

## Collaborative Study of the Method for Counting Microorganisms in Maple Sirup

By AARON E. WASSERMAN (Eastern Regional Research Laboratory, Philadelphia 18, Pa.)

**A proposed procedure for counting bacteria and mold in maple sirup by the membrane filter technique was tested by collaborative assay. Four samples of maple sirup containing varying numbers of bacteria and molds were analyzed by 15 laboratories. Results were treated statistically. There was no apparent significant difference between replicate counts done within the laboratories but a highly significant difference in results obtained among laboratories. Further analysis of differences in procedures brought out by replies to a questionnaire indicate there was no detectable difference in results due to (a) use of glass or stainless steel filter holders, (b) use of glass or plastic petri dishes, (c) dilution of samples in one step or multiple steps, (d) length of storage over approximately a 4-week period before the test was carried out.**

**Further collaborative studies on the procedure were recommended.**

The microbial count of maple sirup could be useful as an indicator of the storage stability of the sirup and as a guide to its future quality. However, no standardized tests have been available for counting yeast, molds, and bacteria in maple sirup. Presumably, when a count was required the slow bacteriological plate count method was used. At the 1961 meeting of the AOAC a rapid

and simple method was proposed for counting microorganisms in maple sirup by a membrane filter technique.

A study with 15 collaborators was set up in 1962 to test the proposed method. Each collaborator received a set of 4 sirups and was requested to carry out duplicate bacterial and mold counts according to the proposed procedure. A questionnaire was also enclosed to obtain some information on possible variations in the manner of conducting the test in each laboratory. This is a report of the results obtained from the study.

### Methods and Materials

The proposed method has previously been described in detail (1), but in brief it consists of filtering an adequate sample of maple sirup through a membrane filter, incubating the filter on an absorbent pad soaked with appropriate medium, then staining (if necessary) and counting the colonies of bacteria, yeasts, or molds growing on the surface of the filter.

### Maple Sirup Samples

Several maple sirups were prepared in the laboratory to contain different concentrations of bacteria and molds. Preliminary assays indicated the dilution range for each of the sirups and this information was given to the collaborators to assist them in planning their experiments.

The sirup samples were prepared as follows:

Sample 1—Commercial maple sirup (dilute 5 or 10 ml of sirup to 100 ml with sterile distilled water and filter through membrane).

Sample 2—Sirup No. 1 inoculated with a culture of organisms obtained from a sirup solution (dilute 1:50,000 and filter 10 ml through membrane).

(There was some difficulty, however, in

<sup>1</sup> Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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preparing this sirup sample. The regular laboratory culture failed to develop when it was needed and another culture of bacteria had to be substituted. Several strains were tested but they lost viability very rapidly in the sirup and could not be detected after 48 hours. Finally, a strain of bacteria isolated from a sucrose sirup was used to inoculate the maple sirup. A count made on the sirup just before the samples were shipped led to the assignment of the 1:50,000 dilution value. Several days after the samples were mailed, another count showed that a large number of viable bacteria were still present. However, when this sample was subsequently assayed by the collaborators at the recommended dilution, very few colonies per filter developed. Apparently the organisms failed to survive in the interval between shipping and testing.)

**Sample 3**—A mixture of sirup No. 1 and contaminated cane sugar obtained through the courtesy of a local sugar refinery (dilute 1:100 and filter 10 ml through the membrane).

**Sample 4**—Sirup No. 1 inoculated with spores from a strain of *Aspergillus* isolated in the laboratory (dilute 1 ml of sirup to 10 ml with sterile distilled water and filter through membrane).

The sirup samples were placed in 2 oz jars that had been sterilized at 121°C for 15 minutes. Sets of the 4 samples, in mailing tubes, were sent *via* air mail to the collaborators. Upon receipt, the sirups were to be stored at 5°C until the predetermined date for carrying out the assays, which had been set for about 12 days after the shipping date so that all of the sirups would have the same incubation time. Upon completion of the counts, the data sheet and a questionnaire designed to cover the operation of the assay were returned to the Associate Referee. Responses were received from fifteen laboratories.

#### Results and Discussion

The collaborators' data for the bacterial counts of the 4 maple sirup samples were analyzed and the statistical analyses of variance are shown in Table 1. Although responses were received from fifteen labora-

tories, the counts reported by one collaborator were so atypical they were not included in the statistical analysis. Other laboratories failed to report values for some of the samples. Therefore the number of values analyzed differ from sample to sample.

