VOLATILE CONSTITUENTS OF MALT VINEGAR

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DECLARATION

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

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Candidate

.....

Director of Studies 3.9.70

CERTIFICATE

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

Signed .....None.....

Date .....3/9/70.....

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SUMMARY

Gas liquid chromatography was used to fractionate the alcohols, esters, carbonyls and acids in commercial malt vinegars. A qualitative and quantitative study was made using a variety of stationary phases. Porapak Q was found to be the column packing material of choice. Acetaldehyde, ethyl acetate, ethyl alcohol, iso-butyl acetate, n-propyl alcohol, n-butyl acetate, iso-butyl alcohol, n-amyl alcohol, acetoin, propionic acid and acetic acid were identified in a variety of malt vinegars.

The origin of these volatiles was investigated by their addition to laboratory acetifications, which enabled an assessment to be made of the probable metabolic routes responsible for the formation and breakdown during acetification.

The esters were found to be formed during the initial alcoholic fermentation and not during subsequent acetification.

In addition three distinct processes of malt vinegar manufacture were examined to ascertain whether these pathways were operative in commercial practice. Both propionic acid and acetoin concentrations were found to be lower by the Fring's and Continuous processes. Increased aeration occurs but lower concentrations were found with laboratory acetifications, otherwise the pattern and character of the volatiles were the same.

Samples obtained during the commercial processes were stored to investigate changes during 'maturing'. No increase was found in the number or concentrations of the volatiles. There appeared to be little change in general volatile characteristics of vinegar during 'the maturation' process, although a decrease was found in the lower boiling point volatiles.

During the growth of mycoderma on wort, n-propyl and n-amyl alcohols increased in concentration. Such an increase would suggest a 'secondary fermentation' by mutant strains of bacteria or yeast arising in the production of both alcohols.

Finally the efficiency of continuous acetification in tower shaped fermentors was investigated in relation to optimum substrate, temperature and aeration.

The estimation of pO_2 was found to be the most suitable parameter for the control of continuous acetification in tower fermentors.

CONTENTS

INTRODUCTION

INTRODUCTION

Vinegar had its recognised place among the products of the Alchemist and was indicated by the symbol ☩ while for distilled vinegar the characteristic ✠ was used. References to Acetum is found in the Classics with the fable of its use by Hannibal to dissolve the Alps.

According to Lemery (1720) there were three sorts of liquors known as Spirit; the Spirit of Animals, the Burning Spirit of Vegetables and the Acid Spirit. The first was typified by Spirit of Hartshorn, the second by spirit of wine, while the last as 'the Spirit of Vinegar, Tartar and Vitriol, is an Acid Essential Salt dissolved and put in fusion by the fire, as I shall prove when I speak of Vinegar'.

The term 'radical' vinegar which survived into the last century (Act of George III, 1818) shows that concentrated acetic acid was prepared from distilled vinegar by neutralisation, concentration and redistillation as far back as the 17th Century.

The earliest description of a process of making vinegar appears to be that published in 1670 in the Transactions of the Royal Society under the heading 'The Way of Making Vinegar in France: Communicated to the Publisher by an Ingenious Physician of that Nation, living at a Place where much of it is Made' (Phil. Trans. Roy. Soc., 1670). This description goes on to describe at some length the process of making wine vinegar.

Since the British were a beer-drinking nation, it was to be expected that the development of the vinegar industry in this country should have come by way of beer rather than from wine. The product of wine became known as 'vin-aigre' (sour wine) and hence by analogy the product derived from beer became known as 'ale-gar'.

The malt vinegar manufacturer evolved out of the brewer because the production of vinegar was the obvious way of disposing of sour beer in the brewery. In the Revenue Act of Charles II (1673) the vinegar produced as a waste product was termed 'Vinegar-Beer' and had to pay a duty of sixpence per barrel.

The date of the establishment of the first vinegar factory is uncertain, but there was a vinegar yard in Castle Street, Southwark in 1641 (History and Antiquities in the Parish of St. Saviour, Southwark, 1795).

Legislation in connection with vinegar had been concerned with the purposes of revenue although interesting details of the industry are given in the Acts of George III who enacted that vinegar makers had to declare whether it was intended to make vinegar from malt, corn, molasses or sugar. The act also classed vinegars into Revenue Proof Acid and numbers were given according to acid strengths. In 1824 the Pharmacopoeia stated that 'the strongest vinegar is termed proof vinegar, and by the manufacturer called No. 24 and it was estimated to contain five per cent of real acetic acid'.

Another explanation of trade numbers was given by Muspratt (1860) who stated that 'proof vinegar has a specific gravity of 1.0085 and containes about five per cent acetic acid. In commerce this vinegar is represented by No. 24'.

It was not until 1864 that Louis Pasteur, the French microbiologist, showed that bacteria present in vinegar were directly responsible for converting alcohol to acetic acid. Since Pasteur's time a number of acid-producing organisms have been isolated from vinegar generators and these have been given the genus name Acetobacter. The manufacture of

vinegar is a two stage process, the first stage being the conversion of sugars to alcohol by yeasts and the second stage where the alcohol is converted to acetic acid by bacteria. Thus vinegar can be produced from any liquid capable of first being converted to alcohol. There are many types of raw materials suitable for use in manufacturing vinegar, but those most widely used are apples and grapes in making cider and wine vinegar, malted barley and oats in making malt vinegar and industrial alcohol in making distilled vinegar. Chemically, vinegar is a dilute solution of acetic acid containing soluble substances of the raw material.

Acetobacter are aerobic organisms and require an abundant supply of air for growth. Vinegar can be produced in a small container but the Acetobacter are active only at the surface of the liquid where air is available. Under these conditions surface area in relation to volume is low and consequently acetification is slow. Starting with the primitive method of placing fruit juices and allowing fermentation and acetification to proceed haphazardly the technology advanced through the old Orleans processes to the relatively modern quick vinegar methods.

The Orleans process consisted of making vinegar in barrels of fifty gallons capacity. The barrels were laid on their sides with two air holes in the side. The barrels were then three-quarter filled with mash prepared by mixing about five gallons of five per cent vinegar with thirty gallons of alcoholic liquid containing five to six per cent alcohol. A small volume of vinegar containing 'mother of vinegar', a mass of vinegar bacteria, was then added to the mash in the barrel as a starter. When the acidity of the vinegar reached five per cent the vinegar was drawn off leaving five gallons of vinegar in the barrel as

an inoculum for the next batch. Three or four months time was required to complete the acetification process.

In the early part of the 18th Century H. Boerhaave, a Dutch technologist, found that the rate of acid production was directly proportional to the amount of surface exposed to air. He modified the Orleans process by using pomace (the residue from grape presses) to loosely pack casks. Wine mash was allowed to trickle down and spread over a large area thereby increasing the volume exposed to aeration. In 1823, Schutzenbach, a German chemist, drilled holes in the Boerhaave generators to admit more air and introduced other types of porous material. This method became known as the quick vinegar process. Most modern generators are based on the principles developed by Schutzenbach. The quick vinegar method reduced the time of acetification from months to days.

The modern quick vinegar process consists of wooden tanks with a perforated false bottom. It is then loosely packed with beechwood shavings or birch twigs over which mash is evenly distributed. The mash is passed through the generator a number of times to complete the acetification.

During 1949 and 1951 a method of acetification called submerged fermentation was developed by Hromatka and Ebner which gave higher production and efficiency rates. No wood shavings or other packing material was required, the bacteria being supplied with air by continuously dispersing air bubbles through the mash. The submerged fermentation method is exemplified by the Frings Acetator designed where the limiting factor of the reaction rate is gas solubility.

Vinegars are often matured by storage for periods ranging from a

few weeks to a few months. During the maturing process it has been held that a complicated pattern of chemical changes occur involving changes in flavour and odour. Traces of alcohols at the end of acetification are held to be largely esterified forming a mixture of trace substances which contribute to character and quality of the final vinegar. The volatile flavour and odour-producing substances contribute largely to the final refined vinegar. One would also expect volatiles to be increased or decreased during manufacture by different processes.

The first reference to the volatile constituents of vinegar was made by Farnsteiner (1908) when he detected a volatile copper reducing substance, resembling an aldehyde, and which was similar to acetole, but differed from it in that its osazone melts at 243°C while that of acetole melts at 145°C. He also noticed that an oily substance was formed with osazone which indicated the presence in the vinegar of another substance that reacted with phenylhydrazine. Farnsteiner concluded that all solutions which had undergone acetification contained a volatile neutral substance that reduced Fehling's solution and which gave a false indication of the presence of sugar to the extent of 0.75 g. per 100 ml.

Gore (1910) described an apparatus for distilling volatile acids in wines and vinegar for subsequent titration with 0.1 Normal alkali and a year later Fincke (1911) described the chemical investigation he made of fermentation vinegar. He found the presence of formic acid which he determined by the reduction of mercuric chloride to mercurous chloride with subsequent weighing of the mercurous chloride.

Balcom (1917) examined distillates obtained from cider vinegar and compared the reducing substances to solutions of diacetyl and acetylmethylcarbinol. He concluded that the volatile reducing substances

of cider vinegar consist largely if not wholly of acetylmethylcarbinol, a normal constituent of cider vinegar.

The volatile acids of vinegar were determined by Fagon (1936) using a distillate of vinegar in which he titrated with alkali. The non-volatile acids being obtained by the difference between that obtained in the direct titration for total acids and the titration of the distillate.

Edwards and Nanji (1938) differentiated between spirit, malt, distilled malt and artificial vinegars by determining the oxidation, iodine and ester values. The ester values were defined as the ml. of 0.01 Normal potassium hydroxide required to saponify the esters in 100 ml. of vinegar under standard conditions. Whitmarsh (1942) using similar methods concluded that malt vinegar had distinctive oxidation and iodine values due to the presence of ethyl alcohol and acetylmethylcarbinol.

Ten years later Pontin (1953) determined the acetone content of vinegars obtained from alcohol in which he used the reaction of salicaldehyde in alkaline solution to detect small quantities of acetone. During the same year Klinc (1953) detected butyric acid in wine and vinegar by oxidation - butyric acid yielding acetone, whilst propionic acid produced acetaldehyde. Wine vinegars contained 50 - 290 mg. of butyric acid per litre and spirit vinegar contained up to 30 mg. per litre.

With the advent of gas liquid chromatography (G.L.C.) in 1952 it was then possible to fractionate and quantify volatiles found in fermentation products. Partition chromatography was introduced by Martin and Synge in 1941 using a liquid moving phase. Liquid-liquid chromatography was further developed by Martin and his co-workers with an elution technique known as 'paper chromatography'. Ultimately Martin together with James

introduced gas-liquid chromatography which was a continuation of the liquid-liquid partition chromatography introduced ten years earlier. While the latter method is mainly applicable to the less volatile lipophilic and hydrophilic substances, G.L.C. was mainly applied to volatile compounds with a boiling point of up to 400°C. In the method of G.L.C. a carrier gas 'mobile phase' flows at a given temperature through a tube containing an inert support impregnated with compounds of various properties (stationary phase). A mixture introduced at the beginning of the column is separated by partition and finally passes in gaseous form through a detector.

With the object of fractionating volatiles during vinegar fermentation Suomalainen and Kangasperko (1960) applied G.L.C. to wine and spirit vinegars. The spirit vinegar contained ethyl acetate, whereas wine vinegar contained acetoin, iso-amyl alcohol, amyl alcohol, iso-butyl alcohol and amyl acetate. They also concluded that the principle amount of esters in commercial vinegars developed during storage.

Quantitative estimation of ethanol in vinegars was made by Morgantini (1962) using a column of twenty per cent polyoxyethylene glycol succinate on celite support at 110°C. Close correlation was obtained between the gas chromatography results and the titrimetric assay for ethyl alcohol.

Cesari, Cusmano and Boniforti (1964) determined acetoin and diacetyl in vinegar by direct comparison of chromatograms of vinegar and standard solutions of acetoin and diacetyl. Accurate and precise results were obtained.

Gas chromatography was used in a study of cider vinegar and distilled vinegar by Kahn et al. (1966) whereby using a suitable liquid phase it was possible to resolve alcohols, esters, acids and 3-hydroxy-2-butanone.

The column was ten feet long containing chromosorb W. The analysis was temperature programmed from 56°C to 120°C. Twelve compounds were found in cider vinegar and five in distilled vinegar. The compounds detected were methanol, ethyl alcohol, sec-butyl alcohol, a C5 alcohol, methyl and ethyl acetate, ethyl lactate, 3-hydroxy-2-butanone, acetic, propionic, iso-butyric and C5 acids. It was possible by this technique by selecting the proper liquid phase, to resolve the alcohols, esters and acids on one chromatogram, although no quantitative assays were made.

Aurand et al. (1966) also atudied the volatile components of cider, wine, tarragon and distilled vinegars by G.L.C. Cider vinegar contained 19 components, wine 17 components and tarragon 20 components with 11 in distilled vinegar. They also found that four compounds were present in all the vinegars, these included acetaldehyde, acetone, ethyl acetate and ethyl alcohol. A six foot glass column with fifteen per cent polyethylene glycol 600 on firebrick or with di-isodecylphthalein on celite with a constant temperature of 105°C was used throughout. In Aurand et al.(1966) work it was found that members of different homologous series of organic compounds may have similar retention volumes requiring functional group analysis for positive identification. Although thirteen components were indicated by the chromatogram fifteen components were actually present. Quantitative data were not obtained but qualitative differences in the individual concentrations amongst a number of samples were noted. It was concluded that the most important carbonyl compound contributing to vinegar flavour was diacetyl. Since esters are known for their aromatic odours it was not surprising that they were the largest group of volatile flavour components. It also appeared that the standards of identity of a particular vinegar could be determined by the relative concentration of the alcohols and esters.

Key et al. (1968) using an extraction procedure with ether and n-pentane studied the flavour components of rice vinegar when fifty one components were fractionated, ten of which were alcohols, eight carbonyl compounds and six esters.

G.L.C. has not been applied to the fractionation of volatiles in malt vinegar although a number of workers have examined volatiles of beer. Since the production of malt vinegar consists of a double fermentation where the basic substrate malt is first fermented by a strain of yeast Saccharomyces under relatively anaerobic conditions to produce an alcoholic liquor one would expect to find a similar pattern of volatiles during vinegar manufacture. With the object of fractionating the volatiles in beer a number of workers have employed G.L.C. The first of these workers Van der Kloot, Tenney and Bavisotto (1958) used a gas chromatograph column consisting of thirty per cent glycerin diluted with methanol on fire brick support to fractionate volatiles contributing to flavour. They were able to detect a number of alcohols and esters, but such components as methyl, propyl and butyl alcohols were obscured by the ethanol peak.

Subsequently Jenard (1959) made a study of the volatile substances of beer in which he distilled the beer giving 0.5 per cent volatiles. The water was removed by bubbling a stream of nitrogen through the distillate and then passing it through a column of potassium carbonate. The volatiles remaining in the carrier gas were then condensed in a trap with liquid air.

Strating and Venema (1961) studied the aroma concentrate from beer by steam distillation in vacuum and extraction of the distillate with a mixture of pentane and diethyl ether. Programmed heating from 55°C to 150°C together with a mixture of two stationary phases were used.

In the same year Harold et al. (1961) showed that the volatile substances which occur in beer originate from malt and also concluded that distilling the sample was detrimental to thermal labile constituents. An ether extraction was recommended. Clarke et al. (1962) also using an ether extract of beer detected eighty one volatile acids.

Maule (1967) examined by gas chromatography the beer volatiles using a copper column packed with ten per cent carbowax on chromosorb W. The carbonyl compounds were acetaldehyde, acetone, diacetyl, 2,3 pentane-dione and acetoin; the alcohols were n-propanol, iso-butanol, 2-methyl butanol and 3-methyl butanol; the esters were ethyl acetate, iso-amyl acetate and ethyl caproate.

Powell and Brown (1966) extracted the volatiles of beer into carbon disulphide which yielded a concentrate. Temperature programming from 60°C to 115°C was used, n-octanol being the internal standard. A further study on beer volatiles was made by Kunitake (1965) without using a concentrating step, the reference volatile being 3-heptanone in 3.5 per cent aqueous ethanol. The peaks were identified by functional group analysis of Hoff and Feit (1964). Hashimoto and Kuroiwa (1966) concentrated by distillation the volatile alcohols and esters of beer with subsequent gas chromatography, the column being twenty five per cent glycerin on shimalite and the temperature 50°C.

The role of gas chromatography in aroma research has been investigated by Teranishi et al. (1963) and Harrison (1963). The variables considered included extraction procedures, distillation and closed loop sweeping with freezing trap separation. The stationary phases examined were polyethylene glycol, polyethylene glycol adipate and diethylene glycol succinate. Suggestions were made to minimise

'drift' and to obtain concentrated bands by using columns of high theoretical plate values.

Although Aurand et al. (1966), Kahn et al.(1966) and Key et al. (1968) have qualitatively assayed alcohols, esters, acids and carbonyls in distilled, rice, cider, wine and tarragon vinegars, none have obtained qualitative or quantitative results with malt vinegar. Since the process of vinegar manufacture consists of a double fermentation where the higher alcohols obtained in the initial fermentation are subjected to acetification, it would be expected that a number of alcohols and their esters would be found in the final product. One object of this work is to identify as far as possible the volatiles in malt vinegar and to relate the findings to the manufacturing processes. An examination is also proposed of the qualitative and quantitative changes in the alcohols, esters and acids during storage. It was felt that gas chromatography would be the technique of choice in such a project.

During the last ten years new processes have been developed which use continuous flow fermentation. Utenkov, a Soviet microbiologist, was working in this field in 1935. His work was not published in 1935 and subsequently in 1950 the mathematical theory of continuous fermentation was published by Novik and Szilard and by Monod. Further development of continuous fermentation was made in the Soviet Union. A complete review and discussion took place at the First International Symposium in Prague in 1958.

Enenkel et al. (1950) described a continuous fermentor for the production of carboxylic acids which consisted of a tube with a height of three metres with a means of introducing air at the bottom which

was the first reference to submerged fermentation in a heterogenous continuous open system using a tubular system. During 1955 Shimwell of British Vinegars Ltd., described a process of vinegar manufacture in which a stainless steel cylindrical vessel approximately three feet in length and two inches in diameter was fitted with a reflux condenser. The rate of aeration was increased during the log phase of growth. An increased pressure was maintained above the surface of the culture so that an increased amount of oxygen was dissolved.

Further advances have been made in the continuous production of vinegar by Greenshields (1968), who designed a tower shaped fermentor its height being at least five times its diameter and, preferably, ten times its diameter. The vessel was fitted at the top end with a separating zone to enable micro-organisms to settle from the rising air and return to the vessel. Heat could be supplied by jackets surrounding the vessel. Compressed air was passed through a sintered glass disc so that small bubbles entered the fermentor. The efficiency was defined 'as the ratio between the volume of liquid added each day and the volume of the fermentation vessel'. Efficiencies of 1.0 were obtained compared to 0.5 achieved for other methods.

Another object of this work is to examine optimum conditions for continuous fermentation in tower fermentors, and the most suitable index for quality control. The tower fermentor is of a heterogeneous nature and is an advantage over other continuous fermentors which are homogeneous. Another advantage of tower fermentors is the long residence time of aerating gas and the availability of the oxygen during acetification.

Such investigations have not been made during malt vinegar manufacture. It is hoped that technological modifications and improvements of less lengthy procedures might be accomplished as a result of this type of investigation.

MATERIALS AND METHODS

SECTION A(I)

GENERAL

MATERIALS AND METHODS

SECTION A (1).

(1) GAS CHROMATOGRAPHY

A Gas Chromatograph (Pye Series 104) equipped with a flame ionization detector was used throughout. The voltage output from the amplifier was attenuated and then indicated on a potentiometric recorder (Leeds and Northrup). The complete instrument used is seen in Fig.1. Accessories included a bubble flowmeter assembly, Fig.1. calibrated from 0 to 25 ml., in 5 ml. divisions and a Disc Integrator, (Model 224, Series D.) obtainable from Jones Chromotography and Co., 71 Ridgeway, Machen, Newport.

(2) COLUMN PACKING MATERIALS

Three distinct packing materials were used.

(a) A 5ft. glass column with a $\frac{1}{4}$" bore was packed with 10% diethylene glycol adipate on acid and alkali washed celite and supplied by W.G.Pye and Co.,Ltd., York Street, Cambridge. Maximum temperature of operation was 175°C. The packing technique consisted of vibrating the column followed by passing the carrier gas nitrogen through the column for a few minutes. The column was then plugged with a 1ft. length of silk thread and preheated in the analyser oven for 24 hr. at a temperature of 200°C. During this time the flame ionization detector was not used.

(b) A 5ft. glass column as above was packed with 10% polyethylene glycol (M.W.1000) and supplied by W.G. Pye and Co.,Ltd., Cambridge on acid and alkali washed celite. Maximum temperature of operation was 100°C., and the preheating temperature 125°C. for 24 hr. The

Fig. I. Pye 104 Gas Chromatograph with bubble flowmeter assembly.

method of packing was similar to that used for diethylene glycol adipate. Polyethylene glycol (PEG) is a very polar stationary phase and recommended by the manufacturer for separating low boiling point alcohols and esters from water. On this column water behaves as an alcohol with a boiling point of 100°C.

(c) A 7ft. glass column with a $\frac{1}{4}$" bore was packed with Porapak Q, Waters Associates Inc. 61 Fountain St., Framingham, Mass., and obtainable from Jones Chromatography, 71 Ridgeway, Machen, Mon. Porapak Q consists of a porous polymer bead 50-80 mesh. The surface area was 634 $m^2/g.$, and the particle diameter 111 Å. The manufacturers claimed that there was no bleed from the packing material; no adsorption of polar compounds; or change in retention times and was stable at high temperatures.

(3) CONDITIONS OF CHROMATOGRAPHY

(a) Apparatus Performance.

The column efficiency was estimated before use by calculation of the theoretical plate number (n) and the peak resolution. Since the diethylene glycol adipate and polyethylene glycol columns were used only during the initial investigations the theoretical calculations were only made for the porapak Q columns. The compounds used for calculation of the theoretical plate number was acetoin and for column performance iso-butyric acid and acetoin. These latter two compounds were chosen because their retention times were near to each other but yet sufficiently separated to permit a satisfactory estimation of their peak width, and also because they were eluted in the chromatogram centrally

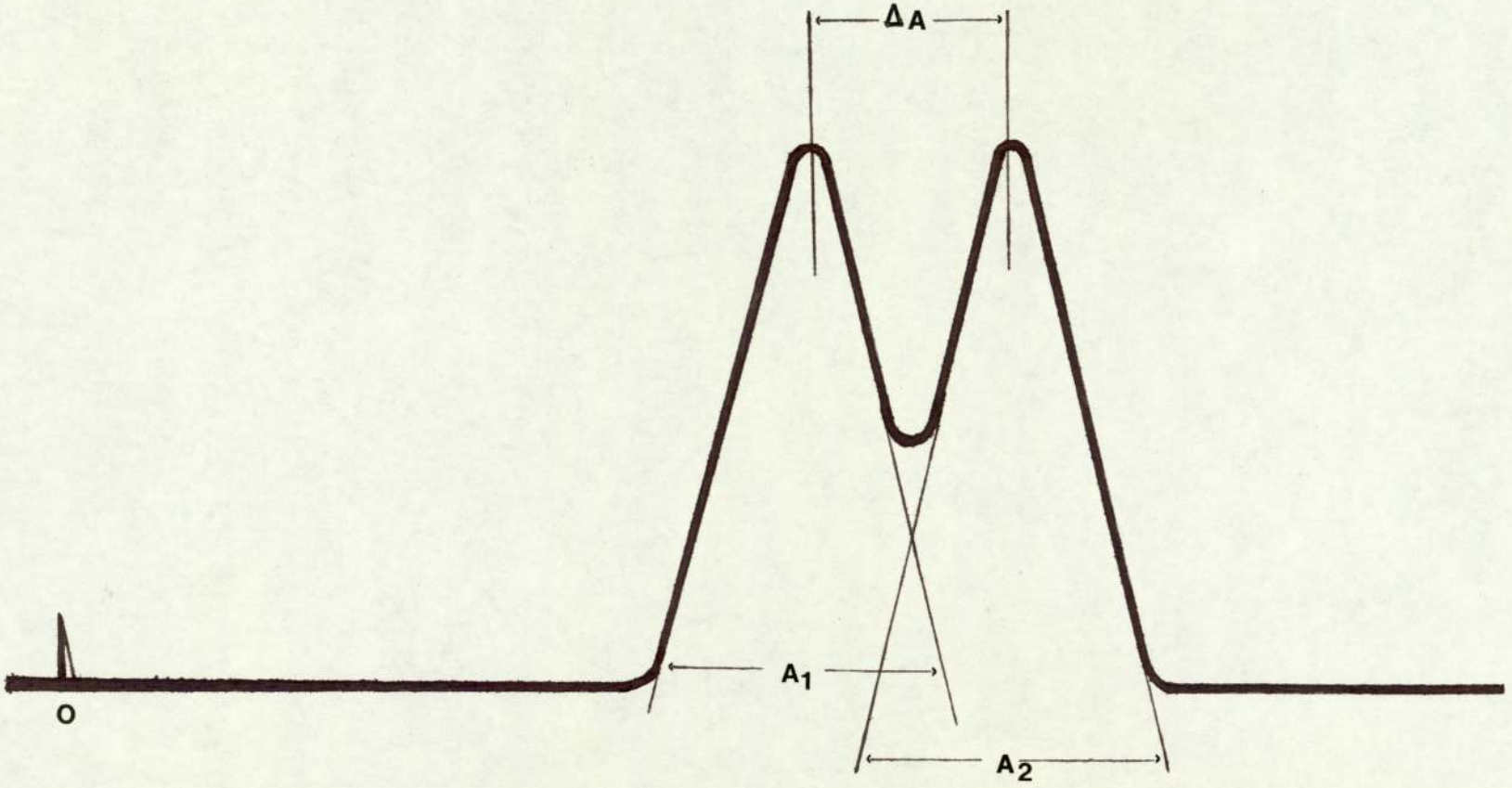


Fig. 2. Peak resolution.

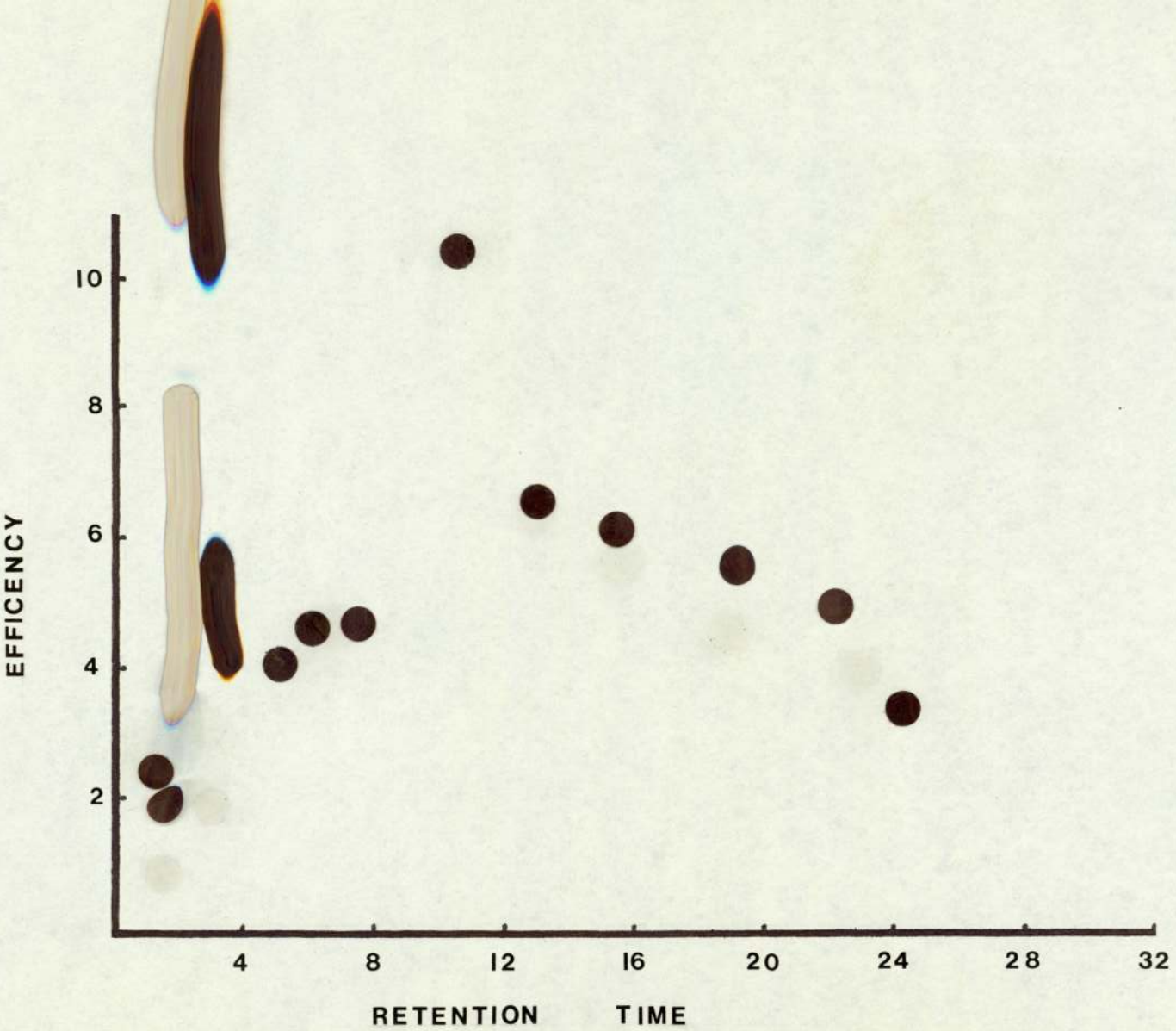


Fig. 3. Column efficiency using Porapak Q.

in relation to the other volatiles. The equation used for the theoretical plate number was

$$n = 16 \times \left(\frac{\text{retention volume}}{\text{peak width}}\right)^2$$

The retention time found for acetoin when using porapak Q was 12.6 cm., and the peak width 19.5 mm.

Therefore $n = 6.4$

The range for n was found to be 6.2 - 6.6.

Peak resolution was calculated from (see Fig.2.).

$$\text{Resolution} = \frac{2 \times \text{difference between retention times}}{\text{sum of peak widths}}$$

$$= 2\Delta A/A1 + A2$$

The peak width of iso-butyric acid was 13 mm. and for acetoin 19.5 mm. The difference between retention times was 20 mm. The range of resolution calculated for six columns was 1.22 - 1.32.

The efficiency was plotted against retention times of all the volatiles detected Fig.3., and Table 1. The efficiency increased up to a retention time of 10.6 cm. (iso-butyric acid). When the efficiency of the column decreased after several months of continuous use, the column was re-packed.

(b) Gas Flow Measurement.

The gas flow measurement kit used was that supplied by the manufacturer (Fig.1). The passage of a soap film between the zero and 10 ml graduation was accurately timed and the gas flow calculated as follows:-

Let the time taken for the film to pass from zero to 10ml. be x. Then volume of gas passing per minute

$$= \frac{60}{x} \times 10$$

The flow rates of both the carrier gas nitrogen and the fuel gas hydrogen were estimated in this manner. The air flow was normally ten times the hydrogen flow and therefore timed from zero to the 25ml. graduation, and calculated from $\frac{60}{x}$ x 25ml.per min.

(4) CONDITIONS FOR THE GAS CHROMATOGRAPHY OF MALT VINEGAR

Initial experiments were made with the object of obtaining optimum temperature, attenuation, sample volume, chart speed, carrier gas flow, and the number of volatiles fractionated.

(a) Diethylene glycol adipate (DEGA).

2 µl. of malt vinegar was chromatographed at an attenuation of x200 at three temperatures 70°C.,85°C., and 100°C. The results obtained are seen in Figs. 4,5 and 6. At a temperature of 70°C., eight clearly defined peaks were obtained, and at 85°C and 100°C., six and four peaks were resolved respectively. The attenuation was then decreased to x100, but no increase in the number of volatiles was detected at this increased sensitivity. Three sample volumes were chromatographed 1 µl., 2 µl., and 3 µl. With the 1 µl. volume two volatiles were not detected and with 3 µl. excessive tailing of the peaks ensued due to column overload. The chart speed giving optimum resolution was 120 cm. per hr. During these experiments the nitrogen and hydrogen flow rate was 45 ml. per min., and the air flow rate was 450 ml. per min. Increasing or decreasing the

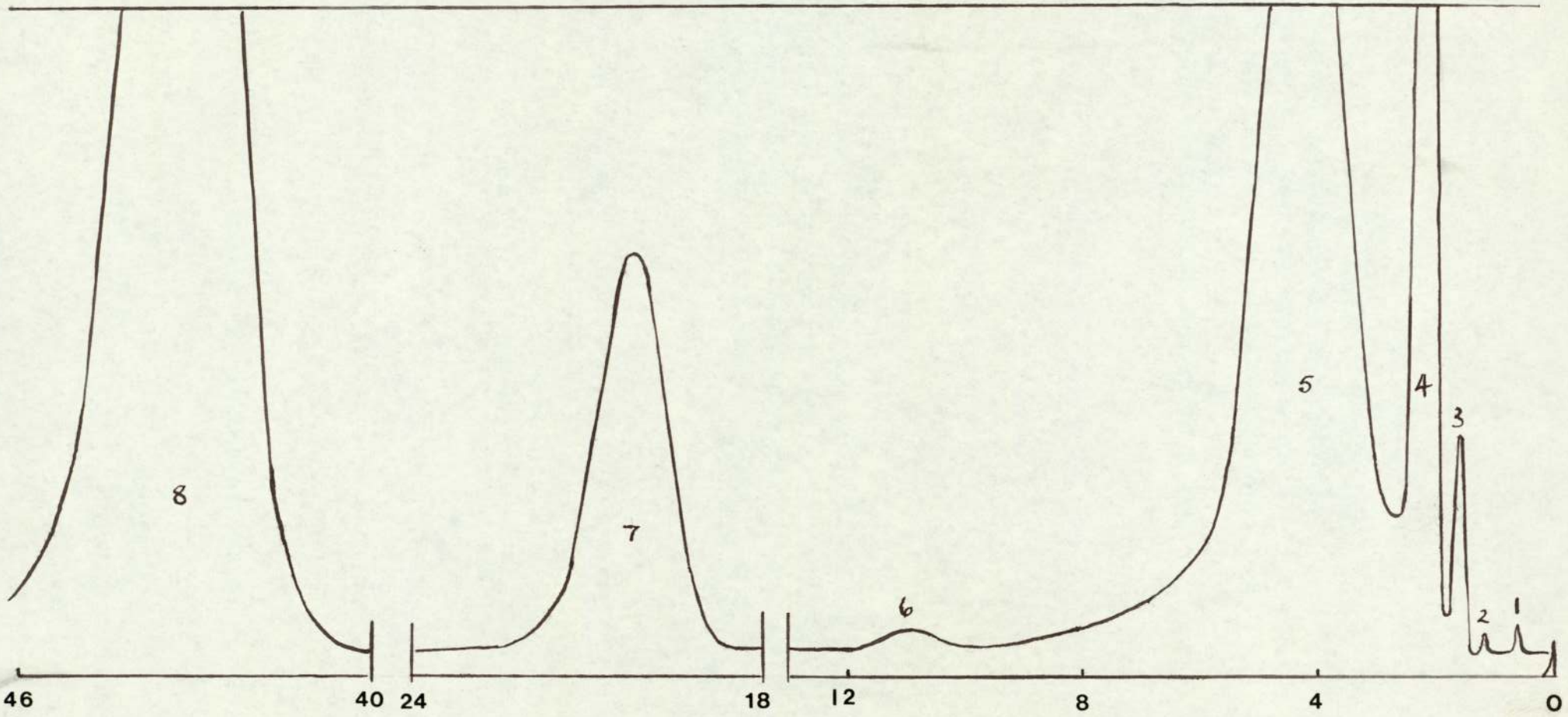
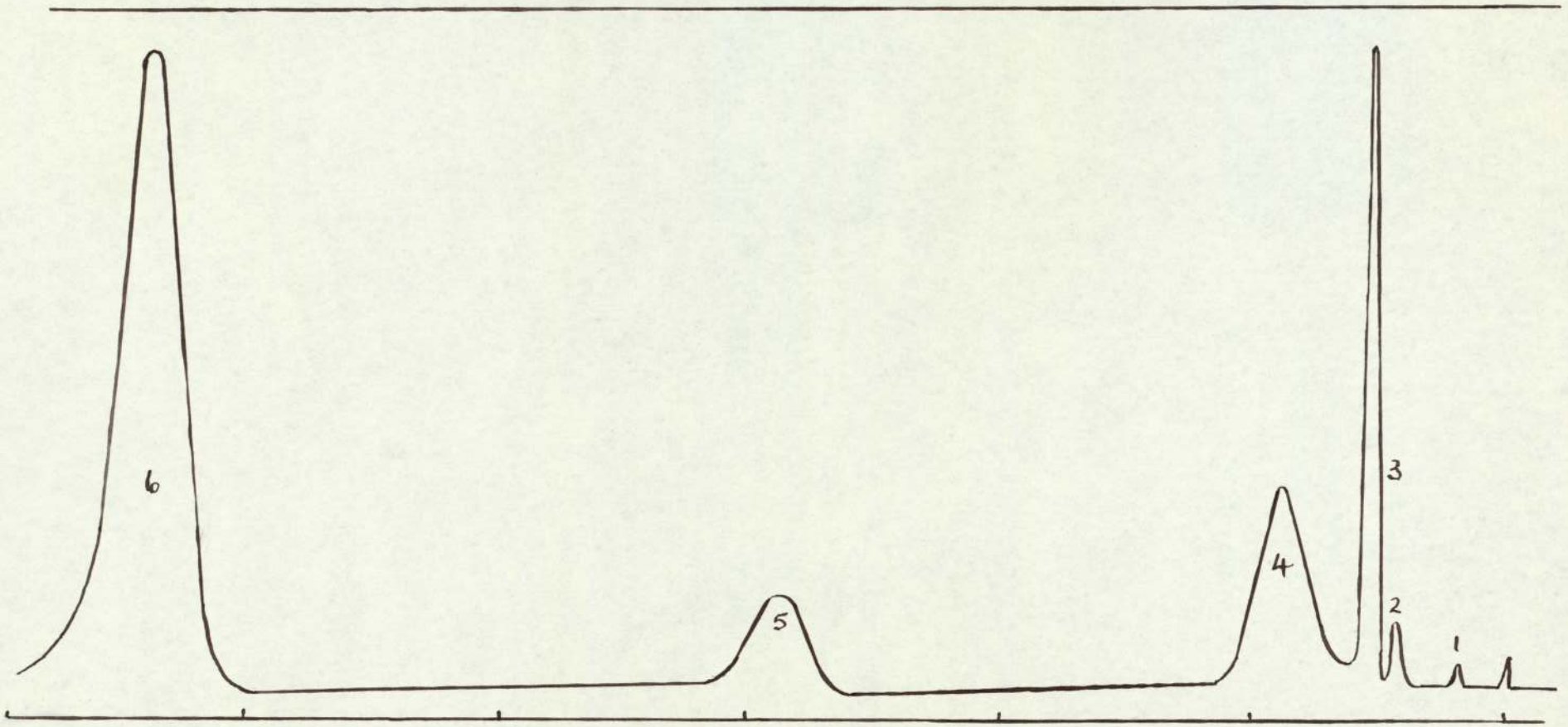


Fig. 4. Volatiles fractionated using DEGA as stationary phase. Temperature 70°C.



1 CM PER MIN

Fig. 5. Volatiles fractionated using DEGA as stationary phase. Temperature 85°C.

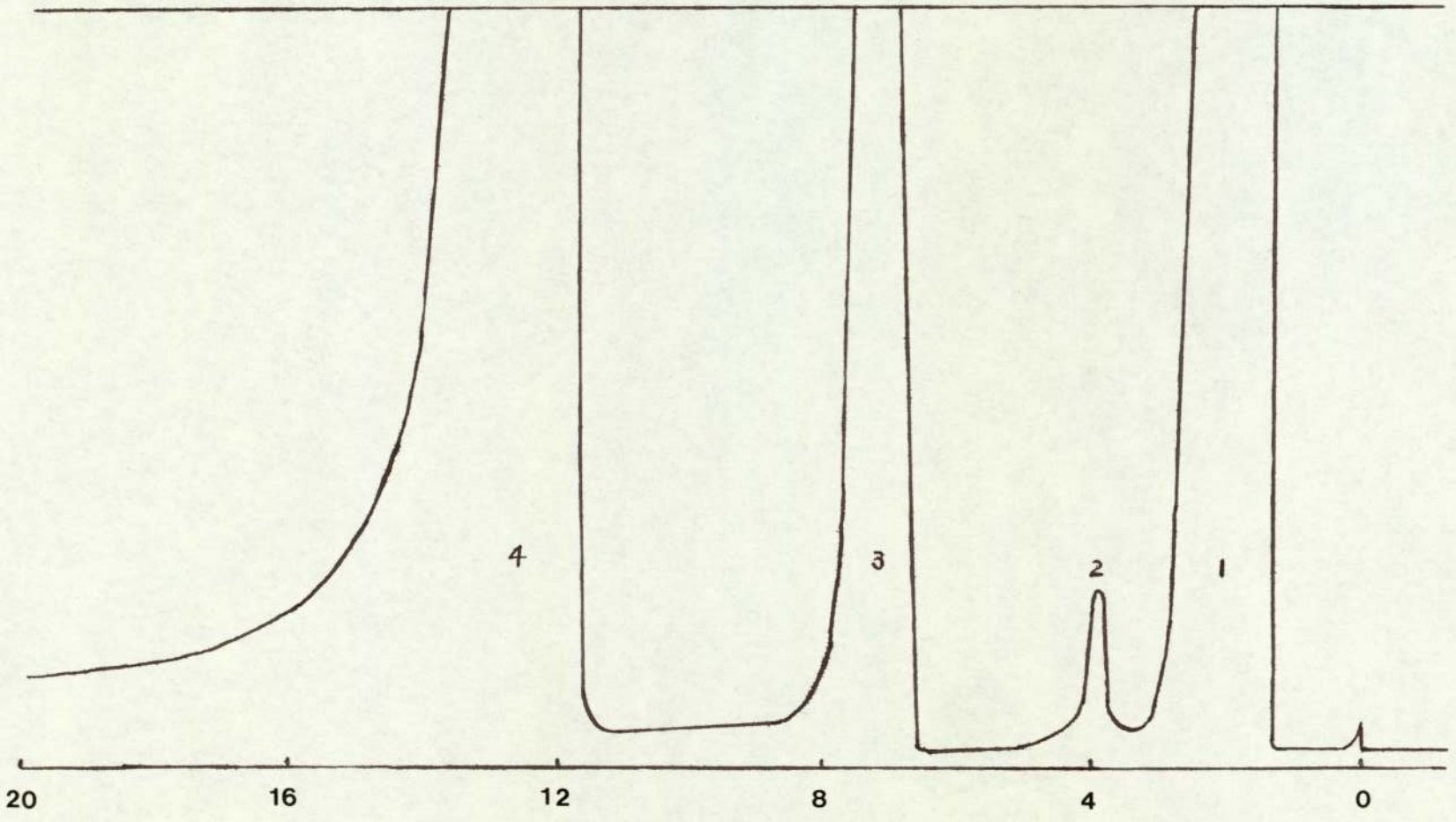


Fig. 6. Volatiles fractionated using DEGA as stationary phase. Temperature 100°C.

carrier gas flow rate did not improve fractionation. The conditions employed for all subsequent experiments with DEGA as stationary phase were as follows:-

Temperature	70°C.
Attenuation	x200
Carrier gas flow rate	45 ml. per min.
Sample volume	2 µl.
Chart speed	2 cm. per min.

(b) Polyethylene glycol (PEG) N.W. 1000

Similar experiments to DEGA were made using PEG as stationary phase. The chromatographs are seen in Figs.7,8 and 9. The optimum conditions found were:-

Temperature	90°C.
Attenuation	x200
Carrier gas flow rate	45 ml. per min.
Sample volume	2 µl
Chart speed	1 cm. per hour.

A greater fractionation of volatiles was obtained with PEG as stationary phase. Ten distinct peaks being detected.

(c) Porapak Q

A seven foot glass column was packed with Porapak Q, and the temperatures used for volatile fractionations were 100°C., 150°C., 200°C., and 225°C. The chromatograms obtained at these temperatures are seen in Figs. 10,11,12,and 13. The temperature chosen for all further experiments was 200°C., when 13 volatiles were detected.

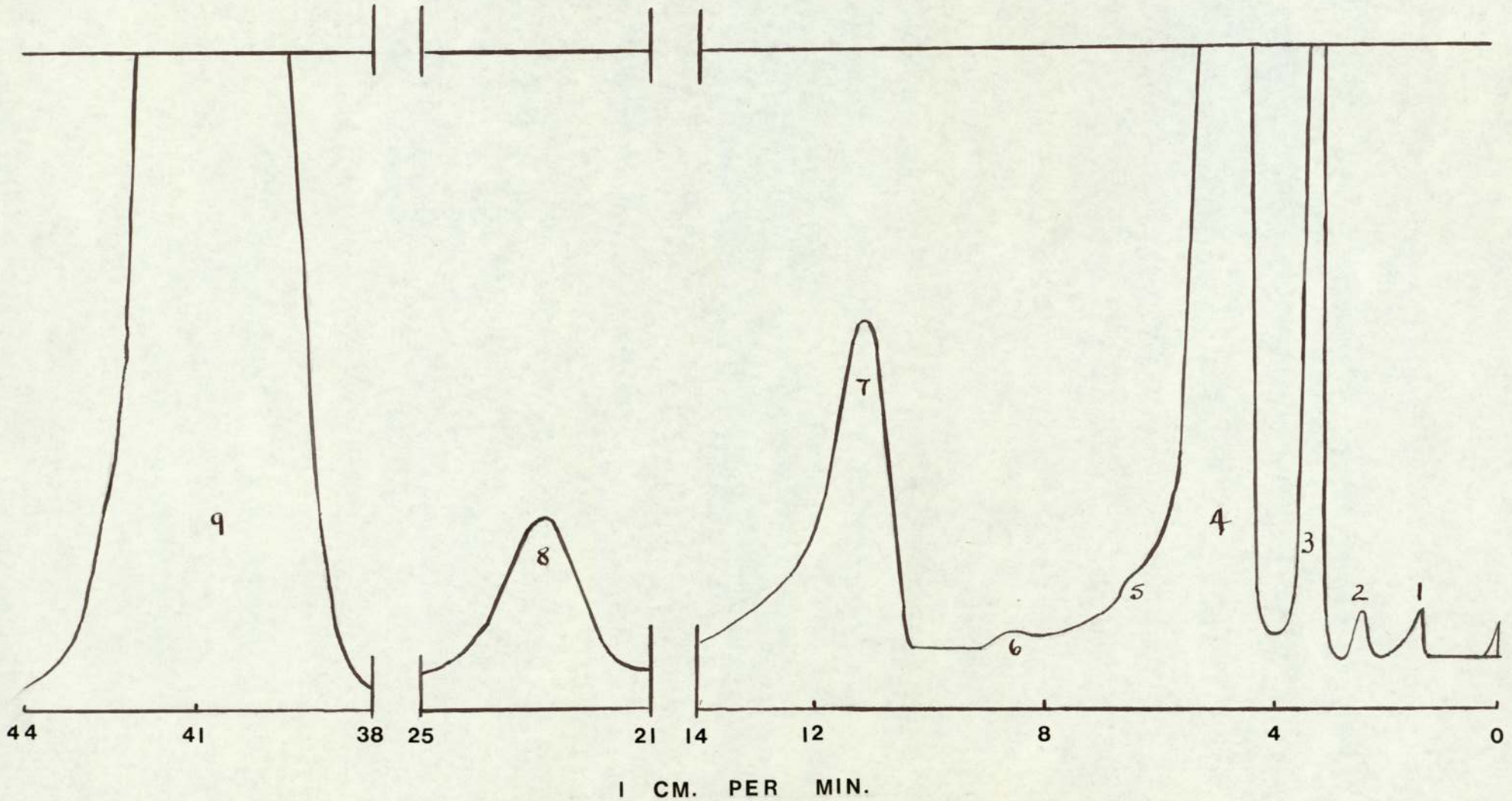


Fig. 7. Volatiles fractionated using PEG 1,000, at a temperature of 60°C.

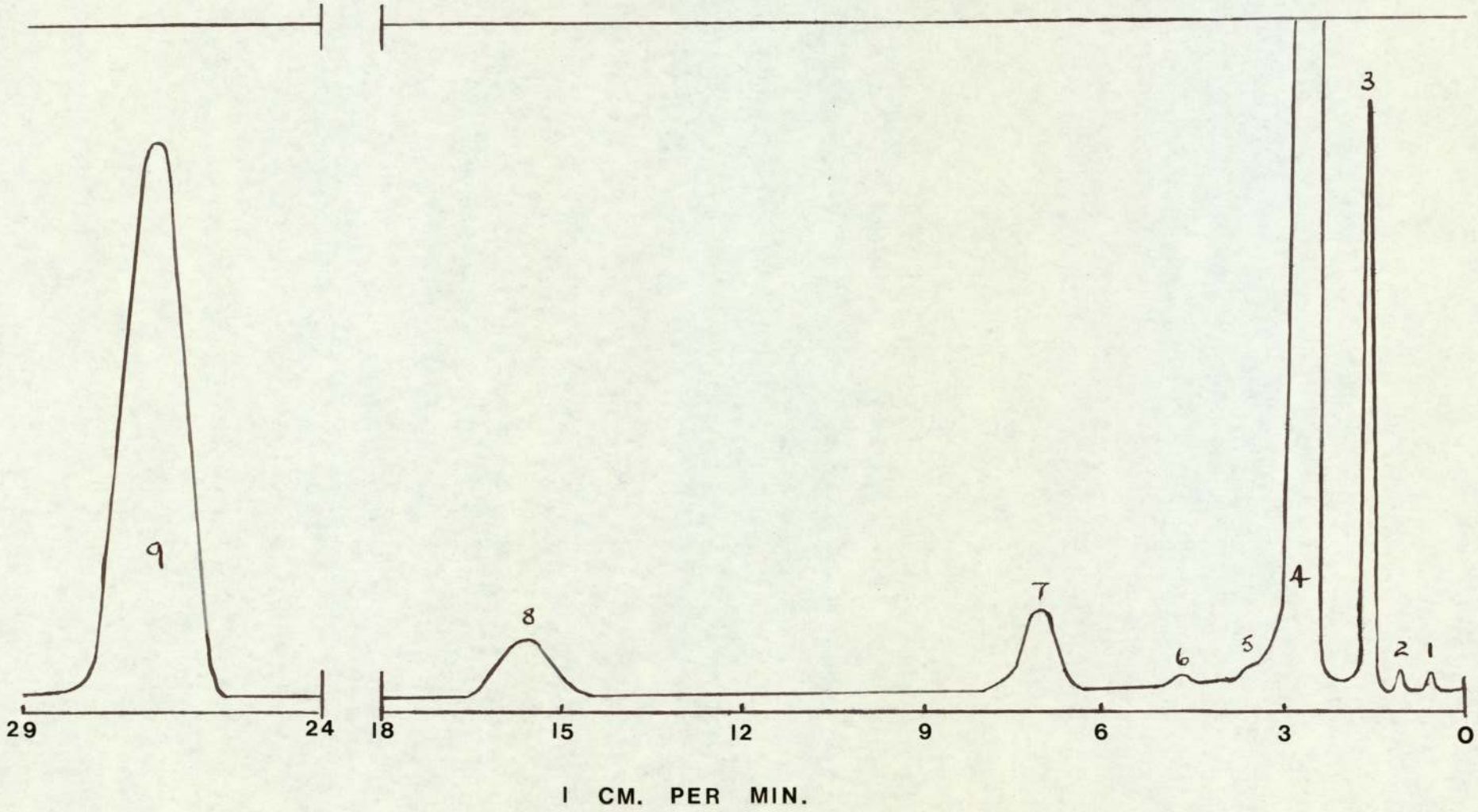


Fig. 8. Volatiles fractionated using PEG 1,000, at a temperature of 70°C.

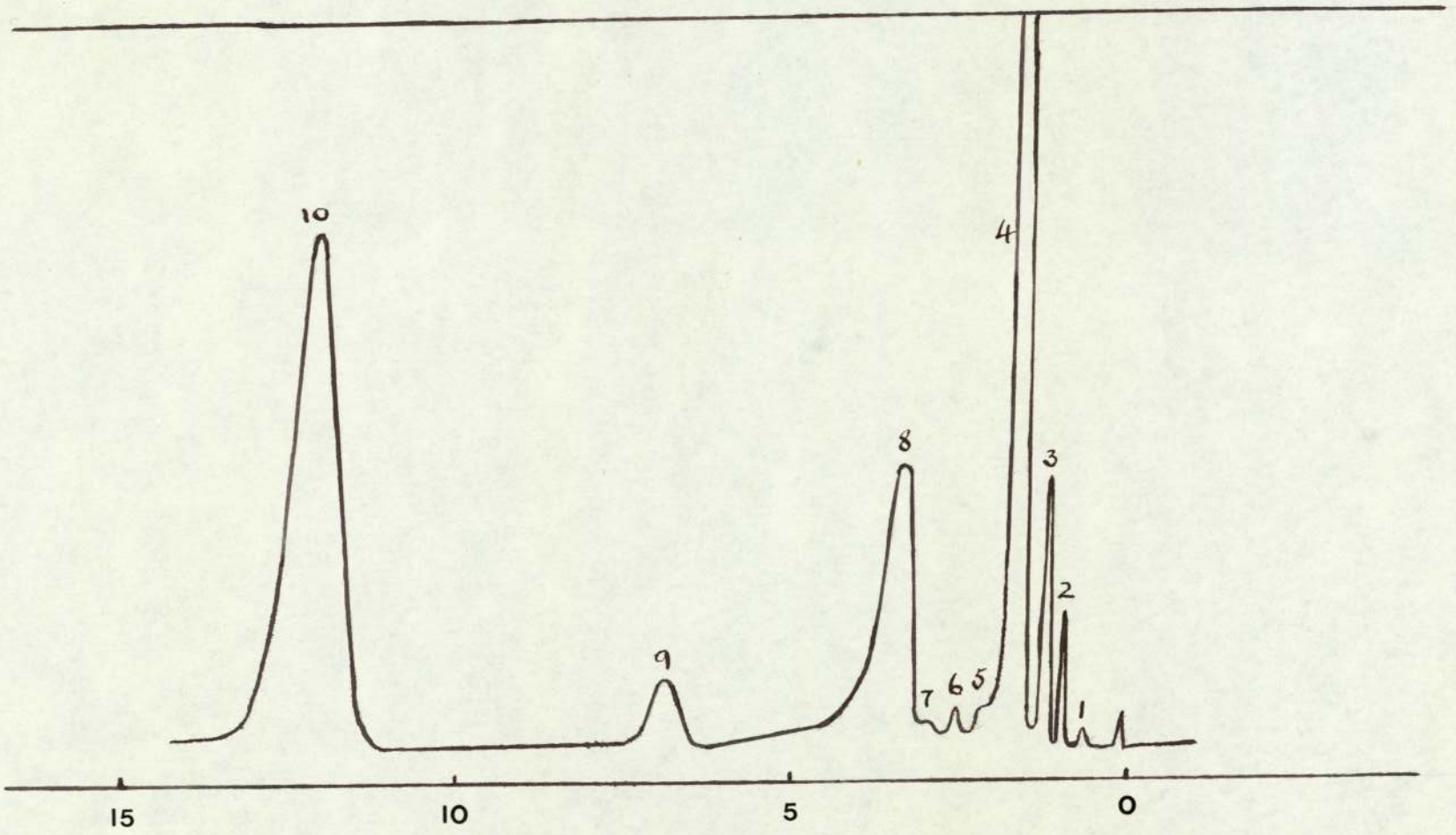


Fig. 9. Volatiles fractionated using PEG 1,000, at a temperature of 90°C.

