on a dairy industry interest and reference for attaining shelf-life prediction results within a reasonable time period.

Significant increments in Psychrotrophic Bacteria Counts generally were experienced ($P<0.01$) with pre-incubation at 21°C for 16,21 and 25 hours (Table 15).

Table 15. Approximate increase in PBC during preliminary incubation (PI) at 21°C for 16,21 and 25 hours without inhibitors or enhancers.

Initial PBC (Range)	Fold increase in PBC Preliminary incubation for		
	16 hours	21 hours	25 hours
$<1.0x10^0$	$4.4x10^3$	$1.3x10^4$	$1.8x10^5$
$1.0x10^0$ -$1.0x10^1$	$9.0x10^3$	$3.2x10^4$	$3.7x10^5$
$1.1x10^1$ -$1.0x10^2$	$3.1x10^3$	$3.1x10^3$	$2.1x10^5$
$1.1x10^2$ -$1.0x10^3$	$1.9x10^4$	$2.2x10^4$	$2.2x10^5$
$1.1x10^3$ -$1.0x10^4$	$5.0x10^1$	$6.0x10^2$	$1.2x10^4$
$1.1x10^4$ -$1.0x10^5$	$2.0x10^3$	$1.6x10^3$	$5.0x10^3$

n = # of samples = 60
PBC: Psychrotrophic Bacteria Count

The real differences in the mean logs of the bacterial count increases under all experimental conditions were tested and are reported in Table 16.

Table 16. Mean of the log of the increase in bacterial counts during preliminary incubation at 21°C for 16,21 and 25 hours without using inhibitors or enhancers.

Bacteria Count	16 Hours	21 Hours	25 Hours	F Value	LSD**	lsd*** α 0.01	lsd*** α 0.05
SPC	1.86	2.32	3.35	14.91*	0.510	0.686	0.522
PBC	2.29	3.10	3.74				

* Significant P<0.01
** LSD=Critical value Waller-Duncan's Bayesian k-ratio t test
*** lsd= Fisher protected least significant difference

In our study, refrigerated storage at 21°C for 21 hours or 21°C for 25 hours without the use of bacterial inhibitors and/or growth enhancers each appeared to be an appropriate method for the preliminary incubation (PI) of milk samples prior to conducting various shelf-life prediction tests. These two different pre-incubation procedures provided high enough levels of psychrotrophic bacteria to be detected by measurable or detectable color

of the redox sensitive dye and significantly favored ($P<0.01$ and $P<0.05$ respectively) increases in Psychrotrophic Bacterial Count.

Looking for the Best Correlation and Accuracy in Predicting Potential Shelf-life

Bacterial enumerations of fresh samples of commercially pasteurized milk were of little value in predicting the milk sample shelf-life. Pre-incubation of milk samples at 21°C for 16, 21 and 25 hours improved the linear relationship of bacterial enumeration to potential shelf-life at 7.2°C (Table 17) (Figures 11,12,13,14).

Table 17. Linear relationship of bacterial enumerations to potential shelf-life of pasteurized whole milk at 7.2°C.

PI	SPC	PBC	MPBC
None	-0.649	-0.726	-0.664
21°C-16 hours	-0.803	-0.829	-0.808
21°C-21 hours	-0.807	-0.806	-0.810
21°C-25 hours	-0.805	-0.764	-0.746

All correlation coefficient significant ($P<0.01$)
PI: Preliminary Incubation
SPC: Standard Plate Count
PBC: Psychrotrophic Bacteria Count
MPBC:Modified Psychrotrophic Bacteria Count

The relative efficiency of the use of bacterial enumerations for classifying the various commercial milk samples by their potential shelf-life was statistically tested; this analysis is summarized in Table 18.

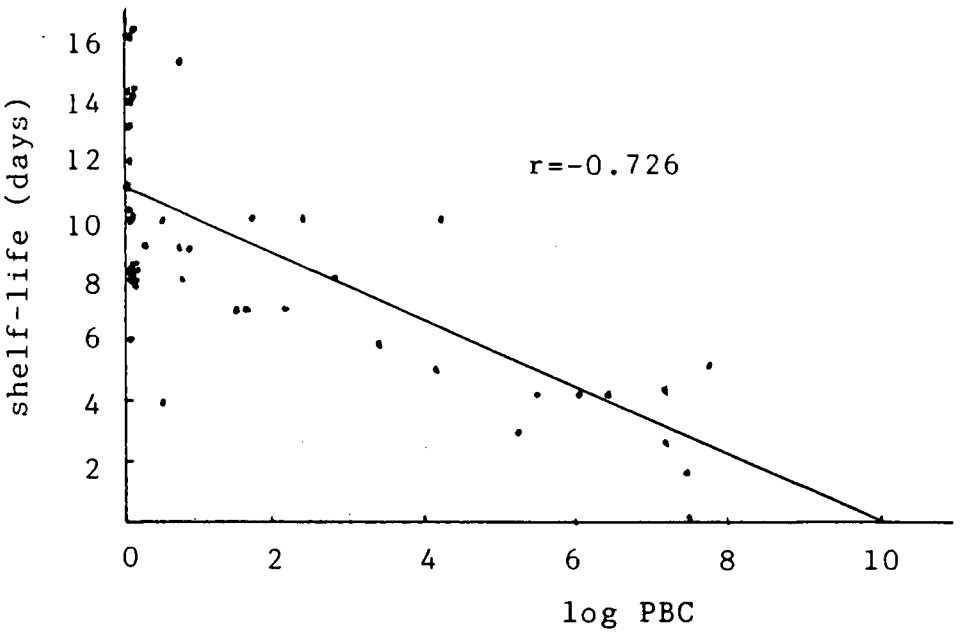

Figure 11. Plot of the log of PBC in fresh milk against potential shelf-life of pasteurized whole milk.

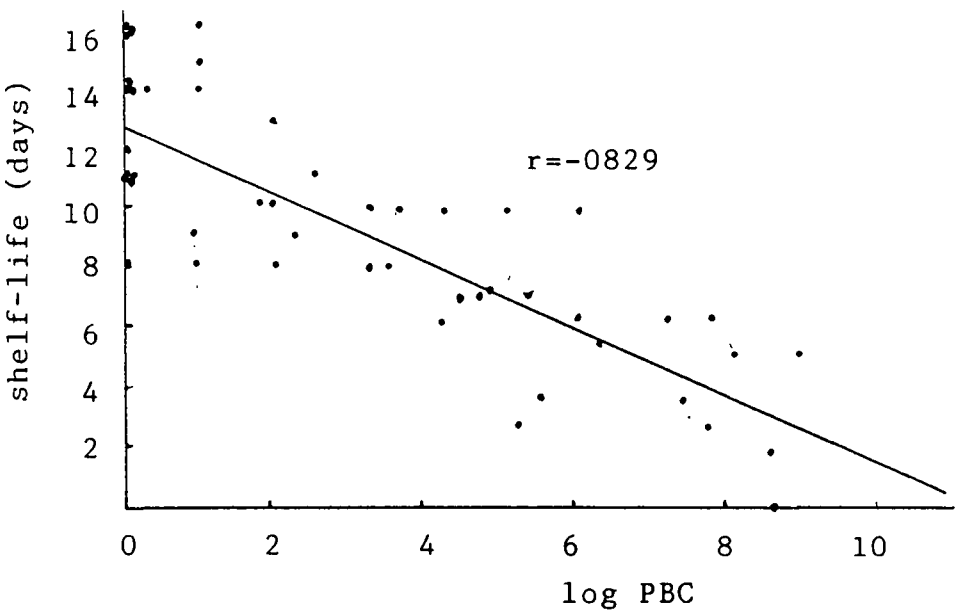

Figure 12. Plot of the log of PBC after preliminary incubation 16 hours at 21°C against potential shelf-life of pasteurized whole milk.

