MALOLACTIC FERMENTATION IN WINES OF MICHIGAN

By

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ABSTRACT

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Malolactic fermentation is the conversion of malic acid to lactic acid and carbon dioxide by certain species of Leuconostoc, Lactobacillus and Pediococcus in wines.

Malolactic fermentation was induced in Foch, DeChaunac and Chancellor wines by using the Leuconostoc oenos strains ML-34, PSU-1 and LS-5A. The musts and wines were subjected to silica gel column chromatography and paper chromatography for determination of the non-volatile acids. Sixteen acids were isolated, out of which twelve were identified. The wines were subjected to sensory evaluation by trained panelists.

The malolactic fermentation was rapid in Chancellor, medium in Foch and slow in DeChaunac by all strains used. Malic acid disappeared while aspartic, glutamic and phosphoric acids were reduced appreciably. There was a considerable loss in titratable acidity with malolactic fermentation. Sensory evaluation revealed that the malolactic fermentation generally improved the quality of the Michigan wines studied in this work.

Dedicated to my parents and my wife, Evangelia.

ACKNOWLEDGMENTS

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Hearty appreciation is also expressed to Dr. Stanley Howell of the Department of Horticulture for his kind and generous help.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

The major acids in grapes are tartaric and malic. Malic acid contributes more to the wine acidity than tartaric, because the latter is present as potassium bitartarate, which is insoluble in ethanol, thus precipitates during the alcoholic fermentation and cold stabilization procedures. Malic acid is also stronger than tartaric acid. The accumulation of malic acid under cool grape growing conditions is the main cause for the high acidity of grapes and wines of these regions (Kunkee, 1974).

In wine making practice it had been early observed that a loss in acidity occurs in new wines which coincides with the growth of certain bacteria. This aspect has been thoroughly studied and referred to as malolactic fermentation, that is a secondary fermentation during which malic acid is converted to lactic acid and carbon dioxide by certain species of the genera Leuconostoc, Lactobacillus and Pediococcus.

There are three aspects to the malolactic fermentation. The first immediate effect is deacidification of the wine, since the dicarboxylic malic acid is converted to the monocarboxylic lactic acid. The second effect is a flavor change in wine and the third effect is an increase in the microbiological stability of wines that have undergone malolactic fermentation.

The occurrence of malolactic fermentation is undesirable in warm

climates as it leads to excessive reduction of acidity and is considered to be a wine spoilage factor, thus precautions are taken to prevent it. In contrast to this, in wines of cool areas a reduction in acidity is desirable and may be accomplished by inducing malolactic fermentation before bottling. Although some authors question the value of malolactic fermentation, winemakers from regions where wines of high acidity are produced have praised this fermentation and declare it essential for the production of premium quality wines.

Wines that are made from grapes grown in Michigan are of high acidity and malolactic fermentation may improve the quality of these wines.

The objective of this research was to study the effect of the malolactic fermentation induced by various bacterial strains on wines produced from different Michigan grape cultivars. Emphasis was placed on chemical changes in the wine and sensory changes in wine quality.

2. LITERATURE REVIEW

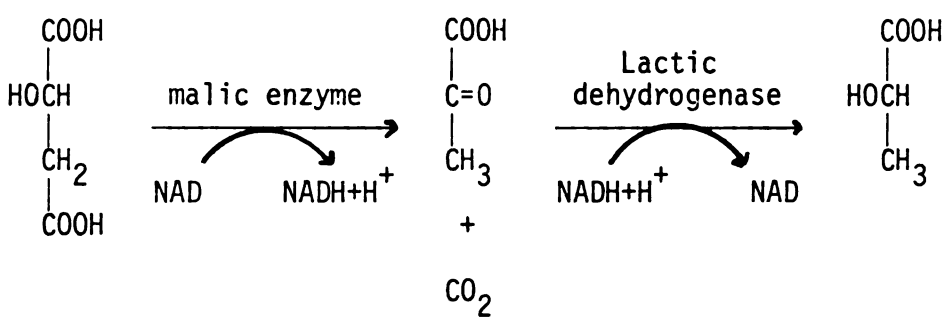
The first to observe a drop in total wine acidity, greater than that expected from the precipitation of tartrates, were Berthelot and DeFlerieu (1864). Ordoneau (1891) suggested that the loss in acidity was due to the conversion of malic acid to another acid.

Pasteur (1858) proved that lactic acid was produced by bacterial action and described the "tourne" disease of wines. Alfred Koch (1900) isolated malolactic bacteria and induced malolactic fermentation in wines. Moslinger (1901) described the malolactic fermentation in the form of a chemical equation. Muller-Thurgau and Osterwaldere (1913) worked on the taxonomy of all bacteria that had been isolated from wines and were capable of carrying malolactic fermentation. Kunkee (1967) stated that the organisms that carry out malolactic fermentation are all from three genera: Leuconostoc, Lactobacillus and Pediococcus. Some other genera cited in older literature are now included in these three genera. Garvie (1967) has given a new classification, according to which all Leuconostocs isolated from wines, are under the name Leuconostoc oneos (from the Greek oinos = wine). Nonomura (1965) presented a new scheme for classifying malolactic Leuconostocs, based on their ability to ferment sugars. Ingraham and Cook (1960) isolated a microorganism from red wines that had a greater ability than others to grow in wines and called it Leuconostoc oenos ML-34. This microorganism was formerly called

Leuconostoc gracile Cf 34. A lot of work on the malolactic fermentation has been done using this strain. Beelman and Gavin (1977) isolated a new strain from French hybrid strains grown in Pennsylvania following a spontaneous malolactic fermentation and named it Leuconostoc oenos PSU-1. Beelman also (1980) compared the two strains ML-34 and PSU-1 and found that PSU-1 induced a more rapid malolactic fermentation. Silver and Leighton (1981) isolated a new strain designated B 44-40 that showed greater tolerance to variations of pH and temperatures than ML-34 and PSU-1.

Many investigators recognize that there is a lot of confusion as far as the mechanism of the conversion of malic acid to lactic acid is concerned. This happens because NAD is being involved as an essential factor in the reaction, but there is not any change in the redox state and the reaction proceeds as a decarboxylation.