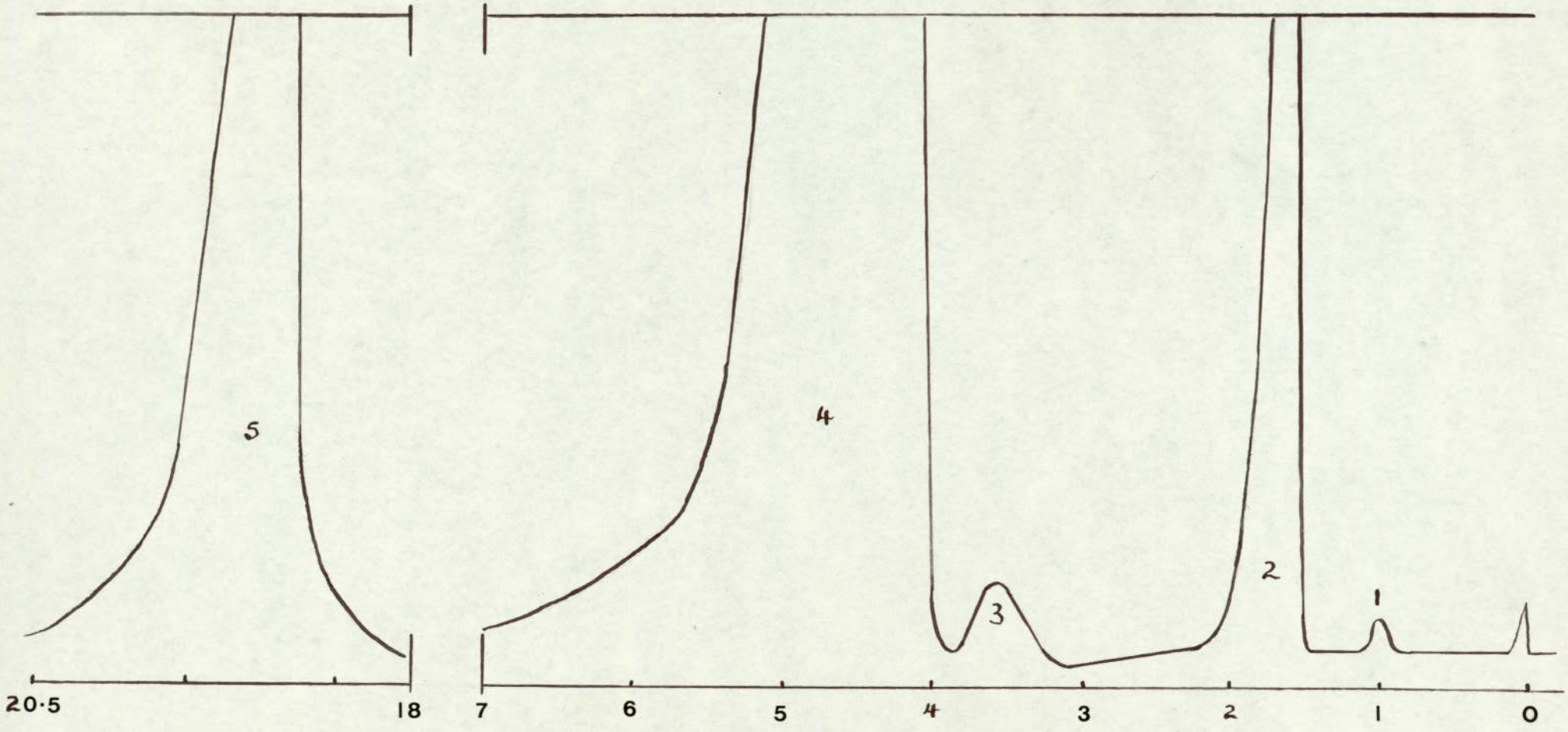


Fig. 10. Volatiles fractionated with Porapak Q at 100°C.

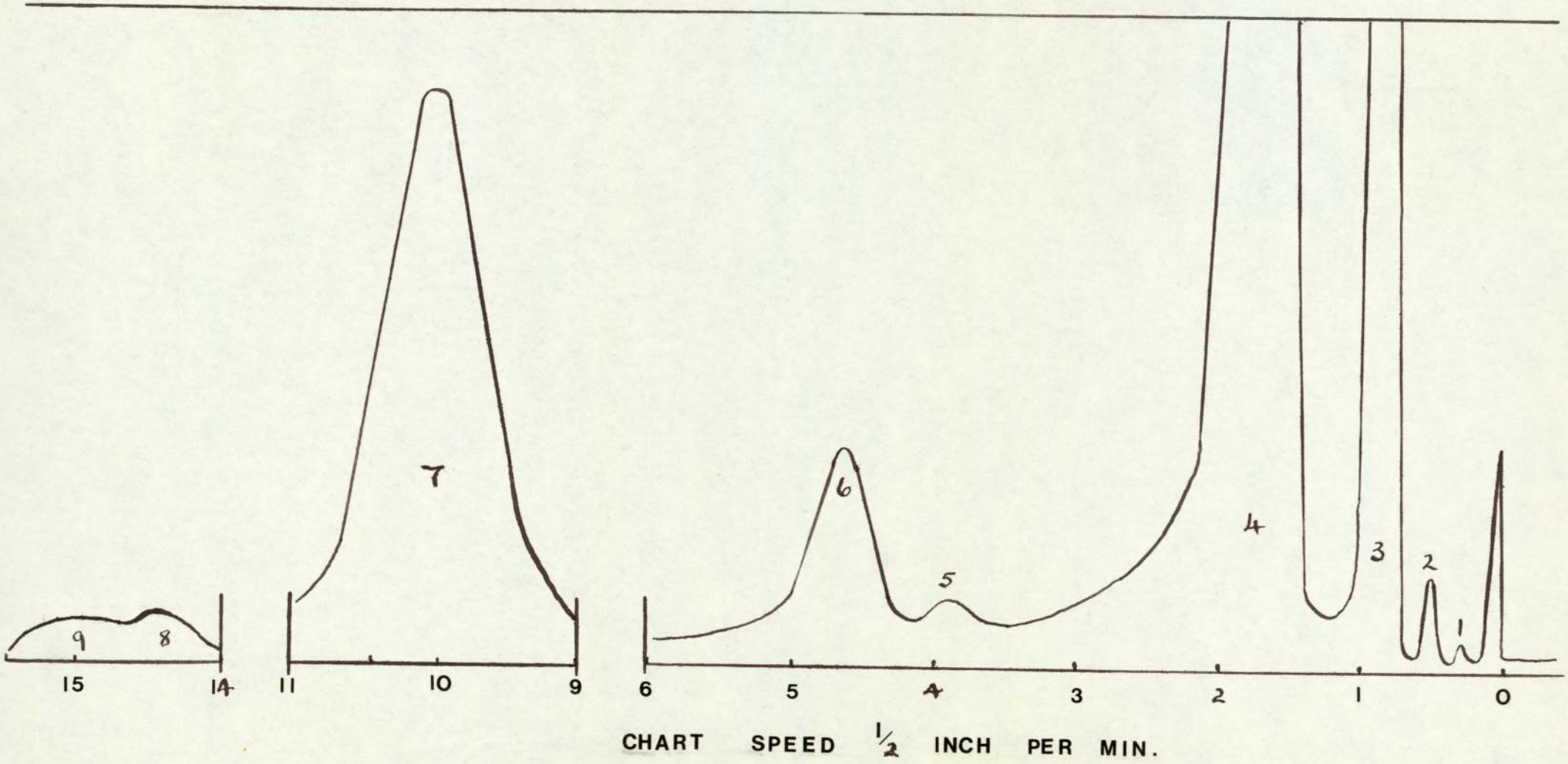


Fig. 11. Volatiles fractionated with Porapak Q at 150°C.

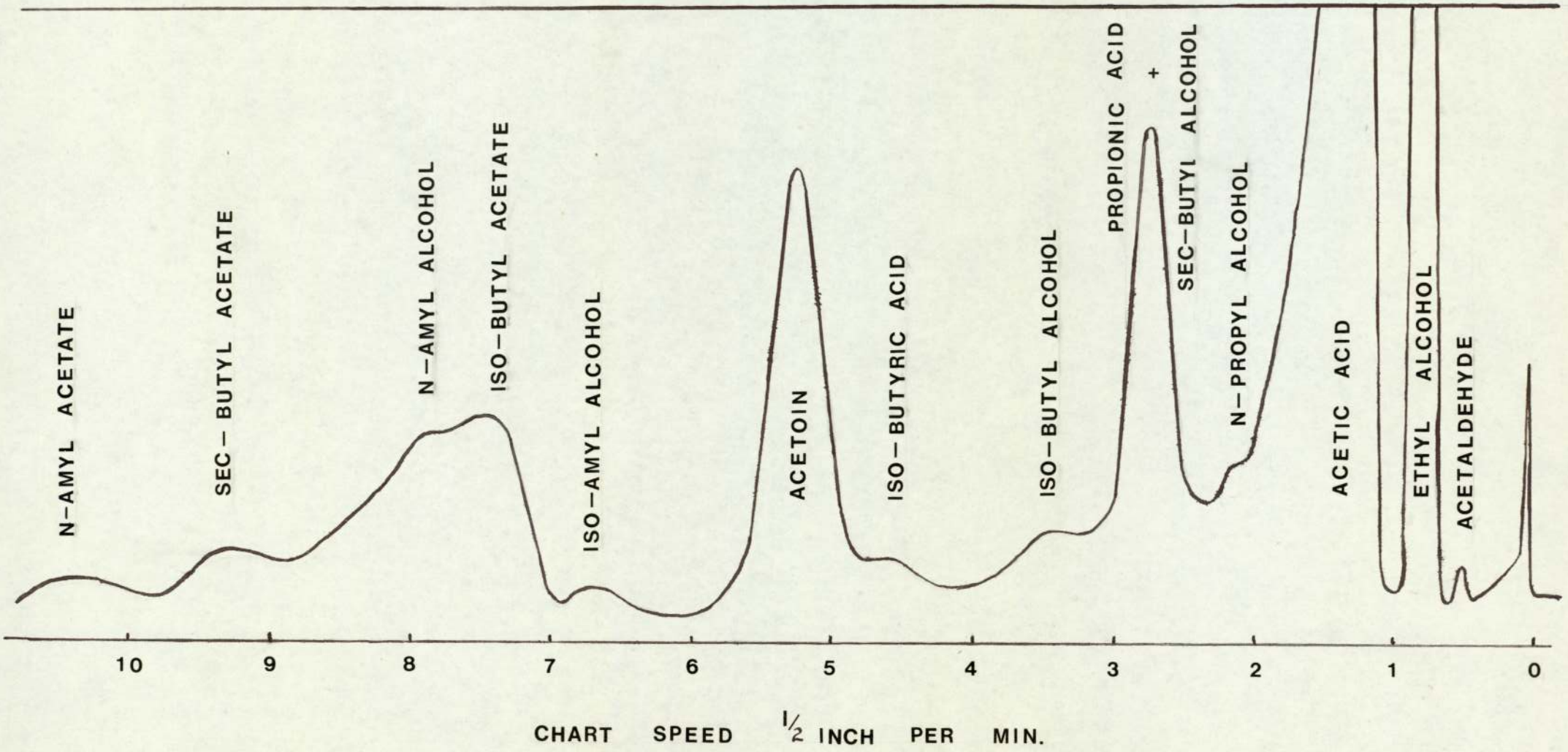


Fig. 12. Volatiles fractionated with Porapak Q at 200°C.

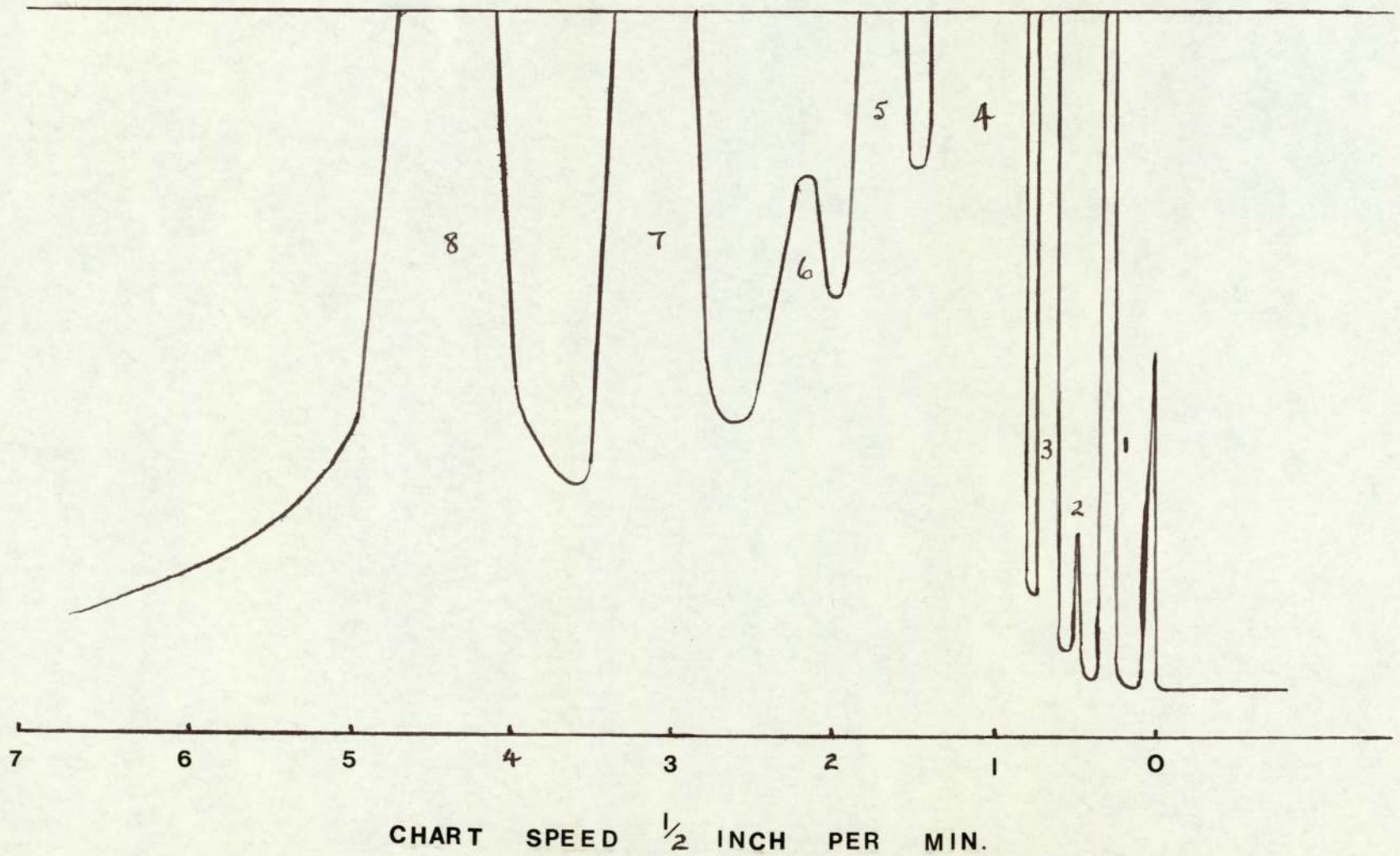


Fig. 13. Volatiles fractionated with Porapak Q at 225°C.

(5) THE EFFECT OF ACETIC ACID ON COLUMN PERFORMANCE.

Both the stationary phases DEGA and PEG 1000 gave unreproduceable retention times due to the high concentration of acetic acid in vinegar. The retention time of acetic acid with PEG 1000 was approximately 1 hr. 20 min., followed by excessive tailing of the peak. It was clear from the initial chromatographs that GLC of neat vinegar on both these stationary phases would be impractical. Two methods of eliminating the acetic acid were employed. In the first case 10 ml.aliquots of malt vinegar were neutralised with sodium hydroxide using phenophthalein as indicator and the sample re-chromatographed. In the second method the volatiles were extracted from 2 x 10 ml. of vinegar samples using 10 ml. of carbon disulphide and 10 ml. of diethyl ether, and the extracts gas chromatographed. Complete extraction of the volatiles was not obtained by carbon disulphide or by diethyl ether. In subsequent work neutralization with sodium hydroxide was used. Porapak Q was not affected by the presence of acetic acid. The retention times of the volatiles after neutralisation were replicable and were comparable to those obtained before neutralisation.

(6) IDENTIFICATION OF VOLATILES

To obtain some indication of the nature of the volatiles present in malt vinegar samples of malt vinegar were treated with potassium permanganate and with strong alkali. A saturated solution of potassium permanganate removed aldehydes and 50% sodium hydroxide removed esters. The volatiles present after treatment were fractionated using PEG as stationary phase. One peak was removed after treatment with potassium permanganate and two other

peaks were removed after alakli treatment.

Further identification of all the volatiles was made by comparing the retention times of diluted aqueous reference compounds with those found in malt vinegar. Subsequently the identification was confirmed by the addition of the respective standards to neutralised vinegar samples, which served two objectives, in the first place to check retention times and, secondly, to determine the recovery of known amounts of standards. All reference compounds as far as possible were of 'AnalaR' quality and obtained from B.D.H.Co.,Ltd., Poole, Dorset. Dilutions were made in Elgastat distilled water.

(7) RETENTION TIMES OF REFERENCE COMPOUNDS

The concentrations of reference compounds varied between 0.2% and 0.05%, the concentration chosen depended upon obtaining peaks within the limit of the chart paper with 2 µl. samples. All the reference compounds examined contained numerous impurities the number ranging between three and thirteen. The concentration used and retention times obtained for each standard solution are listed in Table 2. Further confirmatory experiments were made by adding the respective standards to vinegar samples which served to check retention times and to determine the recovery of known amounts of standards.

(8) CALIBRATION CURVES AND RECOVERY EXPERIMENTS

The concentration of volatiles with lower retention times were calculated from peak height and the volatiles with higher retention times calculated from peak areas.

(a) Methyl formate (Wt./ml. at 20°C. 0.968 g.)

Stock Standard: 0.2 ml. of Methyl formate added to approximately 90 ml. of distilled water and diluted to 100 ml.

Working Standards: The stock solution was diluted 10x to contain 20 mg. per 100 ml. Double dilution of this solution to give 10 mg., 5 mg., and 2.5 mg. per 100 ml. The peak height in mm. was 67,44,23 and 8 respectively. The reproducability was calculated from the formulae

$$S_D = \sqrt{S^2}$$

and the standard error of the mean from

$$\frac{1}{\sqrt{N}} \times S_D$$

and the variance from

$$\frac{1}{\sqrt{N-1}} \times S (x - \bar{x})^2$$

where S_D = standard deviation

S^2 = variance

N = number of values

$S(x - \bar{x})^2$ = sum of the squared deviations

The reproducability results are listed in Table 3. The recovery of methyl formate when added to malt vinegar ranged between 97.4% and 104.8% Table 4.

Methyl formate added	Methyl formate found	Recovery
-	12.2	-
2.5	14.8	104.8%
5.0	17.1	99.0%
7.5	19.6	98.0%

Table 4. Recovery of methyl formate.

(b) Acetaldehyde (Wt.0.778 - 0781 g. per ml. at 20°C.)

Stock standard 0.2% v/v in distilled water.

Working standards. The stock solution was diluted 10x with distilled water and further double diluted to give 10 mg., 5 mg., and 2.5 mg., per 100 ml. The peak heights were 43, 21 and 8 mm. respectively and the standard deviation was 0.39 (Table 3.). The recovery of acetaldehyde when added to vinegar was 96.7% - 101.8% (Table 5).

Acetaldehyde added	Acetaldehyde found	Recovery
-	10.0	-
5.0	15.1	101.8%
7.5	17.6	101.2%
10.0	19.7	96.7%

Table 5. Recovery of acetaldehyde added to a sample of vinegar.

(c) Ethyl acetate

Stock standard, 0.2% v/v (Wt. 0.899 - 0.901 g. per ml. at 20°C)

Working standards, Dilute stock 1/40 with distilled water (5mg. per 100 ml.) and double dilute further to give 2.5 mg. 1.25 and 0.625 mg. per 100 ml. The peak heights in mm were 53,30 and 13. The reproducibility results are seen in Table 3 and the recowery results in Table 6.

Ethyl acetate added	Ethyl acetate found	Recovery
-	6.0	-
2.5	8.2	88.8%
5.0	10.6	92.5%
7.5	13.3	97.3%

Table 6. Recovery of ethyl acetate added to vinegar.

(d) Ethyl alcohol Calculations were made from peak areas (Width at half peak height x height). To obtain on chart readings for ethyl alcohol the attenuation was increased to x 500.

Stock ethyl alcohol. 0.1% v/v (Wt. 0.7897g. per ml. at 20°C).

The working standards were made as follows:-

ml. of distilled water	-	2.5	5.0	7.5
ml. of stock solution	10	7.5	5.0	2.5
mg./100 ml. ethyl alcohol	100	75	50	25
peak area sq.cm.	5.2	3.6	2.6	1.1

The reproducability results are seen in Table 3 and the recovery experiments in Table 7.

Ethyl alcohol added	Ethyl alcohol found	Recovery
-	28.8	-
5	33.5	106%
10	39.6	108%
15	41.9	87.3%

Table 7. Recovery of ethyl alcohol from malt vinegar.

(e) iso-butyl acetate Calculations were made from peak height. Stock iso-butyl acetate 0.2% v/v (Wt. 0.878 - 0.88g per ml at 20°C.)

Working standards were made by diluting the stock solution 20x (10 mg. per 100 ml.) and double diluting further to give final concentrations of 5,2.5, 1.25 and 0.625 mg. per 100 ml. The results obtained for reproducibility are seen in Table 3 and the recovery experiments in Table 8.

iso-butyl acetate added	iso-butyl acetate found	Recovery
-	3.7	-
2	6.0	117%
4	8.0	101%
6	9.0	89.8%

Table 8. Recovery of iso-butyl acetate added to vinegar mg. per 100 ml.

(f) n-propyl alcohol. 0.2% v/v (Wt. 0.803 - 0.806 g. per ml. at 20°C.)

For working standards dilute the stock solution to give 10,5,2.5 and 1.25 mg. per 100 ml. The peak heights were 32,15,7 and 4 mm. respectively. Table 3 lists the reproducibility obtained and Table 9 the recovery results.

n-propyl alcohol added	n-propyl alcohol found	Recovery
0	2.5	0
2	4.8	122%
4	6.8	111.7%
6	8.7	104.5%

Table 9. Recovery of n-propyl alcohol from vinegar.

(g) n-butyl acetate 0.2% v/v (Wt. 0.878 - 0.881 g. per ml at 20°C.)

For working standards dilute stock 30 x (10 mg. per 100 ml.) and further double diluted to give 5,2.5 and 1.25 mg. per 100 ml. The peak heights obtained were 50, 28, 14 and 8 mm. respectively. The

reproducability results are seen in Table 3 and the recovery results in Table 10.

n-butyl acetate added	n-butyl acetate found	Recovery
	7.0	-
2.5	10.1	122%
5.0	12.7	113.4%
7.5	14.6	100.6%

Table 10. Recovery of n-butyl acetate.

(h) amyl alcohol. 0.2% v/v (Wt. 0.810 - 0.813 g. per ml at 20°C) Calculations were made from peak areas. Working standards were made by diluting the stock solution 1/10 (20 mg. per 100 ml.) and further double diluted to give 10.0, 5.0, and 2.5 mg. per 100 ml. The peak areas obtained were 1.4, 0.6and 0.3 sq. cms. The results for reproducibility are seen in Table 3 and the recovery results in Table 11.

Amyl alcohol added	Amyl alcohol found	Recovery
-	5.6	-
2	7.8	108.5%
4	9.5	96.2%
6	11.72	101.1%

Table 11. Recovery of amyl alcohol from vinegar.

(i) Acetoin. 1g./100 ml. Working standards were obtained by diluting the stock solution 10x and further double diluted to give 50 and 25 mg. per 100 ml. Calculations were made by peak area and the attenuation was increased to x 500 to obtain on scale readings. Table 3 lists the results for reproducibility and Table 12 for recovery.

Acetoin added	Acetoin found	Recovery
0	370	0
200	600	115%
400	720	87.5%

Table 12. Recovery of acetoin from vinegar

(9) ETHYL ALCOHOL ESTIMATIONS

The ethyl alcohol estimations by GLC were compared to the results obtained by the Conway (1957) diffusion method. In the Conway method alcohol is diffused into potassium dichromate and sulphuric acid. The alcohol is oxidised to acetic acid and some of the dichromate is reduced. The dichromate remaining was determined iodometrically by adding potassium iodide and titrating the liberated iodine with thiosulphate. The mean of three ethyl alcohol estimations was calculated for both the GLC method and Conway methods. The results for the Conway technique were generally higher, although this would be expected since the method has poor specificity. It was noted however that the sum of the amyl, propyl and ethyl alcohols by GLC approximates the results of the Conway method. Table 13 compares the results of both methods.

(10) ENZYMATIC DETERMINATION OF ACETALDEHYDE

The method employed was that of Klein and Korzis (1958). The reaction is catalyzed by alcohol dehydrogenase

$$CH_3\,CHO + NADH + H^+ \rightleftharpoons C_2H_5OH + NAD$$

The reaction represents an equilibrium which in the presence of excessive NADH and in an acid medium the equilibrium would be to the side of alcohol and NAD. Under these conditions alcohol does not interfere with the determination. The reagents were

were manufactured by C. F. Boerhinger and Soehne Ltd., and aupplied by Courtin and Warner Ltd., Lewes, Sussex. Since the method was originally applied to acetaldehyde determinations in blood, a number of modifications were made. The method, including the modifications made, are given in detail:-

Reagents. Phosphate buffer, -pH 6.0. 12.0 ml. of 1/15 molar $Na_2 HPO4. 2H20$ was added to 88 ml. of 1/15 molar $KH2 PO4$

Reduced NADH - 3.10^{-3} M. NADH dissolved in 1 ml. of 1% Sodium bicarbonate solution.

Alcohol dehydrogenase, -10 mg. enzyme protein per ml.

Methods. Since acetaldehyde is an extremely volatile substance (b.p.20.2°C) preliminary treatment of the sample was performed in closed vessels and at temperatures as low as possible. The measurement itself was performed at room temperature.

To 1.0 ml. of pre-cooled buffer (2°C) is added 1.0 ml. of neutralised vinegar, and mixed. Pipette rapidly 0.05 ml. of NADH, mix and allow to warm up to room temperature within approximately 3 to 5 mins. Read absorbance at 340 nm. (10 mm. cell). Add 0.05 of alcohol dehydrogenase mix and read again after 5 mins.

Calculation. The reaction proceeds stoichiometrically and a difference in absorbancy of Δ Å - 0.1 corresponds (according to the molar absorbancy index of NADH to 0.0161 micro-moles/ml. at 340 mu. This corresponds to the same number of micromoles of acetaldehyde per ml. or with reference to the cell content of 2.0 ml. to 0.0338 micromoles of acetaldehyde.

To obtain mg. per 100 ml. acetaldehyde $= \Delta\text{Å} \times 1.49 \times \frac{x}{10}$

where x = the volume of N 10 NaOH added to neutralize 10 ml. of vinegar. When the acetaldehyde concentration was over 10 mg. per 100 ml., the vinegar was diluted a further 1/10 with distilled water and stored at 2°C.

MATERIALS AND METHODS

SECTION A(2)

SAMPLES OBTAINED DURING

COMMERCIAL VINEGAR MANUFACTURE

SECTION A(2). SAMPLES FROM MALT VINEGAR MANUFACTURERS

(1) MALT VINEGAR SAMPLES

Samples of malt vinegar were obtained locally and in a number of instances directly from the manufacturer. Sixteen different samples were obtained, but further investigations showed that a number of samples were of similar origin. Eleven samples only were proved to be from different manufacturers. The age of the samples could not be ascertained. All were stored in the refrigerator during the investigation. The malt vinegars and their manufacturers were as follows:-

Vinegar Number	Manufacturer
1	Thomas and Evans Ltd.
2	Penistone Pure Malt Vinegar Co.,Ltd.
3	H.P.Sauce Ltd.
4	Derby Malt Vinegar Co., Ltd.
5	Hill Evans Vinegar Co.,Ltd.
6	A.P.T.Ltd.
7	Tesco Ltd.
8	Manor Vinegar Co.
9	Sarsons Vinegar Co.,Ltd.
10	Heinz Vinegar Ltd.
11	Fardon's Vinegar Ltd.

(2) SAMPLES OBTAINED DURING THREE DISTINCT PROCESSES OF MALT VINEGAR MANUFACTURE

(a) The Quick method. Samples were obtained from the Barbourne Brewery, Worcester, who manufacture an all-malt vinegar by the

Quick-vinegar method. The process consists of milling and mashing brewer's malt to produce on cooling a wort of original gravity between 1065 and 1070. The wort is fermented to a gravity of 1000 in 7 days at a temperature between 65 and 75°F. with a brewer's yeast and then transferred to an acetifier containing some acetified liquid from the previous fermentation. It is then sparged over birch twigs with aeration, until the alcohol is oxidised at a temperature of 30°C. On cooling the acetified liquor is stored for two weeks in vats, when it is centrifuged. The vinegar is finally diluted to 5% acetic acid, pasteurised and bottled. Samples were taken during the process as follows:-

Sample Q1. Wort immediately after seeding with yeast.

Sample Q2. The alcoholic wash (charging wort) which had been stored for two weeks prior to acetification.

Sample Q3. Acetified liquid immediately at the end of acetification.

Sample Q4. Vinegar post filtration and pasteurisation..

Sample Q5. A sealed bottle of vinegar at the end of manufacture.

Samples Q1 to Q4 were stored at room temperature for four weeks, sample Q1 being allowed to ferment during this storage period. Using a sterile syringe and needle 2ml. aliquots were taken periodically and chromatographed twice at two attenuations the ethyl alcohol and acetic acid being determined at 10^3 x 20 and all other volatiles at x 500. The stationary phase throughout was Porapak Q.

(b) The Fring's process. Samples were obtained from Hammond's Vinegar Co.,Ltd., Clough Springs Brewery, Wheatley Lane Rd.,

Barrowford who manfacture vinegar by the Fring's process.

The process consists of milling and mashing brewer's malt to produce on cooling a wort having a temperature of 70°F. and an original gravity of between 1045 and 1050. The wort is fermented for three days with brewer's yeast. The alcoholic liquor is then centrifuged to remove yeast, and transferred to an acetator charging vat. The charging wort is acetified in a Fring's type acetator at a temperature of 30°C. to produce vinegar containing approximately 6.5%v/v acid. The vinegar is then stored and blended to give an acid content of 5.0% followed by sterilisation by filtration. Samples were taken during the process as follows:-

Sample F1. Wort obtained before seeding with yeast.

Sample F2. Wort seeded with yeast 48 hrs. prior to sampling.

Sample F3. Fermenting wort at the end of the alcoholic fermentation.

Sample F4. An alcoholic wash 'charging wort' immediately before acetification.

Sample F5. Sample obtained from the Fring's acetifier.

Sample F6. Sample immediately after acetification.

Sample F7. Finished vinegar which had been stored for four days.

Sample F8. Finished vinegar after sterilisation by filtration.

All the samples were stored for 31 days at room temperature. Samples F2, F3, and F5 were allowed to ferment during the storage period in the laboratory. A sterile syringe and needle were used to obtain 2 ml. aliquots of each sample during the laboratory storage. 2 µl. quantity of each aliquot was chromatographed as above.

(c) A Continuous Process. Samples were obtained from Manor Vinegar Co.,Ltd., Lichfield, where a continuous process is employed. The process consists of milling and mashing brewer's malt to produce on cooling a wort having a temperature of 84°F. The specific gravity of the mash was approximately 1056 which is fermented with a brewer's yeast to a gravity of 998 over a period of four to five days. The charging wort is stored for one week before acetification in a continuous fermenter at a temperature of 84°F. The vinegar is then filtered, pasteurised and diluted to 5% acid. Samples were taken as follows:-

Sample C1. A finished sample of malt vinegar blended from both the Quick and Continuous processes.

Sample C2. A sample obtained from the Continuous acetifier.

Sample C3. A sample of 'charging wort' taken after five days fermentation.

Sample C4. The alcoholic wash immediately prior to acetification.

All samples were stored in the laboratory for 33 days at room temperature. Sample C3 was allowed to ferment during this storage period. The aliquots were taken and chromatographed as for the Quick process samples.

(3) SAMPLES OF AGED VINEGARS

Aged vinegars were obtained from the Barbourne Brewery, Worcester and had been stored at room temperature for 4,6 and 12 months respectively. Two sealed bottles of freshly bottled vinegar were also obtained, one of which was stored in the laboratory for 57 days unopened, and the other was stored under similar conditions but unsealed. Aliquots from each bottle were subjected to GLC on the 35,37,41,48, and 57th.days.

SECTION A 3

MATERIALS AND METHODS

LABORATORY ACETIFICATIONS

SECTION A (3). LABORATORY ACETIFICATIONS

Three types of laboratory acetifications were employed to study the volatiles produced in malt vinegar.

(1) SURFACE FLASK. One litre conical flasks with cotton wool plugs were sterilised by autoclaving. To twenty sterilised flasks was added 500 ml. of commercial charging wort. A number of compounds were added to each flask to give final concentrations as listed in Table 14. One flask of each compound was incubated at 22°C. (room temperature), and the other at 30°C. 2 ml. aliquots were taken daily, 1 ml. of which was used for titration with N10 NaOH and phenolphthalein as indicator. The remaining 1 ml. was frozen at -20°C. for subsequent GLC. To each flask was added 10 ml. of an acetifying culture obtained commercially from Beecham's Foods and Drinks, Barbourne Brewery, Worcester. The manufacturer uses an all-malt brew for vinegar manufacture.

(2) AERATED FLASK. A similar culture to the surface culture was prepared but aerated by means of a glass tube containing at one end a sintered glass filter of porosity 2. Air at a flow rate of 20 ml. per minute was passed through this culture. The flask was kept at 22°C. until acetification occurred.

(3) TOWER FERMENTOR. The fermentor used is seen in Fig.14. Two jacketed tower-shaped fermentors each 55 cm. by 9 cm. diameter, aerated from the lower end by means of a sintered glass disc (porosity 2). The first fermentor was used at room temperature while the second was maintained at 30°C. by circulating the water around the jacket from a constant temperature water bath. 1,250 ml.

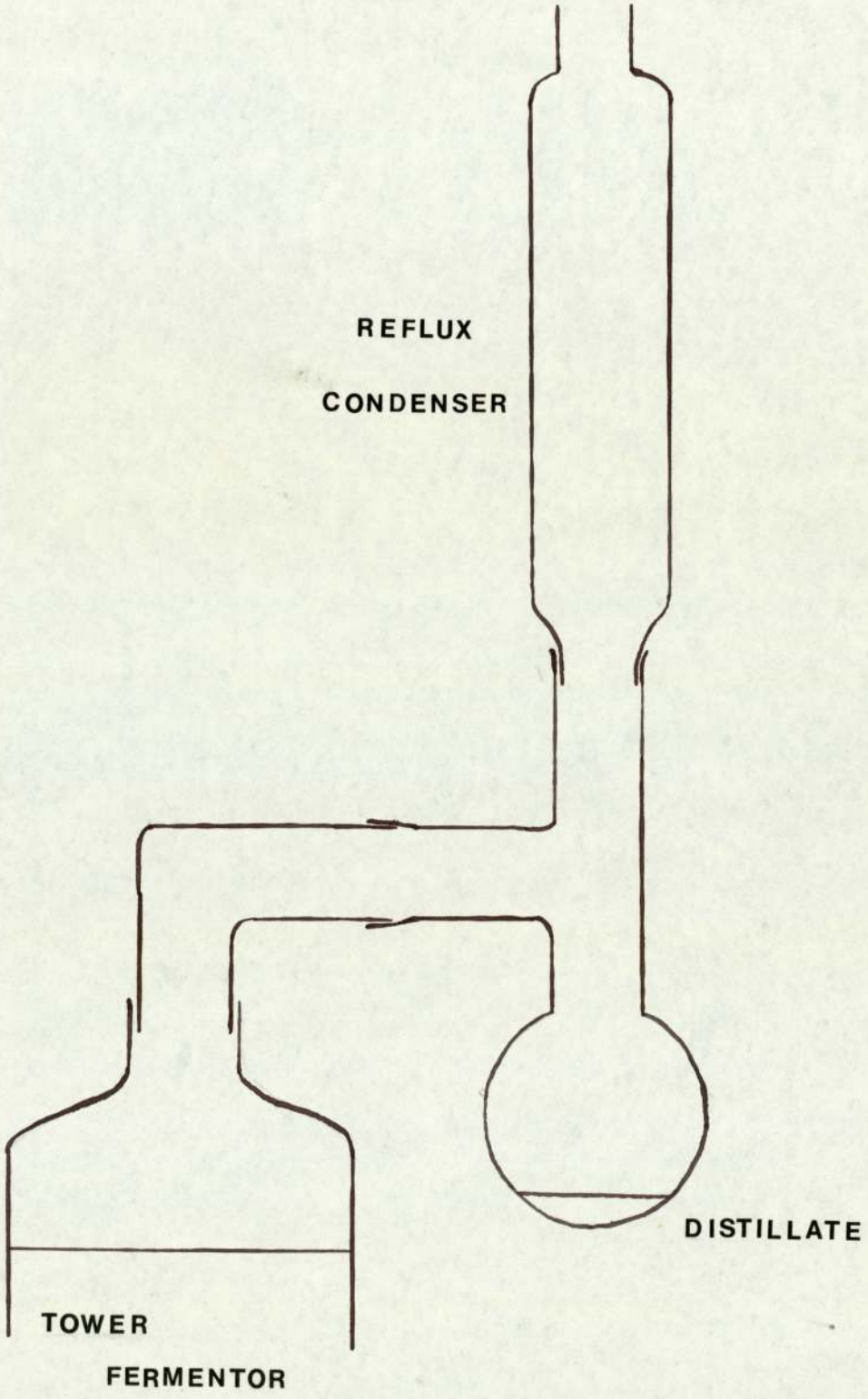


Fig. 15. Method for collection of distillates from tower fermentors.

of charging wort and 50 ml. of acetifying culture was added to both fermentors and aerated at an air flow rate of 40 ml. per minute. Air was passed through the fermenter for seven days during which time 2ml. aliquots were taken daily and examined as for the surface culture method. The compounds added and their concentrations are listed in Table 14.

(4) COLLECTION OF DISTILLATES FROM TOWER FERMENTORS.

Distillates were collected from tower fermentors by attaching a condenser coil (Graham) to a side arm placed on the tower fermentor as shown in Fig.15. The surface area of the condenser was 230 cm., and the length 260 mm. The distillate volume was measured daily and 2 µl subjected to GLC.

(5) AIR FLOW MEASUREMENT

Initial experiments were made with a Gapmeter over the range 25 - 250 cc. per min. The Gapmeter was used as recommended by the manufacturers G.A.Platon Ltd., 281 Davidson Rd., Croydon, Surrey. With flow rates of under 50 ml. per min. the Gapmeter was found to be inaccurate. All further flow rates were determined by using the bubble flow meter as described in (1)e above. The bubble flowmeter was also used to measure the flow rate of 8% CO_2 in O_2 which was used in later experiments.

(6) MYCODERMA CULTURES

Two cultures of mycoderma were used, one of which was obtained from Manor Vinegar Ltd., Lichfield, and the other from Aston University. Both cultures were contaminants of stored charging wort. The cultures were inoculated into unfermented wort and

fermented wort. To conical flasks of 1 litre capacity was measured 500 ml. of wort and a loopful of mycoderma culture was inoculated on to the surface of each flask. Aliquots were taken at 0,7,11,14,19, 24 and 30 days and subjected to GLC.

(7) VOLATILE EXTRACTION WITH ORGANIC SOLVENTS

Three samples of vinegar were extracted separately with chloroform, diethyl ether and carbon disulphide by mixing on a mechanical shaker for 2 hr. 100 ml. samples of malt vinegar were extracted with 20 ml. of each solvent, and the extract gas chromatographed. The extracts were also distilled and both residues and distillates subjected to GLC. It was not possible to decrease the volume of solvent without the formation of an emulsion.

MATERIALS AND METHODS

SECTION A(4)

CONTINUOUS FERMENTATION

SECTION A(4). CONTINUOUS FERMENTATION

(1) $p^{O}2$ ESTIMATIONS

(a) Measurement of $p^{O}2$. A $p^{O}2$ electrode of the Clark-type was used which consisted of a combined platinum cathode and silver/silver chloride anode placed in an electrolyte solution behind a polypropylene membrane of 20 μ thickness. The electrolyte solution was a phosphate buffer to which some potassium chloride had been added to stabilize the potential of the anode (reference electrode). The characteristic of the electrode was strictly linear and was standardised against an oxygen-free solution (i.e containing no free molecular oxygen), 0.01 M Borax solution (pH 9.2) containing 100 mg. of sodium sulphite per 5 ml. of solution. The electrode is housed in a cell circulated with water at constant temperature (30°C) from a constant temperature water bath. The water in the water bath was in equilibrium with atmospheric air due to air being continuously sucked into the thermostat water by means of the built-in suction pump. The water in the water bath was used as a solution with a known $p^{O}2$ after equilibriation for 30 minutes. The $p^{O}2$ of the solution was computed from:-

$$p^{O}2 = (B - a) \times \frac{O2\%}{100}$$

in which 'B' was the barometric pressure in mm. Hg. 'a' the partial pressure of water vapour at 30°C. and 'O_2%' the percentage by volume of oxygen in air when the barometric pressure was 760 mm. Hg and the vapour pressure of water at 30°C. 46 mm. then the $p^{O}2$ would be,—

$$p_{O_2} \quad (760 - 46) \times \frac{20.93}{100}$$

149.4 mm. Hg

where 20.93 is the content of oxygen in air.

The p_{O_2} electrode was obtained from V.A.Howe Ltd., 46, Pembridge Rd., London W 11., and used in conjunction with a pH meter type PH M27 available from the same source. The pH meter was calibrated with three ranges 0 - 120, 80 - 220 and 0 - 1200 mm. Hg and when used in conjunction with a Control Instruments 1 m V Recorder the range 0 - 120 mm. was used.

During continuous monitoring a constant volume of charging wort was pumped through the electrode. The total volume of sample required for p_{O_2} readings was 70 µl. A polyethylene tube (I.D 0.034 and O.D.0.060) was attached to a tygon tube (I.D. 0 .025) and placed in a Quickit 6 Channel Peristaltic Pump. The Peristaltic Pump was obtained from Griffin and George Ltd., Ealing Rd., Alperton, Wembley, Middlesex, and the tygon tubing from Technicon Instruments Ltd., Chertsey, Surrey. The pump speed was set to give a flow rate of 0.2 ml. per min. The chart speed was set at 1" per hr.

Sampling was made at the top, middle and the bottom of the tower fermentor, circulated through the pump and p_{O_2} electrode and then returned to the fermentor. Standardisation was made by placing the sample tube into the oxygen free solution followed by distilled water and water equilibrated with air as standard. The apparatus is illustrated in Fig.14.

(b) Conversion of p_{O_2} measurements to v/v dissolved oxygen.

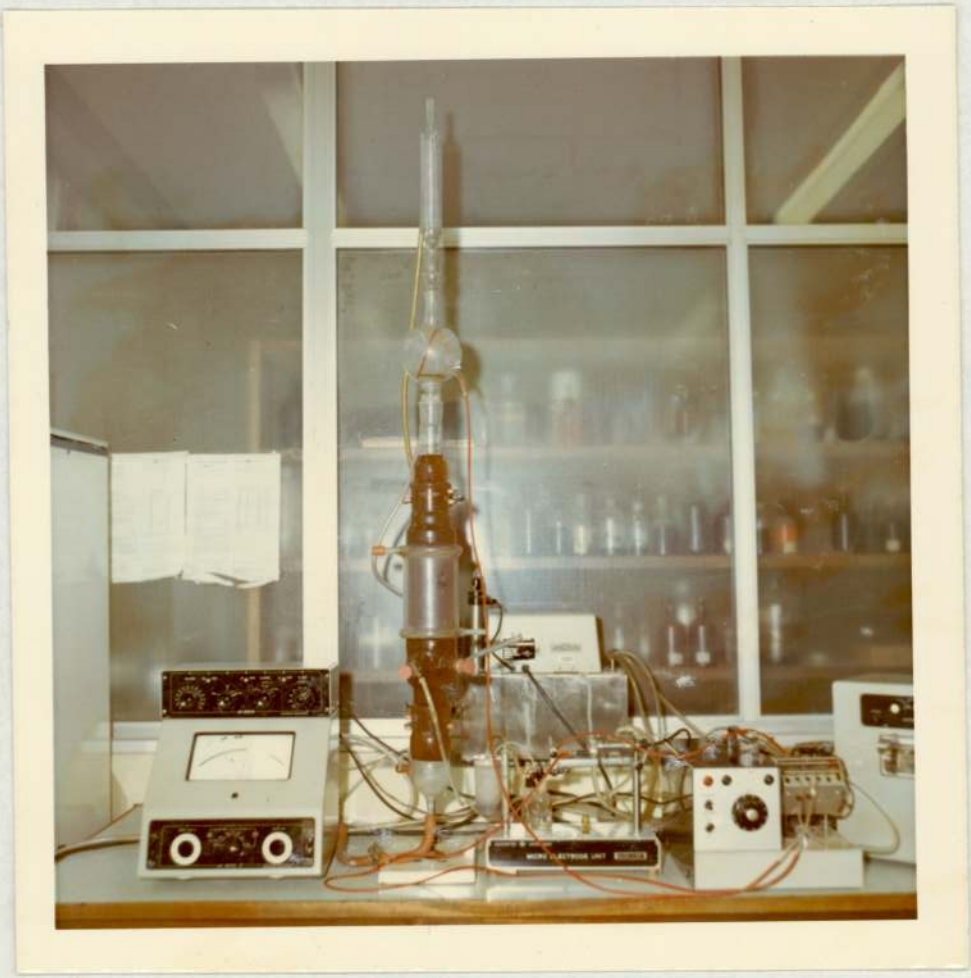


Fig. 14. Astrup Micro-Electrode System used for pO_2 estimations.

To calculate the amount V (volume), at normal temperature and pressure of gas dissolved

$$V = \frac{\alpha \cdot V \cdot P}{760}$$

where α = the absorption coefficient for the gas, V = the volume of liquid, and p = the partial pressure of the gas.

The absorption coefficient (α) for oxygen can be calculated by a formula substitution provided the ml. of dissolved oxygen at saturation is known.

The amount of dissolved oxygen was estimated using the Van Slyke apparatus. The charging wort was mixed in the Van Slyke apparatus with potassium ferricyanide to liberate the oxygen, and caprylic alcohol to prevent frothing. A mixture of oxygen, carbon dioxide, and nitrogen is then obtained, the carbon dioxide being absorbed by sodium hydroxide and the oxygen absorbed with alkaline hydrosulphite. The readings obtained for the measurement of nitrogen was substracted from the result for oxygen plus nitrogen.

If t = temperature in ^{o}C, and B = barometric pressure in mm. of mercury, and V = volume of gas (oxygen + nitrogen or nitrogen) in ml., then the corrected volume of gas at $0^{o}C$. and 760 mm.Hg.

$$= \frac{V\,(B - W)}{760\,(\frac{t}{273} + 1)} \qquad = \frac{V\,(B - W)}{760\,(1 + 0.00367t)}$$

where W = vapour tension of water

Therefore oxygen in ml. per 100 ml. of charging wort (when 2 ml. of charging wort is used).

$$= \frac{V\,(B - W)}{760\,(1 + 0.00367t)} \times \frac{100}{2}$$

The Van Slyke method was as described by Peters and Van

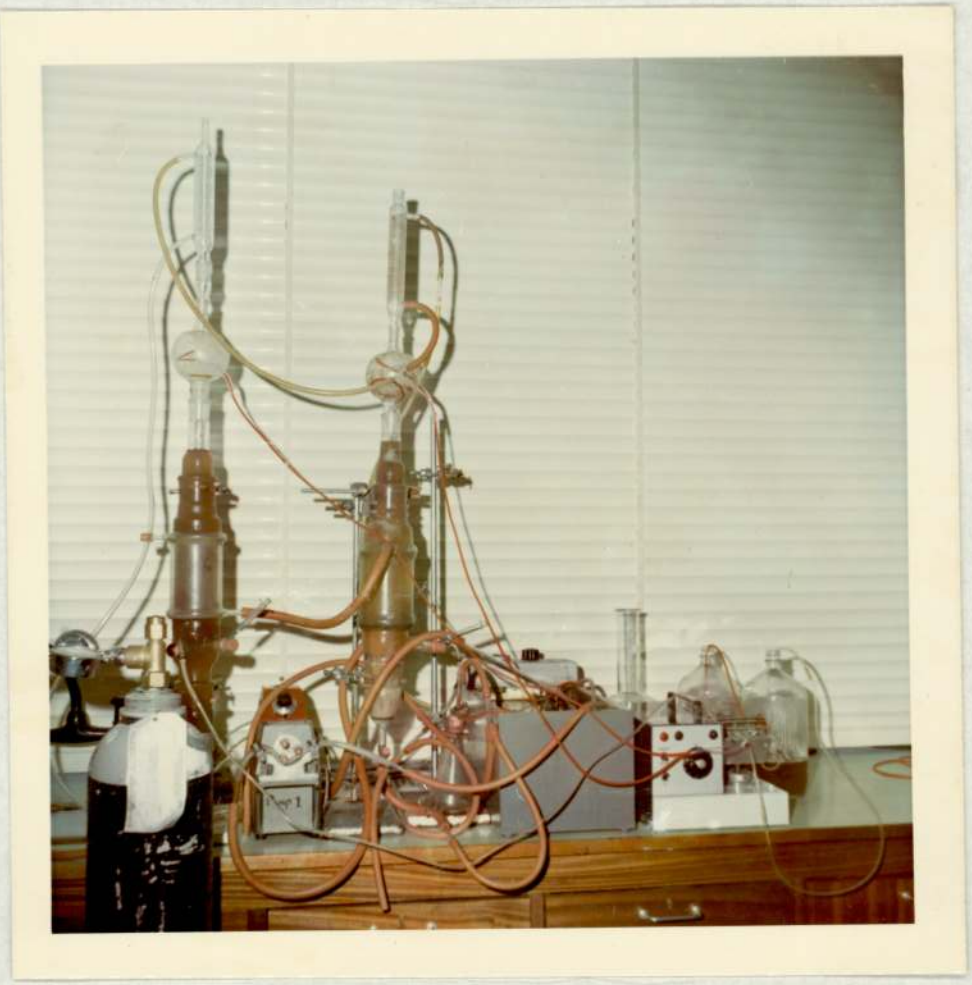


Fig. I6. Continuous fermentation in tower fermentors.

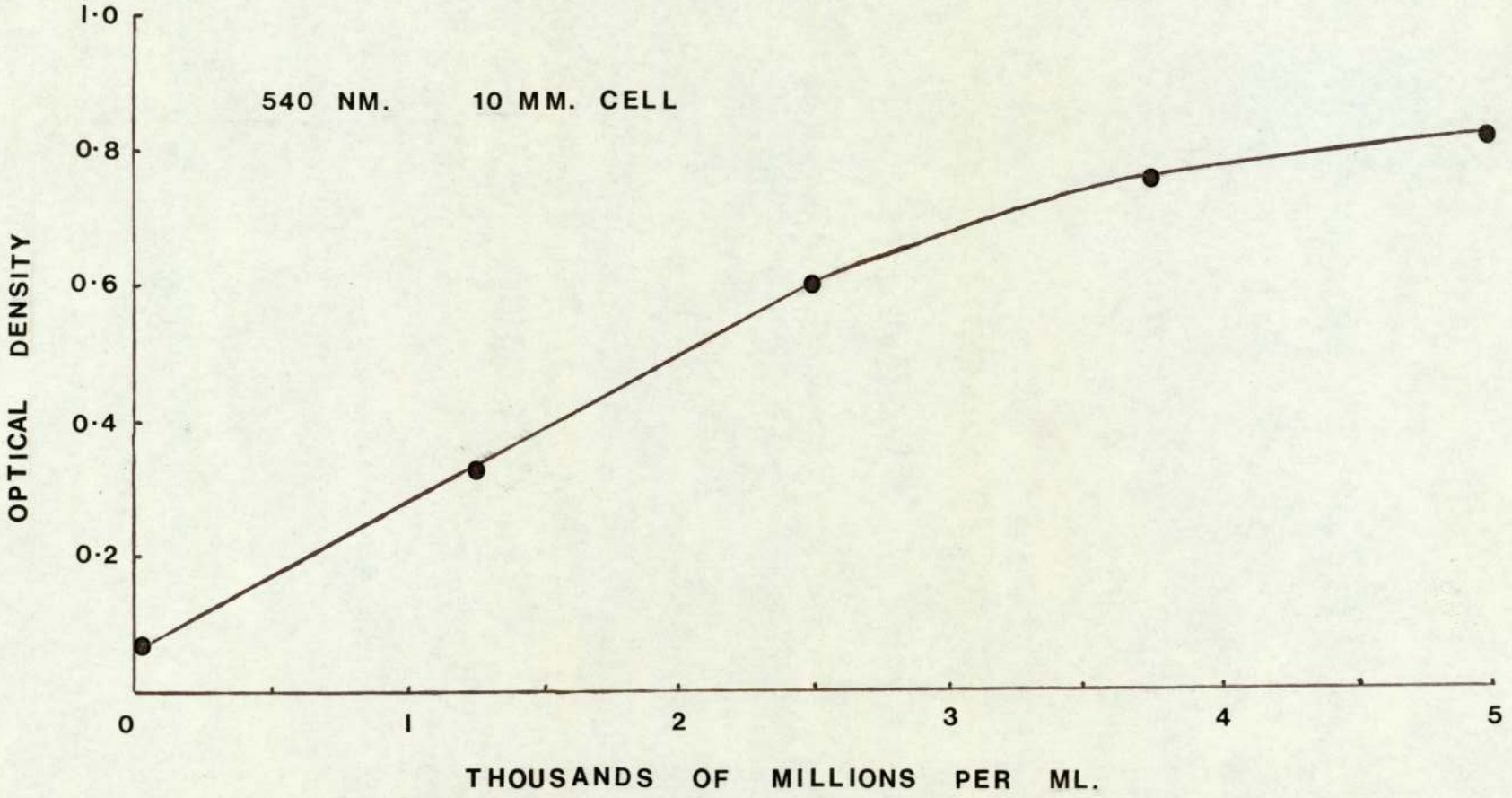


Fig. 17. Standard calibration curve for total bacterial counts.

