CXLIX. STUDIES IN ZYMASIS.

VII. THE ESTIMATION OF THE ETHYL ALCOHOL AND ACETALDEHYDE CONTENT OF APPLES.

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THE following paper presents an account of the methods used in arriving at the alcohol numbers [Fidler, 1933, 1] given in the former papers of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933].

Suggestive information has been gained by previous workers (see literature quoted by Kostytschew [1927] and Thomas [1925]) as to the progress of alcohol production by methods that had not been standardised. Little value, however, can be attached to numbers purporting to represent relations between alcohol and carbon dioxide production. Moreover, we consider that when anaerobic respiration forms one component of the total respiration it is essential to know the accuracy with which the various zymasic products and the respirable substrates have been measured, before any attempt is made to construct a balance sheet.

The results of this critical examination of a specialised analytical technique are presented in summary tables in this paper, and it will be seen that in all cases the standard deviations of the results are known. In view of the detailed discussion of the technique that was to be given in this paper, no mention of the accuracy of the methods was made in the earlier papers of this series, but only significant figures were given in the tables. It will now be seen that we are able to determine whether any experimental treatment can be said significantly to have increased the alcohol number of an apple.

The relation between the actual alcohol number of an apple at the end of an experimental treatment to the production of ethyl alcohol and acetaldehyde during the period of the experiment is discussed in appendices.

I. Estimation of ethyl alcohol and acetaldehyde in physical systems.

(The results of the different sub-sections are given in the summary Table I.)

A. IN AQUEOUS SOLUTIONS.

1. *Acetaldehyde.*

(a) *By the method of Ripper [1900].* Ripper's method is so well known that the details need not be given here. It has been tested, using a pure specimen of acetaldehyde, purchased from Kahlbaum, and found to be accurate to within the limit given by Ripper, namely $\pm 0.3\%$. In four trials of the method, using

different volumes of an acetaldehyde solution made up from a weighed quantity of the pure liquid, the correspondence between the weighed quantity of acetaldehyde and the quantity determined by the fixation with sodium hydrogen sulphite was within 0.1 mg. in quantities ranging from 19.7 mg. to 39.4 mg. of acetaldehyde.

It may be thought that an error would be introduced into the acetaldehyde figure found by this method, due to autoxidation of the sodium hydrogen sulphite. However, using *N*/20 iodine solution in the titrations, no oxidation of the bisulphite solution could be detected in an hour, the liquid standing with a little air in a corked flask in melting ice.

(b) *By oxidation with potassium dichromate.* It has been found that acetaldehyde may be determined by oxidation with potassium dichromate in acid solution, the resulting volatile acid being then distilled off and estimated by titration with standard NaOH solution.

A series of oxidations was carried out with amounts of acetaldehyde ranging from 0.8 to 78.7 mg. under standard conditions, the concentration of sulphuric acid being maintained at 2.5 % by volume and that of potassium dichromate at 5 % by weight.

Aqueous solutions of acetaldehyde of approximately the strengths required were standardised by Ripper's method, and 10 ml. were added to each of three bottles containing 10 g. pure potassium dichromate and 5 ml. pure sulphuric acid, dissolved in 185 ml. of distilled water. The bottles were closed by ground glass stoppers and allowed to stand 18 hours in melting ice, after which the contents were transferred to distilling flasks. The liquids were distilled down to about 50 ml., and water was then added to the contents of the flask. These distillations and additions of water were continued as long as any volatile acid distilled over. Each 500 ml. of distillate was titrated with *N*/10 CO₂-free NaOH, using phenolphthalein as an indicator, and the distillations were continued until 500 ml. of the distillate required an amount of NaOH equivalent to that required by a distillation of a mixture of 10 g. potassium dichromate, 5 ml. sulphuric acid and 200 ml. of water.

This last titration reading is not included in the calculation of the total acidity of the distillates. From the total amount of NaOH required, the acetaldehyde content of the original solution was calculated, on the basis of the equations:

and

and in every case was found to be greater than that found by Ripper's method. Since we may rely on the accuracy of Ripper's method, it is clear that some acidic substance other than acetic acid is produced by the oxidation of acetaldehyde by potassium dichromate in acid solution¹.

Experiments carried out in triplicate in ten different concentrations within the range indicated above gave a mean percentage excess of acetaldehyde by the oxidation method, as compared with Ripper's method, of 10.1 %, with a standard deviation of 2.51. The results are plotted in Fig. 1, where a line has been drawn through the origin, making an angle of 47° 45' with the *x*-axis. The

¹ Part of the distillate, carefully neutralised with CO₂-free NaOH, did not induce turbidity in baryta solution containing barium chloride, so that the excess acidity is not due to SO₂ or CO₂. It may be due to formic acid. Conant and Tongberg [1930] have shown that the normal course of the oxidation of acetaldehyde with ceric sulphate and sulphuric acid is to formic acid, and claim to have demonstrated α -oxidation of acetaldehyde in acid potassium dichromate solution.

tangent of this angle is 1.101. This means that the values of the abscissae exceed those of the ordinates by 10.1 %. This line passes through or very near to the points of the figure. The graph further shows that the deviation from the mean is of the order of 0.5 mg. in the lower concentrations, and never greater than 2.0 mg. in the high concentrations. The sections dealing with estimations of mixtures of ethyl alcohol and acetaldehyde will illustrate the value of the above oxidation method (*e.g.* see III A).

