CCXCV. A NOTE ON THE METHOD OF ESTIMATING VOLATILE ACIDS IN BACTERIAL CULTURES.

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INVESTIGATIONS on the estimation of volatile acids, from both theoretical and practical considerations, have been numerous. Most authors have, however, confined their attention to solutions containing only volatile acids and the literature dealing with the influence of interfering substances on the accuracy of results is small. Thus the empirical methods of Duclaux [1900; 1901], the theoretical considerations of Richmond [1907] and Wiegner [1919] and the work of Dyer [1916] apply to pure solutions only and results based upon these methods become decidedly inaccurate when applied to fluids containing a mixture of organic and inorganic substances such as a bacterial culture.

In general the difficulties of estimating volatile acids in bacterial cultures may be summarised as follows.

(1) Other substances present, particularly lactic acid, may partially distil in steam and add to the titration value of the distillate. Carbon dioxide, whether present as such in the culture fluid or produced as a result of partial decomposition of other substances during the distillation, will have a similar though smaller effect.

(2) The acids contained in the culture may be neutralised, either through the presence of chalk added for that purpose before inoculation or through the simultaneous production of ammonia or other bases as a result of bacterial metabolism. In this case it becomes necessary to add a non-volatile acid to liberate the volatile acids and, as the quantity of the latter is unknown, excess of the former must be used, which means that the effect of p_H and the liberation by the added acid of interfering substances must be considered.

(3) No arbitrary limit can be set to the amount of steam-distillation required to remove all volatile acids since the rapidity with which they are carried over depends on their molecular weight and on the presence of substances, such as soluble salts, which affect their distillation constants. This introduces some difficulty as to knowing when the end-point of distillation is reached.

Foreman and Graham Smith [1928] recommend the steam-distillation of an alcoholic extract previously acidified with hydrochloric acid, and Foreman's method [1928] for eliminating carbon dioxide from this extract minimises errors due to this substance. The use of alcohol, however, greatly increases the bulk of the liquid to be distilled and the method is still subject to many of the difficulties mentioned above. Great accuracy is not in fact claimed, except as regards the method of CO_2 elimination.

Virtanen and Pulkki [1928] recommend for bacterial cultures a preliminary distillation in steam with titration of successive quantities of 200 ml. of distillate, the first fraction being boiled under reflux to remove carbon dioxide. The collected neutralised distillate is then treated with sulphuric acid to liberate volatile

acids and an aliquot portion used for determination of the distillation constant by direct boiling in an apparatus of exact dimensions. No results for actual cultures appear to be given by these authors.

Dyer's method of distilling in steam at constant volume has the advantage that by titrating successive equal quantities of the distillate the end-point may be recognised by the attainment of a small constant titration value after all the volatile acids have been removed. On the other hand the total titration value obtained in this way may represent much more than the volatile acids if there were originally present substances, such as lactic acid, which partially distil. To eliminate this error it is therefore necessary to collect all the fractions of the first distillate and submit them to a second steam-distillation when, as pointed out by Virtanen and Pulkki, the concentration of the lactic acid is so small as to be negligible.

For these reasons the following method was evolved and was found to give satisfactory results:

A suitable quantity of the original culture (100–150 ml.) was acidified with excess of strong sulphuric acid and steam-distilled at constant volume. This was attained by marking the level of the liquid in the distillation flask and heating the latter by a suitably adjusted burner at the same time as a current of steam was passed through the liquid. The distillate was passed through a Liebig's condenser and collected in successive fractions of 100 ml. in measuring cylinders. These fractions were titrated with standard baryta to phenolphthalein and the distillation discontinued when a small constant value was obtained. The successive neutralised fractions were combined, evaporated to a small volume (70–80 ml.), transferred to a flask and the volatile acids liberated by the addition of a quantity of *N* sulphuric acid equivalent to the total amount of baryta used during titration. After shaking thoroughly the flask was left overnight to allow the barium sulphate to settle. The latter was removed by filtration, the precipitate washed and the filtrate made up to a convenient volume (*e.g.* 100 ml.). Carbon dioxide was removed by boiling for 10 min. under a reflux condenser and the liquid again distilled in steam at constant volume, successive portions of the distillate being titrated as before and the process discontinued when a small constant value was attained. The volatile acids were then represented by the total titration value but it was necessary to apply a small correction by subtracting the final small constant value from the titration figure for each 100 ml. fraction of the distillate.

To determine the nature of the volatile acids when required it was found satisfactory to collect and evaporate the neutralised fractions of the second distillation, liberate the volatile acids by the addition of the correct quantity of *N* sulphuric acid, make up the volume of the filtered liquid to 110 ml. and apply Duclaux's methods. Alternatively, the collected fractions of the second distillation could be used for the application of Dyer's method, the liberated acids being steam-distilled from a constant volume of 150 ml.