Slyke (1932).

The absorption coefficient was calculated from the ml. of dissolved oxygen at saturation:-

$$= \frac{760 \text{ x ml. of gas per 100 ml}}{100 \text{ x partial pressure of } O_2}$$

All $p^{O}2$ results were then calculated as θ_2 dissolved as follows:-

$$V = \frac{\alpha \text{ x } 100 \text{ x } p^{O}2}{760}$$

(2) DOSING DEVICES FOR CONTINUOUS FERMENTORS.

Three methods were tested for the continuous addition of charging wort to tower fermentors.

(a) A drip feed was constructed and connected to a 25 litre glass aspirator. The flow was controlled by means of a glass tap. Calibration was made by measuring the volume over a period of 2 mins. using a stop watch and also by counting the number of drops per minute. The 'drip' was connected to the lower end of the tower fermentor.

(b) A Watson Marlow flow inducer was employed to add a constant flow of charging wort to the tower fermentors. Calibration consisted of timing the volume pumped per minute over the range 0.1 ml. per minute to 3.0 ml. per minute. In both methods (a) and (b) gross fluctuations were obtained over a period of 18 hr.

(c) To obtain accurate and precise flow rates a peristaltic pump manufactured by Quickfit and Quartz (as described in 1 (a) above) was employed. The tubes used with the pump were colour coded tygon tubing obtained from Technicon Instruments, Chertsey,

Surrey. Each tube tested was calibrated in ml. per minute at constant pump speed. The internal diameter of the tubes tested were 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05$, 0.065, 0.073, 0.08$, and 0.09 inches. The range covered delivery between 0.06ml. per minute and 1.4 ml. per minute. Deliveries of over 1.4 ml. per minute were obtained by using two pump tubes which were joined by means of a glass 'h' piece.

The theoretical flow was also calculated as follows:-

The rollers move at 'S' cm. per min. and the diameter of the tube = 2r

Then volume per min. = $\pi r^2 S$

Since 'S' is constant then

$$V = K r^2$$

and $K = \pi S$

(3) AIR FLOW RATE MEASUREMENT DURING CONTINUOUS FERMENTATION

Air pressure was maintained by means of an Edward's High Vacuum Compressor of $\frac{1}{8}$ H.P. The flow rate was estimated both by using a Rotameter and by the Gas Chromatograph Bubble flowmeter, as described in A(3) 5 above. An approximate 4% carbon dioxide in oxygen gas was obtained from British Oxygen Co., and an accurate oxygen estimation was made using an Oxygen Analyser Type 0 A 150 obtainable from Servomex Controls Ltd., Crowborough, Sussex. The principle of operation of the Oxygen Analyser is that oxygen is unique amongst common gases in being strongly paramagnetic, whereas most gases are weakly diamagnetic and, consequently, measurement of susceptibility provides a specific indication of the oxygen content of a gas mixture. Since the analyser is a linear measuring device and the

specified accuracy will be obtained over the whole meter scale if the zero is correctly set and the span adjusted with a known gas mixture. The zero was adjusted daily with nitrogen and the span adjusted to 100% with oxygen. As a further check air was also used for calibration of the meter. All the gases tested were passed through a tube containing silica gel to remove water vapour.

(4) CONTINUOUS FERMENTATION IN TOWER FERMENTORS

Two towers were used simultaneously the dimensions of which were as described under A(3) 3 above. To each tower was fitted a 500 ml. Quickfit flask with a side arm one third of the way up the flask. A B34 fitting was placed at the lower end of the flask. To each flask was fitted a reflux condenser cooled with running water. The towers were continusouly pumped with charging wort through the side arm of the flasks. An equivalent amount of vinegar was continuously removed at the base of the tower. At the lower end of the tower a syringe and needle was placed through a rubber bung and left in position. Isolated samples were taken at this point. The continuous towers are seen in Fig.16.

(5) FOAM CONTROL IN CONTINUOUS CULTURE.

Foaming was controlled by the addition of Silicone M S Antifoam Emulsion obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. A final concentration of 0.1% Antifoam in the charging wort controlled foaming during acetification.

(6) CONTROL OF CONTINUOUS FERMENTATION.

(a) Total bacterial counts. A calibration curve was constructed as follows:-

Into 6 test tubes were pipetted the following reagents:-

Tube No.	1	2	3	4	5	6
Distilled Water	9.8	9.8	9.6	9.4	9.2	9.0
Culture	0.2	0.2	0.4	0.6	0.8	1.0

A culture in the log phase of growth was added to Tube No.1 and the tube centrifuged. The supernatant was discarded and the deposit re-suspended in 0.9% NaCL. 0.2 ml. of this suspension was then added to tube No.2. The culture used was grown by the surface culture method for six days, during which time the ethanol concentration had decreased to 0.5 g. per 100 ml.

The turbidity was read on a Perkin Elmer U.V. Spectrophotometer in a 10 mm. cell at a wavelength of 540 nm. A total count was also estimated with a Coulter Counter Model F with an aperture size of 30 microns. Calibration curves were obtained by plotting total bacterial counts against optical density at 540 nm. The standard calibration curve is illustrated in Fig.17.

During the use of 4% CO_2 in oxygen for tower fermentation the charging wort differed in colour from that fermented with air. Consequently the calibration curve was corrected for oxygen fermentation by repeating the total count using a culture grown in oxygen.

The wavelength of maximum absorption was determined on the supernatant obtained by centrifuging the culture by scanning from 350 to 750 nm. The peak absorption was at 580 nm.

It was found subsequently that the absorption of the growth medium altered during the growth of the organisms in oxygen fermentation. Since the optical density changed during growth the cells were washed with distilled water and centrifuged, and the deposit resuspended in distilled water. This suspension was then used for a calibration curve as described above.

(b) pO_2 estimations. Continuous pO_2 estimations were made as described under 17 (a) above.

(c) Total acid estimations. 1 ml. of sample was titrated with N10 sodium hydroxide using phenolphthalein as indicator.

g./100 ml. acetic acid = 0.6 x titration figure (ml.).

(d) Ethyl Alcohol Estimations.

2 µl. aliquots were taken daily for GLC. The attenuation varied depending upon the ethyl alcohol concentration. In continuous fermentation the ethyl alcohol concentration after acetification was approximately 0.1% v/v and the attenuation used for this concentration was x 500.

(7) PHYSICAL SUPPORTS IN TOWER FERMENTORS.

A number of inert solid supports were added to the continuous tower fermentors. These included cellulose powder, crushed filter paper, polystyrene chips, a sheet of filter paper and gauze.

Approximately 5g. of cellulose powder was added to the charging wort, and the turbidity of both the tower and overflow estimated. The object was to estimate any decrease in 'wash out' of bacterial cells. The filter paper used was Whatman's No.42 which was cut

to a size of 30 cm. by 10 cm. The paper was then made into the shape of a tube the edges of which were stapled together. The whole was then placed vertically into the fermentor.

The gauze support was made by winding a 25 cm. wide gauze around a glass cylindrical tube 25 cm. long and 4 cm. diameter. Four layers of gauze were made prior to placing the whole into the fermentor.

RESULTS

SECTION B(1)

VOLATILES OF MALT VINEGAR

THE RESULTS IN THIS SECTION HAVE BEEN

THE SUBJECT OF A PUBLICATION

JONES, D. D. and GREENSHIELDS, R. N., J. Inst. Brewing, (1969), 75, 457.

RESULTS

SECTION B(I) Volatiles of Malt Vinegar

(1) Identification of Volatiles. Preliminary identification was made by G.L.C. after treatment with potassium permanganate which removed the aldehydes leaving ketones and sodium hydroxide which removed esters (Hoff and Feit (1964). The stationary phase was PEG 1000.

Nineteen reference compounds were subjected to G.L.C. and with the exception of diacetyl all were found to contain numerous volatile impurities. Table 16. lists the retention times of the volatiles obtained. There was no problem in identifying the impurities since they were present in minute quantity when compared to the concentration of the actual compound under test.

Although the diacetyl gave no detectable volatile impurities it must be remembered that the concentration employed to obtain an on scale reading was 0.05 per cent when compared to other compounds which ranged between 0.1 per cent and 1 per cent.

Further identification of all the volatiles was made by comparing the retention times of diluted aqueous reference compounds with those found in malt vinegars. Subsequently the identification was confirmed as far as possible by the addition of the respective standards to neutralised vinegar samples. Table 15. lists the results obtained after addition of potassium permanganate and sodium hydroxide.

Four peaks were removed with sodium hydroxide which had retention times of 6, 12, 23 and 25 mm. The peak height of the fourth peak was 4 mm. and when the vinegar sample was diluted with sodium hydroxide the dilution in itself would have eliminated this volatile. The first three peaks were, therefore, presumed to be esters.

After treatment with potassium permanganate a peak with a retention time of 115 mm. was eliminated suggesting the presence of an aldehyde.

Using the results obtained in the above experiments the ten unknown volatiles were identified as ethyl formate, acetaldehyde, ethyl acetate, ethyl alcohol, iso-butyl acetate, n-propyl alcohol, n-butyl acetate, butyl alcohol, amyl alcohol and acetoin. The retention times and the volatiles identified are seen in Table 17.

The number of volatiles detected using Poropak Q. was thirteen an increase of three over PEG as stationary phase. Ethyl formate was not detected by Porapak Q. The volatiles identified in order of retention times were acetaldehyde, ethyl alcohol, acetic acid, n-propyl alcohol, propionic acid, sec-butyl alcohol, isobutyl alcohol, iso-butyric acid, acetoin, iso-amyl alcohol, iso-butyl acetate, n-amyl alcohol, sec-butyl acetate and n-amyl acetate.

The propionic acid and sec-butyl alcohol had similar retention times, (64 mm.) the sec-butyl alcohol being obtained after neutralization of the vinegar sample, and the sample re-chromatographed. Table 18. lists the results obtained with Porapak Q.

The effect of water was noted with both packing materials. The retention time of water was near to that of iso-butyl alcohol with PEG (water = 33.5 and iso-butyl alcohol = 31.5 mm). Quantitative estimation of iso-butyl alcohol with PEG was not possible because of the effect of water. The retention time of iso-butyl alcohol on Porapak Q. coincided with that of acetic acid and the alcohol was quantitatively assayed after neutralisation of the vinegar sample.

Further confirmation was obtained by the method of logarithmic

plotting, Ray (1954) where a plot of the logarithm of the retention volume against the number of carbon atoms yields a straight line.

The plots were made as seen in Fig. 22 and a straight line obtained for the acids and alcohols. Fig. 23. illustrates the plots obtained when log retention volume was plotted against boiling points. Straight lines were obtained for the two groups of compounds which served to confirm further the identification of the volatiles fractionated. Table 19. lists the results obtained and the boiling points of the volatiles on Porapak Q.

(2) A Survey of Malt Vinegars. Sixteen samples of malt vinegar were obtained locally, eleven only were subsequently proven to be from different manufacturers. The age of the samples could not be ascertained. The volatiles present were fractionated on both PEG and Porapak Q. Table 20. lists the concentration of volatiles in the eleven vinegars examined with PEG 1000 and Table 21. lists the volatiles using Porapak Q. Good correlation was found between the quantitative results obtained with both packing materials. The results for Porapak Q. were the means of three determinations.

Due to the effect of water iso-butyl alcohol was not estimated using PEG but this compound was quantitatively assayed by Porapak Q. Ethyl formate and ethyl acetate were not fractionated with Porapak Q. but the volatiles propionic acid, sec-butyl alcohol, iso-butyric acid, iso-amyl alcohol, sec-butyl acetate and n-amyl acetate were fractionated with Porapak Q. It was possible to obtain nine quantitative results for volatiles with PEG and thirteen volatiles with Porapak. Ethyl acetate was not present in two samples tested, iso-butyric acid was present only in three, sec-butyl acetate in four and n-amyl acetate in one sample.

The concentration of acetoin varied greatly between 18.4 mg.

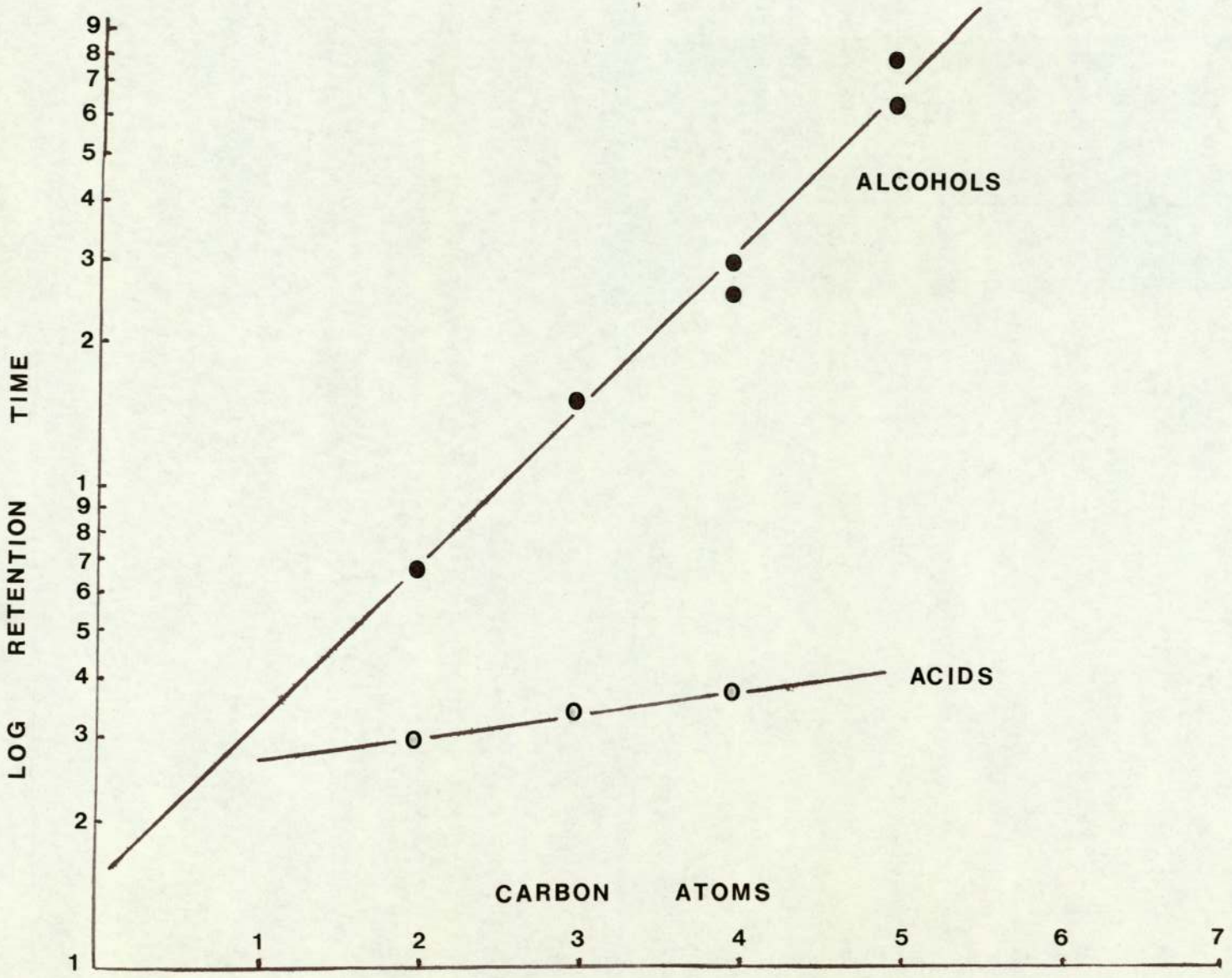
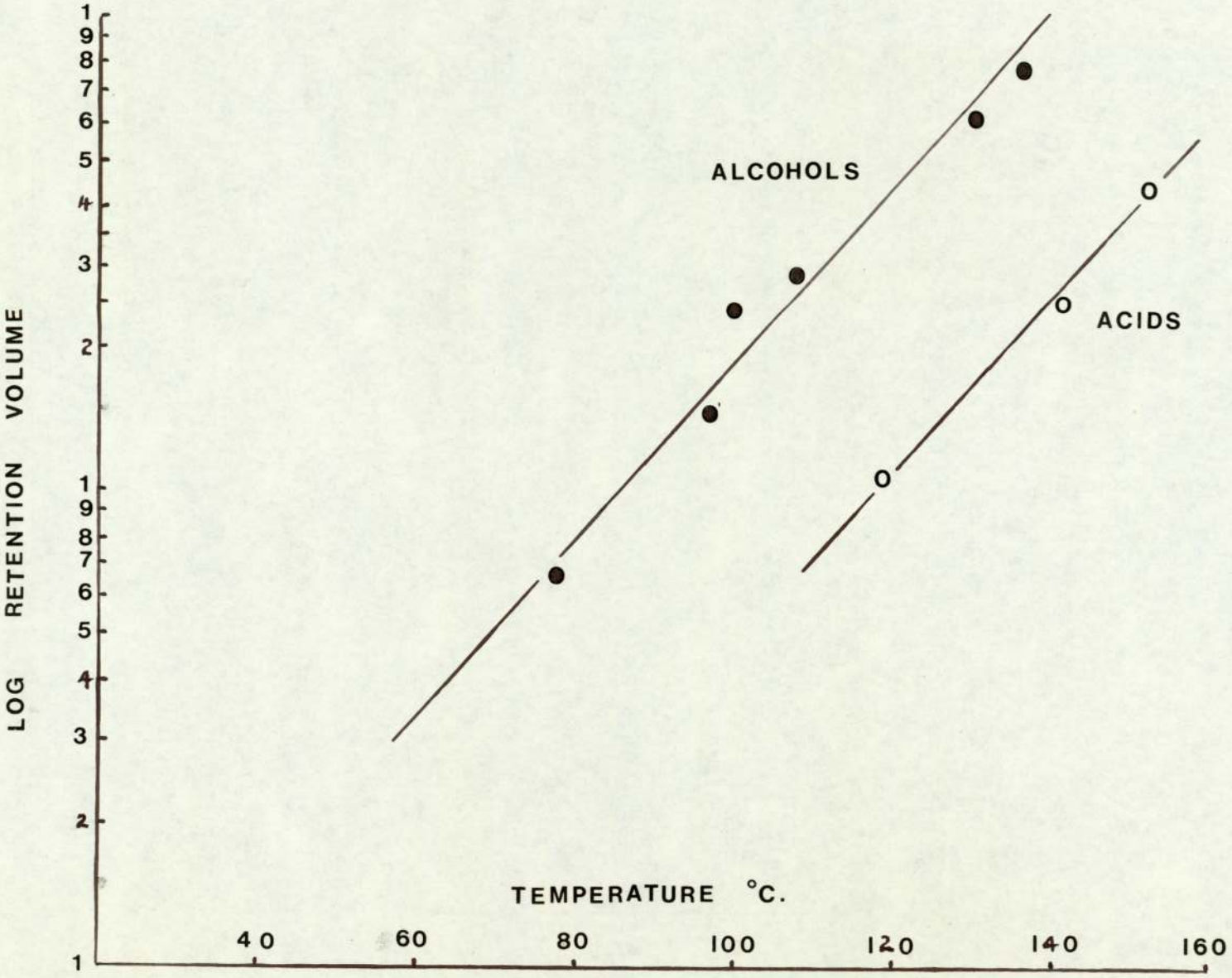


Fig. 22 Log. retention volume plotted against the number of carbon atoms for acids and alcohols.



Fig, 23 Log. retention volume plotted against boiling points of alcohols and acids.

per 100 ml. and 960 mg. per 100 ml. A large variation was also obtained in the concentration of acetaldehyde and ethyl alcohol which suggested different storage periods.

(3) Comparison Between the Ethyl Alcohol Results by G.L.C. and by the Conway Diffusion Method. The estimation of ethyl alcohol in malt vinegar by distillation and subsequent specific gravity measurement was found to be unsuitable because of the low concentrations found. A specific gravity difference of 0.0002 is equal to a difference of 0.13 per cent v/v ethyl alcohol, and the range found by G.L.C. was between 28.8 mg. per 100 ml. and 172.8 mg. per 100 ml.

In consequence the method of Conway (1957) was used. Table 22. lists the results obtained by the two methods. The results of the Conway diffusion bore no resemblance to that found by G.L.C. but the results by the Conway method were reasonably similar to the total alcohol content of the malt vinegars. The difference was significant because P. was less than 0.05 ($t = 10.0$).

The results were to be expected since the diffusion technique has poor specificity when the test is carried out in the presence of other alcohols.

(4) Comparison between Ethyl Alcohol Estimation by G.L.C. and by the Specific Gravity Method. Seven samples were obtained from a manufacturer taken during the process of vinegar manufacture and the ethanol concentrations had been estimated by the S.G. method. These samples were subjected to G.L.C., the results of which are seen in Table 23. Good agreement was obtained between the two methods $P = 0.2$ and $t = 1.42$.

(5) Changes in Volatiles During Storage. Three samples of aged

malt vinegar were obtained from a manufacturer using the Quick-method of vinegar manufacture. All three samples were stored commercially for 4, 6 and 12 months in sealed bottles. A further two bottled samples of freshly made malt vinegar was obtained from the same source, one of which was left open to the air for twenty two days. Aliquots were taken from each bottle on the days shown in Table 24. The results obtained for the aged vinegar stored commercially are seen in Table 25.

During the storage of the two finished samples of vinegar, there was no change in the number of volatiles or their concentration in the sealed bottle. The opened bottle decreased rapidly in their concentration particularly acetaldehyde, which was lost completely in two days. In addition all the volatiles decreased over the twenty two days that the sample was examined.

The three aged vinegars available from the manufacturer did not indicate any changes in the volatile pattern. A detectable loss in all the volatiles was, however, found in the twelve month old vinegar. The largest decrease being found in the lower boiling point volatiles.

(6) Discussion. The examination of malt vinegar by G.L.C. has provided information as to the volatile acids, alcohols, esters and carbonyls present. The compounds detected included acetic, propionic and iso-butyric acids, ethyl, n-propyl, sec-butyl, iso-butyl, iso and n-amyl alcohols; iso-butyl, sec-butyl, ethyl and n-amyl acetates; ethyl formate, acetaldehyde and acetoin.

The identification of all the volatiles was confirmed by adding

suitable standards to vinegar samples before gas chromatography. Porapak Q. was the packing material of choice for fractionating acetic, propionic and iso-butyric acids but in addition, iso-butyl alcohol, iso-amyl alcohol, sec-butyric acetate and n-amyl acetate were detected. Isobutyric acid was found in three samples only, although iso-butyl acetate was found in all the samples. The remaining eight samples did not contain the acid.

Hashimoto and Kuroiwa (1966), Powell and Brown (1966), Kunitake (1966), Morgan (1965) and Harold et al. (1961) have examined by gas chromatography the volatile components of beer. All the alcohols detected in malt vinegar in this investigation have been identified by these workers in beer. As would be expected, the esters and acids are more numerous in malt vinegar owing to acetification.

In the work reported by Aurand et al. (1966) the carbonyls detected were acetaldehyde, acetone, diacetyl, acetoin and isobutyraldehyde. In the present work with malt vinegars, two carbonyls only were detected, acetaldehyde and acetoin. These workers also detected diacetyl both in 'distilled' and grain vinegars. None was detected during the survey suggesting that oxidation of acetoin to diacetyl does not occur in Acetobacter which confirms the work of De Ley (1959).

Acetaldehyde is a product of both yeast and Acetobacter fermentations and since ethyl alcohol is subsequently formed, minor quantities only would be detected. In the present work only one sample was found to have large quantities of acetaldehyde.

Ehrlich (1968) suggests that the higher alcohols are produced during fermentation by deamination and decarboxylation of the amino acids: thus leucine produces iso-amyl alcohol, isoleucine gives active amyl alcohol and valine iso-butyl alcohol. Small amounts only of n-butanol and n-amyl alcohols were detected in these analyses; the corresponding amino acids nor-valine and nor-leucine are of limited natural occurrence.

Iso-amyl alcohol, found in all the vinegars has been found in beer fusel oil by Dupont (1935) and by Webb et al. (1952). It has also been reported by Tanaka (1938) that Acetobacter oxidize: amyl alcohol.

During vinegar fermentation of alcohol, the higher alcohols are partially converted to the corresponding acids and esters. In this survey, only four vinegars contained iso-butyl acetate. Propionic acid and iso-butyric acids are oxidation products of fusel oil components. Propionic acid was found in all the vinegar samples, isobutyric acid being identified in three vinegars only.

The esters isobutyl acetate, sec-butyl acetate and n-amyl acetate could be direct fermentation products or they could be produced by esterification during conditioning or formed during pasteurization Suomalainen (1963).

In alcoholic fermentation, butane-2, 3-diol and acetoin are formed Mahler and Cordes (1968) and in the production of vinegar the diol is oxidised and the acetoin level rises De Ley (1959). Grivsky (1942) describes the oxidation of both the meso and D (-) forms of butane-2, 3-diol in Acetobacter aceti. In addition, two other pathways have been demonstrated in Acetobacter. In the first

Kling (1905) describes the conversion of pyruvate to acetolactate and then to acetoin with the liberation of carbon dioxide. It is not therefore surprising that acetoin was present in large quantities in all the samples investigated, the range being between 0.19 and 0.97 g. per 100 ml.

RESULTS

SECTION B(2)

VOLATILES DURING LABORATORY

ACETIFICATIONS

SECTION B(2)

Volatiles During Laboratory Acetifications

Three distinct methods of laboratory acetification were employed, surface culture, aerated flask and tower fermentor.

(1) Surface Culture. The results obtained for this method are seen in Table 27. 99.4 per cent of the ethyl alcohol was oxidised to acetic acid. 0.4 per cent acetic acid was found in the initial 'charging wort'. Complete oxidation of n-amyl alcohol occurred, n-propyl, iso-butyl, sec-butyl and iso-amyl alcohols were oxidised to a lesser degree. Iso-butyric acid was detected on the sixth day when maximum oxidation of ethyl alcohol had occurred. Propionic acid increased from 32 mg. per 100 ml. to 360 mg. per 100 ml. and subsequently decreased to its original concentration. Maximum acetoin production coincided with maximum oxidation of ethyl alcohol. Both the esters sec-butyl and n-amyl acetates were unchanged. Figs 24, 25 and 26. illustrate graphically the changes in concentrations of acetic acid, propionic acid and acetoin.

(2) Aerated Flask. In this method aeration was continued for thirteen days during which time some evaporation of the ethanol would be expected. The efficiency of oxidation was 89 per cent. Since the surface culture may be considered as a control experiment a total of 1.5 g. per 100 ml. ethyl alcohol was calculated to have been lost in this experiment. The results obtained are seen in Table 28. Maximum acetic acid was produced on the seventh day as in the surface culture technique. The results obtained for the oxidation of the alcohols n-propyl, iso-butyl, sec-butyl and n-amyl were also similar to those obtained for the

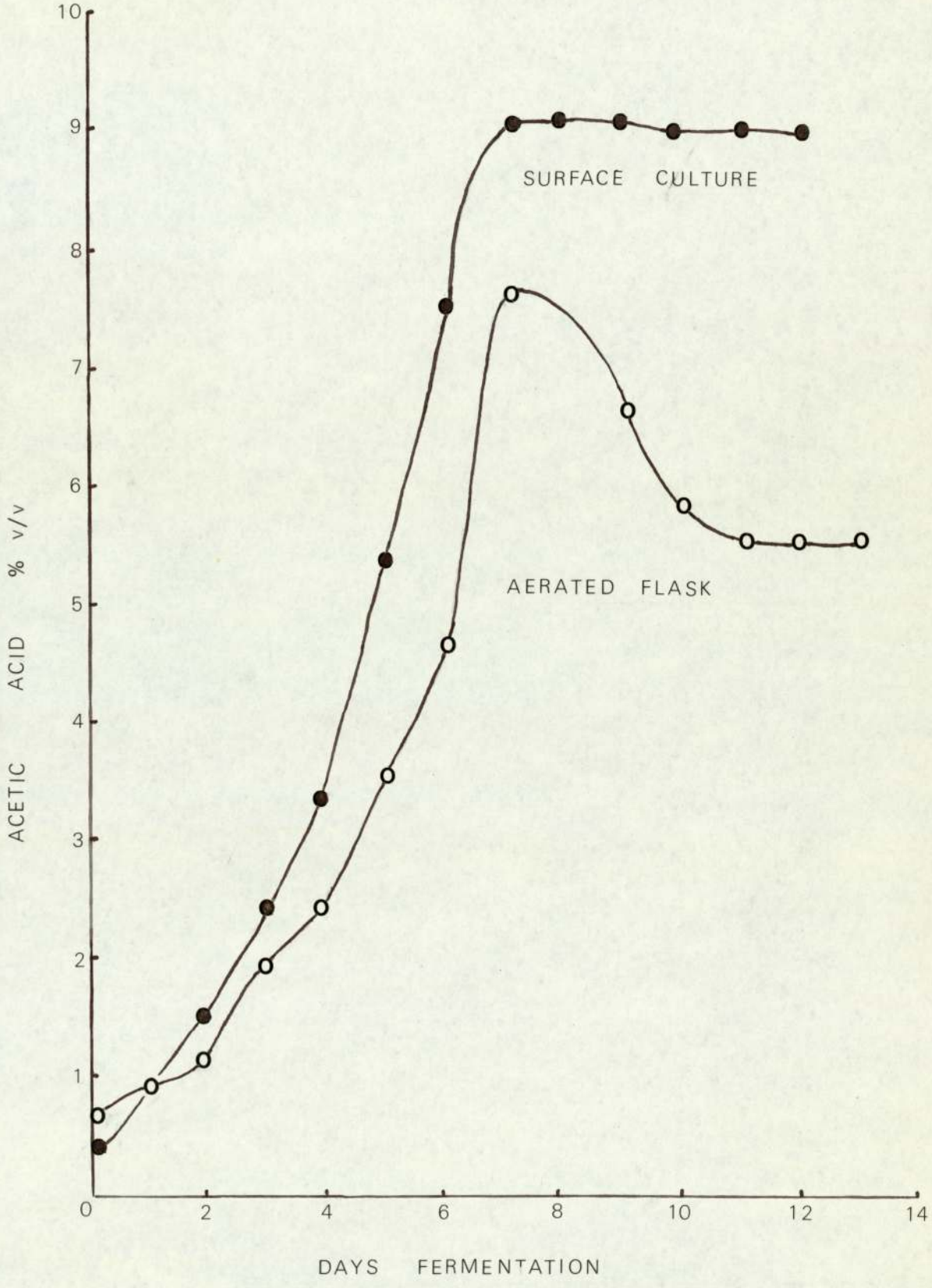


Fig. 24 Acetic acid concentrations during acetification by surface culture and aerated flask.

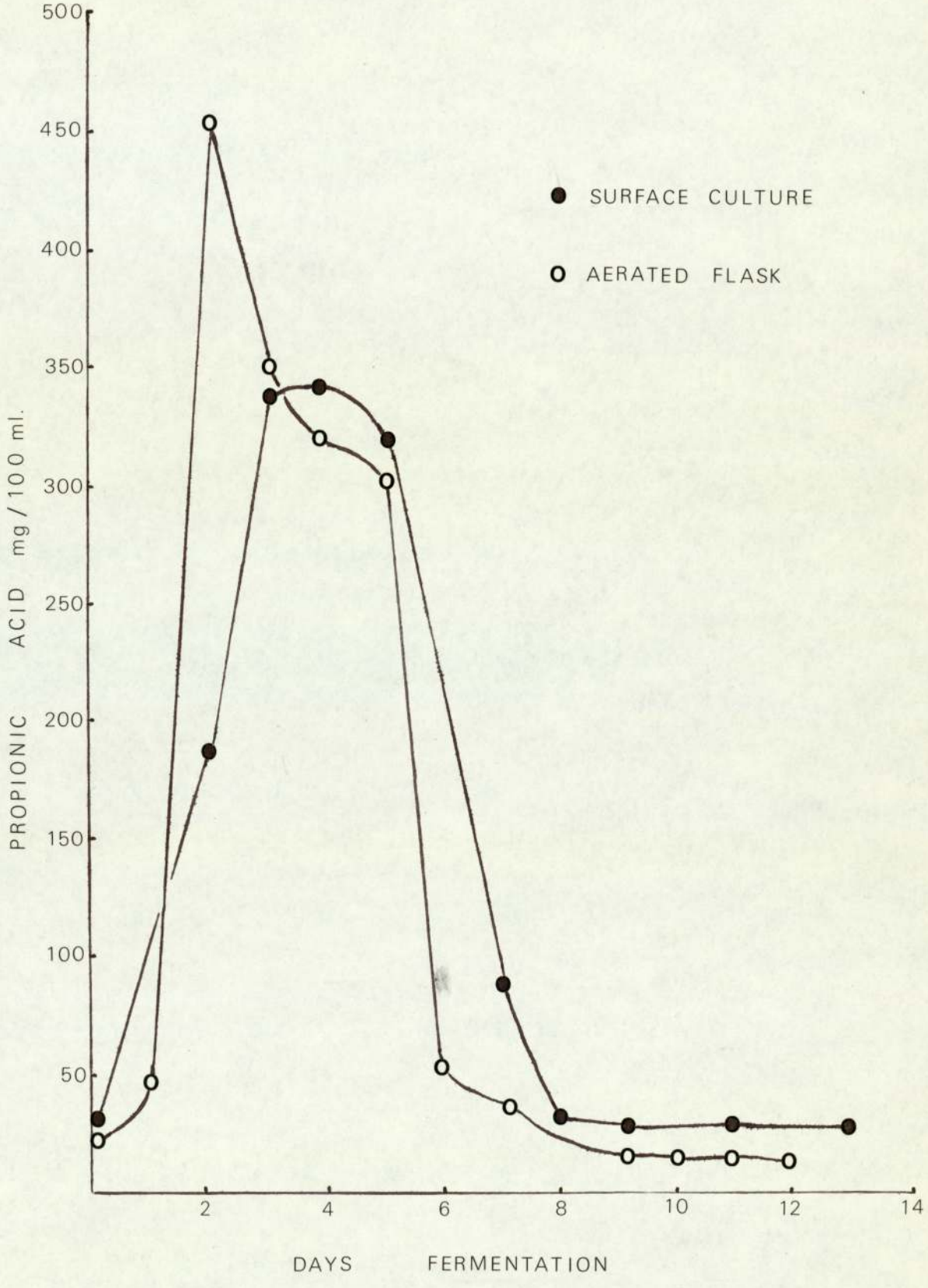


Fig. 25 Propionic acid concentration during acetification by surface culture and aerated flask.

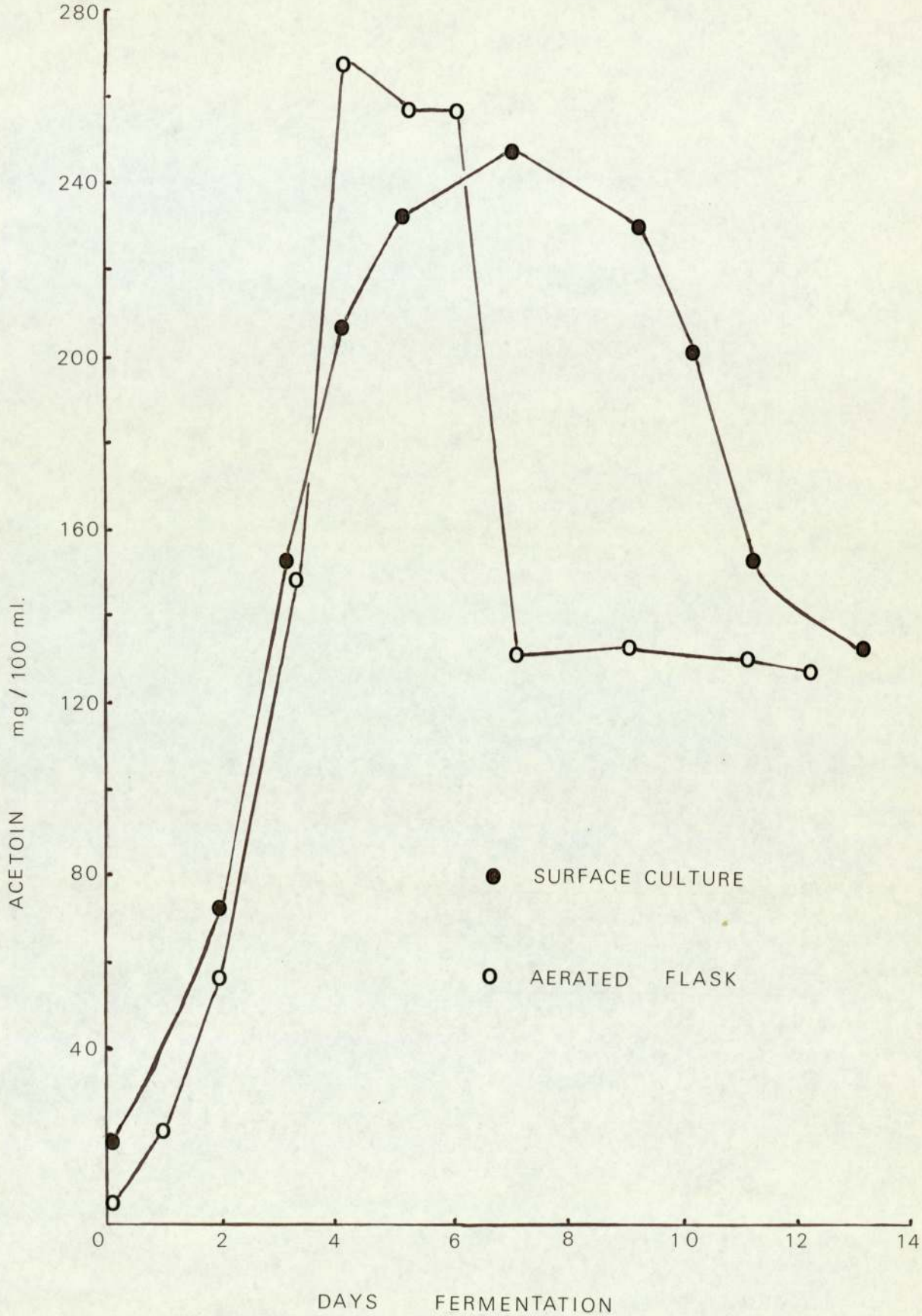


Fig. 26 Acetoin concentrations during acetification by surface culture and aerated flask.

surface culture. On the other hand iso-amyl alcohol oxidation was increased from 30 per cent to 69.3 per cent and isobutyric acid was detected on the first day of acetification. The changes in concentration of acetoin and the esters sec-butyl and n-amyl acetates were also, similar to the surface culture. Fig's 24, 25, 26 illustrate some of the results.

(3) Tower Fermentor. The ethyl alcohol oxidation in the fermentor at 22°C was found to be 93.3 per cent but at a temperature of 30°C it was only 68 per cent. The efficency was lowered in these experiments because of higher temperatures in conjunction with submerged aeration conditions caused considerable evaporation. Oxidation of all the alcohols with the exception of n-propyl alcohol was increased at both temperatures, n-propyl alcohol being reduced from 61.1 per cent to 10.6 per cent at 22°C. Tables 29. and 30. lists the results obtained at both temperatures. Maximum acetic acid production was detected during the fifth day at both 22°C and 30°C. that is, two days earlier than either the surface or aerated flask methods. The changes in the acetic acid concentrations are illustrated in Fig. 27. Both the acids iso-butyric and propionic were in lower concentration, than in the other two techniques. Figs. 28. and 29. graphically illustrate the changes in concentrations of propionic acid and acetoin during this type of acetification.

(4) Discussion. The surface culture acetification may be considered as a control experiment in that no aeration with its accompanying loss of volatiles occurs. In the surface culture method 9.10 per cent v/v acetic acid was produced which suggests that A. aceti was not the only acetifying organism. Henneberg (1897) determined the maximum acetic acid production in various species of Acetobacter; two organisms only were capable of producing 9.1 per cent acid A. ascendens

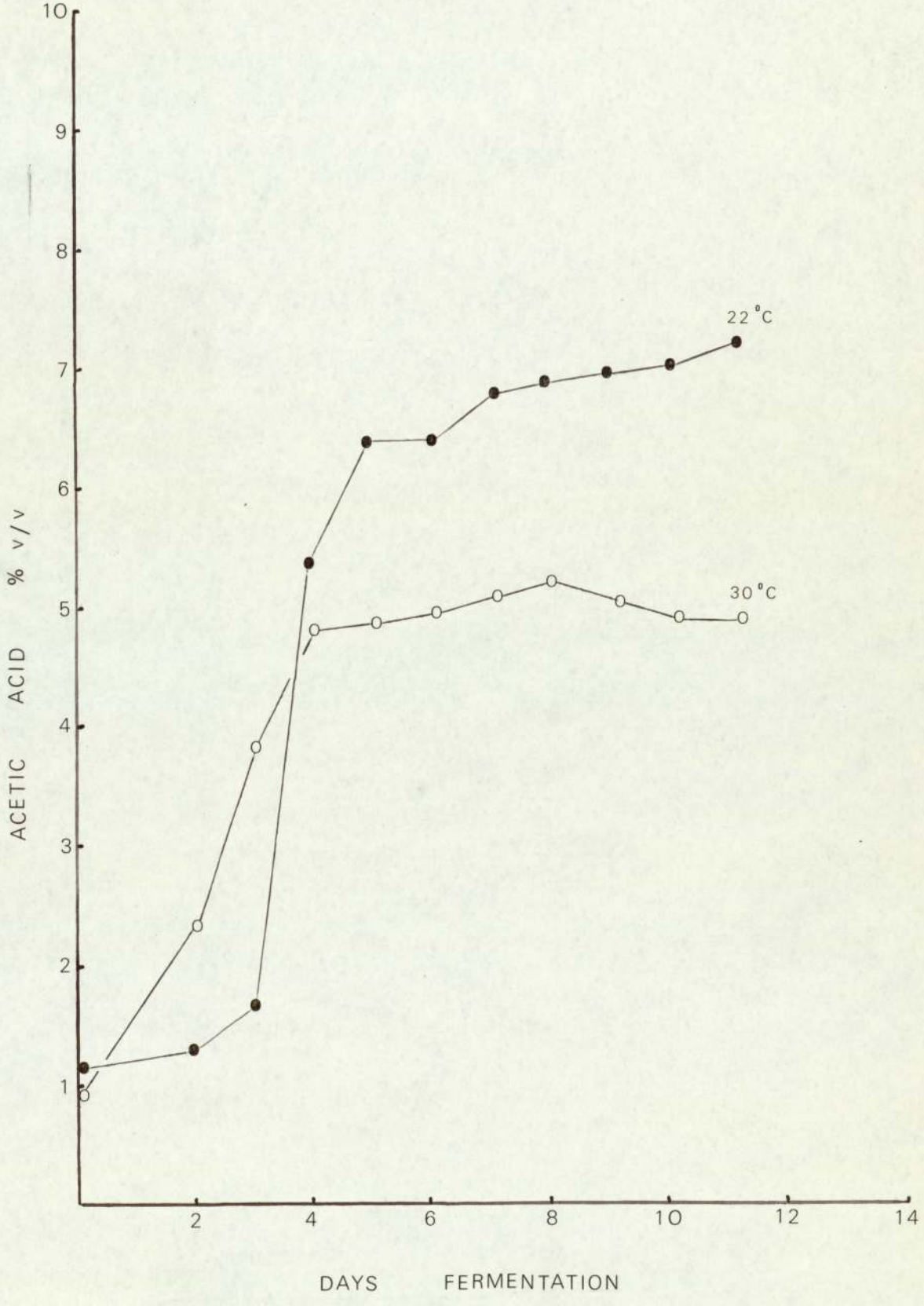


Fig. 27 Acetic acid concentration during acetification in tower fermentors at 22 °C. and 30 °C.

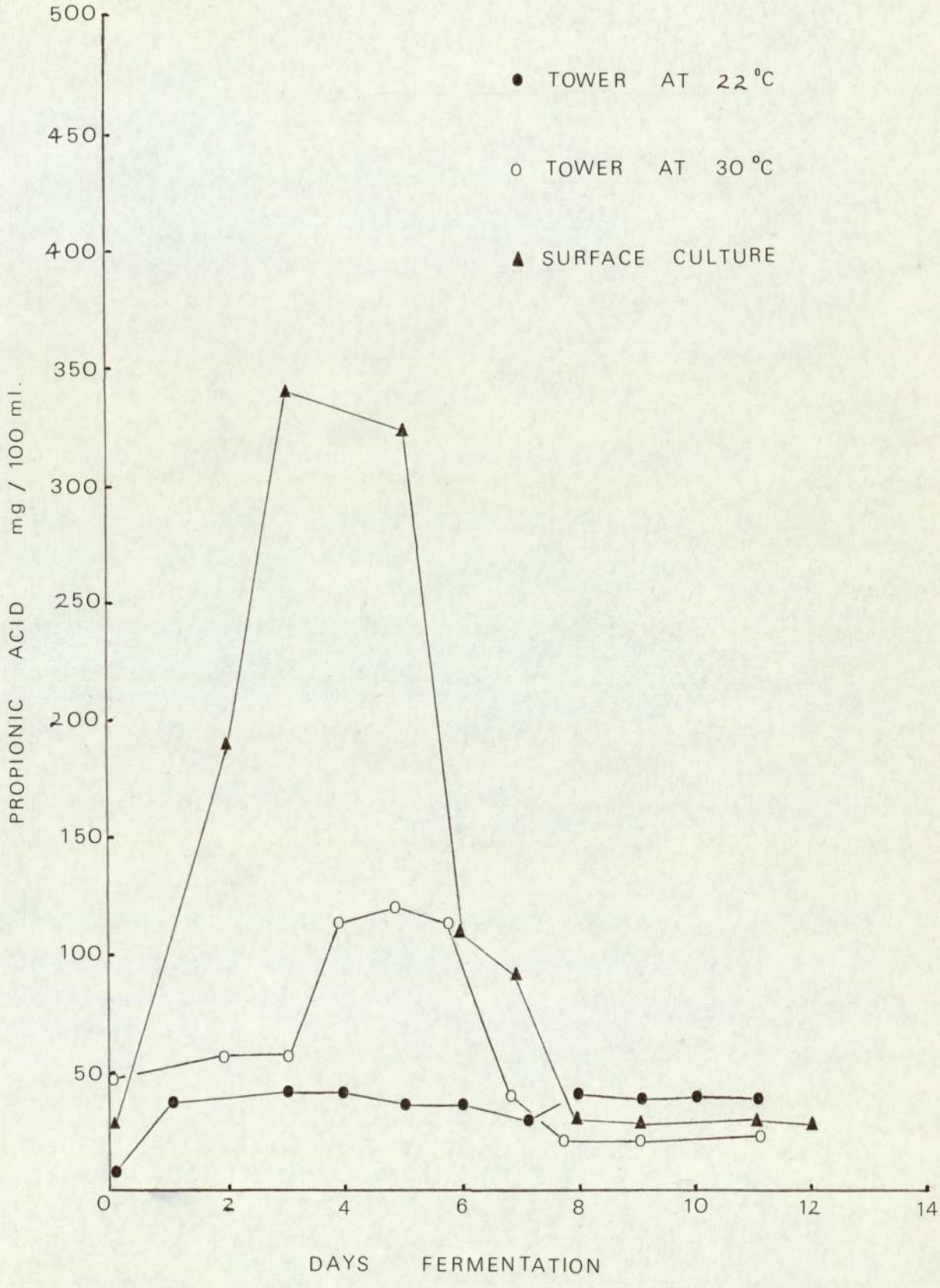


Fig. 28 **Propionic acid concentration during acetification in tower fermentors at 22 °C. and 30 °C. as compared to surface culture.**

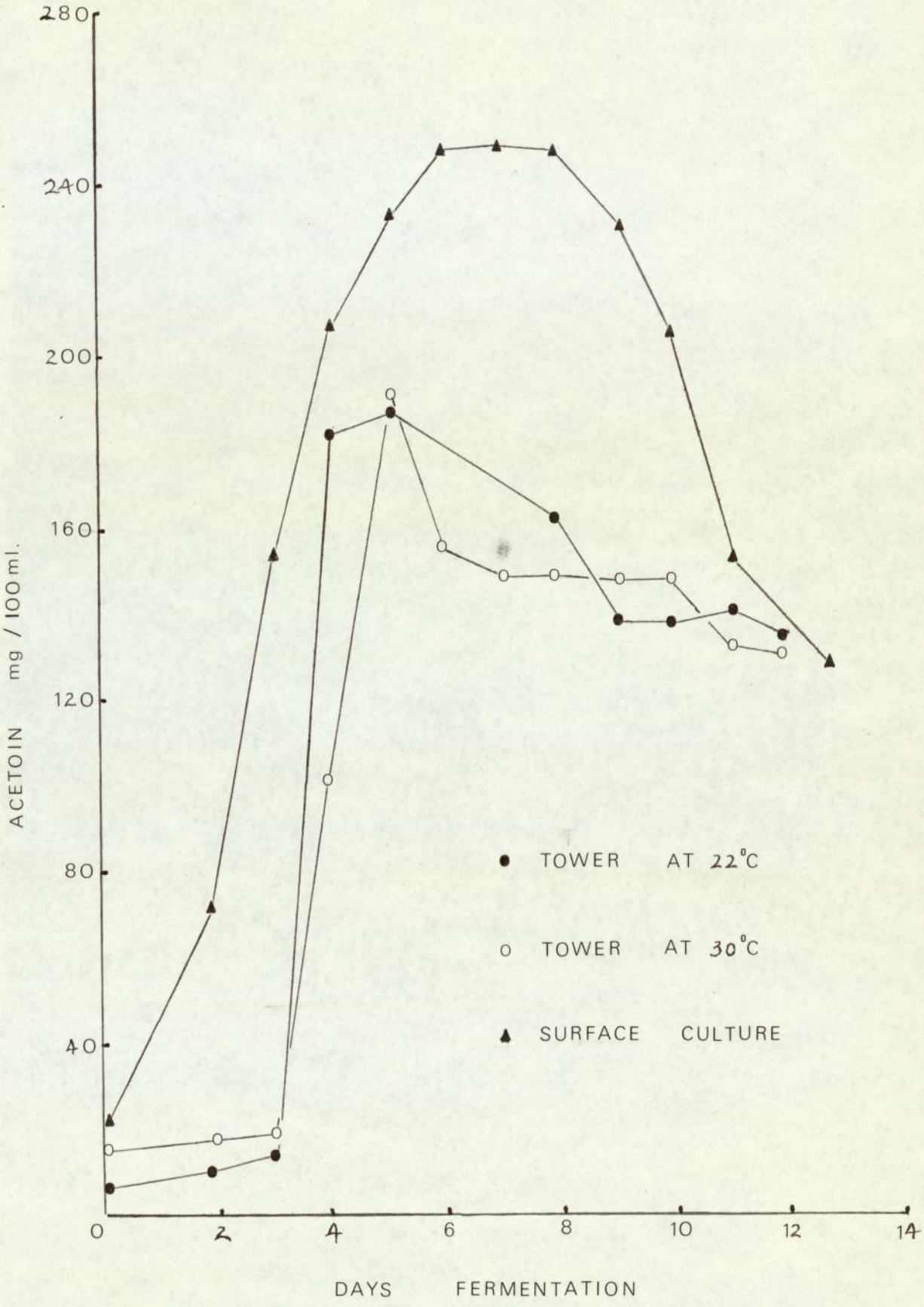


Fig. 29 Acetoin concentration during acetification in tower fermentors at 22 °C. and 30 °C. as compared to that obtained for surface culture.

9.0 per cent and A. schutzenbachi 10.9 per cent.

The oxidation of ethanol has been suggested by Henneberg (1897) to be accomplished in two steps, the first being the oxidation of ethanol to acetaldehyde and the second step is the formation of acetic acid from acetaldehyde. Two distinct reactions have been proposed for the formation of acetic acid from acetaldehyde. Neuberg (1928) suggested that a dismutation reaction occurred in which one mole of acetaldehyde is reduced to ethanol while another mole is oxidised to acetic acid Fig. 30. This reaction proceeds aerobically up to 50 per cent the remaining half of acetaldehyde being oxidised directly to acetic acid. With good aeration the oxidation and dismutation proceed side by side. However, Wieland (1913) reported low dismutation activity in A. ascendens and he proposed a pathway where acetaldehyde was oxidised rather than dismutated to acetic acid (Fig. 31). Furthermore Simon (1930) reported that the ratio of dismutation to direct oxidation was dependant upon pH, with dismutation increasing in basic conditions and decreasing in acid conditions. The results of the present work suggests that a direct oxidation reaction was taking place since conditions were acid and only low concentrations of acetaldehyde were detected in all three acetification techniques (less than 84 mg. per 100 ml.). The speed with which oxidation occurs is dependent upon the amount of available oxygen in the medium. This is illustrated by the fact that the tower fermentor oxidised the ethanol in six days compared to nine days for surface and aerated techniques. However, tower fermentors had lower efficiencies or lower total conversion of ethanol due to evaporation, furthermore when the acetification temperature was increased to 30°C the efficiency was 68 per cent due to a greater evaporation effect.

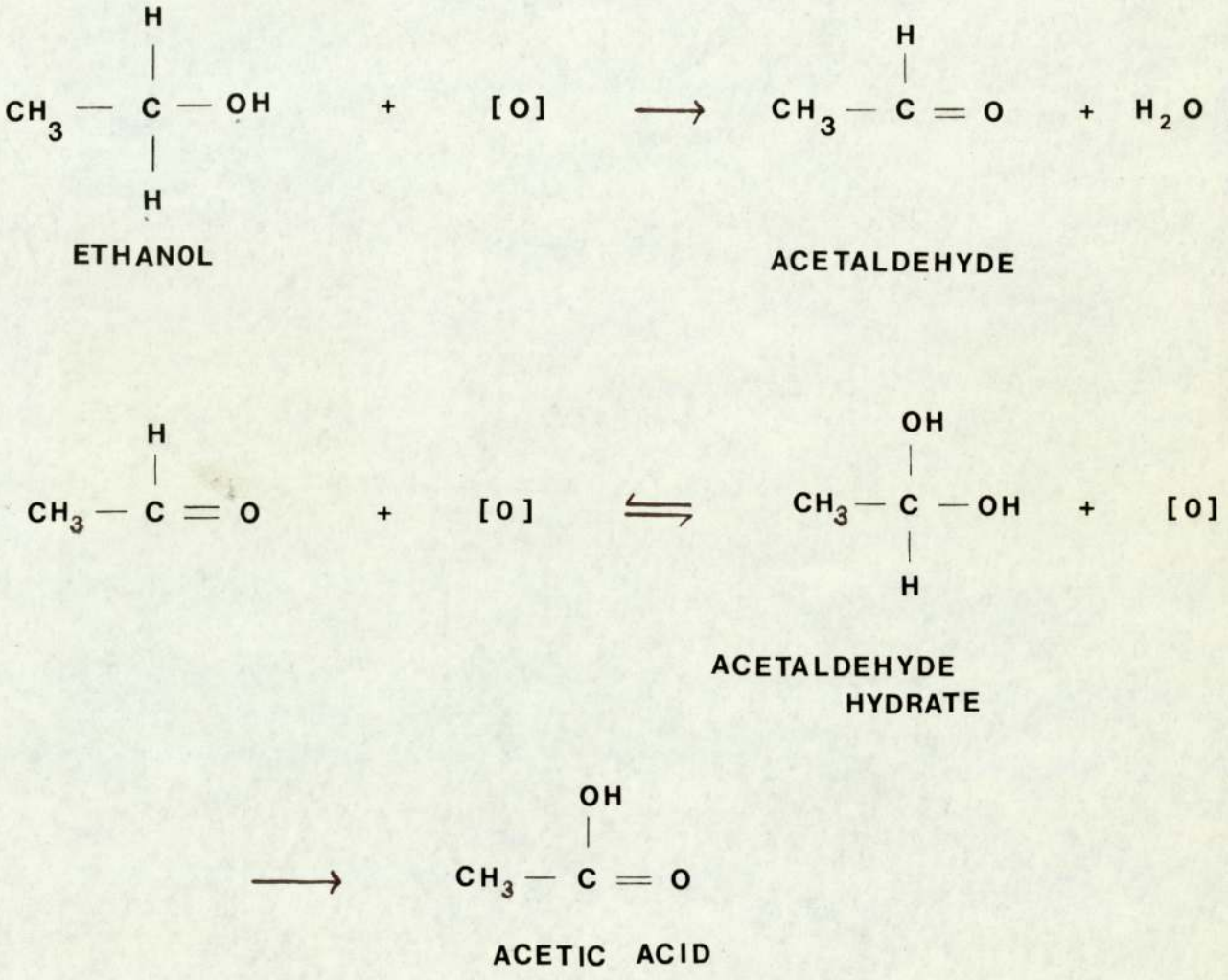


Fig. 31. The direct oxidation of ethyl alcohol to acetic acid.