Ochoa and his coworkers (1950) have proposed that the reaction has two steps:



L (-) Malic acid Pyruvic acid Lactic acid

According to this mechanism, pyruvic acid is either a short-lived, fleeting intermediate, or it is bound to "malic" enzyme, so that as soon as it is formed it is converted to lactic acid by Lactate dehydrogenase. Morenzoni (1974) stated that this concept has led to a

large degree of confusion. Pilone and Kunkee (1970) showed that if carbonic acid or bicarbonate ion were produced, the standard free energy would be -6.2 and -7.2 Kcal/mol, respectively. Morenzoni (1974) stated that malolactic fermentation does not yield enough energy for ATP production by the bacteria and since the produced NADH would immediately be reoxidized to NAD, there is no energy benefit for the microorganism. However, Pilone (1971) has shown an increase in cell yield and growth rate as a result of the malolactic fermentation. Morenzoni (1974) suggests that either something else is produced which the microorganism can use for growth, or that malic acid is toxic to the cell and malolactic fermentation represents a detoxification mechanism. The current concept is that the malic acid utilization system of Leuconostoc oenos ML-34 involves two separate enzyme activities located on the same protein, which act simultaneously on malic acid (Morenzoni, 1974). One activity catalyzes the major reaction:

$$\text{L - Malic acid} \xrightarrow[Mn^{+2}]{NAD} \text{L - Lactic acid} + CO_2$$

and the other activity catalyzes the minor reaction:

$$\text{L - Malic acid} + NAD \longrightarrow \text{Pyruvic acid} + NADH_2 + CO_2$$

In the wine making procedure some important actions may be taken to inhibit malolactic fermentation when it is undesirable. Kunkee (1967) stated that those actions include: 1) Early racking at the end of alcoholic fermentation to prevent autolysis of the yeasts and release of microconstituents; 2) continued monitoring and maintenance

of SO_2 concentrations at a reasonable level depending on pH; 3) maintenance of storage temperatures at less than 18°C; 4) adjustment of acidity to lower the pH (below 3.3 or some other empirically established pH level); and 5) storing the wine in new cooperage or other containers known to be devoid of malolactic bacteria. Other ways to remove malolactic bacteria would be sterile filtration of wines and perhaps pasteurization. Cofran and Mayer (1970) found that fumaric acid can be added to wines at the end of the alcoholic fermentation (about 0.05%) to prevent malolactic fermentation, but Kunkee (1974) does not recommend this technique.

In cases in which the malolactic fermentation is desirable, Kunkee (1974) suggests practices opposite to those for inhibiting it. He suggests storing the wines in cooperage harboring microflora from a previous acceptable malolactic fermentation, or inducing a rapid and clean malolactic fermentation by inoculating the wine with a known strain of malolactic bacteria.

Ardin (1972) has given detailed methods for inducing malolactic fermentation by using commercial starter cultures.

There are many suggestions on the proper time of bacteria inoculation. Kunkee (1974) suggests that inoculation should be made when the wine must is about 5° Brix, because at this time there is no inhibitory effect by the free SO_2 and the alcohol concentration is lower than in the finished wine.

3. METHODS AND MATERIALS

3.1. WINE MAKING PROCEDURES

Grapes of the following cultivars: Foch (KUHLMAN 188.2) from Lawton, Michigan, DeChaunac (SIEBEL 9549) from Sodus, Michigan and Chancellor (SIEBEL 7053) from Lawton, Michigan were harvested when the soluble solids were 19.5, 16.0 and 19.5 ^{o}Brix, respectively.

The grapes were stemmed and crushed with a manual crusher-destemmer, and 20 p.p.m. SO_2 (from potassium metabisulfite) was added to the juice. The soluble solids were adjusted to 21 ^{o}Brix with sugar and the pulp was left with the skins for 24 hours, in plastic containers. The musts were then inoculated with Montrachet yeast and allowed to ferment with the skins for 4 days. The must was pressed in a basket press with hydraulic head at a pressure of 130 psi and the wine was stored in 5-gallon glass containers at 22^{o}C. There were three five-gallon containers for each cultivar, so each one represented a different replicate. After three days the wine musts were transferred into one-gallon containers. There were four one-gallon containers for each replicate to which the treatments were applied: 1) a control (no malolactic cultures were added); 2) inoculated with Leuconostoc oenos strain ML-34; 3) inoculated with L. oenos strain PSU-1; and 4) inoculated with L. oenos strain LS-5A. When the alcoholic fermentation was completed, the wines were racked into one-gallon containers. Upon the completion of the malolactic fermentation,

50 ppm of SO_2 (in the form of potassium metabisulfite) were added and the wines were stored in a cold room where the temperature was -6 to -8°C for cold stabilization. When the cold stabilization procedure ended, the wines were racked again, bottled and labeled.

Preparation of the malolactic starter cultures. Stub cultures of Leuconostoc oenos strains ML-34 and LSU-1 were provided by E. & J. Gallo wineries in California along with a commercial freeze dried culture, "Leucostart-5A."

Inoculum from the stub cultures was transfered to a Rogosa-type broth medium prepared as follows: 60 g "Bacto Rogosa SL Broth" from Difco was rehydrated in 1000 ml of cold distilled water. The mixture was heated to boil so that the medium was completely dissolved. 1.3 ml of glacial acetic acid was added and it was kept boiling for 2-3 minutes. It was then cooled to 40°C. The final pH of the medium was 5.4 at 25°C. The inoculated broth tubes were incubated at room temperature for 3-4 days and subsequently stored in a refrigerator. The starter cultures were transfered into a grape juice medium one week before the wines were ready for inoculation. The grape juice medium was prepared according to Kunkee (1974) by adding 50 ml grape juice, 50 ml water and 0.10 g yeast extract. The pH was adjusted to 4.5 with 0.1 N NaOH. The medium was autoclaved for 20 minutes at 121°C (15 psi). One loopful of inoculum from the Rogosa medium was inoculated in the grape juice medium and incubated at room temperature for 3-4 days. When the soluble solids in the wines reached 4-6 °Brix, 5 ml inoculum from the grape juice was added in each gallon of wine. The process of malolactic fermentation was followed by paper chromatography.

3.2. PAPER CHROMATOGRAPHY

Whatman No. 1 chromatographic paper was cut into 20 x 26 cm rectangles. The wine samples along with the standard acids were spotted on a line approximately 2.5 cm parallel to the long edge. The spots were placed along this line about 2.5 cm apart. Each spot was 10 µl. A cylinder was formed from the paper by stapling the short edge. A wide mouth one-gallon mayonnaise jar served as chromatographic chamber.