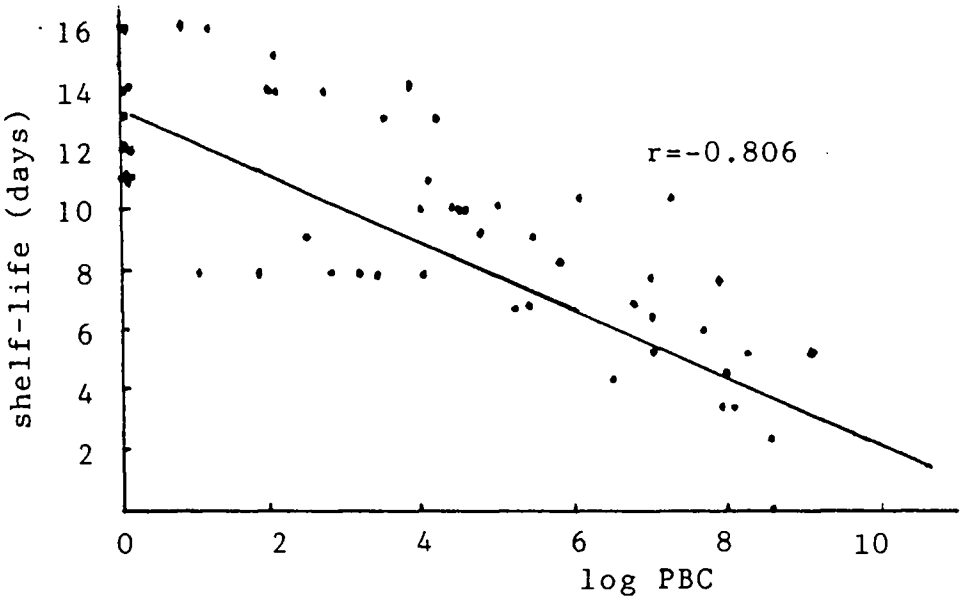

Figure 13. Plot of the log of PBC after preliminary incubation 21 hours at 21°C against potential shelf-life of pasteurized whole milk.

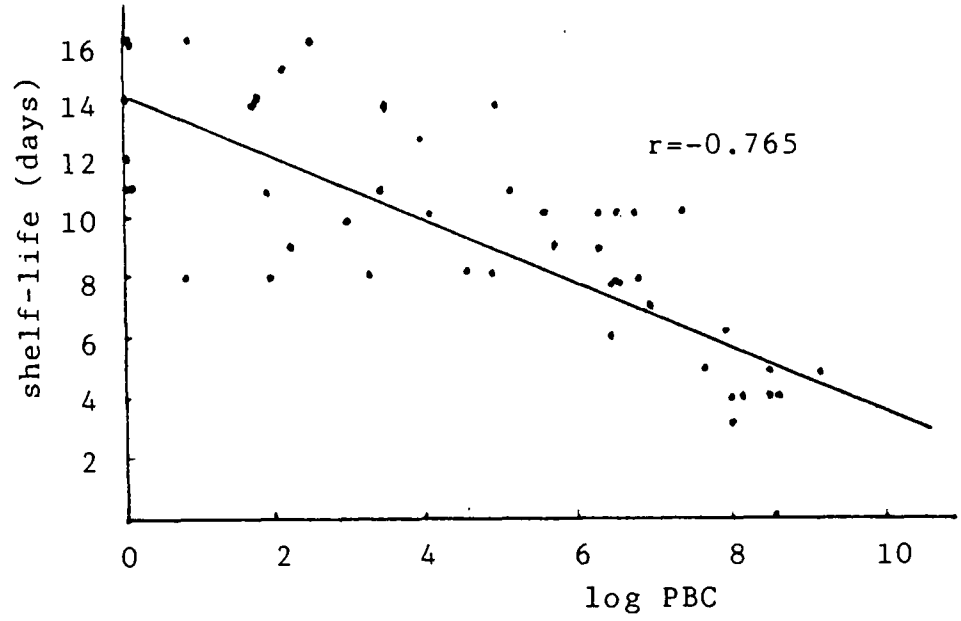

Figure 14. Plot of the log of PBC after preliminary incubation 25 hours at 21°C against potential shelf-life of pasteurized whole milk.

Table 18. Relative efficiency of using bacterial enumerations in predicting potential shelf-life of pasteurized whole milk by categories.

Type of Bacterial Count	Parameter	Preliminary Incubation (PI) None	21°C-16h	21°C-21h	21°C-25h
SPC	χ^2	25.83	27.17	42.04	42.21
	Measure of dependence $\chi^2/n(t-2)$	0.287	0.256	0.389	0.422
	% accuracy	53.3	60.4	68.5	76.0
PBC	χ^2	23.97	32.52	31.50	27.38
	Measure of dependence $\chi^2/n(t-2)$	0.266	0.307	0.292	0.274
	% accuracy	53.3	60.4	66.7	70.0
MPBC	χ^2	29.09	32.52	30.41	27.38
	Measure of dependence $\chi^2/n(t-2)$	0.323	0.307	0.282	0.274
	% accuracy	42.2	60.4	68.5	70.0

All χ^2 significant ($P<0.01$)
SPC: Standard Plate Count
PBC: Psychrotrophic Bacteria Count
MPBC: Modified Psychrotrophic Bacteria Count

Bacterial enumerations after the milk had undergone preliminary incubation (PI) at 21°C for 25 hours appeared to be the most precise method for "categorically" predicting the shelf-life characteristics of commercial pasteurized milk samples. The increase in accuracy of the test method with extended preliminary incubation time was probably due to a marked decrease in the number of results that were false negatives. Psychrotrophic Bacteria Count (PBC) and Modified Psychrotrophic Bacteria Count (MPBC) conducted following pre-incubation at 21°C for only 16 hours failed to detect as many as 26.4% of the milk samples which were determined to have experienced moderate contamination processing (category II). By contrast PBC after PI at 21°C for 21 hours failed to detect 16.7% of the samples that experienced intermediate level of post-pasteurization contamination; and the MPBC (after PI at 21°C for 21 hours) failed to detect 14.8% of the moderately contaminated samples. However, PBC and MPBC conducted after PI at 21°C for 25 hours failed to detect only 12% of the category II milks.

Even though Standard Plate Count results do not differentiate between those organisms that survive the milk product pasteurization process and those that may have gained entrance to the product after pasteurization, the SPC appeared, surprisingly to be the best enumeration method for predicting the shelf-life of pasteurized whole milk, if

bacterial counts were conducted after milk samples had been pre-incubated 25 hours at 21°C (Table 18).

Based on these bacterial enumeration studies, a general guideline for classification of milks by categories of potential shelf-life is presented in Table 19.

The Search for the Most Appropriate Conditions for Preliminary Incubation

No statistical difference ($P>0.05$) was demonstrated for increases in MPBC when milk samples were pre-incubated at 21°C for 21 hours under three different physical conditions: (1) shaking 20 ml volumes of milk sample in 200 x 25 mm screw cap test tube, (2) shaking the contents (20 ml milk sample) in a test tube capped with 0.45 μm filter paper, and (3) maintaining each sample in a quiescent state (Table 20).

Table 19. Classification of pasteurized whole milk by categories of potential shelf-life based on the bacterial counts following preliminary incubation (PI).

CATEGORY	I	II	III
	CONDITIONS OF LEVEL OF POST-PASTEURIZATION CONTAMINATION		
Preliminary Incubation (PI)	Little or no post-pasteurization contamination. (Expected shelf-life > 14 days)	Moderate to intermediate post-pasteurization contamination. (Expected shelf-life, 8-14 days)	Serious post-pasteurization contamination. (Expected shelf-life, ≤ 7 days)
PI 21°C 16H			
SPC	$<1.0\times10^{3}$	1.0×10^{3}-2.0×10^{5}	$>2.0\times10^{5}$
PBC	$<1.0\times10^{0}$	1.0×10^{0}-5.0×10^{4}	$>5.0\times10^{4}$
MPBC	$<1.0\times10^{0}$	1.0×10^{0}-5.0×10^{4}	$>5.0\times10^{4}$
PI 21°C 21H			
SPC	$<3.0\times10^{3}$	3.0×10^{3}-5.0×10^{6}	$>5.0\times10^{6}$
PBC	$<1.0\times10^{0}$	1.0×10^{0}-5.0×10^{5}	$>5.0\times10^{5}$
MPBC	$<1.0\times10^{0}$	1.0×10^{0}-5.0×10^{5}	$>5.0\times10^{5}$
PI 21°C 25H			
SPC	$<3.0\times10^{4}$	3.0×10^{4}-5.0×10^{7}	$>5.0\times10^{7}$
PBC	$<2.0\times10^{0}$	2.0×10^{0}-4.0×10^{6}	$>4.0\times10^{6}$
MPBC	$<2.0\times10^{0}$	2.0×10^{0}-4.0×10^{6}	$>4.0\times10^{6}$

SPC: Standard Plate Count
PBC: Psychrotrophic Bacteria Count
MPBC: Modified Psychrotrophic Bacteria Count

Table 20. Log means of the increase in MPBC after preliminary incubation (PI) at 21°C for 21 hours under three different physical conditions.