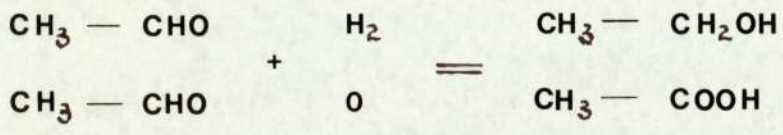


Fig. 30. The formation of acetic acid from acetaldehyde, the dismutation reaction.

The investigations of Ehrlich (1908) suggested that the higher alcohols are produced during fermentation by deamination and decarboxylation according to Fig. 32. The pathways probably responsible for the oxidation products and oxidation of the other volatiles as judged by the present experiments is considered in detail as follows:-

(a) iso-butyl alcohol. This alcohol was found in the charging wort and was oxidised by all the methods examined and also in the commercial process. The further oxidation of isobutyl alcohol to isobutyric acid was observed by Visser't Hooft (1925), Mosel (1932), Asai (1935) and Tanaka (1938). The results of the surface culture and aerated flask confirms the work of Tanaka (1938) who reported that iso-butyl alcohol was oxidised up to 6 per cent relative to ethanol, on the other hand a greatly increased oxidation was obtained in both tower fermentor's at 22°C. and 30°C. Table 31. compares the results obtained by Tanaka with that found in this study. An increased oxidation of this volatile is obtained by good aeration and a temperature of 30°C. The oxidation of isobutyl alcohol at 30°C was 50 per cent and at 22°C 28.3 per cent.

(b) iso-amyl alcohol. Two pathways have been proposed for the production of isoamyl alcohol in the first instance Genevois and Lafon (1957) suggested a pathway Fig. 33. where two molecules of acetate produce acetoacetate which is then decarboxylated to acetone. The acetone is then reduced to iso-propanol. Condensation of acetone with acetaldehyde produces ββ- dimethylacrolein which forms iso-amyl alcohol by reduction. Secondly Wostenholme and O'Connor (1959) have described the formation of sterols and terpenes, some of the intermediates could be reduced to isoamyl alcohol Fig. 34.

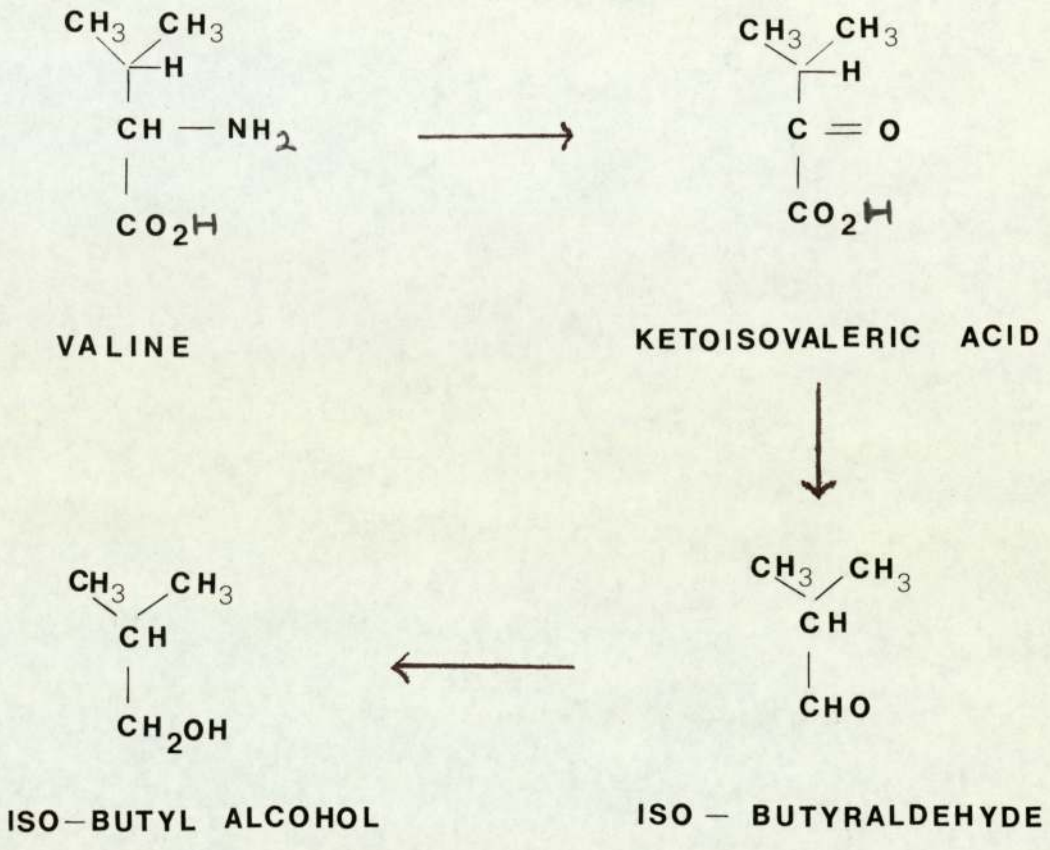


Fig. 32. The formation of iso-butyl alcohol from valine, Mahler and Cordes (1968).

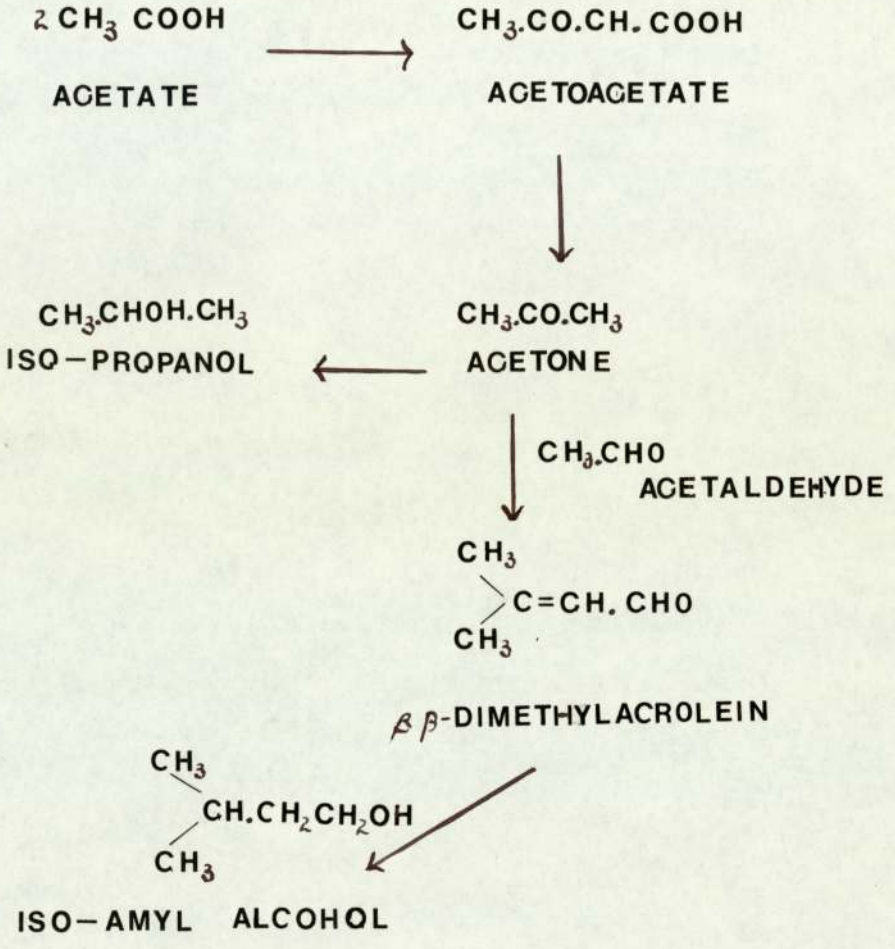


Fig. 33. Pathway for the production of iso-amyl alcohol after Genevois and Lafon (1957).

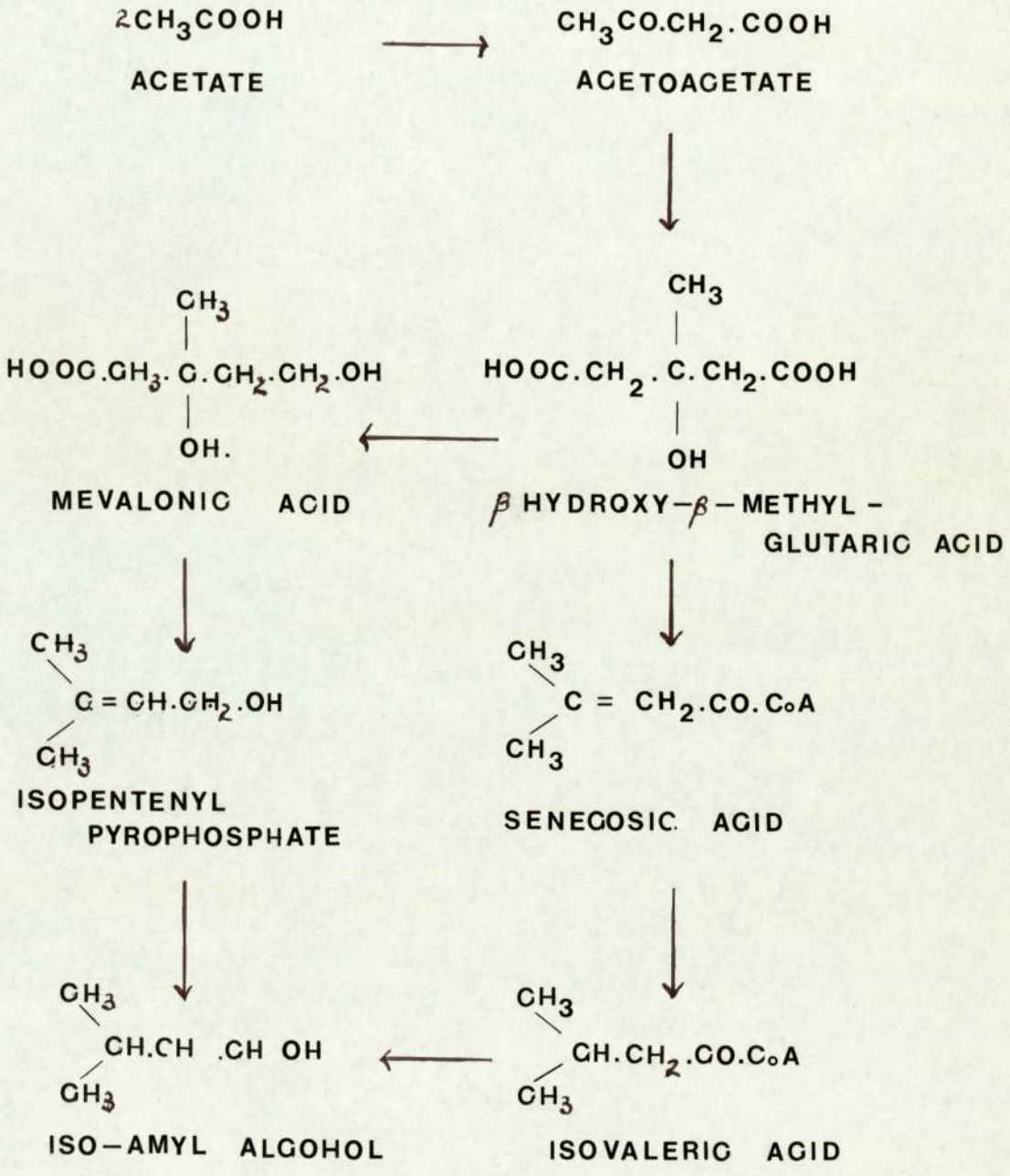


Fig. 34. Production of iso-amyl alcohol from sterol and terpene intermediates after Wostenholme and O'Conner (1959).

It is unlikely that the pathway as postulated by Genevois and Lafon (1957) occurs during acetification since neither acetone nor isopropanol have been detected throughout the investigation. Contrary to the work of Tanaka (1938) isoamyl alcohol was oxidised during acetification in both tower fermentors. The oxidation was found to be 85 per cent relative to ethanol, whereas Tanaka found 30 per cent oxidation. Tanaka also reported the oxidation of isoamyl alcohol with A. peroxydans, A. rancens, and A. aceti to be 15 per cent, 6.3 per cent and 7.0 per cent relative to ethanol. Table 31. lists the oxidation of the volatiles relative to ethanol, The results given with the tower fermentor at 30°C are approximate since loss by evaporation has been shown to be higher at this temperature.

(c) n-propyl alcohol. Guymon et. al. (1961) have presented evidence that n-propyl alcohol is synthesized by yeasts from ∝- ketobutyric acid a known intermediate in the synthesis of isoleucine. Decarboxylation and reduction of this intermediate would result in the formation of n-propyl alcohol. The results of both the surface culture and the aerated flask methods in this investigation confirms the work of Tanaka where 61 per cent of n-propyl alcohol was oxidised, but in both aerated towers the oxidation was greatly reduced from 60 per cent in the aerated flask to 10.6 per cent in the fermentor at 22°C. These results would suggest that n-propyl alcohol oxidation was oxygen dependent in that a decreased oxidation was evident during submerged aeration.

(d) n-amyl alcohol. The biosynthesis of leucine from valine has been investigated by Strassman et. al.(1955) using tracer studies. The pathway proposed includes a series of reactions analogous to the

reactions of the tricarboxylic acid cycle between oxaloacetic acid and ∝ ketoglutaric acid. This evidence was subsequently confirmed by Ingraham and Guymon (1960) who suggested a comparable series of reactions for the biosynthesis of ∝ keto-n-valeric acid from ∝ ketobutyric acid and by decarboxylation and reduction n-amyl alcohol. The oxidation of n-amyl alcohol in this work was similar to that obtained by Tanaka (1938)[2] the results of which are listed in Table 31. There is no evidence to suggest from our results which pathway was utilised in the production of n-amyl alcohol.

(e) sec-butyl alcohol. Genevois and Lafon (1957) proposed a series of reactions which involves the condensation of acetic acid to form isopropyl alcohol and amyl alcohol and the condensation of ethanol to form sec-butyl alcohol. Tanaka (1938)[2] found 8.4 per cent oxidation of sec-butyl alcohol relative to ethanol in A. peroxydans. Similar results were obtained for the surface culture and aerated flasks, but an oxidation of 48.8 per cent was found using the tower fermentor at 30°C. This suggests that sec-butyl alcohol oxidation is dependent upon good aeration as found in the tower fermentor.

(f) acetoin. De Ley (1959) obtained acetoin from D.L. lactate in forty four strains of acetobacter. A. rancens, A. pasteurianus and A. ascendens converted lactate into acetoin up to 74 per cent of the theoretical yield, Kitasato (1928) suggested that acetoin was formed from pyruvate. Acetoin was found to be produced from acetolactate Fig. 35. Wixom (1965) found that acetobacter dehydrated both the isoleucine intermediate ∝, β dihydroxy- methyl-n-valerate and the valine intermediate ∝, β dihydroxyisovalerate. He then suggested that acetobacter can carry out both valine synthesis and acetoin

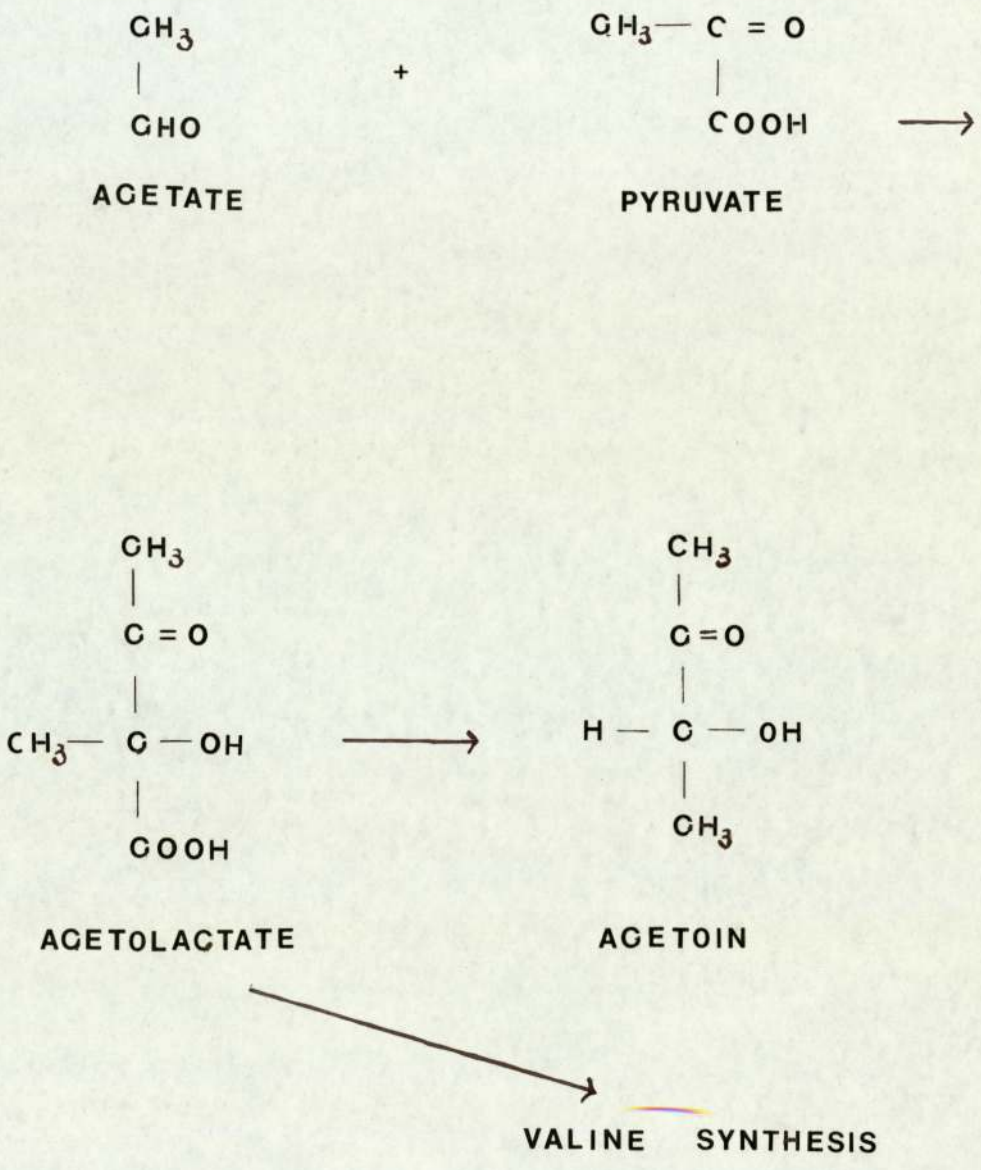


Fig. 35. Pathway of valine synthesis and acetoin formation after De Ley (1959).

formation Fig. 35.

Maximum acetoin formation corresponded with maximum ethyl alcohol oxidation which occured during the seventh day by surface culture, the sixth day in aerated flask and the fourth day by both tower fermentors. The acetoin was present in similar concentration in the surface culture and aerated flask, but in both tower fermentor's the concentration had increased from 186 mg. per 100 ml. to 286 mg. per 100 ml. It is concluded that increased acetoin formation occurs with increased aeration.

(g) Propionic acid. Many micro-organisms are capable of producing propionate (Mahler and Cordes (1968)) from glucose, glycerol, lactic acid and pyruvate.

(i) 3 Hexose $\longrightarrow$ 4 Propionate + 2 Acetic + $2CO_2$

(ii) Glycerol $\longrightarrow$ Propionic acid + Water

(iii) Lactic acid $\longrightarrow$ 2 Propionic + Acetic + CO_2

(iv) Pyruvate + 4H $\longrightarrow$ Propionate

It is unlikely that pathways (i), (ii) or (iii) occur since the fermentation with yeast having gone to completion would leave little residual carbohydrate and neither glycerol or lactic acid have been detected in these experiments. Concentrations in the region of 360 mg. per 100 ml. of propionic acid were reached during acetification both in the surface culture and the aerated flask. The propionic acid concentration obtained for both tower fermentor's were low, the maximum concentration at 30°C being 128 mg. per 100 ml., and at 22°C 50 mg. per 100 ml. These results suggest that the increased propionic acid

concentration is due to the lack of oxygen. The conversion of pyruvate to propionate probably occurs during acetification. The detailed pathway for the conversion of pyruvate to propionate is seen in Fig. 36.

The fate of pyruvate during acetification is dependant upon aeration. This would appear to be the case since pyruvate is metabolised to propionic acid Fig. 36. and also to acetoin.

(h) Esters. The esters iso-butyl acetate, n-amyl acetate and sec-butyl acetate did not increase during acetification.

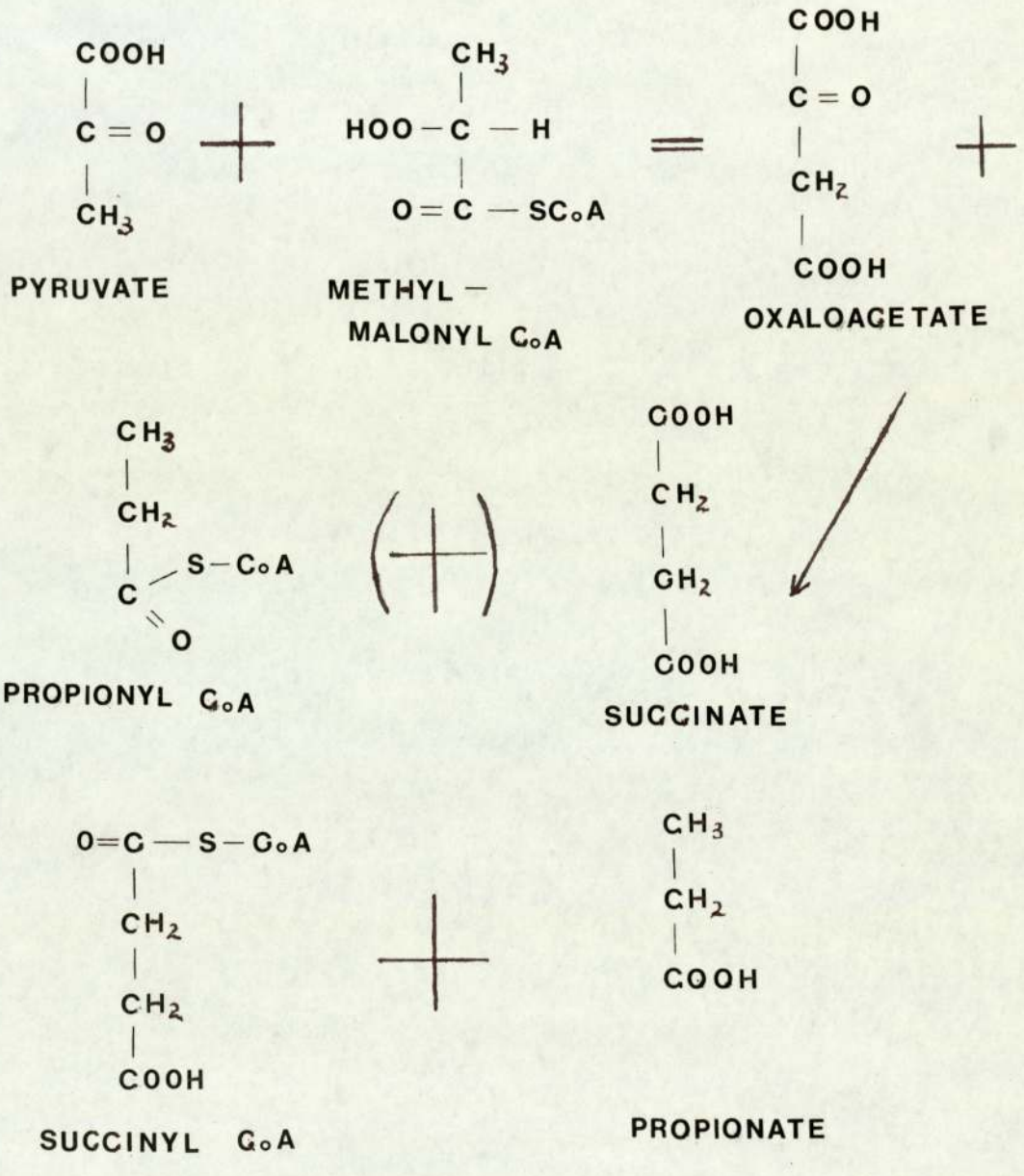


Fig. 36. Pathway of pyruvate to propionic acid.

RESULTS

SECTION B(3)

COMMERCIAL PROCESSES OF

VINEGAR MANUFACTURE

THE RESULTS OF THIS SECTION HAVE BEEN

THE SUBJECT OF TWO PUBLICATIONS

JONES, D. D. and GREENSHIELDS, R. N.

J. Inst. Brew., (1970), 76, 55.

JONES, D. D. and GREENSHIELDS, R. N.

J. Inst. Brew., (1970), 76. 235.

SECTION B(3) Commercial Processes of Vinegar Manufacture

(1) The Quick Process. Four samples were obtained during the manufacturing process, these included wort immediately after seeding with yeast, the alcoholic wash which had been stored for two weeks; acetified liquid at the end of acetification and vinegar post filtration and pasteurization. Table 32. lists the volatiles detected in the fermenting wort. At this stage a number of alcohols were formed,- ethyl, n-propyl, isobutyl iso-amyl and n-amyl. Acetic acid was detected throughout the alcoholic fermentation but no detectable quantities (i.e. over 0.2 mg. per 100 ml.) of either propionic or iso-butyric acids were found. The esters isobutyl, sec-butyl and n-amyl acetates were produced simultaneously with the production of the corresponding alcohols. During the sixteenth day of storage of the charging wort acetification occurred when acetaldehyde, was detected. The volatiles in the alcoholic wash obtained commercially were similar to that found in the fermenting charging wort after it had fermented under laboratory conditions. Acetification again occurred after seven days accompanied by the formation of acetaldehyde and acetoin. Table 33. lists the concentration of the volatiles during the storage of this sample.

The sample obtained after acetification contained all the volatiles detected during the survey of malt vinegars although the concentrations were higher because the stage at which the vinegar was diluted (to approximately 5 per cent) had not been reached. Propionic acid and isobutyric acid were detected and the acetoin concentration had increased from 15.3 mg. to 310 mg. per 100 ml. During storage there was a loss of the lower boiling point volatiles by evaporation as can be seen in Table 34.

The volatiles in the vinegar after filtration and pasteurisation were similar to the sample obtained after acetification (Table 35). although as would be expected after dilution to 5 per cent acetic acid, their concentration was correspondingly lower. During storage the acetaldehyde and ethyl alcohol were completely lost and this loss was accompanied by a decrease in acetic acid, n-propyl alcohol and sec-butyl alcohol.

(2) The Fring's Process. Eleven samples were obtained during the Fring's process of vinegar manufacture the results of the analysis are listed below.

(a) Table 36. lists the volatiles obtained for the sample of wort before seeding with yeast. Three volatiles only were detected in the first aliquot chromatographed, ethyl alcohol, acetic acid and iso-butyl acetate. After nineteen days storage n-propyl alcohol, acetaldehyde, propionic acid, sec-butyl alcohol, iso-butyl alcohol, acetoin, isoamyl alcohol, n-amyl alcohol and sec-butyl acetate were formed. All these volatiles increased in concentration between the nineteenth and thirty first day of storage. Iso-butyric acid and n-amyl acetate were not detected throughout the experiment. An increase of 0.4 per cent v/v of ethyl alcohol occurred during four weeks storage in the laboratory.

(b) Table 37. lists the volatiles detected in the sample of wort seeded with yeast forty eight hours prior to sampling. At this stage of vinegar manufacture a number of alcohols had been formed ethyl, n-propyl, sec-butyl, iso-butyl, iso-amyl and n-amyl. Large quantities 25 mg. per 100 ml. of iso-amyl alcohol was detected after twenty three days subsequent storage in the laboratory. Acetic acid increased to 0.2 g. per 100 ml. but no increase was found in the concentration of propionic acid.

Isobutyric acid was not detected throughout the experiment. Between the twenty third and thirty first day of laboratory storage, acetification took place with the formation of acetaldehyde, acetoin and the esters iso-butyl acetate, sec-butyl acetate and n-amyl acetate. Diacetyl was detected in this sample but decreased in concentration during the first eight days but completely disappeared during acetification.

(c) The volatiles detected in the sample of fermenting wort at the end of alcoholic fermentation are listed in Table 38. Acetic acid was present throughout the sampling period in the laboratory and increased during acetification which occured after twenty three days. Propionic acid and iso-butyric acid, acetaldehyde and acetoin were not detected until acetification had occured. Both the alcohols ethyl and n-propyl were detected in the initial sample, and during storage sec-butyl, iso-amyl and n-amyl alcohols were also produced. The esters iso-butyl acetate, sec-butyl acetate and n-amyl acetate were also formed during storage in the laboratory but were not detected in the initial sample.

(d) The concentration of the volatiles in the charging wort are listed in Table 39. No sec-butyl alcohol, n-amyl alcohol, sec-butyl acetate or n-amyl acetate were detected throughout the laboratory storage period. After eight days storage acetification took place with the formation of acetaldehyde, propionic acid, iso-butyric acid and acetoin. The ester iso-butyl acetate increased from 8.7 mg. per 100 ml. to 12.0 mg. per 100 ml. during acetification.

(e) Table 40. lists the changes during laboratory storage of the sample obtained from the Fring's acetifier. No n-amyl alcohol or n-amyl acetate were detected. The alcohols n-propyl, sec-butyl, iso-butyl and iso-amyl were completely oxidised during the first four days of storage.

(f) Table 41. lists the results obtained during laboratory storage of the acetified sample. The first aliquot taken did not contain detectable quantities i.e. 0.2 mg. per 100 ml. of n-propyl, sec-butyl and isobutyl alcohols. Isobutyric acid, iso-amyl alcohol, n-amyl alcohol and sec-butyl acetate were detected in trace quantities only. The ethyl alcohol was lost by evaporation during the twenty three days storage in the laboratory.

(g) Results obtained for the volatiles in the sample stored commercially for four days are seen in Table 42. The volatiles propionic acid, isobutyric acid and acetoin decreased in concentration, acetaldehyde and ethyl alcohol being lost completely in twenty six days. Both the esters isobutyl acetate and sec-butyl acetate increased from 11.0 mg. per 100 ml. to 13.1 mg. per 100 ml. and 4.5 mg. per 100 ml. to 5.5 mg. per 100 ml. respectively.

(h) Table 43. lists the volatiles detected in finished vinegar obtained after commercial storage of one month. The volatiles acetaldehyde, n-propyl alcohol, sec-butyl alcohol, n-amyl alcohol and n-amyl acetate were not detected. Under laboratory storage conditions both ethyl alcohol and iso-butyl alcohol were lost by evaporation. There was no change in the concentration of propionic acid, acetoin, iso-butyl acetate or sec-butyl acetate.

(i) The results obtained for the concentration of volatiles in the sample of finished vinegar stored commercially for two months are listed in Table 44. No acetaldehyde, n-propyl alcohol, sec-butyl alcohol, n-amyl alcohol, or n-amyl acetate were detected. Iosbutyl alcohol decreased from 6.5 mg. per 100 ml. to trace quantities after four days laboratory storage.

(j) The volatile concentrations obtained for a finished vinegar sample stored commercially for eleven weeks are listed in Table 45. No acetaldehyde, n-propyl alcohol, sec-butyl alcohol, n-amyl alcohol, or n-amyl acetate were detected. Both ethyl alcohol and isobutyl alcohol decreased during six days laboratory storage, no decrease being found with propionic acid, acetoin, isobutyl acetate or sec-butyl acetate.

(k) Table 46. lists the results obtained with a finished sample of vinegar which had not been stored commercially. No n-propyl alcohol, sec-butyl alcohol, iso-butyl alcohol, isobutyric acid, n-amyl alcohol or n-amyl acetate were detected. The ethyl alcohol was lost by evaporation during the twenty six days laboratory storage. Propionic acid decreased from 13.4 mg. per 100 ml. to 10.0 mg. per 100 ml. No change was found in the concentration of acetoin or the esters isobutyl acetate and sec-butyl acetate. Trace quantities only of isoamyl alcohol was detected.

(3) A. Continuous Process

Four samples were obtained from a manufacturer who produces malt vinegar by a continuous process. These consisted of a sample from a blend made from both the Quick and Continuous processes, a finished sample of vinegar from the quick method, a sample from the continuous acetifier, charging wort taken after five days fermentation and alcoholic wash immediately prior to acetification.

(a) Table 47. lists the volatiles obtained for the sample of vinegar blended from the Quick and Continuous processes. Acetaldehyde and n-propyl alcohol were lost completely by evaporation during the thirty

three days laboratory storage. A decrease in concentration of isobutyl alcohol, iso-amyl alcohol, n-amyl alcohol, isobutyl acetate and n-amyl acetate also occurred. No decrease in the concentration of propionic acid or acetoin was detected. No sec-butyl alcohol or sec-butyl acetate were detected.

(b) Table 48. lists the volatile concentrations in vinegar obtained by the Quick method. Acetaldehyde, ethyl alcohol and n-propyl alcohol were lost by evaporation after twenty seven days laboratory storage. The concentrations of isobutyl alcohol, iso-amyl alcohol, isobutyl acetate, n-amyl alcohol, and n-amyl acetate decreased during the laboratory storage period. No sec-butyl alcohol or sec-butyl acetate was detected. No decrease was found in the concentration of either propionic acid or acetoin.

(c) The volatiles detected in the sample obtained from the continuous acetifier are listed in Table 49. Both acetaldehyde and n-propyl alcohol were lost by evaporation. The ethyl alcohol concentration decreased from 0.4 g. per 100 ml. to 0.1 g. per 100 ml. during thirty three days storage. The acetoin concentration was approximately 140 mg. per 100 ml. which was lower than that found for the Quick method 340 mg. per 100 ml.

(d) The volatiles detected in a sample of fermenting charging wort taken after five days fermentation are listed in Table 50. No acetification occurred during the storage period of thirty three days in the laboratory although acetaldehyde and acetoin were detected. n-amyl alcohol increased from 6.0 per 100 ml. to 40 mg. per 100 ml.

(e) Table 51. lists the volatiles detected in the sample of charging wort. No sec-butyl alcohol or sec-butyl acetate was found. An increase in the concentration of isobutyl alcohol and isobutyl acetate however

occurred during laboratory storage.

(4) Discussion. During the fermentation of wort to produce the alcohol for acetification, a number of alcohols in addition to ethyl alcohol were produced, these include n-propyl, sec-butyl, iso-butyl, iso-amyl and n-amyl alcohols. These alcohols were found during examination of the Quick method, the Fring's and the Continuous process of vinegar manufacture. Since malt is used in all three processes it is not surprising that identical results were obtained.

(a) The Quick Method. Since acetic acid was present throughout the fermentation probably due to infection it is not surprising that by esterification sec-butyl and n-amyl acetates were produced. During the third week of storage acetification took place accompanied by an increase in acetic acid and acetoin and the appearance of acetaldehyde. The volatiles found in the fermenting wort have been detected by Hashimoto and Kuroiwa (1966) in beer. The formation of the volatile alcohols and esters is completed by the end of the primary fermentation which is also in agreement with Hashimoto and Kuroiwa (1966) in their study of the volatiles in beer.

During storage of the alcoholic wash it is evident that the esters sec-butyl and iso-butyl acetates are produced during the alcoholic fermentation and not during subsequent acetification. This is contrary to Jenard's (1959) suggestion that they are formed during conditioning and pasteurisation.

A decrease in acetaldehyde and ethyl alcohol was evident after twelve days storage of the acetified sample, a smaller decrease was noted with propionic acid and acetoin. The loss being due to evaporation

and amyl alcohol and the condensation of ethanol to form sec-butyl alcohol. The impression has also persisted, Ingraham et. al. (1960) that the alcohols found in fusel oils are of bacterial rather than yeast origin. There was no evidence in this work to suggest which are responsible for the production of n-amyl alcohol or sec-butyl alcohol. Both alcohols were also detected in the sample of imfermented wort stored in the laboratory for twenty six days and which had not been inoculated with yeast, which was further evidence of a secondary fermentation. The samples obtained during and after the alcoholic fermentation contained only the alcohols n-propyl and isobutyl but during storage at the laboratory sec-butyl, isoamyl and n-amyl were detected. Since wort of lower specific gravity compared to the Quick method was used, the alcohols would be present in lower concentration and would not be detected under the conditions of G.L.C.

The samples during and after acetification were found to contain approximately 80 and 30 mg. per 100 ml. of propionic acid. The concentration of propionic acid found in the Quick method and during the storage of charging wort obtained from the Fring's process were higher. These results suggest that with increased oxygen tension as in the Fring's process the propionic acid is decreased. With the exception of isobutyl acetate, the volatiles were in lower concentrations which would be expected when a more dilute 'charging wort' was used for acetification.

(c) Continuous Process. The sample of fermenting wort and charging wort examined, also suggested that a 'secondary' fermentation had occurred, because an increase was found in the concentration of acetaldehyde, propionic acid, and acetoin. No sec-butyl alcohol or

sec-butyl acetate were detected. It is likely that the mutant strain of yeast or bacteria involved was not capable of synthesizing sec-butyl alcohol.

The propionic acid concentration was lower than that obtained for the Quick method but was in similar concentration to that found for the Fring's process.

The concentration of acetoin by this process was also lower than that found for the Quick process and it is concluded that with increased aeration the acetoin level also decreases. The alcohols iso-butyl, n-amyl and ethyl were in higher concentration in the continuous process than in the quick or Fring's process. This would be expected since in the continuous method complete oxidation does not occur.

During laboratory storage a decrease was noted in the concentration of the lower boiling point volatiles. Similar decreases were found with samples obtained from the Quick and Fring processes.

RESULTS

SECTION B(4)

THE ORIGIN AND OXIDATION OF VOLATILES DURING LABORATORY ACETIFICATIONS

THE RESULTS OF THIS SECTION HAVE BEEN THE SUBJECT OF A PUBLICATION WHICH HAS BEEN SUBMITTED TO THE J. Inst. Brew., (1970)

The results obtained during acetification in the presence of glycerol has been the subject of a British Patent Application No. 5973/70

Greenshields, R. N. and Jones, D. D. Brit. Pat. Appl. No. 5973/70

SECTION B(4).

(1) The Origin and Oxidation of Volatiles During Laboratory Acetifications. Compounds were added to charging wort in known amounts to investigate further the metabolic pathways responsible for the formation of volatiles during acetification. Two methods of laboratory acetification were used, the tower fermentor and surface culture. Both methods were examined at 22°C and at 33°C.

(a) Acetaldehyde. Acetification occurred after twenty six days at 22°C and after fifteen days at 33°C by surface culture. The acetification was delayed when compared to the control experiment which acetified on the twelfth and 7th day respectively. The oxidation of ethanol was not detected until the acetaldehyde concentration had decreased from approximately 1 g. per 100 ml. to 100 mg. per 100 ml. In both tower fermentors acetification occurred on the nineteenth and eleventh days respectively. The acetaldehyde concentration had again decreased before acetification had taken place.

The final acetic acid concentration was higher than the calculated value in all four experiments. The percentage conversion in the surface cultures at 22°C and 33°C was 115 per cent and 100.7 per cent and the tower fermentors were 111 per cent and 109.6 per cent. There was no increase or decrease in the number or concentration of other volatiles when compared to the control experiment. The results obtained are listed in Table 52.(a) and Table 52 (b).

(b) Glycerol. Acetification occurred after six and five days by surface culture and after four days in both tower fermentors. The propionic acid concentration by surface culture increased to a

concentration of 230 mg. per 100 ml. while in the tower fermentors the maximum concentration was approximately 60 mg. per 100 ml. The acetoin concentration in both tower fermentors was 1,300 mg. per 100 ml. compared to concentrations of 400 mg. per 100 ml. by surface culture at 22°C and 350 mg. per 100 ml. at 33°C. The volatile concentrations are listed in Table 52.(a) and Table 52 (b).

(c) Lactic acid. Acetification was detected after eleven days by surface culture at 22°C and nineteen days at 33°C. In both tower fermentors acetification occurred after twelve days fermentation. The propionic acid in the tower fermentors at both temperatures was higher than by surface cultures, values were approximately 200 mg. per 100 ml. and 100 mg. per 100 ml. respectively. The acetoin concentration was approximately 1,100 mg. per 100 ml. and in the control experiment 200 mg. per 100 ml. Acetoin concentration in both fermentors was 500 mg. per 100 ml.

Lactic acid was added in the initial experiments at a concentration of 2000 mg. per 100 ml. and with the exception of the surface culture at 22°C no acetification occurred. Repeat experiments were then made with decreased concentrations of lactic acid to give a final concentration of 500 mg. per 100 ml. when acetification occurred only in the surface culture at 33°C. In both tower fermentors the lactic acid concentration was decreased to 250 mg. per 100 ml. before acetification was possible. The efficiency of ethanol oxidation also decreased to 65 per cent. The results obtained at the three lactate concentrations are listed in Table 53.(a) and Table 53 (b).

(d) Pyruvate. Pyruvate concentrations of 2,000 mg. per 100 ml. inhibited the growth of Acetobacter with the exception of the surface culture at 22°C. The experiments were then repeated with pyruvate

concentrations of 500 mg. per 100 ml. when acetification occurred only in the surface culture at 33°C. Further experiments were made with the tower fermentors at a pyruvate concentration of 100 mg. per 100 ml. Complete inhibition of growth was found in the tower fermentors. In both successful acetifications the acetoin increased from 200 mg. per 100 ml. in the control experiment to 400 mg. per 100 ml. at 33°C and 980 mg. per 100 ml. at 22°C. Table 54 lists the volatiles obtained after the addition of pyruvate.

(e) Glucose. The addition of glucose did not influence the number or concentration of the volatiles detected. The ethanol was oxidised after seven days at 22°C and six days at 33°C both by surface culture methods. Acetification occurred in the tower fermentors after eleven days at 22°C and nine days at 33°C. High concentrations of glucose delayed acetification in tower fermentors. Table 55. lists the concentration of volatiles obtained, by the four methods.

(f) isobutyl alcohol. The isobutyl alcohol oxidation was accompanied by an increase in the isobutyric acid concentration. No increase was found in the concentration of isobutyl acetate. Due to losses by evaporation the final concentration of isobutyric acid by submerged aeration and at a temperature of 33°C was lower than that found for the surface culture at 22°C. Table 56. (a) and Table 56. (b) list the results obtained.

(g) isoamyl alcohol. The results obtained after the addition of isoamyl alcohol are seen in Table 56.(a) and Table 56.(b). The ethanol was oxidised during the eighth day of acetification and the isoamyl alcohol during the eleventh day. No other changes were detected in the volatiles.

(h) n-propyl alcohol. During the oxidation of n-propyl alcohol there was an accompanying increase in propionic acid. An increased oxidation

of n-propyl alcohol was noted at 33°C. The tower fermentor at 33°C oxidised the n-propyl alcohol in nine days and at 22°C in eleven days. Table 57(a) and Table 57 (b) list the volatiles detected after the addition of n-propyl alcohol.

(i) sec-butyl alcohol. sec-butyl acetate was detected in larger quantities than the control experiment immediately after adding the corresponding alcohol. Values of 40 mg. per 100 ml. being detected and in the control experiment trace quantities only were found. The sec-butyl alcohol was not oxidised until all the ethyl alcohol had beem oxidised suggesting that the Acetobacter had a greater affinity for ethyl alcohol as substrate rather than sec-butyl alcohol. The results also suggest that sec-butyl alcohol was metabolised earlier at a temperature of 22°C. Table 57(a) and Table 57 (b) list the results obtained.

(j) n-amyl alcohol. Similar results were obtained for n-amyl alcohol as was obtained for sec-butyl alcohol in that no oxidation occurred before the ethyl alcohol was completely oxidised. An increased concentration of n-amyl acetate was detected immediately on addition of the alcohol. Values of 18 mg. per 100 ml. were found for n-amyl acetate whereas the control experiments contained approximately 5 mg. per 100 ml. The concentrations obtained are seen in Table 53 (a) and Table 53 (b).

(k) Control experiments. The results of the control experiments were similar to the results obtained for the volatiles in the earlier work, when examining acetifications by surface culture and tower fermentors. The results are seen in Table 55.

(2) Discussion.

It would appear that the oxidation of ethyl alcohol to acetic acid is a direct oxidation reaction since there was no increase in the

ethanol concentration during the oxidation of acetaldehyde to acetic acid. This further confirms the work of Simon (1930) who reported that the ratio of dismutation to direct oxidation was dependent upon pH with dismutation increasing in basic conditions. It also seems likely that acetaldehyde when present in increased concentration would be oxidised to acetic acid before ethyl alcohol was oxidised. 78.5 per cent of the acetaldehyde was converted to acetic acid at 22°C and 67 per cent conversion at 33°C. This decrease in conversion at the higher temperature would be expected due to the evaporation of the acetaldehyde.

In the presence of glycerol the acetification period was greatly reduced, the tower fermentation was complete after three days and in the control experiment eight days. Fig. 37. illustrates the speed of acetification in the presence of glycerol. Glycerol may act as a primary energy source thereby reducing the log phase. King et. al. found that A. suboxydans was sensitive to bound forms of pantothenic acid rather than to the free vitamin which is now regarded as a fragment of the coenzyme A. molecule. Glucose oxidation by A. suboxydans is not influenced by coenzyme A deficiency but the oxidation of glycerol was markedly reduced Cheldelin et. al. Widmer et. al. working with cell free extracts of A. suboxydans found three oxidising systems a number of which were phosphate-independant dehydrogenases which oxidised glycerol to the extent of one atom of oxygen per molecule of substrate. Two atoms of oxygen were used per molecule of ethanol. The breakdown product of glycerol was found by Hauge et. al. to be dihydroxyacetone which was then converted to fructose 1, 6 - diphosphate through the action of the isomerase-aldolase system. The fructose 1, 6-diphosphate subsequently joining the reactions of the pentose cycle. Hauge et. al.

has also suggested that glycerol may enter the pentose cycle by two alternative pathways one of which is active at pH 6.0 in independant of A.T.P. and N.A.D. and yields dihydroxyacetone directly. Since the dihydroxyacetone ultimately enters the pentose cycle and the fact that one atom of oxygen only is required per molecule of substrate then it would be expected that the growth of Acetobacter would be enhanced by the presence of glycerol. The effect of glycerol in reducing the log phase of acetobacter growth has been the subject of a patent application.

The propionic acid concentration was higher by the surface culture method than in the tower fermentors 230 mg. per 100 ml. compared to 48 mg. per 100 ml. which suggests that some glycerol was metabolised to propionic acid. It is unlikely that Acetobacter metabolizes glycerol to propionic acid to a large extent since the increased concentration of propionic acid was low.

Rainbow and Mitson (1953) examined the nutritional requirements of acetic acid bacteria and found one group which grew well when glucose but not lactate was supplied as the carbon source. Ammonia failed to support growth when lactate was present except when the growth period was prolonged to seven days. Similar results were obtained in the present work when lactate delayed Acetobacter growth.

Lactate inhibited acetification at concentrations of over 250 mg. per 100 ml. in the tower fermentors. The degree of inhibition increased with both temperature and with increased aeration. Hermann and Neuschul (1932) studied the decomposition of D.L- sodium lactate by thirteen species of acetobacter and found they were all able to produce acetoin. A. rancens and A. ascendens were the most active acetoin producers, giving 31 to 36 per cent of the theoretical yield. The results of the present investigation suggest that both increased temperature and aeration are conducive to acetoin production. 67 per cent of the theoretical yield of acetoin was obtained by surface culture at 22°C and approximately 85 per cent yield at 33°C by surface culture. The mean percentage yield obtained by the

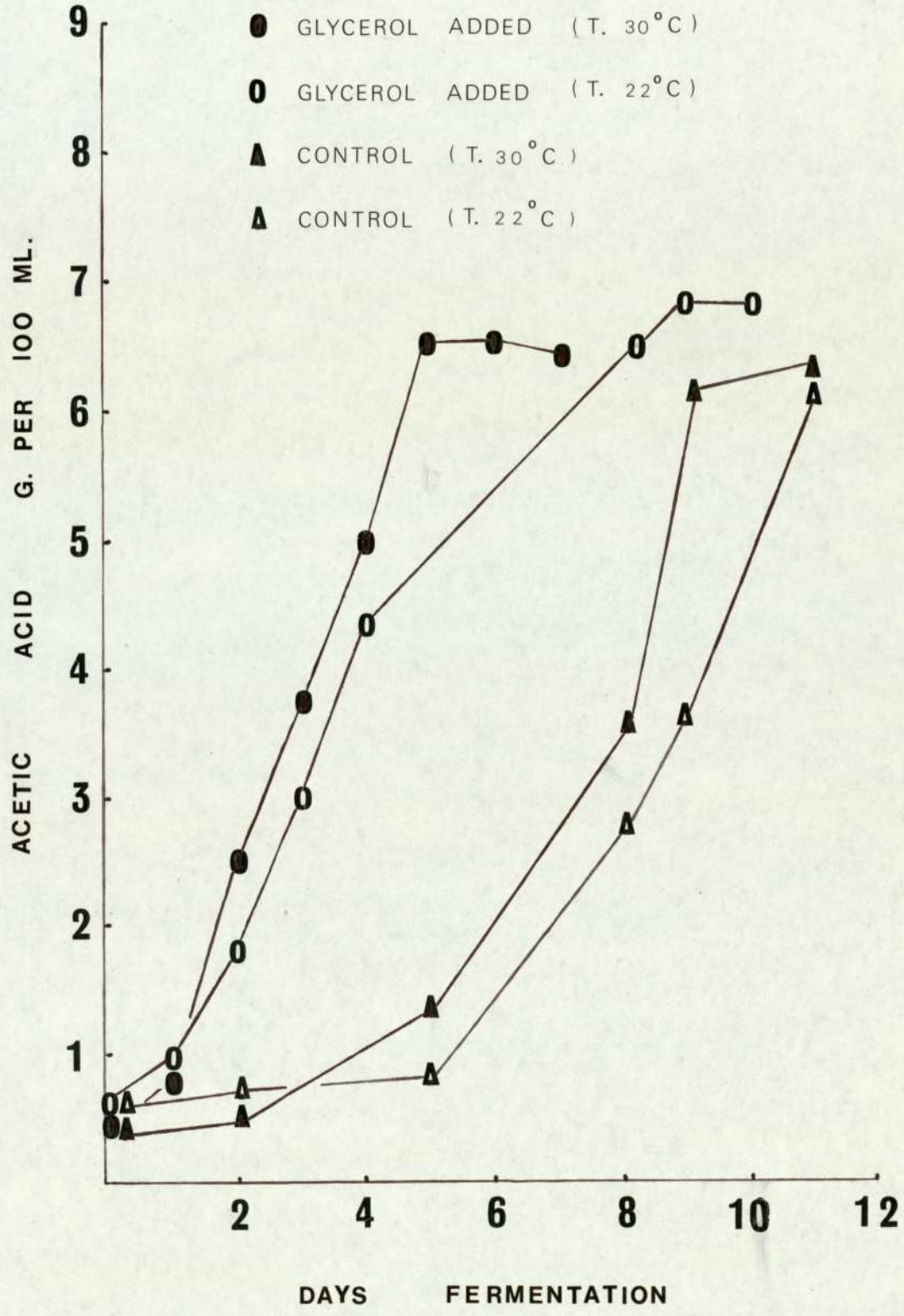


Fig. 37 Acetification in the presence of glycerol in tower fermentors at 22 °C. and 30 °C.

tower fermentors was 84.5 per cent. Both tower fermentors gave increased propionic acid concentration when compared to the control experiments. The increase was 90 mg. per 100 ml. at 33°C and 60 mg. per 100 ml. at 22°C which suggests that some lactate was also being metabolised to propionic acid.

The acetoin level increased in the presence of pyruvate 40 per cent of the theoretical yield being obtained at 22°C and 48 per cent yield at 33°C. King and Cheldelin (1952) reported that pyruvate was oxidised to acetate in two steps, first the pyruvate is decarboxylated to acetaldehyde and secondly the acetaldehyde is oxidised to acetate. The present results do not confirm such an oxidation since the acetaldehyde concentration did not increase in either experiment. Rao (1955) using a strain of A. aceti found that acetaldehyde was not an intermediate in pyruvate breakdown. De Ley (1960) suggested that both soluble and oxidosome linked enzymes of A. peroxydans converted lactate into pyruvate. With lack of oxygen as in our surface culture experiments the pyruvate may be converted into lactate with subsequent formation of acetoin.

Tanaka (1938) measured the oxygen uptake of resting cells of acetic acid bacteria with a number of alcohols as substrates and the results were expressed as the relative activity compared to ethyl alcohol. The tower fermentation methods oxidised the added alcohols in a shorter period of time than by surface culture methods. Tanaka (1938) also found low oxidation activity in Acetobacter for isobutyl alcohol and sec-butyl alcohol but in these experiments activities of 66.5 per cent and 62 per cent were found. Table 58. lists the oxidation found for the added alcohols compared to results obtained by Tanaka (1938) and to the results found during laboratory

acetifications. These results are not directly comparable since Tanaka (1938)[2] measured the oxygen uptake of resting cells and in the previous work the alcohols were not added in high concentrations. Hromatka and Polesofsky (1967) studied the oxidation of n-propyl, n and isobutanol and isoamyl alcohol. In that work the ethanol and acetic acid were first completely oxidised and the amount of acid subsequently liberated was estimated by alkali titration.

With the exception of n-Amyl and isoamyl alcohols the percentage oxidation of alcohols when added to commercial charging wort were higher. Isobutyl alcohol and sec-butyl alcohol were found by Tanaka (1938) to be oxidised only 6 per cent relative to ethanol the results in this work were approximately 60 per cent. Higher oxidation values being found in tower fermentors.