Fig. 1.

Further experiments, in which the concentration of the sulphuric acid was increased to 5 and 15 % and the time of oxidation to 3 days at 15°, gave figures which did not deviate from the mean value of 10.1 % excess by more than $2 \times \text{S.D.}$, so we may conclude that the conditions of the oxidation may be allowed to vary within limits. However, all oxidations of acetaldehyde described in this paper have been carried out in 2.5 % sulphuric acid for 18 hours at 0°.

2. Ethyl alcohol.

(a) *By oxidation with potassium dichromate.* Dox and Lamb [1916] describe a method¹ of estimating small quantities of ethyl alcohol by oxidation to acetic acid and titration of this compound with standard alkali. The method has been tested over a range of alcohol concentrations, and the results are given in Table I. The alcohol added was estimated by determination of the sp. gr. in a pycnometer, and reference to tables. All oxidations were carried out in 500 ml. solution, containing 12.5 ml. concentrated sulphuric acid and 10 g. potassium dichromate. The oxidation mixture was allowed to stand for half an hour at room temperature and then distilled, with repeated additions of water to the contents of the flask. The distillates were titrated against $N/10$ CO_2 -free NaOH until the figure required to neutralise the distillate from the distillation of a mixture of sulphuric acid, potassium dichromate and water was reached. The first titration reading equal to this is included in the calculation of acidity.

¹ First described by Dupré [1867] and given in Allen [1901].

Table I. *Recovery of acetaldehyde and ethyl alcohol from physical systems.*

Method of			No. of different weights of substances added	Total no. of observations	Range of weights of substances added (g.)		Mean percentage recovery	Deviations from mean percentage recovery									
					Standardisation of solution	Estimation of recovery		From	To	In lowest conc.	In highest conc.	Maximum observed	Standard deviation (s.d.)				
Substance added	1	2	3	4	5	6	7	8	+	-	+	-	+	-	+	-	Standard deviation (s.d.)
(1) Estimation in aqueous solution:																	
Acetaldehyde		Weighted amount of acetaldehyde in definite volume of water	Ripper	2	4	0.0197	0.0394	100.25	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
"		Ripper	Oxidation	10	10	0.0008	0.0787	110.1 (a)	3.0	—	—	—	—	—	—	—	2.51
Ethyl alcohol		Pyknometer	"	7	16	0.0421	0.2185	100.1	1.1	1.3	0.4	0.8	1.2	1.3	1.3	0.99	0.99
(2) Following steam-distillation of an aqueous solution:																	
Acetaldehyde		Ripper	Ripper	6	15	0.00120	0.02126	98.0	5.2	1.2	—	0.9	5.2	3.1	1.97		1.97
"		"	Half distillate oxidised. Other half: Ripper	2	8	0.0292	0.0600	97.0 (b)	3.0	5.5	2.2	0.2	3.0	5.5	2.60		2.60
Ethyl alcohol		Pyknometer	Oxidation	7	17	0.0466	0.1718	100.2	1.4	2.3	—	1.5	1.4	2.3	1.09		1.54
Acetaldehyde		Ripper	Ripper	6	24	0.0058	0.0140	98.8	—	2.1	1.9	1.7	2.8	3.3	1.74		1.74
Ethyl alcohol		Pyknometer	Difference	2	24	0.0467	0.1167	100.4	3.0	1.8	0.4	1.3	3.0	1.8	1.03		1.03
Alcohol number		Sum	Oxidation	6+2 (c)	6+24	0.0525	0.1307	100.2	2.4	1.9	1.2	0.9	2.4	1.9	0.97		0.97

(a) Recovery calculated on assumption that all volatile acid is acetic acid.

(b) This figure cannot be compared with (a) since these recoveries were calculated by assuming that the acetaldehyde equivalent of the volatile acid was 100/110.1 (see Fig. 1).

(c) This series includes six experiments in which the recovery of acetaldehyde from the mixture was not estimated, but assumed to be 100 %.

Table I shows that the mean percentage recovery in sixteen trials of the above method, with amounts of ethyl alcohol ranging from 0.0421 to 0.2185 g., was 100.1, and the S.D. of the method was 0.99. This is a high order of accuracy for an organic analysis, and the method is ideally suited to the estimation of ethyl alcohol in low concentrations in aqueous solution.

B. IN DISTILLATES OBTAINED FROM STEAM-DISTILLATION OF AQUEOUS SOLUTIONS.

It is pertinent to the present enquiry to know whether distillation in a current of steam and condensation of the distillates lead to any loss of ethyl alcohol, or acetaldehyde, from aqueous solutions containing these substances, either singly or in mixtures with each other. Details concerning the solutions which have been distilled are given in Table I. All solutions were distilled for 2 hours, the distillates being collected as described in Section III A.

1. *Acetaldehyde.*

It has been found impossible to avoid loss of acetaldehyde in steam-distillation, while taking the greatest care to guard against losses. When the acetaldehyde content of the distillate was determined by Ripper's method, the average recovery in fifteen trials, in which the weight of acetaldehyde ranged between 0.0012 and 0.0213 g., was 98 %, with a S.D. of 1.97. Using the oxidation method of estimation, the average recovery fell to 97 %, and the S.D. rose to 2.6. This error is of the same order as that introduced by the oxidation. So we conclude that, on the average, 3 % of this very reactive substance has been lost, possibly by undergoing some chemical change at the high temperature. It is possible for the loss to be as high as 8 % in extreme cases, although the maximum observed loss was 5.5 %.