The following examples will make the method clear and indicate its usefulness in the elimination of errors:

Exp. 1. Value given by fresh medium acidified with H_2SO_4 . In order to find the value given by a fresh medium containing protein and carbohydrate, but no volatile acid, a medium containing 1% peptone, 5% dextrose and 0.3% yeast extract was prepared. To 100 ml. of this were added 5 ml. of strong sulphuric acid and the volume made up to 150 ml. with water. The duplicate titration values for the first and second distillations are shown in Table I together with the final corrected values for "volatile acids".

Table I. "*Volatile acid*" value given by fresh medium acidified with H_2SO_4 .

Distillate ml.	Titration values in ml. of 0.1196 <i>N</i> baryta			
	First distillation duplicates		Second distillation duplicates	
	I	II	I	II
100	0.34	0.30	0.30	0.28
200	0.35	0.61	0.23	0.20
300	0.35	0.21	0.19	0.16
400	0.25	0.34	0.15	0.14
500	0.20	0.36	0.10	0.10
600	0.19	0.21	0.11	0.07
700	0.20	0.17	0.06	0.06
800	0.21	0.20	0.07	0.06
900	0.21	0.22	—	—
1000	0.28	0.23	—	—
Total	2.58	2.85	1.21	1.07

Corrected values (for second distillation) converted into $N/10$:

I. $\{1.21 - (8 \times 0.06)\} 1.196 = 0.87$.

II. $\{1.07 - (8 \times 0.06)\} 1.196 = 0.71$.

Exp. 2. Influence of lactic acid. The influence of lactic acid in raising the titration value of the distillate and its elimination by the second distillation were shown by adding to the same medium as above 20 ml. of 20 % lactic acid, making up the volume to 150 ml. and distilling as previously. Results are given in Table II. It will be seen that a concentration of rather less than 3 % of lactic acid in the liquid being distilled adds considerably to the titration value of the distillate but that a second distillation eliminates most of the error.

Table II. *Influence of lactic acid.*

100 ml. of medium, 20 ml. 20 % lactic acid + 30 ml. water.

Distillate ml.	Titration values in ml. of 0.1196 <i>N</i> baryta			
	First distillation duplicates		Second distillation duplicates	
	I	II	I	II
100	1.30	1.48	0.68	0.50
200	1.24	1.29	0.40	0.33
300	1.14	1.17	0.30	0.24
400	0.97	1.10	0.22	0.17
500	1.05	1.13	0.13	0.15
600	0.91	1.14	0.09	0.09
700	0.94	1.17	0.11	0.08
800	0.98	1.05	0.06	0.08
900	1.06	1.10	0.07	0.08
1000	1.04	1.09	0.06	0.06
1100	0.98	1.05	—	—
Total	11.61	12.77	2.12	1.78

Corrected values (for second distillation) converted into $N/10$:

I. $\{2.12 - (10 \times 0.06)\} 1.196 = 1.82$ ml.

II. $\{1.78 - (10 \times 0.06)\} 1.196 = 1.41$ ml.

Exp. 3. Determination of acetic acid in presence of lactic acid. To test the efficiency of the method in determining a volatile acid in the presence of lactic acid the same fresh medium was used and to this were added 20 ml. of 20 %

lactic acid and 5 ml. of acetic acid of known strength. Table III shows the results obtained by the same procedure as before. It will be seen that there is good agreement between the duplicates in the second distillation and between these values and the quantity of acetic acid originally added, showing that errors due to the presence of lactic acid and other factors have been eliminated and that the "yield" of volatile acid is very good.

Table III. *Determination of acetic acid in presence of lactic acid.*

100 ml. of medium, 20 ml. 20% lactic acid, 5 ml. acetic acid, 25 ml. water.

Distillate ml.	Titration values in ml. of 0.1196 <i>N</i> baryta			
	First distillation duplicates		Second distillation duplicates	
	I	II	I	II
100	30.80	26.47	24.59	28.54
200	18.30	18.00	15.81	17.58
300	11.23	11.84	10.52	10.03
400	7.48	7.93	6.80	5.89
500	5.26	6.19	4.55	3.79
600	3.65	4.63	3.24	2.57
700	2.71	3.54	2.23	1.69
800	1.75	2.68	1.62	1.20
900	1.34	2.05	1.20	0.66
1000	1.60	1.62	0.89	0.42
1100	1.35	1.45	0.64	0.32
1200	1.42	1.42	0.41	0.23
1300	—	—	0.30	0.17
1400	—	—	0.22	0.10
1500	—	—	0.15	0.06
1600	—	—	0.09	0.05
1700	—	—	0.05	0.07
1800	—	—	0.06	—
Total	86.89	87.82	73.37	73.37

Corrected values (for second distillation) converted into *N*/10:

I. $\{73.37 - (18 \times 0.06)\} 1.196 = 86.46$ ml.

II. $\{73.37 - (17 \times 0.06)\} 1.196 = 86.53$ ml.

Amount of *N*/10 acetic acid added = 86.56 ml.