The experiments with glucose added did not change the number or concentration of the volatiles detected. Asai (1934) examined a number of strains of acetic acid bacteria all of which had strong glucose oxidising activities, gluconic acid was formed to theoretical yield. Gluconic acid would not be detected under the conditions used for G.L.C.

RESULTS

SECTION B(5)

VOLATILES DURING MYCODERMA GROWTH AND AFTER CONCENTRATION WITH ORGANIC SOLVENTS

SECTION B(5).

Volatiles During Mycoderma Growth and after Concentration with Organic Solvents

(1) Volatiles during mycoderma growth. Two cultures of mycoderma were obtained, one from a commercial manufacturer of malt vinegar and the other from the University of Aston. The object was to determine the number and concentration of volatiles during mycoderma growth and the effect upon the volatiles present in malt vinegar. The media used for culture were an unfermented wort and charging wort. Tables 59. and 60. lists the volatiles obtained with both cultures. Similar results were obtained by both cultures of mycoderma. During the first ten days of culture on unfermented wort a volatile pattern similar to that found during acetification occurred. The acetic acid concentration increased from 0.6 g. per 100 ml. to approximately 1.4 g. per 100 ml. after ten days growth. Complete oxidation of ethyl alcohol, acetic acid, n-propyl alcohol, isobutyl alcohol and iso-amyl alcohol occurred after fifteen days culture. Both the acetates iso-butyl and n-amyl increased in concentration during the first five days and subsequently decreased to undetectable quantities. No sec-butyl alcohol or sec-butyl acetate were detected during the experiments. n-propyl and n-amyl alcohol were produced followed by a decrease during their oxidation.

Acetification occurred during the growth of mycoderma on charging wort. The acetoin concentration was five times the concentration of that found using the mycoderma culture from Aston University suggesting the mycoderma flora of the two cultures were dissimilar.

(2) Volatile Concentration by Organic Solvents. One increased volatile

was detected by concentration and extraction with both chloroform and diethyl ether. The volatile was identified by its retention time as being glycerol. The retention time was approximately 545 mm. The peak width was large approximately 10 cm. which gave unreproducable retention times. Other volatiles in low concentration (e.g. n-amyl acetate) when chromatographed on neat vinegar were more pronounced after extraction and concentration.

RESULTS

SECTION B(6)

CONTINUOUS FERMENTATION

SECTION B (6)

CONTINUOUS FERMENTATION

The initial investigations made with towers were batch fermentors to examine and find the optima of various parameters such as air flow, substrate concentration temperature, dosing rate and the monitoring of $p^{O}2$, ethanol, total bacterial counts and acetic acid.

(1) Fermentation parameters and their optima.

(a) Aeration. The $p^{O}2$ electrode as described earlier was standardised against air and an oxygen-free solution of sodium sulphite and boric acid. Air flow was estimated using the soap film method. The $p^{O}2$ was estimated on acetifying liquid with the Acetobacter in the lag phase of growth. Readings were taken at intervals as listed in Tables 61 and 62. The results are seen graphically in Figs. 38 and 39. The maximum $p^{O}2$ attained for all the air flow rates was 137 mm.Hg. This partial pressure of oxygen was obtained when the barometric pressure was 765 mm. Hg. It was then accepted that a p02 of 137 mm. Hg was the maximum attainable for the batch of charging wort used for all the continuous fermentation experiments. A p02 value of 137 mm. would correspond to 0.51 ml. of oxygen dissolved in 100 ml. of charging wort or 12.75 ml. of oxygen dissolved in the tower volume. Flow rates of 120 ml. per minute and over gave rise to excessive foaming. A flow rate of 50ml. per minute was taken as the optimum flow because the maximum p02 was obtained after 1 hr. Flow rates of 8.1 and 5.3 ml. per minute did not attain p02 levels of 137 mm. during the course of the working day. The maximum air flow tested was 800 ml. per minute which attained a p02 of 135 mm. Hg in 0.75 min., but these flow rates were

excessive and were impractical for continuous use.

Continuous monitoring of the pO2 was made during complete acetification in a tower fermentor, the results of which are seen in Fig. 40 and Table 63. Fig. 40 illustrates the pO2 values against total acid concentration. During the log phase of growth or during maximum ethanol oxidation the pO2 value was zero. Immediately the ethanol oxidation decreased during the beginning and end of acetification the pO2 was correspondingly increased. During the period when the pO2 was zero the air flow was increased from 50 ml. per minute to 300 ml. per minute, but no increase in the pO2 value was detected.

Monitoring of pO2 was also made during acetification using 4% CO2 in O2 for aeration. The flow rate chosen was 25 ml. per min. The results obtained are illustrated in Fig.36. Similar results were found with the 4% CO2 in O_2 as found with air, in that rapid ethanol oxidation occurred during the decrease in pO2. The minimum pO2 found was 45 mm.Hg. and the maximum 380 mm. of acetification did not occur for 16 days in the presence of high oxygen tension. With air for aeration, acetification occurred on the 8th day of fermentation.

(b) Substrate Concentration. Ethanol concentrations between 2% and 10% v/v were acetified in a tower fermentor, and the total acid content monitored daily. Figs.41 and 42 and Table 64 list the results obtained. Acetification occurred on the 4th day when using ethanol concentration of 2,3,4,5, 6 and 7% v/v ethanol. The 8% v/v ethanol concentration acetified on the 5th day and the 9% v/v and 10% v/v did not acetify completely during fermentation over

18 days. The results suggest that the maximum ethanol concentration for efficient acetification sould be approximately 7% v/v.

(c) Optimum Temperature. Four temperatures were investigated 20, 25, 30 and 35°C. Fig. 43 and Table 65 illustrate the results obtained. At temperatures of 30 and 35°C. the ethanol was oxidised during the 3rd day of fermentation. The 25°C fermentor acetified on the 5th day and the 20°C. on the 6th day. At this stage a condenser was not used on the fermentor and, at the higher temperatures, a marked loss of ethanol was detected. The efficiency at the four temperatures of 20, 25, 30 and 35°C. were 97%, 79%, 70.6% and 65.1% respectively. Subsequent experiments were made at approximately 33°C., which appeared to be the optimum temperature.

(d) Dosing Rate. Three methods of dosing the tower were investigated drip feed, Watson Marlow pump and a Quickfit pump designed for dosing chromatography columns. Since a volumetric efficiency during continuous fermentation of approximately 2(i.e., the volume of the tower to be acetified every 24 hr.)was required, the dosing rate per minute would be approximately 1.2 ml. per minute. The calibrated curve for the drip feed method is illustrated in Fig.44. A straight line was obtained for flow rates from 0 - 3 ml. per minute. This method was eventually abandoned since large errors were found over periods of 16 hr. (overnight). As the volume of charging wort decreased in the reservoir the dosing rate also decreased rapidly.

A Watson Marlow peristaltic pump was then calibrated Fig.45, a calibration curve constructed between 0.8 ml. per minute and 3.0 ml. per minute. A large error was found in the dosing rate by this method with the small volumes pumped. Overnight on numerous occasions the pumping volume had increased from 1.0 ml. per minute to 1.2 ml. per minute.

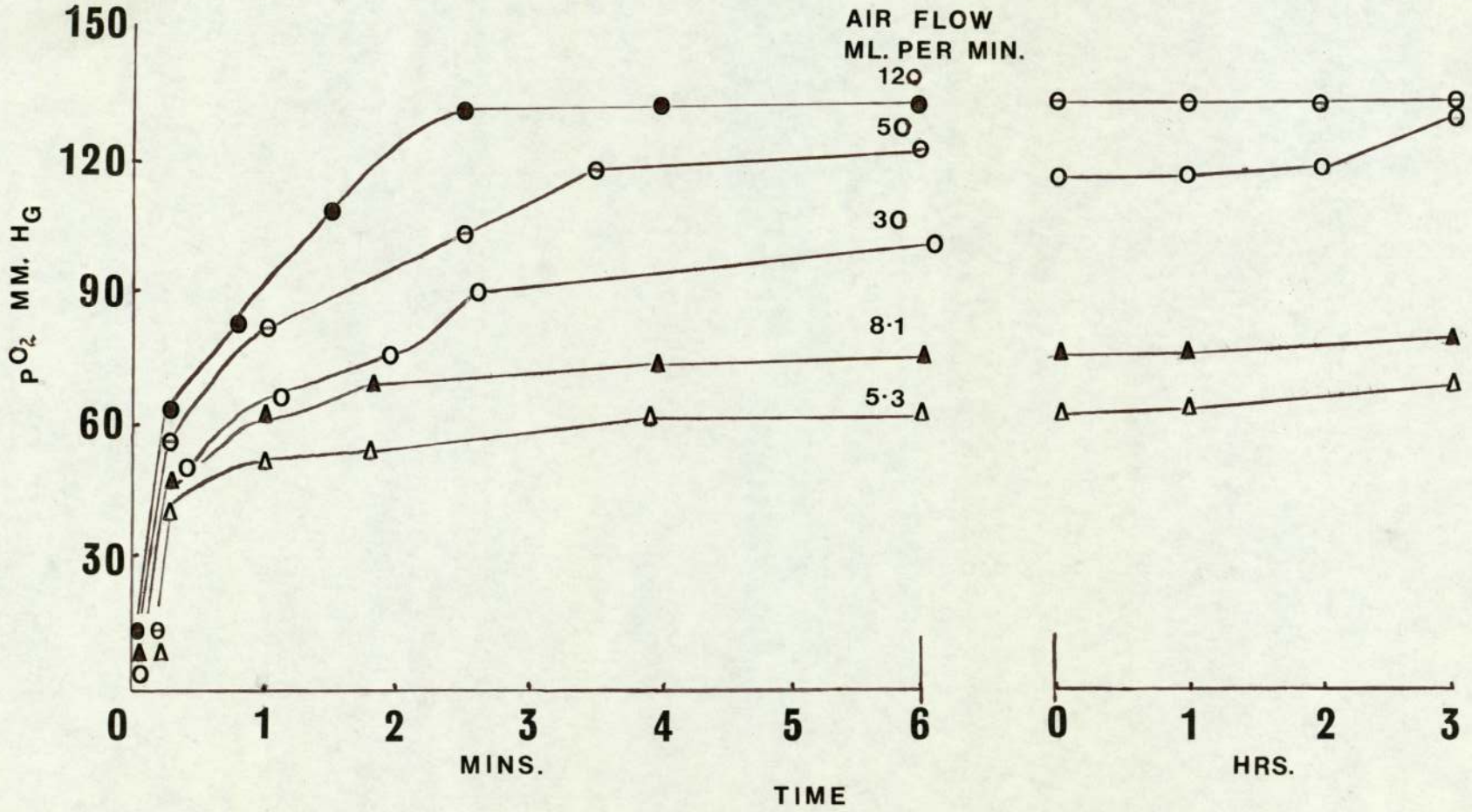


Fig. 38. pO_2 values obtained during increasing air flow.

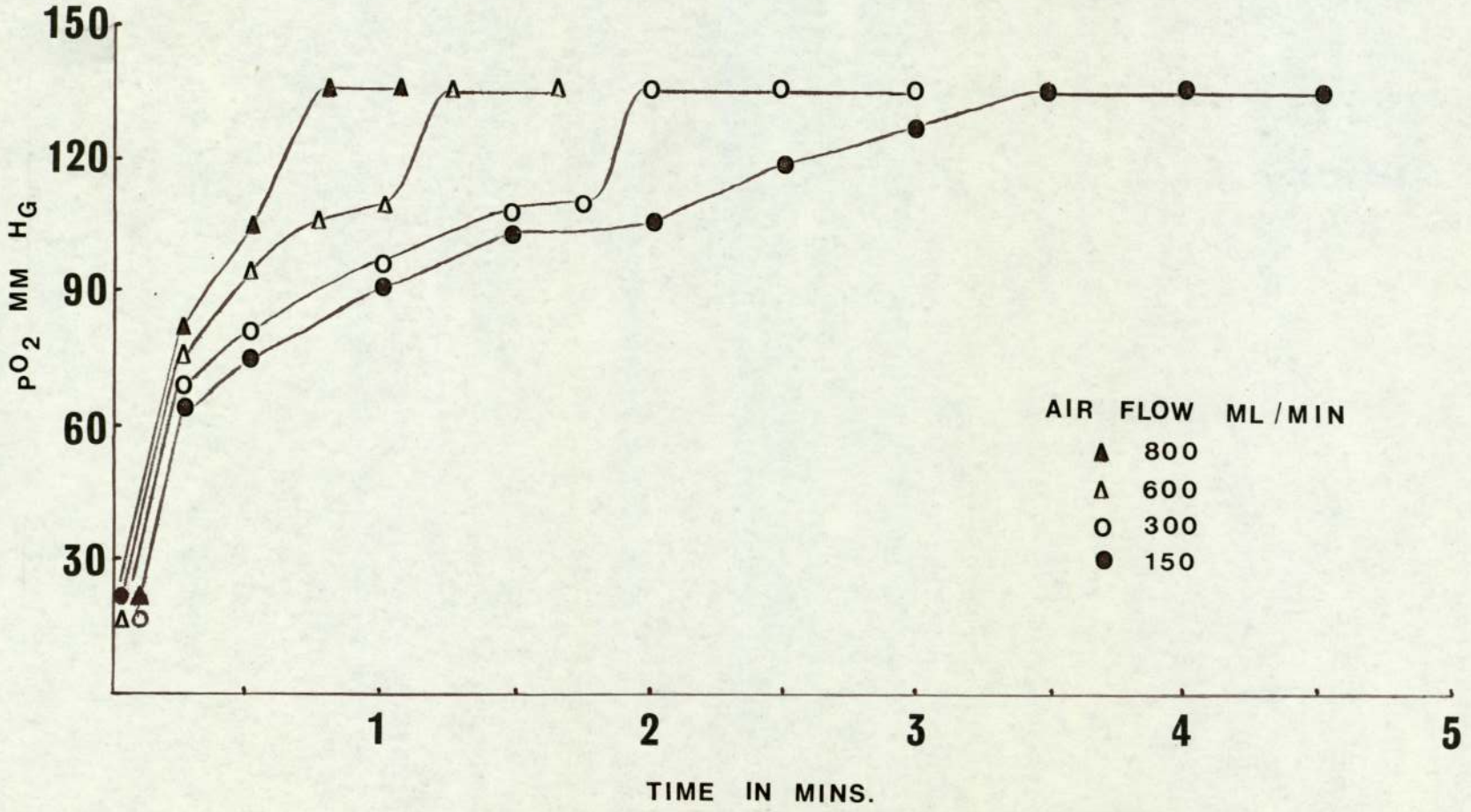


Fig. 39. pO_2 values obtained during increasing air flow.

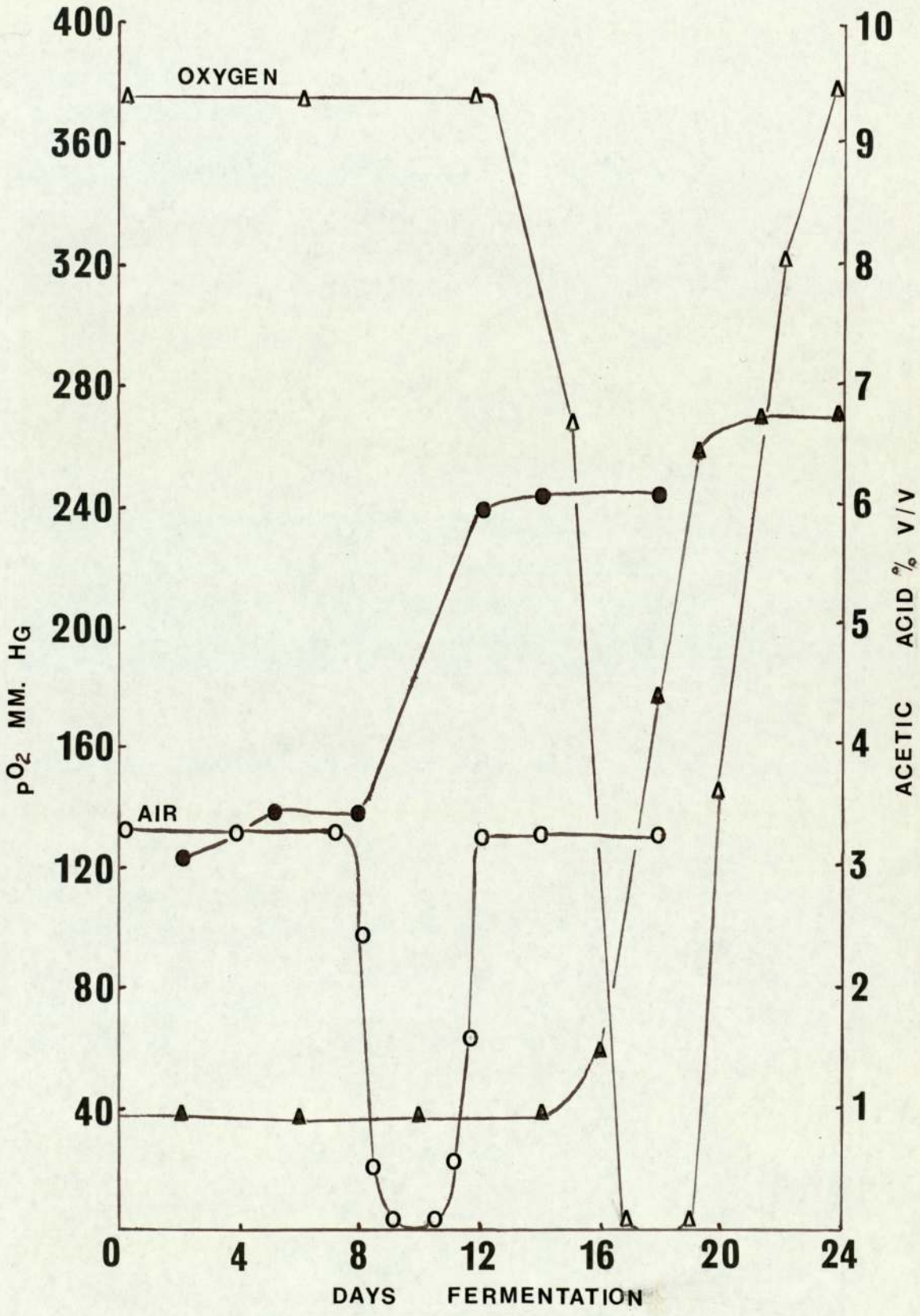


Fig. 40. pO_2 values during acetification in tower fermentors aerated with air and 4% CO_2 in O_2.

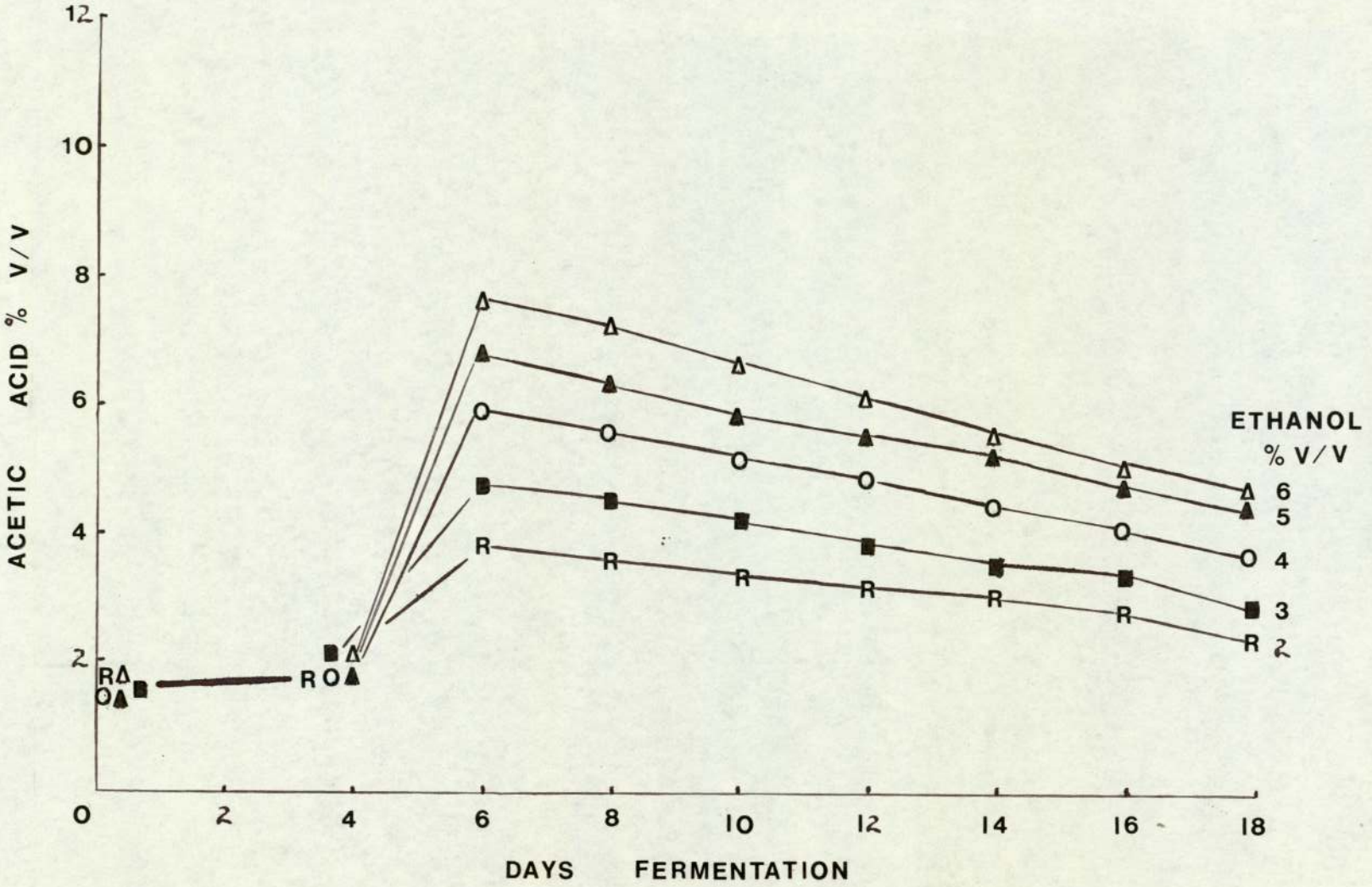


Fig. 41. Acetification in tower fermentors with increasing ethyl alcohol concentrations.

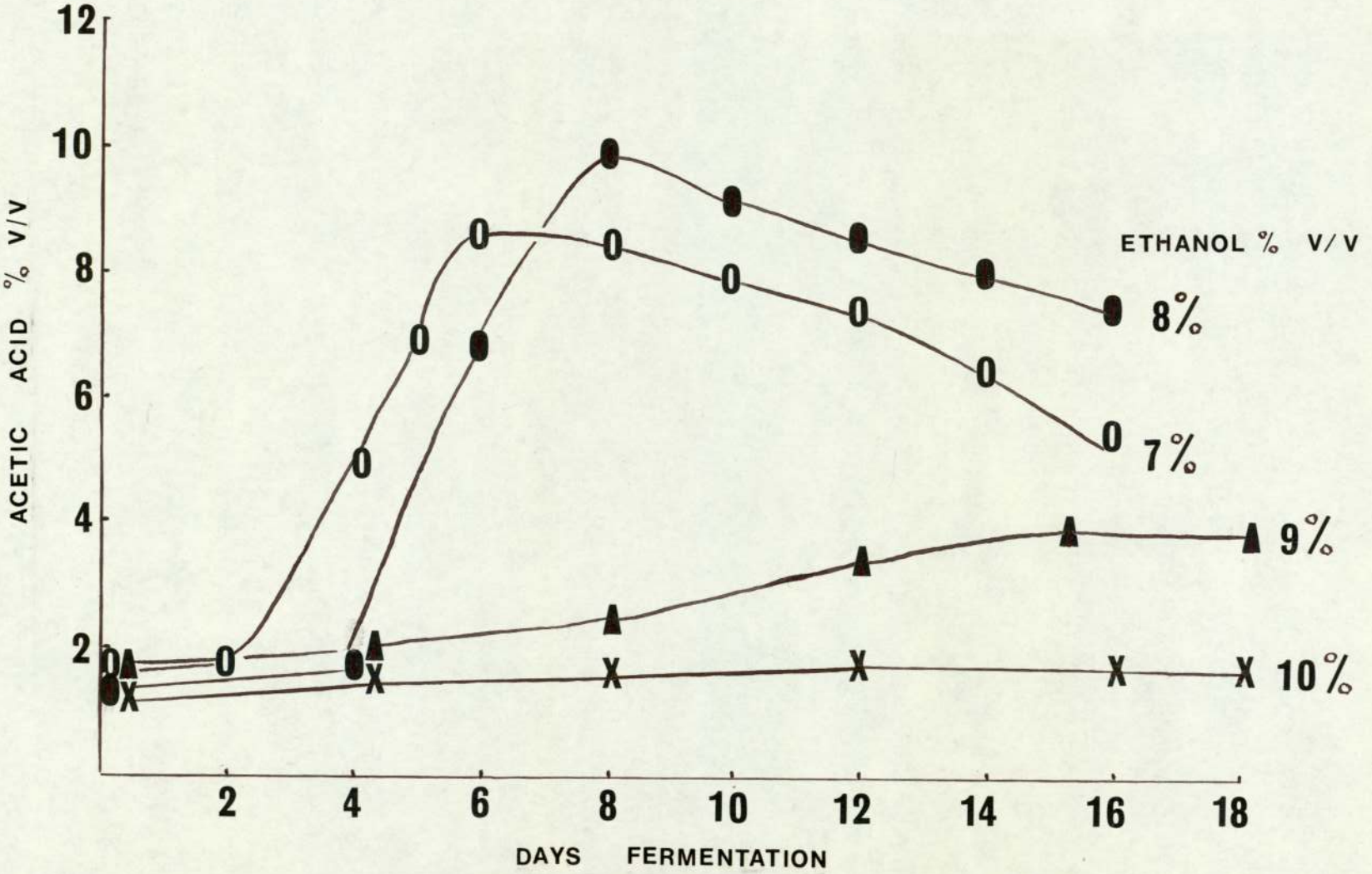


Fig. 42. Acetification in tower fermentors with increasing ethyl alcohol concentrations.

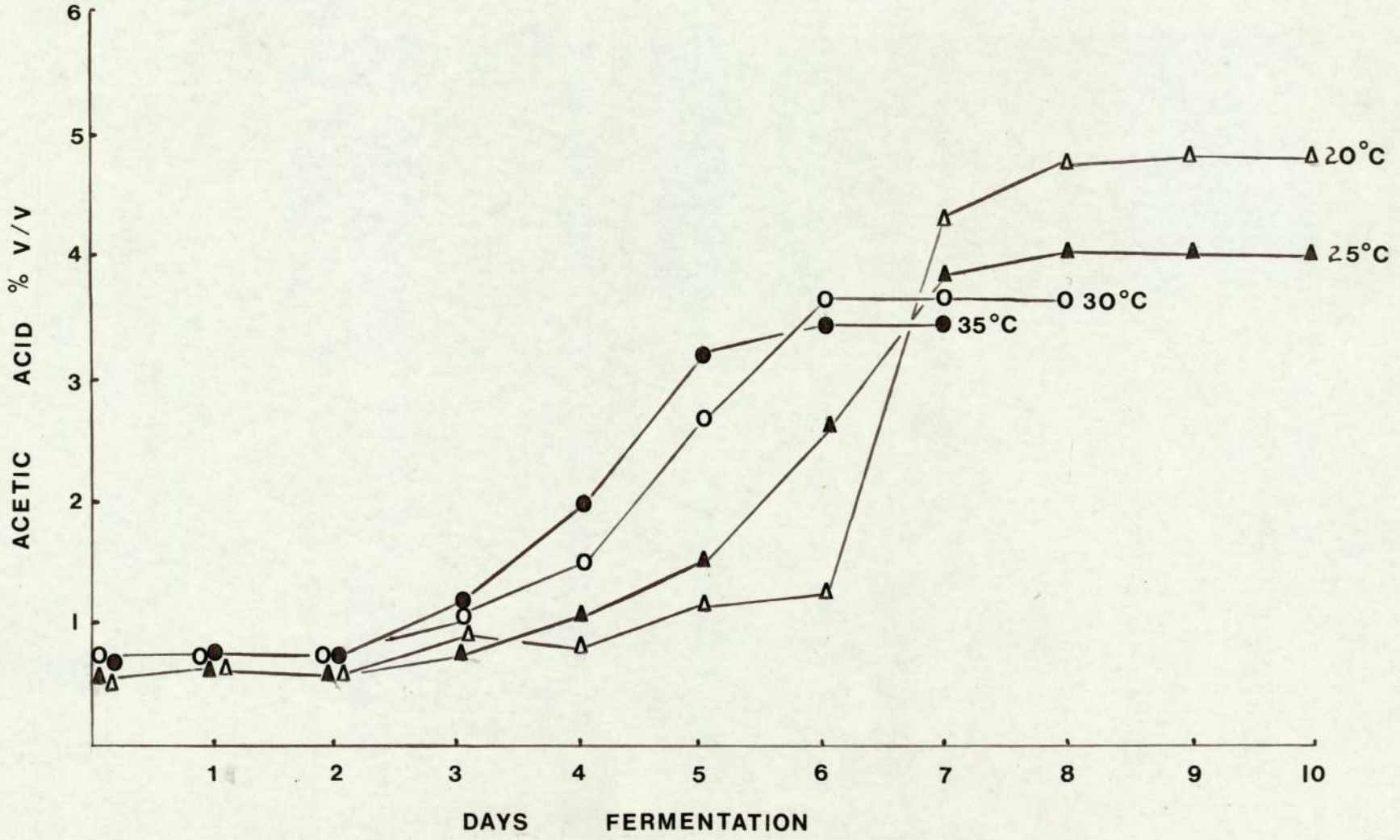


Fig. 43. Acetification in tower fermentors with increasing temperature.

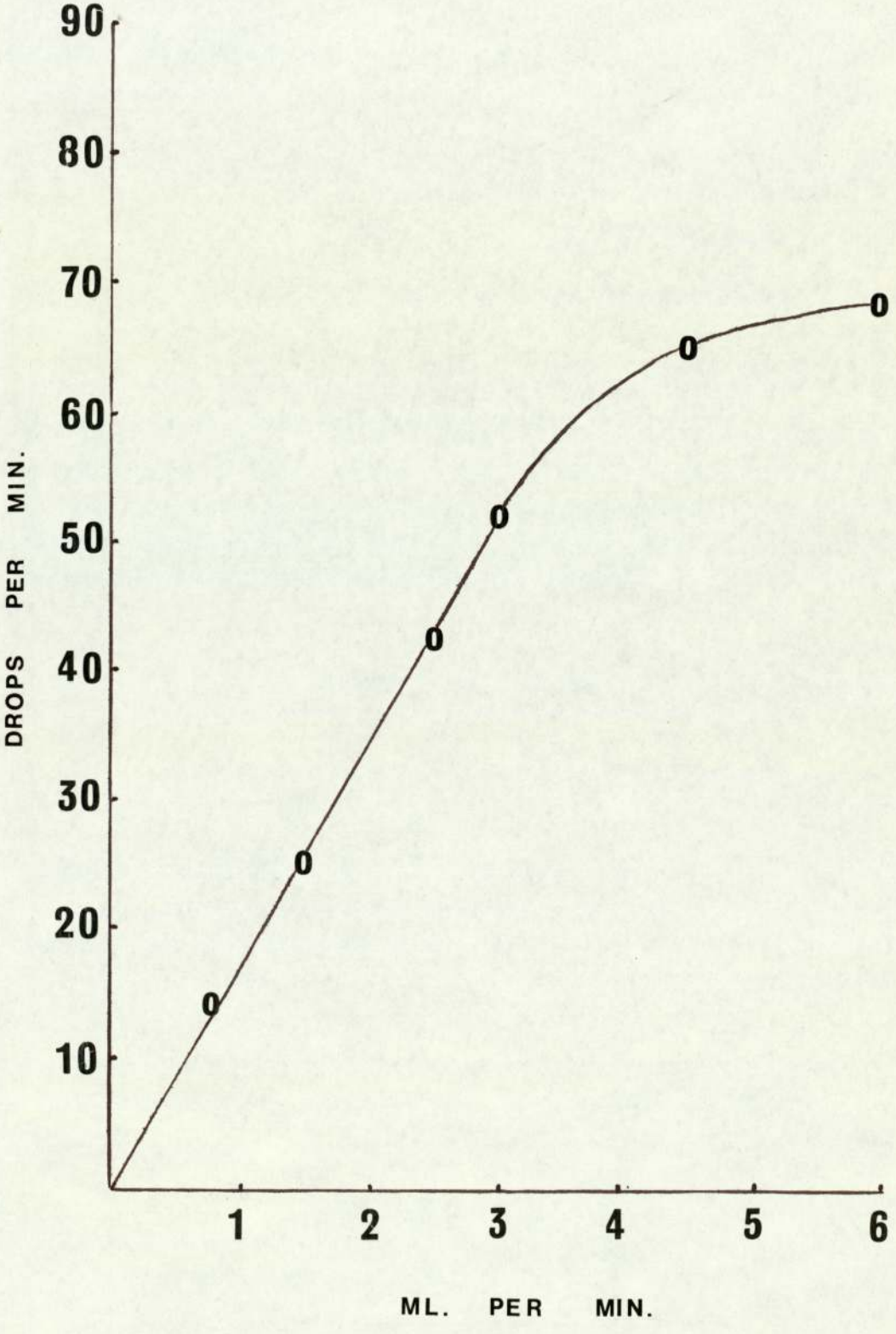


Fig. 44. Calibration of drip feed in ml. delivered per min.

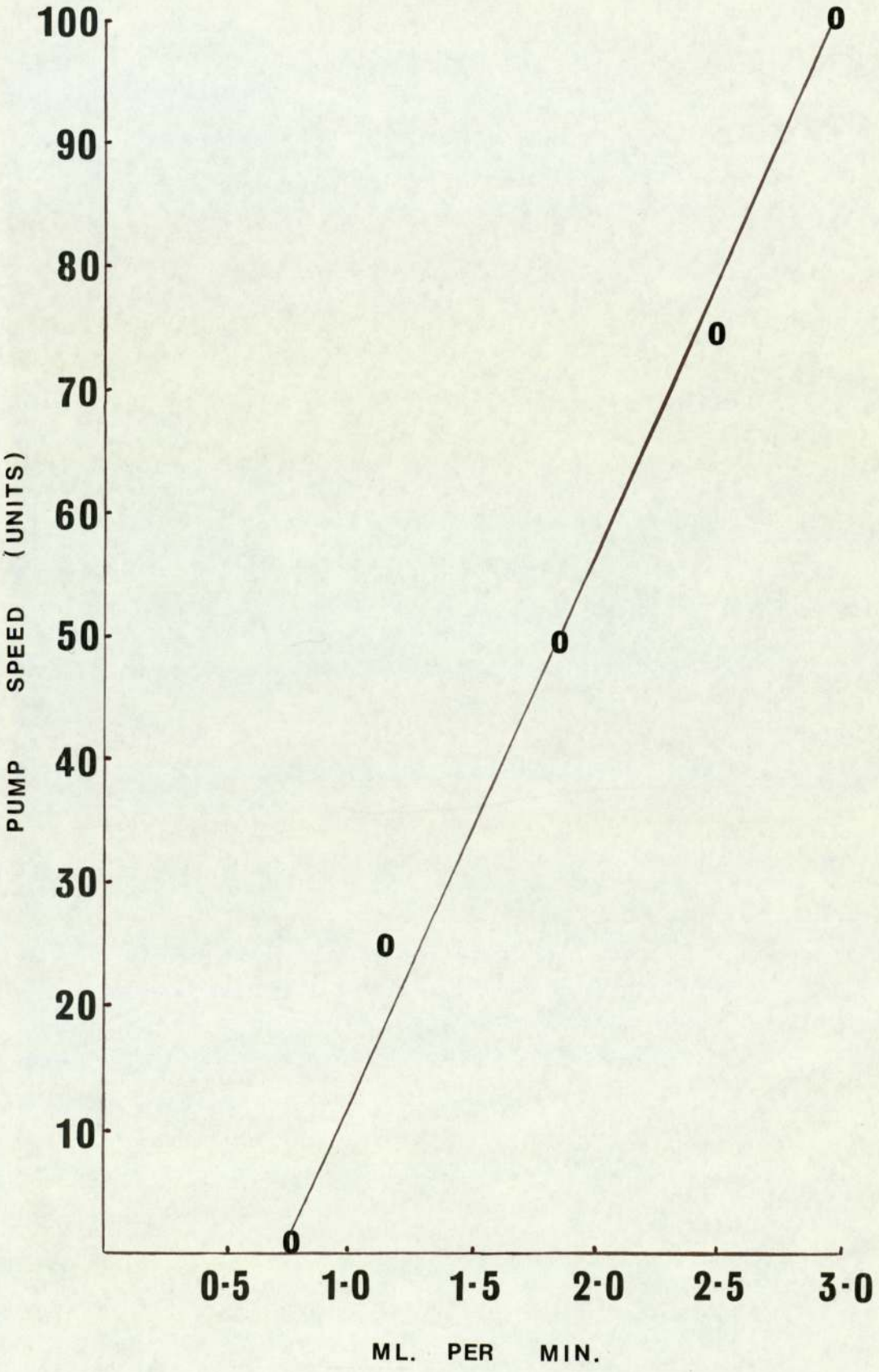


Fig. 45. Calibration of Watson Marlow Flow Inducer Pump.

The third method investigated was found to be accurate and reproducible. A Quickfit pump and Technicon Instruments pump tubing were used. Since two tower fermentors were subsequently used, both being dosed from the same pump, the pump speed was kept constant and the pump tube bores were varied. The calibration curve was linear (Fig.46) in the range tested from 0.025 mm. diameter to 0.11 mm. No fluctuations were detected over long periods of continuous fermentation and the error found over a period of five weeks was 0.01 ml. per minute.

(e) Total Counts. A calibration curve was constructed with the results obtained by reading the O.D. of acetobacter cultures and total counts estimated by means of a Coulter Counter. The Coulter Counter readings were taken as the mean of three determinations. A linear curve was not obtained. Total counts were then estimated by O.D. readings and the results read as thousands of millions per ml. Fig.47.

(f) Foam Control. Foam was not a major problem while using the flow rates found to be optimum in the previous experiments. In a few instances foam was controlled by the addition of 0.5 ml. Silicone M S Antifoam Emulsion.

(g) Position of Sampling Tube in Fermentor. The sampling tube was varied between the top and bottom of the tower fermentor to investigate any variation that may arise in results due to inefficient mixing. The results for ethanol, acid pO2 and turbidity estimations were similar whether the sample was taken at the top, centre or bottom of tower. This was evidence that there was efficient mixing throughout the fermentor.

(h) Oxygen Measurement of Exhaust Gas. The Servomex Oxygen Analyser was used for two purposes. In the first instance it was used to

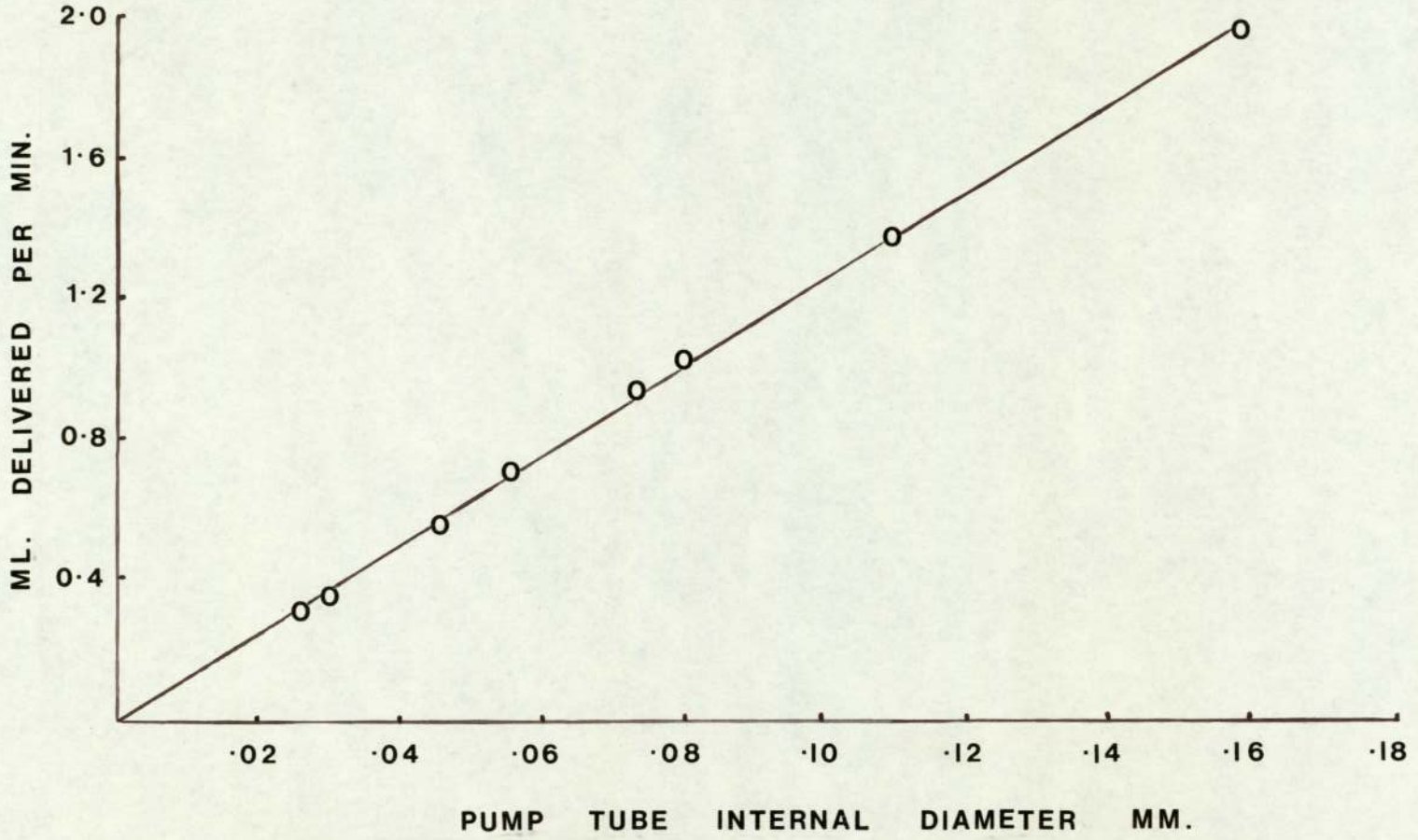


Fig. 46. Calibration of Quickfit Peristaltic Pump for delivery volume of 'charging wort' per minute, using tygon pump tubing.

estimate the quantity of carbon dioxide in the oxygen used. Secondly it was used to estimate the oxygen emitted at the top of the tower. The approximate 4% CO_2 in O_2 was found to range between 3.2% CO_2 and 4.4% CO_2 in eight cylinders tested. The analyser was calibrated in 1% divisions and it was found unsuitable for estimating the oxygen content of the exhaust gas. The oxygen in the exhaust gas would not be expected to decrease by more than 0.1%, at which concentration the analyser would be inaccurate.

(i) Efficiency of Tower Fermentation. To increase the efficiency of acetification a reflux condenser was fitted to the tower fermentor with a side arm to collect the distillate. Samples of the distillate were then subjected to GLC. The volume of the d istillate collected in 24 hrs. ranged between 3 ml. and 6 ml. Table 66 lists the concentration of volatiles detected in the distillate during eleven days acetification. The volatiles detected reflected the state of acetification of the tower. The ethanol concentration during the first day was 9.2% v/v decreasing to 100 mg. per 100 ml. after acetification. The acetic acid content increased during and after acetification. Diacetyl was detected until acetification. None of the esters or n-amyl alcohol were detected due to their higher boiling points. With the increase in propionic acid and acetoin during acetification there was an accompanying increase in the distillate concentration. Subsequent experiments were made with the side arm of the condenser removed so that the distillate was refluxed into the tower fermentor. The efficiency increased from approximately 75% to 97% conversion of ethanol by oxidation.

(2) Continuous Production of Malt Vinegar by Tower Fermentation.

(a) Efficiency. Two tower fermentors were used simultaneously one of which was aerated with air and the other with 4% CO_2 in O_2. Fig. 16 illustrates the complete apparatus used for continuous fermentation. The pO2, turbidity, total acid, and the dosing rate were monitored at 9 a.m. and at 5 p.m. daily. Fig. 47 illustrates the results obtained over a 10 week period. The initial dosing rate was adjusted to give an efficiency of 0.5. No further increase in dosing rate was made until the tower had the time to 'recover' i.e., until such time as the acid content increased after the initial fall.

The optical density and total acid was also estimated on the overflow acetified liquid. The O.D. was found to give an index of the amount of 'wash out' of bacterial cells from the towers. The object was to obtain count levels lower than that found in the tower.

Tower A (Fig. 47) was aerated by means of air, and acetification took place on the 4th day of fermentation at which point dosing began. With each increase in acid production there was a corresponding fall in pO2 and an increase in the total count. With each increase in acid the dosing rate was increased but during this period of ten days the turbidity of the overflow was higher than that found in the tower. During this time the charging wort was being pumped in at the base of the tower and the excess allowed to overflow. With the object of decreasing the 'wash out' of bacterial cells, the charging wort was pumped in at the top of the tower and an equal amount of acetified culture was removed at the base of the tower. Within the sampling area at the base of the tower was found a completely still area where the aeration had no mixing effect. The O.D. of the acetified culture immediately

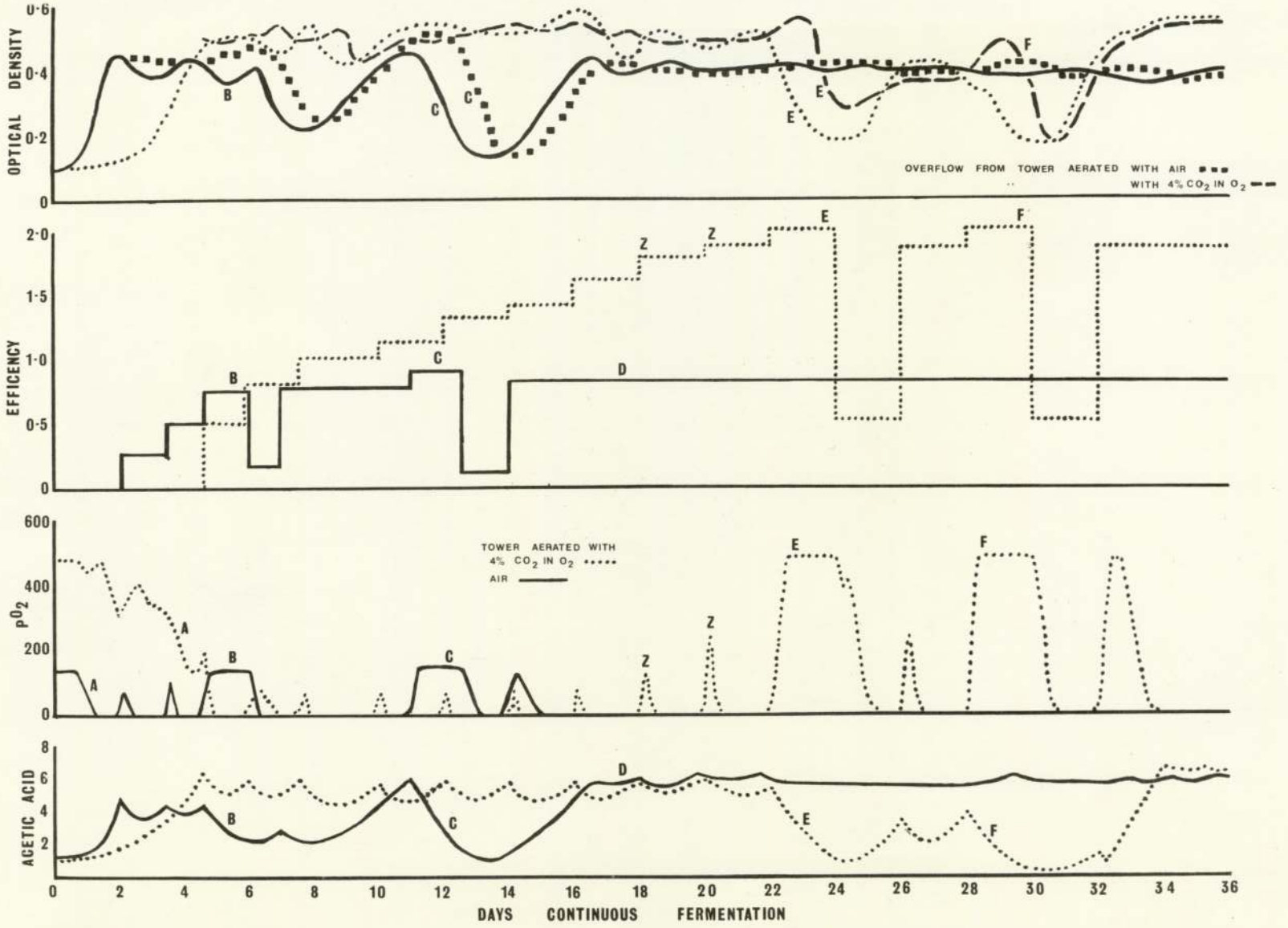


FIG. 47 PARAMETERS INVESTIGATED DURING THE FIRST MONTH OF A THREE MONTH PERIOD OF CONTINUOUS FERMENTATION

(ACETIC ACID G/100 ML., pO_2 MM H_G., EFFICENCY THROUGHPUT PER 24 HRS / VOLUME OF TOWER, OPTICAL DENSITY AT 540 NM)

KEY ON FOLLOWING PAGE

Key to Fig. 47.

A. Acetification during aeration with air was at 1.5 days and with 4 per cent CO_2 in O_2 4 days.

B. Sudden large increase in dosing rate giving increased pO_2 values with decreased acetic acid and optical density values.

C. Dosing rate increased to the point where bacterial cells were washed out from fermentor during which a continuous decrease was found in acetic acid and optical density readings. An increase was found in pO_2 value immediately when dosing rate was increased.

D. Optimum dosing rate obtained with a 24 hour efficiency of 0.8.

E. Wash out of bacterial cells from fermentor when 24 hour efficiency had reached 2.0 with a sudden increase in pO_2 value and a gradual decrease in optical density and acid concentrations.

F. Wash out of bacterial cells as in (E) when the 24 hour efficiency had reached 2.0. Optimum efficiency subsequently found to be 1.8.

Z. With increased dosing rates an immediate increase in pO_2 values without an accompanying decrease in either optical density or acid concentration.

decreased to similar values as found inside the tower. It was subsequently found that the O.D. decreased to a slightly lower figure than that found in the tower.

The pO2 value gave an index of Acetobacter growth. An increase was noted in the pO2 approximately 24 hrs. before a decrease was detectable in the total acid content. A similar decrease was noted in the total bacterial count before the total acid content decreased.

The maximum efficiency obtained with air was 0.8. Further increases in dosing rate immediately stopped acetification, and the O.D. of the acetified culture increased higher than found in the tower culture.

The results obtained for the oxygen tower were similar, in that the pO2 and O.D. changes were better indications than total acid estimations in following the course of acetification. The initial acetification with oxygen was delayed for 4 days while the acetification with air acetified in 2 days.

The maximum efficiency obtained with oxygen was 1.8 double that obtained with air. Although a greater delay occurred with the initial acetification using oxygen for aeration it was compensated when an efficiency of 1.8 was reached and could be maintained.

No foaming was found with the oxygen tower due in the main to the low flow rate of the gas.

Both towers were maintained with continuous flow for 10 weeks and the ultimate reasons for ceasing further acetification was due to the presence of vinegar eel and to the breakdown of the air pump.

(b) The Effect of Glycerol. Since glycerol actuated the growth of Acetobacter in batch culture, its effect was investigated during continuous culture. Glycerol was added to charging wort to give a final concentration of 200 mg. per 100 ml. The concentration was gradually increased in 100 mg. per 100 ml. steps to a concentration of 1000 mg. per 100 ml. The efficiency did not increase with the addition of glycerol, which suggests that other factors such as the oxygen concentration was the limiting factor. Improvement in acetification after the addition of glycerol in batch acetification was a metabolic effect rather than a physical effect.

(c) Solid Supports. A number of solid supports were added to the continuous tower fermentor with the object of increasing the efficiency. The solid support would act as an inert physical means of supporting the acetobacter during multiplication. Both polystyrene and Whatman's No.41 filter paper were cut into pieces of approximately 3 mm. x 3 mm. and 100 ml. volume added to the fermentors. The polystyrene floated to the surface and did not mix throughout the tower. The filter paper on the other hand sank ultimately to the bottom of the tower. No increase in efficiency was obtained with either support.

White absorbent gauze (B.P.C.) approximately 12" in diameter was wrapped around a glass tube of 2" diameter and the whole suspended in the fermentor so that the charging wort was aerated inside and outside the tube.

Filter paper was folded into the shape of a tube of 2" diameter and 12" in length and again suspended in the fermentor, so that complete aeration took place. Both gauze and filter paper

did not increase the efficiency of acetification, although acetobacter growth could be seen to adhere to both supports.

Finally beech twigs cut into lengths of approximately 4" long were placed in the tower fermentor with the object of increasing the efficiency as found in the Quick method of vinegar manufacture. No increased efficiency was found by this method.

(3) Discussion. The optimum substrate concentration was found to be approximately 7% v/v which is the concentration used at present for other methods of vinegar manufacture. The optimum temperature of 33°C. is the temperature normally used by vinegar manufacturers for the quick process.

Acetification occurred earlier with air than with oxygen, which confirmed unpublished work by Mr. Yates of the Manor Vinegar Co., Lichfield, who found similar results. During the use of oxygen the amount of gas dissolved in solution (or available to Acetobacter) was completely utilised. The results suggest that increased efficiencies over that found would only be obtained if the oxygen tension was increased. The pO2 parameter was found to be more sensitive that the total count, total acid, and ethyl alcohol estimations. By estimation of the pO2 value, the condition of the tower could be predicted at least 12 hours before hand. The total acid and ethanol estimations did not indicate such early changes. The wash out of bacterial cells could be quickly ascertained by turbidity measurements.

Glycerol had no effect upon the efficiency of continuous fermentation once the Acetobacter were in the log phase of growth. The advantage of glycerol seems to be to activate the organism when in the lag phase of growth.

During efficient aeration as was obtained in tower fermentors, inert solid supports for Acetobacter growth did not increase efficiency.

GENERAL DISCUSSION

GENERAL DISCUSSION

The examination of malt vinegar by gas chromatography has provided information as to the volatile acids, alcohols, esters and carbonyls present. The compounds detected during the survey included acetic, propionic and iso-butyric acids, ethyl, n-propyl, sec-butyl, iso-butyl, iso and n-amyl alcohols; iso-butyl, sec-butyl, ethyl and n-amyl acetates, ethyl formate, acetaldehyde and acetoin.

All the volatiles detected in malt vinegar in this investigation have been identified by Aurand et al. (1966) in 'distilled', 'grain', 'spirit' and 'natural' vinegars. Since vinegar manufacture consists of a double fermentation it would be expected that the alcohols would have been detected in beer because both during vinegar and beer manufacture entails an alcoholic fermentation by yeast. Hashimoto and Kuroiwa (1966), Powell and Brown (1966), Kunitake (1966), Morgan (1965) and Harold et al. (1961) all have detected the alcohols found in this work in beer.