2. *Ethyl alcohol.*

Seventeen trials of the effect of steam on ethyl alcohol solutions were carried out. In these the weights of ethyl alcohol varied from 0.0466 to 0.1718 g. The average percentage recovery was unaffected by the steam-distillation treatment, and the errors of estimation appeared to be of the same order as those recorded under A (2).

3. *Mixtures of ethyl alcohol and acetaldehyde.*

Mixtures of standard solutions of these substances in different proportions were distilled and the distillates worked up by the standard method described in Section III A. We now encounter for the first time in this discussion variations in the estimates of the "alcohol number." The standard method, it will be seen later, gives a direct measure of the acetaldehyde content and the alcohol number, but the ethyl alcohol concentration is given by the difference between these figures and should therefore be subject to a variation which is a function of the separate variances of the acetaldehyde and alcohol number figures. It is therefore all the more gratifying to find that the mean recovery of ethyl alcohol, as found by difference, was of the same order as that in the modified Dox and Lamb estimation (A (2)) and the standard deviations of the two sets of figures were essentially the same.

The figures in Table I provide evidence that the presence of ethyl alcohol does not affect the recovery of acetaldehyde in a steam-distillation.

II. Estimation of ethyl alcohol and acetaldehyde in biological systems.

Since it was uncertain whether ethyl alcohol and acetaldehyde could be recovered quantitatively from biological material by a steam-distillation technique, a series of experiments was carried out on the recovery of known quantities of these substances from sliced apple tissue.

The sliced tissue (York Imperial, Newton Wonder or Bramley's Seedling apples) was well washed and mixed, and to separate 100 g. samples were added definite volumes of standard solutions of ethyl alcohol or acetaldehyde. The results are given in summary in Table II, and are discussed in some detail in the subsections below. No appreciable differences could be detected between the basal ethyl alcohol or acetaldehyde contents of separate 100 g. samples of the mixed tissue, using the analytical methods described under III A. The tissue was freshly prepared for each series of experiments. Usually four tests were made of any given concentration of the substance studied. All the mixtures stood half an hour at room temperature in a closed distilling flask before the current of steam was started. A distillation time of 2 hours was taken as the standard, and the figures in column 9 of Table II indicate that such a period is a satisfactory one.

The knowledge obtained from the results of the experiments described in I B is very necessary, because at the present time it seems that steam-distillation appears to be the best method of recovering ethyl alcohol and acetaldehyde from plant tissues, and especially from apples, in a short enough space of time to preclude secondary changes.

(A) ACETALDEHYDE AND ETHYL ALCOHOL SUPPLIED SINGLY.

A comparison of the mean percentage recoveries of either of these two substances in purely physical systems and in the presence of apple tissue (Tables I and II) shows that there are no striking differences between the two sets of figures. (In the case of ethyl alcohol the two means are identical.) There appears, however, to be more swing about the mean in the figures obtained from the biological systems. Thus, in the case of the recovery of acetaldehyde, the estimated s.d. of the mean has risen from 1.97 to 2.44, and similarly for ethyl alcohol from 1.09 to 1.56. Thus the maximum deviation to be expected, as a pure matter of chance, once in every twenty estimations, has risen from 4 to 5 % for acetaldehyde, and from 2 to 3 % for ethyl alcohol.

(B) MIXTURES OF ACETALDEHYDE AND ETHYL ALCOHOL.

The alcohol number. In estimations of products of zymasis in fruits, where the size of the sample is so large that sampling errors can be neglected (a state of affairs rarely realised), the results of this subsection will be the most important.

We will consider at this stage only the estimates of the s.d. given in column 16 of Table II. These estimates have been calculated by the same formula as used in the earlier subsections and are comparable with those given in Table I. Although the mean recovery of either acetaldehyde or of ethyl alcohol is not altered by the presence of the apple tissue during the distillation, there is greater deviation from this mean value. Thus the estimated s.d. has risen in each instance. Our published results deal with the alcohol numbers of apples,