Replicate assays of sirups No. 1, 3, and 4 performed in the different laboratories gave values whose difference was not statistically significant. For sample No. 2, however, there was a significant difference at the 95% level between replicate counts, possibly as a result of using the wrong dilution factor.

With all four sirup samples the interlaboratory values gave highly significant differences.

An analysis of variance could not be done for the mold counts. The few reported counts on sirup samples Nos. 1 and 2 were negative, and only a few of the laboratories reported replicate mold counts for Samples No. 3 and 4. Therefore, only the 95% confidence limits were determined, as shown in Table 2.

There are many possible reasons for the lack of reproducibility of the assay between laboratories, e.g., poor sampling technique (both in preparing the original sample and in taking aliquots for assay), shipping conditions, storage conditions, differences in procedure, etc. We were interested in considering these points in some detail to determine why the results differed so widely among the laboratories. Sufficient data were available for some of the factors to permit statistical evaluation of their effect.

#### Preparation of the Sample

Sirups No. 2, 3, and 4 were prepared by the addition of cultures of bacteria or mold to sirup No. 1, with thorough mixing to assure a homogeneous population in the viscous sirup. The three sirups were agitated on a reciprocal shaker for several hours. The bottles containing the sirups were held in a horizontal position to get as great a turbulence effect as possible. It was believed that adequate mixing was obtained, but only replicate assays directly from the sirup would have confirmed this. If the mixing were inadequate, samples of the sirup could contain different populations of microorganisms.

Table 1. Analysis of variance of bacterial counts from maple sirups using membrane filters<sup>a</sup>

	Sirup No. 1				Sirup No. 2 <sup>b</sup>				Sirup No. 3				Sirup No. 4			
	df	SS	MS	F	df	SS	MS	F	df	SS	MS	F	df	SS	MS	F
Total	22 <sup>c</sup>	506.08			26	2513.12			26	1,306,134			20	10,866.2		
Replicates	1	0.94	0.94	2.16	1	187.20	187.20	6.77 <sup>d</sup>	1	2,800	2,800	0.349	1	33.4	33.4	0.34
Labs	11	500.80	45.46	104.2 <sup>e</sup>	13	1994.19	153.34	5.54 <sup>e</sup>	13	1,207,134	96,856	11.58 <sup>e</sup>	10	9,978.6	997.8	10.27 <sup>e</sup>
Error	10	4.34	0.43		12	331.73	27.64		12	96,200	8,016		9	874.0	97.1	
$\bar{X}$	2.22				10.12				211.9				14.55			
$S\bar{E}$	0.1326				0.99				16.94				2.96			
$\bar{X} \pm t_{.05} S\bar{E}$	2.22 $\pm$ 0.27				10,120 $\pm$ 2,082				211.9 $\pm$ 34.7				14.55 $\pm$ 6.10			

df = degrees of freedom    SS = sum of squares    MS = mean squares    F = variance ratio     $\bar{X}$  = mean of sample values  
 $S\bar{E}$  = standard deviation of the mean     $\bar{X} \pm t_{.05} S\bar{E}$  = formula for calculating 95% confidence limits of the mean

<sup>a</sup> Bacterial counts on all tables expressed as number of organisms per ml.  
<sup>b</sup> All data coded by dividing by 10<sup>3</sup>.  
<sup>c</sup> One missing value in all sirup samples was estimated.  
<sup>d</sup> Significant at P = 0.05.  
<sup>e</sup> Significant at P = 0.01.



the samples were mailed, to insure their arrival in time. The collaborators were requested to finish the tests within the week, thus allowing 5 days leeway in scheduling the tests. The reported testing dates actually show a spread of about 4 weeks (5/14/62 to 6/11/62). The laboratories were divided into four groups according to testing dates and an analysis of variance of the data of sirup sample No. 3 was carried out to determine whether the testing date had any effect on the microbial counts. The result is shown in Table 3. There was no detectable difference in the values reported by the various laboratories that can be attributed to the time the assay was carried out.

#### Filter Equipment

Both glass and stainless steel filter holders for the membrane filters are available commercially. The instructions to the collaborating laboratories did not specify the use of one type over the other. Eight laboratories reported using glass filter holders, and seven used the stainless steel. A statistical "t" test of the data for sirup samples No. 1 and 3 was carried out to determine whether the type of filtration equipment influenced the microbial count of the sirup. The results in Table 4 indicate there is no detectable difference between the counts.