Shaken (screw capped tube)	Shaken (Filter paper capped tubes)	Quiescent (screw capped tube)	F Value	LSD**
3.54	3.70	3.32	0.246*	1.57

* Non-significant (1/F = 4.07) P>0.05
**LSD:Critical Value Waller-Duncan's Bayesian k-ratio t test
MPBC: Modified Psychrotrophic Bacteria Count

Despite the fact there were no statistical differences among these three physical conditions of carrying out sample incubation for 20 ml volume milk samples (in 200 x 25 mm screw capped tubes), continuous sample agitation by aid of a wrist action shaker may have merits, since better color endpoints were obtained and fewer false negatives occurred with dye reduction tests, as the result of this sample aeration. Apparently, the hermetically sealed tubes ensured that the reduction reaction was not delayed by oxygen, due to an increase in the oxidation-reduction potential.

Generally, experimental results obtained within the course of these studies indicated that pre-incubation of 20 ml volume milk sample within 200 x 25 mm screw cap tubes

with continuous shaking throughout preliminary incubation at 21°C for either 21 hours or 25 hours were the most satisfactory methods of pre-incubation prior to subjecting samples to a INT - PMS test. However, some study trials indicated that PI at 21°C for 16 hours might also be a satisfactory approach.

DYE REDUCTION TESTS

Parmelee Tube Test for Psychrotrophs

In this study, Parmelee tube test (156) results correlated poorly with 7 day-Moseley keeping quality test results of milk samples (correlation coefficient of only 0.381; significant $P<0.01$). Only 56.8% of the samples were correctly classified into the appropriate categories for potential shelf-life, based on the 7 day-Moseley keeping quality test, with a χ^2 of 10.64 (significant $P<0.05$). This particular method resulted in a markedly high proportion of false positives (37.8%); 18.9% happened to be milk samples with no or low post-pasteurization contamination, but predicted to have an intermediate level of post-pasteurization contamination; 8.1% were milk samples with none or low levels of post-pasteurization contamination, but predicted to have serious keeping quality problems; and 10.8% of the milk samples exhibited an intermediate level of post-pasteurization contamination, but were predicted to have serious keeping quality problems.

Tetrazolium Salt-Resazurin Test Trials

Trials which employed the NTC-R test, as proposed by Bodyfelt (17), did not correlate with 7 day-Moseley keeping quality test results for the milk samples evaluated for shelf-life characteristics in this study ($r=0$, non-significant $P>0.05$). The NTC-R test was able to correctly predict only the shelf-life of milk samples that had experienced substantial post-pasteurization contamination. Milk samples with apparently none or low post-pasteurization contamination, or minor to intermediate levels, were incorrectly classified into the appropiate categories for potential shelf-life (I and II, respectively) due to the occurrence of false positive reactions. These false positive color endpoint were accompanied by bacterial counts greater than 1×10^7/ml at the end of the incubation period (30°C for 16 hours), hence providing a false indication of serious contamination problems.

The use of other tetrazolium salts (different than neotetrazolium chloride) for inhibition of Gram-positive bacteria did not achieve any statistical improvement for the performance of the tetrazolium salt-resazurin test (Table 21). When results of the tetrazolium salt-resazurin test and the 7 day-Moseley keeping quality test were compared, all correlation coefficient were non-significant ($P>0.05$).

Table 21. Linear relationship of the tetrazolium salt-resazurin test to the Moseley keeping quality test (7 days at 7.2°C)

Tetrazolium Salt	r*
Neotetrazolium chloride (NTC)	0.000
Tetrazolium blue chloride (TBC)	-0.137
Tetrazolium violet (TV)	0.000
Triphenyltetrazolium chloride (TTC)	-0.352
NTC + TV	-0.187
NTC + TTC	-0.000
NTC + TBC	-0.274
TV + TBC	+0.203
TV + TTC	-0.445

n: # of samples = 30
r: correlation coefficient
* All r (s) non-significant P>0.05

Trials with the NTC-R test were attempted wherein specific observations of the color development were made with time elapse of up to the 16th hour. It was observed that for this test, color readings (changes) of the endpoint at 10 hours were significantly related to the 7 day-Moseley keeping quality test (correlation coefficient of 0.757; P<0.05) (Figures 15 and 16). Color endpoints of the tetrazolium salt test and the NTC-R test at time intervals

different than 10 hours did not correlate significantly with the results of the 7 day-Moseley keeping quality test ($P>0.05$) (Table 23).

In trials with the NTC-R method, the highest correlation coefficient appeared to occur when readings of the color endpoint were made between 8 and 10 hours of elapsed incubation time. Apparently, at this time in the combined incubation and microbial reduction-oxidation reaction, the effect of color interference by reduction of both dyes (tetrazolium salt and resazurin) was minimal; but regardless of this, color development was difficult to interpret since the color change of the two dyes, due to their response to reduction, was essentially in opposite directions of each other.