Table II. *Recovery of acetaldehyde and ethyl alcohol from biological systems.*

Sub- stance added	Method of Standardi- sation of solution	Variety of apple	No. of different weights of sub- stances added	Total no. of obser- vations	Range of sum of weights of substance added + amount present in tissue (a)		Mean per- centage recovery	Deviations from mean percentage recovery				Standard deviation (s.d.)				
					From	To		In lowest conc.		In highest conc.		Maximum observed	Method 1	Method 2		
								+	-	+	-					
Acetalde- hyde	Ripper	4	5	6	7	8	9	10	11	12	13	14	15	16	17	—
		York Imperial	4	4	0.0092	0.0305	95.2	4.8	—	0.8	—	4.8	4.3	3.75	—	
		Newton Wonder	6	24	0.0025	0.0241	97.8	—	—	0.5	0.7	8.2	3.8	2.64	—	
		Bramley's Seedling	6	24	0.0038	0.0254	98.1	3.5	—	—	0.5	3.5	2.0	1.62	—	
Ethyl alcohol	Pykno- meter	All three varieties	16	52	0.0025	0.0305	97.7	8.3	—	—	3.4	8.3	6.8	2.44	—	—
		York Imperial	6	6	0.0574	0.4257	99.4	0.6	—	—	1.0	1.4	1.0	0.68	—	
		Newton Wonder	4	16	0.0258	0.1728	100.1	2.6	3.6	0.2	0.9	2.6	3.6	1.65	—	
		Bramley's Seedling	5	20	0.0252	0.2396	100.6	1.3	—	2.7	7.5	2.7	7.5	1.95	—	
Acetalde- hyde	Ripper	All three varieties	15	42	0.0252	0.4257	100.2	1.7	—	0.5	1.9	3.1	6.1	1.56	—	—
		Newton Wonder	6	24	0.0053	0.0212	99.6	0.4	5.3	2.9	—	3.8	3.0	2.12	1.77	
		Bramley's Seedling	4	24	0.0054	0.0204	97.8	0.4	3.2	4.2	0.3	4.2	5.2	2.45	2.05	
		Both varieties	10	48	0.0054	0.0212	98.7	1.3	4.4	2.0	0.8	4.7	4.4	2.28	1.92	
Ethyl alcohol	Pykno- meter	Newton Wonder	2	24	0.0255	0.0885	100	5.5	7.0	2.6	2.8	5.5	7.0	3.68	4.16	—
		Bramley's Seedling	2	24	0.0261	0.0986	100.5	4.9	5.5	2.7	4.5	4.9	5.5	2.86	3.16	
		Both varieties	4	48	0.0255	0.0986	100.2	7.5	7.2	2.9	4.2	7.5	7.2	3.33	3.69	
		Newton Wonder	6	24	0.0308	0.1097	99.7	6.1	—	0.3	2.2	6.1	3.4	2.38	1.53	
Alcohol number	Sum	Bramley's Seedling	6	24	0.0315	0.1140	100	3.2	1.6	1.8	3.7	3.7	2.36	2.38	2.00	—
		Both varieties	12	48	0.0308	0.1140	99.8	6.0	—	2.0	3.5	6.0	3.6	2.33	2.00	
		Newton Wonder	6	24	0.0308	0.1097	99.7	6.1	—	0.3	2.2	6.1	3.4	2.38	1.53	
		Bramley's Seedling	6	24	0.0315	0.1140	100	3.2	1.6	1.8	3.7	3.7	2.36	2.38	2.00	

(a) The basal values for acetaldehyde and ethyl alcohol contents of the tissues were determined by the method described in Section III. They are subject to errors of estimation, but it would be an unnecessary refinement to allow for these errors.

(b) Throughout Sections I and II, the actual ethyl alcohol and acetaldehyde contents of the tissues were known, and hence the recoveries of these substances could be expressed as percentage recoveries. Considering any separate series from either of these two Sections (*e.g.* the series of trials of the recovery of ethyl alcohol from Bramley's Seedling apple

tissue) the s.d. is found by the formula $s.d. = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$ where $\sum (x - \bar{x})^2$ = the sum of the squares of the differences between each percentage recovery (x) and the mean percentage recovery of the series ($\bar{x}$), and n = the total number of experiments in the series. Thus $n - 1$ gives the number of degrees of freedom. Only column 16 will be considered in this section.

(c) The method of calculation of column 17, and further reference to this column, is deferred until Section III, in the notes to Table III.

and, if perfectly even sampling is assumed, it will be seen from the s.d. of the alcohol number that a deviation of 4.66 % from the true value is to be expected once in every twenty estimations as a matter of chance.

Up to the present, our studies have not necessitated an accurate knowledge of the ethyl alcohol content of tissues (as distinct from the alcohol numbers), but were we to calculate these figures from the percentage figures for acetaldehyde and the alcohol number, we should expect the odds against any figure deviating by as much as 7 % from the true value to be 19 : 1 (again neglecting sampling error).

III. Variations between small samples.

The data given in the previous section are of use in demonstrating that the projected method is applicable to the assay of alcohol numbers of apples. The figures are of little use, however, in analysis of actual experimental results obtained with whole apples, since the errors introduced by random sampling greatly exceed the errors in the analytical technique. Estimates of total errors (which include sampling errors) for samples of two apples each have been obtained for two varieties of apples. The experiments described in Nos. IV, V and VI of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933] were done with samples ranging from one to three mature fruits, for reasons given in the sixth paper. Thus the samples for the experiments described in this section were of two apples each. As will be seen later, it is easy to calculate the error for a sample of any other size.

The results are given in Table III, and are discussed in Subsection B. Acetaldehyde and ethyl alcohol were formed in the apples by exposing them to the experimental conditions summarised in Table III, in the apparatus described by Fidler [1933, 1], for definite lengths of time. The experiments were arranged in groups, each of which comprised three comparable samples of two apples each. At the end of the experimental treatment, the separate samples of each group were distilled and the acetaldehyde and ethyl alcohol contents of the distillates determined in the manner described in Subsection A.

The figures given in Table III for ethyl alcohol and the alcohol number include the transpired alcohol, as did the figures in the earlier papers. This transpired alcohol is determined as described in Appendix II.

The apples were from the population stored for our use by Dr Cyril West, at the Ditton Laboratory, East Malling, Kent, during the season 1932-3.

A. APPARATUS AND METHOD.

After removal of the apples from the experimental conditions, the alcohol and acetaldehyde were extracted by steam-distillation in the apparatus first used and described by Thomas [1925].