The solvent was prepared by shaking in a separatory funnel 100 ml water, 100 ml n-Butanol, 10.7 ml formic acid and 15 ml 1% bromocresol. After about 20 minutes, the lower (aqueous) phase was drawn off. Seventy ml of the upper phase were placed in the jar. The chromatogram was inserted and the jar lid was attached. The developing time was about 6 hours. The yellow chromatogram was then removed and dried in a ventilated chamber until formic acid was completely volatilized, leaving a blue background with yellow spots of acids.

3.3. DETERMINATION OF THE NONVOLATILE ACIDS IN WINES

Isolation of acids. Twenty ml of wine were mixed with 2 g of polyclar and heated in a steam bath for 5 minutes. The wine was then filtered through a milipore filter (GSWP 047 GS 0.22 µ). The filter and the beaker were rinsed with 15 ml distilled water and the washing was added to the sample, which was then subjected to ion exchange column chromatography as follows:

Ion exchange column chromatography. A column of 0.7 cm internal diameter was packed with 5 g of ion exchange resin Dowex 1 x 8 in the

acetate form, 200-400 mesh, under pressure of 10 inches of water. The column was then washed with 25 ml distilled water followed by 15 ml of ethanol. The eluent was discarded. The elution of the acids was accomplished with 30 ml 8 N formic acid followed by 15 ml (normality) formic acid. The eluent was collected in an evaporation flask and was concentrated in a flash evaporator at 40^{o} C. The concentrate was diluted to 1 ml with water and subjected to silica gel column chromatography as follows:

Silica gel column chromatography. A modification of Zbinovsky and Burris (1954) method was used. Silicic acid (Mallingkrodt 100 mesh) was dried overnight at 110^{o} C. Five g of silicic acid were mixed with approximately 2.7 ml of 0.5 N H_2SO_4 and a slurry was made by adding a mixture of chloroform: n-butanol (15:85 v/v) saturated with 0.5 N H_2SO_4. A small amount of glass wool was placed at the bottom of a chromatographic column (0.7 x 23 cm) and the slurry was poured into the column and packed under pressure of 100 inches of water. Approximately 15 cm of silicic acid was added at a time. The height of the column was 22 cm.

Two blotter disks 7 mm in diameter were cut from a 1 mm thick white filter paper and 0.15 ml of the sample were transfered on each disk, by adding 0.05 ml each time and drying the disk with a hair blower. The disks were then put on the top of the column which was covered with a 1 mm layer of solvent. The disks were pressed evenly with a glass rod. 0.1 ml of solvent was added on top of the disks.

Two solvents were used for elution. The first solvent was chloroform: n-butanol (85:15 v/v) saturated with 0.5 N H_2SO_4 and the second was chloroform: n-butanol (65:35 v/v) saturated with 0.5 N

H_2SO_4. A pressure of 100 inches of water was applied to the column. A 7000 Ultrorac-LKB BROMMA fraction collector was used. The first 40 fractions consisted of 40 drops and the rest of 80 drops each. The fractions were titrated with 0.0171 N NaOH in ethanol as described by Isherwood and Wager (1961). A mixture of 400 ml ethanol and 50 ml water was made slightly alkaline (pale pink to phenolphthalein) with 0.1 N aqueous NaOH and 100 ml of 0.1 N NaOH were added. This solution was stored in a flask open to the air through a CO_2 trap. Ethanolic solution of phenolphthalein was used as an indicator (0.1 5 w/v), that was slightly alkaline. Authentic samples of known acids were used for comparison with the unknowns.

Acid identification by paper chromatography. Fractions corresponding to the column chromatographic peaks of the acid separation were dried in a vacuum oven at 40^{o} C and 2 ml of distilled water were added to the residue. A small amount of ion exchange resin Dowex 50 x 12 (H^{+} form) was added into each fraction and the tubes were shaken vigorously. After the resin settled down, 10 µl aliquots were spotted in Whatman No. 1 sheets, 46 x 57 cm, 2.5 cm apart, 5 cm away from the long edge of the sheet. The sheets were placed into a chromatographic chamber for descending chromatography. The first solvent was n-butanol: formic acid with bromocresol green indicator, prepared in the same way as for the detection of malolactic fermentation in wines and the second solvent was ethanol (96%): ammonium hydroxide: water, 20:1:4, v/v. The chromatograms obtained by the second solvent were sprayed with bromophenol blue indicator in ethanol.

3.4. ANALYSIS OF MUSTS AND WINES

a. Titratable acidity. Five ml sample and 100 ml boiled distilled water were added in a beaker and titrated electrometrically with 0.1221 N NaOH to pH 8.2 end point.

b. Volatile acidity. The volatile acidity was determined using a Cash electric volatile acidity assembly. Ten ml of wine and 250 ml of water were placed in the appropriate parts of the apparatus. The distillation was conducted rapidly until 100 ml has been collected. They were titrated in the distillate with 0.0362 N NaOH with phenolphthalein indicator.

c. Alcohol determination. The alcohol content was determined using a Braun type ebulliometer. The boiling point of water was first determined. The revolving disk of the calculator was set so that the temperature value recorded was just opposite to zero on the outer disk. Twenty-five ml wine were dilluted to 100 with distilled water and 50 ml of the dilluted wine were added to the vessel through the thermometer opening. The sample was boiled until the temperature did not rise any more. The corresponding temperature was located on the inner circle of the calculator and the alcohol content was read opposite to it. This value must be multiplied by the dilution factor.

