A TEST FOR THE KEEPING QUALITY OF PASTEURIZED MILK

E. A. DAY AND F. J. DOAN

The Pennsylvania Agriculture Experiment Station, University Park

(Received for publication November 3, 1955)

A simple color test, capable of detecting pasteurized bottled milk having poor keeping quality under refrigeration, is described. Neotetrazolium dye is mixed with milk in a test tube. The tube is evacuated and sealed, placed in an incubator at 37° C. and examined after four hours. A detectable pink color appearing in the sample is an indication that the milk will undergo flavor spoilage within a four day period. This test apparently measures the psychrophilic activity in the milk.

It has become apparent that bottled pasteurized milk will keep under refrigeration at temperatures below 45°F. for considerable periods of time providing the product is essentially free of psychrophilic bacteria (3, 6, 12). Fortunately these organisms appear to be completely destroyed by pasteurization and those present are consequently the result of post-pasteurization contamination (3, 4, 6, 8, 11, 12).

A very small number of psychrophiles will definitely shorten the refrigerator life of milk. The critical number is so small that counts made on freshly bottled milk are completely futile as an indication of potential keeping quality (3, 6, 12). Furthermore, due to the extended incubation periods required for enumeration of psychrophiles and because of the wide variations in numbers of organisms at the time of flavor spoilage, among different lots of milk, the use of the psychrophilic plate count has not proved very successful in predicting the "keepability." This would seem to indicate that the specific types of psychrophilic organisms present in the milk are more important than total numbers in influencing keeping quality.

The growth of psychrophilic organisms in milk is supported by nutrient material in the medium. Undoubtedly various substances are acted upon and many new compounds formed, some of which are responsible for the array of off flavors associated with low temperature spoilage. The amount of a particular compound required to contribute a flavor is doubtless very minute, making chemical detection uncertain. Because of the variety of flavors encountered it is difficult to correlate changes of particular constituents of milk with eventual flavor spoilage detected organoleptically. Nevertheless, the consumer makes final judgment of the acceptability of the product by organoleptic means. This fact must be considered in the development of a test for predicting the keeping quality of milk.

EXPERIMENTAL AND RESULTS

Studies on Chemical Changes in Refrigerated Milk

In this study, a test for incipient spoilage was sought based on a change in some constituent or property of milk during storage at 40° F. The 29 lots of milk used consisted, for the most part, of commercially bottled pasteurized, homogenized, milk obtained at the bottling machine of a number of milk plants in the vicinity of State College, Pa. All lots of milk were stored at 40°F. and a replicate bottle examined at regular intervals during the storage life of the product. Spoilage was determined organoleptically by two experienced judges, and was based on the appearance of an objectionable flavor,
presumably a result of bacterial action. No samples in the lots of milk examined became oxidized.

Changes in protein stability as measured by the alcohol number, acetone number, and Storrs number (10) were noted during storage of the milk samples but these were too slight, too inconsistent, and occurred too near the time of flavor spoilage to be useful for the purpose in view. Similar conclusions were drawn relative to changes in pH and acidity. Variations in the nitrogen distribution of the non-casein fraction of milk during storage, as determined by the method of Rowland (9) were not well correlated with flavor deterioration. It is interesting to note, however, that the proteose-peptone fraction increased over 100 per cent while the non-protein fraction remained constant up to the time of off flavor detection. Furthermore, no significant change in protein degradation products such as tyrosine and tryptophane were detectable by the method of Hull (5), nor was there any observable change in lactose over the same periods when determined by a picric acid reduction method (7).

Studies on Dye Reduction in Refrigerated Milk

Most information available on the use of oxidation-reduction dyes as a means of measuring keeping quality of refrigerated pasteurized milk has been discouraging, primarily because reduction is so slow that it is difficult to obtain results within a reasonable incubation period (2, 3). It was found, however, that by evacuating the incubation tubes and maintaining the vacuum during the incubation period, the time required for dye reduction can be greatly decreased. Apparently evacuation increases the sensitivity of the dye by removing a large portion of the oxygen from the tube, thus facilitating a more rapid drop in potential of the milk when bacterial action is significant. Thunberg oxidation tubes were found suitable for the purpose but due to their cost, fragility and difficulty of cleaning, regular 15 mm. x 60 mm. culture tubes were later employed. The culture tubes were fitted with number zero, one-hole, rubber stoppers. Glass tubes were fitted into the stopper holes and connected by rubber tubes to a header which in turn led to the vacuum source. Screw clamps were employed on the rubber tubes to maintain the vacuum after the test tubes were removed to the incubator. An ordinary laboratory water aspirator was found satisfactory for generating the vacuum. The milk and dye mixture in the tubes was tempered to between 0° C. and 5° C. using ice water and then evacuated for 3 minutes at 15 mm. pressure as measured by a mercury manometer.