On the other hand it must be borne in mind that "blank" determinations on the same medium *plus* lactic acid, but without any acetic acid, produced figures of 1.82 and 1.41 (Table II)—an average of 1.61. Theoretically, then, the estimations shown in Table III should have given the true value of acetic acid (86.56) plus the figure due to the medium and lactic acid alone (1.61)—or a total figure of 88.17. The explanation of the lower figure obtained in practice may be either (1) that in the presence of appreciable volatile acid the lactic acid shows less tendency to steam-distillation, or (2) that there has been a slight loss of volatile acid sufficient to balance the effect of the lactic acid in increasing the titration figure of the distillate. It should, moreover, be realised that in dealing with a bacterial culture, for which the methods described here are chiefly intended, it is impossible to make a "blank" determination, so that the calculation of results must be made on the assumption that a double distillation removes most of the error due to interfering substances (see calculation of results at foot of Table IV). The results in Table II show that in any case the final error due to this cause is small and the figures in Table III indicate that, when appreciable quantities of volatile acids are present, the error is likely to be still further reduced.

Exp. 4. Determination of volatile acids in a bacterial culture. The ability of the method to give satisfactory duplicates when used for a bacterial culture was tested as follows:

Two separate quantities of 500 ml. of a sterile medium containing 1% peptone, 5% dextrose and 0.3% yeast extract, with 25 g. of added chalk, were inoculated with two different strains of lactobacilli isolated from silage and incubated at 30° for several weeks, the cultures being well shaken each day to facilitate neutralisation of the metabolic acids by the chalk. The residue of chalk was then removed by filtration and washed, the volume of filtrate being made

Table IV. *Estimation of volatile acids in a culture of Lactobacillus (Strain 1).*

Distillate ml.	Titration values in ml. of 0.1300 <i>N</i> baryta			
	First distillation duplicates		Second distillation duplicates	
	I	II	I	II
100	19.82	19.19	11.04	10.60
200	11.38	11.35	6.89	6.79
300	6.47	6.89	3.95	4.01
400	3.23	4.43	2.62	2.65
500	2.28	3.70	1.83	1.66
600	1.54	2.34	0.96	1.18
700	2.00	1.71	0.54	0.86
800	1.80	1.80	0.40	0.68
900	2.02	1.75	0.30	0.45
1000	1.57	1.34	0.25	0.45
1100	1.73	1.72	0.19	0.35
1200	—	—	0.17	0.24
1300	—	—	0.10	0.25
1400	—	—	0.10	—
Total	53.84	56.22	29.34	30.17

Corrected values (for second distillation) converted into *N*/10:

I. $\{29.34 - (14 \times 0.10)\}$ 1.3 = 36.32 ml.

II. $\{30.17 - (13 \times 0.25)\}$ 1.3 = 35.00 ml.

Table V. *Estimation of volatile acids in a culture of Lactobacillus (Strain 2).*

Distillate ml.	Titration values in ml. of 0.1196 <i>N</i> baryta			
	First distillation duplicates		Second distillation duplicates	
	I	II	I	II
100	8.88	11.09	11.17	11.08
200	5.47	5.63	5.41	5.53
300	4.00	4.01	2.85	2.93
400	3.13	2.57	1.63	1.66
500	2.41	1.80	0.91	0.85
600	2.04	1.51	0.72	0.57
700	1.61	1.22	0.44	0.21
800	1.25	1.04	0.25	0.16
900	1.12	0.99	0.16	0.15
1000	1.25	0.99	0.14	0.13
1100	1.00	0.90	0.15	0.14
Total	32.16	31.75	23.83	23.41

Corrected values (for second distillation) converted into *N*/10:

I. $\{23.83 - (11 \times 0.15)\}$ 1.196 = 26.53 ml.

II. $\{23.41 - (11 \times 0.14)\}$ 1.196 = 26.16 ml.

up to 500 ml. Duplicate determinations on each culture were made by adding to 75 ml. of the filtrate 5 ml. of strong sulphuric acid, to liberate the organic acids, and 20 ml. of water and distilling as before. Results are shown in Tables IV and V. Tests of the cultures for other metabolic products showed that Strain 1 produced 1.26% of lactic acid and no other product except acetic acid, whilst strain 2 produced 0.39% of lactic acid, 0.3% of alcohol and the volatile acids were a mixture of acetic and butyric. Table IV shows that the titration values of the first distillate are much higher than those of the second distillate, while in Table V the discrepancy is not nearly so great. This is in agreement with the fact that strain 1 produced a much higher percentage of lactic acid. The agreement between the duplicates in the second distillation is, in the case of both strains, reasonably good. Table IV shows particularly well the errors inherent in a method relying on a primary distillation only.

SUMMARY.

Details are given of a method of estimating volatile acids in bacterial cultures which eliminates most of the errors due to the presence of lactic acid, carbon dioxide and other interfering factors. It has been shown that the "yield" of volatile acid by this method is high and that reasonable consistency between duplicates can readily be obtained.

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