3.5. SENSORY EVALUATION

Sensory evaluation consisted of three tests: a) a twenty point scoring test (Davis), b) a ranked preference test (Amerine, 1976) and c) a paired comparison test (Amerin, 1976).

a. Twenty point scoring test. One bottle of wine from each of the 12 treatments (3 replicates x 4 types of treatments) was taken out of storage several hours before the test. The 12 wines were assigned randomly by a person who did not participate in the panel. Twelve panelists were provided with the score sheets shown in Figures 1 and 2. The scoring scale was clearly defined and understood by all judges.

b. Ranked preference test. In this test 4 judges were asked to compare wines corresponding to the 4 types of treatments (Control, ML-34, PSU-1 and LS-5A), separately for each cultivar. The most preferred wine was given number 1, the second best 2, the third 3 and the fourth 4. This test was done twice. The total score of each wine represents the preference of each wine by all judges. In order to state that a particular wine is different at 0.05 significance level, the sum of the points of this wine in both tests should be out of the range given by Tables (Amerine, 1976). Since this test was done twice the number of judges is considered to be 9 for Foch and 8 for DeChaunac and Chancellor. For 9 judges and 4 wines the range is 15-30 and for 8 judges and 4 wines the range is 13-27. If a wine scores less than the lower limit, it is significantly better than the rest at a 0.05 significance level and if scores more than the higher limit it is significantly worse than the others at the same significance level.

c. Paired comparison test. For each cultivar the most and the least preferred wines of each replicate, as it was determined by the preference test, were paired and randomized (with two digit numbers) by a person who did not participate in the panel. Each judge was asked

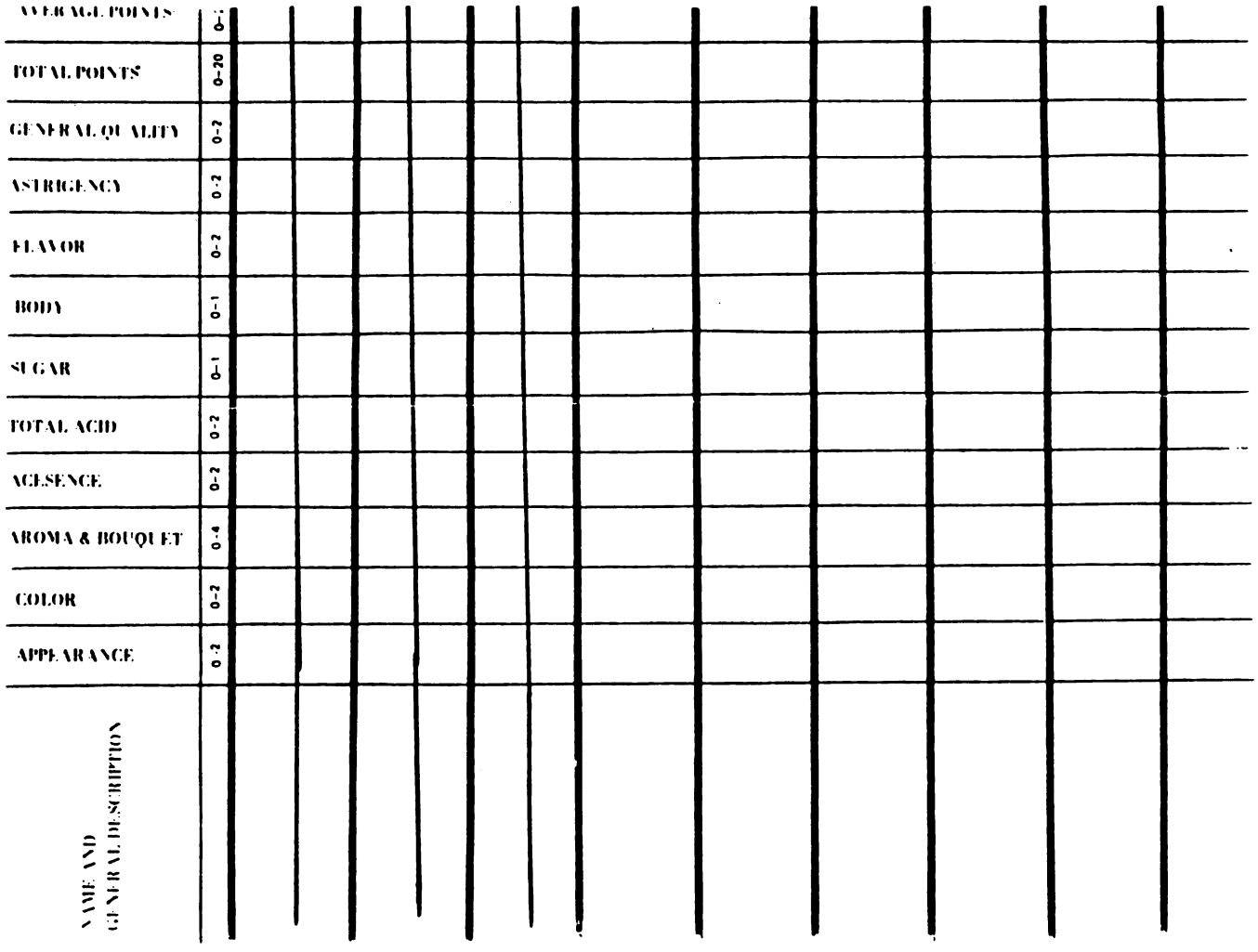


Figure 1. Wine grading score sheet.

Figure 2. GENERAL TERMINOLOGY FOR COMPARATIVE WINE TASTING
A 20 Point System

1) Appearance (0 -2) – Overall visual quality of the wine; including clarity, color, and absence of particles.

2) Color (0–2) – Actual color, hue. Shouldn't be dull or hazy.

3) Aroma and Bouquet (0–4) – The Bouquet is the flowering scent of the wine while the aroma is the lasting smell.

4) Acescence (0–2) – Amount of acetic acid in wine; too much will cause the wine to turn vinegary or leave a grey film.

5) Total Acid (0–2) – Gives wine its tart or tangy quality; too much will bite, not enough will be bland.

6) Sugar (0–1) – Must be in correct proportion to the acids for a rounded taste.

7) Body (0–1) – Body fills the mouth with flavour and lingers as aftertaste.

8) Flavor (0–2) - Individual taste of each bottle or variety of wine.

9) Astringency (0–2) – Sharpness from acid content and tanin sometimes indicates longetivity.

10) General Quality (0–2) – General rating of all points of the wine.

(17–20) Outstanding Quality

(13–16) Good Commercial Wine

(10–12) Commercially acceptible with a noticeable defect

(0–9) Commercially Unacceptable

to indicate which wine in each pair tasted best. This test was done twice for each pair to determine the judge consistency.

The number of correctly chosen wines in each pair were added. This sum was subjected to Testing Hypothesis to determine whether the best wines were randomly selected by the judges.