Passing through the stopper of a 2-litre flask, containing 1500 ml. water, is the narrow stem of a glass cylinder, 30 in. by $2\frac{1}{2}$ in., the top of which is in communication by means of a splash trap with a 10 in. water condenser of the double surface type. The cylinder is well lagged with cotton-wool. The condenser is in turn connected to a tube passing through the stopper of a 1-litre flask to within $\frac{1}{4}$ in. of the bottom. This flask is connected to a second similar receiver. Each receiver contains 50 ml. distilled water and stands in a trough filled with melting ice.

Before use, the apparatus is disconnected at the top of the glass cylinder, and the water in the flask is boiled until the issuing steam has heated the walls of the cylinder. The apples are now cut, each into eight pieces, and dropped into the cylinder, where they are retained by a circle of

perforated zinc, the edges of which are bound with rubber. The apparatus is immediately re-connected and distillation proceeds for 2 hours.

When the distillation is completed, the distillates are combined and made up to 1 litre with water. Half of this liquor is used for the determination of acetaldehyde by Ripper's method¹ and the other half is oxidised by the addition of a solution of 15 ml. concentrated sulphuric acid and 25 g. potassium dichromate in 85 ml. water. The oxidation is allowed to proceed for 18 hours in ice. The conditions may be allowed to vary a little, as indicated in Section I A 1 (a), which also describes the method of distillation of the oxidation-liquors. It is advantageous to use Claisen flasks for these last distillations, and it is important that no traces of colour should be present in the acid distillates. The liquid in the flasks should be distilled to small bulk (about 50–60 ml.) after each addition of water. In this way it is found that three additions and evaporations of water will suffice to carry over very large amounts of volatile acid.

The calculations of the ethyl alcohol content, and the alcohol number are carried out in the following manner.

Let A = ethyl alcohol content of the distillate.

Let B = acetaldehyde content of the distillate (by Ripper's method).

Then the ethyl alcohol equivalent of $B = \frac{44}{46} B$.

On oxidation, B will yield volatile acid equivalent to $\frac{11^*}{10} \times \frac{44}{46} B$, expressed as ethyl alcohol.

C , the total ethyl alcohol equivalent of the distillate from the oxidation

$$= A + \left(\frac{11}{10} \times \frac{44}{46} B \right) \text{ or, } A = C - 1.05 B.$$

The alcohol number $= A + \frac{44}{46} B = A + 0.96 B$.

Of the two alternative methods of approach to the problem of estimation of ethyl alcohol in the presence of acetaldehyde, namely (1) removal of the acetaldehyde as the initial step in the analysis, and (2) oxidation of the whole solution after estimation of the acetaldehyde fraction by Ripper's method, the second has not previously been used. Despite numerous criticisms, it has been the universal practice of workers studying the course of fermentation to follow the first procedure. The acetaldehyde has been removed by, *e.g.* sodium hydrogen sulphite [Kostytschew and Hübbenet, 1912], or *m*-phenylenediamine hydrochloride [Neuberg and Hirsch, 1919]. The solutions are boiled under a reflux condenser to ensure condensation of the reagent with acetaldehyde, neutralised and evaporated at reduced pressure to remove the ethyl alcohol, which is condensed, or collected in sulphuric acid [Thomas, 1925]. The latter is probably the better practice.

Tomoda [1929] reviewing these, and related methods, describes them as "tedious and troublesome in manipulation" and states that loss of ethyl alcohol or incomplete removal of acetaldehyde may readily occur.

¹ *Estimations of acetaldehyde in the presence of HCN and H₂S.* As yet unpublished work by Thomas in 1929–31 and Thomas and Fidler during 1931–3 on the effect of HCN and H₂S on zymesis by apples necessitated slight modifications of Ripper's method. The blue end-point with starch and iodine is impermanent in the presence of HCN, and practice is needed in order to judge the correct end-point. With care, however, this may readily be done. The question is more involved, however, in the presence of H₂S, for iodine is reduced by H₂S. Our procedure when the distillates have contained this substance has been to oxidise one-third only, using one-third for the estimation of H₂S by titration with iodine. An amount of $N/20$ iodine equivalent to that used in this titration was then added to the third portion of the distillate, oxidising the H₂S. H₂S thus being removed, the estimation of acetaldehyde proceeded in the normal manner.

* From Fig. 1.

