

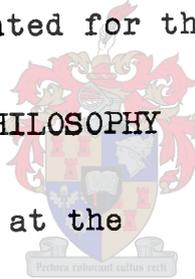
A STUDY OF WINE BOUQUET PRECURSORS IN GRAPES

by

C. S. DU PLESSIS.

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CHAPTER I.INTRODUCTION.

Bouquet is an important facet of wine quality. It is, in effect, the contribution of odourous volatiles in a wine.

In muscat wines, for example, the grape aromas are paramount, and not generally associated with fermentation. (Power & Chesnut, 1921; Sale & Wilson, 1926; Holley, Stoyla & Holley, 1955; Cordonnier, 1956; Webb, Kepner & Maggiore, 1966; Wenzel & De Vries, 1967). On the other hand, the so-called neutral varieties derive their wine bouquet from the fermentation process. These bouquet are generally much more complex than the primary grape bouquet. A considerable amount of work has been reported on the composition of wine volatiles, especially since the advent of gas chromatography (Haagen-Smit, Hirose & Wang, 1949; Kepner & Webb, 1956; Webb & Kepner, 1957; Mecke & De Vries, 1959; Mecke, Schindler & De Vries, 1960; Baraud, 1961; Bayer & Bässler, 1961; Diemair & Schams, 1960; Webb & Kepner, 1961; Drawert, 1962; Webb, 1962; Webb & Kepner, 1962; Webb, Ribereau-Gayon & Boidron, 1963; Lemperle & Mecke, 1964b; Rodopulo & Egorov, 1964; Bayer, 1966; Drawert & Rapp, 1966; Van Wyk, Kepner & Webb, 1967b,c.)

The esters, due to their pleasant odours are generally conceded to contribute to bouquet (Hennig & Villforth, 1942; Chauvet, 1950; Bayer & Reuther, 1956; Peynaud, 1956; Bayer, 1957a, b; Pisarnitskii, 1965). These esters are, no doubt, not the only functional factors; components such as alcohols, acetals and aldehydes have also been reported to contribute in either positive or negative capacities. (Villforth & Schmidt, 1954; Paul, 1958; Vogt, 1958; Amerine, Roessler & Filipello, 1959; Amerine & Cruess, 1960; Ingraham & Guymon, 1960; Morteau, 1960;

Sihto, Nykanen & Suomalainen, 1962; Sihto & Arkima, 1963; Wagener & Wagener, 1968).

Wine bouquet of the neutral varieties originates as a result of fermentation; it consequently shows no relation to grape aroma. It may be influenced by either wine-making procedures or the fermenting yeast (Wahab, Witzke & Cruess, 1949; Tritton, 1952; Cantarelli, 1954, 1955; Vogt, 1958; Amerine & Cruess, 1960). Other reports are not as definite on the influence of the yeast in its contribution to bouquet; this still appears to be a moot point (Fornachon, 1950, Schanderl, 1950; Crowther, 1951-52). However, even if wine-making procedures and yeast are standardized, variations in bouquet still occur from year to year. This is clearly due to variations in pertinent grape components.

Early work in the field of bouquet-forming components tended to concentrate on the free amino acids, probably as a result of the Ehrlich theory, that proteins were the causal agents. (Kutal'ova, 1931; Peynaud, 1939; Shuzui, Narisada & Hiramatsu, 1951; Valaise & Du Pont, 1951; Haehn, 1952; Markh & Scherbakova, 1958; Gadzhiev, 1959; Kazakova, 1959; Amerine & Cruess, 1960). Bidan & Andre (1958) on the other hand examined several wines but could find no correlation here. It may be significant that the amino acid-wine quality aspect has received very little attention over the last few years.

With respect to the formation of bouquet components during fermentation most work has been carried out on the esters. These have, however, been largely limited to ethyl acetate (Peel, 1950, 1951; Davies, Faulkner, Wilkinson & Peel, 1951;

Tabachnick & Joslyn, 1953a, b; Cantarelli, 1955; Peynaud, 1956; Nordström, 1964a). The latter author carried out a series of elegant studies indicating the role of co-enzyme A in ethyl acetate formation. He also reported upon the formation mechanism of higher esters where energy and Co-A were also advanced as critical factors (Nordström, 1962a, 1963, 1963a, 1964b). Pertinent reference to the sensory effect of these components were, however, not noted. Peynaud (1937) and Amerine & Cruess (1960) believed that only ethyl acetate, at concentrations below 200 ppm, was critical in a wine. At this stage, this appears to be an oversimplified statement.

Drawert, Rapp & Ulrich (1965); Drawert et al (1966) and Drawert, Rapp & Ullemeyer (1967) determined the must origin of a few wine volatiles. However, no correlation with the actual contribution of these components were reported.

In spite of the large volume of work on the identification of wine volatiles and also those of other fermented liquors and media, no certainty as to the relevant contribution of all of these components has yet been achieved. (Bayer, 1957a; Wagener & Wagener, 1968). The higher alcohols, which are formed catabolically from amino acids and acetic acid were, at the early stages of this investigation, regarded by many to be probable indicators of bouquet in wines. This surmise was based largely on these components reacting as moieties in chemical esterification. (Villforth & Schmidt, 1954; Thoukis, 1958; Haehn, 1952; Vogt, 1958; Mandel & Bieth, 1960). Lemperle & Mecke (1964a) noted that a relationship could not be achieved between gas chromatograms of wine volatiles and sensory evaluation.

This problem is basically one where functional sensory contributions, either in a singular or concomittant sense, with probable synergistic effects, is not yet understood. In wines there are an exceedingly large number of volatiles. Bouquet analysis is already very complicated. The position is however made still more complex by the many grape volatiles which are carried over into the wine. (Webb, 1962; Chaudhary, Kepner & Webb, 1964; Van Wyk, Webb & Kepner, 1967c). Nawar & Fagerson (1962) have also indicated that interpretation of results of both headspace and concentrate analyses must be approached with caution since one may represent an over-simplified picture and the other an unbalanced and untrue one.

The techniques applied to bouquet investigation have thus far been usually limited to gas chromatographic analyses of the volatiles. An alternate approach would be to utilize simple synthetic fermentation media, which would in the first instance be standard, which is certainly not the case in wines, and induce herein a wine bouquet, by means of added grape components. This would considerably decrease the "background" components. Recently reported work has shown that wine bouquet is significantly decreased by ion exchange treatment of the must (Du Plessis, 1964). These findings, furthermore, indicated a varietal difference in bouquet-forming or inducing components. Ion exchange treatment can absorb i.a. amino acids, ammonia, cations as well as specific vitamins from solutions (Kunin, 1958). The latter components are known to be important in fermentation (Ribereau-Gayon & Peynaud, 1952; Reiff, Kautzmann, Luers & Lindemann, 1960). Consequently, their removal from grape must and re-addition either singly or in combination or at varying concentrations presents a unique method in determining their

influence upon bouquet formation.

The latter technique offered a highly promising approach to this problem. Since it was also indicated that potential bouquet components were held by the resin it consequently offered a lead in the determination of the nature of such substances. The reported methodology was, therefore, applied in this project which was approached as the initial phase of a broad study. The aim was to utilize grape components in a synthetic fermentation medium to achieve a wine bouquet.

C H A P T E R II.METHODS OF ANALYSIS.

The methods of analysis used in this study are given hereunder:-

1. Volatile acidity: Determined with the Cash assembly (Amerine, 1955).
2. Total alcohol: Determined with pycnometer.
3. Higher alcohols: Determined by the A.O.A.C. method (1960).
4. Fermentation rate: Determined by weight loss as a function of time. Since by far the largest part of this weight decrease was due to CO₂ formation, it was also used as a measure of the rate of CO₂ formation.
5. Amino acids.
 - (a) Paper chromatography.

The single dimensional technique was used in preference to the two dimensional. The qualitative method of McFarren (1951) was applied quantitatively. The systems phenol-pH 9.0 buffer, m-cresol-pH 4.0 buffer and benzyl alcohol-butyl alcohol (1:1) - pH 4.0 buffer were used for quantitating aspartic acid, glycine, threonine, glutamic acid, serine, leucine and iso-leucine and phenyl alanine. Other tested systems are noted in Appendix (Table 1). The samples were applied as narrow one inch streaks. This gave better separations than spots.

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The conditions used are set out below:-

Effective length (descending)	16 in. Whatman No. 1.
Equilibration	Overnight (25°C ± 2°).
Run	48 hours (25°C ± 2°).
Drying	2 days in fume cupboard.
Spray	0.5% ninhydrin in acetone containing 2% acetic acid (7% for pH 9 papers). 30 ml per sheet. Dried for 15 min. at 60°C.

To facilitate a uniform flow-through of the solvent the sheets were trimmed with pinking shears.

The separated components were delineated in pencil, cut out and snipped into 10.0 ml methanol.

The coloured extracts were read at 520 mμ in a 1 cm. cuvette (Phenyl alanine, 2cm cuvette). The averaged values of 6 replications were used. Calibration standards were run with each sample. Individual values sometimes showed variations of up to 20%. In some cases where separations were inexplicably poor, chromatograms were re-run.

Prior to analysis, sugars in grape juices were separated from amino acids by cation exchange chromatography (Du Plessis, 1963). The column eluates were concentrated to half the volume of the initial sample and 200⁸00 μl applied.

(b) Automated column procedure.

Where amino acid metabolism was investigated, the former procedure proved too inaccurate and time-consuming for full analysis. An automated system (Beckman, Unichrom) was consequently used for this purpose.

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The parameters applied were as specified by the manufacturers. A sample of 0.5 - 1.0 ml was used.

6. Dry yeast weight.

The yeast strain W.E.1 (Research Institute for Oenology and Viticulture, Stellenbosch) was used throughout this work.

After completion of fermentation at 24-25°C the sample bottles were connected to a nitrogen filled Buchner flask which was in turn connected to a water reservoir with a head of approximately 3 feet. By opening the water reservoir cock nitrogen was forced into the sample bottle. The clear supernatant sample was displaced with nitrogen and made to flow into a nitrogen filled storage bottle. By gently tipping the sample during this procedure practically all the supernatant could be cleanly removed off the yeast.

The yeast was quantitatively washed into graduated centrifuge tubes with 10 ml water and centrifuged at 750 xg for 10 min. The washing was repeated twice with 5 ml ethanol.

The yeast was finally dried at 90°C for 24 hours and weighed.

7. Total nitrogen of yeast.

The yeast sample was digested by standard micro-Kjeldahl technique and samples diluted to contain not more than 120 ppm N. The analyses were carried out on a Technicon Auto Analyzer following the Technicon procedure (Technicon Methodology N-3b). Standards from 20-120 ppm N were run before and after every 10 samples. Concentrations were

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determined from the calibration graph.

8. Thiamine.

The method as described in A.O.A.C. (1960) was followed. The chromatographic tubes each contained approx. 3 gm Decalso. Their flowrates were approx. 0.5 ml min⁻¹.

Twenty and 75 ml quantities of untreated and ion exchange treated grape musts were respectively taken for analysis. Five ml aliquots of the prepared samples were taken for thiamine determinations.

Recovery of thiamine from a synthetic sample was 98.1%.

9. Organoleptic evaluation.

Where sufficient material was available statistical analyses were applied to establish significance. Two and three sample tests were used. (Amerine, Roessler & Filipelo, 1959). In each case only one factor viz. bouquet was examined. Since this unit approach simplified the tests considerably, non-statistical evaluation agreed with statistical evaluation where differences were large. Consequently, where sample volumes were limited, a non-statistical approach was used.

10. Phenols and organic acids by paper chromatography.

Whatman No. 1 and No. 3MM paper was used in all these studies. The solvents used were 6% acetic acid (Bate-Smith, 1964) and n-pentanol: 5M formic acid (1:1) (Buch, Montgomery & Porter, 1952). As the two phases of this latter solvent did not separate readily, the mixture was held overnight

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at 10°C and then separated.

Equilibration was carried out for 4 hours in glass cabinets lined with paper. The 6% acetic and the pentanol: formic acid solvents ran the effective 16 ins. of the chromatogram in 3 and 12 hours, respectively.

The chromatograms were dried over-night in a forced draught convection oven at room temperature.

The chromatograms were examined under U.V. light before and after treatment with ammonia vapour (Seikel, 1962; Whiting, 1964). The hydroxy compounds were visualised by spraying with a 5% phosphomolybdic acid (in methanol) solution, drying at 80°C for 5 min. and saturating with ammonia vapour. (Stahl & Schorn, 1965). The phenols appeared as very dark blue spots on a white background. The acids were visualized by bromophenol blue indicator spray (Buch et al. 1962).

11. Infra-red spectroscopy.

Bands from paper chromatograms were eluted with water. The fractions were dried in a nitrogen stream and held in a vacuum desiccator.

Potassium chloride was used for pressing wafers in a 5mm. vacuum die. (Hannah, 1963). A Beckman IR.7 spectrophotometer with a beam condenser was used for the analysis.

12. Esters.

Esters were quantitatively determined by gas chromatography.

It was initially found that the synthetic fermented media contained, relative to the esters, large quantities of

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higher alcohols. Although several stationary phases viz. beeswax, neo-pentyl glycol succinate, di-ethylene glycol succinate, di-nonyl phthalate, FFAP and Apiezon M. were tested in $\frac{1}{8}$ in. O.D. columns, none were wholly satisfactory. This was primarily due to the tailing of higher alcohols which overlapped the following esters and made their quantitation either inaccurate or impossible. The tailing reducers, sodium capronate (Bayer, 1959), stearic acid and Manoxal O.T. (Harva, Kivalo & Keltakellio, 1959) were subsequently examined with Apiezon M, a stationary phase used in the ester analysis of wines (Wagener et al 1968). Although the latter two effected good separations they caused excessive bleeding at 130-150°C which interfered with the determination of ethyl n-decanoate. Purification of Manoxal O.T. (di-octyl ester of sodium sulphosuccinic acid) by distilling off volatile contaminants, decolourising with charcoal and extracting with sodium bicarbonate solution and pentane, overcame this problem.

The separation parameters used are given below:-

Detector (Dual)	Flame ionisation
Column	10 ft. x $\frac{1}{8}$ in. O.D. s/s
Stationary phase	5% Apiezon M plus 0.8% Manoxal O.T. of Apiezon M.
Solvent	CH ₂ Cl ₂
Packing	Chromosorb G, AW, DMCS, 60-80 mesh
Inlet temperature	230°C
Detector	230°C
Flow rate (Helium)	20 cc/min.
Programme	See Fig. 11
Sample size	25-40 µl

Recoveries were checked in synthetic made-up samples using AR redistilled esters in CH₂Cl₂ and applying the same

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procedure as noted hereunder for the experimental samples. These samples also contained iso-pentanol and 2-phenethanol at 197.05 and 25.0 ppm, respectively. The data are noted in Table 1. Except for iso-amyl acetate the method was satisfactory. The iso-amyl acetate recoveries were unfortunately low but remained relatively constant at 70-72% at the lower concentrations.

One hundred ml synthetic samples containing 2 mg/l ethyl n-nonanoate as internal standard were extracted with ether by the method of Wagener et al (1968). The ethereal extracts were held in a waterbath at 36.5°C in a micro pear-shaped flask connected to a condenser in which water at 2°C circulated. It took about 3 hours to concentrate the sample to the desired volume of approximately 0.2 ml. The concentrates were transferred to small serum-cap stoppered glass containers and held in ice until required. If the sample was not used the same day it was held under liquid nitrogen until required.

A chromatogram of a sample analysis is shown in Fig.11. Results were quantitated from peak areas (height x width at half-height) using the predetermined ester flame ionisation response factors. (See Appendix Table 2).

13. Chlorogenic acid (CGA).

During the course of this study it became necessary to determine chlorogenic acid in the synthetic fermented media and grape juice.

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Table 1.

*Quantitative gas chromatographic analysis
of synthetic esters in ethereal extracts.

Ester.	A.		B.		C.	
	Added.	Recovered.	Added.	Recovered.	Added.	Recovered
i-amyl acetate	2.01	1.25	1.01	0.62	0.51	0.36
Ethyl n-hexoate	0.42	0.40	0.21	0.18	0.11	0.09
Ethyl n-octoate	1.01	0.90	0.51	0.47	0.25	0.24
Ethyl n-decanoate	0.41	0.37	0.21	0.24	0.11	0.09
Phenyl ethyl acetate	2.96	2.98	1.48	1.53	0.74	0.64

* Concentration in ppm.

Two CGA determination methods, viz. that of Zucker & Ahrens (1958) on tobacco and that described in AOAC (1960) on coffee beans proved to be of little value as such. Extraction of CGA, even from synthetic solution with ethyl acetate, (with or without salting out) prior to analysis did not give satisfactory results. The recently published polyamide column method of Lehman, Hahn & Martinod (1967) was next applied.

It was initially found that the treatment of polyamide (Perlon) with dilute alkali resulted in no CGA being adsorbed by such a column. A dilute acetic acid wash however caused CGA to be adsorbed.

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This phenomenon is probably due to several factors where the molecular structure and bonding capacities as well as the effect of ortho di-hydroxy groups (e.g. in CGA) on adsorption by polyamide appear to be relevant (Endres, 1961; Endres & Hormann, 1963; Singleton, 1967). A recent gas chromatographic method of the analysis of CGA isomers similarly utilized a PVP column treatment for initial recovery of the chlorogenates (Wilson, Dunlap & Wender, 1968). All CGA analysis were, therefore, done on PVP columns in contrast to the polyamide used by Lehman et al (1967).

Since leuco-anthocyanidin adsorption by polyamide is not affected by alkali treatment this phenomenon was used as a pre-treatment of grape juice to remove these as well as other interfering compounds prior to CGA analysis (See Appendix, Table 3). Spectra of grape eluates from polyamide and PVP columns (Appendix, Fig. 1) clearly show the marked difference which the polyamide pre-treatment effects and the close similarity of the PVP eluate spectrum with that of CGA.

Polycar AT and polyamide 100-200 mesh (B.S. No. 410/1943) fractions were prepared. After equilibration in water and removal of fines by repeated sedimentation the fractions were kept in 70% methanol solution.

Chromatographic columns (0.4 cm I.D.) were filled by slurring-in the fractions to a height of 11 cm. The columns are readily constructed in the laboratory from easily obtainable materials (Appendix, Fig. 2). A 20 ml syringe with a 4 inch needle is necessary to fill the reservoirs and columns with the washing solution and eluent. These latter solutions contained sulphur dioxide at 150 ppm.

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Grape juice samples were fortified with methanol to 70% by volume and left overnight. Aliquots of the clear centrifuged supernatant, containing 0.5 - 2.0 ml juice but not exceeding 30 µg CGA were loaded onto the in-series columns and quantitatively washed in with 10 ml 70% methanol.

After disconnecting the two columns, the polyamide column is treated with 5 ml alkaline 70% methanol and then again washed with 10 ml 70% methanol. It is ready for use after this step. The PVP column is eluted with 8 ml alkaline 70% methanol and the eluate led by means of Teflon sleeving to beneath the surface of the 50% acetic acid in the 10 ml receiving volumetric flask. A blank using methanol as well as a standard containing 15 µg CGA is done at the same time. The adsorption is read at 324 mµ in a 2 cm path length cell and concentrations determined from standards of synthetic CGA (Appendix, Fig. 3). Grape juice analysis are given in Appendix, Table 4.

The columns were not used for more than 3 determinations prior to reloading them. After more than 3 analyses erratic data and increasing losses were noted.

14. Quinic acid (QA).

QA analysis were to be carried out on fermented media which had received CGA. The method of Levy & Zucker (1960) as used on tobacco leaf and potatoes was applied.

The latter method did, however, not give accurate results on fermented media. This was due to:-

- a) the colour formed with CGA,
- b) the colour formed with unknown non-electrolytes

(Srinivasan & Sprinson, 1959) and

c) rapid clouding of the coloured complex.

The noted method was, therefore, modified to overcome these drawbacks.

To a 50 ml sample (not exceeding $2.0/\text{CGA}$ ^{mM}) is added 10 μl 5N H_2SO_4 , and 0.85 gm Dowex 1 x 8 (sulphate form, 200-400 dry mesh). The supernatant is carefully decanted into a small beaker and the residue washed and centrifuged twice with ca 2 ml water and the wash water added to the initial supernatant.

The pH of this solution is raised to ca 7.5 and quantitatively brought over to a Dowex 1 x 8 (carbonate form, 200-400 dry mesh) resin column (0.5 x 10 cm) and washed in with 10 ml water. The absorbed quinate is eluted with 10 ml 10% ammonium carbonate solution and the eluate dried overnight at 40°C. It was then made up to 5.0 ml.

Aliquots of 0.5 - 2.0 ml (not exceeding 0.04 μ Moles QA) are pipetted into conical centrifuge tubes and the procedure of the above authorities followed but using double quantities and sealing the tubes with marbles.

On complete colour development the tubes are placed in a 50°C waterbath and 50 mg PVP (Polyclar AT, unsifted) added to each. Each tube is shaken for 2 mins. The PVP is centrifuged down (3 min. 750 xg) and the supernatant (colourless) decanted and discarded.

Without washing the precipitate, 1.3 ml dimethylformamide (DMFA) is added and, with intermittent mixing, held in a boiling waterbath for 10 min. again sealing the tubes with marbles. The tubes are centrifuged (3 min. 750 xg) and the supernatant carefully poured into 5.0 ml volumetric flasks.

This step is repeated twice and the flasks filled to the mark with DMFA.

The absorption values of these coloured solutions are measured against a blank in 2 cm cells at 550 m μ . Standard quinic acid samples must be incorporated for each series.

(See Appendix, Fig. 4).

C H A P T E R III.RESULTS AND DISCUSSION.A. Specific grape and wine components.

In the initial series of experiments, H cycle ion exchange treatment was used to remove or decrease amino acids, ammonia and cations from grape juice. The effect of their re-addition upon relevant metabolic products and phenomena and fermentation rate with relation to bouquet formation are noted in the following sub-sections.

Riesling grape juice with 150 ppm sulphur dioxide, was treated in an ion exchange column (commercial Amberlite IR 120 H form, 400 gm, I.D. 3.2 cm) at a flowrate of $2.5 \text{ ml cm}^{-2} \text{ min.}^{-1}$. The first 250 ml was discarded and subsequent effluent collected at a constant pH of 1.87. This was designated as pH 2 must.

The pH 2 must was mixed with untreated must to achieve groups of pH 3.2, 2.9 and 2.6. The percentages of pH 2 must and control (untreated) must in the groups are noted in Table 2. The pH of all the treated groups were increased to that of the control by the addition of 5N alkali (1:1, NaOH: KOH) to eliminate the pH effect in subsequent studies.

Since this ion exchange treatment would entail a strong lowering of grape juice pH an initial test was carried out to determine the effect of a temporary high hydrogen ion concentration increase upon wine bouquet. The same must that was to be used in the initial experiment, was decreased to pH 2 by the addition of concentrated HCl. The pH was again raised to its initial value after 4 hours by the addition of sodium hydroxide. Another sample of the same must received an

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equivalent amount of sodium chloride. These samples were fermented; their fermentation characteristics were the same. An organoleptic evaluation of their wines by the triangular test (5 judges, 2 replications) showed that there was no significant difference between them. It was, therefore, accepted that the temporary strong decrease of must pH was not detrimental to wine bouquet formation.

Table 2.

*Percentage ion exchange treated and untreated must in pH groups.

Group.	Actual pH.	% Control must.	% pH 2 must.	Added alkali ml % must.
Control	3.8	100.0	Nil	Nil
pH 3.2	3.17	59.7	40.3	0.25
pH 2.9	2.87	46.7	53.3	0.35
pH 2.6	2.60	28.6	71.4	0.42
pH 2.0	1.87	Nil	100.0	0.58

* To differentiate between these groups they are referred to as the pH 3.2, pH 2.9 groups etc. Their pH values prior to fermentation were all brought to that of control by the alkali addition shown in the last column.

1. Ammonia and Amino Acids.

The amino acids were determined in the control sample; their concentrations are noted in Table 3. The amino acids in the pH 2 must were very low and could not be accurately determined. However, they were approximated at less than 2 ppm on spot size and colour intensity. The amino acids analysed were those which were important in yeast metabolism (Schultz & Pomper, 1948; Mandel & Bieth, 1960).

Table 3.

Analysis of several amino acids in
Riesling must.

Amino Acid.	Concentration mg/l.
Alanine	159.1
Aspartic acid	39.1
Glutamic acid	97.2
Glycine	22.7
Leucine and iso-Leucine	60.8
β -Phenyl alanine	50.1

The ammonia concentration of the pH 2 must was determined at 3.4 and that of the control at 146.7 ppm.

Each pH group was divided into four sub-groups where each sub-group contained two duplicate 500 ml samples. L-amino acids were added to specific samples to bring their values up to that of the control. These additions were based on the percentage pH 2 must in the mixture. The same system was applied in ammonia additions to the relevant samples containing ion exchange treated must. The plan for this series is given in Table 4.

Table 4.

Scheme of treatment upon and additions
made to ion exchange treated must.

Sub- Group.	Additions.	pH Group.			
		pH 3.2	pH 2.9	pH 2.6	pH 2.0
A	Nil	+	+	+	+
B	Ammonia	+	+	+	+
C	Amino acids.	+	+	+	+
D	Ammonia and Amino acids.	+	+	+	+

The fermentation of these samples ^{showed} marked differences with the fermentation rate generally dropping as the percentage ion exchange increases (Fig. 1).