4. RESULTS AND DISCUSSION

4.1. WINEMAKING PROCEDURE

Approximately 20 ppm SO_2 were added as potassium metabisulfite during crushing. Actually the malolactic strains used in this work can induce the fermentation in a 50 ppm SO_2 level. However, the initial pH of all crushes was very low and less SO_2 was sufficient to protect the wines from spoilage microorganisms without inhibiting the starter cultures from inducing a rapid and clean malolactic fermentation. The starter cultures were transfered from a Rogosa-type medium to a grape juice medium before added in the wines for two reasons: 1) to avoid shocking the bacteria by transfering them from their optimum pH to wines that had a very low pH and 2) to avoid the addition of large amounts of Rogosa medium into the wine. The above mentioned medium has a distinct odor that could affect the wine flavor.

4.2. ACID CONTENT OF MUSTS AND WINES

The musts and wines contain compounds which may clog the silicic acid column, resulting in low flow rates and bad resolution of the acids. Most of the phenolic compounds present in the samples were removed with polyclar and the ethanol wash of the ion exchange column.

A further clean up of the sample was accomplished by ion exchange column chromatography. The acids were bound to the resin while the

irrelevant compounds were eluted with water and ethanol. Finally the acids along with some anthocyanin were eluted with formic acid. The eluent was concentrated in a flash evaporator at temperature not exceeding 40^{0} C, so that lactic acid will not form anhydrites nor evaporate.

In preparing the silica gel column it was found that 2.7 ml of 0.5 N H_2SO_4 added to 5 g silicic acid was sufficient to prevent excessive breaking of the column and obtain a good resolution of the acids.

The silica gel chromatograms are as shown in Figures 3 through 11. Table 1 gives the identification code of the acids in these chromatograms. The acid content of musts and the wines of the three cultivars are as shown in Tables 2 through 7.

The identification of the acids was based on the effluent volume of the acid peaks, in comparison with the effluent volume of standard acids in silica gel column chromatography and on paper chromatography of the acids in two solvents. The Rf values of the unknown acids were compared with those of standard acids (Table 8).

Paper chromatography revealed that the fifth fraction contained mainly shikimic acid along with an unknown, but for simplicity the whole fraction was expressed as shikimic acid. There were also two minor peaks that were not identified and a rather significant peak (#12) which could not be identified. This acid according to Carles (1969) could be dimethylglyceric acid, but there was no standard acid available for comparison. Carles, however, stated that dimethylglyceric acid can be found in amounts of 5-10 meq/lit. He also stated that in paper chromatography this acid had a high Rf value

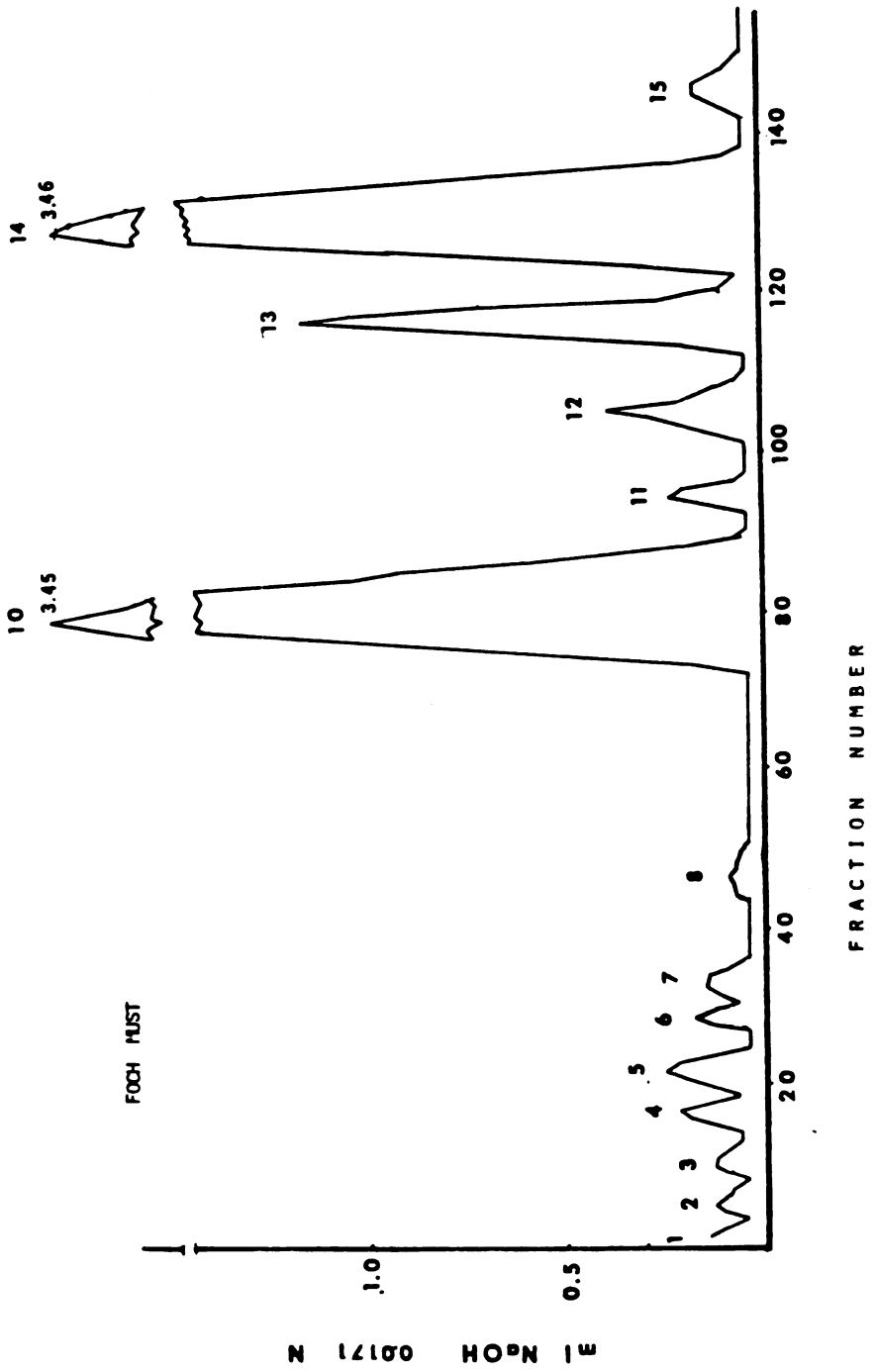


Figure 3. Silica gel column chromatography of the acids of Foch must.