Table III. *Errors in estimations with samples of two apples each.*

Variety of apple	No. of exp. order	Date exp. begins	Initial weight of sample (g.)	% oxygen in atmosphere of exp.	State of apples	Duration of exp. (hrs.)	g. per 100 g. apple per 100 hrs.			Increase in % alc. no. in 100 hrs. exposure to gas	CO ₂ : alcohol no. (both expressed as gram-molecule equivalents)	s.d. (a)					2 √2 (s.d.) (b)										
							Acet-aldehyde	Ethyl alc. (allowing for transpired alc.)	% alc. no.			Alc. no. or ethyl alc. %	CO ₂ %	Alc. no. or ethyl alc. %	CO ₂ : alc. no. %	Acet-aldehyde %	Alc. no. or ethyl alc. %	CO ₂ %	CO ₂ : alc. no. %								
1	2			5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21							
Newton Wonder	1 a	17. xi. 32	275-0	7-0	Green	96-0	0-0003	0-0117	0-0120	—	0-2389	—	—	—	—	—	—	—	—	—							
	b	"	290-0	"	"	"	0-0003	0-0091	0-0094	—	0-2461	—	—	—	—	—	—	—	—	—							
	c	"	279-0	"	"	"	0-0003	0-0117	0-0120	—	0-2475	—	—	—	—	—	—	—	—	—							
	2 a	26. x. 32	225-0	5-0	Green	72-0	0-0011	0-0137	0-0148	—	0-2315	—	—	—	—	—	—	—	—	—							
	b	"	232-0	"	"	"	0-0011	0-0138	0-0149	—	0-2169	—	—	—	—	—	—	—	—	—							
	c	"	235-0	"	"	"	0-0008	0-0134	0-0142	—	0-2137	—	—	—	—	—	—	—	—	—							
	3 a	19. x. 32	260-0	Nil	Green	119-0	0-0013	0-1521	0-1534	0-1500	0-2538	100:56-5	—	—	—	—	—	—	—	—							
	b	"	306-0	"	"	"	0-0014	0-1764	0-1778	0-1778	0-2760	100:60-4	—	—	—	—	—	—	—	—							
	c	"	296-0	"	"	"	0-0017	0-2009	0-2026	0-1992	0-3160	100:65-9	—	—	—	—	—	—	—	—							
	4 a	7. ii. 33	222-5	3-5	Yellow	71-5	0-0027	0-2177	0-2204	—	0-3077	—	—	—	—	—	—	—	—	—							
b	"	213-0	"	First sign of wrinkling	"	0-0035	0-2290	0-2325	—	0-3164	—	—	—	—	—	—	—	—	—								
c	"	217-5	"	"	"	0-0027	0-2210	0-2237	—	0-3080	—	—	—	—	—	—	—	—	—								
5 a	10. xi. 32	226-5	Nil	Green	95-5	0-0042	0-2442	0-2485	0-2430	0-3430	100:67-8	—	—	—	—	—	—	—	—								
b	"	236-0	"	"	"	0-0036	0-2455	0-2485	0-2428	0-3153	100:73-6	—	—	—	—	—	—	—	—								
c	"	235-0	"	"	"	0-0033	0-2418	0-2451	0-2394	0-3294	100:69-5	—	—	—	—	—	—	—	—								
Bramley's Seedling	6 a	16. xii. 32	256-0	21-0	Green to yellow-green	96-0	0-0030	0-0611	0-0641	—	0-4033	—	—	—	—	—	—	—	—	—							
	b	"	240-5	"	"	"	0-0026	0-0566	0-0592	—	0-3922	—	—	—	—	—	—	—	—	—							
	c	"	256-0	"	"	"	0-0026	0-0620	0-0645	—	0-3748	—	—	—	—	—	—	—	—	—							
	7 a	1. xii. 32	261-5	7-6 then in air 10 days at 15°	Green	48-5 + 240	0-0047	0-0998	0-1045	—	0-3280	—	—	—	—	—	—	—	—	—							
	b	"	281-5	"	"	"	0-0045	0-0920	0-0965	—	0-2638	—	—	—	—	—	—	—	—	—							
	c	"	250-0	"	"	"	0-0046	0-0964	0-1010	—	0-2927	—	—	—	—	—	—	—	—	—							
	8 a	1. ii. 33	223-5	3-5	Yellow-green	95-0	0-0031	0-1376	0-1407	0-2638	7-5	4-4	8-9	6-3	7-5	4-4	25	18	21	12							
	b	"	228-5	"	"	"	0-0027	0-1151	0-1178	—	0-2444	—	—	—	—	—	—	—	—	—							
	c	"	222-5	"	"	"	0-0026	0-1234	0-1260	—	0-2601	—	—	—	—	—	—	—	—	—							
	9 a	24. xi. 32	260-5	Nil	Green	95-5	0-0040	0-3633	0-3673	0-3632	0-4085	100:85-0	—	—	—	—	—	—	—	—							
b	"	265-0	"	"	"	0-0030	0-3269	0-3299	0-3258	0-3625	100:86-0	—	—	—	—	—	—	—	—								
c	"	262-5	"	"	"	0-0034	0-3649	0-3683	0-3642	0-3958	100:92-1	—	—	—	—	—	—	—	—								
							Considering both varieties together ...													12-1	7-7	6-2	5-6	34	22	18	16

(a) *The s.d.* In the experiments described in this section where the variation between samples of two apples, considered in groups of three samples, is studied, the actual contents of acetaldehyde and ethyl alcohol were unknown. Thus deviations can only be expressed, within each group, as deviations from the mean value for the group. The formula used in Sections I and II (see note (b) to Table II) for estimating the s.d. cannot be used in this case. We have instead to determine the square of the difference between each individual value within a group and the mean value of the group, and to sum the resulting figures for the whole series. We thus obtain an expression: $2\bar{x}^2 = \Sigma(x - \bar{x}_1)^2 + \Sigma(x - \bar{x}_2)^2 + \dots + \Sigma(x - \bar{x}_m)^2$, where x is any individual value and $\bar{x}_1, \bar{x}_2$, and $\bar{x}_m$ are the different mean values for the different groups. The s.d. is given by $\sqrt{\frac{\Sigma \bar{x}^2}{n-m}}$ where n = total number of experiments, m = total number of groups of experiments, and $n-m$ = number of degrees of freedom.