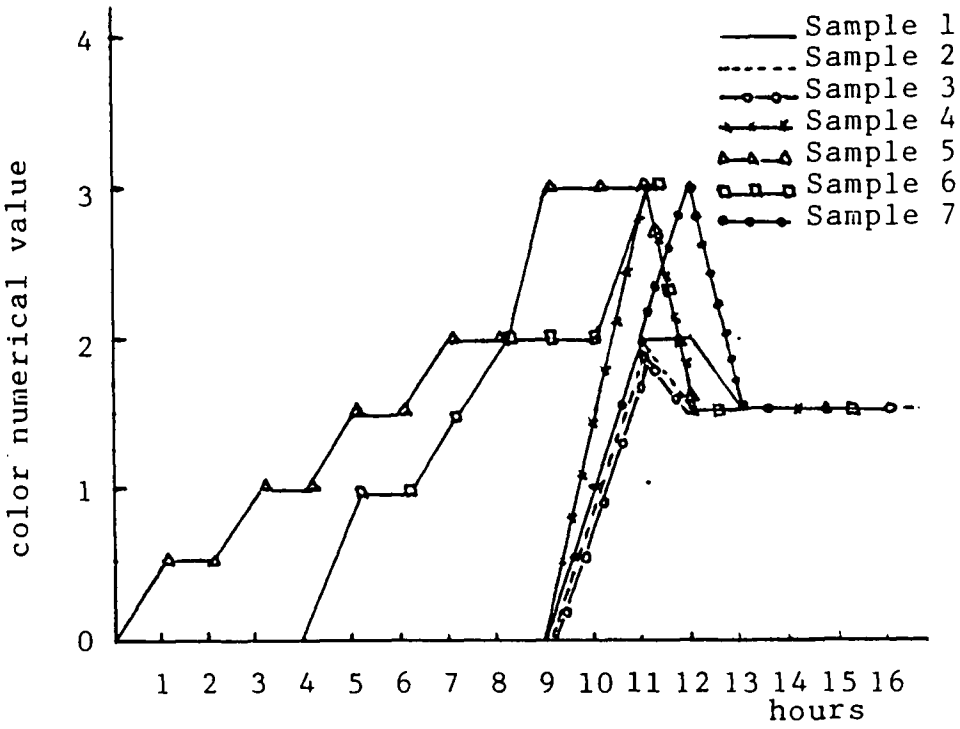

Figure 15. NTC-R test. Color development during 16 hours

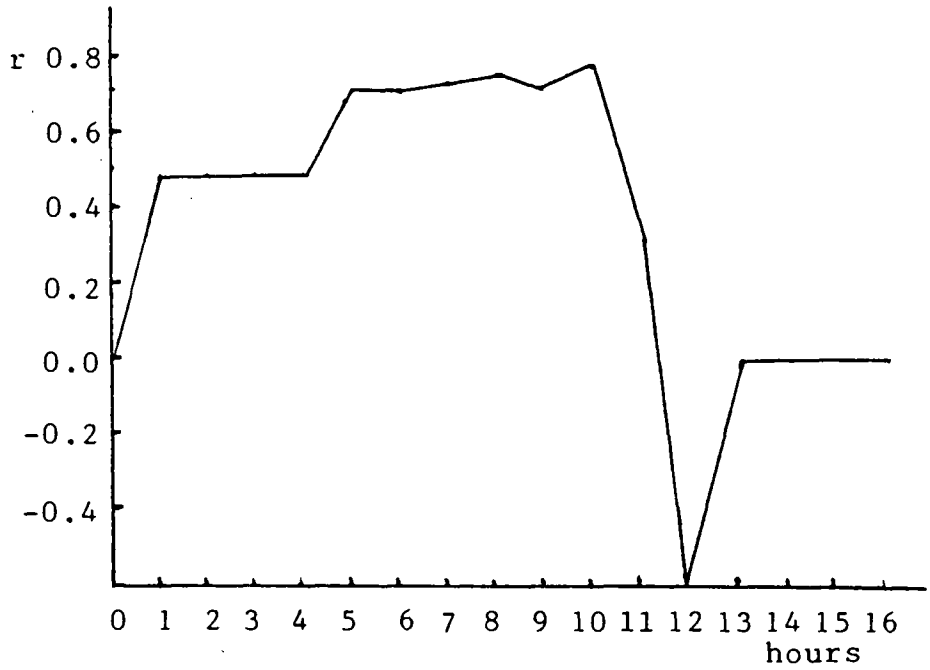

Figure 16. Linear relationship of the NTC-R test with color readings at different times to the Moseley keeping quality test.(7 days at 7.2°C).

Table 22. Linear relationship of the tetrazolium salt-resazurin test with color reading at different times to the Moseley keeping quality test (7 days at 7.2°C).

Time (hours)	Tetrazolium Salt								
	NTC	TBC	TV	TTC	NTC TV	NTC TTC	NTC TBC	TV TBC	TV TTC
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474
2	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474
3	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474
4	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474	0.474
5	0.715	0.715	0.715	0.715	0.715	0.715	0.715	0.715	0.715
6	0.715	0.715	0.715	0.715	0.715	0.715	0.715	0.715	0.715
7	0.724	0.724	0.724	0.724	0.724	0.724	0.724	0.724	0.724
8	0.734	0.734	0.734	0.734	0.734	0.734	0.734	0.734	0.734
9	0.715	0.715	0.715	0.715	0.715	0.715	0.728	0.734	0.715
10	0.757*	0.611	0.504	0.542	0.598	0.441	0.528	0.734	0.284
11	0.284	0.468	0.474	0.120	-0.141	0.373	-0.332	-0.154	-0.154
12	-0.491	-0.099	-0.154	0.242	0.598	0.091	-1.043	0.114	0.000
13	0.000	-0.298	0.000	0.435	0.000	0.000	-0.033	0.321	-0.150
14	0.000	-0.128	0.000	0.734	0.000	0.000	0.000	0.458	-0.154
15	0.000	0.114	0.000	0.734	0.000	0.000	0.000	0.546	-0.154
16	0.000	0.133	0.000	0.734	0.000	0.000	0.000	0.546	-0.154

* Significant ($P<0.05$). All other r (s) non-significant ($P>0.05$).

NTC: Neotetrazolium chloride; TBC: Tetrazolium blue chloride;

TV: Tetrazolium violet; TTC: Triphenyltetrazolium chloride.

The NTC-R test on the basis of the color endpoints at 10 hours permitted categorization of the milk samples as to their potential shelf-life with an accuracy of 71.4%. The color endpoints (time) of the tetrazolium salt-resazurin test that provided the highest accuracy in predicting potential shelf-life of milk are shown in Table 23.

Table 23. Tetrazolium salt-resazurin test. Color endpoints (time) that provided the highest accuracy in predicting potential shelf-life of milk.

Tetrazolium Salt	Time (Hours)	% Accuracy
Neotetrazolium chloride (NTC)	10	71.4
Tetrazolium blue chloride (TBC)	5-10	57.1
Tetrazolium violet (TV)	5-11	57.1
Triphenyltetrazolium chloride(TTC)	6-9	57.1
NTC+TV	5-10	57.1
NTC+TTC	5-9	57.1
NTC+TBC	5-10	57.1
TV+TBC	5-10	57.1
TV+TTC	5-16	57.1

Tetrazolium salt test

The tetrazolium salt test was not related to the 7 day-Moseley keeping quality test. All correlation coefficients between the color endpoints of the tetrazolium salt test and the results of the 7 day-Moseley keeping quality test were non-significant (P>0.05) (Table 24).

Table 24. Linear relationship of the tetrazolium salt test to the Moseley keeping quality test (7 days at 7.2°C).

Tetrazolium Salt	Correlation Coefficient
Neotetrazolium chloride (NTC)	0.416
Tetrazolium blue chloride (TBC)	0.289
Tetrazolium violet (TV)	0.197
Triphenyltetrazolium chloride (TTC)	0.492
NTC+TV	0.472
NTC+TTC	0.322
NTC+TBC	0.415
TV+TTC	0.399

n: # of samples = 15
All r (s) non-significant P>0.05

The tetrazolium salt test was a poor predictor

of potential shelf-life by categories (Table 25).

Table 25. Relative efficiency of the tetrazolium salt test in predicting potential shelf-life of milk by categories.

Tetrazolium Salt	% Accuracy
Neotetrazolium chloride (NTC)	60.0
Tetrazolium blue chloride (TBC)	26.7
Tetrazolium violet (TV)	40.0
Triphenyltetrazolium chloride (TTC)	60.0
NTC+TV	60.0
NTC+TTC	53.3
NTC+TBC	66.7
TV+TTC	60.0

The combination NTC+TBC appeared to be the most adequate tetrazolium salts to be used with the tetrazolium salt test as dye reduction indicators and Gram-positive bacteria inhibitors ($\chi^2 = 12.04$, significant $P < 0.05$).

Modified Parmelee Tube Test (MPTT)

The modified Parmelee tube test in which tetrazolium

salts were used as redox potential indicators was not related to the 7 day-Moseley keeping quality test. All correlation coefficients between the color endpoints of the modified Parmelee tube test and results of the 7 day-Moseley keeping quality test were non-significant (P>0.05) (Table 26).

Table 26. Linear relationship of the modified Parmelee tube test to the Moseley keeping quality test (7 days at 7.2°C).

Tetrazolium Salt	Correlation Coefficient
Neotetrazolium chloride (NTC)	-0.092
Tetrazolium blue chloride (TBC)	-0.092
Tetrazolium violet (TV)	-0.092
Triphenyltetrazolium chloride (TTC)	-0.180
NTC+TV	0.021
NTC+TTC	-0.092
NTC+TBC	-0.092
TV+TTC	-0.092
TV+TBC	-0.092

n: # of samples = 15
All r (s) non-significant P>0.05

The modified Parmelee tube test was also a poor predictor of potential shelf-life by categories with strong

evidence of independence between variables (Table 27).

Table 27. Relative efficiency of the modified Parmelee tube test in predicting potential shelf-life of milk by categories.