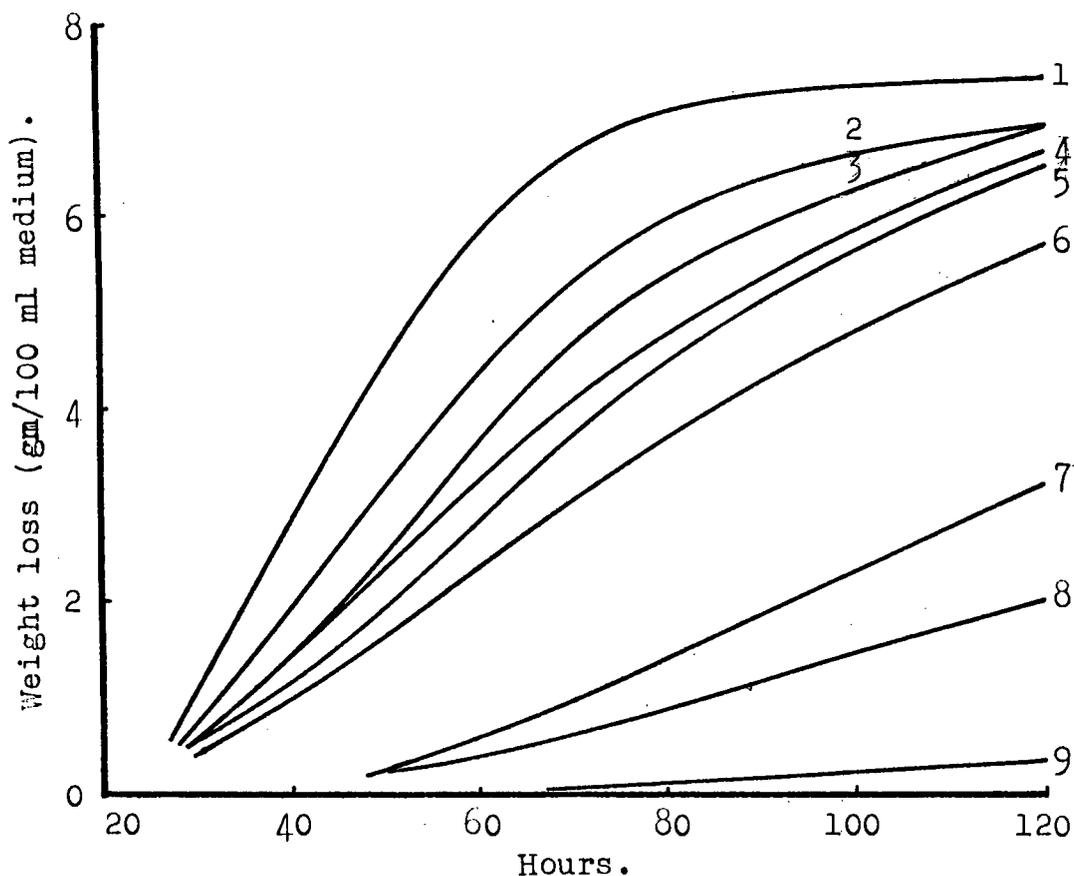


Fig. 1. Fermentation of untreated and ion exchange treated (IET) must with ammonia and amino acid additions. (1: untreated control; 2: pH 3.2, A, B, C & D (40.3% IET must); 3: pH 2.9, B & D (53.3% IET must); 4: pH 2.9, A & C (53.3% IET must); 5: pH 2.6, B & D (71.4% IET must); 6: pH 2.6, A & C (71.4% IET must); 7: pH 2.0, B & D (100% IET must); 8: pH 2.0, C (100% IET must); 9: pH 2.0, A (100% IET must). A = nil addition, B = ammonia, C = amino acids, D = ammonia plus amino acids).

The pH 2.0 A, B, C and D samples took 49, 42, 19 and 16 days, respectively, to complete fermentation. The other groups all fermented out within 12 days. This period decreased pro-rata the lower the degree of ion exchange treatment, the control sample completing fermentation in 5 days.

The analytical data of these samples are given in Table 5. The alcohol content show variations only in the 2.0 group which are slightly lower than any other. The volatile acidities of this group are also clearly higher than any other sample. In the sub-groups of this group, those samples containing ammonia (with or without amino acids) manifested the lowest volatile acidity values (Fig. 2).

Table 5.

* Analysis of wines of ion exchange treated Riesling must containing additions of untreated must, ammonia and amino acids.

Sample and Treatment.	pH.	Total Alcohols vol. %.	Vol. Acidity gm/l.	Higher Alcohols mg.%
Control	3.7	9.3	0.24	24.1
pH 3.2 A	3.7	9.4	0.24	27.0
B	3.6	9.3	0.21	22.3
C	3.7	9.3	0.23	26.9
D	3.6	9.3	0.23	22.8
pH 2.9 A	3.8	9.3	0.24	30.7
B	3.7	9.3	0.24	24.9
C	3.8	9.3	0.24	29.5
D	3.7	9.3	0.24	24.5
pH 2.6 A	3.8	9.3	0.28	37.2
B	3.6	9.3	0.24	27.9
C	3.7	9.3	0.25	34.5
D	3.6	9.3	0.23	25.4
pH 2.0 A	3.5	9.1	0.69	47.9
B	3.6	9.1	0.49	40.7
C	3.6	9.1	0.69	40.5
D	3.5	9.2	0.41	36.5

* See Table 4 for specific additions.

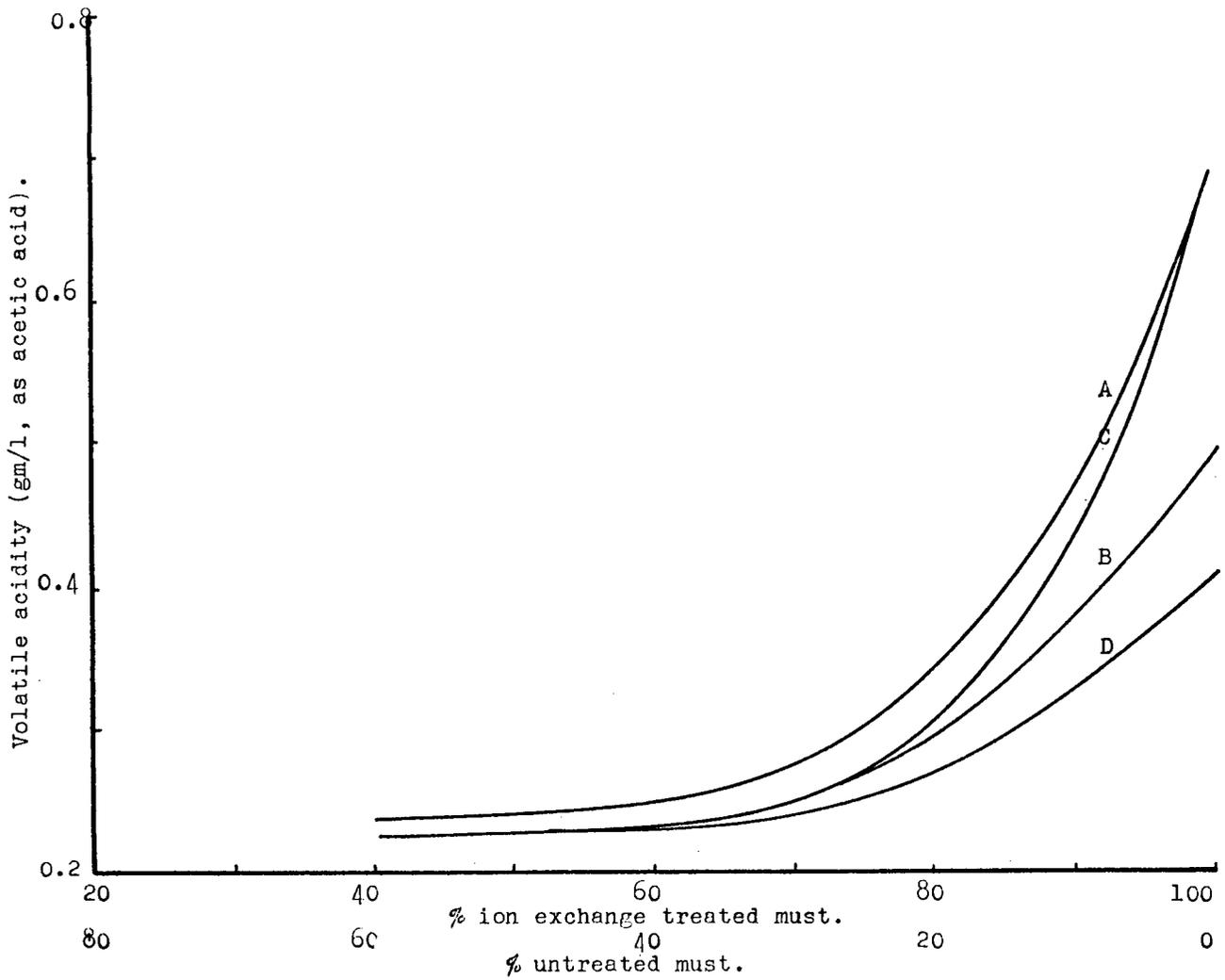


Fig. 2. Effect of ammonia and amino acids on formation of acetic acid in fermented ion exchange treated must. (A, Nil addition; B, Ammonia; C, Amino acids; D, Ammonia plus amino acids).

The higher alcohol concentrations showed a more pronounced pattern over the whole series (Table 5, Fig. 3). These components all increased with increased ion exchange treatment. The higher alcohols of the A sub-groups, i.e. where only pH was corrected increased linearly; these values were also, per pH group, the highest. In the amino acid series (C) the same pattern in the higher alcohol increments were noted. In these latter samples though, the individual higher alcohol values were lower per pH group (relative to A) and the rate of increase, relative to ion exchange treatment, also lower (Fig. 3). With the addition of ammonia (B series) a marked drop in higher alcohol formation occurred but with the rate of increase up to the pH 2.6 group (71.4% ion exchange treated must) very similar to that of the C series. After this point, however, a sharp increase occurred in the maximum ion exchange treatment with the final value of this B series equalling that of the C series. The combination of amino acids with ammonia (D series) gave a similar pattern to that of B but with the difference that the higher alcohol values were lower in the pH 2.6 and pH 2.0 samples, i.e. 71.4 and 100% ion exchange treated must, respectively.

Of prime importance here was whether there would be significant bouquet differences in these wines, i.e. whether the addition of ammonia and/or amino acids could bring about bouquet formation relative to where they were not added (A sub-groups). This sensory evaluation was done on a paired sample system where only two samples were tasted at a time. The tasters were asked to differentiate only on bouquet and always to compare the second sample with the first of a pair.

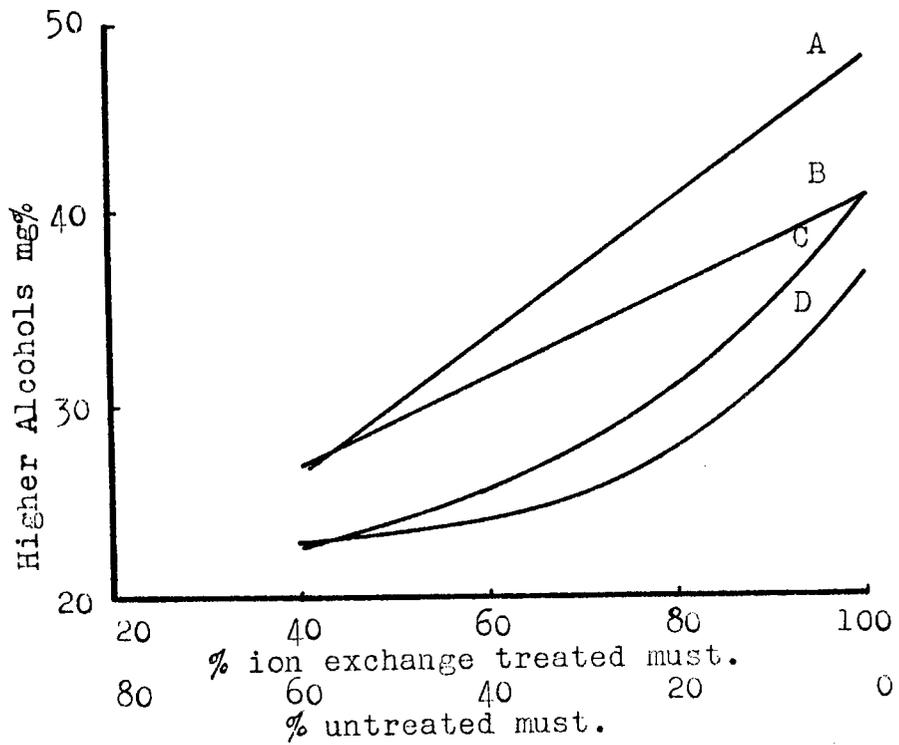


Fig. 3. Effect of ammonia and amino acid additions on formation of higher alcohols in fermented ion exchange treated must. (A, Nil Addition; B, Ammonia; C, Amino acids; D, Ammonia plus Amino acids).

In the first evaluation, an intra-comparison of each sub-group was done where the degree of formation of bouquet was judged against the nil-addition sample (A). In the second evaluation all samples (except the extremely slow fermenting pH 2.0 A and C samples) were similarly compared against the control (untreated fermented grape juice). Each pair occurred twice, e.g. control - A and A - control i.e. in reverse tasting order.

In the sub-group organoleptic analysis no clear consistent differences between any of the A samples and addition samples (B, C or D) were found. (Table 6(a)).

Table 6(a)

* Bouquet evaluation of sub-groups of ion exchange treated musts' wines against nil-addition sample.

Group.	Sub-group.	No difference.	prefer.	B,C, D prefer.	Treatment vs A (Treated preferred)
pH 3.2	B	3	4 (NS)	9 (NS)	(0.05)
	C	8	3 (NS)	5 (NS)	
	D	8	2 (NS)	6 (NS)	
pH 2.9	B	5	6 (NS)	5 (NS)	NS
	C	5	1 (NS)	10 (0.05)	
	D**	3	11 (0.05)	2 (NS)	
pH 2.6	B	11	1 (NS)	4 (NS)	NS
	C	7	3 (NS)	6 (NS)	
	D	3	5 (NS)	8 (NS)	
Total treatment over whole series.			36	55	(0.05)

* pH 2.0 group not included since A sample had not yet completed fermentation after 40 days.

Significance noted in brackets, NS = not significant.

** Malodorous characteristic in sample pH 2.9 D, caused preference of sample pH 2.9 A. These data are, therefore, disregarded here.

The no-difference data also tend to reflect this. When all the additions are taken as one treatment, significance is shown only by the pH 3.2 group. The malodorous characteristic which had formed in the pH 2.9 D sample strongly affected the evaluation; significance may have been shown had it not been present.

Taking the treatments over the whole series, significance was achieved in that the treated samples were preferred (Table 6(a)).

These data are regarded indicative in that the addition of the nitrogenous components do tend to improve bouquet. However, the effect appears to be very slight.

In the second evaluation the control sample was clearly preferred to the A samples of the sub-groups. (Table 6(b)). On the other hand the control sample was not always significantly preferred to the ammonia and/or amino acid addition samples. Taking all the treatments as a unit the control sample was again significantly preferred. The same trend was shown when a specific addition was taken over all the groups.

In this latter evaluation it was again indicated that an improvement in bouquet formation of the addition samples over that of the A samples was achieved. This was again partial confirmation of the indications achieved in the initial tasting. However, the important finding in these tastings was that the addition of the nitrogenous components tended to improve bouquet relative to where it was not added. Improvement was, however, not so marked that it consistently equalled the control.

Table 6(b).

* Bouquet evaluation of sub-groups of ion exchange treated musts' wines against that of untreated must.

Group.	Control equals sub-groups.				Control vs Sub-Groups.											Control vs treatments B, C and D.	
	K=A	K=B	K=C	K=D	K	vs	A	K	vs	B	K	vs	C	K	vs		D
3.2	1	6	2	2	10	(0.01)	1	6	NS	0	9	(0.05)	1	6	NS	4	(0.05)
**2.9	2	0	3	1	9	(0.05)	1	7	NS	5	6	NS	3	11		0	NS
2.6	0	1	4	2	10	(0.05)	2	10	(0.01)	1	7	NS	1	8	NS	2	(0.001)
***2.0	-	3	-	0	-		-	9	(0.01)	0	-		-	12	(0.001)	0	(0.001)
Total treatment over whole series.					29	(0.001)	4	28	(0.001)	6	22	(0.001)	5	26	(0.001)	6	

* K = Untreated sample (Control); significance (P) given in brackets in terms of control preference; NS = not significant.

** The 2.9 D sample inexplicably manifested a malodorous characteristic. It is, therefore, not included in significant determinations.

*** The A and C sub-groups were not included, their fermentation was not complete at this stage.

2. Cations and Vitamins.

With the ion exchange treatment of a must with a cation resin, not only ammonia and amino acids are removed. Other cations, e.g. magnesium, zinc, copper etc. are also affected (Rankine, 1955, Rankine & Bond, 1955). It has also been reported that certain synthetic cation exchange resins can remove thiamine from its solutions (Myers, 1943; Herr, 1945; Winters & Kunin, 1949). Consequently, the next series of tests carried out incorporated ammonia, amino acids, cations and vitamins.

The must utilized in this series was obtained from Riesling grapes; healthy clusters were harvested and the must expressed. This must, as before, received 150 ppm sulphur dioxide. It was stored in 10 liter glass containers sparged with nitrogen and held at -10°C until required.

The amino acids and ammonia of this must were again determined. The amino acid concentrations are given in Table 7 and did not differ radically from that of the must used in the first series. The ammonia concentration was determined at 163.4 ppm.

Table 7.

Analysis of several amino acids in
Riesling must.

Amino Acid.	Concentration mg/l.
Aspartic acid	39
Glutamic acid	123
Glycine	22
α -Alanine	109
Leucine)	
iso-Leucine)	57
β Phenyl alanine	45
Methionine *	8
Valine *	61

* Not added to musts.

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The vitamin concentrations added were the same as utilized by Van Zyl (1958); these concentrations all fell within the ranges determined in grapes (Radler, 1957; Peynaud & Lafourcade, 1956, 1957a, b; Ournac & Flanzky, 1957). The cations, calcium, magnesium, zinc and manganese, were also added in the concentrations as used by Van Zyl (1958). The vitamin concentrations are given below:-

Thiamine	0.5 ppm
Pyridoxine-HCl	0.5 ppm
Nicotinamide	2.5 ppm
Ca-pantothenate	2.5 ppm
Biotin	25 µg/l.

In this series all the must received maximum ion exchange treatment with no addition of control must. The effluent must was collected when it attained its lowest constant pH, i.e. below 2. Its pH was subsequently increased to that of the influent must by the addition of alkali as before. Samples were prepared as set out in Table 8 and in duplicate.

The fermentation rates of these samples were found to differ significantly (Fig. 4). These rates could be grouped into four classes. The slowest fermenting was the B sample. This was followed by the group C, D6 and D8 - D11. Samples D1 - D5 and D7 fermented still faster and constituted the third group. The control sample (A) showed the highest fermenting rate.

The analytical data of these samples are recorded in Table 9. The pH values and total alcohols do not differ significantly. Although, it would be expected that the omission of thiamine or cations would decrease the alcohol yield this did not obviously occur (cf Fukui, Tani & Kishibe, 1958). On the other hand the acetic acid (volatile acidity) and higher alcohol contents differed markedly as a result of vitamin and cation additions (Figs. 5 & 6).

Table 8.

Scheme of additions made to ion exchange treated Riesling must.

Sample. Ammonia. Amino acids. Cations. Thiamine. Pyridoxine. Nicotinamide. Pantothenate. Biotin.

* A (Control)	-	-	-	-	-	-	-	-	-
B	+	+	-	-	-	-	-	-	-
C	+	+	+	-	-	-	-	-	-
D1	<hr/>			+	+	+	+	+	+
2				+	+	+	+	+	-
3				+	+	+	+	-	+
4				+	+	-	+	+	+
5				+	-	+	+	+	+
6	All - Ammonia, Amino			-	+	+	+	+	+
7	acids, Cations			+	-	-	-	-	-
8	added as in C.			-	+	-	-	-	-
9				-	-	+	-	-	-
10				-	-	-	+	-	-
11				-	-	-	-	-	+

* Untreated must.

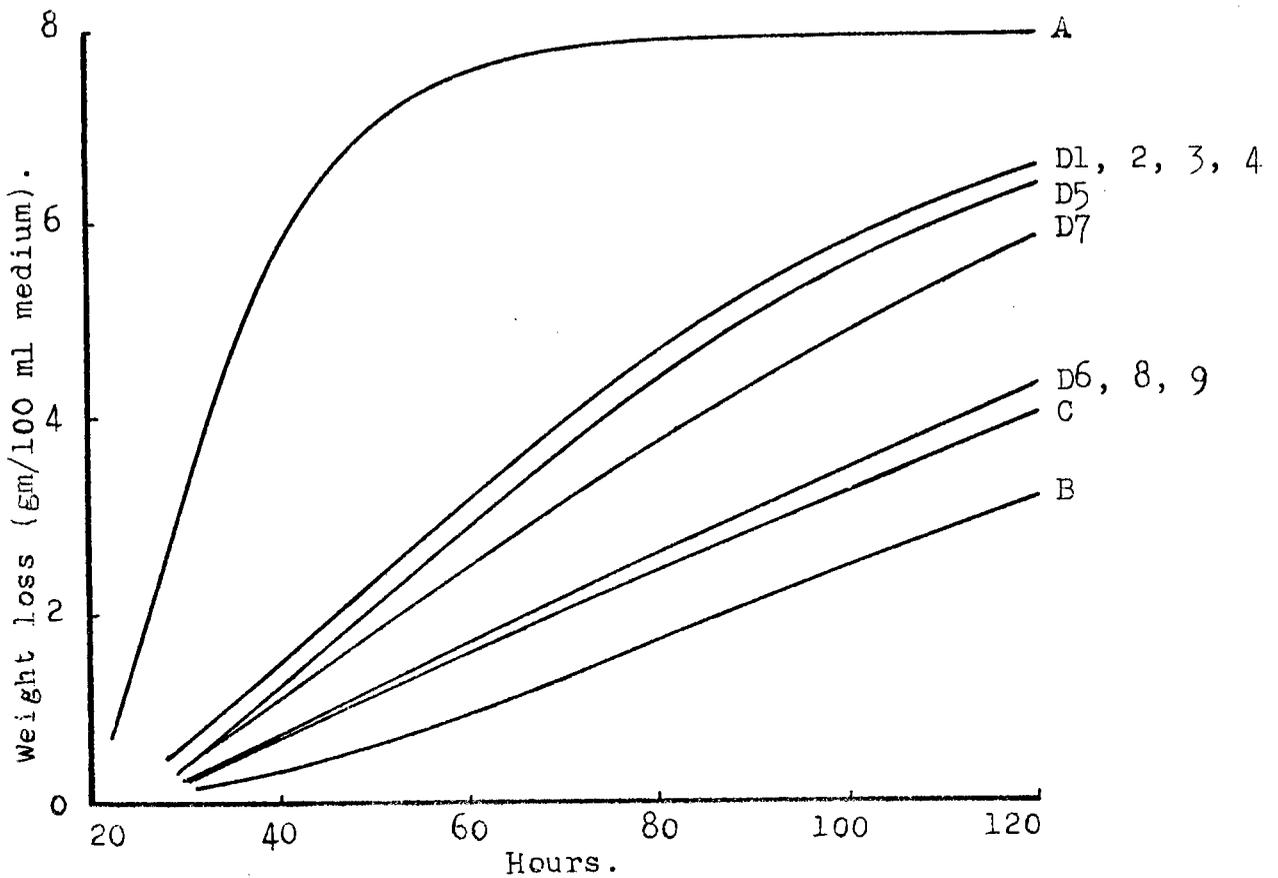


Fig. 4. Fermentation of ion exchange treated musts indicating thiamine effect, with and without other nutrilites (A, Control untreated must; D1 -D5, D7, thiamine addition; D6, D8-11, Nil thiamine; C, Nil thiamine; B, Nil thiamine, Nil cations) (See Table 8 for specific additions).

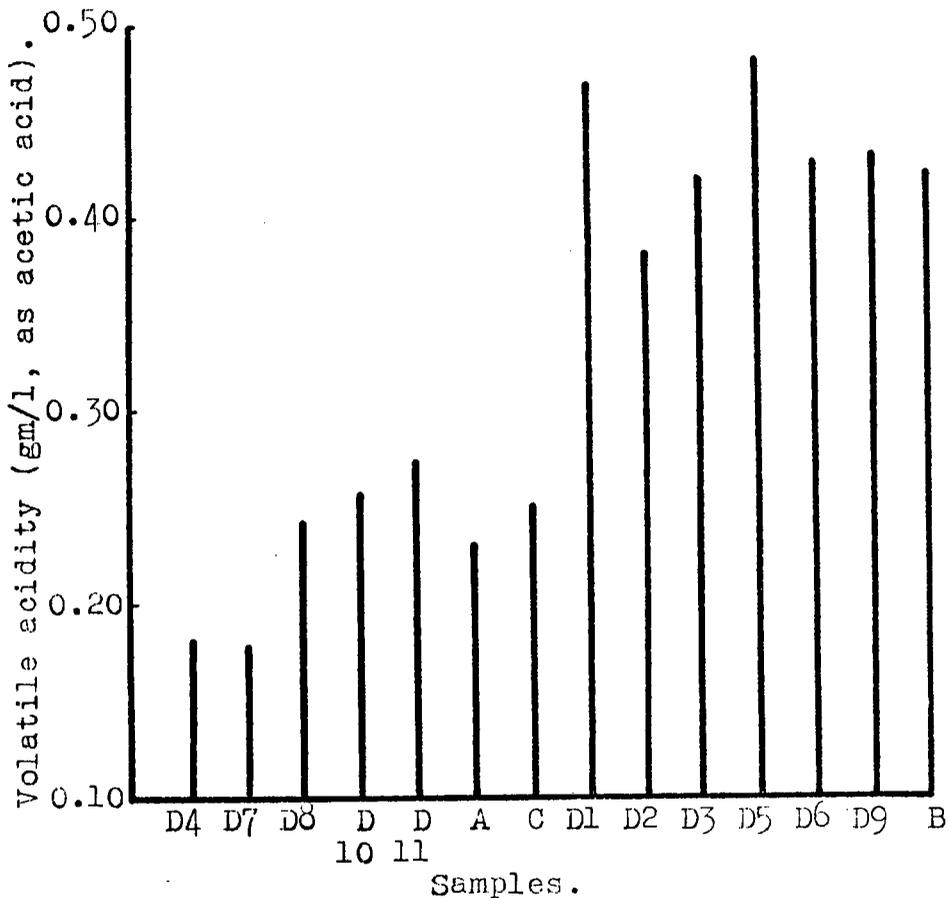


Fig. 5. Acetic acid concentrations (volatile acidity) of wines of ion exchange treated musts indicating thiamine and nicotinamide effects. (A, Control untreated; D4, D7, thiamine addition; D8, D10, D11, Nil thiamine or nicotinamide additions; D1, D2, D3, D5, D6, D9, nicotinamide with or without thiamine; C, Nil thiamine; B, Nil thiamine; Nil cations) (See Table 8 for specific additions).