Table 4. Statistical "t" test of the difference between bacterial counts of maple sirups No. 1 and No. 3 obtained with glass and stainless steel filter holders

Calculations	Sirup No. 1		Sirup No. 3	
	Glass	Stainless Steel	Glass	Stainless Steel
n	13	10	17	10
$\bar{X}$	0.915	3.45	151.29	379.5
Variance ( $s^2$ )	3.86	44.75	27,123	31,338
$t^0$	1.163		1.864	

$$t = \frac{\bar{X}_S - \bar{X}_G}{\sqrt{\frac{s_S^2}{n_S} + \frac{s_G^2}{n_G}}}$$

where G = values for glass filter holders, and  
S = values for stainless steel filter holders.

#### Equipment Sterilization

In the proposed procedure, sterilization of the filter holder was not advocated, but it was suggested that the holder be washed in very hot water after each filtration. Thirteen laboratories reported sterilizing the filter holder before starting the test; twelve laboratories further washed the holder with very hot or boiling water as recommended; three laboratories washed the equipment with sterile water. Two laboratories indicated the use of antiseptics or alcohol, presumably followed by a water wash, between each filtration. In only three instances was there any indication of gross contamination. There is a wide latitude in defining the term "very hot water" used in washing the filter holders, and there is a possibility that samples will be contaminated if the water is not above 190°F. Ideally, sterile filtration equipment should be used for each operation, but this is not practical when large numbers of samples have to be tested. The use of the "hot water," or better yet "boiling water," rinse is justified by the fact that there was no detectable difference between replicate assays within each laboratory.

#### Petri Dishes

After removal from the filter holders, the membrane filters were incubated on pads soaked in culture medium in sterile petri dishes. Glass petri dishes have long been employed in the bacteriology laboratory for determining the number of viable organisms, but in recent years plastic dishes have been used in ever-increasing numbers. The type of petri dish was not specified for these experiments and the questionnaire returns showed that six laboratories used glass dishes whereas nine laboratories preferred plastic. Although the increasing use of the plastic dishes indicates acceptance for use in bacteriological procedures, it was possible that some of the chemical components of the plastic might affect the growth of the organisms. The bacterial counts of sirup samples No. 1 and 3 were analyzed to determine the possible effect of the use of glass or plastic petri dishes on the number of viable organisms. The results in Table 5 indicate there was no detectable difference between the

**Table 5. Statistical "t" test of the difference between bacterial counts of maple sirups No. 1 and No. 3 obtained with glass and plastic petri dishes**

Calculations	Sirup No. 1		Sirup No. 3	
	Glass Dishes	Plastic Dishes	Glass Dishes	Plastic Dishes
n	11	12	11	16
$\bar{X}$	0.84	3.06	214.7	221.8
Variance ( $s^2$ )	4.285	37.762	36,909	58,879
t*	1.182		0.85	

$$t = \frac{\bar{X}_P - \bar{X}_G}{\sqrt{\frac{s_P^2}{n_P} + \frac{s_G^2}{n_G}}}$$

where G = values for glass dishes and  
P = values for plastic dishes.

counts on the filters incubated in the two types of petri dishes.

Another possible source of error is in taking representative aliquots of the original sirup and subsequently diluting them before filtration. If the microbial population of a sirup is not homogeneous, replicate samples taken from the sirup may detect this, but it cannot be determined with replications of the dilution obtained from the one sample of the original sirup. The effect of variations in sampling procedure in this study could not be determined because the submitted information was not complete; some laboratories sampled their sirups by either of the above methods.

Dilution of the microbial population for counting also involves a possible source of error. Clumps of bacteria that survive a one-step dilution procedure and develop as a single colony may separate into smaller groups or individual bacteria as a result of the agitation received in making dilutions in several steps. The bacterial count under these conditions will be greater. Seven laboratories reported that sirup sample No. 3 was prepared by making the required dilution in several steps; eight laboratories reported diluting this sirup in a single step. The analysis of variance in Table 6 shows there was no detectable difference in the re-

**Table 6. Analysis of variance of the effect of diluting sirup in one step vs. diluting in multiple steps on the bacterial counts in maple sirup No. 3**

Calculations	Single Step Dilutions	Multiple Step Dilutions	df	SS	MS	F
n	14	14	26	1,290,036	62,374	1.27
$\bar{X}$	221.7	202.14	1	62,374	49,106	
			25	1,227,662		

sults of the bacterial counts obtained by the two procedures.

While fourteen of the fifteen respondents carried out the assays at least in duplicate, one laboratory took three samples from each sirup and assayed three aliquots of each sample—a total of nine assays for each sirup. The results of the analysis of these data are shown in Table 7. In sirup samples No. 2, 3, and 4 there appears to be no detectable difference between the samples taken from the sirups.