Tetrazolium Salt	% Accuracy
Neotetrazolium chloride (NTC)	35.7
Tetrazolium blue chloride (TBC)	28.6
Tetrazolium violet (TV)	28.6
Tr phenyltetrazolium chloride (TTC)	28.6
NTC+TV	35.7
NTC+TTC	28.6
NTC+TBC	28.6
TV+TTC	35.7
TV+TBC	28.6

In this study, the modified Parmelee tube test could only correctly classify those milk samples with no or minor post-pasteurization contamination. Milks with moderate and severe post-pasteurization contamination appeared as false negatives, because 16 hours incubation was apparently insufficient time for the redox potential to descend to a point where the required color changes occur. When the time interval of incubation prior to making the readings

was increased to 24 hours, the accuracy demonstrated by this method was 83.3%, when the tetrazolium salts NTC, TV, TTC, NTC+TBC and TV+TBC were used; only 50% when TBC was used and 66.7% when using the combinations NTC+TV, and NTC+TTC, and TV+TTC.

INT-PMS Test

Sources of light, either artificial or natural (380-750 nanometers) exerted a pronounced effect on dye reduction when p-Iodonitrotetrazolium violet (INT) was employed. However this reduction - oxidation indicator was less susceptible in milk samples to photo-reduction by light at laboratory conditions (indirect sunlight + artificial light) than the other tetrazolium salts.

p-Iodonitrotetrazolium violet (INT) was also more rapidly reduced than other tetrazolium salts. After milk samples had undergone preliminary incubation, the addition of INT permitted a more rapid reading of the color endpoints ($\leqslant$ 20 minutes).

INT-PMS Test at 21°C. Conduct of the INT-PMS test at 21°C after the milk had undergone PI at 21°C for 16,21 and 25 hours appeared to be a fair indicator of potential shelf-life (Table 28). The physical conditions of conducting the test method which provided the highest correlation coefficients were: PI at 21°C for 25 hours, determining the color endpoint 20 minutes (r=-0.759)

and 40 minutes (r =-0.804) after addition of the dye.

Table 28. Linear relationship of the INT-PMS test at 21°C to potential shelf-life of pasteurized whole milk.

Preliminary Incubation (PI)	Color Development Time (Minutes)	r
21°C 16 hours	10	-0.557
	20	-0.603
	30	-0.675
	40	-0.693
21°C 21 hours	10	-0.687
	20	-0.655
	30	-0.680
	40	-0.717
21°C 25 hours	10	-0.679
	20	-0.759
	30	-0.700
	40	-0.804

n : # samples = 60
All r (s) significant P<0.01
r: Correlation coefficient

The best conditions for conducting the INT-PMS test at 21°C for prediction of milk shelf-life (in terms of accuracy) were PI at 21°C for 25 hours followed by determining the color endpoint 20 minutes (76.6%) and 30 minutes (76.4%) after addition of the dye (Table 29).

Table 29. Relative efficiency of the INT-PMS test at 21°C in predicting potential shelf-life of pasteurized whole milk.

Preliminary Incubation (PI)	Color Development Time (Min.)	χ^2*	Measure of Dependence $\chi^2/n(t-1)$	% Accuracy	% False Positives	% False Negatives
	10	40.65	0.344	69.5	16.9	13.6
	20	52.18	0.492	73.6	22.6	3.8
21°C 16 hours	30	51.55	0.469	72.7	20.0	7.3
	40	28.13	0.320	68.2	18.2	3.4
	10	18.68	0.283	69.7	18.2	12.1
	20	38.02	0.317	70.0	21.7	8.3
21°C 21 hours	30	51.33	0.306	70.2	17.9	11.9
	40	42.99	0.371	72.4	17.2	10.4
	10	51.71	0.369	74.3	17.1	8.6
	20	57.83	0.452	76.6	18.7	4.7
21°C 25 hours	30	64.22	0.446	76.4	16.7	6.9
	40	28.26	0.393	72.2	19.4	8.3

n: # of samples = 60
All χ(s) significant $P<0.01$

The occurence of false positives reactions for the test was a definite reason for the reduced efficiency of this method. However, it was noted that a singular brand of milk (A) was responsible for 81.5% of the total false positive test results during the course of this study. The processor of Brand A pasteurizes milk at 175°F (79.4°C) for 22 seconds, a relatively high heat treatment, compared with the minimum requirement for fluid Grade A milk (161°F = 71.6°C for 16 seconds per the USPHS PMO (FDA)). This higher heat treatment is probably responsible for substantial denaturation of the whey proteins, which are quite heat sensitive due to their high content of sulfhydryl and disulfide bonds and subsequent release of reducing substances (-SH groups) that undoubtedly affect dye reduction time.

Application of the INT-PMS method to sources or brands of milk other than brand A, resulted in a marked increase in the correlation coefficient of this method to the potential shelf-life (Table 30). When brand A was excepted as a source of milk samples the INT-PMS method also showed an increase in accuracy and dependence between variables (Table 31). So the INT-PMS method in this case was a most reliable indicator of potential shelf-life, with correlation coefficients of up to 0.930 and 92.3% accuracy.

Table 30. Linear relationship of the INT-PMS test at 21°C to potential shelf-life of pasteurized whole milk. (Brand A excepted).

Preliminary Incubation (PI)	Color Development Time (Minutes)	r*
21°C 16 hours	10	-0.679
	20	-0.751
	30	-0.808
	40	-0.823
21°C 21 hours	10	-0.881
	20	-0.673
	30	-0.789
	40	-0.813
21°C 25 hours	10	-0.830
	20	-0.848
	30	-0.898
	40	-0.930

r : Correlation coefficient
n : # of samples = 35
* All r (s) significant P<0.01

Table 31. Relative efficiency of the INT-TMS test at 21°C in predicting potential shelf-life of pasteurized whole milk (Brand A excepted).

Preliminary Incubation (PI)	Color Development Time (Min.)	χ^2*	Measure of Dependence $\chi^2/n\ (t-1)$	% Accuracy	% False Positives	% False Negatives
	10	26.39	0.458	72.4	6.9	20.7
	20	35.13	0.586	80.0	13.3	6.7
21°C 16 hours	30	36.82	0.541	79.4	11.8	8.8
	40	29.57	0.510	79.3	13.6	17.3
	10	6.58*	0.299	63.6	9.1	27.3
	20	26.98	0.540	84.0	8.0	8.0
21°C 21 hours	30	39.19	0.490	77.5	7.5	15.0
	40	23.36	0.403	72.4	10.3	17.3
	10	49.21	0.632	84.6	5.1	10.3
	20	43.68	0.840	92.3	3.8	3.9
21°C 25 hours	30	40.97	0.585	82.9	2.9	14.2
	40	36.75	0.755	91.7	0.0	8.3

n: # of samples $\simeq$ 35
* non-significant $P>0.05$.
All others χ^2 significant $P<0.01$.

The INT-PMS test at 21°C (brand A excepted) proved to be the most accurate technique for predicting milk shelf-life (92.3%), when PI at 21°C for 25 hours was used and interpretation of the color endpoint was made after the elapse of 20 minutes. This was associated with a correlation coefficient of 0.840 (Figure 17).