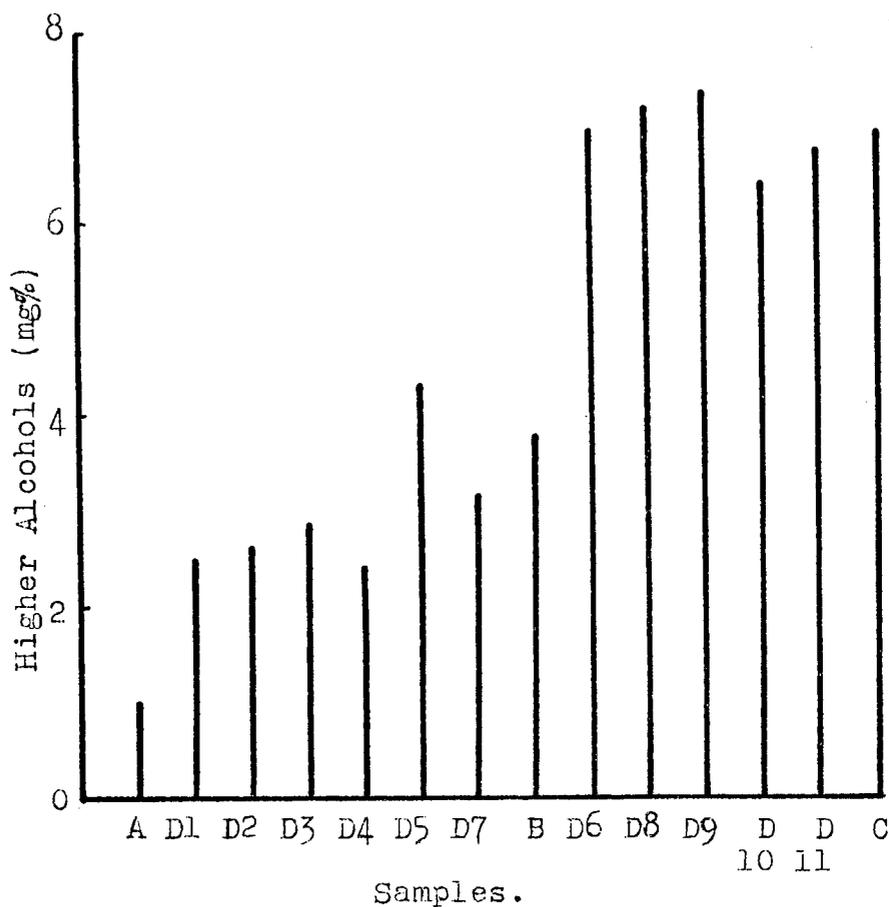


Fig. 6. Higher alcohol concentration of wines of ion exchange treated musts indicating thiamine effect. (A, Control, untreated; D1-D5, D7, thiamine addition; D6, D8-D11, Nil thiamine; C, Nil nutrilites; B, Nil nutrilites, Nil cations; See Table 8 for specific additions).

Table 9.

* Analyses of wines of ion exchange treated Riesling must containing several added vitamins.

Sample.	pH	Total alcohol vol. %.	Vol. acidity gm/l.	Higher alcohols mg. %
A (Control)	3.6	10.1	0.23	24.9
B	3.5	10.0	0.42	38.8
C	3.4	10.0	0.25	54.3
D 1	3.6	9.9	0.47	32.2
2	3.6	10.0	0.38	33.0
3	3.6	9.9	0.42	34.0
4	3.6	10.1	0.18	32.0
5	3.6	10.0	0.48	41.6
6	3.4	9.8	0.42	54.7
7	3.6	9.9	0.18	35.6
8	3.5	9.9	0.24	55.9
9	3.4	9.8	0.43	56.6
10	3.4	9.9	0.25	51.9
11	3.4	9.9	0.27	53.5

* See Table 8 for specific additions.

In the acetic data (Table 9, Fig. 5) the effect of cation addition was clearly manifested. With cation addition to B (=C), a sharp drop in acetic acid formation occurred. The depressing effect of cations on the formation of acetate was marked under these conditions of study. In the samples to which vitamins had been added three groups were shown in the magnitude of acetic acid formation. The lowest content group contained samples D4 and D7. This was followed by D8, D10 and D11 as intermediate with D1, D2, D3, D5, D6 and D9 falling in the highest content group.

In the low acetic acid content samples, D4 contained all the vitamins except nicotinamide whereas D7 contained only thiamine. In these two samples thiamine was common to both but

both also did not contain nicotinamide. In the intermediate group, the samples D8, D10 and D11 did not contain biotin, pantothenate and pyridoxine. Sample C, to which these vitamins were added, also gave the same value as the latter group. This indicates that, under these conditions, the latter vitamins had little or no effect on acetic acid formation. From this it can be deduced that the addition of thiamine, as in D4 and D7 had a depressing effect on the formation of acetic acid. In the high acetic acid group, viz. D1, D2, D3, D5, D6 and D9 thiamine was present in the first four. This appeared to have no effect in significantly depressing acetic acid formation. Since biotin, pyridoxine and pantothenate have already been shown to be ineffective here, the cause of this phenomenon must have been due to nicotinamide. Where only nicotinamide was present a high acetic acid content was also shown. All the former samples contained this vitamin and its effect was such that it either "overrode" the relatively small depressing influence of thiamine or increased acetic acid to such an extent that it masked the effect of thiamine if it were present.

The higher alcohols showed strong variations. (Table 9, Fig.6). The control sample was clearly the lowest with the other samples all higher. These also differed in that a high and a low group could be noted. Sample B fell in the low group with sample C clearly in the higher. This orientation was just the reverse of that shown by these two samples in the acetic acid analyses data (Table 9, Fig. 5). The addition of cations, therefore, increased higher alcohol formation. The samples which had received vitamins, viz. D1 - D5 and D7 were, as a group, lower in higher alcohols than D6 and D8 - D11. From Table 8 it will be noted that none of the latter group contained thiamine, whereas

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the former all did. Since sample C contained no vitamins and also showed the same high higher alcohol concentration as the D6, D8 - 11 group it was obvious that nicotinamide, biotin, pantothenate and pyridoxine were not functional in this metabolism under these conditions. Thiamine was clearly the only important vitamin here; its presence caused an decrease and its absence an increase in higher alcohol formation.

The sensory evaluating system was simplified here in that a ranking was first done (five judges) to gain an initial indication of strong differences. Samples D1 - D6 and D7 - D11 were examined as two different groups. Samples B and C were also subjected to a triangular taste test; no significant difference could be established (4 correct decisions out of 8). Sample B was, therefore, included in the subsequent statistical evaluation.

In the initial ranking of the two former sample groups, two unanimous decisions were noted, viz. D5 and D7 were clearly the best in their individual sets. It was also indicated that D6 and D9 were the poorest with a strong difference of opinion on D4. The balance of the samples were classed as being very much the same (Table 10).

Triangular tasting was carried out on A, B, D4, D5 and D7; each trio appeared twice in the series, once as e.g. ABB and once as BAA. The data are noted in Table 11. It has been noted that the samples which had not and had received cations (B and C, respectively), showed no significant differences. Furthermore, B was found to be poorer than A (Control) (Table 11). The higher alcohol and especially acetic acid concentrations of these two samples (B and C) showed strong differences; these did not affect bouquet formation markedly. However, since the

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fermentation was improved by the addition of cations, they were consequently added to all further samples.

Table. 10.

* Bouquet - ranked position of wines of
ion exchange treated musts.

Wine.	Judge.			
	A	B	C	D
	<u>Ranked position.</u>			
D 1	2	4	2	2
D 2	2	3	3	5
D 3	2	3	3	3
D 4	6	2	1	4
D 5	1	1	1	1
D 6	2	5	4	6
D 7	1	1	1	1
D 8	2	2	2	2
D 9	2	5	3	5
D10	2	3	2	2
D11	2	3	3	2

* Samples D1 - D6 and D7 - D11 were examined in two separate evaluations.

The addition of vitamins brought about a marked improvement in some samples (cf B - D5, Table 11) whilst in others, notably D6 and D9 a decrease was recorded (Table 10). The former sample (D6) contained all the vitamins except thiamine and the latter only nicotinamide. These were the only samples which contained nicotinamide in the absence of thiamine. Their analyses were almost identical with both having high acetic acid and higher alcohol concentrations; their fermentation rates were also very similar. There were no other samples in which the two analysed components were both orientated in the high content groups. It, therefore, does appear that nicotinamide in the absence of thiamine tends to lead to formation of less bouquet,

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which in turn and under the conditions of this study appears to be correlated to the degree of higher alcohol and acetic acid formation.

Table 11.

*Bouquet evaluation of several wines of ion exchange treated musts.

Preference for	Sample pairs.					
	A - B	A - D4**	A - D5	A - D7	B - D5	D5 - D7**
A (Control)	7	6	3	2		
B		1			0	
D4			1			
D5				5	8	4
D7					6	3
Sample better-						
P	0.01	0.01	NS	0.05	0.001	NS

* See Table 8 for specific additions to must.

** One decision on bouquet preference not given.

NS = Not significant; P = significance.

The good bouquet samples D5 and D7 were not much different from the control or significantly better and also did not significantly differ from each other (Table 11). Both of these samples contained thiamine, the former also having nicotinamide. The fermentation rates of these two samples were very much the same. Their analyses, however, differed in that the acetic acid value in D5 was considerably higher than that in D7. As far as this component is concerned here, there appears to be no contribution to bouquet. The higher alcohol values of these two samples, on the other hand both fell in the low group. This does also not appear to be a critical factor in relation to bouquet since D4, which had both low acetic acid and low higher alcohols, the same as D7, was significantly poorer than control whereas

D7 was clearly better.

It could have been surmised that, merely on the basis of analysis, D4 and D7 could have been organoleptically similar. However, as shown this was not so and the functional factors in this manifestation was apparently the vitamins where thiamine and nicotinamide were indicated to be important. The role of the latter vitamin here appears to be complex; poor bouquet resulted when nicotinamide was added without thiamine. However, good results were also achieved by the addition of only thiamine.

These findings showed no clear relationship of analyses of acetic acid and higher alcohols with bouquet formation. The only factor where some indication was achieved was fermentation where the best samples showed high rates. This was, however, also not specific since samples with poor bouquet also fermented at a relatively high rate (Fig. 4). Since thiamine alone could effect a bouquet increase it was added as standard procedure to all further samples.

3. Thiamine with cations, ammonia and amino acids.

Since thiamine addition to ion exchange treated musts has been shown to bring about a marked improvement in the wines' bouquet a final series was done where its concentration was increased to a higher value than in the original must. The thiamine concentrations were consequently determined before and after ion exchange treatment. Concentration in several musts were also determined. These analyses are recorded in Table 12. The data show that the resin treatment removes thiamine. The thiamine values of the grape juice samples were more or less in the range determined by Paynaud & Lafourcade (1957a) viz. 0.25-0.5ppm.

Table 12.

*Thiamine concentrations in ion exchange treated
and untreated grape musts.

Cultivar.	Thiamine $\mu\text{g}/100\text{ml}$.
Riesling a	17.7
Riesling b	37.0
Riesling c	30.1
Riesling d	33.9
Steen a	38.7
Steen b	41.4
St. Emillion a	27.6
St. Emillion b	22.4
St. Emillion c	20.6
Green grape a	23.5
Green grape b	35.3
Ion exchange treated Riesling	0.0
Cabernet Sauvignon	35.1

* Musts are of different origins, vintages or harvestings.

The plan of this study is given in Table 13. A simultaneous examination of ammonia and amino acids in the presence of thiamine was also done. The same must as used in the previous study was used here. It was treated as before and the effluent, at constant pH (1.9), collected.

The relevant alcohol, acetic acid and higher alcohol analyses are recorded in Table 14.

The alcohol values were, to all intents, much the same. The acetic acid values were high in groups B and D and lower in A and C where overall intra-group differences were not large. The values in groups B and D approximated each other as a whole; the same was the case with A and C. The values in the latter two groups were generally higher than the control values. The higher alcohols followed the same pattern as the acetic acid data but with the values being slightly lower in the D group

than those in B.

The fermentation data are set out in Fig. 7. The typical group incremental pattern was again achieved; all the treated groups fermented slower than the control with rates decreasing in the order A, C, B and D.

Table 13.

Scheme of additions to ion exchange treated Riesling must to examine effect of thiamine in presence and absence of ammonia and amino acids.

Sample.	Thiamine ug %.	Ammonia mg/l.	Amino acids*
K	0	0	-
A 1	34	160	AA
2	68	160	AA
3	34	320	AA
4	34	160	AA x 2
5	68	320	AA x 2
6	34	160	AA, Nil PA
B 1	34	-	AA
2	34	-	AA, PA x 2
3	68	-	AA, PA x 2
4	34	-	PA x 2, Nil Leu
5	34	-	AA, Nil PA
C 1	34	160	-
2	34	320	-
3	68	320	-
D 1	34	-	-
2	68	-	-

* AA Amino acid concentrations are, unless otherwise specified, the same as given in Table 7.

PA = Phenyl alanine.

Leu = Leucine.

K = Control (untreated).

Table 14.

* Analyses of wines of ion exchange treated Riesling must containing added thiamine, ammonia and amino acids.

Sample.	pH	Total alcohol vol. %.	Volatile acidity gm/l.	Higher alcohols mg. %.	Dry yeast weight gm/l wine.
K (Control)	3.5	9.4	0.21	26.4	3.87
A 1	3.4	9.1	0.25	17.9	2.29
2	3.4	9.2	0.24	21.3	2.43
3	3.3	9.2	0.30	18.5	2.35
4	3.4	9.2	0.29	20.0	2.40
5	3.4	9.2	0.27	20.8	2.69
6	3.4	9.2	0.24	20.5	2.41
B 1	3.4	9.3	0.39	46.1	1.53
2	3.4	9.2	0.36	41.1	1.71
3	3.4	9.3	0.36	46.1	1.75
4	3.4	9.2	0.37	41.8	1.60
5	3.4	9.3	0.37	46.5	1.54
C 1	3.4	9.1	0.27	18.4	2.18
2	3.3	9.1	0.25	18.9	2.38
3	3.3	9.1	0.27	19.9	2.36
D 1	3.5	9.3	0.44	32.6	1.26
2	3.5	9.3	0.39	31.6	1.29

* See Table 13 for specific additions.

Sensory evaluation was done by ranking; statistical significance was not determined. It was found in previous tests that only where differences were large, did individual non-statistical tastings correlate with statistical panel evaluations. Since the statistical setting up of a test required considerable time and material it was considered, in view of the latter findings, to concentrate more on individual response. If the results were regarded as significant they would serve as a guide to subsequent experiments and could also be confirmed by replicate studies. All future sensory analyses were, therefore, utilised as pilot tests and further work generally carried out on a large-difference system.

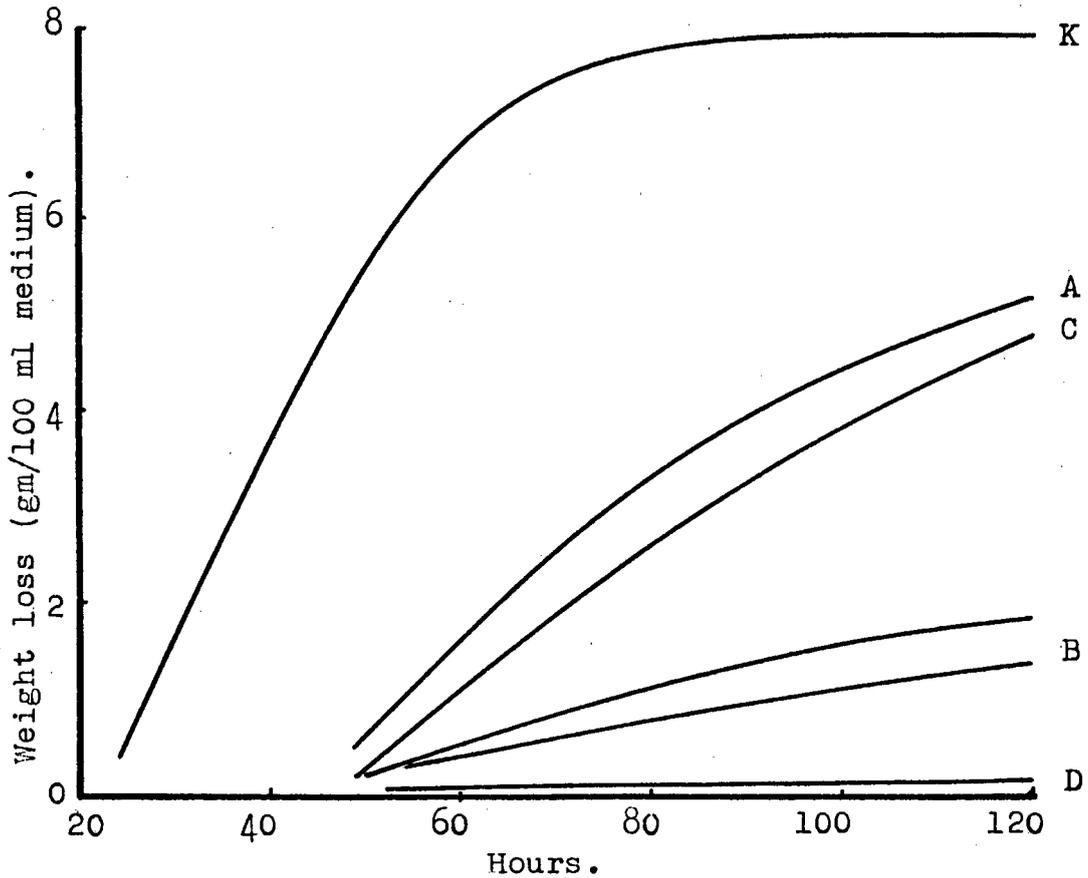


Fig. 7 Fermentation of ion exchange treated Riesling musts indicating effect of ammonia and amino acids with thiamine. (K, Control, untreated; A, thiamine, ammonia and amino acids; B, thiamine, amino acids, Nil ammonia; C, thiamine, ammonia, Nil amino acids; D, only thiamine. See Table 13 for specific additions).

In the ranking of these samples the control was classed as the best. The groups A and C were found to have very much the same character, not as good as the control, but clearly better than B or D samples. Although, there were individual A and C samples which appeared better than the rest in their group this was not regarded as being significant on the basis of individual response.

In this series it was found that increasing the thiamine, ammonia or amino acids, individually or collectively, to twice their must values effected no marked difference on bouquet formation, fermentation or analyses. When amino acids were excluded the same effect was shown. The bouquet in this latter group (Table 13) which, together with that in A, could perhaps be more aptly termed as vinous, was, therefore, not influenced by addition of amino acids. Ammonia on the other hand was critical in this respect; its exclusion, irrespective of any other additions was clearly detrimental to bouquet formation as evidenced by the B and D samples. It was also of interest to note that thiamine alone (D sample) was ineffective in forming bouquet.

4. Acetic Acid.

Acetic acid has been associated with bouquet through the probability of ester formation. In all three of the studies in this series it was noted that marked acetic acid (volatile acidity) formation occurred. In normal fermentation acetate is not an end, but rather an intermediate product (Genevois, 1961). After its formation in the initial stages of fermentation from one third to one half can again be taken up by the yeast (Peynaud, 1938). Added acetate is readily utilized by the yeast

in the formation of e.g. succinate, amyl alcohol, higher fatty acids, etc. (Genevois, 1961). This is accomplished by the "activation" of acetate to acetyl CoA (Decker, 1959) (Fig. 8). It is an important mechanism and no organism apparently lacks it. Yeast deficient in the Co-A moiety, pantothenate, as e.g. Saccharomyces cerevisiae, does not utilize acetate as efficiently as in the presence of pantothenate. (Novelli & Lipmann, 1947; Ribereau-Gayon, Peynaud & Lafon, 1959; Nordström, 1962a). Sebrell & Harris (1954) considered the activation step a necessity for perhaps all acetyl groups.

(a) The influence of ammonia.

The data in Table 14 show that where a nil or amino acid addition was made acetic acid increased relative to an ammonia addition. Thiamine additions at the noted concentrations had no effect.

It has been noted that retarded fermentations generally cause an increase in acetic acid formation. (Ribereau-Gayon, Peynaud & Lafon, 1956). It has also been reported that yeast growth limitation through nitrogen or magnesium deficiencies similarly increases the acetic formation (Nordström, 1966a). The latter findings have been confirmed by these studies (Fig. 1, 7 vs 8 vs 9; Fig. 2, A & C vs B & D, Table 5; Fig. 9, A & C vs B & D, Table 14; Fig. 5, B vs C, Table 9).

Two probable pathways for acetic acid formation have been reported viz. oxidation of acetaldehyde, through yeast aldehyde dehydrogenase and hydrolysis of acetyl-co-enzyme A. (Gergely, Hele & Ramakrishnan, 1958; Nordström, 1966a) (Fig. 8). No certainty as to the relative contribution of either of these pathways upon acetic acid formation has been noted.

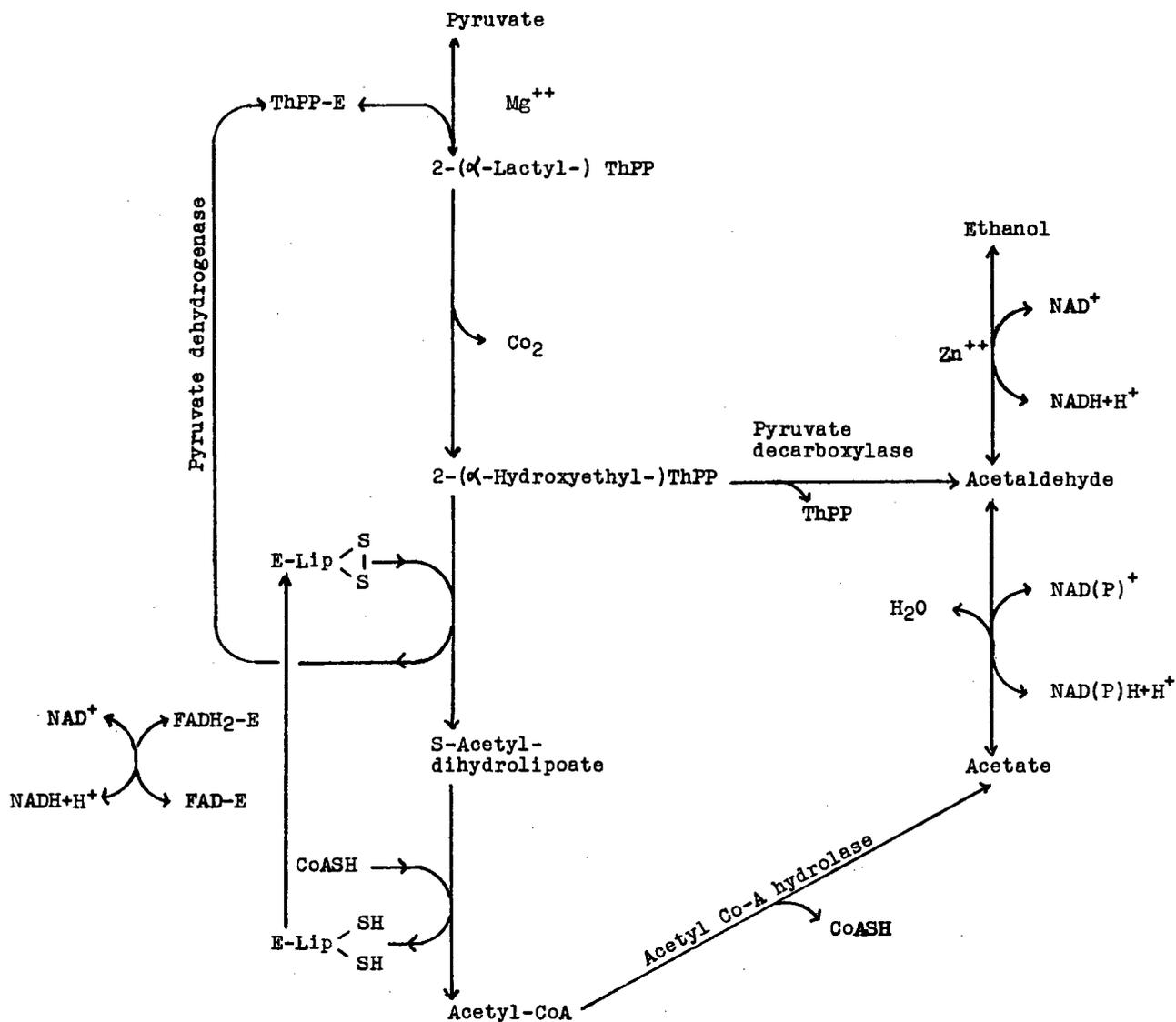


Fig. 9. Pyruvate-acetylCoA pathway showing formation of acetaldehyde ethanol and acetate. (Marr, Forti, Cocucci, Ferrini, Elviri & Michal).

Nordström (1966a) reported that in limited yeast growth the acetate formation generally increases after fermentation. He furthermore, expresses the view that acetate formation is dependant upon intracellular acetaldehyde. Lafon (1959) similarly reported that acetate derives from the dismutation between ethanol and acetaldehyde.

The amino acids added to the treated must in relatively high concentrations were reported to be as readily assimilable by the yeast cell as ammonia (Thorne, 1941). Nordström (1962a) also found that the use of ammonium sulphate or alanine made no difference to cell substance formed when used as sole nitrogen source. On this basis it is therefore interesting that acetic acid formation was decreased to such a degree by ammonia addition. The addition or non-addition of amino acids had little effect (Table 14). Contrary to these studies, Ribereau-Gayon, Peynaud & Guimberteau (1959) reported that increases in acetic acid formation resulted from ammonia or amino nitrogen additions. On the other hand Amerine & Cruess (1960) noted that California musts are high in nitrogen but acetic acid formation in the wines is low. This appears to contradict the former authorities and outlines the foregoing remarks on this phenomenon. It is clear that the formation mechanism of acetate by yeasts under the conditions of study is complex. It appears also to be coupled to yeast growth where this property is influenced here by ammonia to a stronger degree than would be normally expected.

(b) The influence of thiamine and nicotinamide.

