

# THE VALUE OF THE RESAZURIN TEST FOR THE DETERMINATION OF MILK QUALITY.

By W. F. SCHUBERT, B.A., B.Sc., A.Ed. (Bacteriologist) and PATRICIA M. NAGLE, B.Sc.  
(Assistant) Dairy Research Branch, Division of Dairying.

## SUMMARY.

*Both the ten-minute and the one-hour resazurin tests were compared with the "modified" methylene blue test, the standard plate count at 37°C., the direct microscopic count, and the leucocyte count.*

*Neither of the resazurin tests has any advantage over the "modified" methylene blue test for the examination of milk received at depots.*

## INTRODUCTION.

The work reported in this paper was prompted by the interest displayed by market milk organizations in the resazurin test and a desire to introduce it as a routine test at milk factories in preference to the "modified" methylene blue test. An effort has been made to determine its value in relation to other standard milk quality examinations on a relatively large and representative number of milk samples.

Most market milk grading programmes combine organoleptic classification with a dye reduction test. Though both the "old" methylene blue test and the "modified" methylene blue test of Wilson (1935) are widely used as indices of bacteriological quality, in recent years there has been a revival of interest in the resazurin test, which was first publicised by Ramsdell, Johnson and Evans (1935). Resazurin was reported by these investigators to be slightly more electro-positive than methylene blue, thus requiring a shorter incubation period. As the time lag involved in the methylene blue test has proved a serious disadvantage, the search for a shorter test has been a major factor in the stimulation of renewed interest in the resazurin test.

## MATERIAL AND METHODS.

Three hundred and forty farmers' milks were sampled in the routine way on the receiving platform of a Brisbane milk depot and submitted to:—

- (1) The Ten-minute Resazurin Test;
- (2) The One-hour Resazurin Test;
- (3) The "Modified" Methylene Blue Test;
- (4) The Standard Plate Count at 37 deg. C.;
- (5) The Direct Microscopic Count; and
- (6) The Leucocyte Count.

No controlled ageing of the milk samples in the laboratory was attempted. The average age of samples on testing was approximately three hours, due to the time elapsing between milking of the herd and arrival of the milk at the depot.

The following methods were followed:—

1. *Resazurin Test*.—The resazurin testing was carried out according to the technique outlined by the Ministry of Agriculture and Fisheries of Great Britain (1943). The dye used was prepared by the National Aniline and Chemical Company Inc. for use as an indicator of reduction in milk and distributed by Eastman Kodak Company, of Rochester, New York State, U.S.A. A 0.05 per cent. stock solution was prepared and kept stored in a refrigerator. This was diluted 1 to 10 as required. A Lovibond flat-type resazurin comparator was used for reading the tests.

2. *“Modified” Methylene Blue Test*.—The methylene blue testing was carried out according to the modified technique of Wilson (1935). B.D.H. methylene blue tablets as approved by the Ministry of Agriculture and Fisheries of Great Britain were used throughout the tests.

3. *Standard Plate Count at 37 deg. C.*—The standard plate counts were made on tryptone-glucose-extract-milk agar as recommended by the American Public Health Association (1941), with incubation at 37 deg. C. for 48 hours.

4. *Direct Microscopic Counts*.—These were made according to the recommendations of the American Public Health Association (1941). Twenty fields were counted.

5. *Leucocyte Count*.—Twenty fields of each direct microscopic count smear, referred to in (4), were counted.

## RESULTS.

### The Ten-minute Resazurin Test.

#### 1. *Comparison with the “Modified” Methylene Blue Test.*

An examination of Table 1 reveals that of the 282 samples giving a “modified” methylene blue test reading of four hours or greater, 280 were covered by resazurin test grades 6 and 5. Of the 58 milks which failed a 4-hour “modified” methylene blue standard, only six were covered by a resazurin disc reading of less than 5. Of these six milks, four were rejected on the platform by organoleptic grading as being unsuitable for inclusion in the bulk. All milks showing a resazurin grade of 3 or lower were included in the reject classification. It will be observed also that all of the latter milks were included in the half-hour “modified” methylene blue group. Thus, organoleptic examination, the ten-minute resazurin test and the “modified” methylene blue test were equally efficient in detecting the grossly inferior milks.

**Table 1.**

COMPARISON OF TEN-MINUTE RESAZURIN TEST AND "MODIFIED" METHYLENE BLUE TEST.

Resazurin Disc Number in 10 minutes.	" Modified " Methylene Blue Test (hours).					Total Number of Samples.
	5.	4-5.	2½-3½.	1-2.	½.	
6 .. .. .	140	99	35	3	0	277
5 .. .. .	21	20	13	1	0	55
4 .. .. .	1	1	0	2	..	4
3 .. .. .	..	..	..	..	1	1
2 .. .. .	..	..	..	..	2	2
1 .. .. .	..	..	..	..	..	0
0 .. .. .	..	..	..	..	1	1
Total .. ..	162	120	48	6	4	340

2. Comparison with the Standard Plate Count at 37 deg. C.

The method of expression of the data in Table 2 and subsequent tables is that of Golding and Jorgensen (1945). These workers prefer the method on the grounds that the calculation of the mean and standard deviation for each resazurin grade 0 to 6 inclusive does not supply enough practical information and that a spot graph would be too indefinite. Consequently, the geometric means of the standard plate counts for each grade have been worked out and the distribution of the samples for each resazurin grade arranged on the basis of the percentage of their plate counts.