Aurand et al. (1966) found qualitative differences amongst samples of vinegars and they suggested that the quality of a particular vinegar could be determined by the relative concentration of the alcohols and esters. The results of the survey of commercially available malt vinegars did not indicate possible standards of quality of a particular vinegar, although large differences in concentration of acetoin and propionic acid were obtained. Furthermore a number of malt vinegar manufacturer's employ more than one process of vinegar manufacture, the final product often being blended from separate batches obtained from two distinct processes. It was, therefore, difficult to compare the volatile concentrations found in vinegar

samples obtained from different manufacturers. Indeed later experiments demonstrated that both acetoin and propionic acid concentrations were found to vary with different processes.

During acetification of alcohol, the higher alcohols are partially converted to the corresponding acids and esters. It would therefore be expected that some of these chemical changes would be due partially or wholly to the acetification process employed, the storage period, the maturing process and pasteurisation.

With the object of clarifying the chemical changes, commercial samples were obtained during three distinct processes of malt vinegar manufacture. Samples were obtained during both the alcoholic fermentation and during acetification by the Quick, the Frings and a Continuous process.

The charging wort examined in all three processes contained the alcohols ethyl, n-propyl, iso-butyl and iso-amyl. The esters iso-butyl, sec-butyl and n-amyl acetate were produced simultaneously with the production of the corresponding alcohols. An important point to emerge was that acetic acid (0.4% v/v) was present throughout the yeast fermentation therefore it would be likely that esters would be formed at this stage of vinegar manufacture. Later experiments confirmed that this was the case contrary to the work of Suomalainen and Kangasperko (1963) who suggested that esters would be formed during conditioning and pasteurization. Hashimoto and Kuroiwa (1966) on the other hand, in their study of beer volatiles found that the formation of esters was complete by the end of the primary fermentation.

The charging wort obtained from the Frings process did not contain sec-butyl alcohol or n-amyl alcohol but during storage both

alcohols and the corresponding esters were detected. This would suggest a 'secondary' fermentation by yeast or bacteria. Ingraham et. al. (1960) reported the formation of n-amyl alcohol by a mutant strain of Saccharomyces cerevisiae and Genevois and Lafon (1957) suggested a series of reactions that could produce sec-butyl alcohol. Examination of the charging wort from the Continuous process did not reveal the presence of sec-butyl alcohol or its ester sec-butyl acetate. It is probable that the mutant strain of yeast or bacteria involved were not capable of synthesizing sec-butyl alcohol.

During acetification, acetaldehyde, acetic acid, and acetoin increased in concentration together with the formation of propionic acid and iso-butyric acids. Both acetoin and propionic acid concentrations were in lower concentration with increased aeration and therefore values were lower by the Continuous process than by the Quick and Fring processes. De Ley (1959) found that the addition of acetaldehyde to pyruvate under anaerobic conditions resulted in a considerable increase of acetoin. With increased aeration in the Continuous process the acetoin would therefore be in lower concentration.

No increase in the concentration or the number of volatiles was detected during storage of the samples from the three processes. A decrease was detected in the concentration of the lower boiling point volatiles. When sealed vinegar bottles were stored the decrease was detected after twelve months storage only. There appeared to be no change in the general characteristics of vinegar during the 'maturation' process. This confirmed the evidence gained from practical experience by Mr. M. Phillips, Head Brewer, Barbourne Brewery, Worcester.

The metabolic pathways involved during malt vinegar manufacture were investigated by the addition of known quantities of compounds

(which were likely to yield such information) to charging wort prior to acetification.

Tanaka (1938)[2] measured the oxygen uptake of resting cells of A. peroxydans, A. rancens and A. aceti with a variety of alcohols as substrates. It was found that the acetic acid bacteria oxidised ethyl alcohol most rapidly and n-propyl, n-butyl, n-amyl, iso-amyl, iso-butyl and sec-butyl in rates decreasing in that order. In the present study the % oxidation relative to ethanol was found to be dependent upon aeration and temperature. The oxidation of n-propyl alcohol decreased with increased aeration and iso-butyl alcohol oxidation increased with both increased aeration and temperature. Generally the alcohols were oxidised in decreasing order from primary to secondary alcohols and also as the carbon chains become longer.

Neuberg (1928) suggested that the oxidation of ethyl alcohol to acetic acid consisted of a dismutation reaction and Wieland (1913) proposed a pathway where acetaldehyde was oxidised to acetic acid. The acetaldehyde added prior to acetification was converted to acetic acid without a detectable increase in ethyl alcohol concentration. In the acetifications studies the high concentration of acetaldehyde inhibited acetification which suggests that a direct oxidation of ethyl alcohol was taking place.

Lactate was converted into acetoin giving an approximate yield of 84 per cent. An increase was also found in the propionic acid concentration. The conversion of lactate to acetoin confirms the work of Herman and Neuschul (1932) who obtained an yield of 34 per cent. Acetoin was also found to be increased by the addition of pyruvate, approximately 44 per cent yield was obtained. Acetobacter therefore can metabolize both lactate and pyruvate to acetoin.

The propionic acid was found to be produced from lactate, pyruvate, glycerol and during the oxidation of n-propyl alcohol. The yields obtained were 9.2 per cent, 15 per cent, 8 per cent and 84 per cent respectively.

n-Amyl alcohol and sec-butyl alcohol were detected only after storage of the charging wort obtained from the Frings and the Continuous Process, an attempt was made to examine the effect of mycoderma growth on fermented and unfermented wort. It was found that both alcohols were produced during the growth of mycoderma although the results did not indicate whether bacteria or a mutant strain of yeast was involved. A summary of the metabolic pathways as suggested by the results of this investigation are illustrated in Fig. 48.

During the last twenty years continuous flow fermentations have been developed Enenkel (1950), Shimwell (1955) and Greenshields (1968). The system used by Greenshields (1968) consists of a tower shaped fermentor and aerated at the lower end through a sintered glass filter. The whole of which was surrounded by a jacket for maintaining constant temperature. The advantage of this system over other continuous systems is that no physical means of mixing is required, the air in its passage through the tower would in itself ensure efficient mixing. Other advantages was the long residence time of the air in the fermentor and the higher concentration of organisms present.

The parameters for such a system were investigated which included optimum substrate concentration, temperature and aeration. With increased aeration and temperature the efficiency of ethanol oxidation decreased due to loss of the ethanol by evaporation. The evaporation

was overcome by fitting a reflux condenser at the top end of the fermentor, so that the distillate would return to the tower. The optimum substrate, temperature, aeration with air and aeration with 4 per cent CO_2 in O_2 were 7 per cent v/v ethanol, 33°C., 50 ml. per minute and 25 ml. per minute respectively. The efficiency of ethanol oxidation to acetic acid under optimum conditions was 97.4 per cent using air and 98.8 per cent with 4 per cent CO_2 in O_2.

The parameters investigated for the control of acetification in tower fermentors included total bacterial counts as determined by optical density measurements, total acid, and pO_2 measurements. The optical density measurements and total acid estimations did not indicate the viability of the acetobacter. On the other hand the pO2 values were indicative of changes in the fermentor at least twelve hours earlier than acid or optical density readings.

The total bacterial count results were of value when estimated on both the fermentor and the acetified liquor, to determine the degree of 'wash out' of bacterial cells. The optimum found for acetification was when the ratio of O.D. of the overflow to that found for the fermentor was one or less than one. The total bacterial count in the tower fermentor during aeration with air was approximately 1.5 thousand million per ml. and with 4 per cent CO_2 in O_2 2.4 thousand million per ml. with the greatly increased twenty four hour efficiency using 4 per cent CO_2 in O_2 for aeration it would be expected that the total bacterial count would also be greatly increased.

The maximum efficiency of fermentation as determined by the throughput of vinegar per twenty four hours was found to by 0.8 when aerated with air and 1.8 when aerated with 4 per cent CO_2 in O_2. The whole continuous fermentation in tower fermentors was found to be

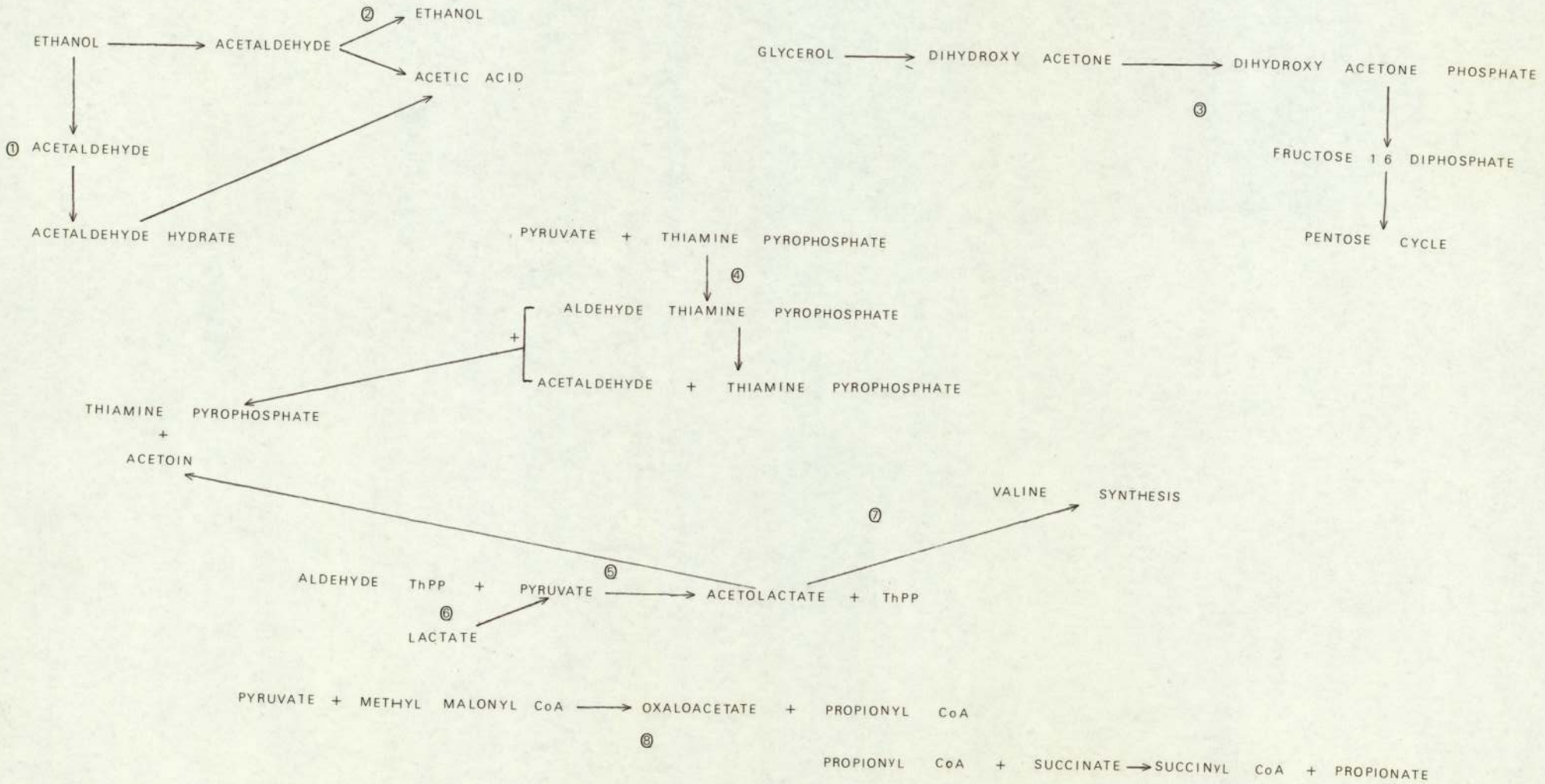


FIG 48 SUMMARY OF THE METABOLIC PATHWAYS FOUND IN ACETOBACTER. INDEX ON FOLLOWING PAGE

Index to Fig. 48.

(1) Direct oxidation of ethyl alcohol, Henneberg (1897)

(2) Oxidation of ethyl alcohol the dismutation reaction, Neuberg (1928)

(3) Oxidation of glycerol, King and Cheldelin (1952)

(4) Pathway of pyruvate to acetoin, Asai (1960)

(5) Pathway of pyruvate to acetoin, Asai (1960)

(6) Pathway of lactate to acetoin, De Ley (1960)

(7) Pathway of pyruvate to acetoin and valine synthesis, De Ley (1960)

(8) Pathway of pyruvate to propionic acid, Mahler and Cordes (1968)

governed by the amount of dissolved oxygen which was monitored by pO_2 estimations. Increased efficiences would only be obtained by increasing the oxygen dissolved in the acetifying solution. The maximum volume of dissolved oxygen during aeration with air was 0.51 ml. per 100 ml. and with 4 per cent CO_2 in O_2 1.8 ml. per 100 ml. of charging wort. During the log phase of growth, the acetobacter utilize completely the oxygen dissolved.

Aeration with 4 per cent CO_2 in O_2 at a rate of 25 ml. per minute would theoretically oxidize 1,300 ml. of 7 per cent v/v solution of ethyl alcohol in twenty four hours and aeration with air at a rate of 50 ml. per min. would oxidize a similar quantity of ethyl alcohol in 2-3 days. The results obtained were similar to the theoretical values calculated in that the oxygen fermentor would theoretically be 1.84 times as efficient as the fermentor aerated with air and during this investigation it was found to be 1.8 times efficient. These efficiencies were obtained with 98.9 per cent oxidation of ethanol to acetic acid.

The values for dilution rate (D), maximum growth rate (u max) and the steady state (S) were calculated at intervals during the continuous fermentation. For these calculations the specific growth rate(u) was assumed to be equal to u max, since the substrate concentration was in sufficiently high concentration Malek and Fencl,(1966).

During continuous fermentation at maximum efficiency with air for aeration D was found to be 0.0332 $hour^{-1}$ and u max to vary between - 0.1178 and + 0.1178 and the steady rate between - 0.1510 and + 0.085. During aeration with 4 per cent CO_2 in O_2 D was found to

be 0.075 hour^{-1} and μ max to vary between - 0.0488 and + 0.0488; the steady state varied between + 0.0488 and + 0.262. Tempest et. al. (1967) found that most organisms growing at their optimum temperature and pH gave values for μ max of less than 0.05. Aeration with air gave μ max results outside this limit whereas aeration with 4 per cent CO_2 in O_2 gave results for μ max within this limit. This would not be surprising since the larger volume of air used for aeration, than that used for 4 per cent CO_2 in O_2 would not be conducive with good steady state conditions.

At points E. F, B. and C. in Fig. 47 a gross 'wash out' of bacterial cells was detected. The dilution rates at these points were 0.0832, 0.082, 0.0312, and 0.037 hour^{-1} respectively. Large values were obtained for μ max and were - 0.5108, - 0.8109, - 0.1866 and 0.8708 respectively. At these points the maximum growth rate had been exceeded and the bacterial population would diminish.

Efficiencies of present commercial processes used for the manufacture of malt vinegar are difficult to obtain, but the results obtained during this investigation with tower fermentors are sufficiently encouraging to recommend them for commercial production of malt vinegar.

The present work has investigated the volatiles found during malt vinegar manufacture both on a laboratory and commercial scale. A number of other investigations are seen to be possible for future work on malt vinegar. Volatiles present in micro quantities and fractionated by other stationary phases and the enzymes involved during the alcoholic fermentation and subsequent acetification could be investigated. Acetification in tower fermentors on an industrial pilot scale and increased oxygen tension has much to recommend them in any future work of this type.

APPENDIX

VOLATILE	RETENTION TIME	PEAK WIDTH	EFFICIENCY
	cm.	mm.	
Acetaldehyde	1.0	2.5	2.5
Ethyl alcohol	1.6	7.0	0.9
Acetic Acid	3.0	8.5	1.9
n-Propyl alcohol	5.0	10.0	4.0
Propionic acid	6.5	12.0	4.6
iso-Butyl alcohol	7.0	13.0	4.6
iso-Butyric acid	10.6	13.0	10.4
Acetoin	12.6	19.5	6.4
iso-Amyl alcohol	15.6	26.0	5.7
iso-Butyl acetate	18.6	34.0	4.6
n-Amyl alcohol	22.8	45.0	4.0
sec-Butyl acetate	24.1	52.0	3.4

Table 1. Column efficiency for the volatiles detected in malt vinegar.

Compound	Concentration (% v/v)	Retention Time (m.)
Acetaldehyde	0.05	1.0
Ethyl Alcohol	0.2	1.6
Acetic acid	0.2	3.0
n-Propyl alcohol	0.08	5.0
Propionic acid	0.1	6.7
iso-Butyl alcohol	0.1	7.0
iso-Butyric acid	0.1	10.6
Acetoin	0.2	12.6
iso-Amyl alcohol	0.1	15.6
iso-Butyl acetate	0.1	18.6
n-Amyl alcohol	0.1	22.8
sec-Butyl acetate	0.1	24.1

TABLE 2. Concentrations and retention times for reference compounds

Volatile	$\bar{x}$ mean of 10 determinations	S_D	$S_D/\sqrt{N}$
Methyl formate	12.3	0.4986	0.1577
Acetaldehyde	9.66	0.3868	0.1223
Ethyl acetate	6.0	0.1547	0.0489
Ethyl alcohol	30.1	1.7960	0.5679
iso-Butyl acetate	3.7	0.4230	0.1340
n-Propyl alcohol	2.4	1.0720	0.3390
n-Butyl acetate	7.1	0.4230	0.1331
Amyl alcohol	5.65	0.1870	0.0630
Acetoin	370.0	22.4	7.10

Table 3. Reproducability tests for the volatiles estimated

Vinegar No.	Ethyl Alcohol by GLC mg/100ml.	Total Alcohol by GLC mg/100ml.	Ethyl Alcohol by Conway Diffusion mg/100ml.
1	38.7	66.2	64.0
2	53.3	68.7	72.0
3	40.3	56.9	54.0
4	172.8	195.4	200.0
5	119.5	134.5	135.0
6	84.9	100.4	105.0
7	28.8	54.91	56.0
8	50.4	68.31	70.6
9	74.9	92.4	97.0
10	30.2	52.8	53.0
11	67.6	65.5	85.0
Mean ($\bar{x}$)	69.2	86.9	90.1

Table 13. Results obtained for the estimation of ethyl alcohol by GLC and Conway diffusion methods.

COMPOUND ADDED	FINAL CONCENTRATION ADDED TO SURFACE CULTURE AND TOWER FERMENTOR. mg. per 100 ml.
Glucose	2,000
Acetaldehyde	1,000
Glycerol	2,000
iso-Butyl alcohol	2,000
Lactic acid	2,000, 500, 250
iso-Amyl alcohol	250
Pyruvic acid	2,000, 500, 200
n-Propyl alcohol	50
sec-Butyl alcohol	200
n-Amyl alcohol	200

Table 14. Concentrations of compounds added to charging wort during laboratory acetifications.

Peak Retention Time	Retention after Treatment with NaOH	Retention time after treatment with potassium permanganate
6	-	6.5 (ester)
9	9	9
12	-	12 (ester)
16.5	16.5	16.5
23	-	-
25	-	-
29	28	-
33	33	35
68	70	70
115	132	- (aldehyde)

TABLE 15. Results after the addition of potassium permanganate and sodium hydroxide to vinegar samples

TABLE 16. Concentrations used for standards and the volatile retention times obtained.

Compound	Concentration	Peak Retention Times
Ethyl alcohol	1%	7, 8.5, 17*, 30, 32.5, 39, 53, 118
Methyl alcohol	0.2%	7, 13.5*
Amyl alcohol	neat	10, 12, 17, 25, 29, 33, 42, 62*
Pentan-2-ol	neat	10, 12.5, 15, 17, 25, 30, 36*, 45.5, 61
Butan-2-ol	neat	8, 11, 12, 14, 16, 18, 22*, 30, 37, 48, 63
Iso-butyl alcohol	neat	11, 15, 19, 21, 24, 33* 43, 64
Butan-1-ol	neat	15, 22, 29, 40, 48*, 66, 78, 87
Acetaldehyde	neat	7*, 14, 33, 39, 46
Acetoin	0.4%	6, 8, 15, 26, 32, 40, 47, 67, 115*
Butyl methyl ketane	0.1%	6, 10, 14, 17, 21*, 28, 31
Diacetyl	0.05%	17*
Ethyl acetate	2%	10.5*, 18
N-amyl acetate	0.1%	6, 17, 27, 32, 41, 47*, 85

Continued overleaf.

TABLE 16. continued

Compound	Concentration	Peak Retention Times
Iso-butyl acetate	0.1%	17, 20*, 27, 31, 47.5
N-propyl alcohol	0.1%	14, 15.5, 20, 22.5, 24*, 31
Ethyl n-butyrate	0.1%	7, 11, 15, 18, 20, 22.5*, 32
Ethyl formate	0.1%	4.5, 6.5, 9*, 13.5, 22.5
Methyl formate	0.1 %	6*, 10.5, 14, 16, 22.5, 33
N-butyl acetate	0.1%	6, 7.5, 11, 15, 17, 22, 28*, 38, 64

* Retention time for actual compound

Peak Number	Retention time (mm.)	Volatile
1	6	ethyl formate
2	9	acetaldehyde
3	11.5	ethyl acetate
4	16	ethyl alcohol
5	22	isobutyl acetate
6	25	n-propyl alcohol
7	29.5	n-butyl acetate
8	31.5	effect of water and
	33.5	isobutyl alcohol
9	69	amyl alcohol
10	114	acetoin

TABLE 17. Retention Times of Identified Volatiles with PEG 1000 As Stationary Phase

VINEGAR NUMBER	RETENTION TIMES m.m.												
1	12	17	29	38	64	75	114	124	162	180	189	216	237
2	10	17	29	38	65	78	-	124	160	181	192	-	-
3	12	17	29	38	64	78	-	122	164	184	-	-	-
4	12	18	29	38	63	76	-	124	162	181	193	-	-
5	12	18	29	38	64	76	-	127	162	181	192	-	-
6	12	18	29	38	64	75	-	124	162	181	192	-	-
7	12	18	29	38	64	75	-	125	164	181	192	-	-
8	12	18	29	38	64	77	-	127	160	181	188	197	-
9	12	18	29	38	63	77	117	125	160	184	192	199	-
10	12	18	29	38	63	76	116	125	160	181	191	204	-
11	12	18	29	38	65	76	-	125	160	181	191	-	-
Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13

TABLE 18.

Retention times of volatiles detected with Porapak Q.

Peak 1, acetaldehyde; 2, ethyl alcohol; 3, acetic acid; 4, n-propyl alcohol; 5, propionic acid and sec-butyl alcohol; 6, iso-butyl alcohol; 7, iso-butyl acid; 8, acetoin; 9, iso-amyl alcohol; 10, iso-butyl acetate; 11, n-amyl alcohol; 12, sec-butyl acetate; 13, n-amyl acetate.

Compound	Log Rv	B.P.°C.
Ethyl formate	0.7782	34
Acetaldehyde	0.9542	20.2
Ethyl acetate	I.0607	77
Ethyl alcohol	I.2041	78.4
iso-butyl acetate	I.3424	118
n-propyl alcohol	I.3979	98
n-butyl acetate	I.5119	108
amyl alcohol	I.8388	130
acetoin	2.0569	144

TABLE 19. Log Rv and boiling points of the volatiles detected.

TABLE 20

CONCENTRATION OF VOLATILES IN ELEVEN VINEGAR SAMPLES USING PEG 1000
(mg.per 100 ml).

Compound	t_R per (mon.)	V_R(ml.) per min.)	Vinegar number 1	2	3	4	5	6	7	8	9	10	11	S.D.	% Recovery
Ethyl formate	6	27	3.26	18.52	10.89	9.0	15.67	10.89	5.62	18.47	10.62	18.54	9.0	0.4986	97.4-104.8
Acetaldehyde	9	40.5	30.18	4.21	4.21	6.18	17.55	16.66	14.04	17.55	12.28	17.55	114	0.3868	96.7-401.8
Ethyl acetate	11.5	517.5	0.98	8.32	-	8.51	5.0	6.88	9.72	12.96	6.84	-	5.26	0.1547	88.8-97.3
Ethyl alcohol	16	720	38.7	53.3	40.3	172.8	119.5	84.9	28.8	50.4	74.9	30.2	67.7	1.786	87.3-108
Iso-butyl acetate	22	990	0.25	0.99	0.71	1.98	1.42	1.98	0.71	4.03	0.71	2.0	2.0	0.423	89.8-117
n-Propyl alcohol	25	1.125	3.95	0.99	4.93	2.95	2.95	2.46	2.46	2.46	2.95	2.95	19.7	1.072	104.5-122
n-Butyl alcohol	29.5	1.327	0.4	3.56	0.4	7.17	3.56	6.72	1.35	16.63	6.72	3.56	0.79	0.423	100.6-122
Isobutyl alcohol and/or effect of water	32.5	1.462	-	-	-	-	-	-	-	-	-	-	-	-	-
n-Amyl alcohol	69	3.115	4.86	3.65	5.5	4.54	4.86	3.65	9.04	6.80	3.56	6.48	6.8	0.187	96.2-108.5
Acetoin	114	5.13	190	20	57	370	690	770	520	940	450	550	970	0.024	87.5-115

TABLE 21

CONCENTRATION OF VOLATILES IN VINEGAR USING PORAPAK Q (mg/100 ml).

Compound	t_R (min.)	V_R	Vinegar number 1	2	3	4	5	6	7	8	9	10	11
Acetaldehyde	12	480	30.42	4.46	4.50	6.0	17.0	16.64	13.2	17.75	12.0	16.82	106
Ethyl alcohol	17	680	42.0	50.3	42.4	185.7	121.4	84	26.2	52.0	75.0	29.8	74.4
Acetic acid (g/100ml.)	29	1160	5.4	5.1	5.1	5.25	5.2	5.1	5.5	5.2	5.1	5.1	5.1
n-Propyl alcohol	38	1520	3.22	0.41	4.27	2.40	2.30	2.20	2.71	2.21	2.00	2.70	2.20
Propionic acid (g./100 ml.)	64	2560	1.20	2.50	0.10	0.21	0.15	0.50	0.14	0.14	0.15	0.14	0.16
sec-Butyl alcohol	64	2560	6.21	8.47	2.50	11.10	4.62	5.11	4.62	5.20	2.55	2.55	3.0
Isobutyl alcohol	74	2960	8.40	2.19	3.18	2.70	2.30	3.81	8.46	3.00	8.46	8.21	4.2
Isobutyric acid	116	4640	1.25	-	-	-	-	-	-	-	5.0	4.62	-
Acetoin	124	4960	198	18.4	58.2	374	710	765	524	940	440	540	960
Iso-amyl alcohol	160	6400	1.42	0.92	1.4	1.82	0.91	0.87	1.4	1.4	0.84	3.2	2.4
Isobutyl acetate	180	7200	0.25	1.10	0.74	1.90	1.32	1.88	0.80	4.0	0.81	2.1	1.98
n-Amyl alcohol	192	7680	4.92	3.41	5.32	4.68	4.86	3.54	8.92	6.1	3.62	6.0	6.1
sec-Butyl acetate	205	8200	5.84	-	-	-	-	-	-	6.71	5.42	5.56	-
n-Amyl acetate	237	9480	5.2	-	-	-	-	-	-	-	-	-	-

Vinegar number	Ethyl Alcohol by GLC (mg. per 100ml.)	Total Alcohol by GLC (mg. per 100 ml.)	Ethyl Alcohol by Conway diffusion (mg. per 100 ml.)
1	38.7	66.17	64.0
2	53.3	68.7	72.0
3	40.3	56.9	54.0
4	172.8	195.4	200.0
5	119.5	134.5	135.0
6	84.9	100.4	105.0
7	28.8	54.91	56.0
8	50.4	68.31	70.6
9	74.9	92.4	97.0
10	30.2	52.8	53.0
11	67.6	65.5	85.0

t = 10.0

P = 0.05

TABLE 22. Results Obtained for the Estimation of Ethyl Alcohol by GLC and Conway Diffusion

Sample	Values given by Manufacturer	Values by G.L.C.
1. Separated wort	6.5	6.45
2. Acetifier 3	2.2	2.3
3. Acetifier 15	0.48	0.78
4. Acetifier 4	2.7	2.48
5. Charging wort	5.8	5.76
6. Acetifier 14	0.49	0.8
7. Acetifier 10	'low'	0.043
t	1.42	
P	0.2	

TABLE 23. Ethanol estimations by G.L.C. compared to estimations made by manufacturer (g/100ml.).

VOLATILE	UNITS	UNOPENED BOTTLE DAYS STORED						OPENED BOTTLE DAYS STORED				
		0	35	37	41	48	57	0	2	6	13	22
Acetaldehyde	mg/100 ml	15.0	15.0	14.0	13.2	14.2	14.8	15.0	14.0	-	-	-
Ethyl alcohol	"	165	185	160	160	165	160	160	150	120	80	32
Acetic acid	g/100ml	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.4	4.0	3.8	3.4
n-Propyl alcohol	mg/ 100ml	1.8	1.9	1.9	1.9	1.8	1.8	1.5	1.4	1.4	0.3	0.2
Propionic acid	"	125	128	125	125	129	125	125	120	120	120	122
sec-Butyl alcohol	"	2.4	2.4	2.0	1.8	1.8	2.0	2.4	2.4	1.6	1.4	1.0
iso-Butyl alcohol	"	3.0	3.0	3.2	3.0	3.0	3.0	3.0	3.0	3.0	3.8	2.8
iso-Butyric acid	"	3.5	3.1	3.8	3.8	3.2	3.2	3.8	3.1	2.8	2.8	2.6
Acetoin	"	200	210	208	210	210	210	200	215	208	200	184
is o-Amyl alcohol	"	0.4	0.6	0.6	0.6	0.4	0.6	0.5	0.6	0.6	0.4	0.2
iso-Butyl acetate	"	1.5	1.5	1.8	1.5	1.5	1.8	1.8	1.6	1.6	1.8	1.6
n-Amyl alcohol	"	4.8	4.6	4.6	4.2	4.8	4.5	5.0	4.5	4.4	4.6	4.4
sec-Butyl acetate	"	8.2	7.8	8.4	8.2	8.2	8.6	8.8	8.8	8.0	8.0	7.7
n-Amyl acetate	"	-	-	-	-	-	-	-	-	-	-	-

TABLE 24. Volatiles detected in two bottles of finished vinegar, one of which was not sealed.

Volatile	Units	AGE OF VINEGAR		
		4 months	6 months	12 months
Acetaldehyde	mg/100 ml.	20.8	18.5	12.7
Ethyl alcohol	"	182	172	162
Acetic acid	g/100 ml.	5.0	5.0	4.8
n-Propyl alcohol	mg/100 ml.	2.0	4.2	1.6
Propionic acid	"	146	123	115
sec-Butyl alcohol	"	4.4	5.0	5.0
iso-Butyl alcohol	"	3.8	4.2	2.1
iso-Butyric acid	"	7.4	7.4	5.0
Acetoin	"	160	150	154
iso-Amyl alcohol	"	1.4	1.5	1.2
iso-Butyl acetate	"	1.5	1.3	1.2
n-Amyl alcohol	"	3.8	3.8	3.6
sec-Butyl acetate	"	6.0	4.4	4.4
n-Amyl acetate	"	+	+	+

TABLE 25. Volatiles detected in aged vinegars obtained from manufacturer

VOLATILE	Units	DAYS 0	2	3	4	5	6	7	8	9	10	11	12	15
Acetaldehyde	mg/100ml	2.0	14.0	23.4	36.6	39.1	42.0	36.4	6.0	6.0	3.0	2.9	2.0	2.0
Ethyl alcohol v/v	~~g/100ml~~	8.75	7.0	5.3	5.2	4.6	2.0	1.3	014	0.2	0.14	0.12	.09	.01
Acetic acid v/v	~~g/100ml~~	0.4	1.5	2.4	3.35	5.3	7.5	9.0	9.1	9.1	9.0	8.9	8.8	8.8
n-propyl alcohol	mg/100ml	1.8	1.6	0.9	1.2	1.0	0.9	0.8	0.9	0.7	0.7	0.8	0.7	0.7
propionic acid	"	32	200	360	360	336	88	88	40	32	32	32	32	32
sec-butyl alcohol	"	1.4	1.6	1.6	1.6	1.4	1.8	1.4	1.4	1.6	1.4	1.4	1.4	1.4
iso-butyl alcohol	"	15.2	13.4	13.2	10.4	10.0	14.0	13.0	13.0	13.5	13.1	12.6	13.2	13.2
iso-butyric acid	"	-	-	-	-	-	2.0	1.2	1.2	1.2	2.0	2.2	2.4	2.2
Acetoin	"	3	76	152	206	232	246	246	246	224	206	146	130	106
iso-amyl alcohol	"	20	18	18.2	18.4	18.0	18.0	16.4	16.6	14.0	12.0	10.2	10.4	11.0
iso-butyl acetate	"	2.8	3.0	2.6	2.6	2.9	2.9	2.6	2.6	2.6	2.6	2.6	2.8	2.4
n-amyl alcohol	"	4.8	6.2	3.8	2.3	2.0	1.7	1.8	1.8	0.7	-	-	-	-
sec-butyl acetate	"	5.0	5.2	5.2	5.0	4.2	5.0	4.2	4.2	4.8	5.1	5.2	5.6	2.6
n-amyl acetate	"	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 27 Volatiles during acetification by surface culture.

VOLATILES		DAYS												
	Units	0	1	2	3	4	5	6	7	9	10	11	12	13
Acetaldehyde	mg/100ml	2.0	4.8	14.6	14.2	18.0	18.0	6.0	6.0	6.0	6.0	6.0	4.4	4.4
Ethyl alcohol √/√	g/100ml	8.65	8.3	7.7	4.6	4.2	2.9	2.0	0.71	.20	.06	.03	.03	.01
Acetic acid √/√	g/100ml	0.4	0.85	1.1	1.9	2.4	3.4	4.5	7.6	6.5	5.8	5.6	5.6	5.6
n-propyl alcohol	mg/100ml	1.8	1.8	1.8	1.6	2.0	1.6	1.1	0.8	0.8	0.8	1.0	0.8	0.8
propionic acid	"	32	44	480	360	336	320	51	40	28	24	23	24	24
sec-butyl alcohol	"	1.4	1.4	1.2	1.8	1.4	1.4	1.6	1.6	1.8	1.4	1.4	1.4	1.4
iso-butyl alcohol	"	14.2	14.0	13.0	13.5	13.5	13.5	13.5	13.5	13.2	13.0	13.0	13.1	12.8
iso-butyric acid	"	-	0.5	0.8	1.8	3.4	5.8	6.0	6.0	8.0	4.0	3.5	3.0	3.0
Acetoin	"	3	21	58	151	266	256	252	128	132	132	132	132	132
iso-amyl alcohol	"	15.0	15.0	15.0	12.0	6.0	4	3.6	4.6	4.2	3.2	3.0	1.6	1.4
iso-butyl acetate	"	2.4	2.5	3.2	3.2	2.5	2.7	2.7	2.5	2.7	2.9	2.8	2.6	2.5
n-amyl alcohol	"	4.8	6.2	6.2	4.8	4.8	2.8	2.0	0.6	-	-	-	-	-
sec-butyl acetate	"	3.0	3.1	3.0	3.0	3.3	3.5	3.3	3.3	3.5	3.5	3.5	3.5	2.5
n-amyl acetate	"	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 28. Volatiles during acetification in an aerated flask.

VOLATILES		DAYS										
	Units	1	2	3	4	5	6	7	8	9	10	11
Acetaldehyde	mg/100ml	-	-	10	10	10	22	35	20	-	-	-
Ethyl alcohol	v/v %/100ml	7.5	7.3	7.2	3.0	2.6	1.2	0.4	0.2	0.1	0.1	-
Acetic acid	v/v %/100ml	1.08	1.29	1.59	5.17	6.26	6.29	6.56	6.7	6.8	6.8	7.0
n-propyl alcohol	mg/100ml	2.8	2.8	2.5	2.5	2.8	2.5	2.2	2.5	2.5	2.5	2.4
propionic acid	"	10	42	48	42	36	42	40	50	50	50	50
sec-butyl alcohol	"	3.4	3.4	3.0	3.0	3.0	3.0	2.8	2.2	2.2	2.2	2.2
iso-butyl alcohol	"	12.0	12.0	12.0	10.0	10.0	8.9	8.8	8.8	10.0	10.0	9.2
iso-butyric acid	"	2.0	1.8	1.8	1.4	0.8	0.8	0.6	0.8	0.8	0.8	0.8
Acetoin	"	-	10	14	180	184	142	140	160	134	134	134
iso-amyl alcohol	"	24	20	20	20	10.0	10.0	8.0	3.0	2.2	1.6	1.4
iso-butyl acetate	"	2.9	2.9	2.9	2.9	2.2	2.4	2.4	2.6	2.8	2.8	2.8
n-amyl alcohol	"	5.8	5.8	5.6	4.8	4.8	4.4	4.0	2.6	0.6	-	-
sec-butyl acetate	"	2	20	20	20	20	20	18	20	20	20	18
n-amyl acetate	"	-	-	-	-	-	-	-	-	-	-	-

TABLE 29 Volatiles during acetification in a tower shaped fermentor at 22°C.

VOLATILES		DAYS										
	Units	1	2	3	4	5	6	7	8	9	10	11
Acetaldehyde	mg/100ml	5	22	22	35	52	84	15	15	10	10	4
Ethyl alcohol	v/v %/100ml	7.5	7.2	5.0	4.0	3.0	0.7	0.2	0.2	0.12	0.05	-
Acetic acid	v/v %/100ml	1.1	2.4	3.7	4.7	4.8	4.9	4.9	5.1	4.9	4.9	4.8
n-propyl alcohol	mg/100ml	2.4	2.4	2.4	2.0	2.0	2.2	2.0	2.0	2.0	2.0	2.2
propionic acid	"	56	56	56	120	128	122	40	34	32	32	32
sec-butyl alcohol	"	3.4	3.6	3.4	3.4	3.0	1.8	2.0	2.0	1.8	1.8	1.8
iso-butyl alcohol	"	14.0	14.0	12.0	7.0	7.0	7.0	7.0	7.2	7.2	6.0	6.2
iso-butyric acid	"	2.2	1.8	1.8	5.4	5.0	4.6	4.0	3.1	3.0	2.8	2.8
Acetoin	"	14.0	18.0	18.0	98	186	155	146	144	144	142	130
iso-amyl alcohol	"	20.0	14.0	10.0	10.0	10.0	5.0	4.0	4.0	3.0	3.0	2.0
iso-butyl acetate	"	1.7	2.2	2.2	2.5	2.4	2.4	2.6	2.4	2.5	1.6	1.6
n-amyl alcohol	"	6.2	5.8	5.0	2.4	2.6	0.6	-	-	-	-	-
sec-butyl acetate	"	20	30	32	22	24	22	20	20	20	20	20
n-amyl acetate	"	-	-	-	-	-	-	-	-	-	-	-

TABLE 30 Volatiles during acetification in a tower shaped fermentor at 30oC.

SUBSTRATE	% OXIDATION RELATIVE TO ETHANOL						
	A. PEROXYDANS	A. RANCENS	A. ACETI	SURFACE CULTURE	AERATED FLASK	TOWER AT 22°C	TOWER AT 30°C
Ethyl alcohol	100	100	100	100	100	100	100
Acetaldehyde	47.3	53.6	40.5	85.8	66.6	100	88.4
n-propyl alcohol	70.0	76.6	96.0	61.1	60.0	10.6	16.6
iso-butyl alcohol	14.0	6.1	6.0	11.1	4.8	28.3	50.0
sec-butyl alcohol	8.4	0.8	0.7	11.1	11.1	35.3	48.8
n-amyl alcohol	60.0	64.0	85.0	85.4	87.5	100	100
iso-amyl alcohol	15.0	6.3	7.0	30.0	69.3	87.5	85.0

TABLE 31. The relative activity of Acetobacter after Tanaka (1938), relative to activities found in the present study

Volatile (mg/100 ml.)	Storage time (Days) 1	3	5	7	9	14	16	23	27
Acetaldehyde	-	-	-	-	-	-	-	10	110
Ethyl alcohol (%/100ml.). v/v	1.05	6.8	6.3	7.85	7.75	7.65	7.55	6.75	6.6
Acetic acid (%/100ml.). v/v	0.4	0.5	0.5	0.5	0.6	0.6	0.7	1.1	1.3
n-Propyl alcohol	-	1.6	1.8	3.4	3.3	2.4	2.4	2.3	2.6
Sec-butyl alcohol	-	-	-	4.0	3.8	3.8	4.5	4.0	4.0
Iso-butyl alcohol	-	2.1	2.1	2.4	2.1	2.1	3.2	14.0	18.2
Acetoin	-	4.6	4.8	4.8	5.2	5.0	8.0	10.1	15.3
Iso-amyl alcohol	-	-	2.4	2.8	3.6	3.4	4.0	4.7	5.0
Iso-butyl acetate	-	0.5	1.5	1.5	1.5	1.5	1.8	2.5	2.5
n-Amyl alcohol	-	-	-	15.0	18.0	34.8	40.2	40.0	38.0
Sec-butyl acetate	-	-	-	5.0	5.0	4.8	5.0	5.0	5.0

TABLE 32. Volatiles detected in Fermenting Charging Wort. Sample Q.1.

Volatile (mg/100 ml.)	Storage time (days)								
	1	3	5	7	9	14	16	23	27
Acetaldehyde	-	-	-	15.0	20.2	40.6	80.1	120.4	130.7
Ethyl alcohol (g/100ml.)	6.8	6.7	6.7	5.5	5.4	5.4	5.25	4.3	4.1
Acetic acid (g/100ml.)	0.75	0.8	0.8	1.65	1.7	1.7	2.3	2.55	2.7
n-Propyl alcohol	1.6	1.8	2.0	1.6	1.5	1.4	1.4	1.2	0.8
Sec-butyl alcohol	4.5	4.5	4.8	5.0	4.5	5.0	5.2	5.5	5.6
Iso-butyl alcohol	19.5	19.0	19.4	18.0	16.5	17.1	16.3	16.0	15.0
Acetoin	5.0	6.2	8.0	10.4	15.0	15.1	20.7	25.4	20.5
Iso-amyl alcohol	5.0	4.8	5.0	4.0	6.0	4.7	6.9	5.7	5.4
Iso-butyl acetate	1.5	1.5	1.8	1.5	1.8	1.9	1.5	1.9	2.0
n-Amyl alcohol	45.2	45.0	48.1	40.7	38.6	40.4	36.5	36.5	36.0
Sec-butyl acetate	5.8	10.2	10.4	9.6	9.6	9.6	10.2	10.1	10.1

TABLE 33. Volatiles detected in the alcoholic wash, stored for four weeks. Sample Q.2.

VOLATILE (mg./100 ml.)	STORAGE TIME (DAYS)								
	1	3	5	7	9	14	16	23	27
Acetaldehyde	15.3	18.3	8.0	8.0	5.6	4.2	4.0	4.0	4.0
Ethyl alcohol (g/100ml).	0.3	0.3	0.35	0.35	0.3	0.3	0.3	0.2	0.1
Acetic acid (g/100ml).	9.3	9.4	9.4	9.2	8.15	7.4	6.9	6.25	6.1
n-Propyl alcohol	1.8	1.8	1.6	1.6	1.3	1.6	1.5	1.9	0.8
Propionic acid	145	148	156	150	150	143	145	140	140
Sec-butyl alcohol	2.7	2.0	1.8	1.8	1.9	2.5	2.1	2.7	1.8
Iso-butyl alcohol	5.7	5.1	4.9	4.9	5.0	5.0	5.7	5.5	5.5
Iso-butyric acid	4.8	4.0	4.0	5.2	5.4	4.8	4.8	5.0	4.8
Acetoin	310	315	312	302	308	304	294	280	276
Iso-amyl alcohol	1.5	1.5	1.8	0.5	0.4	0.5	0.5	0.4	0.5
Iso-butyl acetate	1.4	1.5	1.5	1.9	1.5	1.5	1.6	1.8	1.6
n-Amyl alcohol	8.3	5.4	8.4	4.8	5.4	5.4	5.2	5.0	5.2
Sec-butyl acetate	10.2	9.8	9.8	10.0	10.2	10.0	10.0	9.8	9.2

TABLE 34. Volatiles detected in Sample Q 3 obtained after acetification

VOLATILE (mg./100 ml.)	SURVEY RESULTS	STORAGE TIME (DAYS)								
		1	3	5	7	8	14	16	23	27
Acetaldehyde	30.4	12.6	8.2	5.2	4.8	4.8	4.0	3.8	-	-
Ethyl alcohol	42.0	130	110	110	110	100	42	35	22	-
Acetic acid	5.4	5.4	5.45	5.4	5.3	5.2	5.0	4.5	4.2	4.2
n-Propyl alcohol	3.22	1.8	1.4	1.4	1.8	1.3	1.3	1.3	1.8	1.7
Propionic acid	120	135	130	130	128	128	135	130	130	130
Sec-butyl alcohol	6.2	1.8	1.0	1.4	1.8	1.3	1.5	1.0	1.2	0.8
Iso-butyl alcohol	8.4	3.5	3.5	3.8	3.4	3.4	3.5	3.9	3.6	3.6
Iso-butyric acid	1.25	3.6	3.4	4.2	3.4	3.4	3.4	3.4	3.2	3.0
Acetoin	198	200	214	216	230	220	220	220	208	204
Iso-amyl alcohol	1.4	0.8	0.6	0.8	0.7	0.8	0.8	0.8	0.5	0.5
Iso-butyl acetate	0.3	1.6	1.6	1.3	1.7	1.1	1.3	1.6	1.6	1.6
n-Amyl alcohol	4.9	4.6	4.6	4.3	4.7	4.1	4.3	4.6	4.6	4.6
Sec-butyl acetate	5.8	7.0	7.4	7.0	7.2	7.4	7.0	7.0	7.0	7.4

TABLE 35. Volatiles detected after filtration and pasteurization compared to results obtained during the previous survey. Sample Q 4

Volatile	Units	Laboratory Storage (Days)							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	0	0	1.0
Ethyl alcohol	g/100 ml.	0.1	0.1	0.1	0.2	0.4	0.6	0.4	0.4
Acetic acid	g/100 ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3
n-Propyl alcohol	mg/100 ml.	0	0	0	0	±	±	3.8	3.6
Propionic acid	"	0	0	0	0	±	±	4.6	4.6
sec-Butyl alcohol	"	0	0	0	0	0	0	3.1	3.1
iso-Butyl alcohol	"	0	0	0	0	±	±	2.1	2.5
iso-Butyric acid	"	0	0	0	0	0	0	0	0
Acetoin	"	0	0	0	0	20	28	124	237
iso-Amyl alcohol	"	0	0	0	0	0	0	2.0	2.2
iso-Butyl acetate	"	0.3	0.3	0.2	0.3	3.4	3.6	4.2	3.8
n-Amyl alcohol	"	0	0	0	0	0	0	10	46
sec-Butyl acetate	"	0	0	0	0	7.7	8.2	8.9	8.7
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 36. Volatiles detected in wort obtained before seeding with yeast during laboratory storage. Sample F.I.

Volatile	Units	Laboratory Storage (Days)							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	28	52.5	52.5
Ethyl alcohol	g/100 ml.	6.5	6.5	6.5	6.6	7.1	6.9	6.8	4.25
Acetic acid	"	0	0	0	0.1	0.15	0.25	0.40	2.0
n-Propyl alcohol	mg/100 ml.	4.0	6.2	6.1	6.0	6.0	4.0	4.0	4.0
Propionic acid	"	16	16	15	16	58	84	169	167
sec-Butyl alcohol	"	0	4.0	4.8	4.8	5.4	4.6	3.4	3.4
iso-Butyl alcohol	"	4.1	6.2	6.8	10.4	8.3	4.2	4.1	4.1
iso-Butyric acid	"	0	0	0	0	0	0	0	0
Acetoin	"	0	0	0	0	0	±	11.4	11.4
iso-Amyl alcohol	"	0	±	5.0	12.5	17.5	25.0	16.0	16.0
iso-Butyl acetate	"	0	0	0	±	±	±	±	±
n-Amyl alcohol	"	0	0	2.1	10.0	8.4	8.4	6.0	6.0
sec-Butyl acetate	"	0	0	0	0	0	0	2.4	2.4
n-Amyl acetate	"	0	0	0	0	0	1.5	3.6	3.6

Table 37. Volatiles detected in a 48 hr. old fermenting wort during laboratory storage. Sample F2.

Volatile	Units	Laboratory Storage (Days)							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	2.5	9.0	14.0
Ethyl alcohol	g/100 ml.	4.6	4.7	4.8	4.8	5.1	4.8	4.8	4.9
Acetic acid	"	0.3	0.3	0.3	0.3	0.4	0.7	0.9	1.0
n-Propyl alcohol	mg/100 ml.	1.6	2.1	2.4	3.9	4.1	4.0	3.8	2.0
Propionic acid	"	0	0	0	0	4	19	70	42
sec-Butyl alcohol	"	0	0	0	3.0	3.0	3.1	4.2	4.0
iso-Butyl alcohol	"	5.0	7.2	8.4	13.0	12.5	8.2	4.8	4.0
iso-Butyric acid	"	0	0	0	0	0	0	138	154
Acetoin	"	0	0	0	37.5	148	138	172	175
iso-Amyl alcohol	"	0	±	5.2	8.5	16.5	16.7	16.8	8.0
iso-Butyl acetate	"	0	0	0	±	8	21	41	85
n-Amyl alcohol	"	0	0	0	3.6	10.0	10.4	10.7	11.1
sec-Butyl acetate	"	0	±	±	1.7	1.7	2.1	4.4	6.7
n-Amyl acetate	"	0	0	0	0	±	±	±	±

TABLE 38. Volatiles detected in a sample of fermenting 'charging' wort during laboratory storage. Sample. F.3.

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	28	50	48	34
Ethyl alcohol	g/100 ml.	5.0	5.3	5.4	5.3	2.9	1.4	1.2	0.4
Acetic acid	"	0.2	0.2	0.2	0.6	2.3	4.8	4.6	4.5
n-Propyl alcohol	mg/100 ml.	2.0	2.4	2.3	2.3	2.1	1.8	1.1	0.7
Propionic acid	"	0	0	0	28	176	178	104	42
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	3.8	4.5	4.4	4.3	4.1	4.0	3.6	3.4
iso-Butyric acid	"	0	0	0	0	0	0	15.0	18.2
Acetoin	"	0	0	0	4.6	75	112	142	189
iso-Amyl alcohol	"	2.6	2.6	2.4	2.6	2.8	1.2	1.2	1.2
iso-Butyl acetate	"	8.7	8.9	8.9	10.4	10.4	10.6	12.0	12.0
n-Amyl alcohol	"	1.0	1.6	1.6	1.4	1.4	1.6	1.6	1.6
sec-Butyl acetate	"	4.4	4.4	4.8	4.8	4.6	4.6	4.6	4.8
n-Amyl acetate	"	±	±	±	±	±	±	±	±

TABLE 39. Volatiles detected in a sample of alcoholic wash prior to acetification. Sample F.4.

Volatile	Units	Day's Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	15.4	20.6	30.1	15.1	15.1	15.0	4.0	0
Ethyl alcohol	g/100 ml.	2.6	1.8	0.9	0.3	0.2	0.1	0.1	0.1
Acetic acid	"	2.9	3.4	4.6	5.3	5.9	5.9	5.8	5.9
n-Propyl alcohol	mg/100 ml.	0.4	0.3	0	0	0	0	0	0
Propionic acid	"	30	38	52	89	88	87	87	45
sec-Butyl alcohol	"	2.0	2.0	0	0	0	0	0	0
iso-Butyl alcohol	"	3.5	0.4	0	0	0	0	0	0
iso-Butyric acid	"	0	0	0	0.6	0.8	1.1	1.3	3.7
Acetoin	"	88	102	108	92	92	82	77	74
iso-Amyl alcohol	"	3.0	2.7	0	0	0	0	0	0
iso-Butyl acetate	"	14.0	14.0	14.0	14.8	14.8	14.8	16.0	16.1
n-Amyl alcohol	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	5.4	5.4	4.5	4.5	3.3	3.3	3.3	3.5
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 40. Volatiles detected in a sample obtained from Fring's acetifier. Sample F.5.

Volatiles	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	18.0	18.0	16.4	15.4	9.1	6.1	2.8	0
Ethyl alcohol	g/100 ml.	0.2	0.2	0.2	0.2	0.2	0.1	0	0
Acetic acid	"	5.8	5.8	5.8	5.8	5.7	5.6	5.7	5.6
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	25	25	25	25	26	26	28	30
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyric acid	"	±	±	0	0	0	0	0	0
Acetoin	"	68	68	65	72	82	82	85	85
iso-Amyl alcohol	"	±	±	±	±	±	±	±	±
iso-Butyl acetate	"	10.6	11.1	10.8	10.8	10.0	12.0	12.5	15.0
n-Amyl alcohol	"	±	±	±	±	±	±	±	±
sec-Butyl acetate	"	±	±	±	±	±	±	±	±
n-Amyl acetate	"	0	0	0	0	0	0	±	±

TABLE 41. Volatiles detected in a sample of vinegar immediately after acetification. Sample F.6

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	5.6	1.4	0	0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.8	0
Acetic acid	"	5.7	5.7	5.6	5.7	5.7	5.7	5.6	5.4
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	23	25	22	17	17	17	14	14
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	4.0	4.0	4.0	±	±	±	±	±
iso-Butyric acid	"	5.1	4.8	4.8	4.4	4.7	4.6	4.3	4.3
Acetoin	"	72	74	72	72	68	62	65	62
iso-Amyl alcohol	"	±	±	±	±	±	±	±	±
iso-Butyl acetate	"	11.0	11.5	11.5	11.9	13.1	13.0	12.2	12.2
n-Amyl alcohol	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	4.5	4.8	4.5	4.5	5.5	5.4	5.5	5.5
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 42. Volatiles detected in a sample of finished vinegar stored comercially for 4 days. Sample F.7.

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.1	0.1	0.1	0.1	0.1	0	0	0
Acetic acid	"	4.7	4.8	4.8	4.8	4.7	4.8	4.7	4.7
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	10.0	10.4	10.3	10.3	10.4	10.4	10.3	10.3
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	4.2	0	0	0	0	0	0	0
iso-Butyric acid	"	5.6	5.4	5.1	5.2	5.1	5.5	5.4	5.0
Acetoin	"	78	82	82	72	84	85	80	80
iso-Amyl alcohol	"	3.0	3.0	3.0	3.0	±	±	±	±
iso-Butyl acetate	"	11.6	11.1	11.8	11.8	11.4	11.2	11.6	11.4
n-Amyl alcohol	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	4.5	4.8	4.5	4.7	4.7	4.9	4.5	4.5
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 43. Volatiles detected in a sample of finished vinegar stored comercially for four weeks.

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0
Acetic acid	"	5.7	5.8	5.9	5.8	5.9	5.6	5.8	5.8
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	8.4	8.2	8.2	8.7	8.8	8.9	8.9	9.0
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	6.5	±	±	±	±	±	±	±
iso-Butyric acid	"	3.7	3.8	3.1	2.7	2.8	2.7	2.9	3.0
Acetoin	"	100	95	87	88	85	82	88	83
iso-Amyl alcohol	"	±	±	±	±	±	±	±	±
iso-Butyl acetate	"	8.1	8.2	7.5	7.4	7.5	7.5	7.5	7.4
n-Amyl alcohol	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	4.5	3.3	3.7	3.3	3.3	3.3	3.5	3.5
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 44. Volatiles detected in a finished vinegar sample stored comercially for nine weeks.