In the second study of this series acetic acid variations also occurred. This manifestation was not directly due to either ammonia or amino nitrogen since these components were common to

all the samples. The findings again confirm the complexity of factors influencing the formation of the compound. However, it has been shown that the resin removes thiamine. The di-valent magnesium ion will also be strongly absorbed, if not totally removed (Bauman, Eichorn, 1947; Kunin, 1958). Thiamine (and magnesium) functions in the decarboxylation of pyruvate (Fig. 8) (Haehn, 1952; Kirchoff, 1960). With a deficiency of this nutriline, pyruvate increases. This has been confirmed by Ribereau-Gayon, Peynaud & Lafon (1956). The data in Fig. 4 clearly show that rate of CO₂ formation is decreased in those samples without thiamine (D6, D8 - 11). (Trevelyan & Harrison, 1954 a, b.)

Disregarding the samples which contained nicotinamide (alone or with other vitamins) for the present, the effect of thiamine addition was clearly to decrease the acetic acid (Fig.5). Samples D8, 10 and 11 were also those which in all probability contained a higher pyruvate concentration than D4 and 7, which two had received thiamine. The former group of samples, furthermore, were all higher in acetic acid than were the latter. Lafon (1959) reported that acetate does not derive directly from the decarboxylation of pyruvate but from the dismutation between ethanol and acetaldehyde.

It has been reported (Nordström, 1966a) that with increased pyruvate the acetaldehyde will decrease. This would in all probability have been so in samples D8, 10 and 11 (cf Fig. 8). If the degree of conversion of acetaldehyde to acetate by NAD⁺ were only a question of aldehyde concentration then it could be expected that samples D8, 10 and 11 should have lower acetic acid concentrations than D4 and 7. This was not found to be so, in

fact, the reverse was the case i.e. where pyruvate was no doubt high, as a result of lowered decarboxylation rate, acetate was also high and vice versa.

Nordström (1966a, 1968a) noted that the fraction of acetaldehyde oxidized to acetate decreased when the cellular concentration of acetaldehyde increased; a larger proportion of the acetaldehyde was reduced to ethanol. It was computed that above a specific acetaldehyde concentration i.e. increasing acetaldehyde concentration, the fraction of acetaldehyde oxidized decreases and will subsequently give a lower acetate concentration. He moreover determined that a thiamine deficiency will increase the pyruvate which subsequently leads to increased acetate. These results were achieved in synthetic media and were similar to those found in the ion exchange treated grape juice media. It appears that the same mechanism could have been operative here.

It is also of interest to note that sample C contained no added vitamins, only the cations i.e. including magnesium. When these cations were omitted in sample B, the acetic acid rose again, in all probability as a result of increased pyruvate and low acetaldehyde (cf low carbon dioxide formation in B, Fig.4).

In the ion exchange treatment of must, nicotinamide (or nicotinic acid) is strongly decreased (Table 15). With the addition of nicotinamide, irrespective of whether thiamine or any other nutrilites were present a marked increase in acetic acid formation occurred (Fig. 5). Acetate formation appears to be largely the result of aldehyde dehydrogenase (Lafon, 1959; Nordström, 1968a). This requires NAD (P) (See Fig. 8), Nicotinamide is readily converted to NAD (or NAD(P) by yeast (White, Handler & Smith, 1964). An increase in the concentration

of nicotinamide in a nicotinamide-deficient medium, such as in ion exchange treated must, could be expected to increase acetate formation. Acetate increases were, in fact, noted (cf Nordström, 1966a). It is advanced that NAD increase through nicotinamide addition is a logical explanation of this phenomenon.

Nordström (1962a) also reported on a similar phenomenon, viz. that the increase of the Co-A moiety, pantothenate, leads to a corresponding decrease in acetic acid formation (See Fig. 8).

Table 15.

*Absorption of nicotinic acid and nicotinamide from saturated potassium bitartrate solution by cation exchange resin (H form).

	Initial concentration mg %.	Final concentration mg %.	Removed %.
Nicotinic acid	0.5	0.21	57.2
Nicotinamide	0.5	0.078	84.4

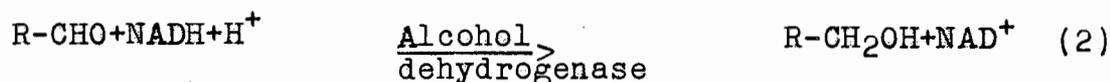
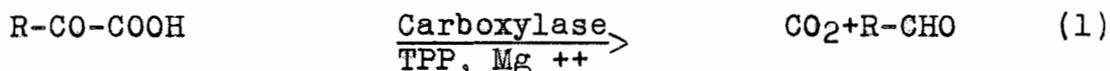
* 1 gm moist Dowex 50W x 8 (200-400 dry mesh) contacted with 10.0 ml 0.5 mg % solutions for 5 min; supernatant filtered off and resin washed with approx. 8 ml sat. KHT soln. Final vol. 20.0 ml, measured at 261.5 m μ , 5 cm path length.

5. Higher alcohols.

(a) Influence of ammonia, amino acids, thiamine and nicotinamide.

The formation of higher alcohols are intimately connected with the amino acid metabolism in yeasts. Their formation can occur catabolically via the well-known Ehrlich mechanism or anabolically from the main carbon source (Villforth & Schmidt, 1954; Thoukis, 1958; Suomalainen & Kahanpää, 1963). In both of these mechanisms α -keto acids are important intermediates

Castor & Guymon, 1952; Guymon, Ingraham & Crowell, 1961; Suomalainen & Keranen, 1967; Ayräpää, 1967). These keto acids are decarboxylated, by means of carboxylase, to the aldehydes which are subsequently reduced to the analogous alcohols by the hydrogen transfer system, as indicated below (Sentheshanmuganathan, 1960):-



In the second study thiamine was the only vitamin to have a marked effect on higher alcohol formation. Samples which had the lowest values had received thiamine whereas D6 and D8 - 11, which had not, were the highest (Table 9). On the basis of the latter equations the opposite to what would be expected was noted. It has been shown under acetic formation that this same unexpected pattern occurred. This latter phenomenon was reported by Nordström (1968a) to be due to the inverse effect of a high acetaldehyde concentration in acetic acid formation. Since aldehydes are also the immediate precursors of higher alcohols it appears that the same type of mechanism may be functional in both instances. However, proof of this occurrence has not been gained nor has any pertinent work on it been noted.

It was surprising that nicotinamide did not, contrary to its marked influence on acetic acid formation, show a greater effect. The maintenance of the redox balance after the addition of nicotinamide to cation exchange treated musts and the surmised reduction of increased NAD, was expected to be maintained by at least a small increase in higher alcohols. (It will be noted

that it was in this same study that acetic acid formation was increased by added nicotinamide). This phenomenon is not understood. However, Nordström, (1968b) indicated that under anaerobic conditions, redox balance can be maintained by the molar equivalent reduction of dihydroxyacetone phosphate to glycerol for aldehyde not reduced to ethanol. If this occurred more readily than higher aldehyde reduction then it should have been reflected in glycerol data. This was however, not done. Since the sugar concentrations of the grape juice was high (ca 20°B), the ethanol concentrations were also too high to reflect the influence of nicotinamide.

Of further interest was the effect of cations on higher alcohol formation. Their addition produced a concentration of higher alcohols similar to that in the samples which contained all the vitamins except thiamine (Fig. 6). This was further confirmation on the relative ineffectiveness of the relevant vitamins. However, with the omission of the cations a clear decrease in higher alcohol formation occurred (Fig. 6,B). If the influence of magnesium is similar to that of thiamine, which appeared to be the case in acetic acid formation and which would not be an unreasonable assumption in higher alcohol formation, it is surprising that the effect is actually the reverse of that of thiamine. In fact, it appears that the absence of magnesium or the presence of thiamine have the same influence. This can not readily be credited. The cations and not necessarily only magnesium could perhaps be active in an enzymatic mechanism, possibly specific alcohol dehydrogenases. However, this is not known and relevant literature has not been noted where this phenomenon has been fully investigated. The function of the

cations in higher alcohol formation appear to be more complex than is the singular approach based on the role of magnesium in the decarboxylation step. The data of the control sample (Fig. 6, A) also show the lowest higher alcohol concentration in this study. This tends to confirm that all relevant factors have not yet been clearly defined. Confirmatory findings have been reported (Nordström & Carlsson, 1965).

In the initial study increases in higher alcohol occurred in all the sets (Fig. 3). Äyräpää (1967) carried out studies on the higher alcohol formation in synthetic media at different ammonia and amino acid levels. He noted the increase in higher alcohol formation with a decrease in nitrogen content of the medium. These findings were confirmed in our data.

The shape of the curves are also of interest in that the ammonia addition curve (Fig. 3, B) is typical of that of an anabolic metabolism as found by Äyräpää (1967). The addition of amino acids plus ammonia (Fig. 3, D) again reflects the same pattern as B but is lower due to increased total nitrogen content. The linear relationships in C appears to be typical for the yeast utilized in this study. Äyräpää (1968) found similar tendencies but also others which varied with yeast species and strain.

The reason for the varying shapes of these curves is still unknown. The higher alcohol decreasing effect of ammonia is well-known and is clearly shown in the lower values of the samples which received it (Villforth & Schmidt, 1954). The thiamine effect has also been reported upon above; its influence is not readily apparent from these data.

In the final study of this series the effect of thiamine was

examined in the presence of ammonia and/or amino acids. Doubling the thiamine concentration had no noticeable effect on the higher alcohols, it was clearly present in the must at a sufficiently functional level so that it was not influenced by further increases (Table 14). The same was the case with ammonia and amino acids or ammonia alone as in group C.

In general the higher alcohol formation suppressing effect of ammonia was again shown. No further significant effects, due to the influence of thiamine, could be determined.

Generalisation.

The approach to this series of experiments was partly based on the commonly accepted contribution of esters to bouquet. It was for this reason, as noted previously, that ester moieties were analysed. Although they apparently did in some cases correlate with bouquet formation, the pattern was not consistent. They could, therefore, not be used as standards in bouquet prediction.

Nordström (1962a, 1963b, 1966b, 1966c) showed subsequent to this work that the formation of various esters was apparently not one which was critical to the existence of the yeast cell, but was an energy-requiring, and probably an enzymatic process. Formation of e.g. ethyl acetate by chemical esterification was found to be insignificant in relation to the enzymatic formation.

The latter authority also reported that a shortage of e.g. nitrogen or phosphorous would lead to a decrease in ester formation. It is also generally known that fermentation rate will be retarded under the latter conditions. This has been shown to be especially true for nitrogen. Where fermentation was markedly

slow, bouquet was also found to be poor; the best bouquet formation occurred in those samples which showed the highest fermentation rate. But this was not consistent and, therefore, no clear criterion. There were very obviously other factors which were not examined here, which did not necessarily affect the fermentation but did influence bouquet. In partial confirmation of this it was found that synthetic samples containing all the tested components in a sugared medium showed no recognisable vinous bouquet. The initial series showed that all future media must contain, as basic ingredients, ammonia, the noted amino acids and thiamine. Not one of the other vitamins were shown to be a necessity.

B (1) Cation and anion exchange resin eluates from grape juice loaded columns.

The initial studies showed that a cation resin can adsorb components from musts which are critical in bouquet formation. However, it was also clear that there could have been components, other than those already examined, which were also functional. This series was carried out to confirm validity of the foregoing.

(a) Macro fractionation with HCl and NH₄OH.

Riesling must was passed down ion exchange columns as before. The columns were washed with distilled water and the absorbed components eluted ($0.6 \text{ ml cm}^{-2} \text{ min}^{-1}$) as noted (Table 16). The eluates were concentrated with repeated water addition in vacuo at 50-60°C to remove excess acid and alkali. These concentrates were added to the ion exchange treated must (pH 2 must) and pH adjusted as before to untreated must value. Since it was not certain that thiamine was present in the concentrate it was added at 34 µg %. The control sample was untreated.

The samples all contained 250 ml must which was brought to a final volume of 300 ml by the addition of concentrate and/or water.

Table. 16.

Sample plan indicating fractionation system.

Sample.	Must volume (liter).	Resin quantity gm (moist).	Eluent.	Fraction and vol. (ml).	Fraction volume used (ml).
1	1.5	200	N HCl	1 1000	250
2				2 500	250
3	1.5	200	2N HCl	1 500	250
4				2 1000	250
*5	6	800	5N HCl	Total	$\frac{1}{3}$
6				bulked (5 liter)	$\frac{1}{2}$
7	3	400	2N NH ₄ OH	1 1000	250
8				2 1000	250
9				3 1000	250

* Approximately $\frac{1}{3}$ and $\frac{1}{2}$ of total fraction used.

The fermentation data are given in Fig. 9. The fermentation rates generally showed the typical variations achieved in the preceding work with both fast and slow fermenting samples. Of interest, however, was the marked increase of tempos in the bulked 5N HCl eluate samples (5, 6); they were very close to that of the control and very much like that of the pH 3.2 samples of the first study (Fig. 1). Furthermore, the first fractions of the N HCl, 2N HCl and 2N NH₄OH eluents all fermented faster than did the second or subsequent fractions in the relevant groups. Although initially lagging, the first fraction of the 2N HCl eluate rapidly overtook the second (Fig. 9, 3 vs 4).

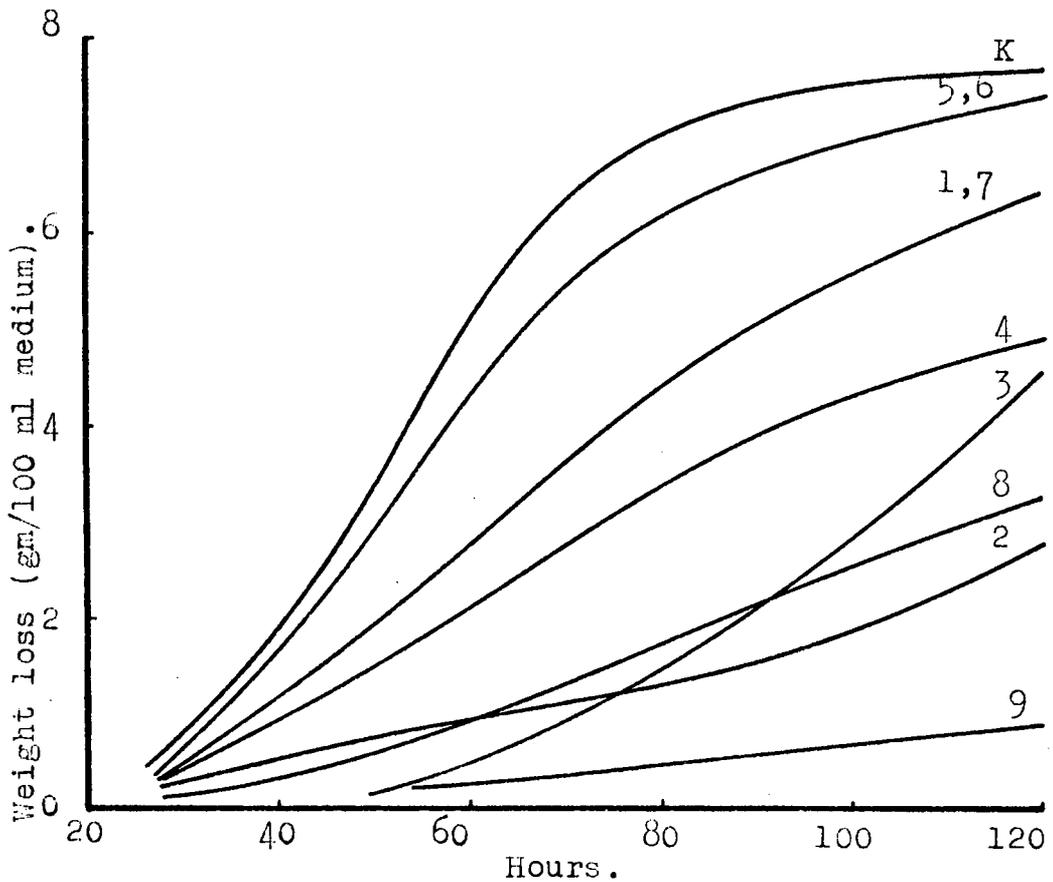


Fig. 9. Fermentation of ion exchange treated must with additions of fractions of resin absorbed must components. (K, Control, untreated; 1, 2: first and second fractions, 1N HCl eluate, respectively; 3, 4: first and second fractions, 2N HCl eluate, respectively; 5, 6: $\frac{1}{3}$ and $\frac{1}{2}$ of bulked 5N HCl eluate, respectively; 7, 8, 9: first, second and third fractions of 2N NH_4OH eluate, respectively. See Table 16 for specific additions).

The first series showed that ammonia addition raised the fermentation rate. Furthermore, the effect of these additions were also reflected in a lowering of the higher alcohols. Ammonia would have been present at a higher concentration in the first 1N and 2N HCl acid eluate fractions (Table 16).

(Samuelson, 1953). In both of these samples a confirmation of this appeared to be achieved in an increased fermentation rate and lowered higher alcohol concentration (Fig. 9, Table 17). On the other hand the alkali eluate fractions, where ammonia elution was not pertinent, also differed in fermentation rate, with the first fraction higher than the second. This finding, together with the initial retarded fermentation of the first 2N HCl fraction and the very high and similar rates of the bulked eluate samples indicated that there were unknowns being eluted from the columns which affected fermentation.

Table 17.

Analyses of wines of ion exchange treated Riesling must containing fractions of cation resin eluate.

Sample.	pH.	Total alcohol vol. %	Volatile acidity gm/l.	Higher alcohols mg/100 ml.
* C	3.7	10.4	0.27	22.3
1	3.7	10.2	0.27	20.3
2	3.8	10.3	0.37	33.4
3	3.6	9.9	0.72	18.4
4	3.8	10.1	0.36	27.0
5	3.6	10.3	0.42	20.7
6	3.6	10.3	0.54	18.0
7	3.9	10.2	0.69	26.6
8	3.8	10.2	0.41	24.6
**9	-	-	-	-

*C = Control, untreated must. (See Table 16 for treatments).

** Fermentation extremely prolonged; data not included.

The sensory evaluation of these wines were carried out by three judges where samples were ranked only on bouquet and against the control as standard. The judges were unanimous, in that the sample containing the initial fraction of the NH_4OH eluate (7) manifested an unpleasant character. They furthermore, all agreed that the sample containing one third the total bulked 5N HCl eluate (5) was the best. The rankings of the first four samples are given in Table 18.

Table 18.

Bouquet ranked position of wines of ion exchange treated musts.

Judge.	Ranked position of sample.			
	1.	2.	3.	4.
A	5	1	3,K	6
B	5	3	1	K
C	5	6	K	1

The analytical findings relative to the sensory showed no consistent pattern. With additional analytical data a meaningful relationship may have been found. However, this aspect was not investigated further and these analysis were, therefore, discontinued. Fermentation rates, relative to bouquet formation, were up to this stage, the only readily determinable factor which kept to a pattern in that the samples manifesting the higher fermentation rates normally contained those with the better bouquet.

-61-

(b) Macro fractionation with 5N HCl.

Since the 5N HCl bulked eluate gave promising results it appeared that a fractionation of it. i.e. not bulking, could also produce acceptable bouquet formation. Should such a cut exist it would simplify later identification of critical bouquet forming or relevant components. Also included in this series were combinations of fractions of eluates of the former study. The additions are set out in Table 19.

Table 19.

* Sample plan indicating volume (ml) of eluate fractions used.

Sample.	Fraction.		Fraction.		5N HCl bulked.	2N NH OH Fraction.		**	5N HCl Fraction.			
	1.	2.	1.	2.		1.	2.		1.	2.	3.	4.
1	250	250	-	-	-	-	-	-	-	-	-	-
2	-	-	250	250	-	-	-	-	-	-	-	-
3	-	-	-	-	-	250	250	-	-	-	-	-
4	-	-	-	-	1/6***	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	50	-	-	-	-
6	-	-	-	-	-	-	-	200	-	-	-	-
7	-	-	-	-	-	-	-	-	250	-	-	-
8	-	-	-	-	-	-	-	125	125	-	-	-
9****	-	-	-	-	-	-	-	-	-	250	-	-
10	-	-	-	-	-	-	-	80	80	80	-	-
11	-	-	-	-	-	-	-	50	50	50	150	-
12	-	-	-	-	-	-	-	-	-	-	-	-
(Control)												

* First four eluates are of previous series (See Table 16).

** Volumes collected for fractions 1 - 4 were 500, 500, 500, 1,500 ml, respectively.

*** 1/6 of total bulked eluate.

**** 160 ppm ammonia added.

This pilot macro-fractionation was carried out using the same parameters as in the previous study. Thiamine was added at 34 µg to all samples. Ammonia was added to sample 9 since it was doubtful whether it contained sufficient.

The organoleptic evaluation was carried out by four judges, again comparing against the control as standard. The best bouquet was noted in 4 with 5 also good. The bouquet of sample 6 tended to be weaker than that of control. The weakest sample was number 3 which contained the combined alkali eluates. The remarks of the judges on bouquet are noted in Table 20.

Table 20.

Bouquet-ranked position of wines of ion exchange treated must.

Sample.	Judges.			
	A.	B.	C.	D.
4	good	the best	very good	very fruity.
5	equal to control.	better than control.	clean	clean
6	weaker than control.	better than control.	very little bouquet.	weak bouquet (like 2).

The remaining samples showed no marked formation of bouquet. Clearly, the 5N HCl eluate was important in this phenomenon; it confirmed the results of the previous tests.

The fermentation rates are not shown but again differed strongly. Sample 11, e.g. fermented just as fast as the control whereas 4 was intermediate between these samples with 6 the slowest fermenting. In this determination, fermentation rate failed as an indication of bouquet formation

(c) Individual and combined effects of amino acids.

The high hydrogen ion concentration in the 5N HCl eluant had as effect the strong elution of the amino acids held on the absorbing resin column. Since it was the aim to later dispense with the pH 2 must as base and use a synthetic medium the effect of individual amino acids was investigated. The strong acid eluate concentrate contained high concentrations of amino acids; it was at this stage not sure which of these were critical to bouquet formation.

(i) The high concentration amino acids of musts.

Ion exchange treated Riesling must (pH 2 must) was again used with addition of ammonia, thiamine and cations as before. The amino acids determined (Table 7) were examined. To these samples were added either all the amino acids or all but one of them. The readily assimilable amino acid pairs viz. alanine-glutamic acid, glutamic acid - aspartic acid and alanine - aspartic acid were also added to some samples (Schultz & Pomper, 1948).

The findings on bouquet formation in this series showed that none of these samples differed markedly, one from the other. The only significant finding was that the absence of glutamic acid slowed down fermentation slightly; this had no obvious effect on bouquet which was poor in all of the samples.

(ii) Low concentration (activating) amino acids of musts.

In the analyses of amino acids in ion exchange treated must it was noted that small quantities of amino acids (less than approximately 2 ppm) were present. Since there was apparently a slight leakage of amino acids it was probably that these were the acidic ones; they would have been eluted prior to the more basic amino acids. On the basis that micro quantities of these

latter components could have influenced bouquet formation, this series was planned to incorporate them together with the already used amino acids. The micro amino acids are also noted to be activating (Brunner, 1960). Also investigated in this series were samples which contained the full complement of amino acids as reported and determined in musts (See Table 16) (Luthi & Vetsch, 1953; Castor, 1953; Castor & Archer, 1956; Lafon-Lafourcade & Peynaud, 1959; Drawert, 1963; Van Wyk & Venter, 1965) as well as those determined in yeast cells (Mojonnier, Hedrick & Porter, 1955; Brunner, 1960). Sample 1 and 2 amino acid additions are given in Table 21. The balance of the sample plan follows the latter table.

Although in some cases DL components were added only the L form was taken into account.

Table 21.

Amino acid concentration in grape must and yeast cells as used in Samples 1 and 2.

	<u>Sample 1.</u> Concentration as in must (mg/l).	<u>Sample 2.</u> Concentration as in yeast cells. (mg/l medium)*
L-Aspartic acid	40	170
L-Glutamic acid	120	160
Glycine	20	65
DL-Threonine	150	104
DL-Serine	100	65
L-Histidine	25	25
DL- β -Phenyl alanine	120	104
L- α -Alanine	250	67.5
DL-Valine	120	145
DL-Methionine	120	30
L-Leucine	60	92.5
L-iso-Leucine	60	52
L-Arginine	1,000	75
L-Lysine	15	112.5
L-Tyrosine	30	45
L-Ornithine	15	160**
L-Asparagine	100	170**
DL- γ -Amino butyric acid	300	8**

Cont./

Table 21 continued.

	<u>Sample 1.</u> Concentration as in must (mg/l).	<u>Sample 2.</u> Concentration as in yeast cells. (mg/l medium)*
DL-Proline	140	104
L-Tryptophane	16	30
L-Cystine	8	2

* Dry yeast weight taken as 2.5 gm/liter medium.

** From data of Brunner, (1960).

Samples 1 and 2:- See Table 21.

Sample 3:- aspartic acid, glutamic acid, glycine, α -alanine, leucine, iso-leucine, β -phenyl alanine in concentrations as given in Table 7 together with threonine, histidine, valine, lysine and tryptophane each at 1 ppm (with respect to the L-form).

Sample 4:- The same initial seven amino acids as in 3 but with methionine, lysine, arginine and asparagine at 1 ppm.

Sample 5:- Combination of 3 plus 4.

Sample 6:- Amino acids listed under 1 and 2 in concentrations of 1 ppm.

Sample 7:- As in 6 but at 0.5 ppm

Sample 8:- As in 6 but at 0.25 ppm.

Sample 9:- No amino acids.

Sample 10:- Untreated must (Control).

The fermentations of these samples varied strongly; sample 1 fermented as fast as the control with the others all at a slower rate (No data given). This was, however, not reflected in the bouquet for sample 1 was markedly malodourous. This was also determined in 2 whilst 6 - 9 lacked bouquet. In relation to the latter samples, the bouquet of 3 - 5 were much better lacking strong malodours. Sample 5 was the best of these three. Although the formation of bouquet did not occur to such a

strong degree here as had already been noted previously it was evident that the concentrations of amino acids used, were, under these conditions, conducive to an organoleptically cleaner sample.

A further two series of 50 samples were carried out where the high concentrations of the initial seven amino acids (see sample 3 above) were each in turn decreased to 1 ppm. Pairs of these amino acids were similarly treated. All these samples contained the low concentration (1 ppm) amino acids as in sample 5 above. Not one of these samples showed any bouquet improvement over that of 5. These latter concentrations of amino acids were, therefore, used in all subsequent work and denoted as the macro and micro amino acids. It was found at a later stage that if the ratios of the macro amino acids was held the same, the concentrations could be decreased to half the original value without affecting the bouquet formation. These concentrations of the macro amino acids were consequently used in all the latter studies.