It is useful to know how far any result may differ from the mean value of the population, and 2 (s.d.) will give the percentage deviation from the mean within which 95 % of the observations will lie, as a matter of chance.

(b) It is often useful in biological work to know the probability of two independent observations differing by a given amount. The odds are 19 to 1 against any two random samples from the same population of apples differing by more than the percentage values given in this column. The well-known expression for this is $2\sqrt{2}$ (s.d.).

(c) All experiments were carried out at 23°.

The alternative procedure of oxidising the whole solution has the merit of being simple in manipulation, involving only those operations which would be necessary in estimating the alcohol content of a solution were it first of all freed from acetaldehyde. Little apparatus is necessary and, since the oxidation proceeds overnight, no time is lost.

B. RESULTS.

The results of the experiments on Newton Wonder and Bramley's Seedling apples are given in Table III.

When the estimates of s.d. given in Table III are compared with those given in column 17 of Table II, it is immediately evident that random sampling has introduced an error greatly in excess of that due to the actual estimations.

From Table III it is seen that there must be very great differences between the values for any zymasic product in different samples of two apples each, before it can be said with certainty that the values are significantly different. Thus *e.g.* the alcohol numbers of two samples, each of two Bramley's Seedling apples, from different experimental conditions, must differ by more than 23 % before we are certain that the different conditions of the experiments have resulted in different values for the alcohol numbers.

Fortunately, there exists a well-known method for reducing the error introduced by random sampling. If the s.d. of a single observation is known, then the s.d. of the mean of n observations from comparable samples is $\frac{\text{s.d.}}{\sqrt{n}}$. This formula may be used in another way. Thus the s.d. of a sample of N apples, when the s.d. of a sample of size n is known is given by $\text{s.d.}_N = \frac{\text{s.d.}_n}{\sqrt{N/n}}$. If *e.g.* the sample size is increased to 20 apples, the s.d. of a single observation will be reduced to a value which is $\frac{1}{\sqrt{10}}$ times that of an observation from a sample of two apples.

The values for columns 14 to 21 of Table III for a sample of N apples may be found by multiplying the values given in the table by $\frac{1}{\sqrt{N/2}}$. It will be seen that the s.d. of an observation from a sample of 20 apples is relatively small. If the standard size of the sample were increased to such a great extent, it would, of course, be desirable to redetermine by experiment the value of the s.d.

SUMMARY.

1. Commencing with the estimation of acetaldehyde and ethyl alcohol in purely physical systems, and then passing to biological systems, a technique has been evolved which is applicable to the assay of these substances in apples.
2. The standard deviations of the method described have been determined.
3. The method finally adopted is fairly rapid, and its accuracy is sufficient for the work reported in previous papers.
4. In brief, the method consists of a preliminary steam-distillation for 2 hours to rid the fruit of ethyl alcohol and acetaldehyde. Half the distillate is then oxidised to acetic acid, giving a measure of the alcohol number, while the acetaldehyde in the other half is estimated by Ripper's method. The ethyl alcohol figure is found by subtraction.
5. Apples should be distilled as soon as possible after removal from experimental conditions, as otherwise losses of zymasic products may occur (see Appendix I).

In addition to the acknowledgments to Mr Meirion Thomas, the Department of Scientific and Industrial Research, and to Armstrong College, which have been made previously [Fidler, 1933, 1], I wish to record my indebtedness to Mr J. Wishart, Reader in Statistics in the University of Cambridge, for his helpful criticisms of the methods of calculation of the standard errors.

Appendices.

THE RELATION BETWEEN THE PRODUCTION OF ACETALDEHYDE AND ETHYL ALCOHOL DURING THE COURSE OF THE EXPERIMENT AND THE AMOUNTS FOUND IN THE FRUIT AT THE TIME OF ANALYSIS.

The alcohol number of apples at the end of an experiment, determined by analysis, is not a true measure of the production of ethyl alcohol and acetaldehyde during the course of the experiment, especially when the analysis is deferred until some time after the fruit has been removed from the experimental conditions.

It has been shown that acetaldehyde disappears from apple tissue on exposure to air¹. The results of experiments demonstrating this are given in Appendix I.

Ethyl alcohol is transpired by apples, but the loss from the fruit may be measured. The method of estimation and the magnitude of the escape are given in Appendix II.

APPENDIX I.

The loss of acetaldehyde from apples exposed to air.

The work described in this appendix was carried out solely with a view to determining how long analyses of apples could be delayed at the end of an experiment without affecting the acetaldehyde content.

The results may later be useful in considering the metabolism of acetaldehyde².

1. *Method.*

In many cases, a definite amount of acetaldehyde solution (standardised by Ripper's method) was added to a known weight of sliced apple tissue, the acetaldehyde content of which had been determined. In the majority of the experiments, the acetaldehyde was formed in the apples by exposing them to nitrogen or carbon dioxide-oxygen mixtures as described by Thomas [1925]. Analyses were carried out on samples immediately on re-exposure to air, and after different periods of aeration. All analyses of fruit were carried out by steam-distillation followed by acetaldehyde estimation by Ripper's method.

2. *Results.*

The results of the investigation are given in Table IV.