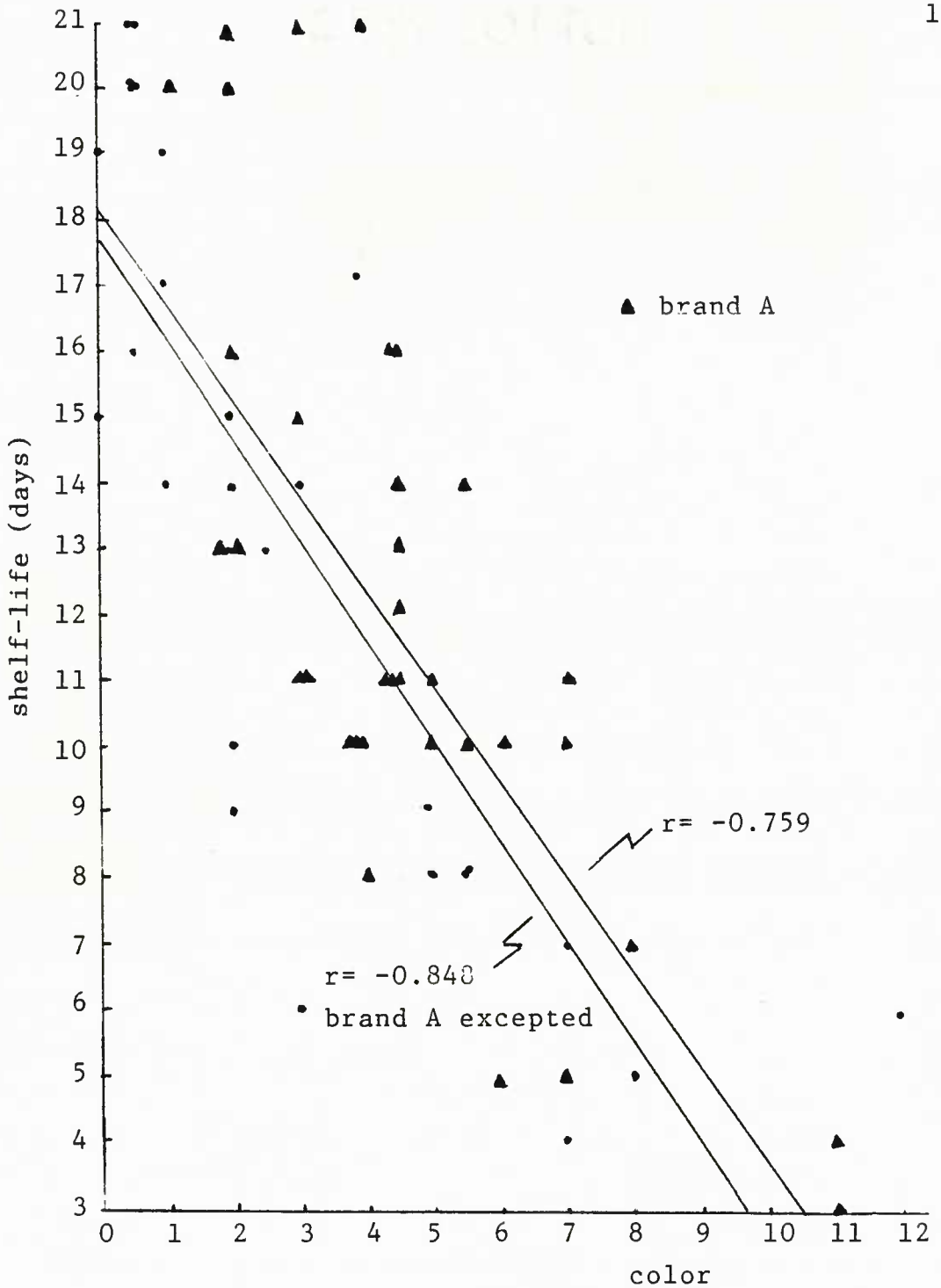

Figure 17. Plot of the dye color development, INT-PMS test at 21°C for 20 minutes with preliminary incubation (PI) at 21°C for 25 hours against potential shelf-life of pasteurized whole milk

The INT-PMS test at 21°C with PI at 21°C for 25 hours and interpretation of the endpoint at 20 minutes could perhaps be used with satisfactory accuracy to predict the potential shelf-life for pasteurized fluid milk products keeping in mind the following interpretation (based on experimental results from this investigation):

Table 32. INT-PMS test interpretation, with preliminary incubation (PI) at 21°C for 25 hours. Dye reduction test conducted at 21°C for 20 minutes.

Category	Color Numerical Value	Color Change*	Indication of Keeping Quality
I	0	P12A1	Low or no post-pasteurization
	1	P12B1	contamination. Expected shelf-life greater than 14 days.
	2	P12C1	Minor to intermediate
	3	P12D1	post-pasteurization contamination.
II	4	P12E1	Expected shelf-life between
	5	P12F1	8 and 17 days.
	6	P12G1	
	7	P12H1	
	8	P13I2	
	9	P12I1	Serious post-pasteurization
III	10	P12J1	contamination. Expected
	11	P13K8	shelf-life 7 days or less.
	12	P14L8	

* Dictionary of color by Maerz and Paul (117)

Typical color reactions when running this test are shown in figures 18, 19, 20 and 21. However, these photographs should not be used to interpret color development of the INT-PMS test since exposure of these samples to direct sunlight to take the pictures resulted in a slight change of the actual color of the reaction in the dark. The best interpretation of endpoints should be attained by following colors given by the Dictionary of Color (117).

Sensitivity: In general the extent of development of color change could possibly be used as an indicator of the psychrotrophs in pasteurized fluid milk, however color reduction of tetrazolium salts is not only apparently related to the total bacterial count, but to the differences in reducing ability of these microoorganisms. The INT-PMS test at 21°C for 20 minutes with PI at 21°C for 25 hours was able to detect less than 1 initial PBC/ml and less than 5.0×10^1 total colony forming units (CFU)/ml in fresh pasteurized whole milk, which were able to grow up to 1 PBC/ml and 1.0×10^3 total CFU/ml during preliminary incubation. In other words, this method demonstrated sufficient sensitivity to bacterial counts of 1.0×10^3/ml. Any change in color from PL2A1 (white) to PL2B1 appeared to be associated with at least 1 PBC/ml and 1.0×10^3 total CFU/ml at the end of the PI period. Any change in color from PL2B1 to PL2C1 appeared to be associated with

at least 2PBC/ml and 3.0×10^4 total CFU/ml at the end of the PI. Any change in color from PL2F1 to PL2G1 appeared to be associated with at least 4.0×10^6 PBC/ml and 5.0×10^7 total CFU/ml.

Table 33 indicates that there was a significant relationship between the particular color development by this method and the bacterial counts after PI at 21°C for 25 hours.

Table 33. Linear relationship of the INT-PMS test at 21°C for 20 minutes after preliminary incubation (PI) at 21°C for 25 hours to bacterial counts after preliminary incubation (PI) at 21°C for 25 hours.

	Bacterial Counts		
	PBC	SPC	MPBC
	Correlation Coefficient (r)		
Brand A considered	0.581	0.699	0.534
Brand A not considered	0.725	0.712	0.621

All r: significant $P<0.01$

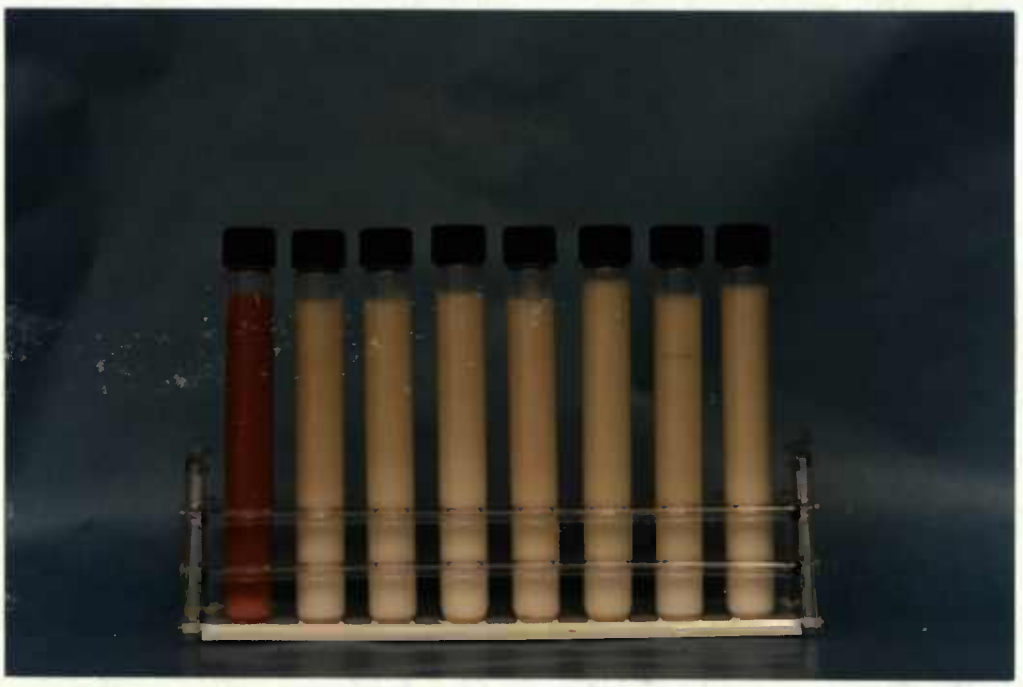

Figure 18. Typical development of the INT-PMS test at 21°C for 20 minutes with preliminary incubation (PI) at 21°C for 25 hours. Milks with low or no post-pasteurization contamination. (A category III milk was used as a control).