**Table 2.**

COMPARISON OF TEN-MINUTE RESAZURIN TEST AND STANDARD PLATE COUNT AT 37°C.

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	1	0	2	1	4	55	277
Mean Standard Plate Count (1,000 per ml.)* ..	25,840	..	13,360	9,440	875	135	80
Percentage of Samples with Plate Count below—							
10,000 .. .. .	..	..	..	..	..	..	6.1
50,000 .. .. .	..	..	..	..	..	25.5	40.1
100,000 .. .. .	..	..	..	..	25	40.0	59.9
500,000 .. .. .	..	..	..	..	25	83.6	87.7
1,000,000 .. .. .	..	..	..	..	50	90.9	92.9
10,000,000 .. .. .	..	..	..	100	100	100	100
50,000,000 .. .. .	100	..	100	..	..	..	..

\* Geometric means.

An examination of Table 2 reveals a high inverse relationship between resazurin disc number and mean standard plate count. The table also shows the advantages to be gained by the rejection of certain resazurin grades. The rejection of all milks having a resazurin grade reading below 5 would imply a mean standard plate count of less than 100,000 per ml. for the accepted milk.

### 3. Comparison with the Direct Microscopic Count.

Though the experimental error in the compilation of Table 3 is high, especially at the right-hand end of the table where the counts are low, the overall picture is much the same as in Table 2. The rejection of all milks having a resazurin grade reading below 5 would give the accepted milk a mean direct microscopic count of less than 50,000 per ml.

**Table 3.**

COMPARISON OF TEN-MINUTE RESAZURIN TEST AND DIRECT MICROSCOPIC COUNT.

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	1	0	2	1	4	55	277
Mean Direct Microscopic Count (1,000 per ml.)* ..	93,940	..	9,759	4,715	268.1	39.81	38.41
Percentage of Samples with a Count below—							
50,000 .. ..	..	..	..	..	..	..	78.3
100,000 .. ..	..	..	..	..	..	72.7	86.3
500,000 .. ..	..	..	..	..	75	85.5	96.4
1,000,000 .. ..	..	..	..	..	100	100	98.9
10,000,000 .. ..	..	..	50	100	..	..	100
100,000,000 .. ..	100	..	100	..	..	..	..

\* Arithmetic means.

### 4. Comparison with the Leucocyte Count.

Table 4 is open to the same criticism as Table 3. Though on the whole, high resazurin grades are associated with relatively low leucocyte counts (28.9 per cent. below 500,000, 67.1 per cent. below 1,000,000 in the case of resazurin grade 6), it has to be admitted that in the case of the better quality milks the overall picture is rather complicated owing to the lack of knowledge regarding the proportionate parts played by the bacteria and the reducing systems associated with the leucocytes in the reduction of resazurin.

**Table 4.**

COMPARISON OF TEN-MINUTE RESAZURIN TEST AND LEUCOCYTE COUNT.

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	1	0	2	1	4	55	277
Mean Leucocyte Count (1,000 per ml.)* .. ..	3,080	..	966	322	1,754	972	846.5
Percentage of Samples with Count below—							
100,000 .. ..	..	..	..	..	..	..	1.1
500,000 .. ..	..	..	50	100	..	16.4	28.9
1,000,000 .. ..	..	..	50	..	25	60	67.1
10,000,000 .. ..	100	..	100	..	100	100	100

\* Arithmetic means.

5. *Conclusions.*

(a) This test has real value in detecting milks of very inferior quality. However, both the "modified" methylene blue test and organoleptic grading were as efficient in this respect.

(b) The test showed no refinement in the grading of the better class of milks as indicated by the "modified" methylene blue test.

(c) The test showed reasonably good correlation with the standard plate count and the direct microscopic count.

(d) The connection between the resazurin grade and the reducing systems associated with the leucocytes was not particularly clear in the case of the better class milks.

**The One-hour Resazurin Test.**

1. *Comparison with the "Modified" Methylene Blue Test.*

An examination of Table 5 shows that greater discrimination was obtained with the one-hour resazurin test than with the ten-minute resazurin test. Of the 340 milks examined, 154 fell in the 6-5 category, 176 in the 3-4 category and 10 in the 2-1-0 category. Acceptance of resazurin grades 4, 5 and 6 would mean the inclusion of 265 of the 282 milks giving a "modified" methylene blue test of four hours or greater. The one-hour test thus introduces greater refinement into the grading.

**Table 5.**

COMPARISON OF ONE-HOUR RESAZURIN TEST AND "MODIFIED" METHYLENE BLUE TEST.