2.4) Anion exchange, resin eluates from grape juice loaded columns.

With the use of a synthetic medium as objective it was necessary that effective anions be determined. To this end the anion eluate concentrates (AE) were incorporated in a pilot series. The AE's were prepared both directly from control must or from cation treated must by eluting the anion resin column (Amberlite IRA-410, hydroxide form) with 3N HCl and concentrating in the same manner as for the cation eluate concentrates (CE). The AE turned dark brown during the concentration process.

In this series only three samples received AE, the balance (19) were control or those to which the anions (sulphate, borate, molybdate and phosphate) were added (Wickerham, 1951). Apart

from the control the only samples which fermented after one day were those three which had received the AE addition.

Since fermentations were strongly retarded this series was repeated. However, a weak base resin (Amberlite IR 45 - free base form) was used instead of the initially applied strong base resin. It is known that the latter resin type, in contrast to the former can adsorb glucose (Khym, Zill & Cohn, 1957) and this was probably the cause of the very dark brown colour of the anion eluate due, no doubt, to caramelisation at the temperature of vacuum concentration (50°C). Weak base resins do not absorb glucose but could be less effective (Khym et al, 1957).

A 310 gm resin column was treated with 1.2 liter cation resin (H cycle) treated must and, after washing, eluted with 1.5 liter 3N HCl. The additions made to the aqueous samples are given in Table 22. These data showed that only those samples which received the AE commenced fermentation normally after 24 hours.

These results were unexpected and indicated that the medium containing synthetic anions was incomplete. It was initially assumed that inositol was not necessary in the medium. This did, however, not appear to be the case for the addition of this component to the non-fermenting samples caused fermentation to commence normally.

These findings indicated first, that the yeast required inositol for normal fermentation and secondly that it was strongly retained by a weak base resin but could be desorbed by the HCl eluent. Subsequent samples, which did not receive the AE, therefore, all received inositol at 2 ppm (Wickerham, 1951).

Table 22.

*Additions of cation and anion eluates made to aqueous media and effect on fermentation.

Cation eluate must.	**Vol. cation eluate added.	**Vol. anion eluate added.	Fermentation.
Riesling, 1962	150	150	+
Riesling, 1962	75	150	+
Riesling, 1961	150	150	+
Riesling, 1961	75	150	+
White French, 1962	150	150	+
White French, 1962	75	150	+
Green grape, 1960	150	150	+
Green grape, 1960	75	150	+
Riesling, 1961	150	Nil	-
Riesling, 1961	75	Nil	-
White French, 1962	150	Nil	-
White French, 1962	75	Nil	-
Green grape, 1960	150	Nil	-
Green grape, 1960	75	Nil	-

* Sample size 150 ml.

** Concentrate from given must volumes (approximate)
Anion eluates from same must.

(b) Anion with cation resin eluates in aqueous media.

The sample plan for this series is noted in Table 23. Fermentation in all these samples occurred normally. The bouquet formation occurred strongest in sample No. 3. One judge noted it to have a flowery bouquet but nothing much else.

These findings were of much interest since this specific flowery bouquet could be induced from cation and anion extracts added to an aqueous glucose solution. The use of treated must as basal medium was, therefore, discontinued.

Table 23.

* Additions of varying concentrations of cation and anion resin eluate concentrates to aqueous and ion exchange treated must.

Sample.	<u>CE</u> ≈ ml must.	<u>AE</u> ≈ ml must.
1	100	100
2	100	50
3	50	50
4	25	100
5	25	50
6	25	25
7	100	100
8	100	50
9	50	50
10	25	100
11	25	50
12	25	25

*Samples 1 - 6 were added to 18% aqueous glucose solution containing inositol (2 ppm) vitamin B1 and ammonia in previously noted concentrations.

Samples 7 - 12 were added to cation and anion resin treated must containing vitamin B1, inositol and ammonia in previously noted concentrations.

Additions were made to 100 ml samples.

CE: cation eluate concentrate; AE: anion resin eluate concentrate.

The effect of the AE checked in the following series but with its influence relative to varietal property. The varietal CE was simultaneously examined. The sample plan is noted in Table 24.

The fermentation rates of these samples were to all intents the same. The most marked delicate bouquet formation occurred in samples 1, 2 and 3 with samples 5 - 12 not differing very much but with poorer bouquets. Samples 13 - 16 manifested a readily perceivable full character not in keeping with a delicate white wine.

Table 24.

*Sample plan in determining the varietal effect of cation and anion resin eluate concentrates on bouquet formation.

Anion eluate concentrates (cultivar).	Cation eluate concentrates (cultivar).			
	Riesling.	White French.	Green Grape (ex Tulbagh).	Green Grape (ex Franschoek).
Riesling	+ (1)	+ (5)	+ (9)	+ (13)
White French	+ (2)	+ (6)	+ (10)	+ (14)
Green Grape (ex Tulbagh).	+ (3)	+ (7)	+ (11)	+ (15)
Green Grape (ex Franschoek).	+ (4)	+ (8)	+ (12)	+ (16)

* (i) Sample numbers are given in parenthesis next to each mixture.

(ii) 100 ml samples, containing concentrate from approx. 50 ml must; the basal aqueous medium contained 18% glucose, inositol (2 ppm), vitamin B₁ and ammonia in previously stated concentrations.

These data tentatively indicated that the cation eluate concentrates were important in bouquet formation; in fact apparently more so than anion eluate concentrates. Furthermore, the best results were achieved with the Riesling variety. This was consequently used in all further studies and combined with any eluate except that from Green grape ex Franschoek.

It must be stated, however, that these data were indicative only and in keeping with the pilot nature of most of the foregoing work. The variations which could occur in bouquet was partly confirmed when the bouquet of the latter samples (held in half full nitrogen sparged containers) were found to be considerably weaker at a re-evaluation one week later.

3. Fractionation of cation eluate concentrate and use in synthetic media.

Since the CE appeared to contain unidentified components contributing to bouquet formation it was fractionated in an attempt to narrow the field of investigation.

Dry Riesling CE lots (≈ 100 ml) were extracted with polar and relatively non-polar solvents viz. ethanol, acetone and ether (1 x 25 ml). The solvent-free extracts were tested for bouquet formation in a 18% glucose media which contained Riesling AE, macro and micro amino acids, vitamin B₁, and ammonia in previously noted concentrations. The acetone fraction had an excellent bouquet, in fact it was judged to be better than that of the control (Riesling CE and AE).

The latter series were repeated using a synthetic medium which varied from that of Wickerham (1951) in that amino acids, ammonia, glucose and vitamins were added as already noted. Tartaric acid was also added at 3.5 gm/l and the pH, as in all subsequent work adjusted to 3.2 with NaOH. Bouquet formation did not occur in the acetone fraction as reported before, in fact, this sample was rated as poor with control (Riesling CE and AE) being clearly better.

The CE used for extraction was subsequently increased (≈ 250 ml must) and its extract examined by the same procedures as above for bouquet formation. Bouquet now occurred strongly; one judge noted that it had perhaps too much. A check on these findings in a repeat test gave negative results.

The results achieved here were no doubt, due to unknown factors and highly unpredictable. The system for obtaining this fraction was, therefore, regarded as unsatisfactory and

discontinued. It was, however, determined that wine bouquet could be induced in a completely synthetic medium by a fraction obtained from grape juice.

C. Ion exclusion system in recovery of relevant grape components.

The fractionation of the CE by means of various solvents achieved no clear pattern in the formation of a good bouquet and were largely based on the apparent elution of electrolytes from the resin column by means of ion exchange.

In addition to ion exchange all such types of resins also have the property of absorbing compounds in the resin liquid phase (Simpson & Bauman, 1954). This, in the case of non-electrolytes, is ascribed to the so-called Donnan effect (which restricts penetration of electrolytes into the resin structure) and polar interactions and van der Waal force effects between the resin matrix and the non-electrolyte. (Reichenberg, 1957a). Under suitable conditions the distribution coefficient of a non-electrolyte between the intra and extra-resin phases, is normally larger than that of an electrolyte.

A charged resin may consequently be eluted with water since no ion exchange is operative. Interesting separations of ketones, aldehydes, alcohols and ethers have been carried out in synthetic solutions (Rieman, 1961). However, in this study the charging of the resin column (H form) with grape juice involved, no doubt, more complex processes than in synthetic solutions. The resin was in this instance used simultaneously as an agent for removing cations and absorbable components and for absorbing probable bouquet precursors. These latter components, if indeed such were held, could then be readily removed by a water eluant

without desorbing the absorbed must cations. Since resin capacity for ion exclusion is normally low it was surmised that in the present studies, this would be further lowered as a result of concomittant ion exchange.

Grape juice contains considerable quantities of sugars. This was, however, not regarded as a problem since these sugars, viz. glucose and fructose are highly soluble in water and their distribution coefficients would no doubt be small. The sugar solutes would in all probability, therefore, assist in salting out less soluble components into the resin liquid phase.

1.(a) Water eluate in synthetic media.

The must used in this and all subsequent series was preserved with ca 2000 ppm sulphur dioxide and held at room temperature. It was surmised that oxidation in the adversely cold stored must (used in the earlier studies) could have occurred by incursion of air and affected the results. The excessive sulphur dioxide did not interfere in any of the subsequently used extraction procedures or fermentations.

In the initial study one liter of Riesling grape juice was passed through 400 gm of Amberlite 1R 120 (commercial grade, H cycle) at a slow flow rate ($0.25 \text{ ml cm}^{-2} \text{ min}^{-1}$). The must was washed through with water (75% of bed volume) and one liter water eluate subsequently collected. This was evaporated under vacuum at 25°C until nearly dry. The concentrate was extracted with two ca 10 ml portions of acetone as was done in the case of the CE. The extract free from acetone, had a herb-like odour, was used in toto in the modified Wickerham (1951) medium. The residue was also utilized in a similar sample.

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Bouquet formation occurred in both of these samples. However, that in the acetone extract sample was markedly more pronounced and also cleaner than in its residue containing counterpart.

This phenomenon was checked using what was regarded as a poorer quality must (Green grape - ex Franschoek) and applying the same technique as before. A further 500 ml water eluate was collected after the liter fraction and also tested. Bouquet formation occurred to the highest degree in the sample containing the liter fraction but not of as high an order as in the first of this series. The extract of the second water eluate fraction also showed bouquet formation but very much less than that in the latter sample. The completely synthetic control sample showed no vinous characteristics at all; it had a typical "cabbage" odour.

The water eluate effect was re-tested with good Riesling must. Since acetone extraction of the concentrated fractions gave good results a pilot extraction of Riesling must with acetone was also included. Two hundred and fifty ml Riesling must was concentrated (vacuum, 25°C) to approximately 100 ml to increase the "salting out" effect of glucose. Twenty-five ml acetone was added to this and intermittently shaken for four days. This fraction as well as the standard water eluate fraction were tested for bouquet formation in the normal manner but none of them gave positive results. The water eluate fraction sample now manifested a full "honey" odour, as sometimes noted in dessert wines but which is regarded as undesirable in delicate dry white wines.

The water eluate fraction was again checked but again gave no delicate bouquet. This was repeated but in this case dispensing with the acetone extraction of the water eluate concentrate. Two-thirds of this fraction was used in one sample and one-third in another. Bouquet formation occurred in both samples but more strongly in the sample with the largest addition. This latter sample also manifested an "bitter almond" back-ground characteristic. The bouquet concentration in the one-third added sample was low and was readily shaken off. The completely synthetic control sample was again malodourous. Since this latter phenomenon occurred regularly in all subsequent studies and it will hereafter not be noted.

A repeat of the latter study confirmed bouquet formation although the one-third sample manifested the better results. A fraction containing an appreciable quantity of must i.e. collected just prior to the water eluate fraction gave the best bouquet formation. A subsequent test gave a similar result. The further investigation of this fraction would however, have served no purpose since elimination of ineffective bouquet-inducing components was clearly not the case here as was in the standard water eluate fraction.

A further five studies were carried out after the latter. These tests included solubilization chromatography (Rieman, 1961) with 25% ethanol as well as acetone-formic acid-water and ethanol-formic acid-water (40-30-30) eluants. The acetone eluant fraction had a pronounced peach-apricot odour, most probably an artifact, which carried over into the fermented sample. The common ion i.e. hydrogen ion concentration was also increased in the must to pH 0.5 with HCl prior to passage

through the resin column. The overall effect on the must cations would be a decreased exchange and probable slightly increased "salting out" effect on non-electrolytes.

In not one of these experiments was the high bouquet concentration achieved as that in the earlier studies. The effective components were apparently being influenced by an unknown factor or factors.

Fractionation of water eluate.

Since the water eluate, like the cation eluate gave erratic results it appeared that either the bouquet relevant components were losing activity during the recovery process or their effect was masked by other simultaneously recovered substances.

Although the parameters and conditions for and of recovery were kept as constant as possible it was not certain that variations were not occurring. Since this was unknown it was initially assumed that the latter reactions did not occur. Consequently the initial step was a fractionation of the water eluate where the first approach was limited to the effect of acids. This was based on the premise that, especially the higher fatty acids, could react as ester moieties (Nordström, 1964b). Since concentration of water eluates etc. by evaporation was applied in the recovery processes it was assumed that these acids were not highly volatile. That they, as well as aldehydes, could be obtained by ion exclusion has been reported in the review and studies by Simpson et al (1954), Reichenberg (1957b) and Rieman (1961).

(i) Ion exchange chromatography.

The pilot fractionation of the acids was initially carried out by the method used in the quantitative determination of grape acids, utilizing a strong base resin (Du Plessis, 1968). The water eluate was concentrated as normally done and loaded on the columns. Forty-four x 10 ml fractions were collected and every three bulked. The concentrated fractions were each checked for bouquet formation. The typical "cabbage" odour, the same as the control sample, appeared in each fraction's sample. Clearly, therefore, this method was ineffective in separating bouquet precursors or inducers.

(ii) Precipitation.

In the second approach the acids were separated by precipitation. They were neutralized in the concentrated water eluate (ca 50 ml) to pH 7.8 by addition of 5% barium hydroxide. The clear yellowish brown solution was concentrated to ca 10 ml and 10 volumes of 96% ethanol added. Riesling must gave a voluminous yellow green precipitate and White French practically none. This precipitate was centrifuged down and the supernatant decanted and retained. The precipitate was dissolved in the minimum amount of water, normally about 25 ml for 1 liter Riesling must. The pH of this aqueous phase was decreased to ca 2.5 with dilute sulphuric acid and the precipitated barium sulphate centrifuged off and discarded.

The supernatant was used and will be referred to as the "acid" fraction in subsequent studies. The initial alcoholic phase will be referred to as the "alcohol" fraction. The small amount of barium in this fraction was also removed as noted above and the alcohol-free fraction used.

In the first series the "acid" fraction of 1 liter Riesling must was used as before (See B.3) in 100 ml synthetic samples. Fermentation rate of these samples did not differ from that of the control. The bouquet of the acid fraction sample manifested a most pronounced flowery bouquet. It was noted by one judge that it was like a cold-fermented wine. It will be recalled that these samples were fermented at 24-25°C. Another pleasing aspect of the "acid" fraction sample was its "clean" odour; it had none of the "cabbagy" smell of most of the other samples. The alcohol fraction manifested no bouquet but had a characteristic "flour bag" odour. This was typical; it occurred consistently whenever this fraction was used.

A repeat series was carried out using one-third and two-thirds portions of the acid and alcohol fractions. The two-thirds portion of the former fraction gave a good bouquet formation, the one-third less so. The alcohol fractions gave the typical noted odour.

The effect of the bouquet-inducing acid fraction was again checked. The judges noted that the bouquet of this sample had a strong flower bouquet (very clearly so), pure flower bouquet, very strong and the strongest bouquet in the whole of this specific series of seventeen samples.

These results appeared promising in that strong bouquet formation occurred with a high consistency in four replicate series. Although the fraction effecting this phenomenon was noted as the "acid" fraction it certainly contained components other than acids. This was already clear during the neutralization with barium hydroxide where its colour changed to a yellowish brown. Furthermore, during the alcohol precipitation step the insoluble

barium fraction had a greenish yellow colour. These two reactions are typical of phenols. Such components were, therefore, no doubt, also included in this fraction. The following step here was clearly a further separation of, and examination of possible critical bouquet inducing factors in the "acid" fraction.

An important finding during this study was the loss of activity of this fraction during the storage of grape juice. An attempt to obtain an effective fraction from grape juice of the previous vintage was not successful. The juice used in obtaining the active acid fraction was again used 9 months later. No bouquet formation was achieved. The volume must used to obtain this fraction was ultimately increased to 4 liters. This was also unsuccessful. These findings clearly showed that the critical components were of an unstable nature. Fresh juice not exceeding 3 months age was consequently used in future work.

(b) The alcohol soluble water eluate fraction.

The alcoholic supernatant fraction, remaining after the removal of the insoluble barium compounds (potential A fraction) consistently gave, as noted, a "flour-bag" characteristic in the bouquet of the fermented sample. The effect of this fraction was also checked with CGA, acids and alcohols (See Chapter III C.3 (a) (i), (ii)). The results were all negative relative to bouquet increases.

This fraction was suspected to contain aldehydes which subsequent checks with 2, 4 dinitrophenyl hydrazine indicated to be the case (Vogel, 1951). These compounds were, therefore, removed as their hydrazones (Pippen, Nonaka, Jones & Stitt, 1959). The excess reagent was removed by extracting with ethyl acetate.

The bouquet effect was checked with the hydrazine treated alcohol fraction. It was determined that the "flour-bag" smell had disappeared. However, very little else of interest remained in the bouquet of these samples. Further investigation into this fraction was, therefore suspended.

2. The "acid" fraction (A fraction).

(a) Fractionation by steam distillation.

In the foregoing procedures concentration of large aqueous volumes were carried out by evaporation under vacuum. The more volatile components could have been lost under these circumstances. It was, therefore, doubtful if they or the slightly less volatile components were critical. Several series were run where the ice-cooled condensate from fractions were exhaustively extracted with ether (liquid: liquid, 40 hours) and tested for bouquet. Negative results were achieved. However, since it was assumed that the higher fatty acids could have been functional the lower volatility of these substances was noted and steam distillation applied as an initial fractionating step.

The "A" fraction from 1 liter Riesling must (normal pH approximately 2) was steam distilled and two successive 500 ml fractions of distillate collected. These were concentrated by vacuum evaporation and checked for bouquet formation by fermentation in synthetic media as before. The sample which contained the residue i.e. after steam distillation, was found to have the most bouquet. The first 500 ml of distillate fraction also manifested a bouquet but to a much weaker degree than the residue sample and was also slightly malodorous like the control sample. The second distillate sample was very poor and

therefore discarded. An experienced judge, who up to this stage had not participated in the evaluations was asked to examine these samples on bouquet, being told that they were synthetic wine samples. This was done to carry out an interim impartial check. He noted that the control was malodorous, the residue sample was the cleanest and had wine character whilst the first distillate sample tended to both of the latter. These findings agreed wholly with that previously found viz. that a fermented sample was malodorous whereas addition of the A fraction prior to fermentation induced a wine bouquet.

(b) Fractionation by paper chromatography.

Bouquet formation occurred in both the residue (R) and steam distillate fraction (S) samples of the A fraction. It was, therefore, indicated that bouquet precursors or inducers were present in both of the former fractions. On this premise, these two fractions were further fractionated by paper chromatography to determine whether identical components appeared on the chromatograms of these two fractions and whether they could induce bouquet formation.

The steam distilled residue and distillate of an A fraction (2 liters Riesling must) was paper chromatographically fractionated on Whatman No. 3 mm paper with the pentanol:formic acid solvent. The R_m values of the visualized components are given in Table 25. Chromatograms were cut between visualized bands and all strips i.e. including "blank" areas, eluted with water (See Fig. 10).

Table 25.

*R_m values of A fraction steam distillate and residue components.

Residue**				Distillate***		
Band.	R _m .	Range.	Colour.	Band.	R _m .	Colour.
1	0.10	0.10	F(y)	1	0.10	F
2	0.17	0.17	F(y)			
3	0.24	0.23 - 0.26	F(y)	2	0.27	F
4	0.43	0.41 - 0.45	Y			
5	0.61	0.59 - 0.64	F,Y	3	0.61	F
6	0.74	0.72 - 0.75	F,Y			
7	0.90	0.87 - 0.92	F			
8	1.0	1.0	Y			
9	1.16	1.15 - 1.17	F			
10	1.24	1.22 - 1.26	Y			
11	1.34	1.30 - 1.37	F	4	1.15	F
12	1.95	1.92 - 1.99	F,Y			
13	2.29	2.28 - 2.29	F	5	2.33	F
14	2.37	2.37 - 2.38	Y			
15	2.54	2.54	F			

*R values relative to malic acid standard.

**Average R_m value for tartaric acid reference = 0.46.

***Unsprayed duplicate of chromatogram used for elution of bands.

F = Fluoresces.

Y = Yellow with indicator spray.

(y) = No clean separation shown with indicator spray.

These eluates were checked for bouquet formation by the standard procedure in synthetic media. No marked bouquet formation was determined in any of the corresponding distillate and residue bands (Table 25, cf residue 1, 3, 5, 11, 13 with distillate 1, 2, 3, 4, 5 bands). This test was rechecked and similar findings noted.

It was concluded that detrimental chemical charges were apparently being caused by the fractionation and elution system. This technique was consequently discontinued.

3. Chlorogenic acid.

The promising A fraction contained many components (Fig.10). Apart from these shown, it could no doubt also have contained others which were not visualized here. The technique applied to obtain it could, for example, also have included acyl co-enzyme A compounds (Lynen, Reichert & Rueff, 1951; Gregory, Novelli & Lipmann, 1952). However, band 12 (Fig. 10), cut 11) which had no corresponding distillate band (Table 25) gave a fermented sample which showed a slight bouquet development. It also manifested a decrease in malodorous characteristics.

Band 12 showed acidic properties. It furthermore fluoresced bright blue; after ammonia treatment it was bright green under U.V. light (Seikel, 1962). It furthermore, showed strong reducing properties when treated with phosphomolybdic acid solution and ammonia (Feigl & Anger, 1966). However, based on its spot size its concentration was the highest on the chromatogram. Chlorogenic acid gives all the reactions noted above. It is furthermore known to be present in grapes in relatively high concentration (Appendix Table 3)(Sondheimer, 1958; Jurics, 1967). Moreover, it is also adsorbed on a cation exchange resin by van der Waals' forces (Whiting, 1964) and can be eluted with water.

On the basis of the foregoing an initial test of its effect at approximately 2 mM on bouquet formation in a synthetic medium was done. The fermented sample was found to have no malodorous characteristic. In the sensory evaluation it was noted as clean. Its effect on the bouquet of fermented media was similar to that of band 12 and it was consequently examined in greater detail.

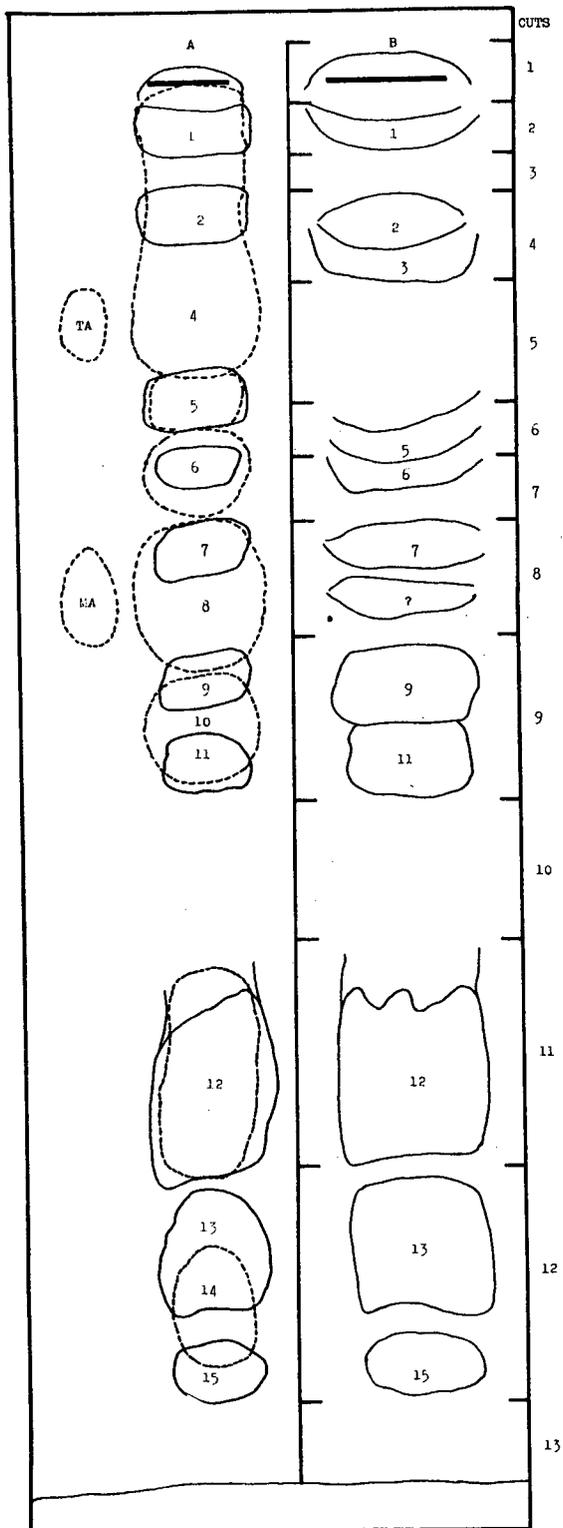


Fig. 10. One dimension chromatogram of A fraction steam distillation residue; A, 10% B, 30% of total concentrate. Broken lines indicate acidic components, unbroken lines border fluorescing compounds. Cuts made in B are shown. TA and MA are, respectively, tartaric and malic acid reference. Band numbers in A & B correspond to data in Table 25.

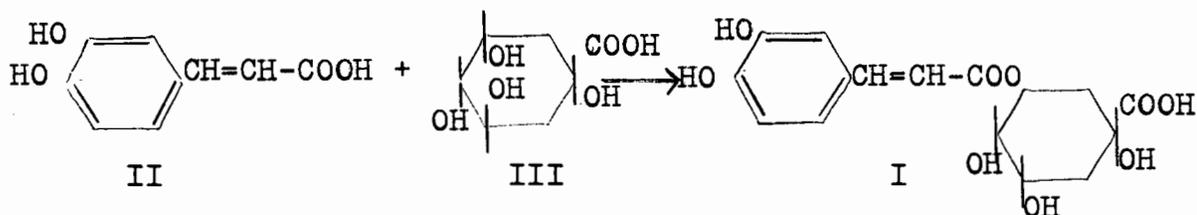
(a) Effect on bouquet formation in synthetic media.