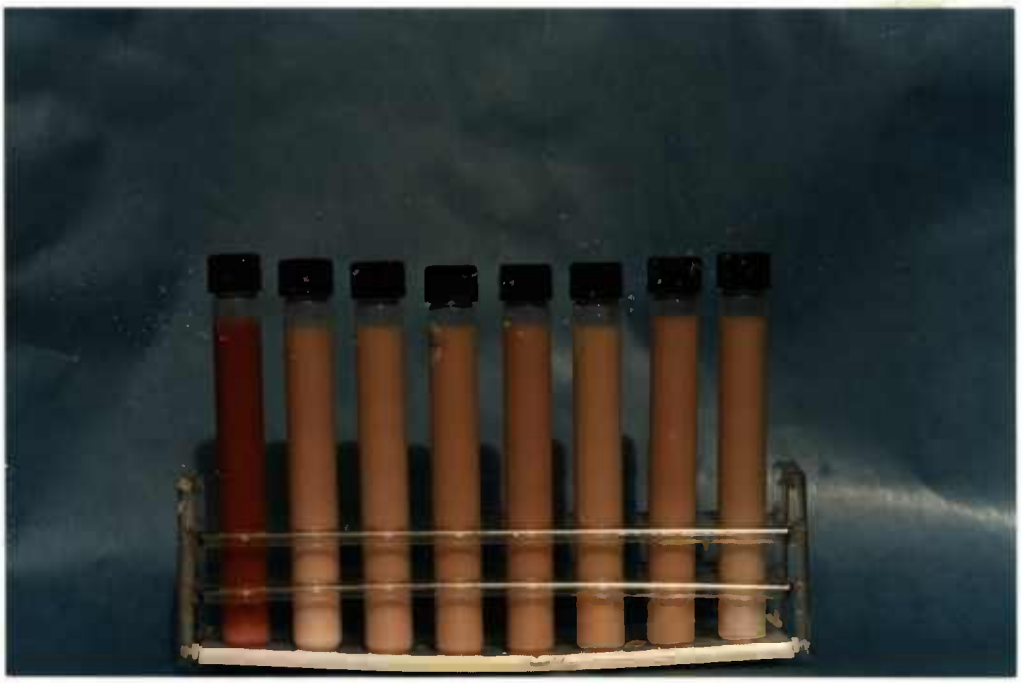

Figure 19. Typical color development at the INT-PMS test at 21°C for 20 minutes with preliminary incubation (PI) at 21°C for 25 hours. Milks with minor to intermediate post-pasteurization contamination. (A category III milk was used as a control).

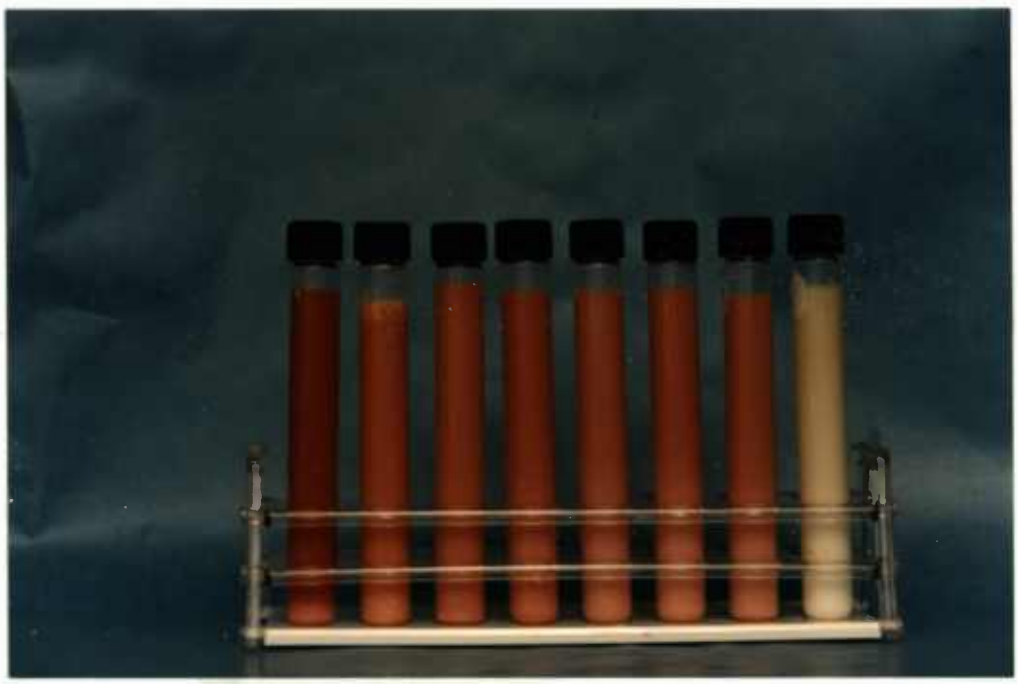

Figure 20. Typical color development of the INT-PMS test at 21°C for 20 minutes with preliminary incubation (PI) at 21°C for 25 hours. Milks with serious post-pasteurization contamination. (A category I milk was used as a control).

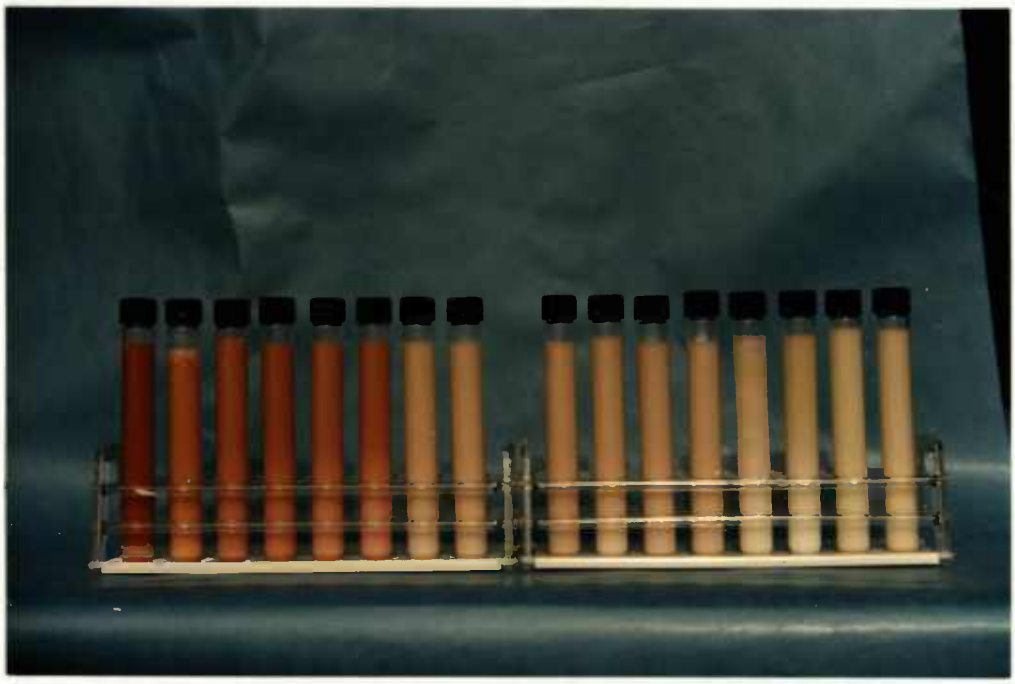

Figure 21. Typical development of the INT-PMS test at 21°C for 25 hours. Milks with serious; minor to intermediate; and low or no post-pasteurization contamination.

Costs: The INT-PMS method appears to be a relative inexpensive test, since reagents would be expected to cost only 4.54 to 9.64 cents per sample (Table 34).

Table 34. Reagent costs used in the INT-PMS test.