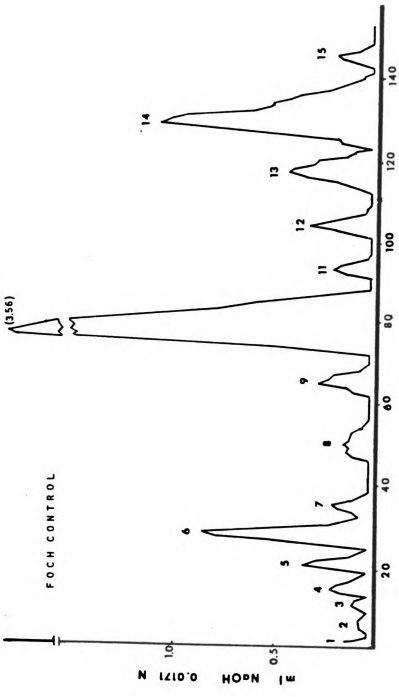


Figure 4. Silica gel column chromatography of the acids of Foch control wine (no malolactic cultures added).

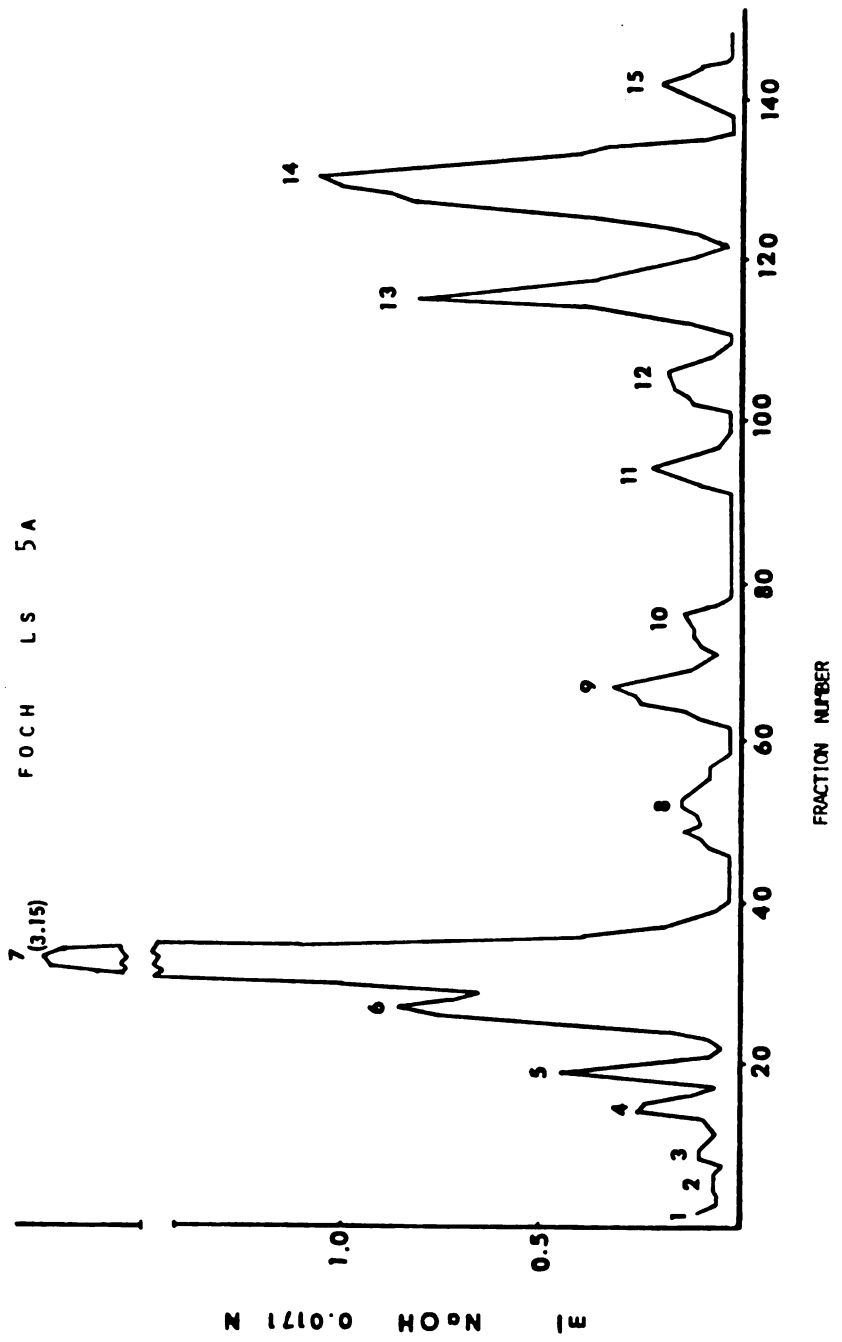


Figure 5. Silica gel column chromatography of the acids of Foch wine treated with Leuconostoc oenos LS-5A.