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.1	0.1	0.1	0	0	0	0	0
Acetic acid	"	5.9	5.8	5.8	5.8	5.9	5.9	5.9	5.8
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	8.2	9.2	9.4	9.4	9.4	10.0	10.0	10.0
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	2.1	2.0	1.1	0	0	0	0	0
iso-Butyric acid	"	3.6	3.8	3.8	3.3	3.3	3.3	3.0	3.0
Acetoin	"	120	115	114	120	120	127	123	122
iso-Amyl alcohol	"	3.0	3.0	3.0	3.0	2.0	2.6	2.6	2.6
iso-Butyl acetate	"	11.0	11.8	11.6	11.4	11.4	11.0	11.0	11.0
n-Amyl alcohol	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.3
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 45. Volatiles detected in a finished vinegar sample stored comercially for eleven weeks.

Volatile	Units	Days Storage							
		2	4	6	8	19	23	26	31
Acetaldehyde	mg/100 ml.	0	0	0	0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0
Acetic acid	"	5.6	5.6	5.6	5.7	5.7	5.7	5.7	5.5
n-Propyl alcohol	mg/100 ml.	0	0	0	0	0	0	0	0
Propionic acid	"	13.4	13.0	13.0	10.0	9.7	9.7	9.8	10.0
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	0	0	0	0	0	0	0	0
iso-Butyric acid	"	0	0	0	0	0	0	0	0
Acetoin	"	63	61	60	62	60	62	60	62
iso-Amyl alcohol	"	±	±	±	±	±	±	±	±
iso-Butyl acetate	"	10.1	10.1	9.6	10.7	10.7	10.8	10.2	10.4
n-Amyl acetate	"	0	0	0	0	0	0	0	0
sec-Butyl acetate	"	3.5	3.5	3.5	3.3	3.3	3.5	3.3	3.3
n-Amyl acetate	"	0	0	0	0	0	0	0	0

TABLE 46. Volatiles detected in a finished vinegar sample after sterilisation and dilution. Sample F.8.

Volatile	Units	Laboratory Storage (Days)						
		3	10	14	17	23	27	33
Acetaldehyde	mg/100 ml.	2	2	2	3	2.5	0	0
Ethyl alcohol	g/100 ml.	0.4	0.3	0.3	0.3	0.1	0.1	0.1
Acetic acid	"	6.8	6.8	6.7	6.4	6.2	6.2	6.2
n-Propyl alcohol	mg/100 ml.	1.4	1.5	0.9	0.7	0.9	0.4	0
Propionic acid	"	40	32	32	34	38	38	38
sec-Butyl alcohol	"	0	0	0	0	0	0	0
iso-Butyl alcohol	"	9.0	7.5	7.5	7.5	7.5	4.5	3.1
iso-Butyric acid	"	5.5	5.8	6.0	6.0	6.0	4.8	4.2
Acetoin	"	234	245	274	258	250	258	245
iso-Amyl alcohol	"	8.6	8.4	7.5	4.3	5.0	5.4	5.2
iso-Butyl acetate	"	60	55	55	56	55	50	42
n-Amyl alcohol	"	10.8	10.0	10.0	8.7	9.0	9.0	8.9
sec-Butyl acetate	"	0	0	0	0	0	0	0
n-Amyl acetate	"	14	14	14	14	13.4	8.2	6.0

TABLE 47. Volatiles detected in a finished sample of vinegar blended from both the Quick and Continuous processes. Sample C.1.

Volatile	Units	Laboratory Storage (Days)						
		3	10	14	17	23	27	33
Acetaldehyde	mg/100 ml.	3.0	3.0	3.0	3.0	3.0	0	0
Ethyl alcohol	g/100 ml.	0.1	0.1	0.1	0.1	0.1	0	0
Acetic acid	"	6.7	6.7	6.8	6.7	6.7	6.6	6.6
n-Propyl alcohol	mg/100 ml.	0.8	0.7	0.6	0.4	0.3	0.3	0
Propionic acid	"	45	45	45	40	44	44	40
sec-Butyl alcohol	"	0	0	0	0	0	0	0
iso-Butyl alcohol	"	10.2	10.5	10.5	9.5	9.0	9.2	8.5
iso-Butyric acid	"	2.3	2.4	2.4	2.7	1.8	1.2	1.2
Acetoin	"	325	340	340	330	325	320	312
iso-Amyl alcohol	"	10.0	8.2	7.5	7.5	7.5	8.0	7.8
iso-Butyl acetate	"	87	82	82	82	84	78	74
n-Amyl alcohol	"	7.0	8.1	6.9	6.8	6.8	4.8	4.8
sec-Butyl acetate	"	0	0	0	0	0	0	0
n-Amyl acetate	"	9.4	9.0	9.1	9.0	9.0	9.1	8.4

TABLE 48. Volatiles detected in a finished vinegar produced by the Quick method

Volatile	Units	Laboratory Storage (Days)						
		3	10	14	17	23	27	33
Acetaldehyde	mg/100 ml.	3.0	2.0	1.0	0	0	0	0
Ethyl alcohol	g/100 ml.	0.4	0.3	0.3	0.3	0.3	0.2	0.1
Acetic acid	"	6.4	6.5	6.6	6.6	6.8	6.8	6.7
n-Propyl alcohol	mg/100 ml.	0.6	0.6	0.5	0.5	0	0	0
Propionic acid	"	30	34	31	32	34	34	32
sec-Butyl alcohol	"	0	0	0	0	0	0	0
iso-Butyl alcohol	"	6.2	6.0	5.8	6.0	4.8	5.1	4.8
iso-Butyric acid	"	2.4	2.5	2.7	2.7	2.7	2.4	2.1
Acetoin	"	140	138	142	135	120	125	125
iso-Amyl alcohol	"	10.0	11.2	10.0	10.5	10.1	8.6	8.6
iso-Butyl acetate	"	30	38	38	30	28	28	21
n-Amyl alcohol	"	10.0	10.0	10.0	8.3	8.5	8.5	8.0
sec-Butyl acetate	"	0	0	0	0	0	0	0
n-Amyl acetate	"	8.6	8.6	9.0	8.5	8.5	8.2	7.8

TABLE 49. Volatiles detected in a vinegar sample produced by the Continuous method. Sample C.2.

Volatile	Units	Laboratory Storage (Days)						
		3	10	14	17	23	27	33
Acetaldehyde	mg/100 ml.	0	10.0	15.0	15.0	15.0	6.0	6.0
Ethyl alcohol	g/100 ml.	5.8	5.8	5.8	5.8	5.7	5.4	5.4
Acetic acid	"	0.7	0.7	0.7	0.6	0.5	0.5	0.5
n-Propyl alcohol	mg/100 ml.	0.8	0.8	0.8	0.8	0.7	0.6	0.6
Propionic acid	"	18	25	76	83	60	60	64
sec-Butyl alcohol	"	0	0	0	0	0	0	0
iso-Butyl alcohol	"	11.5	12.1	11.5	11.3	11.3	10.5	10.2
iso-Butyric acid	"	0	0	0	0	0	0	0
Acetoin	"	0	28	23	23	21	22	20
iso-Amyl alcohol	"	18	19	26	16	16	14	14
iso-Butyl acetate	"	36	38	38	40	38	36	36
n-Amyl alcohol	"	6	16	36	40	40	40	38
sec-Butyl acetate	"	0	0	0	0	0	0	0
n-Amyl acetate	"	12	10	10	14	15	15	15

TABLE 50. Volatiles detected in a sample of fermenting wort after five days fermentation. Sample C.3.

Volatile	Units	Laboratory Storage (Days)						
		3	10	14	17	23	27	33
Acetaldehyde	mg/100 ml.	3.0	3.5	3.5	2.0	2.5	2.5	2.0
Ethyl alcohol	g/100 ml.	7.2	7.0	6.9	6.8	6.5	6.5	6.5
Acetic Acid	"	0.4	0.4	0.4	0.4	0.4	0.3	0.3
n-Propyl alcohol	mg/100 ml.	0.7	0.7	0.7	0.7	0.5	0	0
Propionic acid	"	8	13	20	20	22	30	45
sec-Butyl alcohol	"	0	0	0	0	0	0	0
iso-Butyl alcohol	"	9.1	6.2	6.0	6.5	6.5	6.8	6.8
iso-Butyric acid	"	0	4.5	4.5	2.5	2.5	2.5	2.5
Acetoin	"	28	30	35	37	35	37	38
iso-Amyl alcohol	"	28	19	18	20	20	16	14
iso-Butyl acetate	"	41	50	48	52	54	76	88
n-Amyl alcohol	"	35	34	34	35	30	30	30
sec-Butyl acetate	"	0	0	0	0	0	0	0
n-Amyl acetate	"	12	14	18	15	15	14	14

TABLE 51 Volatiles detected in a sample of 'charging' wort immediately prior to acetification. Sample C.4.

VOLATILE	NUMBER OF DAYS FERMENTATION WITH ADDED ACETALDEHYDE																	
	SURFACE CULTURE AT 22°C											SURFACE CULTURE AT 33°C						
	0	5	6	7	10	12	15	19	21	26	32	0	5	6	7	10	12	15	19
Acetaldehyde mg/100ml	980	990	980	960	500	150	80	6	4	8	2	1008	970	940	810	800	750	110	12
Ethyl alcohol % v/v	5.2	5.1	5.1	5.0	5.1	5.0	5.0	4.9	4.9	0.4	2	5.6	5.6	5.5	5.6	5.4	1.8	0	0
Acetic Acid % v/v	0.4	0.4	0.4	0.4	0.5	0.8	1.2	1.4	1.4	1.4	1.5	0.4	0.4	0.6	0.6	0.7	3.6	6.8	7.2

	NUMBER OF DAYS FERMENTATION WITH ADDED GLYCEROL																	
	0	5	6	7	10	12	15	19	-	-	-	0	5	6	7	10	12	15	19
Ethyl a lcohol % v/v	5.6	3.3	1.2	0.1	0.1	0	0	0	-	-	-	4.8	0.8	0	0	0	0	0	0
Acetic acid % v/v	0.4	1.8	4.8	5.6	5.8	5.8	4.5	3.4	-	-	-	0.4	4.9	4.4	4.2	2.5	1.8	0.6	0.2
Propionic acid ,g/100 ml	38	168	182	200	232	160	120	64	-	-	-	62	166	240	230	210	160	180	150
Acetoin mg/100ml	62	278	354	374	400	450	-	-	-	-	-	68	350	300	276	270	275	200	185

TABLE 52 (a) Volatiles detected during acetification with added acetaldehyde and glycerol.

VOLATILE	NUMBER OF DAYS FERMENTATION WITH ADDED ACETALDEHYDE															
	TOWER FERMENTOR AT 22°C								TOWER FERMENTOR AT 33°C							
	0	2	5	8	9	11	19	21	0	2	5	8	9	11	19	21
Acetaldehyde mg/100 ml	980	970	960	710	740	25	52	40	1050	980	940	412	305	84	15	16
Ethyl alcohol % v/v	5.1	5.2	5.1	5.1	5.0	4.9	0.2	0	5.2	5.4	5.2	5.0	3.9	0.2	0	0
Acetic acid % v/v	0.5	0.6	0.8	1.1	1.4	2.4	5.6	6.6	0.5	0.6	0.8	1.4	2.4	5.2	6.4	6.6

	NUMBER OF DAYS DERMENTATION WITH ADDED GLYCEROL															
	0	2	3	4	5	6	7	8	0	1	2	3	4	5	6	7
Ethyl alcohol % v/v	6.2	3.6	2.6	0.6	0.2	0	0	0	6.2	6.0	3.7	2.2	0.6	0	0	0
Acetic acid % v/v	0.4	1.8	3.0	4.4	5.5	6.3	6.5,6.9		0.4	0.8	2.5	3.8	5.0	6.5	6.5	6.4
Propionic acid mg/100 ml	42	24	24	22	21	22	25	25	38	38	46	62	34	30	30	28
Acetoin mg/100 ml	54	188	424	980	304	287	294	280	85	168	1110	1200	1214	1200	980	988

TABLE 52 (b) Volatiles detected d uring acetification with added acetaldehyde and glycerol

NUMBER OF DAY'S FERMENTATION WITH ADDED PYRUVATE

VOLATILE	SURFACE CULTURE AT 22°C								SURFACE CULTURE AT 33°C								
	0	4	7	8	11	13	15	19	0	4	7	8	11	13	15	19	34
Ethyl alcohol % v/v	5.6	5.6	5.5	4.0	2.1	0.5	0.1	0.1	5.4	5.2	5.4	5.4	5.0	5.0	4.8	4.8	0.3
Acetic acid % v/v	0.4	0.4	0.5	1.7	3.4	5.3	6.3	6.3	0.5	0.7	0.6	0.6	0.6	0.5	0.5	0.8	5.5
Propionic acid mg/200ml	22	25	30	35	84	92	84	64	14	18	20	22	22	24	34	92	86
Acetoin mg/100ml	30	55	54	424	982	880	600	490	31	28	30	62	67	62	64	255	424

TABLE 54 Volatiles detected during acetification with added pyruvate

NUMBER OF DAY'S FERMENTATION WITH ADDED N-AMYL ALCOHOL

VOLATILE	SURFACE CULTURE AT 22°C									SURFACE CULTURE AT 33°C							
	0	5	6	7	10	12	15	19	21	0	5	6	7	10	12	15	19
Ethyl alcohol % v/v	5.8	5.6	5.4	5.0	3.8	1.1	0.2	0.1	0	5.2	5.0	3.8	1.1	0.3	0.3	0	0
Acetic scid % v/v	0.7	0.9	1.2	1.2	2.1	4.6	5.2	5.4	5.3	0.5	0.9	2.1	4.6	5.2	5.4	5.3	5.3
n-Amyl alcohol mg/100 ml	254	218	180	170	175	169	120	46	14	238	228	218	180	165	106	82	64

NUMBER OF DAYS FERMENTATION WITH ADDED LACTATE

	0	4	7	8	11	13	15	19	0	4	7	8	11	13	15	19	34
Ethyl alcohol % v/v	5.2	5.1	5.0	2.8	0.2	0.1	0	0	5.4	5¼4	5.3	5.1	5.0	5.1	4.8	2.2	0.4
Acetic acid % v/v	0.5	0.6	0.6	2.3	4.5	4.8	4.8	4.8	0.4	0.5	0.5	0.4	0.6	1.5	1.7	4.7	5.2
Propionic acid mg/100 ml	38	40	44	50	76	50	56	54	24	28	28	32	41	41	46	92	104
Acetoin do	40	40	88	1325	1705	1580	1430	1430	50	64	62	60	75	78	270	780	810

TABLE 53 (a) Volatiles detected during acetification with added n-amyl alcohol and DL lactate

NUMBER OF DAYS FERMENTATION WITH ADDED N-AMYL ALCOHOL

VOLATILE	TOWER FERMENTOR AT 22°C									TOWER FERMENTOR AT 33°C								
	0	2	3	4	6	8	9	10	11	0	2	3	4	6	8	9	10	11
Ethyl alcohol % v/v	5.8	5.6	5.4	5.0	3.8	1.1	0.2	0.1	0	5.5	5.5	5.0	4.7	3.1	1.0	0	0	0
Acetic acid % v/v	0.4	0.9	1.3	1.3	2.3	2.7	4.6	5.2	5.4	0.6	0.8	1.0	1.1	2.2	4.8	5.3	5.3	5.3
N-Amyl alcohol mg/100ml	210	200	190	194	180	154	86	52	38	204	200	186	190	180	140	66	22	14

NUMBER OF DAY S FERMENTATION WITH ADDED LACTATE

	TOWER FERMENTOR AT 22°C								TOWER FERMENTOR AT 33°C							
	0	6	8	9	10	12	14	15	0	6	8	9	10	12	14	15
Ethyl alcohol % v/v	5.8	5.4	3.8	2.1	2.1	0.6	0.2	0.2	5.9	5.7	3.9	2.0	1.8	0.6	0.2	0.2
Acetic acid % V/v	0.4	0.8	1.8	2.6	3.8	4.2	4.2	4.1	0.4	0.8	1.8	2.8	3.9	4.3	4.2	4.1
Propionic acid mg/100ml	28	51	156	176	193	224	212	204	15	62	145	160	170	192	170	164
Acetoin mg/100ml	12	46	460	500	500	480	400	450	24	56	510	500	480	432	412	400

TABLE 53 (b) Volatiles detected during acetification with added n-amyl alcohol and DL Lactate

	GLUCOSE								CONTROL EXPERIMENTS							
	Surface culture 22°C		Surface culture 33°C		Tower fermentor 22°C		Tower fermentor 33°C		Surface culture 22°C		Surface culture 33°C		Tower fermentor 22°C		Tower fermentor 33°C	
DAY	0	19	0	19	0	21	0	11	0	17	0	19	0	10	0	10
Acetaldehyde mg/100ml	2	2	2	2	8	12	62	18	4	5	2	4	6	2	6	11
Ethyl alcohol % v/v	5.2	0	5.2	0	6.2	0	6.4	0	5.4	0	5.4	0	5.5	0.2	5.4	0
Acetic acid %v/v	0.4	3.5	0.4	3.8	0.4	6.5	0.4	6.3	0.4	5.6	0.4	5.6	0.4	5.5	0.4	5.6
n-propyl alcohol mg/100ml	0.8	-	2.2	-	1.7	-	1.9	-	1.8	-	1.9	0.4	1.8	-	1.6	0.4
propionic acid mg/100ml	43	45	44	52	43	90	32	30	40	45	44	62	52	75	54	43
sec-butyl alcohol mg/100ml	3.1	1.2	2.1	0.2	2.4	2.0	2.4	0.6	2.4	1.3	2.7	1.8	2.5	0.6	2.7	0.8
iso-butyl alcohol mg/100ml	7.8	4.0	6.8	3.0	7.7	6.4	6.4	1.8	6.8	3.1	6.0	3.3	7.2	2.4	7.0	1.8
iso-butyric acid mg/100ml	5.4	4.6	4.3	2.5	2.1	3.9	2.7	3.1	6.1	8.2	5.0	8.0	7.0	4.1	7.1	3.8
Acetoin mg/100ml	67	75	65	85	40	140	54	130	64	82	66	80	54	150	60	172
iso-amyl alcohol "	12	9.7	10.4	6.4	16.3	14.3	19.4	9.4	8.4	6.4	9.1	7.4	9.0	8.1	8.8	5.0
iso-butyl acetate "	42	40	54	50	37	38	30	27	35	34	36	30	28	35	30	34
n-amyl alcohol "	16.1	11.1	12.4	11.0	34	48	28	15	10.2	10.4	14.2	9.4	14.8	8.8	13.0	5.7
sec-butyl acetate "	-	-	-	-	-	-	-	0	-	0	-	-	-	0	-	0
n-amyl acetate "	8.4	8.0	4.4	5.1	5.4	7.4	5.0	4.4	8.1	7.4	9.2	6.8	9.0	8.2	8.4	6.7

TABLE 55 Volatile concentrations at beginning and end of experiments with added glucose and with control experiments.

NUMBER OF DAYS FERMENTATION WITH ADDED ISO-BUTYL ALCOHOL

VOLATILE	SURFACE CULTURE at 22°C								SURFACE CULTURE AT 33°C							
	0	5	6	7	10	12	15	19	0	5	6	7	10	12	15	19
Ethyl alcohol % v/v	5.2	3.3	1.2	0.1	0.1	0	0	0	5.6	3.3	1.3	0.1	0.1	0	0	0
Acetic acid % v/v	0.4	1.8	4.3	5.6	5.8	5.8	4.5	3.4	0.4	1.9	4.4	5.7	5.8	5.8	4.5	3.4
Iso-butyl alcohol mg/100 ml	215	210	185	126	54	12	9	2.4	204	190	174	168	50	8.2	4.4	1.8
Iso-Butyric acid mg/100 ml	21	40	120	128	138	210	230	240	16	38	62	98	176	190	184	188

NUMBER OF DAYS FERMENTATION WITH ADDED ISO-AMYL ALCOHOL

	0	4	7	8	11	13	15	19	0	4	7	8	11	13	15	19	34
Ethyl alcohol % v/v	5.3	3.4	1.4	0.4	0.2	0.1	0	0	5.2	3.4	1.0	0.4	0.2	0	0	0	0
Acetic acid % v/v	0.4	2.1	4.2	5.2	5.8	6.1	5.5	3.5	0.4	2.0	4.1	5.2	5.7	6.0	4.5	3.5	3.0
Iso-Amyl alcohol mg/100 ml	220	210	180	164	100	52	22	12	250	233	240	210	156	94	94	82	74

TABLE 56)(a)

Volatiles detected during acetification with added iso-butyl alcohol and iso-amyl alcohol

NUMBER OF DAYS FERMENTATION WITH ADDED ISO-BUTYL ALCOHOL

VOLATILE	TOWER FERMENTOR AT 22°C									TOWER FERMENTOR AT 33°C								
	0	2	3	4	5	6	7	8	10	0	2	3	4	5	6	7	8	10
Ethyl alcohol % v/v	5.2	5.2	5.2	3.7	1.8	1.4	0.6	0.2	0.1	5.3	4.7	3.4	1.6	0.6	0.2	0.1	0	0
Acetic acid % v/v	0.4	0.4	1.3	2.1	3.2	3.8	4.5	4.9	4.9	0.4	1.4	2.1	3 .3	4.7	4.9	4.9	4.9	4.9
iso-Butyl alcohol mg/100 ml.	184	174	170	170	162	150	40	31	12	204	195	170	150	95	40	30	8	10
iso-Butyric acid mg/100 ml	8.4	9.4	10.1	84	100	182	174	185	160	7.4	8.1	18	68	145	168	172	170	174

NUMBER OF DAYS FERMENTATION WITH ADDED ISO-AMYL ALCOHOL

	0	6	8	9	10	12	14	15	0	6	8	9	10	12	14
Ethyl alcohol % v/v	5.1	3.0	0.6	0.4	0.2	0	0	0	5.2	2.5	0.6	0.4	0.2	0	0
Acetic acid % v/v	0.4	2.1	4.2	5.3	5.8	6.1	4.4	3.3	0.4	2.6	4.7	5.0	5.2	5.2	5.2
iso-Amyl alcohol mg/100 ml	236	220	210	224	185	40	25	30	254	234	210	152	112	84	36

TABLE 56 (b) Volatiles detected during acetification with added iso-butyl alcohol and iso-amyl alcohol.

NUMBER OF DAYS FERMENTATION WITH ADDED N-PROPYL ALCOHOL

VOLATILE	SURFACE CULTURE AT 22°C								SURFACE CULTURE AT 33°C								
	0	4	7	8	11	13	15	19	0	4	7	8	11	13	15	19	34
Ethyl alcohol % v/v	5.6	5.6	5.4	5.6	3.4	2.1	0.4	0.1	5.8	5.4	3.8	2.4	0.1	0.1	0	0	0
Acetic acid % v/v	0.4	0.5	0.5	0.5	2.9	3.4	6.0	6.1	0.4	0.6	2.3	3.3	6.0	6.1	4.5	3.5	3.0
N-Propyl alcohol mg/100ml	54	50	50	50	45	40	20	5.1	57	59	57	42	20	8	1.2	-	-
Propionic acid mg/100ml.	32	32	38	38	38	95	144	160	41	56	112	145	210	162	152	150	78

NUMBER OF DAYS FERMENTATION WITH ADDED SEC-BUTYL ALCOHOL

	0	5	6	7	10	12	15	19	21	0	5	6	7	10	12	15	19
Ethyl alcohol % v/v	5.8	5.6	4.0	2.2	0.4	0.1	0.1	0.1	0	5.8	5.6	3.4	0.1	0	0	0	0
Acetic acid %v/v	0.7	0.9	1.8	3.6	5.6	5.6	5.2	5.3	3.3	0.4	2.0	2.4	5.2	5.3	5.3	4.7	4.1
Sec-butyl alcohol	210	200	195	200	186	85	45	42	40	198	180	184	178	120	110	92	84

TABLE 57(a) Volatiles detected during acetification with added n-propyl alcohol and sec-butyl alcohol

NUMBER OF DAYS FERMENTATION WITH ADDED N-PROPYL ALCOHOL

VOLATILE	TOWER FERMENTOR AT 22°C						TOWER FERMENTOR AT 33°C					
	0	4	8	9	10	12	0	4	8	9	10	12
Ethyl alcohol % v/v	5.6	5.1	2.7	1.6	0.5	0.1	5.7	5.1	2.2	0.6	0.1	0
Acetic acid % v/v	0.4	0.6	3.1	4.2	4.9	5.4	0.4	0.8	3.2	4.8	5.6	5.7
N-propyl alcohol mg/100ml	48	50	50	36	24	15	52	48	46	28	6.5	1.2
Propionic acid mg/100ml	32	32	100	148	154	152	34	34	85	124	164	172

NUMBER OF DAYS FERMENTATION WITH ADDED SEC-BUTYL ALCOHOL.

	0	3	4	6	8	9	10	11	0	2	3	4	6	8	9	10
Ethyl alcohol % v/v	5.7	5.5	5.2	3.9	1.0	0.2	0.2	0	5.8	5.8	5.6	4.7	3.2	0.6	0.2	0.1
Acetic acid % v/v	0.4	1.3	1.3	2.3	4.7	5.3	5.5	5.3	0.5	0.7	1.2	1.5	2.2	4.7	5.3	5.3
Sec-butyl alcohol mg/100ml	212	200	208	195	190	165	152	96	200	206	198	188	175	130	126	104

TABLE 57 (b) Volatiles detected during acetification with added n-propyl alcohol and sec-butyl alcohol

% OXIDATION RELATIVE TO ETHANOL

SUBSTRATE	A. Peroxydans	A. Rancens	A. Aceti	Results obtained in our previous communication				Results obtained in the present study			
				Surface culture 22°C	Aerated flask 22	Tower at 22°C	Tower at 33oC	Surface culture 22°C	Surface culture 33°C	Tower at 22°C	Tower at 33°C
Acetaldehyde	47.3	53.6	40.5	85.8	66.6	100	88.4	94.4	99.2	98.1	98.4
n-propyl alcohol	70.0	76.6	96.0	61.1	60.0	10.6	16.6	84.4	79.2	81.1	80.4
iso-butyl alcohol	14.0	6.1	6.0	11.1	4.8	28.3	50.0	65.0	62.0	67.1	72.4
sec-butyl alcohol	8.4	0.8	0.7	11.1	11.1	35.3	48.8	56.0	60.4	65.2	67.4
n-amyl alcohol	60.0	64.0	85.0	85.4	87.5	100	100	67.4	67.0	76.0	78.0
iso-amyl alcohol	15.0	6.3	7.0	30.0	69.3	87.5	85.0	44.6	43.7	56.7	58.9

TABLE 58

The relative activity of acetobacter after Tanaka (1938) compared to values obtained during laboratory acetifications with an increased alcohol content.

		MYCODERMA FROM MANUFACTURERS					FROM ASTON				
DAYS		0	5	10	15	21	0	5	10	15	21
Acetaldehyde	mg/100ml.	0	15	0	0	0	0	0	48	15	0
Ethyl alcohol	g/100ml.	0.2	0.1	0	0	0	0.2	0.3	0.3	9.1	0
Acetic acid	g/100ml.	0.6	0.6	0.9	0.1	0.1	0.6	0.8	1.4	0.2	0
n-Propyl alcohol	mg/100ml.	0.8	1.8	3.8	0	0	0.8	0	0	0	0
Propionic acid	"	3.1	15.0	4.0	0	0	3.1	13	26	0	0
sec-Butyl alcohol	"	0	0	0	0	0	0	0	0	0	0
iso-Butyl alcohol	"	-	1.6	-	0	0	-	7.5	0	0	0
iso-Butyric acid	"	0	0	-	0	0	0	3.8	0	0	0
Acetoin	"	25	25	24	35	27	25	25	12	14	0
iso-Amyl alcohol	"	3.6	2.6	0	0	0	3.6	1.0	0	0	0
iso-Butyl acetate	"	160	34	30	0	0	160	55	50	0	0
n-Amyl alcohol	"	3.7	8.5	8.3	3.7	0	3.7	0	0	0	0
sec-Butyl acetate	"	0	0	0	0	0	0	0	0	0	0
n-Amyl acetate	"	6.2	26.4	43	8.0	0	6.2	35.4	40.0	0	0

TABLE 59. Volatiles during mycoderma growth on unfermented wort.

MYCODERMA

	FROM MANUFACTURER							FROM ASTON						
DAYS	0	7	11	14	19	24	30	0	7	11	14	19	24	30
Acetaldehyde	0	0	0	0	0	0	0	0	8	8	5	5	5	0
Ethyl alcohol	7.4	5.4	0.1	0.1	0	0	0	7.4	6.3	4.9	3.6	0.5	0	0
Acetic acid	0.3	2.1	7.8	7.0	3.5	1.2	0.5	0.3	0.3	0.3	0.4	3.5	4.4	3.7
n-Propyl alcohol	7.7	8.7	9.0	1.5	1.3	1.3	0.8	7.7	7.8	6.8	5.8	0	0	0
Propionic acid	13	190	25	25	25	13	22	13	70	70	76	128	90	48
sec-Butyl alcohol	0	-	-	0	0	0	0	0	0	0	0	0	0	0
iso-Butyl alcohol	7.5	15	7.5	7.5	5.2	2.5	1.1	7.5	7.5	7.4	7.5	8.1	5.2	4.8
iso-Butyric acid	-	-	32	44	0	0	0	-	-	-	-	0	0	0
Acetoin	27	350	462	548	550	610	600	27	25	35	38	121	104	92
iso-Amyl alcohol	32.5	12.5	10.0	6.0	0	0	0	32.5	17	18	17	10	10	8
iso-Butyl acetate	38	38	41	41	41	17	15	38	41	48	48	96	85	124
n-Amyl alcohol	5.2	11.6	3.4	2.1	0	0	0	5.2	22.0	20.1	6.4	4.0	0	0
sec-Butyl acetate	0	0	-	0	0	0	0	0	0	0	0	0	0	0
n-Amyl acetate	5.0	6.2	7.0	5.0	6.0	6.1	6.1	5.0	10	8.4	9.0	8.2	5.0	5.0

TABLE 60 Volatiles during mycoderma growth in 'charging wort'

Air Flow (per minute) 5.3 ml.

Time Interval mins.	pO_2 mm. Hg.	Time Interval mins.	pO_2 mm. Hg.
0	45.0	2.5	59.5
1.0	57.0	3.0	62.0
1.5	58.5	6.0	66.0
2.0	59.0	180.0	70.0

Air Flow 8.1 ml. per min.

0	45.0	2.5	74.0
1.0	66.5	3.0	74.0
1.5	71.0	6.0	74.0
2.0	73.5	180.0	76.0

Air Flow 50 ml. per min.

0	45	2.5	104
1.0	88	3.0	115
1.5	97	6.0	128
2.0	101	60.0	135

Air Flow 120 ml. per min.

0	45	2.5	136
1.0	90	3.0	137
1.5	110	4.0	137
2.0	132	6.0	137

TABLE 61. pO_2 measurements of charging wort at air flow rates from 5.3 ml. per min. to 120 ml. per min.

Air Flow (per minute) 150 ml.

Time Interval mins.	pO_2 mm. Hg.	Time Interval mins.	pO_2 mm. Hg.
0	45.0	2.5	137
1.0	90	3.0	137
1.5	110	4.0	137
2.0	137	6.0	137

Air Flow (per minute) 300 ml.

0	45	2.5	137
1.0	97	3.0	136
1.5	110	4.0	137
2.0	137	6.0	137

Air Flow (per minute) 600 ml.

0	45	1.0	110
0.25	76	1.5	136
0.5	93	2.0	136
0.75	104	3.0	135

Air Flow (per minute) 800 ml.

0	45	1.0	135
0.25	86	1.25	135
0.5	107	1.5	135
0.75	135	1.75	135

TABLE 62. pO_2 measurements of charging wort at air flow rates from 150 ml. per min. to 800 ml. per minute.

Days Fermentation	pO_2 mm Hg.	Acetic Acid g/100 ml.
0	125	3.2
1	130	3.2
2	135	3.05
3	137	3.0
4	137	3.0
5	137	3.5
6	138	3.25
7	137	3.5
8	98	3.6
9	0	4.1
10	0	4.6
11	14	5.2
12	128	5.4
13	-	-
14	135	5.4
15	137	5.3

TABLE 63. pO_2 values obtained during acetification in a tower shaped fermentor.

	DAYS FERMENTATION																	
Ethanol	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2½%	1.7	1.7	1.7	2.8	3.9	3.9	3.8	3.7	3.6	3.5	3.5	3.4	3.4	3.2	3.0	3.0	2.9	2.7
3%	1.7	1.8	1.8	3.0	4.5	4.9	4.8	4.6	4.5	4.4	4.3	4.1	3.6	3.8	3.6	3.4	3.2	2.7
4%	1.7	1.7	1.7	3.0	5.6	5.9	5.7	5.6	5.4	5.3	5.1	4.9	4.7	4.5	4.2	4.0	2.9	3.8
5%	1.7	1.7	1.7	3.2	6.0	6.8	6.5	6.3	6.0	5.9	5.8	5.5	5.3	5.2	5.1	5.0	4.8	4.8
6%	1.7	1.7	1.8	2.1	5.5	7.6	7.4	7.3	6.9	6.6	6.2	6.0	5.8	5.5	5.2	5.0	4.8	4.7
7%	1.7	1.7	1.8	2.0	7.0	8.5	8.6	8.6	8.2	8.0	7.8	7.4	7.0	6.5	6.1	5.6	6.8	4.8
8%	1.7	1.7	1.7	1.9	3.0	7.0	9.5	9.8	9.4	9.2	8.9	8.7	8.5	8.1	7.9	7.7	7.4	7.2
9%	1.7	1.7	1.7	1.8	2.0	2.1	2.2	2.5	2.7	2.9	3.2	2.6	3.8	4.0	4.0	4.1	4.1	4.2
10%	1.7	1.7	1.7	1.8	1.8	1.8	1.8	2.0	2.0	2.0	1.8	2.0	2.0	2.0	2.0	2.1	2.1	2.1

TABLE 64. Acetic acid concentrations (% v/v) during acetifications with increasing substrate concentrations at 33°C.

	TEMPERATURE			
Day	20°C	25°C	30°C	35°C
0	1.08	1.08	1.08	1.08
1	1.09	1.08	1.08	1.08
2	1.09	1.09	1.08	1.09
3	1.17	1.2	1.44	1.6
4	1.26	1.5	2.1	2.75
5	1.29	2.1	3.7	4.5
6	1.56	3.75	4.7	4.7
7	5.76	5.1	4.8	4.65
8	6.3	5.6	4.9	4.65
9	6.48	5.65	4.9	4.65
10	-	-	-	
11	-	-	-	
12	7.02	5.75	5.1	4.75
Efficiency	97%	79%	70.6%	65.1%

TABLE 65. Acetic acid concentrations (% v/v) during acetifications at varying temperatures

	DAYS FERMENTATION					
	1	3	4	5	8	11
Acetaldehyde	29	29	235	68	8	1.8
Ethanol	9.2	8.5	8.4	2.8	0.1	.01
Acetic	138	51	53	200	400	900
Diacetyl	30	25	22	28	-	-
n-Propyl alcohol	-	-	-	-	-	-
Propionic acid	8	8	60	54	30	22
Sec-Butyl alcohol	-	-	-	-	-	-
iso-Butyl alcohol	10	2.5	2.0	2.2	7.0	7.2
iso-Butyric acid	-	-	-	-	-	-
Acetoin	2.1	7.0	6.0	3.4	130	138
iso-Amyl alcohol	31	31	12	6	2	-
iso-Butyl acetate	-	-	-	-	-	-
n-Amyl alcohol	-	-	-	-	-	-
sec-Butyl acetate	-	-	-	-	-	-
n-Amyl acetate	-	-	-	-	-	-
Volume of distillate	3 ml.	4 ml.	3.8 ml.	5 ml.	5.4 ml.	4.2 ml.

TABLE 66. Volatiles detected in distillates from tower fermentors. Concentrations in mg/100 ml. with the exception of ethyl alcohol % v/v

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VOLATILE CONSTITUENTS OF VINEGAR. I. A SURVEY OF SOME COMMERCIALLY AVAILABLE MALT VINEGARS

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Gas liquid chromatography was used to fractionate the alcohols, esters, carbonyls and acids in commercial malt vinegars. A qualitative and quantitative study was made using two different stationary phases; methyl formate, acetaldehyde, ethyl acetate, ethyl alcohol, isobutyl acetate, n-propyl alcohol, n-butyl acetate, isobutyl alcohol, amyl alcohol, acetoin, propionic acid and acetic acid were identified and estimated in eleven different malt vinegars.

The results of this survey were compared with the information previously found for malt and other types of vinegars. A preliminary assessment has been made of the origins of these volatiles.

Introduction

Malt vinegar has been used as a condiment for over 100 years but it is only in the last 50 years that its chemical nature has been investigated. Since the process of vinegar manufacture consists of a double fermentation where the alcohols obtained in the initial fermentation are subjected to acetification, it could be expected that a number of related alcohols, acids and esters would be found in the final product.

Difficulty was originally encountered in fractionating the volatiles in vinegar because the methods available were not sufficiently specific; analyses were therefore restricted to determinations of oxidation values, iodine values and ester values. Klinc[12] detected butyric acid in wine and vinegar and during the same year Pontin[19] determined acetone by its reaction with salicylaldehyde in alkaline solution.

With the advent of gas liquid chromatography (GLC) in 1952,[9] a new dimension in analytical technique was available for this type of investigation and was soon applied to fermentation products. A study of the volatile substances in beer was made by several workers,[10,23,7,20,16] all of whom used different concentration steps or stationary phases. Ribereau[21] determined ethyl acetate in wine using polyethylene glycol as stationary phase and Webb,[27] using both polyethylene glycol succinate and carbowax as stationary phases, detected acetone, isobutyraldehyde, isovaleraldehyde, isopropyl, isobutyl, iso-amyl and hexyl alcohols, methyl acetate and ethyl esters of formic, acetic, propionic, valeric, caproic, oenanthic, caprylic, capric, lauric, salicylic and cinnamic acids.

Application of GLC to vinegar began in 1962 with the quantitative estimation of ethanol in malt vinegar by Morgantini[18] who found a close correlation with the titrimetric assay. The volatiles of wine and spirit vinegars were investigated by Suomalainen,[22] who found that spirit vinegar contained only ethyl acetate whereas wine vinegar contained acetoin, iso-amyl alcohol, amyl alcohol, isobutyl alcohol and amyl acetate. He also concluded that an increased amount of esters developed in commercial vinegars during storage. Kahn *et al.*[11] examined cider and distilled vinegar and, using a suitable liquid phase, it was possible to resolve alcohols, esters, acids and 3 hydroxy-2-butanone on one gas chromatogram. Twelve compounds were found in cider vinegar and five in distilled vinegar. The compounds detected were methanol, ethyl alcohol, sec-butyl alcohol, a C-5 alcohol, methyl and ethyl acetate, ethyl lactate, 3 hydroxy-2-butanone, and acetic, propionic, isobutyric and a C-5 acid. Aurand *et al.*[1] also studied the volatile constituents of "distilled", "grain," "spirit" and "natural" vinegars. Cider vinegar contained nineteen components, wine contained seventeen, tarragon twenty, and distilled vinegar eleven. Four compounds

were common to all the samples examined: acetaldehyde, acetone, ethyl acetate and ethyl alcohol. Quantitative data were not obtained but qualitative differences were observed in the individual concentrations amongst a number of samples. It was concluded that the most important carbonyl compound contributing to vinegar flavour was diacetyl. Since esters are known for their aromatic odours, it was not surprising that they were the largest group of volatile flavour compounds. Moreover, the quality of a particular vinegar could be determined by the relative concentration of the alcohols and esters.

Experimental

Vinegar samples.—Samples of malt vinegar were obtained locally or, in a number of instances, directly from the manufacturers. Although sixteen samples were obtained, only eleven proved to be from different manufacturers. The age of the vinegar samples could not be ascertained.

Conditions for gas liquid chromatography.—A Pye series 104 Chromatograph equipped with a flame ionization detector was employed. Two glass columns, bore $\frac{1}{4}$ in., were used with lengths of five and seven feet. The 5-ft. column was packed with 10% polyethylene glycol (PEG) molecular weight 1000, on 85–100 mesh acid washed celite. The 7-ft. column was packed with porous polymer beads 50–80 mesh Porapak Q.

In both instances the rate of flow of carrier gas nitrogen was 45 ml. per min., hydrogen 45 ml. per min. and air 500 ml. per min. A constant temperature of 90° C. was used with the PEG 1000 and 200° C. with the Porapak Q. In all the experiments the sample volume was 2 μl. The amplifier attenuation was normally 500 but when the larger volatile constituents were estimated the attenuation was increased to 2000.

The retention time of acetic acid on PEG 1000 was in the region of $1\frac{1}{2}$ hr., and, as would be expected, excessive tailing of the peak ensued. To eliminate the effect of acetic acid, sodium hydroxide was added to neutralize the acids. 10 ml. of vinegar was titrated with normal sodium hydroxide solution using a pH meter to monitor the pH change. A titration curve was plotted and the corresponding amount of alkali added to the vinegar before gas chromatography.

Standards.—Reference compounds, as far as possible, were of "AnalaR" quality and were obtained from B.D.H. Co. Ltd., Poole, Dorset. Dilutions were made in distilled water. Table I lists the concentrations of reference compounds used for quantitative analyses.

Identification of volatiles.—To obtain some indication of the volatiles present, a preliminary treatment of two neutralized vinegar samples was made. To one sample was added a saturated solution of potassium permanganate which removed aldehydes, leaving

TABLE I

Concentration of Standard Solutions for Calibration

Standard	Concentrations for calibration (mg. per 100 ml.)			
Ethyl formate	2·5	5	10	20
Acetaldehyde	2·5	5	10	20
Ethyl acetate	1·25	2·5	5·0	7·5
Ethyl alcohol	25	50	75	100
Isobutyl acetate	0·625	1·25	2·5	5·0
n-Propyl alcohol	1·25	2·5	5·0	10
n-Butyl acetate	1·25	2·5	5·0	10
n-Amyl alcohol	2·5	5·0	7·5	10
Acetoin	25	50	75	100
sec-Butyl alcohol	1·25	2·5	5·0	10
Isobutyl alcohol	1·25	2·5	5·0	10
Isobutyric acid	0·625	1·25	2·5	5·0
Iso-amyl alcohol	1·25	2·5	5·0	10
sec-Butyl acetate	1·25	2·5	5·0	10
n-Amyl acetate	0·625	1·25	2·5	5·0
	(g. per 100 ml.)			
Acetic acid	1·0	2·0	4·0	6·0
Propionic acid	0·125	0·25	0·5	1·0

ketones and to the second sample was added 2 ml. of 50% sodium hydroxide per 10 ml. of vinegar, to remove esters. The volatiles present after treatment were fractionated using PEG 1000 as stationary phase. The ninth peak, which was subsequently confirmed as being acetoin, was removed after treatment with potassium permanganate. Both the ester peaks (ethyl formate and ethyl acetate) were removed after treatment with the alkali. Table II illustrates the results obtained.

TABLE II

RESULTS AFTER PRELIMINARY TREATMENT WITH POTASSIUM PERMANGANATE AND STRONG ALKALI (Stationary phase PEG 1000)

Peak retention time	Retention after treatment with NaOH	Retention time after treatment with potassium permanganate
(mm.)	(mm.)	(mm.)
6	—	6·5 (ester)
9	9	9
12	—	12 (ester)
16·5	16·5	16·5
23	—	—
25	—	—
29	28	—
33	33	35
68	70	70
115	132	— (aldehyde)

Further identification of all the volatiles was made by comparing the retention times of diluted aqueous reference compounds with those found in malt vinegars. Subsequently the identification was confirmed by the addition of the respective standards to neutralized vinegar samples. This served two objectives—to check retention times and to determine the recovery of known amounts of standards. Figs. 1 and 2 illustrate typical chromatographs with vinegar number four using PEG 1000 and Porapak Q respectively.

Table III lists the retention times of the volatiles detected using PEG 1000 as stationary phase and Table IV lists the volatiles detected using Porapak Q. Ethyl formate and ethyl acetate were not detected with Porapak Q. In addition to the volatiles detected with PEG 1000, propionic acid, isobutyl alcohol, isobutyric acid, iso-amyl alcohol, sec-butyl acetate and n-amyl acetate were detected in measurable quantities with Porapak Q.

TABLE III

RETENTION TIMES OF IDENTIFIED VOLATILES WITH PEG 1000 AS STATIONARY PHASE

Peak number	Retention time (mm.)	Volatile
1	6	ethyl formate
2	9	acetaldehyde
3	11·5	ethyl acetate
4	16	ethyl alcohol
5	22	isobutyl acetate
6	25	n-propyl alcohol
7	29·5	n-butyl acetate
8	31·5 and 33·5	effect of water and isobutyl alcohol
9	69	amyl alcohol
10	114	acetoin

The effect of water was noted with both stationary phases. The retention time of water was near to that of isobutyl alcohol with PEG 1000; water = 33·5 and isobutyl alcohol = 31·5 mm. Quantitative estimation of isobutyl alcohol with PEG 1000 was not possible because of the effect of water. The retention time of isobutyl alcohol on Porapak

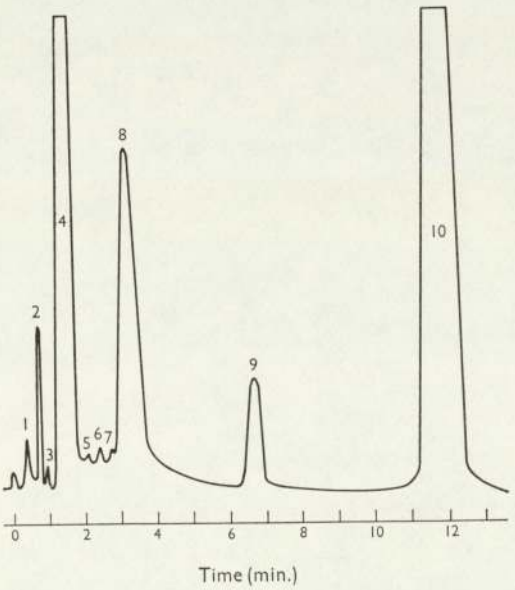

Fig. 1.—Gas chromatogram of neutralized vinegar on PEG 1000. 2 μl. of sample. Chart speed 1 cm. per hr. For identification of peaks, see Table III.

TABLE IV

RETENTION TIMES OF VOLATILES DETECTED WITH PORAPAK Q

(Peak 1 acetaldehyde; 2 ethyl alcohol; 3 acetic acid; 4 n-propyl alcohol; 5 propionic acid and sec-butyl alcohol; 6 isobutyl alcohol; 7 isobutyric acid; 8 acetoin; 9 iso-amyl alcohol; 10 isobutyl acetate, 11 n-amyl alcohol; 12 sec-butyl acetate; 13 n-amyl acetate)

Vinegar number	Retention times (mm.)												
1	12	17	29	38	64	75	114	124	162	180	189	216	237
2	10	17	29	38	65	78	—	124	160	181	192	—	—
3	12	17	29	38	64	78	—	122	164	184	—	—	—
4	12	18	29	38	63	76	—	124	162	180	193	—	—
5	12	18	29	38	64	76	—	127	162	180	192	—	—
6	12	18	29	38	64	75	—	124	162	180	192	—	—
7	12	18	29	38	64	75	—	125	164	180	192	—	—
8	12	18	29	38	64	77	—	127	160	180	188	197	—
9	12	18	29	38	63	77	117	125	160	184	192	199	—
10	12	18	29	38	63	76	116	125	160	180	191	204	—
11	12	18	29	38	65	76	—	125	160	180	191	—	—
Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13

Q coincided with that of acetic acid and the alcohol was quantitatively assayed after neutralization of the vinegar sample.

Quantitative results.—Table V lists the concentration of volatiles in the eleven vinegars examined with PEG 1000; VI gives the mean of three results using Porapak Q. Close correlation was found between the quantitative results obtained with both stationary phases.

The ethyl alcohol concentration obtained by gas liquid chromatography was compared to that obtained by the method of Conway.[2]

The mean of three ethyl alcohol estimations by the Conway technique was calculated. Table VII lists the results obtained by both methods. The Conway method gave higher values but this would be expected, since the method has poor specificity. It was noted, however, that the sum of the amyl, propyl and ethyl alcohols by GLC approximates to the results of the Conway diffusion methods,

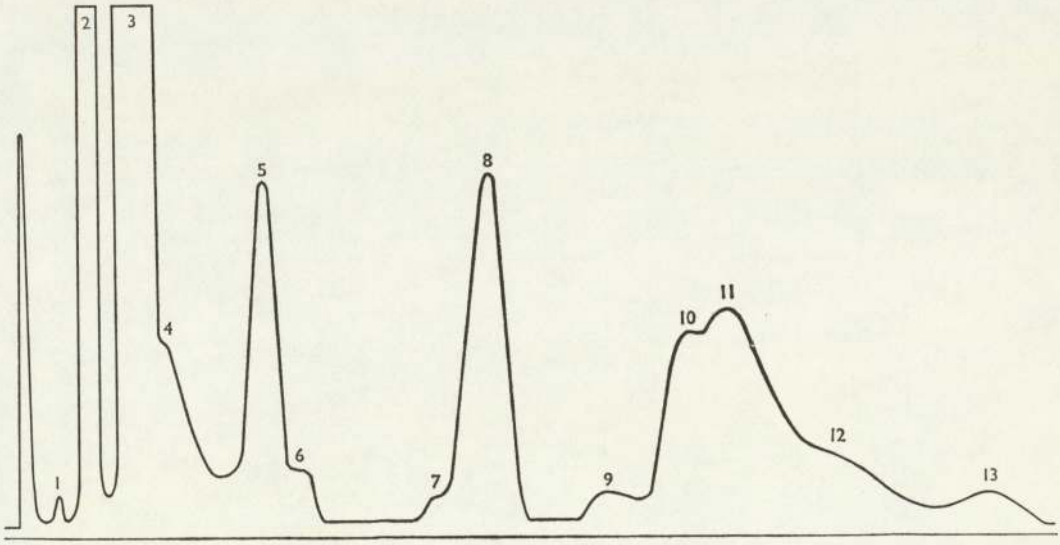

Fig. 2.—Gas chromatogram of vinegar on Porapak Q. 2 μl. of sample. Chart speed 30 in. per hr. Identification of peaks, see Table IV.

CONCENTRATION OF VOLATILES IN ELEVEN VINEGAR SAMPLES
(mg. per 100 ml.)

Peak number	Compound	t_R (min.)	v_R (ml. per min.)	Vinegar number											S.D.	% Recovery
				1	2	3	4	5	6	7	8	9	10	11		
1	Ethyl formate	6	27	3·26	18·52	10·89	9·0	15·67	10·89	5·62	18·47	10·62	18·54	9·0	0·4986	97·4–104·8
2	Acetaldehyde	9	40·5	30·18	4·21	4·21	6·18	17·55	16·66	14·04	17·55	12·28	17·55	114	0·3868	96·7–101·8
3	Ethyl acetate	11·5	517·5	0·98	8·32	—	8·51	5·0	6·88	9·72	12·96	6·84	—	5·26	0·1547	88·8–97·3
4	Ethyl alcohol	16	720	38·7	53·3	40·3	172·8	119·5	84·9	28·8	50·4	74·9	30·2	67·7	1·796	87·3–108
5	Isobutyl acetate	22	990	0·25	0·99	0·71	1·98	1·42	1·98	0·71	4·03	0·71	2·0	2·0	0·423	89·8–117
6	n-Propyl alcohol	25	1·125 lit.	3·95	0·99	4·93	2·95	2·95	2·46	2·46	2·46	2·95	2·95	19·7	1·072	104·5–122
7	n-Butyl acetate	29·5	1·327	0·4	3·56	0·4	7·17	3·56	6·72	1·35	16·63	6·72	3·56	0·79	0·423	100·6–122
8	Isobutyl alcohol and/or effect of water	32·5	1·462	—	—	—	—	—	—	—	—	—	—	—	—	—
9	n-Amyl alcohol	69	3·115	4·86	3·65	5·5	4·54	4·86	3·65	9·04	6·80	3·56	6·48	6·8	0·187	96·2–108·5
10	Acetoin	114	5·13	190	20	57	370	690	770	520	940	450	550	970	0·0224	87·5–115

TABLE VI

CONCENTRATION OF VOLATILES IN VINEGAR USING POROPAK Q
(Results, mg. per 100 ml., as the Means of Three Determinations)

Peak number	Compound	t_R (min.)	v_R	Vinegar number										
				1	2	3	4	5	6	7	8	9	10	11
1	Acetaldehyde	12	480	30·42	4·46	4·50	6·0	17·0	16·64	13·2	17·75	12·0	16·82	106
2	Ethyl alcohol	17	680	42·0	50·3	42·4	185·7	121·4	84	26·2	52·0	75·0	29·8	74·4
3	Acetic acid (g./100 ml.)	29	1160	5·4	5·1	5·1	5·25	5·2	5·1	5·5	5·2	5·1	5·1	5·1
4	n-Propyl alcohol	38	1520	3·22	0·41	4·27	2·40	2·30	2·20	2·71	2·21	2·00	2·70	2·20
5(a)	Propionic acid (g./100 ml.)	64	2560	1·20	2·50	0·10	0·21	0·15	0·50	0·14	0·14	0·15	0·14	0·16
5(b)	sec-Butyl alcohol	64	2560	6·21	8·47	2·50	11·10	4·62	5·11	4·62	5·20	2·55	2·55	3·0
6	Isobutyl alcohol	74	2960	8·40	2·19	3·18	2·70	2·30	3·81	8·46	3·00	8·46	8·21	4·2
7	Isobutyric acid	116	4640	1·25	—	—	—	—	—	—	—	5·0	4·62	—
8	Acetoin	124	4960	198	18·4	58·2	374	710	765	524	940	440	540	960
9	Iso-amyl alcohol	160	6400	1·42	0·92	1·4	1·82	0·91	0·87	1·4	1·4	0·84	3·2	2·4
10	Isobutyl acetate	180	7200	0·25	1·10	0·74	1·90	1·32	1·88	0·80	4·0	0·81	2·1	1·98
11	n-Amyl alcohol	192	7680	4·92	3·41	5·32	4·68	4·86	3·54	8·92	6·1	3·62	6·0	6·1
12	sec-Butyl acetate	205	8200	5·84	—	—	—	—	—	—	6·71	5·42	5·56	—
13	n-Amyl acetate	237	9480	5·2	—	—	—	—	—	—	—	—	—	—

suggesting that the diffusion technique estimates all the alcohols found in malt vinegars.

Quantitative assays of all volatiles fractionated with Porapak Q were made from measurements of peak heights because no tailing of the peaks occurred. Porapak Q also eliminated the undesirable effects of water.