Reagent	Amount/Sample (g)	Cost/Sample* ($)
Sodium Citrate	0.2400	0.0032-0.0084
p-Iodonitrotetrazolium Violet (INT)	0.0024	0.0400-0.0830
Phenazine Methosulfate (PMS)	0.0006	0.0022-0.0050
	TOTAL	0.0454-0.0964

* Variation in cost due to quantities of chemicals purchased.

Limitations: The accuracy of this method appears to be affected markedly by the heat treatment (pasteurization process) given to the milk. Pasteurization temperatures above 170°F (76.6°C) would likely increase the frequency of incurring false positive results.

Cautions: Certain precautions must be undertaken when carrying out this procedure. Disposable rubber gloves must be worn when working with phenazine methosulfate (PMS) (possible carcinogenic effects). Sterile filtration

should be used to sterilize INT and PMS, since each reagent is extremely heat sensitive. Reagents, working solutions and milk samples that contain added INT and PMS must be protected at all times from either direct, indirect sunlight or artificial light, (with aluminum foil or heavy black cloth); these reagents are susceptible to photoreduction. Phenazine methosulfate and INT reagents must be stored at temperatures under 4° C to keep the reliable characteristics of the fresh product. Working solutions of sodium citrate should be tempered at 21°C before it is added to the milk for its clarification; otherwise, the large amount of sodium citrate (40 ml) could change the temperature of the system, and thus alter the results of the dye reduction test.

INT-PMS Test at 30°C. Conduct of the INT-PMS test at 30°C did not appear as effective as performance at 21°C. The physical conditions of conducting the test method which provided the highest correlation coefficient were: PI at 21°C for 25 hours, determination of the color endpoint after 10 minutes ($r = -0.745$) and after 30 minutes ($r = -0.720$) from addition of the dye (Table 35,36).

Table 35. Linear relationship of the INT-PMS test at 30°C to potential shelf-life of pasteurized whole milk.

Preliminary Incubation (PI)	Color Development Time (Minutes)	r*
	10	-0.344
	20	-0.575
21°C 16 hours	30	-0.560
	40	-0.500
	10	-0.478
	20	-0.683
21°C 21 hours	30	-0.634
	40	-0.707
	10	-0.745
	20	-0.634
21°C 25 hours	30	-0.720
	40	-0.704

n: # of samples = 60
r: correlation coefficient
* All r (s) significant P<0.01

Table 36. Relative efficiency of the INT-PMS test at 30°C in predicting potential shelf-life of pasteurized whole milk.

Preliminary Incubation (PI)	Color Development Time (Min.)	χ^2*	Measure of Dependence $\chi^2/n(t-1)$	% Accuracy	% False Positives	% False Negatives
	10	18.59	0.166	66.1	14.3	19.6
	20	22.18	0.252	59.1	18.2	22.7
21°C 16 hours	30	42.76	0.305	70.0	20.0	10.0
	40	30.87	0.250	62.9	21.7	12.3
	10	21.49	0.276	79.5	7.7	12.8
	20	51.43	0.373	71.0	20.3	8.7
21°C 21 hours	30	55.21	0.358	72.7	16.9	10.4
	40	33.83	0.384	70.5	15.9	13.6
	10	25.95	0.209	58.1	16.1	25.8
	20	43.13	0.337	71.9	21.9	6.2
21°C 25 hours	30	39.73	0.310	68.7	17.2	14.1
	40	31.36	0.356	68.2	22.7	9.1

n: # of samples = 60
* All χ^2 significant $P<0.01$

A study of the INT-PMS method by sampling milks (except brand A) produced an increase in the correlation coefficient of this method to the potential shelf-life (Table 37). And it also showed better performance, in most of the cases, in correctly predicting the shelf-life of pasteurized whole milk (Table 38).

Table 37. Linear relationship of the INT-PMS test at 30°C to potential shelf-life of pasteurized whole milk. (Brand A excepted).

PI	Color Development Time (Minutes)	r*
21°C 16 hours	10	-0.658
	20	-0.601
	30	-0.653
	40	-0.536
21°C 21 hours	10	-0.802
	20	-0.850
	30	-0.797
	40	-0.844
21°C 25 hours	10	-0.848
	20	-0.871
	30	-0.820
	40	-0.817

n : # of samples = 35
* All r (s) significant P<0.01

Table 38 Relative efficiency of the INT-PMS test at 30°C in predicting potential shelf-life of pasteurized milk (Brand A excepted).

Preliminary Incubation (PI)	Color Development Time (Min.)	χ^2*	Measure of Dependence $\chi^2/n(t-1)$	% Accuracy	% False Positives	% False Negatives
	10	15.82	0.273	62.1	6.9	31.0
	20	11.67*	0.208	57.2	10.7	32.1
21°C 16 hours	30	17.64	0.226	59.0	23.1	17.9
	40	26.53	0.402	75.7	6.1	18.2
	10	23.68	0.592	75.0	5.0	20.0
	20	26.15	0.436	73.4	13.3	13.3
21°C 21 hours	30	35.65	0.482	75.7	5.4	18.9
	40	32.10	0.553	82.8	3.4	13.8
	10	27.21	0.400	73.5	5.9	20.6
	20	24.78	0.459	77.8	11.1	11.1
21° 25 hours	30	21.09	0.391	70.4	3.7	25.9
	40	35.35	0.609	82.8	10.3	6.9

n: # samples = 35
* Significant $P<0.05$. All others $\chi^2(s)$ significant $P<0.01$.

CONCLUSIONS

Bacterial enumerations of fresh samples of commercial pasteurized milk were of little value in accurately predicting shelf-life of commercial samples of pasteurized milk. Pre-incubation of milk samples at 21°C for either 16, 21 or 25 hours improved the linear relationship of bacterial counts to the potential shelf-life as determined by storing at 7.2°C and using sensory evaluation to determine the elapsed time prior to product palatability expired.

Incubation of 20 ml volume milk samples in continuously shaken tubes at 21°C for 25 hours (without using microbial inhibitors or growth enhancers) provided the meaningful estimates of psychrotrophic bacteria populations; this approach was also the best indicator of potential shelf-life. PI at 21°C for 25 hours served as a suitable pre-incubation method for conduct of subsequent dye reduction tests.

The Parmelee tube test (PTT) demonstrated a significant correlation with the 7 day-Moseley keeping quality test, but it was not a reliable predictor of the potential shelf-life of pasteurized milk samples.

The tetrazolium salt-resazurin test as proposed by Bodyfelt (17) and the tetrazolium salt test did not correlate with the 7 day-Moseley keeping quality test;

furthermore they demonstrated poor abilities for predicting the potential shelf-life of commercial milk samples. A modification of the tetrazolium salt-resazurin test, wherein readings of the color endpoints were made after 10 hours of incubation at 30°C provided significant relationships between the NTC-R test and the 7 day-Moseley keeping quality test; it also classified milk samples as to their potential shelf-life with an accuracy of 71.4%.

Results of the modified Parmelee tube test (MPTT) did not correlate with the 7 day-Moseley keeping quality test; the MPTT could not appropriately predict the potential shelf-life of commercial fluid milk samples. However, when the time interval of incubation prior to making the reading was increased to 24 hours, the accuracy demonstrated by this method was improved to 83.3%.

A method referred to as the INT-PMS test conducted at 21°C (20 minutes reaction time) after preliminary incubation at 21°C for 25 hours, appeared to be the most sensitive and reliable dye reduction procedure for indicating milk potential shelf-life ($r=-0.840$). This method demonstrated some potential as a simple, rapid, inexpensive and accurate method for determining post-pasteurization contamination of fluid milk products. This dye reduction method demonstrated to be: (1) accurate (92.3%), (2) rapid (<26 hours), (3) simple to perform, and

(4) inexpensive (4.54 to 9.64 cents/sample).

Unfortunately, the interpretation of the color endpoints of the INT-PMS test could be affected by the intensity of heat treatment (pasteurization) applied to the milk by dairy processors. Presumably, temperatures above 170°F (76.6°C) could serve to increase the frequency of false positive test results and hence limit the applicability of this method as a reliable shelf-life projection tool. Further studies to inactivate or somehow negate the effect of heat liberated -SH groups are recommended. This migh be achieved by using reagents such as iodoacetic acid, N-ethylmaleimide and iodoacetamide.

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