Resazurin Disc Number in 1 hour.	"Modified" Methylene Blue Test (hours).					Total Number of Samples.
	5.	4-5.	2½-3½.	1-2.	½.	
6 .. .. .	9	1	..	..	..	10
5 .. .. .	82	53	9	..	..	144
4 .. .. .	66	54	27	..	..	147
3 .. .. .	8	7	11	.2	1	29
2 .. .. .	..	2	1	4	..	7
1 .. .. .	..	..	..	..	..	0
0. .. .. .	..	..	..	..	3	3
<b>Total .. .. .</b>	<b>165</b>	<b>117</b>	<b>48</b>	<b>6</b>	<b>4</b>	<b>340</b>

2. *Comparison with the Standard Plate Count at 37 deg. C.*

Table 6 shows the advantages to be gained by extending the incubation period to 60 minutes. The rejection of all grades below 5 would give the accepted milk a mean standard plate count of less than 60,000 per ml. as compared with a mean standard plate count of less than 100,000 per ml. for the ten-minute test.

**Table 6.**

COMPARISON OF ONE-HOUR RESAZURIN TEST AND STANDARD PLATE COUNT AT 37°C.

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	3	0	7	29	147	144	10
Mean Standard Plate Count (1,000 per ml.)* .. ..	16,640	..	1,489	284.9	103.5	69.33	18.32
Percentage of Samples with a Plate Count below—							
10,000 .. ..	..	..	..	..	2.7	8.3	20
50,000 .. ..	..	..	..	24.1	34.0	41.0	70
100,000 .. ..	..	..	..	27.6	53.7	61.8	100
500,000 .. ..	..	..	14.3	58.6	86.5	93.8	..
1,000,000 .. ..	..	..	28.6	71.0	93.8	96.5	..
10,000,000 .. ..	..	..	100	100	100	100	..
50,000,000 .. ..	100	..	..	..	..	..	..

\* Geometric means.

*3. Comparison with the Direct Microscopic Count.*

The rejection of all milks with a resazurin grade below 5 in Table 7 would give the accepted milk a mean direct microscopic count of less than 40,000 per ml. as compared with 50,000 per ml. for the ten-minute test.

**Table 7.**

COMPARISON OF ONE-HOUR RESAZURIN TEST AND DIRECT MICROSCOPIC COUNT.

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	3	0	7	29	147	144	10
Mean Direct Microscopic Count (1,000 per ml.)* ..	20,750	..	347.9	94.93	38.86	32.66	23.00
Percentage of Samples with a Count below—							
50,000 .. ..	..	..	51.7	75.5	84.7	100	100
100,000 .. ..	..	14.3	58.6	85.7	91.7	..	..
500,000 .. ..	..	42.9	86.2	98.6	98.6	..	..
1,000,000 .. ..	..	100	93.1	100	98.6	..	..
10,000,000 .. ..	33.3	..	100	..	100	..	..
50,000,000 .. ..	66.6	..	..	..	..	..	..
100,000,000 .. ..	100	..	..	..	..	..	..

\* Geometric means.

*4. Comparison with the Leucocyte Count.*

Though in Table 8 the highest mean leucocyte count is associated with the lowest resazurin grade reading, the relationships in the higher resazurin grades are by no means regular. This is believed to be due to the complications listed in connection with Table 4.

**Table 8.**  
COMPARISON OF ONE-HOUR RESAZURIN TEST AND LEUCOCYTE COUNT

Resazurin Grade.	0.	1.	2.	3.	4.	5.	6.
Number of Samples ..	3	0	7	29	147	144	10
Mean Leucocyte Count (1,000 per ml.)* .. .. .	1,670	..	1,110	1,211	95.1	729.8	680.8'
Percentage of Samples with a Leucocyte Count below—							
100,000 .. .. .	..	..	..	..	.7	2.8	..
500,000 .. .. .	33.3	..	..	10.3	22.4	36.1	30
1,000,000 .. .. .	33.3	..	42.9	41.4	59.8	77.8	70
10,000,000 .. .. .	100	..	100	100	100	100	100

\* Arithmetic means.

5. *Conclusions.*

(a) This test is more discriminating than the ten-minute test for milks tested within a few hours of production.

(b) Since the basic need in the bacteriological testing of milk is simplicity, it is felt that the one-hour resazurin test has no advantages to offer over the "modified" methylene blue test as a platform grading test.

(c) The test is reasonably well correlated with the standard plate count and the direct microscopic count.

(d) The relation between the resazurin grade and the leucocyte count was much the same as in the case of the ten-minute test.

**GENERAL CONCLUSIONS.**

It appears from the tests made that neither the ten-minute nor the one-hour resazurin test is superior to the "modified" methylene blue test for bacteriological examination of milk received at depots. Either could be employed as a correlative test, as the ten-minute test is under the British National Milk Testing and Advisory Scheme (Great Britain: Ministry of Agriculture and Fisheries, 1942.)

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