TABLE VII

RESULTS OBTAINED FOR THE ESTIMATION OF ETHYL ALCOHOL BY GLC AND CONWAY DIFFUSION

Vinegar number	Ethyl alcohol by GLC	Total alcohol by GLC	Ethyl alcohol by Conway diffusion
	(mg. per 10 ml.)	(mg. per 100 ml.)	(mg. per 100 ml.)
1	38·7	66·17	64·0
2	53·3	68·7	72·0
3	40·3	56·9	54·0
4	172·8	195·4	200·0
5	119·5	134·5	135·0
6	84·9	100·4	105·0
7	28·8	54·91	56·0
8	50·4	68·31	70·6
9	74·9	92·4	97·0
10	30·2	52·8	53·0
11	67·6	65·5	85·0

DISCUSSION

The examination of malt vinegar by gas chromatography has provided information as to the volatile acids, alcohols, esters and carbonyls present. The compounds detected include acetic, propionic and isobutyric acids, ethyl, n-propyl, sec-butyl, isobutyl, iso- and n-amyl alcohols; isobutyl, sec-butyl, ethyl and n-amyl acetates; ethyl formate, acetaldehyde and acetoin.

The identification of all the volatiles was confirmed by adding suitable standards to vinegar samples before gas chromatography. Porapak Q was the packing material of choice for fractionating acetic, propionic and isobutyric acids but in addition, isobutyl alcohol, iso-amyl alcohol, sec-butyl acetate and n-amyl acetate were detected. Isobutyric acid was found in three samples only, although isobutyl acetate was found in all the samples. The remaining eight samples did not contain the acid.

In the work reported by Aurand *et al.*[1], the carbonyls detected were acetaldehyde, acetone, diacetyl, acetoin and isobutyraldehyde. Three further carbonyls, isovaleraldehyde, methyl valeraldehyde and methyl isobutyl ketone were detected in distilled vinegars. The alcohols were ethyl, propyl, n-butyl, sec-butyl, amyl, iso-amyl and sec-active amyl alcohol. The acetate esters of these alcohols were also fractionated and in addition ethyl formate, methyl formate and ethyl propionate were detected. Kahn *et al.*[11] also examined cider and distilled vinegars detecting in addition acetic, propionic and isobutyric acids. In the present work with malt vinegars, two carbonyls only were detected, acetaldehyde and acetoin. Although n-butyl alcohol was not detected in malt vinegar, the iso-alcohol was. The esters, ethyl propionate and ethyl formate, were not fractionated by either stationary phase.

Hashimoto & Kuroiwa,[8] Powell & Brown,[20] Kunitake,[14] Morgan[17] and Harold *et al.*[7] have examined by gas chromatography the volatile components of beer. All the alcohols detected in malt vinegar in this investigation have been identified by these workers in beer. As would be expected, the esters and acids are more numerous in malt vinegar owing to acetification.

In alcoholic fermentation, butane 2,3 diol and acetoin are formed[15] and in the production of vinegar the diol is oxidized and the acetoin level rises.[3] These two compounds are formed when pyruvate condenses with co-enzyme A to give α-acetolactic acid; decarboxylation of this acid gives acetoin and thence butane 2,3 diol by reduction. Grinsky[6] describes the oxidation of both the meso and D(−) forms of butane 2,3 diol in *Acetobacter aceti*. In addition, two other pathways have been demonstrated in *Acetobacter*. In the first, Kling[13] describes the conversion of pyruvate to acetolactate and then to acetoin with the liberation of carbon dioxide. It is not therefore surprising that acetoin was present in large quantitites in all the samples investigated, the range being between 0·19 and 0·97 g. per 100 ml. Aurand *et al.*,[1] in their work, detected diacetyl both in "distilled" and "grain" vinegars. None was detected in the present work, suggesting that oxidation of acetoin to diacetyl does not occur in *Acetobacter*. This confirms the work of De Ley.[3]

Acetaldehyde is a product of both yeast and *Acetobacter* fermentations and since acetoin is subsequently formed, minor quantitites only would be detected. In the present work only

one sample was found to have large quantities of acetaldehyde.

Ehrlich[15] suggests that the higher alcohols are produced during fermentation by deamination and decarboxylation of the amino acids: thus leucine produces iso-amyl alcohol, isoleucine gives active amyl alcohol and valine isobutyl alcohol. Thorne[25] has confirmed Ehrlich's work. Small amounts only of n-butanol and n-amyl alcohols were detected in these analyses; the corresponding amino acids nor-valine and nor-leucine are of limited natural occurrence.

Of the alcohols containing four carbon atoms, isobutyl alcohol is the most widely distributed in fermentation products and was found in all samples. Sec-butyl alcohol has also been characterized in beer and was detected in measurable quantities when the propionic acid was neutralized. In all the samples of vinegar examined, n-propyl alcohol was detected; this has also been found in beer. All species of *Acetobacter* are able to oxidize n-propanol to propionic acid.

Iso-amyl alcohol, found in all the vinegars, has been found in beer fusel oil by Dupont[4] and by Webb *et al.*[27] It has also been reported by Tanaka[24] that *Acetobacter* oxidizes amyl alcohol.

During vinegar fermentation of alcohol, the higher alcohols are partially converted to the corresponding acids and esters. In this survey only four vinegars contained isobutyl acetate. Propionic and isobutyric acids are oxidation products of fusel oil components. Propionic acid was found in all the vinegar samples, isobutyric being identified in three vinegars only.

During the conversion of ethyl alcohol to acetic acid many of the compounds in fermented wort are chemically changed, completely or in part; thus sec-butyl alcohol and propyl alcohol produce isobutyric and propionic acids respectively.

The esters isobutyl acetate, sec-butyl acetate and n-amyl acetate could be direct fermentation products or they could be produced by esterification during conditioning or formed during pasteurization.[22]

It is hoped to relate the findings of the present work to the process of vinegar manufacture, in order to provide an understanding of the complex changes that occur during the storage of the alcoholic wash, the acetification process, and the storage and ageing of vinegar.

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VOLATILE CONSTITUENTS OF VINEGAR. II. FORMATION OF VOLATILES IN A COMMERCIAL MALT VINEGAR PROCESS

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Gas liquid chromatography was used to fractionate the alcohols, esters, carbonyls and acids formed in a commercial process of vinegar manufacture. Ethyl, n-propyl, sec-butyl, iso-butyl, n-amyl and iso-amyl alcohols, and iso-butyl, sec-butyl and n-amyl acetates were produced during the initial alcoholic fermentation. During acetification, propionic acid, iso-butyric acid and acetoin were produced. There was no increase in the number of volatiles or in their concentration during the maturing process of the charging wort or the subsequent treatments and storage period of the vinegar after acetification. A loss in the lower boiling point volatiles occurred during the storage of all the samples examined. An assessment has been made of the formation of these volatiles both before and after acetification.

Introduction

A study of the formation of volatiles during the initial alcoholic fermentation of the malt vinegar process requires an understanding of the alcohols and esters that have been found in ale beer. Both qualitative and quantitative investigations have been made on beer and beer headspace both by distillation and extraction techniques prior to gas liquid chromatography (GLC). Enebo[7] was the first to apply GLC to the analysis of the volatile alcohols and esters of beer. Later, Harold *et al.*[10] reported that several of the volatiles originated from the malt and thirty-seven components were identified after ether extraction of wort and beer.

The selectivity of liquid substrates for the analysis of lower alcohols and esters of lower fatty acids was investigated by Kamibayashi *et al.*[17] who also analysed the lower

fatty acids and fusel oils in fermentation products. Van der Kloot *et al.*[23] detected iso-amyl alcohol, active amyl alcohol, ethyl acetate and amyl acetate in a steam distillate of beer. Bavisotto *et al.*[5,6] also recorded the formation of these volatiles in bottled beer during storage, and noted the changes in acetaldehyde, acetone and ethanol in wort during fermentation, a relationship being found between the fermentation temperature, wort composition, pitching rate and yeast strain. Arkima & Sihto[1] investigated the change in the volatile substances during fermentation of various beers and Sihto & Arkima[21] found a correlation between fusel oil and the flavour of Finnish beers.

The formation of fusel alcohols was correlated with yeast growth by Nordström[20] who had also earlier investigated the formation of esters from alcohols and acyl CoA. Lüers & Opekar[18] and Bavisotto *et al.*[5,6] reported an increase in volatiles during lagering while Glimm & Stentzel[9] and Arkima & Sihto[1] found no such increase. Hashimoto & Kuroiwa[11] confirmed the formation of esters during lagering and showed that their concentration remained constant. Glimm & Stentzel[9] reported an increase in higher alcohols with higher temperatures of fermentation.

Little is known of the volatiles or their formation in the acetification process. Kahn *et al.*,[16] who investigated the volatiles of cider vinegar by GLC, suggested that yeast fermentation of cider yields fusel alcohols, which in turn are partially converted into the corresponding acids, methyl and ethyl acetates also being produced during acetification. Aurand *et al.*[4] examined the volatile components in the vapours of natural and distilled vinegars; tarragon vinegar containing twenty components and distilled vinegars containing eleven.

In our previous work acetaldehyde, ethyl, n-propyl, n-amyl, sec-butyl, iso-butyl and iso-amyl alcohols, iso-butyl, sec-butyl and n-amyl acetates, acetic, iso-butyric and propionic acids and acetoin were detected and measured in a variety of commercial malt vinegars. To determine how these volatiles are formed in malt vinegar, samples were obtained during a process of vinegar manufacture and examined by GLC.

Experimental

Gas chromatography.—A Pye series 104 gas chromatograph equipped with a flame ionization detector was used. The columns the packing material, Porapak Q, 50–80 mesh and the operating conditions were similar to those used in our previous communication.[15]

Process.—Samples were obtained from the Barbourne Brewery, Worcester which manufactures an all malt vinegar by the Quick-vinegar method. The process consists of milling and mashing brewer's malt to produce on cooling a wort of original gravity between 1065 and 1070. The wort is fermented to a gravity of 1000 in 7 days at a temperature between 65 and 75° F. with a brewer's yeast and then transferred to an acetifier containing some acetified liquid from the previous fermentation. It is then sparged over birch twigs with aeration until the alcohol is oxidized to approximately 85% at a temperature of 35° C. On cooling the acetified liquor is stored for two weeks in vats, when it is centrifuged. The vinegar is finally diluted to 5% acetic acid, pasteurized and bottled.

Samples.—Samples were taken at the following points in the process:

Sample 1. Wort immediately after seeding with yeast.

Sample 2. The alcoholic wash (charging wort) which had been stored for 2 weeks prior to acetification.

Sample 3. Acetified liquid immediately at the end of acetification.

Sample 4. Vinegar post filtration and pasteurization.

Samples 5 and 6. Two sealed bottles of vinegar at the end of manufacture.

Samples 7, 8 and 9. Vinegar samples obtained from the manufacturer. These were 4, 6 and 12 months old respectively.

Samples 1 to 4 were stored at room temperature for 4 weeks, Sample 1 being allowed to ferment during this storage period. Using a sterile syringe and needle, 2 ml. aliquots were taken periodically (Tables I to IV).

Samples 5 and 6 were stored for 5 weeks. Aliquots were taken at the beginning and end of this storage period. At the end of 5 weeks the stopper of Sample 5 was replaced with a sterile bung. A sterile needle was used to pierce the bung which was left with the syringe in position. By this means aliquots were taken intermittently as shown in Table V. Sample 6 was stored without any form of seal.

TABLE I

VOLATILES DETECTED IN FERMENTING CHARGING WORT. SAMPLE 1

Volatile (mg./100 ml.)	Storage time (days)								
	1	3	5	7	9	14	16	23	27
Acetaldehyde	—	—	—	—	—	—	—	10	110
Ethyl alcohol ($\times 10^{-3}$)	1·05	6·8	6·3	7·85	7·75	7·65	7·55	6·75	6·6
Acetic acid ($\times 10^{-3}$)	0·4	0·5	0·5	0·5	0·6	0·6	0·7	1·1	1·3
n-Propyl alcohol	—	1·6	1·8	3·4	3·3	2·4	2·4	2·3	2·6
Sec-butyl alcohol	—	—	—	4·0	3·8	3·8	4·5	4·0	4·0
Iso-butyl alcohol	—	2·1	2·1	2·4	2·1	2·1	3·2	14·0	18·2
Acetoin	—	4·6	4·8	4·8	5·2	5·0	8·0	10·1	15·3
Iso-amyl alcohol	—	—	2·4	2·8	3·6	3·4	4·0	4·7	5·0
Iso-butyl acetate	—	0·5	1·5	1·5	1·5	1·5	1·8	2·5	2·5
n-Amyl alcohol	—	—	—	15·0	18·0	34·8	40·2	40·0	38·0
Sec-butyl acetate	—	—	—	5·0	5·0	4·8	5·0	5·0	5·0

Each aliquot was chromatographed twice at two attenuations, the ethyl alcohol and acetic acid being determined at $10^3 \times 20$ and all other volatiles at $\times$ 500.

RESULTS

Table I lists the volatiles detected in the fermenting wort. At this stage of vinegar manufacture a number of alcohols were formed—ethyl alcohol, n-propyl, iso-butyl, iso-amyl and n-amyl alcohols. Acetic acid was detected throughout this alcoholic fermentation but no detectable quantities (*i.e.*, over 0·2 mg. per 100 ml.) of either propionic or iso-butyric acids were found. The esters iso-butyl, sec-butyl and n-amyl acetates were produced simultaneously with the production of the corresponding alcohols. During the 16th day of storage some acetification occurred when acetaldehyde, which was not detected earlier, was found.

The volatiles in the alcoholic wash obtained commercially (Sample 2) were similar to Sample 1, after it had fermented under laboratory conditions. Acetification again occurred after 7 days accompanied by the formation of acetaldehyde and acetoin. Table II lists the concentration of the volatiles during the storage of this sample.

Sample 3, obtained after acetification, contained all the volatiles detected in our previous survey although the concentrations were higher because the stage at which the vinegar was diluted (to approximately 5%) had not been reached. Propionic and iso-butyric acids were detected and the acetoin concentration had increased from 15·3 mg. to 310 mg. per 100 ml. During storage there was a loss of the lower boiling point volatiles by evaporation as can be seen in Table III.

The volatiles in the vinegar after filtration and pasteurization (Sample 4 in Table IV) were similar to the Sample 3, although, as would be expected after dilution to 5%

TABLE II

VOLATILES DETECTED IN THE ALCOHOLIC WASH, STORED FOR FOUR WEEKS. SAMPLE 2

Volatile (mg./100 ml.)	Storage time (days)								
	1	3	5	7	9	14	16	23	27
Acetaldehyde	—	—	—	15·0	20·2	40·6	80·1	120·4	130·7
Ethyl alcohol ($\times 10^{-3}$)	6·8	6·7	6·7	5·5	5·4	5·4	5·25	4·3	4·1
Acetic acid ($\times 10^{-3}$)	0·75	0·8	0·8	1·65	1·7	1·7	2·3	2·55	2·7
n-Propyl alcohol	1·6	1·8	2·0	1·6	1·5	1·4	1·4	1·2	0·8
Sec-butyl alcohol	4·5	4·5	4·8	5·0	4·5	5·0	5·2	5·5	5·6
Iso-butyl alcohol	19·5	19·0	19·4	18·0	16·5	17·1	16·3	16·0	15·0
Acetoin	5·0	6·2	8·0	10·4	15·0	15·1	20·7	25·4	20·5
Iso-amyl alcohol	5·0	4·8	5·0	4·0	6·0	4·7	6·9	5·7	5·4
Iso-butyl acetate	1·5	1·5	1·8	1·5	1·8	1·9	1·5	1·9	2·0
n-Amyl alcohol	45·2	45·0	48·1	40·7	38·6	40·4	36·5	36·5	36·0
Sec-butyl acetate	5·8	10·2	10·4	9·6	9·6	9·6	10·2	10·1	10·1

TABLE III

VOLATILES DETECTED IN SAMPLE 3 OBTAINED AFTER ACETIFICATION

Volatile (mg./100 ml.)	Storage time (days)								
	1	3	5	7	9	14	16	23	27
Acetaldehyde	15·3	18·3	8·0	8·0	5·6	4·2	4·0	4·0	4·0
Ethyl alcohol ($\times 10^{-3}$)	0·3	0·3	0·35	0·35	0·3	0·3	0·3	0·2	0·1
Acetic acid ($\times 10^{-3}$)	9·3	9·4	9·4	9·2	8·15	7·4	6·9	6·25	6·1
n-Propyl alcohol	1·8	1·8	1·6	1·6	1·3	1·6	1·5	1·9	0·8
Propionic acid	145	148	156	150	150	143	145	140	140
Sec-butyl alcohol	2·7	2·0	1·8	1·8	1·9	2·5	2·1	2·7	1·8
Iso-butyl alcohol	5·7	5·1	4·9	4·9	5·0	5·0	5·7	5·5	5·5
Iso-butyric acid	4·8	4·0	4·0	5·2	5·4	4·8	4·8	5·0	4·8
Acetoin	310	315	312	302	308	304	294	280	276
Iso-amyl alcohol	1·5	1·5	1·8	0·5	0·4	0·5	0·5	0·4	0·5
Iso-butyl acetate	1·4	1·5	1·5	1·9	1·5	1·5	1·6	1·8	1·6
n-Amyl alcohol	8·3	5·4	8·4	4·8	5·4	5·4	5·2	5·0	5·2
Sec-butyl acetate	10·2	9·8	9·8	10·0	10·2	10·0	10·0	9·8	9·2

acetic acid, their concentration was correspondingly lower. During storage the acetaldehyde and ethyl alcohol were completely lost and this loss was accompanied by a decrease in acetic acid, n-propyl alcohol and sec-butyl alcohol.

Table V lists the results obtained during storage of two finished samples of vinegar. No change in the number of volatiles or in their concentrations was detected in the sealed bottle. The opened bottle decreased rapidly in its concentration, particularly of acetaldehyde which was lost completely in two days. In addition, all the volatiles decreased over the 22 days that the sample was examined.

The three aged vinegar samples available from the manufacturer, Samples 7, 8 and 9 did not indicate any changes in the volatiles pattern. A detectable loss in all the volatiles was, however, found in the 12-month old vinegar.

DISCUSSION

During the fermentation of wort to produce the alcohol for acetification, a number of alcohols in addition to ethyl alcohol are produced; these include n-propyl, sec-butyl, iso-butyl, iso-amyl and n-amyl alcohols. Since acetic acid was present throughout the fermentation, probably owing to infection, it is not surprising that by esterification sec-butyl and n-amyl acetates were produced. During the third week of storage, acetification took place, accompanied by an increase in acetic acid and acetoin and the appearance of acetaldehyde. The volatiles found in the fermenting wort have been detected by Hashimoto & Kuroiwa[11] in beer. The formation of the volatile alcohols and esters is completed by the end of the primary fermentation which is in agreement with Hashimoto, in his study of the volatiles in beer.

During the storage of the alcoholic wash

TABLE IV

VOLATILES DETECTED AFTER FILTRATION AND PASTEURIZATION COMPARED TO RESULTS OBTAINED DURING THE PREVIOUS SURVEY.[15] SAMPLE 4

Volatile (mg./100 ml.)	Survey results	Storage time (days)								
		1	3	5	7	8	14	16	23	27
Acetaldehyde	30·4	12·6	8·2	5·2	4·8	4·8	4·0	3·8	—	—
Ethyl alcohol	42·0	130	110	110	110	100	42	35	22	—
Acetic acid ($\times 10^{-3}$)	5·4	5·4	5·45	5·4	5·3	5·2	5·0	4·5	4·2	4·2
n-Propyl alcohol	3·22	1·8	1·4	1·4	1·8	1·3	1·3	1·3	1·8	1·7
Propionic acid	120	135	130	130	128	128	135	130	130	130
Sec-butyl alcohol	6·2	1·8	1·0	1·4	1·8	1·3	1·5	1·0	1·2	0·8
Iso-butyl alcohol	8·4	3·5	3·5	3·8	3·4	3·4	3·5	3·9	3·6	3·6
Iso-butyric acid	1·25	3·6	3·4	4·2	3·4	3·4	3·4	3·4	3·2	3·0
Acetoin	198	200	214	216	230	220	220	220	208	204
Iso-amyl alcohol	1·4	0·8	0·6	0·8	0·7	0·8	0·8	0·8	0·5	0·5
Iso-butyl acetate	0·3	1·6	1·6	1·3	1·7	1·1	1·3	1·6	1·6	1·6
n-Amyl alcohol	4·9	4·6	4·6	4·3	4·7	4·1	4·3	4·6	4·6	4·6
Sec-butyl acetate	5·8	7·0	7·4	7·0	7·2	7·4	7·0	7·0	7·0	7·4

TABLE V

VOLATILES DETECTED IN TWO BOTTLES OF FINISHED VINEGAR, ONE OF WHICH WAS NOT SEALED. SAMPLES 5 AND 6

Volatile (mg./100 ml.)	Unopened bottle: days stored						Opened bottle: days stored				
	0	35	37	41	48	57	0	2	6	13	22
Acetaldehyde	15·0	15·0	14·0	13·2	14·2	14·8	15·0	14·0	—	—	—
Ethyl alcohol	165	185	160	160	165	160	160	150	120	80	32
Acetic acid ($\times 10^{-3}$)	4·8	4·8	4·8	4·8	4·8	4·8	4·8	4·4	4·0	3·8	3·4
n-Propyl alcohol	1·8	1·9	1·9	1·9	1·8	1·8	1·5	1·4	1·4	0·3	0·2
Propionic acid	125	128	125	125	129	125	125	120	120	120	122
Sec-butyl alcohol	2·4	2·4	2·0	1·8	1·8	2·0	2·4	2·4	1·6	1·4	1·0
Iso-butyl alcohol	3·0	3·0	3·2	3·0	3·0	3·0	3·0	3·0	3·0	3·8	2·8
Iso-butyric acid	3·5	3·1	3·8	3·8	3·2	3·2	3·8	3·1	2·8	2·8	2·6
Acetoin	200	210	208	210	210	210	200	215	208	200	184
Iso-amyl alcohol	0·4	0·6	0·6	0·6	0·4	0·6	0·5	0·6	0·6	0·4	0·2
Iso-butyl acetate	1·5	1·5	1·8	1·5	1·5	1·8	1·8	1·6	1·6	1·8	1·6
n-Amyl alcohol	4·8	4·6	4·6	4·2	4·8	4·5	5·0	4·5	4·4	4·6	4·4
Sec-butyl acetate	8·2	7·8	8·4	8·4	9·2	8·4	8·6	8·8	8·0	8·0	7·7

it is evident that the esters, sec-butyl and iso-butyl acetates, are produced during the alcoholic fermentation and not during subsequent acetification. This is contrary to Jenard's[14] suggestion that they are formed during conditioning and pasteurization.

A decrease in acetaldehyde, ethyl alcohol and acetic acid was evident after 12 days' storage of the acetified sample, and a smaller decrease was noted with propionic acid and acetoin. This loss was due to the evaporation of the lower boiling point volatiles. The gross increase in the acetoin level during acetification confirms the work of Garino-Canina[8] who also reported the oxidation of butane 2,3 diol; n-propyl alcohol was identified throughout the process although Henneberg[12] has shown that n-propyl alcohol was oxidized to propionic acid by *Acetobacter*. n-Amyl alcohol was oxidized during the acetification, a finding which is in agreement with Asai & Shoda[2] who reported rapid oxidation of this alcohol by *A. ascendens* and *A. aceti*. In this investigation, iso-butyl alcohol was oxidized to isobutyric acid, and similar oxidation has been reported by Visser't Hooft,[13] Mosel,[19] Asai[3] and Tanaka.[22] Iso-amyl alcohol was also oxidized but to a lesser extent; this confirms the work of Asai & Shoda.[2] Propionic acid was not converted to oxalic acid (as reported by Henneberg[12]) the concentration of which was constant throughout the period of storage. Both n-propyl and sec-butyl alcohols formed during the alcoholic fermentation were only partially oxidized and this agrees with the findings of Tanaka.[22]

The sample obtained after filtration and pasteurization did not show an increase in the number or concentration of volatiles. The same was true for the stored samples of finished vinegar and the aged vinegar. As may be seen from Tables V and VI, the only detectable change was due to evaporation of

TABLE VI

VOLATILES DETECTED IN AGED VINEGARS OBTAINED FROM MANUFACTURER. SAMPLES 7, 8 AND 9

Volatile (mg./100 ml.)	Age of vinegar		
	4 months	6 months	12 months
Acetaldehyde	20·8	18·5	12·7
Ethyl alcohol	182	172	162
Acetic acid ($\times 10^{-3}$)	5·0	5·0	4·8
n-Propyl alcohol	2·0	4·2	1·6
Propionic acid	146	123	115
Sec-butyl alcohol	4·4	5·0	5·0
Iso-butyl alcohol	3·8	4·2	2·1
Iso-butyric acid	6·4	7·4	5·0
Acetoin	160	150	154
Iso-amyl alcohol	1·4	1·5	1·2
Iso-butyl acetate	1·5	1·3	1·2
n-Amyl alcohol	3·8	3·8	3·6
Sec-butyl acetate	6·0	4·4	4·4

TABLE VII

SUMMARY OF THE VOLATILES DETECTED IN A PROCESS OF VINEGAR MANUFACTURE. RESULTS ARE CALCULATED FOR DIRECT COMPARISON ON THE BASIS OF A VINEGAR CONTAINING 5% ACETIC ACID

Volatile (mg./100 ml.)	Wort	Charging wort	Acetified sample	Sample after filtration and pasteurization	Survey results*	Stored sample (6 months)
Acetaldehyde ..	—	—	7·8	12·2	28·5	18·5
Ethyl alcohol ($\times 10^{-3}$)	5·0	5·0	0·2	0·11	0·40	0·17
Acetic acid ($\times 10^{-3}$)	0·32	0·6	5·0	5·0	5·0	5·0
n-Propyl alcohol ..	2·2	1·5	1·1	1·6	3·0	4·2
Propionic acid ..	—	—	80	125	112	123
Sec-butyl alcohol ..	2·6	3·5	1·5	1·6	6·0	5·0
Iso-butyl alcohol ..	1·5	14·4	3·0	3·3	8·0	4·2
Iso-butyric acid ..	—	—	2·8	3·4	1·1	7·4
Acetoin	3·0	5·9	162	184	180	150
Iso-amyl alcohol ..	1·8	3·7	0·8	0·7	1·3	1·5
Iso-butyl acetate ..	0·96	1·3	0·8	1·4	0·2	1·3
n-Amyl alcohol ..	9·6	35·6	4·3	4·2	4·3	3·8
Sec-butyl acetate ..	3·2	7·7	5·4	6·7	5·4	4·4

* n-Amyl acetate (5·0 mg./100 ml.) also present.

the lower boiling point volatiles. The head brewer of this brewery, Mr. M. Phillips, has obtained evidence from practical experience that there appears to be little change in general characteristics of vinegar during the "maturation" process which is now confirmed by this investigation.

The formation of the volatiles is summarized in Table VII. The results have been recalculated for comparison on the basis of a vinegar containing 5% acetic acid. It is concluded that no loss of volatiles would ensue from vinegar while the storage container was sealed for at least 6 months.

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VOLATILE CONSTITUENTS OF VINEGAR. III. FORMATION AND ORIGIN OF VOLATILES IN LABORATORY ACETIFICATIONS

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The volatiles produced during commercial malt vinegar manufacture have been examined using laboratory scale acetifications. The formation and changes of these volatiles in surface culture, aerated and submerged fermentations were found to be substantially similar to those found in the commercial process. Losses resulted with higher temperatures and submerged aeration. With maximum aeration the levels of propionic acid and acetoin decreased. The oxidations of n-propyl, isobutyl, sec-butyl, n-amyl and iso-amyl alcohols relative to ethanol have been compared to those found by Tanaka. The metabolic pathways which are probably responsible for the origin of these volatiles are discussed.

Introduction

Investigations into the formation of alcohols, esters, acids and carbonyls in commercial malt vinegars and in a commercial process of malt vinegar manufacture have already been reported.[11,12] The investigation on the manufacturing process was made on isolated samples throughout the process, which made the interpretation of the origin of these volatiles difficult. In this paper methods are described for the continuous examination of the volatiles produced during acetification on a laboratory scale using commercial charging wort and acetifying cultures to allow a more definitive attempt to clarify the metabolic pathways responsible for the volatiles found in malt vinegar.

Materials and Methods

Three distinct techniques, which are described below, were employed in acetifying a commercial alcoholic wash. The wash or charging wort was derived from an all-malt brew and obtained from a malt vinegar manufacturer who employs the Quick method for acetification. The acetifying culture was obtained from the same source directly from commercial acetaters.

Surface culture.—One litre of charging wort was inoculated with 50 ml. of the acetifying culture. A two-litre flat bottomed conical flask was used so that a maximum surface area was obtained giving a volume to surface area ratio of 2·5 to 1. The flask was kept at 22° C. without agitation for 13 days. 2-ml. samples were removed daily using a sterile pipette from which 2-μl. aliquots were examined by gas liquid chromatography (GLC).

Aerated flask.—A similar culture was prepared and aerated by means of a glass tube containing at one end a sintered glass filter of porosity 3. Air at a flow rate of 20 ml. per min. was passed through this culture. The flask was kept at 22° C. for 13 days and daily samples were taken and examined as in the surface culture technique.

Submerged aeration technique.—Two jacketed tower-shaped fermentors each 55 cm. by 9 cm. diameter were aerated from the lower ends by means of sintered glass discs (porosity 3). The first fermentor was used at room temperature and the second was maintained at 30° C. by circulating attemperated water around the jacket. 1250 ml. of charging wort and 50 ml. of acetifying culture were added to both fermentors which were aerated at a flow rate of 40 ml. per min. Air was passed through for seven days; 2-ml. samples were taken daily and examined by GLC.

Gas chromatography.—A Pye 104 chromatograph equipped with a flame ionization detector was employed throughout. Conditions for GLC were as described previously.[11,12] Ethyl alcohol and acetic acid were estimated

at an attenuation of $10^3 \times 20$ and all other volatiles at $\times 500$.

Results

Surface culture.—The results obtained for this method are seen in Table I. 99·4% of the ethyl alcohol was oxidized to acetic acid. 0·4% acetic acid was found in the initial charging wort. Complete oxidation of n-amyl alcohol occurred and n-propyl, isobutyl, sec-butyl and iso-amyl alcohols were oxidized to a lesser extent. Isobutyric acid was detected on the sixth day when maximum oxidation of ethyl alcohol had occurred. Propionic acid increased from 32 mg. per 100 ml. to 360 mg. per 100 ml. and subsequently decreased to its initial concentration. Maximum acetoin production coin-

TABLE I

Volatiles During Acetification by Surface Culture

Volatile (mg./100 ml.)	Days												
	0	2	3	4	5	6	7	8	9	10	11	12	15
Acetaldehyde	2·0	14·0	23·4	36·6	39·1	42·0	36·4	6·0	6·0	3·0	2·9	2·0	2·0
Ethyl alcohol ($\times 10^3$)	8·75	7·0	5·3	5·2	4·6	2·0	1·3	0·4	0·2	0·14	0·12	0·09	0·01
Acetic acid ($\times 10^3$)	0·4	1·5	2·4	3·35	5·3	7·5	9·0	9·1	9·1	9·0	8·9	8·8	8·8
n-Propyl alcohol	1·8	1·6	0·9	1·2	1·0	0·9	0·8	0·9	0·7	0·7	0·8	0·7	0·7
Propionic acid	32	200	360	360	336	88	88	40	32	32	32	32	32
sec-Butyl alcohol	1·4	1·6	1·6	1·6	1·4	1·8	1·4	1·4	1·6	1·4	1·4	1·4	1·4
Isobutyl alcohol	15·2	13·4	13·2	10·4	10·0	14·0	13·0	13·0	13·5	13·1	12·6	13·2	13·2
Isobutyric acid	—	—	—	—	—	2·0	1·2	1·2	1·2	2·0	2·2	2·4	2·2
Acetoin	3	76	152	206	232	246	246	246	224	206	146	130	130
Iso-amyl alcohol	20	18	18·2	18·4	18·0	18·0	16·4	16·6	14·0	12·0	10·2	10·4	10·0
Isobutyl acetate	2·8	3·0	2·6	2·6	2·9	2·9	2·6	2·6	2·6	2·6	2·6	2·8	2·4
n-Amyl alcohol	4·8	6·2	3·8	2·3	2·0	1·7	1·8	1·8	0·7	—	—	—	—
sec-Butyl acetate	5·0	5·2	5·2	5·0	4·2	5·0	4·2	4·2	4·8	5·1	5·2	5·6	5·6

Traces of n-amyl acetate also present throughout.

TABLE II

Volatiles During Acetification in an Aerated Flask

Volatiles (mg./100 mg.)	Days												
	0	1	2	3	4	5	6	7	9	10	11	12	13
Acetaldehyde	2·0	4·8	14·6	14·2	18·0	18·0	6·0	6·0	6·0	6·0	6·0	4·4	4·4
Ethyl alcohol ($\times 10^3$)	8·65	8·3	7·7	4·6	4·2	2·9	2·0	0·71	0·20	0·06	0·03	0·03	0·01
Acetic acid ($\times 10^3$)	0·4	0·85	1·1	1·9	2·4	3·4	4·5	7·6	6·5	5·8	5·6	5·6	5·6
n-Propyl alcohol	1·8	1·8	1·8	1·6	2·0	1·6	1·1	0·8	0·8	0·8	1·0	0·8	0·8
Propionic acid	32	44	480	360	336	320	51	40	28	24	23	24	24
sec-Butyl alcohol	1·4	1·4	1·2	1·8	1·4	1·4	1·6	1·6	1·8	1·4	1·4	1·4	1·4
Isobutyl alcohol	14·2	14·0	13·0	13·5	13·5	13·5	13·5	13·5	13·2	13·0	13·0	13·1	12·8
Isobutyric acid	—	0·5	0·8	1·8	3·4	5·8	6·0	6·0	8·0	4·0	3·5	3·0	3·0
Acetoin	3	21	58	151	266	256	252	128	132	132	132	132	132
Iso-amyl alcohol	15·0	15·0	15·0	12·0	6·0	4	3·6	4·6	4·2	3·2	3·0	1·6	1·4
Isobutyl acetate	2·4	2·5	3·2	3·2	2·5	2·7	2·7	2·5	2·7	2·9	2·8	2·6	2·5
n-Amyl alcohol	4·8	6·2	6·2	4·8	4·8	2·8	2·0	0·6	—	—	—	—	—
sec-Butyl acetate	3·0	3·1	3·0	3·0	3·3	3·5	3·3	3·3	3·5	3·5	3·5	3·5	2·5

Traces of n-amyl acetate also present throughout.

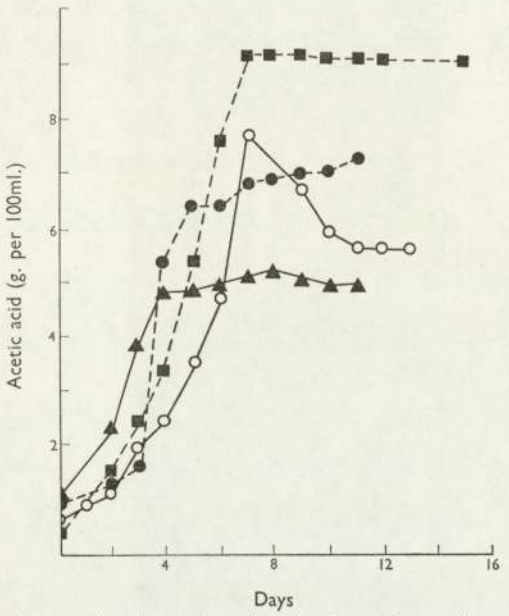

Fig. 1.—Acetic acid production in surface culture ○, aerated flask ■, tower fermentor at 22° C. ● and tower fermentor at 30° C. ▲.

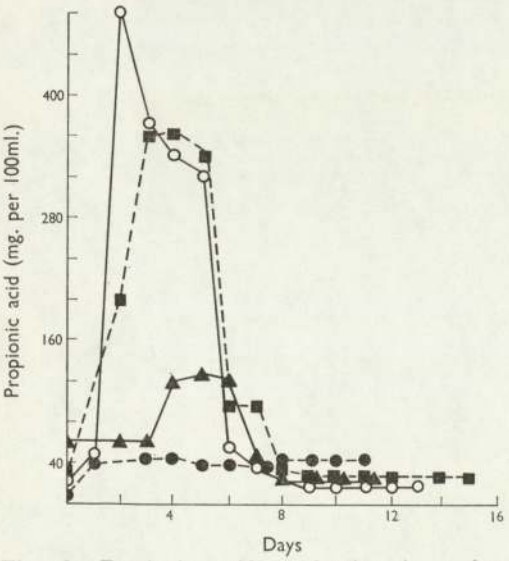

Fig. 2.—Propionic acid production in surface culture ○, aerated flask ■, tower fermentor at 22° C. ● and tower fermentor at 30° C. ▲.

cided with the maximum oxidation of ethyl alcohol. Both sec-butyl and n-amyl acetates were unchanged. Figs. 1, 2 and 3 illustrate the changes noted in concentrations of acetic acid, propionic acid and acetoin.

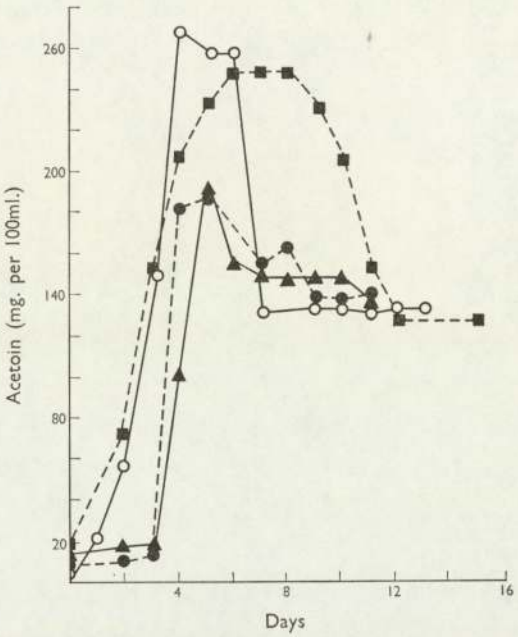

Fig. 3.—Acetoin formation in surface culture ■, aerated flask ○, tower fermentor at 22° C. ● and tower fermentor at 30° C. ▲.

Aerated flask.—In this method aeration was continued for 13 days during which time some evaporation of the ethanol would be expected. The efficiency of oxidation was 89%. Since the surface culture was considered as a control experiment, by comparison a total of 1·5 g. per 100 ml. ethyl alcohol was calculated to have been lost in this experiment. The results obtained are given in Table II. Maximum acetic acid was produced on the seventh day as in the surface culture technique. The results obtained for the oxidation of n-propyl, isobutyl, sec-butyl and n-amyl alcohols were also similar to those obtained for the surface culture. On the other hand, iso-amyl alcohol oxidation was increased from 30% to 69·3% and iso-butyric acid was detected on the first day of acetification. The changes in the concentrations of acetoin and sec-butyl and n-butyl acetates were also similar to the surface culture.

Tower-shaped fermentor.—The ethyl alcohol oxidation in the fermentor at 22° C. was found to be 93·3% but at 30° C. it was only 68%. The efficiency was lowered in these experiments because the higher temperature in conjunction with submerged aeration

conditions caused considerable evaporation. Oxidation of all the alcohols with the exception of n-propyl was increased at both temperatures, n-propyl being reduced from 61·1% to 10·6% of 22° C. Tables III and IV list the results. Maximum acetic acid production was detected during the fifth day at both 22° C. and 30° C., *i.e.*, 2 days earlier than in either the surface or the aerated flask method. The changes in acetic acid concentrations are shown in Fig. 1. Both isobutyric and propionic acids were in lower concentration than in the other two techniques. Figs. 2 and 3 illustrate the changes in concentrations of propionic acid and acetoin during this type of acetification.

Discussion

The surface culture acetification may be considered as a control experiment in that no deliberate aeration with its accompanying loss of volatiles was used. In the surface culture method 9·1% acetic acid was produced which suggests that *Acetobacter aceti* was not the only acetifying organism. Henneberg[8] determined the maximum acetic acid production in various species of *Acetobacter*; two only were capable of producing 9·0% acid (*A. ascendens* 9·0% and *A. schutzenbachii* 10·9%).

The oxidation of ethanol is suggested by Henneberg[7] to be accomplished in two steps, the first being the oxidation of ethanol to acetaldehyde and the second the formation of acetic acid from acetaldehyde. Two distinct reactions have been proposed for the formation of acetic acid from acetaldehyde. Neuberg[16] suggested that a dismutation reaction occurred in which one mole of acetaldehyde is reduced to ethanol while another mole is oxidized to acetic acid. This reaction proceeds aerobically up to 50%, the remaining half of acetaldehyde being oxidized directly to acetic acid. With good aeration the oxidation and dismutation proceed side by side. However, Wieland[20] reported low dismutation activity in *A. ascendens* and he proposed a pathway where acetaldehyde was oxidized rather than dismutated to acetic acid. Furthermore, Simon[17] reported that the ratio of dismutation to direct oxidation is dependent upon pH, with dismutation

TABLE III

Volatiles During Acetification in a Tower-shaped Fermentor at 30° C.

Volatile (mg./100 ml.)	Days										
	1	2	3	4	5	6	7	8	9	10	11
Acetaldehyde	5	22	22	35	52	84	15	15	10	10	4
Ethyl alcohol (× 10^3) ..	7·5	7·2	5·0	4·0	3·0	0·7	0·2	0·2	0·12	0·05	—
Acetic acid (× 10^3) ..	1·1	2·4	3·7	4·7	4·8	4·9	4·9	5·1	4·9	4·9	4·8
n-Propyl alcohol ..	2·4	2·4	2·4	2·0	2·0	2·2	2·0	2·0	2·0	2·0	2·2
Propionic acid	56	56	56	120	128	122	40	34	32	32	32
sec-Butyl alcohol ..	3·4	3·6	3·4	3·4	3·0	1·8	2·0	2·0	1·8	1·8	1·8
Isobutyl alcohol ..	14·0	14·0	12·0	7·0	7·0	7·0	7·0	7·2	7·2	6·0	6·2
Isobutyric acid ..	2·2	1·8	1·8	5·4	5·0	4·6	4·0	3·1	3·0	2·8	2·8
Acetoin	14·0	18·0	18·0	98	186	155	146	144	144	142	130
Iso-amyl alcohol ..	20·0	14·0	10·0	10·0	10·0	5·0	4·0	4·0	3·0	3·0	2·0
Isobutyl acetate ..	1·7	2·2	2·2	2·5	2·4	2·4	2·6	2·4	2·5	1·6	1·6
n-Amyl alcohol	6·2	5·8	5·0	2·4	2·6	0·6	—	—	—	—	—
sec-Butyl acetate ..	20	30	32	22	24	22	20	20	20	20	20

Traces of n-amyl acetate present throughout.

TABLE IV

VOLATILES DURING ACETIFICATION IN A TOWER-SHAPED FERMENTOR AT 22° C.

Volatile (mg./100 ml.)	Days										
	1	2	3	4	5	6	7	8	9	10	11
Acetaldehyde	—	—	10	10	10	22	35	20	—	—	—
Ethyl alcohol (× 10^3) ..	7·5	7·3	7·2	3·0	2·6	1·2	0·4	0·2	0·1	0·1	—
Acetic acid (× 10^3) ..	1·08	1·29	1·59	5·17	6·26	6·29	6·56	6·7	6·8	6·8	7·0
n-Propyl alcohol ..	2·8	2·8	2·5	2·5	2·8	2·5	2·2	2·5	2·5	2·5	2·4
Propionic acid	10	42	48	42	36	42	40	50	50	50	50
sec-Butyl alcohol ..	3·4	3·4	3·0	3·0	3·0	3·0	2·8	2·2	2·2	2·2	2·2
Isobutyl alcohol ..	12·0	12·0	12·0	10·0	10·0	8·9	8·8	8·8	10·0	10·0	9·2
Isobutyric acid ..	2·0	1·8	1·8	1·4	0·8	0·8	0·6	0·8	0·8	0·8	0·8
Acetoin	—	10	14	180	184	142	140	160	134	134	134
Iso-amyl alcohol ..	24	20	20	20	10·0	10·0	8·0	3·0	2·2	1·6	1·4
Isobutyl acetate ..	2·9	2·9	2·9	2·9	2·2	2·4	2·4	2·6	2·8	2·8	2·8
n-Amyl alcohol	5·8	5·8	5·6	4·8	4·8	4·4	4·0	2·6	0·6	—	—
sec-Butyl acetate ..	20	20	20	20	20	20	18	20	20	20	18

Traces of n-amyl acetate present throughout.

increasing in basic conditions and decreasing in acidic conditions. The results of the present work suggests that a direct oxidation reaction was taking place since conditions were acid and only low concentrations of acetaldehyde were detected in all three acetification techniques (less than 84 mg. per 100 ml.). The speed with which oxidation occurs is dependent upon the amount of available oxygen in the medium. This is illustrated by the fact that the tower fermentor oxidized the ethanol in 6 days compared to 9 days for surface and aerated techniques. However, the tower fermentors had lower efficiencies or lower total conversion of ethanol due to evaporation; furthermore when the acetification temperature was increased to 30° C. the efficiency was even lower because of greater evaporation.

The investigation of Ehrlich[14] suggested that the higher alcohols are produced during fermentation by deamination and decarboxylation. The pathways probably responsible for the oxidation products and oxidation of the other volatiles as judged by the present experiments are considered in detail as follows:

Isobutyl alcohol.—This alcohol was found in the charging wort and was oxidized in all the methods examined and also in the commercial process. The further oxidation of isobutyl alcohol to isobutyric acid was observed by Visser't Hooft,[9] Mosel,[15] Asai[1] and Tanaka.[19] The results of the surface culture and aerated flask confirms the work of Tanaka[19] (see Table V) who has reported that isobutyl alcohol was oxidized up to 6% relative to ethanol; on the other hand, a greatly increased oxidation was obtained in both tower fermentors at 22° C. and 30° C. An increased oxidation of this volatile is obtained by good aeration and a temperature of 30° C. The oxidation of isobutyl alcohol at 30° C. was 50% and at 22° C., 28·3%.

Iso-amyl alcohol.—Two pathways have been proposed for the production of iso-amyl alcohol. In the first Genevois & Lafon[4] suggested a pathway where two molecules of acetate produce aceto-acetate which is then

TABLE V

RELATIVE ACTIVITY OF ACETOBACTER SPP. ON PURE SUBSTRATES (TANAKA) COMPARED TO ACTIVITIES FOUND IN THE PRESENT STUDY

Substrate	% Oxidation relative to ethanol						
	A. peroxydans	*A. rancens*	*A. aceti*	Surface culture	Aerated flask	Tower at 22° C.	Tower at 30° C.
Ethyl alcohol ..	100	100	100	100	100	100	100
Acetaldehyde ..	47·3	53·6	40·5	85·8	66·6	100	88·4
n-Propyl alcohol ..	70·0	76·6	96·0	61·1	60·0	10·6	16·6
Isobutyl alcohol ..	14·0	6·1	6·0	11·1	4·8	28·3	50·0
sec-Butyl alcohol ..	8·4	0·8	0·7	11·1	11·1	35·3	48·8
n-Amyl alcohol ..	60·0	64·0	85·0	85·4	87·5	100	100
Iso-amyl alcohol ..	15·0	6·3	7·0	30·0	69·3	87·5	85·0

decarboxylated to acetone. The acetone is then reduced to isopropanol. Condensation of acetone with acetaldehyde produced $\beta\beta$-dimethylacrolein which forms iso-amyl alcohol by reduction. Secondly, Wostenholme & O'Connor[22] have described the formation of sterols and terpenes and in their scheme some of the intermediates could be reduced to iso-amyl alcohol.

It is unlikely that the pathway postulated by Genevois & Lafon[4] occurs during acetification since neither acetone nor isopropanol have been detected throughout the investigations. Contrary to the work of Tanaka,[19] iso-amyl alcohol was oxidized during acetification in both tower fermentors. The oxidation was found to be 85% relative to ethanol whereas Tanaka[19] found only 30% oxidation. Tanaka[19] also reported the oxidation of iso-amyl alcohol relative to ethanol with *A. peroxydans*, *A. rancens* and *A. aceti* to be 15%, 6·3% and 7·0% respectively. Table V lists the oxidation of all the alcohols relative to ethanol although the results given with the tower fermentors at 30° C. are approximate since loss by evaporation has been shown to be high at this temperature.

n-Propyl alcohol.—Guymon *et al.*[6] have presented evidence that n-propyl alcohol is synthesized by yeasts from α-ketobutyric acid, a known intermediate in the synthesis of isoleucine. Decarboxylation and reduction of this intermediate would result in the formation of n-propyl alcohol. The results of both the surface culture and the aerated flask methods in this investigation confirm the work of Tanaka[19] (61% of n-propyl alcohol oxidized) but in both aerated towers the oxidation was reduced, from 60% in the aerated flask to 10·6% in the fermentor at 22° C. These results would suggest that n-propyl alcohol oxidation was oxygen dependent in that a decreased oxidation was evident during submerged aeration.

n-Amyl alcohol.—The biosynthesis of leucine from valine has been investigated by Strassman *et al.*[18] using tracer studies. The pathway proposed includes a series of reactions analogous to the reactions of the tricarboxylic acid cycle between oxaloacetic acid and α-ketobutyric acid. This evidence was subsequently confirmed by Ingraham & Guymon[10] who suggested a comparable series of reactions for the biosynthesis of keto-n-valeric acid from α-ketobutyric acid and, by decarboxylation and reduction, n-amyl alcohol. The oxidation of n-amyl alcohol in this work was similar to that obtained by Tanaka[19] (see Table V). There is no evidence to suggest from our results which pathway was utilized in the production of n-amyl alcohol.

sec-Butyl alcohol.—Genevois & Lafon[4] proposed a series of reactions which involve the condensation of acetic acid to form isopropyl alcohol and amyl alcohol and the condensation of ethanol to form sec-butyl alcohol.

Tanaka[19] found 8·4% oxidation of sec-butyl alcohol relative to ethanol in *A. peroxydans*. Similar results were obtained for the surface culture and aerated flasks but an oxidation of 48·8% was found using the tower fermentors at 30° C. This suggests that sec-butyl alcohol oxidation is dependent upon good aeration, as is found in the tower fermentor.

Acetoin.—De Ley[3] obtained acetoin from D,L-lactate with 44 strains of *Acetobacter*. *A. rancens*, *A. pasteurianus* and *A. ascendens* converted lactate into acetoin up to 74% of the theoretical yield. Kitasato[13] suggested that acetoin was formed from pyruvate. Acetoin was also found to be produced from acetolactate. Wixom[21] found that *Acetobacter* dehydrated both the isoleucine intermediate, dihydroxy-β-methyl-n-valerate and the valine intermediate, dihydroxy-isovalerate. He then suggested that *Acetobacter* can carry out both valine synthesis and acetoin formation.

Maximum acetoin formation corresponded with maximum ethyl alcohol oxidation which occurred during the seventh day in surface culture, the sixth day in aerated flask and the fifth day in both tower fermentors. The acetoin was present in similar concentrations in the surface culture and the aerated flask, but in both tower fermentors the concentration had increased from 186 mg. per 100 ml. to 286 mg. per 100 ml. It is concluded that maximum acetoin formation occurs with increased aeration.

Propionic acid.—Many micro-organisms are capable of producing propionate[14] from glucose, glycerol, lactic acid and pyruvate, as shown below.

(*a*) 3 Hexose = 4 propionate + 2 acetic acid + 2 CO_2.

(*b*) Glycerol = propionic acid + water.

(*c*) Lactic acid = 2 propionic acid + acetic + CO_2.

(*d*) Pyruvate + 4H = propionate.

It is unlikely that pathways (*a*), (*b*) or (*c*) occur since complete fermentation with yeast would leave little residual carbohydrate and neither glycerol nor lactic acid has been detected in these experiments. Concentrations of propionic acid in the region of 360 mg. per 100 ml. were reached during acetification both in the surface culture and the aerated flask. Results for both tower fermentors were low, the maximum concentration at 30° C. being 128 mg. per 100 ml. and, at 22° C., 50 mg. per 100 ml. These results suggest that the increased concentration is due to lack of oxygen.

The conversion of pyruvate to propionate probably occurs during acetification by the following pathway:

(*a*) Pyruvate + methyl malonyl CoA = oxaloacetate + propionyl CoA.

(*b*) Succinate + propionyl CoA = succinyl CoA + propionate.

The fate of pyruvate during acetification is dependent upon aeration. This would appear to be the case since pyruvate is metabolized to propionic acid and also to acetoin.

Esters.—The esters isobutyl acetate, n-amyl acetate and sec-butyl acetate did not increase during acetification, which confirms our previous work.[12]

Of the volatiles found in charging wort (ethyl alcohol, acetic acid, n-propyl alcohol, sec-butyl alcohol, isobutyl alcohol, acetoin, iso-amyl alcohol, isobutyl acetate, n-amyl alcohol, sec-butyl acetate and n-amyl acetate) only ethyl alcohol, acetic acid, n-propyl alcohol, sec-butyl alcohol, isobutyl alcohol, acetoin, iso-amyl alcohol and n-amyl alcohol are altered during acetification. It would appear that in the commercial process the results resemble those found in the laboratory experiments conducted at 22° C. There has been no investigation on cider, spirit or wine vinegar to correlate such changes but the results agree with Tanaka's work with *Acetobacter* on pure substrates. The important feature of this investigation is that all the volatiles found are approximately in the same concentration in the acetified liquor regardless of the origin or type of acetification. This is also the case with the results of the commercial vinegars examined earlier.[12] The reason for this is not fully understood since both temperature and aeration influence the metabolic pathways used by *Acetobacter*. However, the process of acetification would appear to produce a pattern and concentration of volatiles in the external environment which is in equilibrium with the bacterial cell's most favourable metabolic balance providing conditions (albeit variable) are suitable for bacterial growth and fermentation. This is

TABLE VI

CONCENTRATION OF VOLATILES IN ACETIFIED LIQUIDS WHEN ETHYL ALCOHOL CONCENTRATION IS 0·2%

Volatile (mg./100 ml.)	Surface culture	Aerated flask	Tower at 22° C.	Tower at 30° C.	Commercial process	Survey
Acetaldehyde ..	6·0	6·0	15·0	20·0	4·0	30·4
Ethyl alcohol ($\times 10^3$)	0·2	0·2	0·2	0·2	0·2	0·2
Acetic acid ($\times 10^3$) ..	9·1	6·5	4·9	6·7	6·3	5·4
n-Propyl alcohol ..	0·7	0·8	2·0	2·5	1·9	3·2
Propionic acid ..	32	28	40	50	140	1·2
sec-Butyl alcohol ..	1·6	1·8	2·0	2·2	2·7	6·2
Isobutyl alcohol ..	13·5	13·2	7·0	8·8	5·5	8·4
Isobutyric acid ..	1·2	8·0	4·0	0·8	5·0	1·25
Acetoin	224	132	146	160	280	198
Iso-amyl alcohol ..	14·0	4·2	4·0	3·0	0·4	1·4
Isobutyl acetate ..	2·6	2·7	2·6	2·6	1·8	0·3
n-Amyl alcohol ..	0·7	—	—	2·6	5·0	4·9
sec-Butyl acetate ..	4·8	3·5	20·0	20·0	9·8	5·8
n-Amyl acetate ..	±	±	±	±	±	5·2

further illustrated by Table VI which compares the results obtained for acetified liquids when the alcohol is at approximately 0·2%.

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