Resazurin, methylene blue, and p, p′-diphenylenebis -2- (3, 5-diphenyltetrazolium chloride), the latter commonly called neotetrazolium, were examined to determine the dye most suitable for detecting psychrophilic activity in refrigerated milk. Resazurin

| Table 1 — Predictability of Flavor Spoilage of Commercially Bottled Milk by the Neotetrazolium Test During Storage at 40° F. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Milk lot Number | Reduction of neotetrazolium | 25°C Bacterial plate count on day of first positive test | 25°C Bacterial plate count at time of flavor spoilage | Total keeping period |
| | Day of positive test | Days in advance of spoilage | | |
| 1 | 7 | 3 | 2.5M | 11M | 10 |
| 2 | 9 | 4 | 1.8M | 17M | 13 |
| 3 | 0 | 5 | 45T | 25M | 5 |
| 4 | 5 | 7 | 400T | 110M | 12 |
| 5 | 7 | 3 | 1.4M | 210M | 10 |
| 6 | 8 | 6 | 4.0M | 72M | 14 |
| 7 | 5 | 4 | 1.8M | 19M | 9 |
| 8 | 8 | 2 | 12M | 200M | 10 |
| 9 | 7 | 3 | 3.6M | 250M | 10 |
| 10 | 10 | 5 | 12M | 32M | 15 |
| 11 | 8 | 3 | 14M | 70M | 11 |
| 12 | 4 | 4 | 750T | 320M | 8 |
| 13 | 5 | 4 | 380T | 30M | 9 |
| 14 | 7 | 2 | 4.3M | 7.0M | 9 |
| 15 | 11 | 1 | 65M | 34M | 12 |
| 16 | 19 | 4 | 1.3M | 27M | 23 |
| Average | 7.50 | 3.75 | | 11.25 |

*M = Million
T = Thousand
Although data on bacterial counts are not presented for the 29 lots of milk examined in the first study of this report, the results were similar to those presented in the table. The growth curve, as determined by the average of logarithms of counts, up to the time of flavor spoilage, was typical of several such curves previously published. However, individual lots of milk varied considerably from the average and there was no consistency in total count at the time of flavor deterioration. These data together with those in the table again emphasize the fact that total numbers of organisms in refrigerated milk do not correlate well with keeping quality. The fact that physical and chemical changes do not correlate with flavor deterioration might be expected in view of the number of different types of off flavors encountered. The dye reduction test employing neotetrazolium and tube evacuation on the other hand, while not correlating with bacterial populations does appear to give evidence of bacterial action leading to flavor spoilage and consequently lends itself to a method for detecting the onset of spoilage. When used according to the procedure described below, it will predict flavor deterioration an average of almost four days prior to the appearance of the defect in the majority of instances.

Neotetrazolium in the oxidized form contributes no color to milk while the completely reduced form imparts a magenta color. During the 4 hour incubation period employed in this test, the milk normally changes only to a strong pink color. The reason for the various shades of pink is that the reduced form of neotetrazolium (diformazan) is insoluble in aqueous solutions and exists as a colloidal suspension. The degree of color considered as a positive test is slightly darker than the phenolphthalein end point obtained in the lactic acid titration of milk. A deeper shade after four hours incubation would only indicate that the milk is of more inferior keeping quality. The reduction of neotetrazolium to the diformazan is an irreversible reaction which is helpful in eliminating some difficulties encountered in the use of reversible dyes.

In the course of the work it was observed that some freshly pasteurized lots of milk reduced neotetrazolium. The reason for this is not definitely known but may be due to a lowering of the oxygen tension as a result of pasteurization. This is a temporary sit-
TION and disappears by the third day of storage. To avoid false positive tests, however, the keeping quality test should not be made until after the milk has been held for a three day period.

There are several ways in which this test may be used and some of the details doubtless could be studied further in order to develop procedures to fit the specific needs of various laboratories interested in the problem of milk keeping quality.

One procedure would be to obtain sufficient replicates of a lot of milk for periodic examinations and store it at refrigeration temperatures. If the milk is examined at daily intervals, after the third day of storage, it is possible to predict flavor spoilage at least 3 days prior to the appearance of off flavors in the majority of instances. If replicates are examined periodically but not daily, it is possible to predict at each examination that the milk will either keep or spoil during the following 3 or 4 day period.

Perhaps the simplest way for a milk plant laboratory to use the test would be first to determine the maximum number of days their product might be held before being consumed. This interval would then be employed as the period of time a sample would be held before applying the test. A negative test at this point might well be considered satisfactory, for it would indicate a keeping period of at least 3 days more than that absolutely required.

**SUMMARY OF PROCEDURE FOR THE TEST**

**Reagents and Equipment**

1. p,p'-Diphenylenebis -2-(3,5 - diphenyltetrazolium chloride) (neotetrazolium). 0.2 per cent aqueous solution.
2. Incubation tubes, either Thunberg oxidation tubes or tubes so fitted and set up as to be easily evacuated and to be capable of maintaining the vacuum during the incubation period.
3. A means of evacuating the tubes such as a laboratory water aspirator or vacuum pump.
4. Thermostatically controlled water bath incubator (37° C.).
5. Mercury manometer or a vacuum gauge to test the apparatus and assure satisfactory evacuation of the tubes before incubation.

**Procedure**

1. Pipette 0.5 ml of neotetrazolium (0.2% aqueous solution) into a clean dry incubation tube (preferably sterile).
2. Pipette 5.0 ml of milk to be tested into the tube and mix.
3. Temper the contents of the tube to 0° to 5° C.
4. Evacuate the tube at 15 mm pressure for 3 minutes and then seal the tube.
5. Temper to 37° C. and incubate at this temperature for 4 hours after which observe for a change in color of the milk from white to pink.

**Interpretation of results**

Reduction of the dye to a definitely discernable pink color at the end of 4 hours incubation at 37° C. is the criterion for a positive test. Such a result obtained on bottled milk after a minimum of 3 days refrigerated storage, would predict spoilage within a 3 to 4 day period.

**REFERENCES**