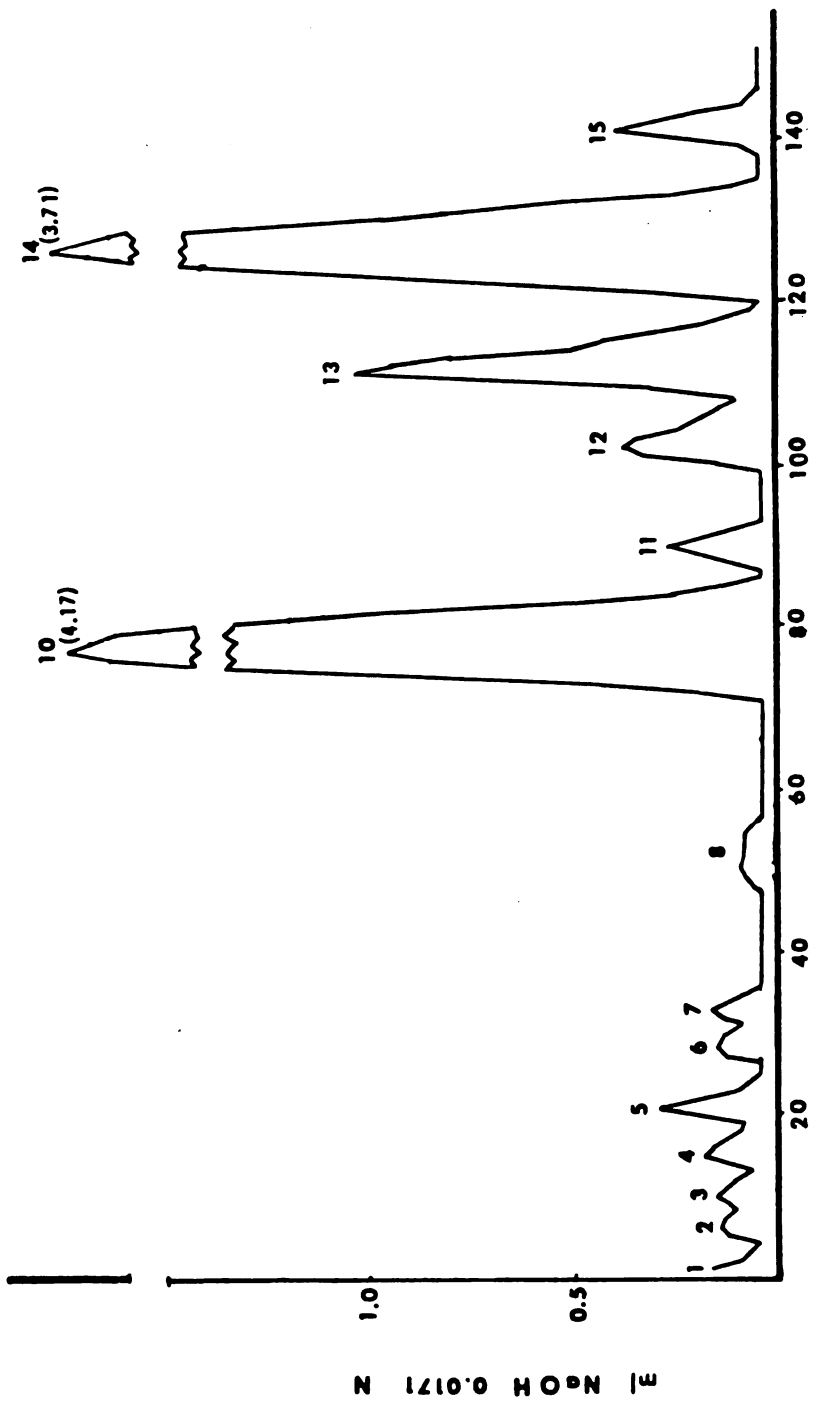


Figure 6. Silica gel column chromatography of the acids of DeChaunac must.

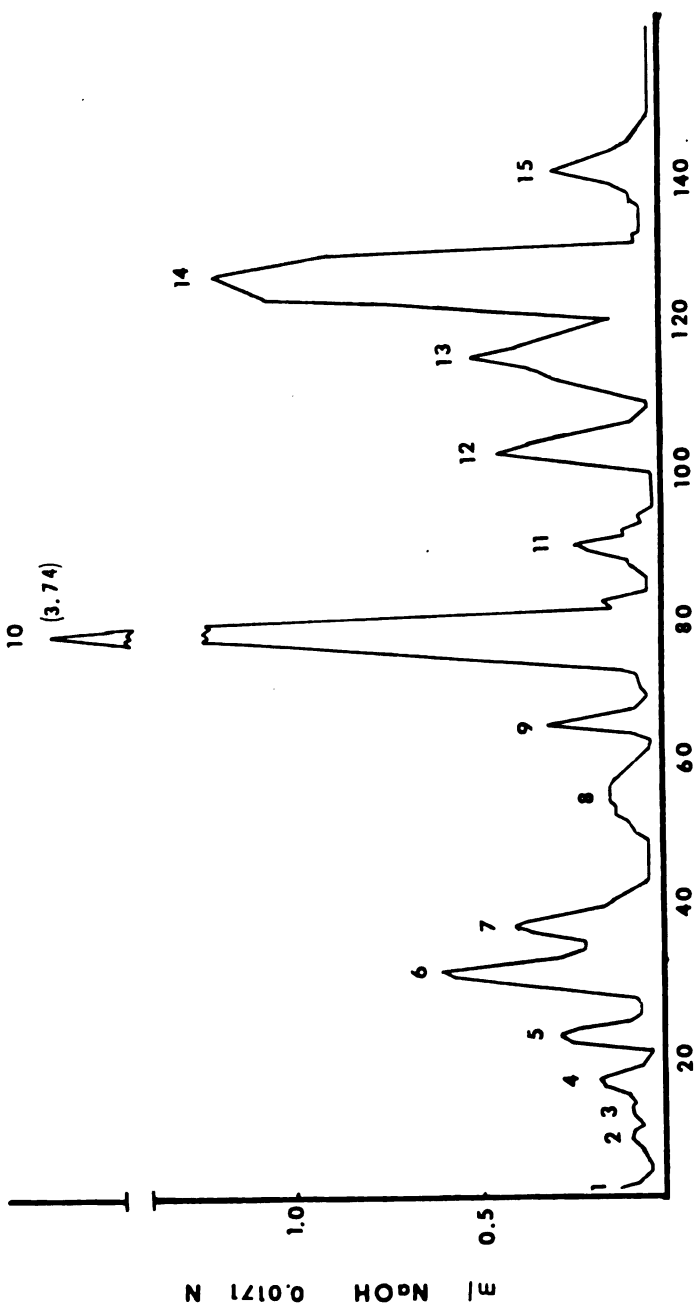


Figure 7. Silica gel column chromatography of the acids of DeChaunac control wine (no malolactic cultures added).