Three laboratories carried out standard bacteriological plate counts on the sirup samples in addition to using the membrane filters. A comparison of the results of one of the laboratories is shown in Table 8. Bacterial counts in sirup samples No. 2 and 3 and mold counts in sirup sample No. 4 were compared. From the "t" test analysis

of the counts obtained by both the membrane filter and plate counts there appears to be no detectable difference between the methods. The data of the second laboratory are not shown because replicates were not reported, but the results of the single assay were essentially the same as above. While no great significance should be attached to the analysis of so few values, it is still interesting as an indicator of the possible correlation between the two techniques.

The third laboratory also reporting plate counts found some discrepancies between filter and plate counts. In the discussion of the results (2) it was stated that one inherent advantage of the membrane filter technique over plating techniques was the fixed composition of the growth medium regardless of sample dilution or quantity filtered. If the carbohydrate content of the medium is

**Table 7. Analysis of variance of replicate bacterial counts of maple sirups No. 2, No. 3, and No. 4 by the membrane filter technique**

	Sirup No. 2				Sirup No. 3				Sirup No. 4			
	df	SS	MS	F	df	SS	MS	F	df	SS	MS	F
Total	8	26.00			8	433.56			8	157.56		
Replicates	2	2.66	1.33	0.341	2	203.56	101.78	2.65	2	4.22	2.11	0.83
Error	6	23.34	3.89		6	230.0	38.33		6	153.34	25.58	
$\bar{X}$	2.3				18.22				10.22			
$S\bar{x}$	1.97				2.06				1.68			
$\bar{X} \pm t_{.05} S\bar{x}$	2.33 ± 1.52				18.22 ± 4.75				10.22 ± 3.88			

**Table 8. Statistical "t" test of the difference between bacterial and mold counts in maple sirup obtained by the membrane filter technique or standard plate counts**

	Sirup No. 2*		Sirup No. 3		Sirup No. 4	
	Filter	Plate	Filter	Plate	Filter	Plate
n	2	2	2	2	2	2
$\bar{X}$	8.75	17.5	500	535	135	47
Variance ( $s^2$ )	28.12	0.5	0	92,450	4,075	61
t <sup>b</sup>	2.045				1.93	

\* Bacterial counts were done on sirups No. 2 and No. 3; mold counts were done on Sirup No. 4.

$$t = \frac{\bar{X}_F - \bar{X}_P}{\sqrt{\frac{s_F^2}{n_F} + \frac{s_P^2}{n_P}}}$$

where F = values for filter and  
P = values for plate.

limiting for growth, the sugar added with the sample may become an important factor in the number of colonies developing on the plate. The effect of using a plating medium with high sugar concentration has been discussed (3).

Thus, a number of factors have been considered in an effort to determine the cause for the lack of conformity in microbial counts among fifteen laboratories. Some of these factors involved differences in procedure which were made apparent by replies to specific questions submitted to the collaborators. Undoubtedly there are still many differences in procedure, as a result of the training and experience of the individual collaborator, which cannot be anticipated in a questionnaire. Any of these factors may be sufficiently critical to affect the results of the counts.

Variability in counts obtained by the membrane filter technique has been reported previously (4). An analysis of some of the factors of the experimental procedure failed to reveal the source of bias. The author, however, indicated that variation in the culture medium or in the filter itself probably was responsible for the differences in the counts. Although this work involved only replicate assays within one laboratory it illustrates some of the factors that should be considered in setting up further collaborative studies on the determination of microbial counts in maple sirup with the membrane filter technique.

#### Recommendation

It is recommended that further collaborative studies on the method be continued.

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R. N. Costilow, Department of Microbiology and Public Health, Michigan State University, East Lansing, Mich.

G. K. Crowell, Bureau of Food and Chemistry, State Department of Health, Concord, N. H.

D. R. Davis, Department of Horticulture, Ohio Agricultural Experiment Station, Wooster, Ohio

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D. A. Muentener, Michigan Department of Agriculture, Lansing, Mich.

D. Pomar, Vermont Health Department, Burlington, Vt.

I. Schurman, Food and Drug Administration, Chicago, Ill.

L. W. Slanetz, Department of Bacteriology, University of New Hampshire, Durham, N.H.

S. Stachenko, Canadian and Dominion Sugars Company, Ltd., Montreal, Quebec, Canada

B. Witlin, Department of Bacteriology, Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

C. A. Wood, Food and Drug Administration, Philadelphia, Pa.

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