In this series CGA was added to the basic synthetic media in concentrations of 2.1, 1.4, 0.7 and 0.3 mM (75, 50, 25, 10 mg%, w/v). Also included here was the basic medium (control) and one sample containing the A fraction (from 1 liter Riesling must).

The control was again malodourous with the A fraction having an excellent and very strong bouquet. The 2.1 and 1.4 mM CGA samples had markedly less bouquet than the A fraction samples; but with the malodorous "cabbagy" control characteristic absent. Below these CGA concentrations i.e. for 0.7 and 0.3 mM the control characteristic again appeared.

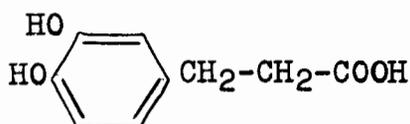
The inoculum used up to this stage was 2% of a 50% diluted standard sterilized grape juice. It was not certain what effect the grape juice components had in conjunction with the CGA. A series was therefore done where the four CGA concentrations were checked in counterpart samples, inoculated with the normal grape juice propagating media and one where a loopful of yeast from the latter was propagated in the basic synthetic media. The 2.1 and 1.4 mM CGA samples again gave the "cleanest" effect; the propagating media made no difference. All subsequent inoculation was, therefore, done with the grape juice media but with the inoculum decreased to 1%.

Since chlorogenic acid (I) is an acid ester consisting of the moieties caffeic (II) and quinic acids (III), it appeared probable that the CGA effect was due to one of these moieties.

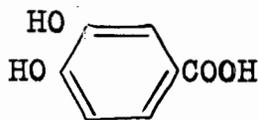


Samples of basic media, each containing 1.6 mM CGA, caffeic acid and quinic were checked for bouquet. The former two gave clean samples whereas the latter was the same as the control i.e. it was as malodourous. The effect of CGA was clearly due to its caffeate moiety.

It was furthermore of interest to determine whether specific groups in the caffeate molecule were effective in this phenomenon. As a first approach the influence of the conjugated aliphatic side chain was investigated. One to 1.6 mM concentrations of dehydro caffeic acid (IV), the ethyl ester of this acid and protocatechuic acid (V) were tested in synthetic media.



IV

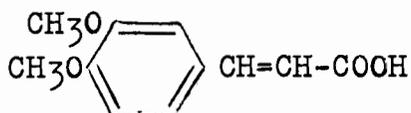


V

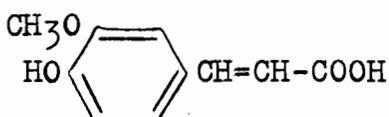
If the unsaturated side chain was indeed effective here, its replacement by a saturated one would not cause the clean bouquet to develop. It was found that good results were achieved in each case. On these findings, the indications were that this group had but little effect. The fermentations, relative to weight losses, were also quite normal.

The ortho orientated phenolic di-hydroxy groups are known to be readily oxidisable (Swain, 1962) and are also effective as inhibitors. The hydroxy group effect of caffeic acid was therefore, pertinently examined by using components which did not

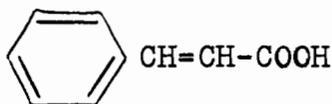
contain them or where they were replaced by methoxy groups viz. with 3, 4 - dimethoxy cinnamic acid (VI), ferulic acid (VII) and cinnamic acid (VIII).



VI



VII



VIII

It was not possible to add these components in as high a concentration as wished since they were not readily soluble in the medium. The di-methoxy cinnamic acid was added to 1.0 mM; this did, however, not completely dissolve, the ferulic acid was not fully soluble at 1.25 mM and the cinnamic acid was also not wholly soluble at 1.0 mM.

In comparison with caffeeate samples all these were markedly inferior in bouquet. The di-methoxy cinnamic acid sample had the typical "cabbagey" characteristic of the control sample.

The ferulic acid sample had an unpleasant odour quite different from the control. The cinnamic acid sample would not ferment and in a subsequent series of lower concentrations viz. 0.07, 0.17, 0.34 and 0.68 mM were used. Fermentation occurred normally in the first two, was retarded in the third and did not occur in the last. The fermented samples all had particularly

vile odours. These were found to be carry-overs from the unfermented media which manifested this characteristic upon addition of the cinnamic acid.

The fermentations, i.e. weight loss, of both the di-methoxy cinnamic and ferulic acid samples were also clearly retarded but not as strongly as in the case of cinnamic acids. De Greef & van Sumere (1966) also reported similar findings for cinnamic acid, and o-hydroxy and ferulic acids which inhibited yeast growth strongly at 0.3 and 0.6 mM, respectively.

These data clearly indicate the importance of the o-di-hydroxy orientation in bouquet formation. It appeared at this stage that the caffeates could be functional as potent reducing agents due to their relative ease of oxidation. If this were indeed the case then good results could also be expected with reducing agents such as sulphur dioxide, ascorbic acid or cysteine. Samples were consequently prepared containing 75 and 150 ppm sulphur dioxide and 50 ppm ascorbic acid all with and without 1.5 mM caffeic acid. All those samples which did not contain caffeic acid were found to have the malodourous characteristic. Clearly the reducing agents had no effect during fermentation and caffeic acid was, therefore, not functional in this respect.

(i) With ester moieties.

Since this approach was based on a mono-function of caffeate it appeared probable that its effect, relative to bouquet increase, could have been in conjunction with other factors. It seemed possible that this influence could be upon ester moieties and which was not influenced by reducing agents. To this end caffeate (or CGA) was used together with several acids and alcohols occurring in wines and grapes and their esters (See Webb,

1962). A pilot study was done using n-butyric acid, i-butyric acid, n-valeric acid, i-valeric acid, 3 methyl butan-2-ol, i-butanol and phenethanol with 1.0 mM caffeic acid (or 2.0 mM CGA) in concentrations ranging from/- 2 mM.

These additions were found to have no clear influence upon increase in bouquet formation. Furthermore, the odour of especially the alcohols often masked the bouquet. The effect of CGA was examined quantitatively upon ester formation. These results are reported under section 3.(b).

(ii) With amino acids.

As a corollary to the work reported in Chapter III (B. 1(c)), the effect of amino acids was checked in conjunction with CGA. The influence of these components upon wine quality has, to our knowledge not yet been fully investigated (Bidan & André, 1958; Kazakova, 1959). Drawert (1963) noted that amino acids apparently attained a plateau in grapes at optimum maturity. Further effects of di-hydroxy phenolic acids in inhibiting decarboxylation of amino acids (in bacteria) and influencing the incorporation of these compounds into yeast protein have been reported (Kimura, Kuwano & Hikino, 1958; van Sumere & de Greef, 1966).

The sample plan is given in the Appendix (Table 5). Basically the first 19 samples were set up to check the effect of CGA upon higher alcohol formation and subsequent bouquet. The remaining 14 samples were to determine the effect of several higher alcohol precursor amino acids (iso-leucine, valine and tyrosine) in the presence of the Wickerham (1951) amino acids (Mandel & Bieth, 1960).

No clear bouquet improvement was achieved. The only obvious effect was that of CGA in decreasing the malodorous characteristic.

(b) Effect on ester formation in synthetic media.

It has been noted that the addition of CGA to the synthetic media causes the disappearance of the malodorous characteristic in the fermented media. The bouquet formation which occurs is relatively slight and clearly not as pronounced as that achieved by the use of the A fraction. However, it was not yet known if CGA does indeed cause an ester, and consequently a bouquet increase. In the malodorous samples these esters can not be perceived. A gas chromatogram of ester separations is shown in Fig. 11.

In this series CGA was added at 0.01, 0.1, 1.0 and 2.0 mM concentrations to samples containing all the amino acids. Samples were also included which contained no CGA or CGA with and without amino acids. Where no amino acids were added, ammonia was the sole nitrogen source (Table 26). These data showed that the highest individual ester concentrations in the CGA additions occurred in samples 2 and 4; the remaining two were slightly lower. There was no marked trend here and clearly did not show that these esters increased with concomitant CGA increases. The actual differences were, however, also very small, especially if compared to ester concentrations in natural wines (cf Wagener et al, 1968). It was, therefore, very doubtful if the small increases would contribute significantly to bouquet.

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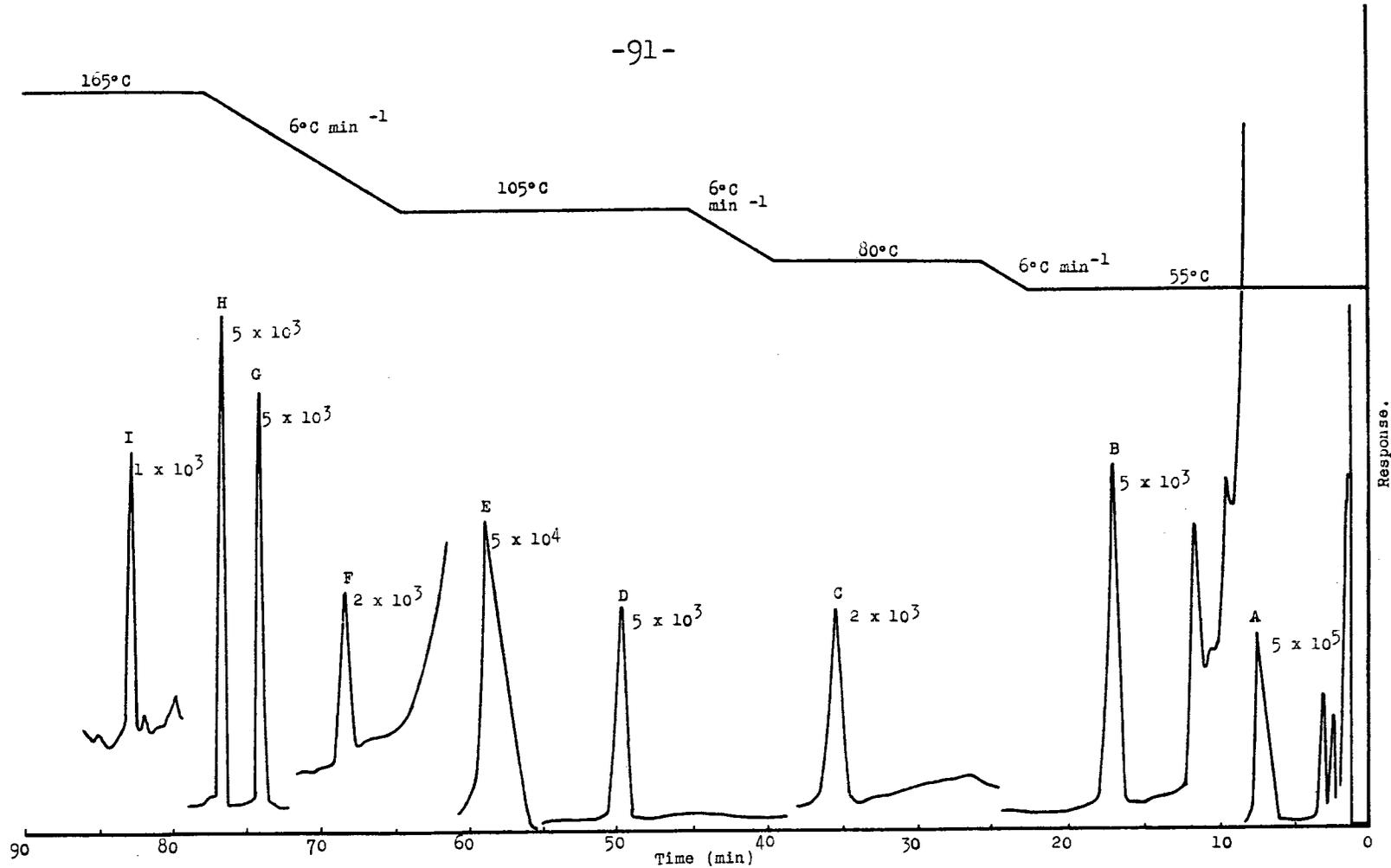


Fig. 11. Chromatogram of ester separation for quantitation in synthetic fermented media and temperature programme. Attenuation noted at each peak. (A = iso-pentanol; B = iso-amyl acetate; C = ethyl n-hexoate; D = ethyl n-heptoate, internal standard; E = 2-phenethyl alcohol; F = ethyl n-octoate; G = phenyl ethyl acetate; H = ethyl n-nonanoate, internal standard; I = ethy n-decanoate).

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Table 26.

The effect of CGA and amino acids on formation of esters by yeast fermentation.

No.	Pre-fermentation treatment.	Esters (μ Moles).											
		Iso-amyl acetate.		Ethyl n-hexanoate.		Ethyl n-octanoate.		Ethyl n-decanoate.		Phenyl ethyl acetate.		Total.	
		(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
1	0.01 mM CGA	1.84	2.00	1.25	1.18	1.16	0.93	0.25	0.20	2.42	6.36	6.93	10.66
2	0.1 mM CGA	3.00	2.09	2.08	1.46	1.80	0.93	0.25	0.20	3.88	6.48	11.00	11.75
3	1.0 mM CGA	2.07	2.07	1.25	1.25	1.10	0.93	0.20	0.20	2.85	6.06	8.47	10.51
4	2.0 mM CGA	2.84	2.38	1.80	1.46	1.39	1.10	0.25	0.20	3.08	6.42	9.37	11.56
5	Nil CGA	2.38	2.30	1.46	1.53	1.28	1.16	0.25	0.20	2.91	6.06	8.27	11.25
6	Nil CGA												
	Nil AA*	0.66	1.54	0.62	1.18	0.70	0.70	0.25	0.12	0.42	0.48	2.66	4.02
7	2.0 mM CGA												
	Nil AA*	0.66	1.77	0.62	1.25	0.75	0.58	0.25	0.12	0.36	0.55	2.65	4.27

(i) First (ii) Repeat series

* AA - Amino acids.

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The organoleptic evaluation showed that all samples except 4 (2.0 mM CGA - Table 26, 4(i) were malodorous. The 0.1 mM CGA (2) sample showed slightly higher ester concentrations than the 2.0 mM CGA (4) and the sensory effect of them in 2 could have been masked by the malodour. However, since the ester differences here were so small they were regarded as insignificant. The sample which contained no CGA (5) did also not differ markedly in ester concentrations from the first four. These data confirmed the ineffectivity of CGA to induce ester synthesis.

The final two samples (6 and 7) were, except for the ethyl n-decanoate, clearly lower in the balance of the esters than were any of the others. Since the factor common to these two samples was the absence of amino acids, it was the latter which caused the drop in ester concentration. The fact that CGA was present in one and not the other had little, if any, effect on this phenomenon.

The latter series were repeated; increases in CGA again showed no clear and consistent ester increments (Table 26, (ii) data). The Nil CGA sample (5 (ii) also, as before showed practically no difference from that of the CGA samples. It will, therefore, be apparent that CGA up to 2.0 mM, had no influence on increasing ester content.

The samples containing no amino acids (6(ii), 7(ii); Table 26) i.e. repeats of 6(i) and 7(i) (Table 26) generally showed, except for phenyl ethyl acetate, slightly lower values than the CGA samples. This difference was however, not as marked as in the previous series. The only clear agreeing differences were the low phenyl ethyl acetate values in both series. It has been reported that phenyl alanine is probably the

precursor of phenethyl alcohol and formed by a mechanism similar to that advanced by Ehrlich for iso amyl alcohol etc. (Mandel & Bieth, 1960; Åyräpää, 1967; Ussigho-Tomasit, 1967). If the phenyl alanine is therefore initially low (cf 7 vs 8, Table 31), it could be surmised that phenyl ethyl alcohol would also be low. This could also be expected in the case of the other higher alcohols formed by the same mechanism. Nordström (1964c, 1966c) noted that acetate formation (esters) occurred when alcohols were added to media and fermented. However, it was also reported that yeast species differed as to the activity of their ester forming mechanisms (Nordstrom, 1966d). In the latter two series it appeared, therefore, that the activity of the phenyl ethyl acetate forming mechanism was consistently low. This apparently also was the case with the majority of the other esters, but, as noted above, not as marked in the latter series.

The CGA additions were done to a maximum of 2.0 mM. This was its approximate maximum solubility in the cold medium. A further series was, therefore, carried out to check increased CGA concentration effect in the media. The potassium salts of CGA, as well as caffeic acid, were prepared and both added at 6.0 mM. The ester concentrations determined in the fermented media are given in Table 27. The nil CGA sample served as a control.

The 6.0 mM CGA sample was slightly higher in every ester than the 2.0 mM CGA control. This phenomenon did not occur consistently in the high caffeate sample. Since the effect of these two components were shown to be the same in other respects it appeared strange that their influence was also not similar here.

Table 27.

The effect of high CGA and caffeic acid concentrations on
formation of esters by yeast fermentation.

No.	Prefermentation treatment.	Esters (μ Moles).					Total.
		iso-amyl acetate.	ethyl n-hexoate.	ethyl n-octoate.	ethyl n-decanoate.	phenyl ethyl acetate.	
1	2.0 mM CGA	6.38	2.57	1.16	0.85	9.93	20.89
2	6.0 mM CGA**	8.53	3.12	2.09	1.30	12.66	27.70
3	1.0 mM CA*	7.22	2.64	1.97	1.0	12.60	25.43
4	6.0 mM CA**	6.76	3.05	1.97	0.95	10.30	23.03
5	Nil CGA	4.69	2.15	1.57	0.85	9.15	18.40

*CA = Caffeic acid

** Added as potassium salt.

In any case, the ester increments in the high CGA sample were again relatively small and did not show on the organoleptic evaluation. The high caffeate sample had a strong medicinal smell which effectively masked every other odour. In general, it was determined that where ester increments occurred they were of such a low order as to be organoleptically insignificant. These findings again confirm those of the previous series in that marked bouquet improvement does not occur with CGA addition.

It has been reported that a low oxygen tension in a medium resulted in a marked inhibition of ester formation (Cowland & Maule, 1966). Furthermore, where media were prepared with reduced oxygen it was found that fermentation proceeded much slower than with normal oxygen content. Considering only the fermentation rate it appeared feasible to surmise that ester losses would be less at a low rate. Since at this stage it also appeared that oxygen was important in the CGA effect (ready oxidation of CGA) a series was done where this factor was pertinently examined. The findings are recorded in Table 28.

As in the preceding ester analyses these findings did not show variations which correlated with organoleptic evaluations. The influence of CGA with or without oxygen did not show a clear pattern; to all intents a 75% decrease in oxygen did not influence the latter ester concentrations under these conditions

(c) The effect of fermentation on chlorogenic acid.

Sikovec (1966, a, b) suggested that phenols, including CGA, could be partly assimilated by yeasts as carbon source. Yeasts and especially yeast-like fungi have been reported as being able to utilize phenols and phenolic compounds also, i.e. as carbon sources (Harris & Ricketts, 1962; Westlake & Spencer, 1966).

Table 28.

The effect of oxygen concentration in the presence and absence of CGA upon ester formation in yeast fermentation.

No.	Prefermentation treatment.	Esters (μ Moles).					Total.
		iso-amyl acetate.	ethyl n-hexoate.	ethyl n-octoate.	ethyl n-decanoate.	phenyl ethyl acetate.	
1	0.01 mM CGA ± 8 ppm O ₂	4.01	1.60	0.81	0.55	8.24	15.26
2	0.10 mM CGA ± 8 ppm O ₂	4.00	1.66	0.87	0.55	9.15	16.22
3	1.0 mM CGA ± 8 ppm O ₂	6.76	2.15	1.16	0.70	10.96	21.73
4	2.0 mM CGA ± 8 ppm O ₂	2.54	1.25	1.16	0.65	5.82	11.41
5	0.01 mM CGA 2 ppm O ₂	8.76	2.29	1.28	0.65	5.63	18.61
6	0.10 mM CGA 2 ppm O ₂	4.99	2.22	1.22	0.98	4.24	13.65
7	1.0 mM CGA 2 ppm O ₂	5.15	2.01	1.16	0.85	4.66	13.83
8	2.0 mM CGA 2 ppm O ₂	4.23	1.53	1.05	0.85	9.63	17.27
9	Nil CGA ± 8 ppm O ₂	4.23	1.73	0.93	0.55	8.85	16.28
10	Nil CGA ± 2 ppm O ₂	5.68	2.95	1.16	0.98	5.51	15.48

*Normal O₂ content of media at atmospheric pressure varied between 7.5 - 9.7 ppm.

Since it was not yet known in what manner CGA affected the bouquet of synthetic media and this could well be effected by CGA utilization by the yeast, the fate of this compound was consequently investigated.

CGA was analyzed by the described method. The data are given in Table 29. The most marked decreases were recorded in the 0.1 - 2.0 mM samples (columns 2 and 3). The concentration of CGA in sample 1 was very low and losses could not be readily determined. It was assumed that hydrolysis of CGA occurred and if this was the case then quinic acid would no doubt also be present. This component was determined by the described method and data recorded in Table 29. Quinic acid (QA) was found in all the CGA samples. Although not shown, positive results were also determined in samples which did not receive CGA. These values varied, per series, from 0.011 - 0.016 mM. They were subtracted as blank values. However, sample 1 (Table 29) showed a very low QA value which increased in the other samples as CGA concentration was raised. If these values are taken as hydrolyzed CGA then the losses of CGA increased as its concentration was increased (Table 29, column 5). (The QA values of sample 1 were so small that they were not taken into account here). Sample 2 CGA losses were regarded here as insignificant.

Caffeic acid, if it is present, is determined as CGA by the applied method. Assuming that hydrolysis occurred, then a more accurate CGA recovery value will be obtained by first subtracting the QA (= CA) values prior to their being added on again. The percentage CGA losses shown by this approach is given in Table 29, column 6. Losses now appear in samples 2 - 4 with those in the latter two being consistently so,

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relative to the data in column 5.

Table 29.

The concentration of CGA in synthetic medium before and after fermentation.

Sample.	CGA added mM.	a CGA recovered mM.	QA determined mM.	% CGA loss or gain (corrected).	
				b (i)	c (ii)
1	0.01	0.01	0.001	+ 10.0	0
2	0.10	0.093	0.0086	+ 1.60	- 7.0
3	1.00	0.95	0.0198	- 3.02	- 5.0
4	2.00	1.84	0.0455	- 5.73	- 8.0

a. Caffeic acid is determined along with CGA

b. Determined from difference between sum of columns 3 and 4 and known values in column 2.

c. Assuming caffeic acid was present at same concentration as quinic acid then column 4 data was first subtracted from corresponding column 3 data prior to determining difference as in a.

QA = Quinic acid.

It was, therefore, clear that the 1.0 and 2.0 mM CGA fermented samples showed repeated losses which could, under the latter assumptions not be regarded as being due to hydrolysis. A check was, therefore, made on the presence of related phenols (reducing substances) in the fermented media by paper chromatography. The R_c values i.e. R_f values relative to CGA of the discrete spots of these paper chromatograms are noted in Table 30. A schematic chromatogram is given in Fig. 12. One hundred ml samples were extracted with n-propanol and salted out (Dickinson & Gawler, 1954; Parkinson, 1954). The chromatograms were viewed under UV light and sprayed with methanolic phospho-molybdic acid solution (Stahl & Schorn, 1965). The data in Table 30 show that two spots viz. 3 and 8 were common to the fermented CGA

samples. Both these were reducing and fluoresced blue. Spot 8 appeared only in samples 3 and 4 i.e. those with high CGA concentrations. This consistent pattern agreed with that determined in the CGA losses in Table 29, columns 5 and 6.

From the latter it appeared that spot 8 could be caffeic acid. Caffeic acid samples were run one dimensional in the two solvents; its R_f values were for the 6% acetic acid and n-pentanol:formic acid 0.59 (range 0.54 - 0.62) and 1.33 (range 1.30 - 1.36), respectively. The caffeic acid spot is shown schematically in Fig. 12. Two hundred ml 2.0 mM CGA fermented sample was also extracted as before and applied as a 12 inch streak. It was developed in one dimension with the acetic acid solvent. The R_f of spot 8 here was 0.50. This spot was eluted with 70% ethanol (containing 100 ppm SO_2 as anti-oxidant), concentrated in vacuum and developed again with the pentanol solvent. It gave one spot with $R_f = 1.50$. From these R_f values this component would have shifted from the position of spot 8 (Fig. 12) to a position intermediate to it and the caffeic acid. From this marked shift it appeared probable that spot 8 could perhaps well be caffeic acid. A further i.r. check was also done by extracting 500 ml 2.0 mM CGA fermented sample, separating spot 8 in the acetic acid solvent and eluting. The concentrate was mixed into KCL and a micro wafer pressed as described under methods of analysis. Although the wafer assumed a light brown colour it gave no i.r. spectra. There was clearly insufficient component present (See Beckman application data sheet, IR-88-M1).

Table 30.

*Rc values of fermented and un-fermented media, with and without CGA.

Spot.	Sample No. - Treatment.															
	1 0.01 mM CGA.		2 0.1 mM CGA.		3 1.0 mM CGA.		4 2.0 mM CGA.		5 Nil CGA.		6 Medium 2.0 mM CGA.		7 2.0 mM CGA (Aqueous).			
	Rc		Rc		Rc		Rc		Rc		Rc		Rc			
	I	II	I	II	I	II	I	II	I	II	I	II	I	II		
1	1.17	0.19	1.20	0.21	-	-	-	-	-	-	-	-	-	-		
2					1.16	0.25	1.06	0.29	-	-	1.05	0.31	1.0	0.31		
3	1.17	0.63	1.09	0.64	1.10	0.60	1.04	0.63	-	-	-	-	-	-		
4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	-	1.0	1.0	1.0	1.0		
5	1.16	1.0	1.16	1.03	1.20	0.98	1.17	1.15	-	-	1.27	1.04	1.21	1.02		
6	1.06	1.25	1.08	1.13	1.08	1.28	1.04	1.40	-	-	1.02	1.33	-	-		
7	1.18	1.93	1.22	1.72	1.04	1.58	0.99	1.89	-	-	1.01	1.81	1.02	1.81		
8	-	-	-	-	0.48	1.48	0.49	1.70	-	-	-	-	-	-		

Rc = Rf value relative to CGA.

I = First dimension, 6% AcOH.

II = Second dimension, n-Pentanol: 5M Formic acid (1:1).