It is to be concluded from Table IV that loss of acetaldehyde may occur from apples exposed to air, and that this loss may be serious if the aeration is prolonged. Therefore analyses of apples for acetaldehyde should be carried out as soon as possible after removal from experimental conditions.

¹ Experiments by Thomas [1925] indicated that there was no loss of ethyl alcohol after several days' exposure to air, but work now being carried out seems to suggest that disappearance of ethyl alcohol may take place in special circumstances.

² The oxidation of acetaldehyde is one of the aspects of the current researches in this series. It is for this reason that a discussion of the cause of the acetaldehyde loss is not entered upon here.

Table IV. *The change in concentration of acetaldehyde in apple tissue.*

Variety of apple	State of apple	Condition of exp.	No. of hours during which mixture of acetaldehyde and apple tissue was exposed to air	Amount of acet-aldehyde present in system (g.)		Percentage loss of acet-aldehyde	Whether or not the loss is a significant one
				At beginning of exp.	At end of exp.		
York Imperial	—	Acetaldehyde added in soln.	0.5	0.0092	0.0093	Nil	—
"	—	"	0.5	0.0305	0.0277	9.1	Yes
"	—	"	0.5	0.0183	0.0175	4.5	No
"	—	"	0.5	0.0124	0.0117	5.6	No
"	Senescent	"	24	0.0266	0.0205	23.0	Yes
"	"	"	44	0.0325	0.0166	49.0	Yes
Newton Wonder	Young	"	48	0.0362	0.0334	8.5	Yes
"	"	Acetaldehyde formed in N ₂	72	0.0033	Nil	100.0	Yes
"	4 months in store	Acetaldehyde formed in 70 % CO ₂ -30 % O ₂	97	0.0034	0.0001	97.0	Yes
"	"	"	97	0.0046	0.0012	74.0	Yes
"	5 months in store	"	72	0.0104	0.0085	18.2	No
"	"	"	72	0.0225	0.0136	39.1	No
"	7 months in store	"	48	0.0275	0.0111	59.6	Yes
Winesap	—	"	72	0.0140	0.0106	24.3	No
Lord Suffield	Very young	"	73	0.0320	0.0147	54.0	Yes
"	"	"	101	0.0097	0.0034	64.9	Yes

APPENDIX II.

Transpiration losses.

A. *Ethyl alcohol.* Earlier workers on the subject of carbon dioxide : alcohol ratios realised that the experimental material may lose ethyl alcohol by transpiration. Kostytschew in his text-book [1927] cites the use of an ice-cooled wash-bottle containing distilled water to trap ethyl alcohol. In the researches already described in this series, concentrated sulphuric acid has been used successfully for this purpose.

1. *Method.*

As described in an earlier paper [Fidler, 1933, 1], the gas stream issuing from the fruit chamber passed through a wash-bottle containing 50 ml. of concentrated sulphuric acid. This treatment was shown to rid the gas of all appreciable amounts of ethyl alcohol, the method being checked as follows. A solution of ethyl alcohol in water was standardised by the oxidation method, and definite volumes were measured out into wash-bottles. Air was bubbled through each of these bottles at about 3 litres per hour, for definite time periods at room temperature (12–15°). The issuing air passed through each of two wash-bottles containing 50 ml. of concentrated sulphuric acid. The results are given in Table V.

Table V shows that all the ethyl alcohol escaping from the solution is trapped in the first wash-bottle containing sulphuric acid. The original Dox and Lamb [1916] aeration method of estimating ethyl alcohol made use of the formation of the non-volatile ethyl hydrogen sulphate in this manner.

Table V. *Trapping of ethyl alcohol in sulphuric acid.*

Amount of ethyl alcohol in wash-bottle (g.)		Loss of ethyl alcohol from wash-bottle (g.)	Period (days)	Ethyl alcohol trapped in sulphuric acid (g.)		Net loss or gain of ethyl alcohol (g.)
Beginning of aeration	End of aeration			First wash- bottle	Second wash- bottle	
0.1880	0.1022	0.0858	3	0.0853	Nil	-0.0005
0.1880	0.1290	0.0590	3	0.0605	„	+0.0015
0.1880	0.1650	0.0230	1	0.0230	„	Nil
0.1880	0.1650	0.0230	1	0.0248	„	+0.0018
0.1880	0.0924	0.0956	5	0.0943	„	-0.0013
0.1880	0.0819	0.1061	5	0.1055	„	-0.0006

2. *The ratio alcohol content: alcohol escape.*

There is no need to give a detailed account of the magnitude of the escape of ethyl alcohol from apples, but a few examples will be of interest in indicating the values we have observed.

In our experiments on Newton Wonder and Bramley's Seedling apples at 23° during 1930-33, the mean value for the transpired alcohol was 8 % of that contained in the apple. During November and December 1933, an experiment was carried out on Sturmer Pippins in nitrogen at 20°. When the ethyl alcohol content of the tissues had risen to 0.2 % (after 12 days) the escape totalled 0.006 %, but when the alcohol content reached a value of 0.6 % (after 45 days) the escape totalled 0.076 %, or 15 % of the content. This figure has been exceeded with Bramley's Seedling apples.

Thus large errors may be introduced if no account is taken of ethyl alcohol loss by transpiration.

B. *Acetaldehyde*. We have, as yet, few figures for acetaldehyde escape, but such as we have indicate that there is little probability that appreciable error would be introduced into the alcohol figures by this means.

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