*All samples except 6 and 7 were fermented prior to chromatographing.

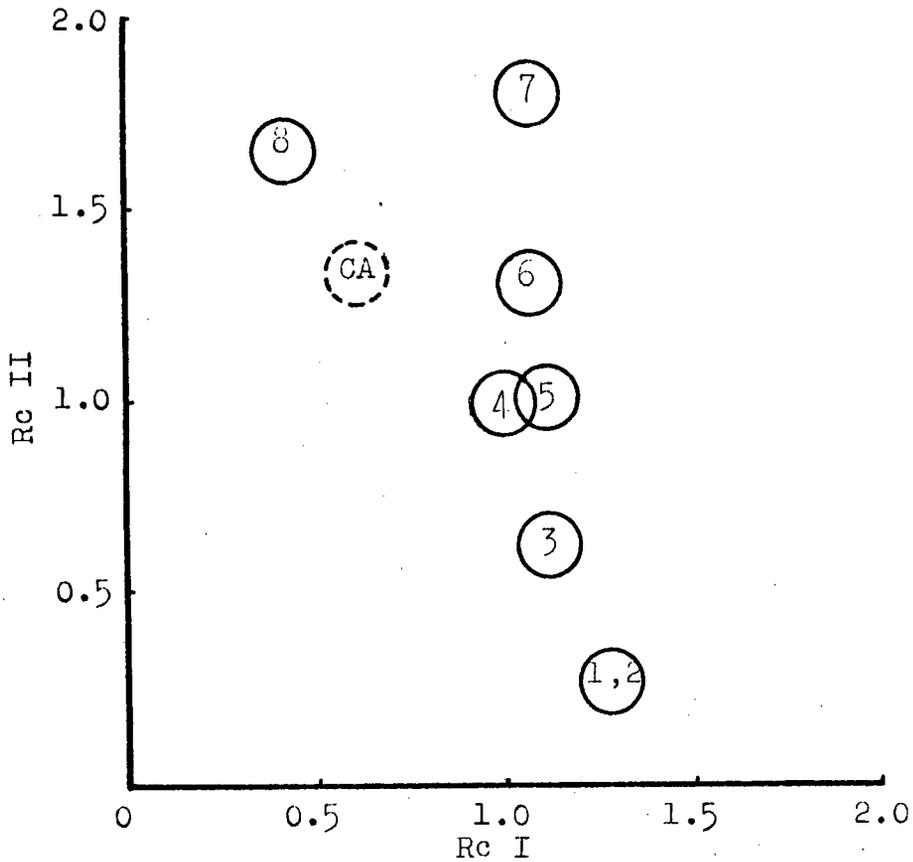


Fig. 12. Schematic illustration of paper chromatogram of fermented synthetic media containing chlorogenic acid (4 = chlorogenic acid). CA = Caffeic acid. Rc = Rf values relative to chlorogenic acid. I, II First and second dimensions; Solvent I: Acetic acid, Solvent II: n-Pentanol: 5M Formic acid. (1:1).

In general it appeared that CGA is influenced during yeast fermentation. This is most marked at a relatively high concentration. Apart from hydrolysis, which, according to the presented facts does apparently occur, a further portion is either lost, utilized or converted during this process. The formation of spots unique to samples containing CGA and fermented clearly indicate this. However, the utilized portion is exceedingly small and it is doubtful if it is of any practical value, relative to bouquet formation. This, in effect confirms the foregoing remarks on bouquet.

(d) Effect on Nitrogenous components.

Residual amino acid concentration.

Amino acids have been examined in the foregoing studies on the composition of a basal medium and their effect upon bouquet in conjunction with CGA. The findings here, however, do not exclude their function as possible co-factors, such as e.g. thiamine, in bouquet formation. CGA clearly affects reactions during fermentation; the mechanism of its effect is as yet unknown. With the foregoing in mind an investigation into its influence upon 22 amino acids and ammonia during fermentation was done.

(i) Ammonia as sole nitrogen source.

Under the conditions of study the yeast could synthesize all 22 amino acids.

The majority of these showed no clear pattern between CGA or Nil CGA samples. However, α -alanine, leucine and γ -amino butyric acid increased by 50%, 600% and 80%, respectively, with CGA addition (Table 31). At this stage there were already indications that this component affected amino acid metabolism. In terms

of total nitrogen dispersion its effect also appeared to be on amino acids and not ammonia (Table 32).

(ii) Chlorogenic acid increments: 0.01 to 2.0 mM.

The overall effect of CGA in this series was to increase the net uptake of largely the acid and neutral amino acids where these were initially added in relatively large quantities, and to depress the synthesis of those which were not added or added in small quantities. (Table 31, aspartic acid, threonine, serine, asparagine, sarcosine, glutamic acid, glycine, α -alanine, valine and phenyl alanine). The basic amino acids, including γ -amino butyric acid but excluding ornithine, were, conversely higher in the presence of a fairly high CGA concentration. Except for the latter group this CGA effect was manifested even at 0.01 mM concentration; increasing this 200 times made very little difference to the final result.

6.0 mM.

These data are noted in Table 33. With the threefold increase of CGA from 2.0 to 6.0 mM increases were manifested in aspartic acid, serine, asparagine, glutamic acid, phenyl alanine, valine, cystine, iso-leucine, leucine, γ -amino butyric acid, ornithine, histidine, alanine, sarcosine, methionine, tyrosine, lysine and arginine but being small in the latter six amino acids and large in the initial six.

In the previous series it will be recalled that CGA up to 2.0 mM, tended to increase the net uptake of most of the neutral/^{and}acidic amino acids or depress the synthesis of those amino acids which were either not added or added in small quantities. With the further increase of CGA to 6.0 mM the opposite effect was noted

Table 31.

* Amino acid concentrations in media with chlorogenic acid and low oxygen content.

Amino Acids.	Samples.							
	Medium	CGA 0.01 mM	CGA 1.0 mM	CGA 2.0 mM	*CGA 2.0 mM 2 ppm O ₂	Nil CGA	Nil CGA Nil AA	CGA 2.0 mM Nil AA.
	1	2	3	4	5	6	7	8
Aspartic acid	279.0	24.0	20.9	23.1	79.9	32.6	20.1	18.2
Threonine	27.6	21.8	20.2	21.5	29.4	27.6	24.9	26.6
Serine	1.9	17.1	15.6	16.5	27.5	28.4	22.4	23.0
Asparagine	16.5	37.3	36.0	38.4	47.9	51.6	37.6	39.5
Sarcosine	Nil	24.4	18.7	22.3	61.4	36.3	39.7	38.5
Proline	Nil	27.4	34.6	26.6	24.5	43.0	44.1	42.0
Glutamic acid	971.8	129.8	114.5	124.0	487.7	201.7	50.0	55.4
Glycine	399.5	252.5	271.7	271.7	426.8	363.9	103.7	127.0
α-Alanine	2,080.0	829.0	842.4	822.6	1,417.0	1,189.7	137.6	194.2
Valine	Nil	8.7	8.1	6.5	15.3	11.6	7.4	8.8
Half Cystine	Nil	9.9	7.4	7.1	19.6	8.1	3.7	8.6
Cystathionine	Nil	+	+	+	+	+	+	+
Methionine	39.0	12.0	13.3	8.7	22.7	12.0	11.1	9.7
iso-Leucine	309.6	8.3	8.0	8.7	68.4	11.6	7.7	5.0
Leucine	267.3	16.8	12.6	11.4	77.8	14.2	17.0	10.7
Tyrosine	Nil	2.9	2.6	2.4	9.8	3.6	3.6	4.2
β-Phenylalanine	717.3	35.1	32.0	34.3	265.3	57.6	5.3	7.5
γ-Amino-butyric acid	Nil	36.5	39.7	76.8	7.7	34.2	10.4	18.4
Ornithine	+	53.6	53.3	55.6	41.2	51.2	35.4	39.6
Lysine	7.6	47.3	44.9	75.7	58.7	42.4	33.4	38.9
Histidine	9.6	11.9	10.6	16.9	15.5	12.2	11.4	15.7
Arginine	5.8	34.8	28.3	47.9	38.9	27.7	29.8	27.8
Total	5,132.5	1,641.1	1,635.4	1,718.7	3,243.0	2,261.2	656.3	759.3

*: Concentration: μ Molar

** Not fermented, all others fermented.

*** O₂ content decreased before fermentation to this value; all others normal content at ca 8 ppm.

CGA: Chlorogenic acid.

AA: Amino acids.

Table 32.

* The effect of chlorogenic acid and oxygen on nitrogen content and cell substance of yeast.

Sample.	Treatment.			Nitrogen content.					Dry yeast weight.		
	CGA mM	**O ₂	Amino acids.	Yeast mg/l medium.	Medium mg/l.	Balance mg/l.	Yeast %	***Medium %	Balance %	mg/gm dry yeast.	mg/100 ml medium.
1	0.01	Normal	All	105.0	41.3	52.1	52.9	20.8	26.3	522.8	80.6
2	0.1	"	"	103.6	46.9	47.9	52.2	23.6	24.2	517.8	80.3
3	1.0	"	"	102.5	40.0	55.9	51.7	20.2	28.1	488.0	85.1
4	2.0	"	"	99.1	51.1	48.2	49.9	25.8	24.3	495.6	81.7
5	2.0	2 ppm	"	49.3	73.8	75.3	24.8	37.2	38.0	447.0	44.9
6	Nil	Normal	"	124.8	54.0	18.6	62.9	27.2	9.9	624.4	82.1
7	Nil	"	Nil (only NH ₄ ⁺)	83.8	18.2	29.8	63.6	13.8	22.6	523.7	64.0
8	2.0	"	Nil (only NH ₄ ⁺)	87.3	18.1	26.4	66.2	13.7	20.1	513.7	67.9

* Samples correspond with those referred to in Table 31; samples of same treatment (not numbers) were identical.

** Normal O₂ content approx. 8 ppm.

*** Total amino acids and ammonia.

and clearly manifested in aspartic acid, serine, asparagine, glutamic acid, valine and phenyl alanine. The effect, therefore, was to depress uptake of most of the acidic and neutral amino acids, or increase synthesis in the initially absent components. The latter group of amino acids are specifically those whose final concentrations were lowered by addition of even 0.01 mM CGA.

This phenomenon indicated that there appeared to be two effects of CGA; one at the lower i.e. 0.01 - 2.0 mM and the other at a higher concentration. The basic amino acids and including γ -amino butyric acid, however, manifested precisely the same tendency as that noted in the 2.0 mM media viz. increased residual concentrations (lower net uptake). Although, ornithine, γ -amino butyric and histidine show this effect clearly that in arginine and lysine appears more indicative than significant.

Since caffeic acid was shown to be the effective moiety of CGA it was surmised that its physiological effect would be similar to that of CGA. It will be noted firstly that, except for ornithine, histidine, γ -amino butyric acid and proline the 2.0 mM CGA and 1.0 mM caffeic acid samples' amino acids were to all intents practically equal. Furthermore, when caffeic acid concentration was also increased to 6.0 mM the same residual amino acid concentration pattern as in its CGA counterpart was also achieved in aspartic acid, threonine, serine, asparagine, sarcosine, glutamic acid, valine, iso-leucine, leucine, phenyl alanine, ornithine and lysine. This effect was expected and it also confirms the "reversed" influence of the higher, relative to the lower CGA concentrations. It is of interest to note also that most of the values of the high concentration caffeic acid

samples, were markedly higher than those of CGA. This is especially noteworthy in serine, glutamic acid, leucine and phenyl alanine. It is not clear at this stage why this should be so. It can be surmised, however, that since the probable reactive structures of CGA and caffeic acid are similar, the higher activity of caffeic acid in depressing net amino acid uptake could possibly be ascribed to decreased spatial hindrance in this compound.



R=3, 4 di-hydroxy phenol, R^1 = hydroxy (caffeic acid)
 R^1 =3-quinic acid (CGA)

(iii) The effect of oxygen at 6.0 mM chlorogenic acid concentration.

As a further step in defining the CGA effect it was noted that the 3,4 di-hydroxy phenols are readily oxidizable (Swain, 1962; Pierpoint, 1966). It was therefore surmised that since the relevant factor could be oxidation-reduction, a similar effect as noted above could be achieved by oxygen manipulation. The ratio of 0.25 mM O_2 /6.0mM CGA (=0.04) was taken as an arbitrary standard and the O_2 in the standard medium (normal O_2 content 0.25 mM) reduced to 0.062 mM. This ratio viz. 0.062 mM O_2 /2.0 mM CGA (=0.03) approximated that of the latter.

The amino acid data of this sample in the final series is recorded in Table 33. The majority of the amino acids which were positively affected by CGA and caffeic acid (aspartic acid, serine, sarcosine, glutamic acid, valine, iso-leucine, leucine and phenyl alanine) were also similarly affected by decreased oxygen. In every one of the latter amino acids, except asparagine, higher concentrations (i.e. relative to the 2.0 mM

Table 33.

* Amino acid concentrations in media with chlorogenic acid, caffeic acid, phloridzin and low oxygen content.

Amino Acids.	Samples.						
	** 1	2	3	4	5	6	7
	Medium.	CGA 2.0 mM.	CGA 6.0 mM.	CA 1.0 mM.	CA 6.0 mM.	Phloridzin.	CGA 2.0 mM 2 ppm O ₂
Aspartic acid	287.3	24.6	44.8	20.4	58.6	35.5	56.7
Threonine	32.4	27.0	25.1	25.3	28.3	28.2	26.1
Serine	3.2	14.5	58.5	12.3	96.3	22.5	19.6
Asparagine	18.9	108.5	131.1	98.2	173.9	131.0	105.9
Sarcosine	Nil	10.1	13.7	9.7	21.4	12.5	22.0
Proline	Nil	47.1	28.2	31.7	44.4	41.2	39.4
Glutamic acid	1,168.0	93.2	171.6	98.8	381.7	273.8	321.7
Glycine	416.0	315.5	280.9	365.0	350.2	437.3	393.1
α -Alanine	1,774.0	1,164.0	1,199.0	1,065.0	1,539.0	1,265.0	1,086.0
Valine	Nil	6.9	11.9	5.1	15.0	9.2	11.5
Half Cystine	Nil	3.7	7.6	4.1	-	6.1	-
Cystathionine	Nil	+	+	+	+	+	+
Methionine	32.9	13.5	16.4	11.4	8.1	4.6	8.3
iso-Leucine	250.3	2.7	5.0	2.5	7.1	3.4	8.3
Leucine	264.6	5.5	13.1	3.6	37.0	24.8	17.2
Tyrosine	Nil	1.7	2.3	1.5	2.0	1.0	2.3
β -Phenylalanine	716.8	24.7	33.4	27.5	110.0	88.1	255.7
γ -Amino-butyric acid	Nil	21.7	80.8	11.1	12.1	10.0	7.7
Ornithine	5.8	43.5	79.9	26.7	51.2	33.0	21.5
Lysine	5.0	27.2	31.7	25.4	39.8	29.9	32.3
Histidine	4.8	8.3	14.1	5.0	5.5	8.6	14.1
Arginine	6.0	19.3	21.5	20.5	28.2	23.9	31.9

* Concentrations μ Molar

** Not fermented; all others fermented.

*** O₂ content decreased before fermentation to this value; all others, normal concentration at ca 8ppm.

CGA: Chlorogenic acid.

CA: Caffeic acid.

CGA control) were manifested in the fermented medium.

The basic amino acids did not generally show consistent effects and no marked trend could be noted (Table 33).

γ -Amino butyric acid concentration was clearly depressed by the low O₂ concentration. This was determined in both series. It was similarly also depressed by 1.0 and 6.0 mM caffeic acid but increased by CGA.

In a brief report van Sumere & de Greef (1966) noted the inhibitory effect of phenolic acids on the amino acid uptake by Saccharomyces cerevisiae and the incorporation of penetrated amino acids into protein. The latter authors reported that the influence of phenolic acids on phenyl alanine and glutamic acid was similar to that of the uncoupling agent 2, 4-di-nitro phenol. This would, in effect, result in a decreased uptake of amino acids i.e. a higher residual concentration, similar to the findings reported in Table 33 on CGA addition or oxygen decrease. Siekevitz (1952) determined that the uptake of amino acids by liver slices were inhibited by the exclusion of oxygen or the addition of 2, 4-di-nitro phenol. This relates uncoupling phenols and oxygen. Phloridzin, which is known to react like 2,4-di-nitro phenol was used in its stead. The findings on amino acid uptake was similar to that of CGA (6.0 mM) and oxygen decrease (Table 33). Meyerhof & Wilson (1948) showed that in yeast this component impairs phosphate transfer from phosphoenol pyruvate to ADP. It, therefore, appears that the CGA effect upon amino acid uptake in the fermentation of a synthetic medium centers around phosphorylation.

Although clear effects of CGA upon amino acids were determined, they showed no relationship to the ester or bouquet findings. In relation to the 80-90% utilization of added amino

acids the CGA effect appeared very small. Sikovec (1966 a, b) has reported on similar general findings on the influence of chlorogenic acid on yeast.

Although the effect of CGA upon removing the malodorous characteristic of synthetic fermented media was not determined, this latter, as yet unknown, property was one of the most critical in this study. In the investigation of bouquet the unpleasant odour of synthetic fermented media prevented a meaningful sensory examination of such products. Consequently these di-hydroxy phenols were regarded as absolutely necessary as e.g. thiamine in this study. It was, in fact, a key to the successful examination of wine bouquet in synthetic media.

Christensen & Caputi (1968) determined di-hydroxy phenols, without chlorogenates, to be about 100 mg/l in red wine. De Wet (unpublished data) recently found these to be in the order of 140 mg/l in Steen must. The former data plus the determined chlorogenates in must (Appendix, Table 4) are close to those of De Wet. These values agree with the 150 mg/l of protocatechuic acid or 180 mg/l caffeic acid required to effect a clean bouquet in synthetic media. Chlorogenates require approximately 350 mg/l to produce the same result, but, as shown, it is only the caffeate moiety which is effective. However, there appears to be no reason why the di-hydroxy phenols can not also play this role in grape juice and wine. It is probable that a shortage of these components in musts could also cause unpleasant odours to form in wines. This appears to be an aspect which is worth while investigating.

C H A P T E R IV.

Bouquet component "X".

(a) Formation in synthetic media.

During the checking of an efficient eluate for bouquet precursors, N HCl was used on the basis of "salting out" chromatography. The same parameters as applied in obtaining the wafer eluates were utilized. The aldehydes of the alcohol fraction of this eluate were removed, as described in Chapter III C(b) prior to checking for bouquet. Of the sixteen samples in this specific series the one containing this fraction was markedly different from the rest. It had a bouquet which was heavy and sweet and definitely not delicate. It was, however, not of the "honey" or "oxidized" type and was also noted by the judges as not delicate but associated with wine. The causal component of this odour, was termed "X".

Although two repeat series were carried out, this effect could not again be achieved. However, since it had been perceived in dry white wines they were subsequently used for further investigation of it.

(b) Separation from commercial wines.

As initial approach it was assumed that "X" had a relatively high vapour pressure. A suitable system for its separation was developed on this basis.

The wine to be used was firstly increased in pH to ca 6.5 to prevent CO₂ blocking the liquid nitrogen cooled trap. The counter-current extraction system is shown with parameters in Appendix Fig. 5. With fine jets, the bubbles formed are very small and give and maintain a large gas-liquid interface.

The nitrogen gas, used for sweeping, was held to a total flow rate of approximately 100 cc min^{-1} . The volatiles were first passed through a salt-ice cooled trap (ca minus 19°C , glass helix-Vigreux type) prior to being collected in the final trap.

This trap consisted of a $15 \times \frac{1}{4}$ " OD s/s U-tube filled with small glass helices and with Nupro stainless steel needle valves at the inlet and outlet. On completion of a collecting run the valves were closed and Hamilton septum-piercing needle attached to a Luer lock adapter on the inlet side needle valve. The 10 cc sample loop of the gas chromatograph was filled through its sealing septum. A chromatogram of a separation, using the parameters given here-under, is shown in Fig. 13.

The following columns and conditions were found to give a satisfactory separation:-

First column: 3ft x $\frac{1}{8}$ OD copper, 25% diglycerol on chromosorb W, 60-80 mesh.

Second column: 2 ft x $\frac{1}{8}$ OD copper, 35% di-nonyl phthalate on celite 60-80 mesh.

(These two columns are connected in series)

Oven temperature: 0°C , iso-thermal.

Detector: F.I.D., 40°C , 10% of upstream splitter to flame, 90% vented.

Flowrate: 14 cc/min.

Carrier gas: Helium.

Repetative collection of fraction "X" from the gas chromatograph was done in a 5 ft coiled $1/16$ " O.D. thin-walled s/s tube cooled in liquid nitrogen. Since this trap had a relatively large volume (1.7 cc) the fraction was transferred to a small 12" melting point glass, glass-bead filled, U-tube (in liquid nitrogen) with helium.

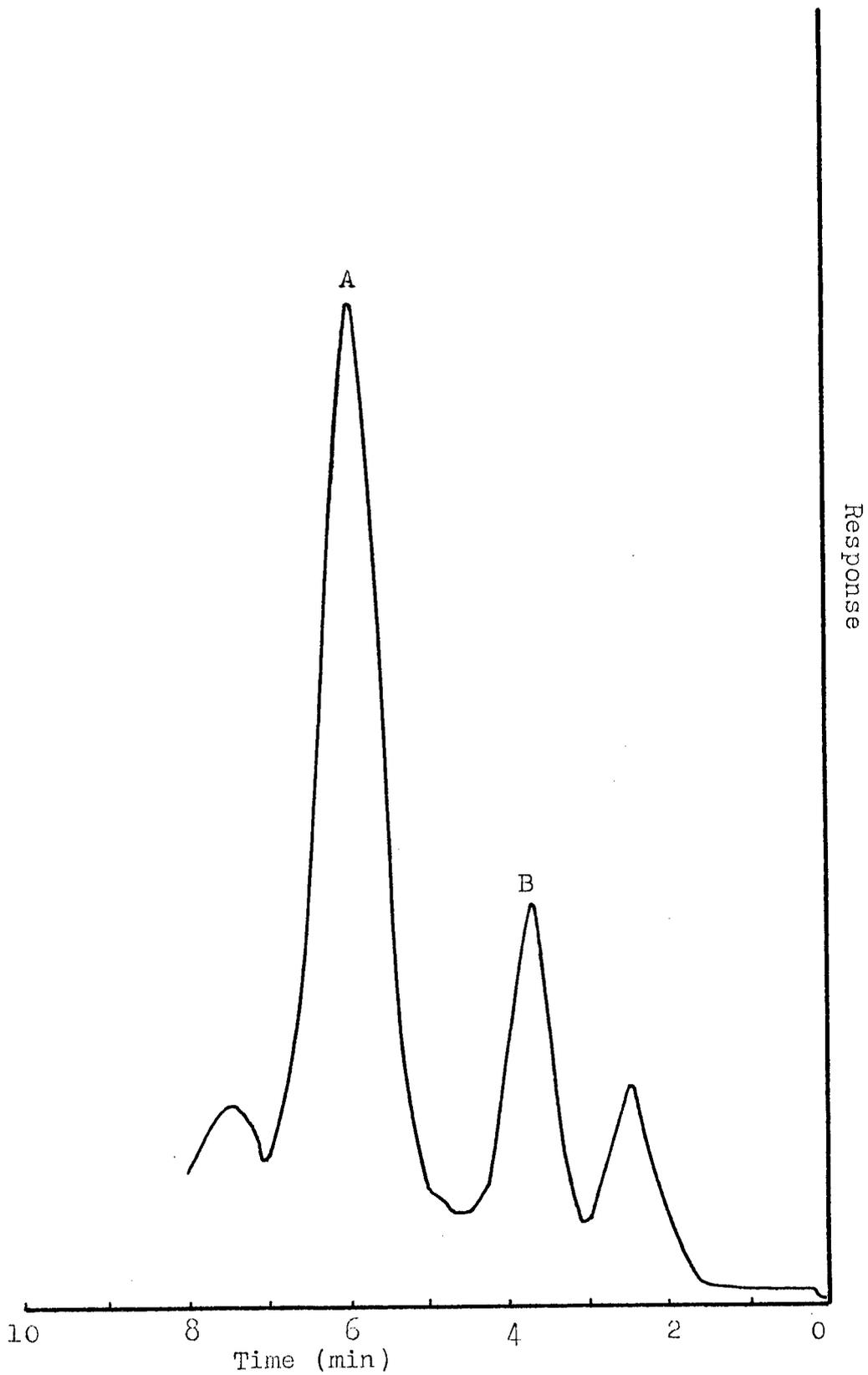


Fig. 13. Chromatogram showing separation of heavy sweet odourous wine bouquet component. (Peak A). Peak B is acetaldehyde. Attenuation = 50. (See text for parameters of separation).

The transfer technique worked well with 2 μ l ether and the sample could be transferred to an i.r. micro gas cell (8 mm path length) and, in conjunction with a beam condensor, give a 20% transmission at 1140 cm^{-1} . The same procedure was carried out with fraction "X" from 5 liter wine. Although the fraction could be readily smelled in the cell it gave no i.r. spectra. Its concentration was clearly too low.

Two samples, one sealed in a small melting point tube and the other in the stainless steel trap were sent for mass spectrometer analysis. Both determinations were not successful. These findings tend to confirm that this substance is present in very low concentration.

Although success has not been achieved in identifying this component it is interesting in that it apparently also stems from a grape component. Furthermore, it is practically the opposite in tone of the delicate fruity odour of table wines and can, in our opinion, contribute in a meaningful manner to wine bouquet. Its tendency would no doubt be to give a fuller but not unpleasant character to this wine quality facet of dry white table wines.

C H A P T E R V.

Conclusion.

It has been shown that a medium which contains the basic requirements for a normal yeast fermentation is not necessarily that which would induce bouquet. Nordström (1962a) noted that a nitrogen, phosphorous or magnesium shortage lead to reduced ester synthesis. This study has also indicated bouquet improvement in wines by the addition of ammonia and amino nitrogen to cation resin treated musts' wines. This aspect is not of great practical importance since grapes normally contain more than sufficient of these components. The amino acid concentrations used in the basal medium were indeed far lower than that found in grapes.

Vitamin deficiencies were similarly shown to influence bouquet. The effect here appeared to be more pronounced than that of nitrogen. Thiamine and nicotinamide (or nicotinic acid) were strongly removed from grape juice by cation resin treatment. Under these conditions marked effects were recorded on the metabolic products of acetic acid and higher alcohols. Nordström (1966a) similarly reported upon their effect in synthetic media. It was, however, thiamine which was most functional in bouquet formation. This Nutrilite is, however, regarded as a basic component of a medium since its presence in a full synthetic medium will not induce bouquet. Although it has been reported that thiamine addition to grape juice accelerated fermentation no mention was made of bouquet improvement. In general, the thiamine content of grapes is normally, like the nitrogenous components, adequate and not directly contributory to

bouquet. The thiamine analysis of several grape samples tended to confirm this in that no clear pattern in the content of this component could be gained. Furthermore, doubling the thiamine content of a grape juice also made no appreciable difference to its bouquet.

A marked effect on the bouquet of a fermented synthetic medium was found to be brought about by the di-hydroxy phenols. The malodorous characteristic of a synthetic medium which negated sensory evaluation was completely removed by these compounds. The 3, 4 di-hydroxy phenol grouping was critical; replacing the hydroxy groups by one or two methoxy groups cancelled out this effect. A clean bouquet was achieved with 180 mg/l caffeic acid, 350 mg/l chlorogenic acid and 150 mg/l protocatechuic acid. To our knowledge this is the first report on this important role of these compounds. However, not one of these latter compounds are present in such high concentrations in grape musts. Chlorogenates were found to vary between 30-50 mg/l and were far too low to be effective as such. Clearly the good effect of e.g. chlorogenate alone in a synthetic medium must be in grape juice, at least, an additive one of this species of compounds. Reported findings showed that these di-hydroxy phenolics (except chlorogenates) in red grapes were in the order of 100 mg/l and 140 mg/l in a Steen must. These values appear high enough to be effective in the juice. It was clear that the di-hydroxy phenols such as chlorogenic acid were on integral part of a synthetic medium for the investigation of bouquet formation.

The only marked influence of chlorogenic acid which could be determined on the components of a post-fermentation synthetic medium, was its effect upon amino acid uptake.

At a concentration of 0.01 to 2.0 mMolar the net uptake of those neutral and acidic amino acids which were added to the medium in relatively large quantities showed an increasing tendency.

On the other hand synthesis of amino acids which were either not added or added in very small quantities were depressed. With the increase of chlorogenic acid to 6.0 mM the opposite effect was noted. This appeared to be due to the influence of the di-hydroxy phenols upon phosphorylation. These findings could not be related to either bouquet or ester analysis.

Theoretically there is no reason why, at least as regards bouquet, a synthetic wine can not be made in the laboratory. This has been, relative to the bouquet facet of wine quality a fundamental approach of this study. A technique was developed by which a fraction could be obtained from grape juice which could induce a pronounced wine bouquet when used in a synthetic medium. The actual bouquet forming or inducing components in this fraction or grapes have not yet been identified. There are still many problems to overcome, as preliminary separatory techniques indicated that these components appeared to unstable. Furthermore, a long storage of grape juice under sulphur dioxide also caused a marked loss of activity. However the field of probables has been considerably narrowed. The critical grape juice fraction contains only a few grape components in relation to the juice itself.

Wine bouquet has been induced in a synthetic medium by the addition of a grape juice fraction. Two types of bouquet were achieved, the one of a delicate flowery, ester-like nature, reminiscent of a cold-fermented wine, and the other of a fuller type. Although not proven, these appear, relative to bouquet,

complimentary to each other. However, a stage has clearly been reached where wine volatiles analysis must be primarily channelled into objective bouquet definition. This study has indicated a readily applicable approach from both a bouquet aspect as well as determining quality-facet precursors in grapes.

A P P E N D I X.

Table 1.

The systems examined for one dimensional separation of specific amino acids (McFarren, 1951).

1. Phenol-pH 12 buffer:	Tailing and reddish coloured spots.
2. Phenol - pH 9.0 buffers:	(a) Good separation of aspartic acid, glycine and threonine. (b) Glutamic acid and serine lie relatively close together.
3. m-Cresol - pH 8.4 buffer:	Tailing of phenyl alanine.
4. m-Cresol - pH 4.0 buffer:	(a) Good separation of valine, methionine, tyrosine and phenyl alanine. (b) Spots are more compact than in 3.
5. Benzyl-butyl alcohol (1:1) -	(a) Good separation of phenyl alanine. (b) Leucine and i-leucine do not always separate cleanly, they could, however, in such cases still be analysed.
6. Benzyl-butyl alcohol (1:1) - pH 8.4 buffer:	(a) Good separation of phenyl alanine. (b) Separation of leucines not as good as in 5.

Table 2.

Response factors for a flame ionisation detector for several esters.

Ester.	*Response factor (F) (cm μg^{-1}).
i-amyl acetate	0.496
ethyl n-hexoate	0.452
ethyl n-octoate	0.403
ethyl n-decanoate	0.510
phenyl ethyl acetate	0.443
ethyl n-heptoate	0.363
ethyl n-nonanoate	0.405

*Attenuation 5×10^3 ; 25.0 μl injections; average of five replications.

The concentrations of esters were determined with the following formula:-

$$\text{Concentration in ppm} = \frac{I_c \cdot A_x \cdot F_i}{A_i \cdot F_x}$$

where

- Ic = concentration internal standard (ppm)
- Ai = Peak area of internal standard (cm^2)
- Ax = " " " ester (cm^2)
- Fi = Response factor of internal standard
- Fx = " " " " ester.

Table 3.

Quantitative analyses of chlorogenic acid in several grape juices with a poly (vinylpyrrolidinone) column with a pre-fractionating polyamide column.

Cultivar and standard.	Quantity	Absorption (324 m).	
		*Polyamide column eluate.	Poly (vinyl pyrrolidinone) column eluate.
Chlorogenic acid A	15 µg	0.010	0.256
Hermitage	0.8 ml	0.111	0.63
White French	1.31 ml	0.143	0.468
Chlorogenic acid B	15 µg	0.009	0.257
Riesling	2.0 ml	0.140	0.182
Steen	2.0 ml	0.236	0.217

* No chlorogenic acid is absorbed by this column.

Table 4.

*Replicate analyses of total chlorogenates in Riesling grape juice by the modified Lehmann, Hahn and Martinod method.

Vintage.	30/5/68.	3/6/68.	24/5/68.	29/5/68.
** 1964	11.97	12.81	-	-
** 1967	-	-	50.3	51.1

* Concentration in mg/l.

** Samples preserved with ca. 2000 ppm SO₂

Table 5.

*Sample plan for determination of effect of amino acids and chlorogenic acid upon bouquet.

No.	CGA.	WA.	MaA.	MiA.	NA.	Leu.	i-Leu.	Val.	Tyr.
1	+	+	+	+	-	+	+	+	-
2	+	+	+	+	-	+	-	-	-
3	+	+	+	+	-	-	+	-	-
4	+	+	+	+	-	-	-	+	-
5	+	+	+	+	-	+	+	-	-
6	+	+	+	+	-	+	-	+	-
7	+	+	+	+	-	-	+	+	-
8	+	+	+	+	-	-	-	-	-
9	+	+	-	+	+	-	-	-	+
10	+	-	-	+	+	-	-	-	+
11	+	-	-	+	+	-	-	-	-
12	-	+	+	+	-	+	-	-	-
13	-	+	+	+	-	-	+	-	-
14	-	+	+	+	-	+	+	-	-
15	-	+	-	-	-	-	-	-	-
16	-	+	-	+	+	-	-	-	+
17	-	+	-	+	+	-	-	-	-
18	-	+	-	+	+	-	-	-	+
19	-	-	-	+	+	-	-	-	-
20	*	-	+	+	-	+	-	-	-
21	*	-	+	+	-	-	+	-	-
22	*	-	+	+	-	-	-	+	-
23	*	-	+	+	-	+	+	-	-
24	*	-	+	+	-	+	-	+	-
25	+	-	+	+	-	-	+	+	-
26	*	-	+	+	-	+	+	+	-

CGA = Chlorogenic acid; WA = Wickerham's amino acids (See text); MaA = Macro amino acids (See B (c)(i)); MiA = Micro amino acids (See B (c)(ii)); Leu-Leucine; i-Leu = iso-Leucine; Val = Valine; Tyr = Tyrosine; NA = Normal amino acids (B(c) (i), (ii)).

* Samples duplicated, one half received CGA (2.0 mM), the counterparts not.

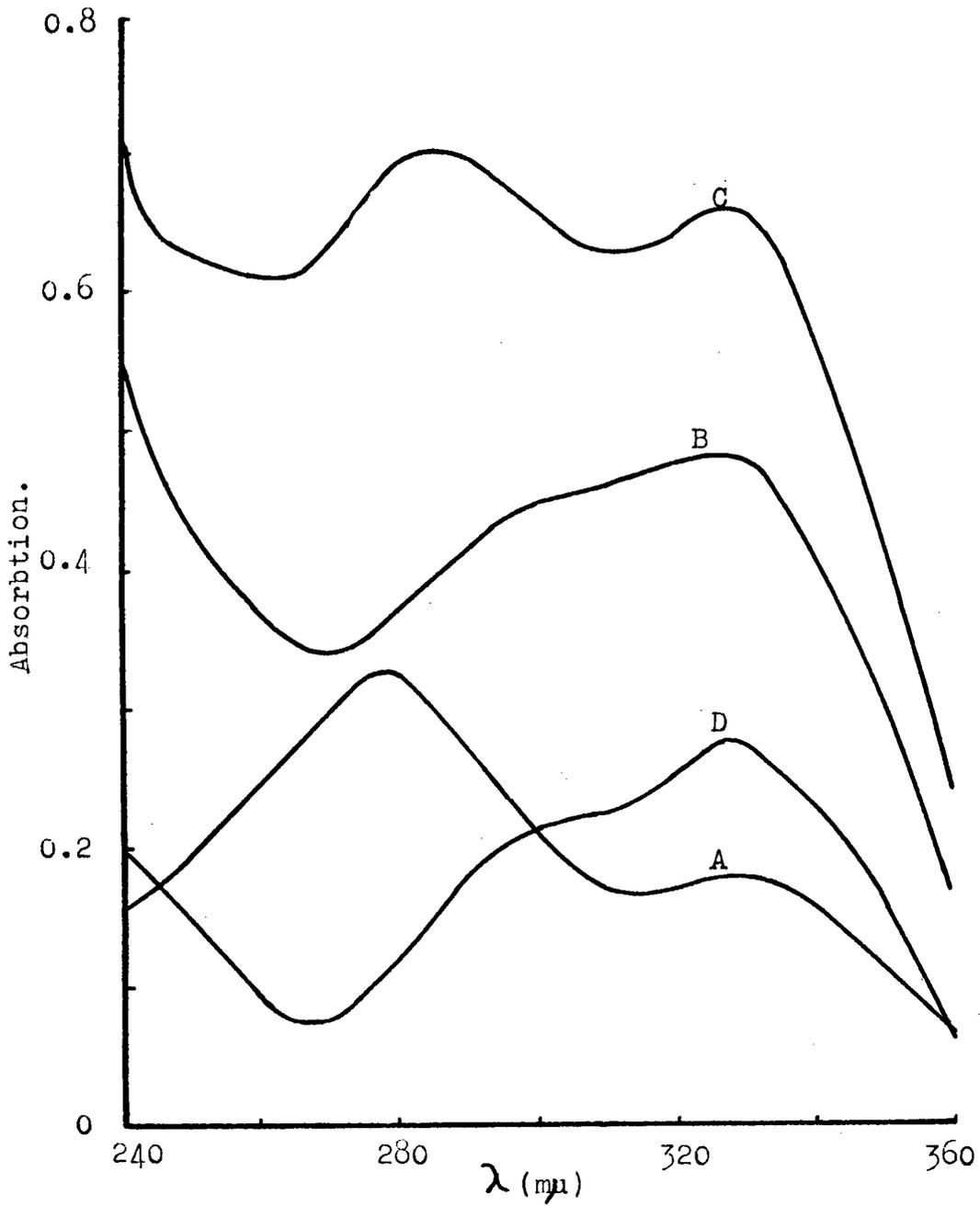


Fig. 1. Spectra of polyamide and poly(vinylpyrrolidinone) column eluates of Riesling grape juice. (A, Polyamide column eluate; B, Poly(vinylpyrrolidinone) column eluate; C, Total eluate; D, Chlorogenic acid).

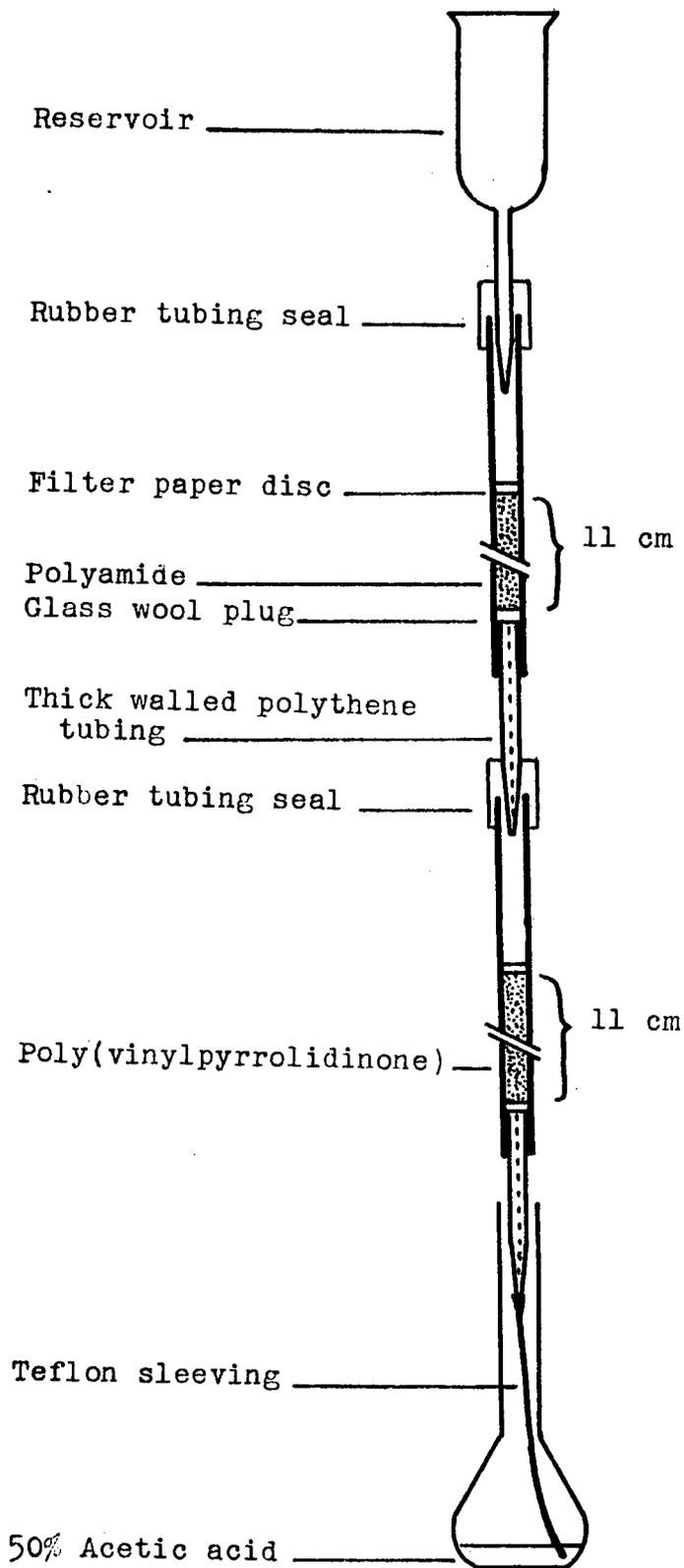


Fig. 2. Combined polyamide and poly(vinylpyrrolidinone) column for chlorogenate determination.

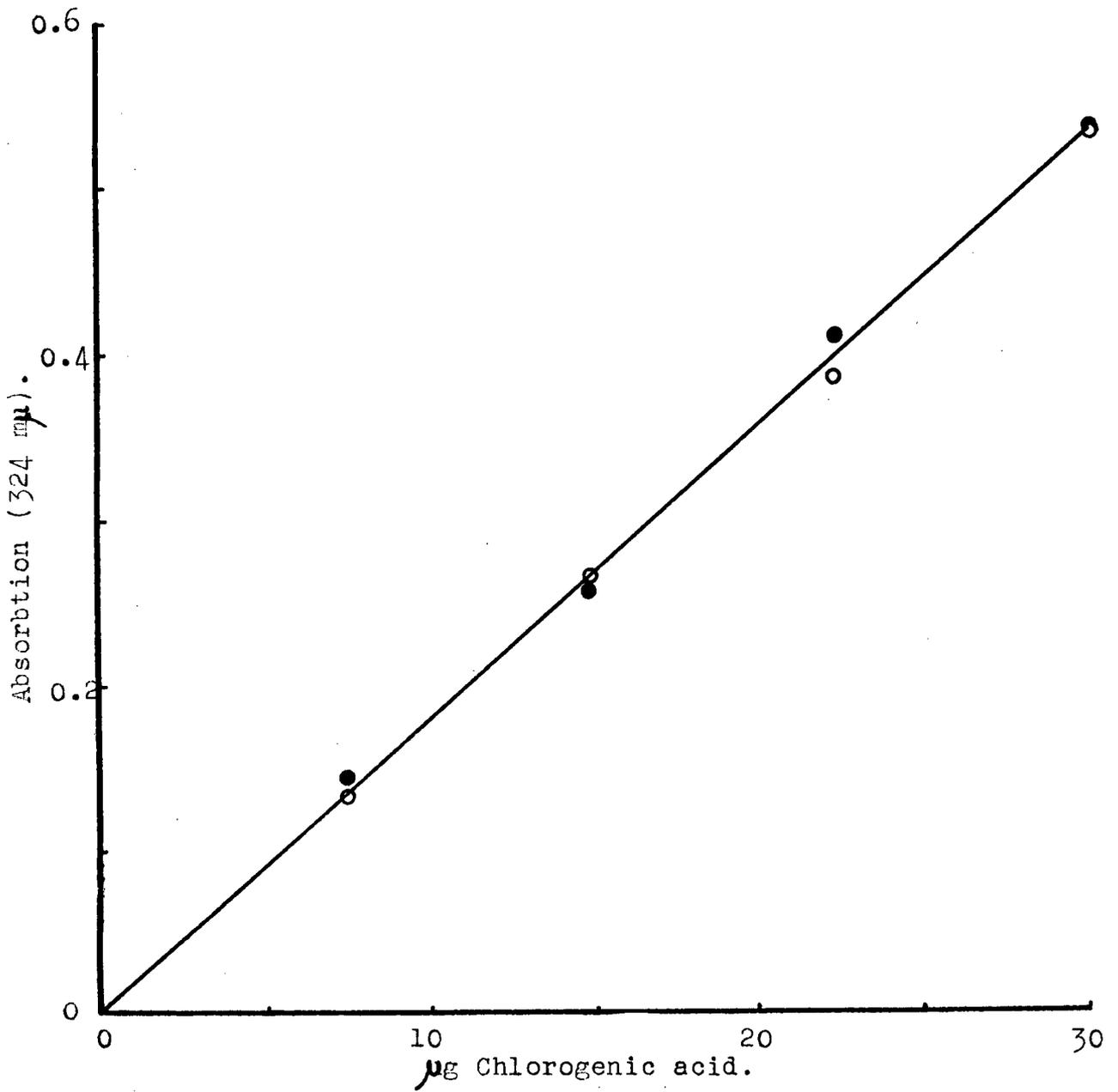


Fig. 3. Calibration graph for chlorogenic acid determination (Plots o and ● are for two different days).

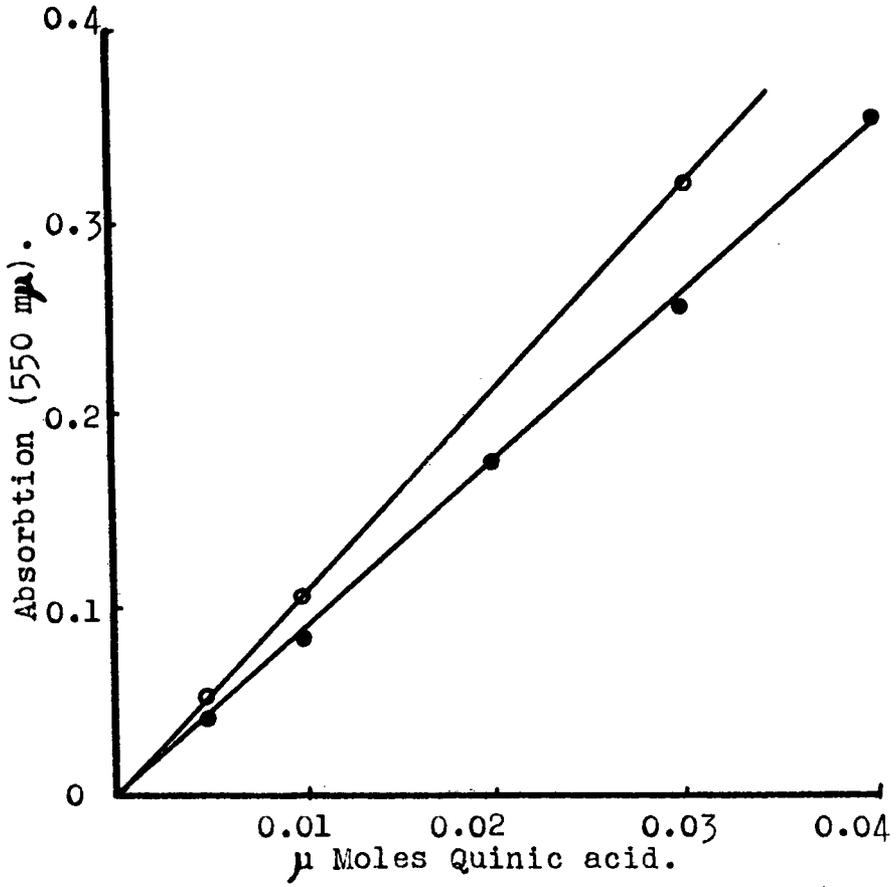


Fig. 4. Calibration graphs for quinic acid determination. (Plots o and ● are for two different days).

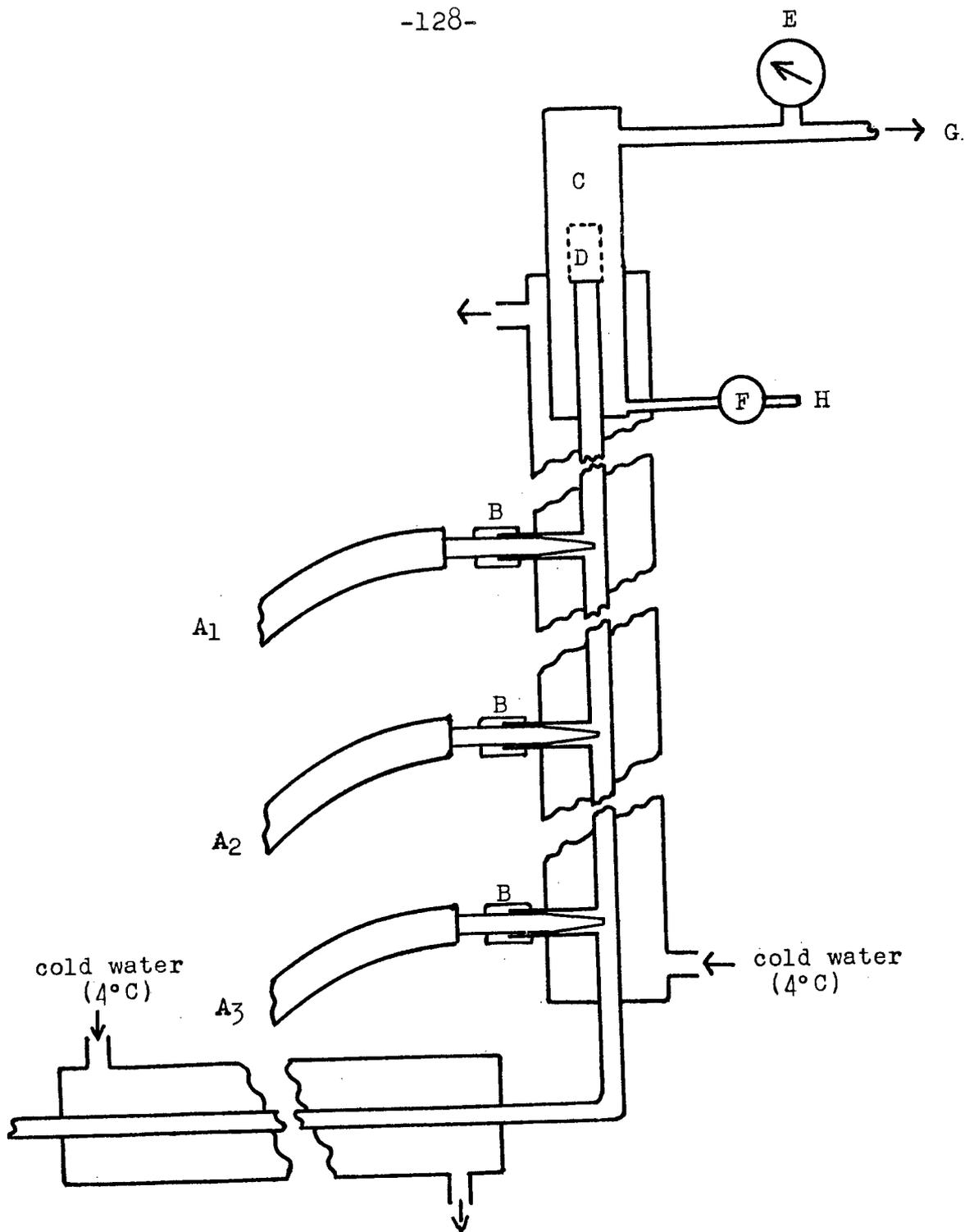


Fig. 5. Diagram of system used for recovery of highly volatile wine components. (A₁, A₂, A₃: nitrogen nozzles, N₂ flow 30cc min⁻¹ at 20 psi each; B: Latex tubing seals; C, Vapour chamber; D: Sintered glass head; E, Pressure gauge, to monitor trap blockage; F: Variable restrictor, set to maintain sealing layer in C; G: Volatiles to traps; H: To waste or recycle; I: Wine, metered flow 10 cc min⁻¹).

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