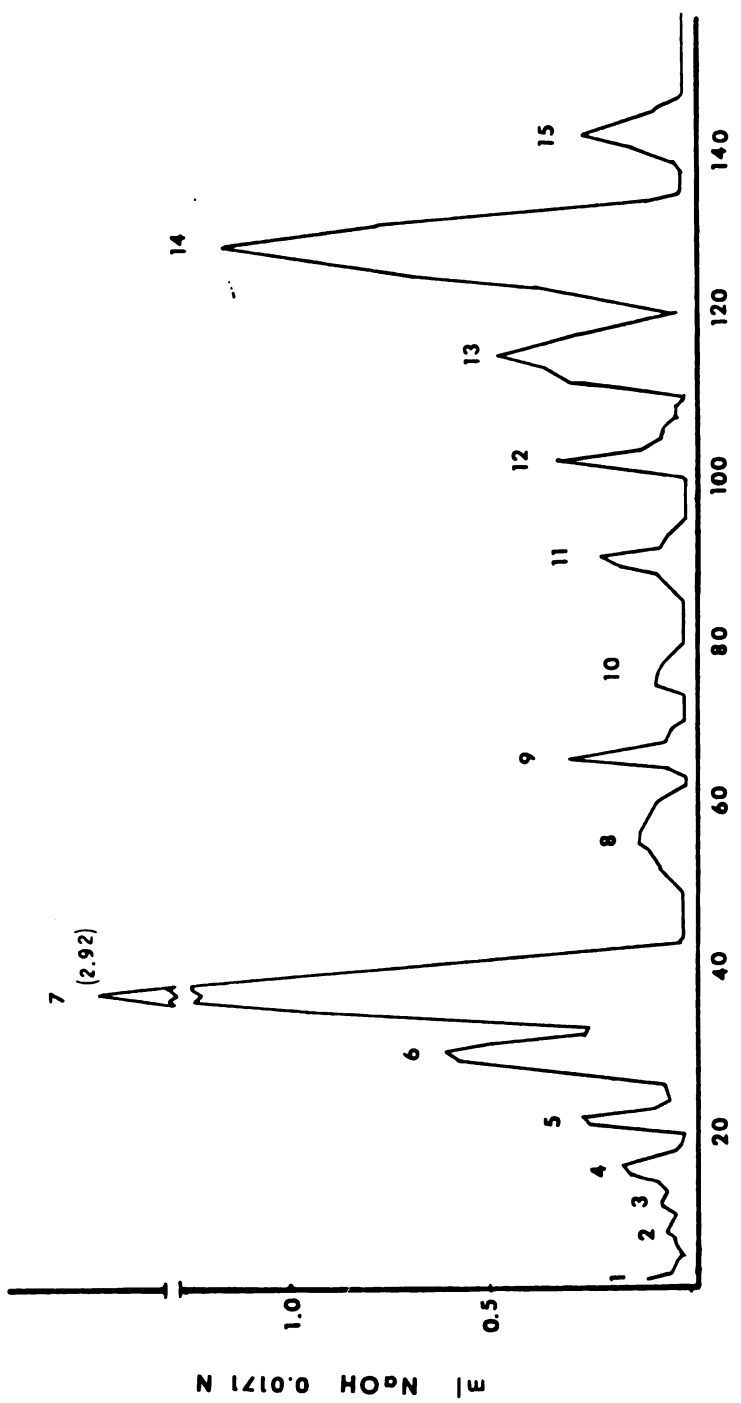


Figure 8. Silica gel column chromatography of the acids of DeChaunac wine treated with L. oenos PSU-1.

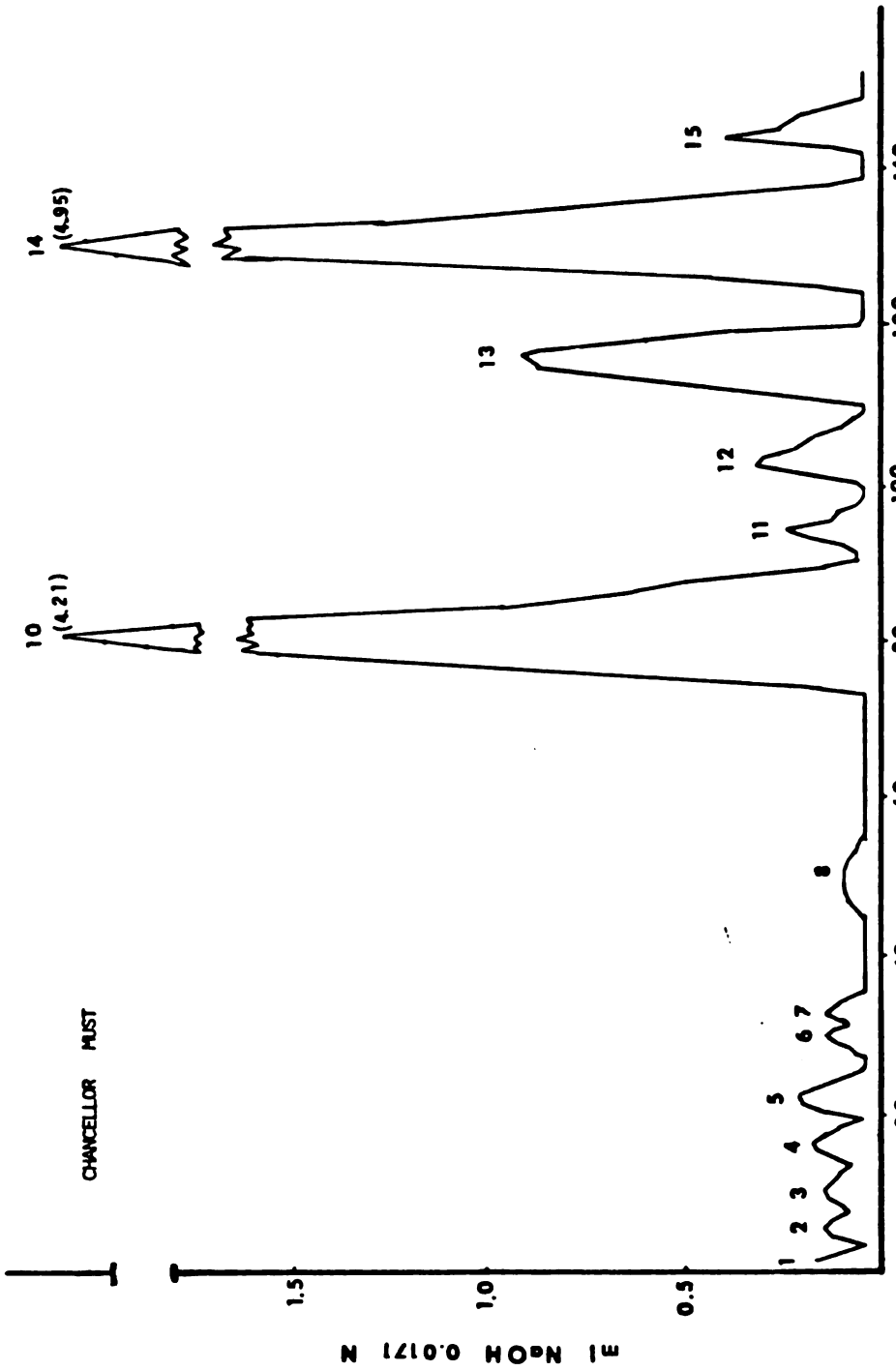


Figure 9. Silica gel column chromatography of the acids of Chancellor must.

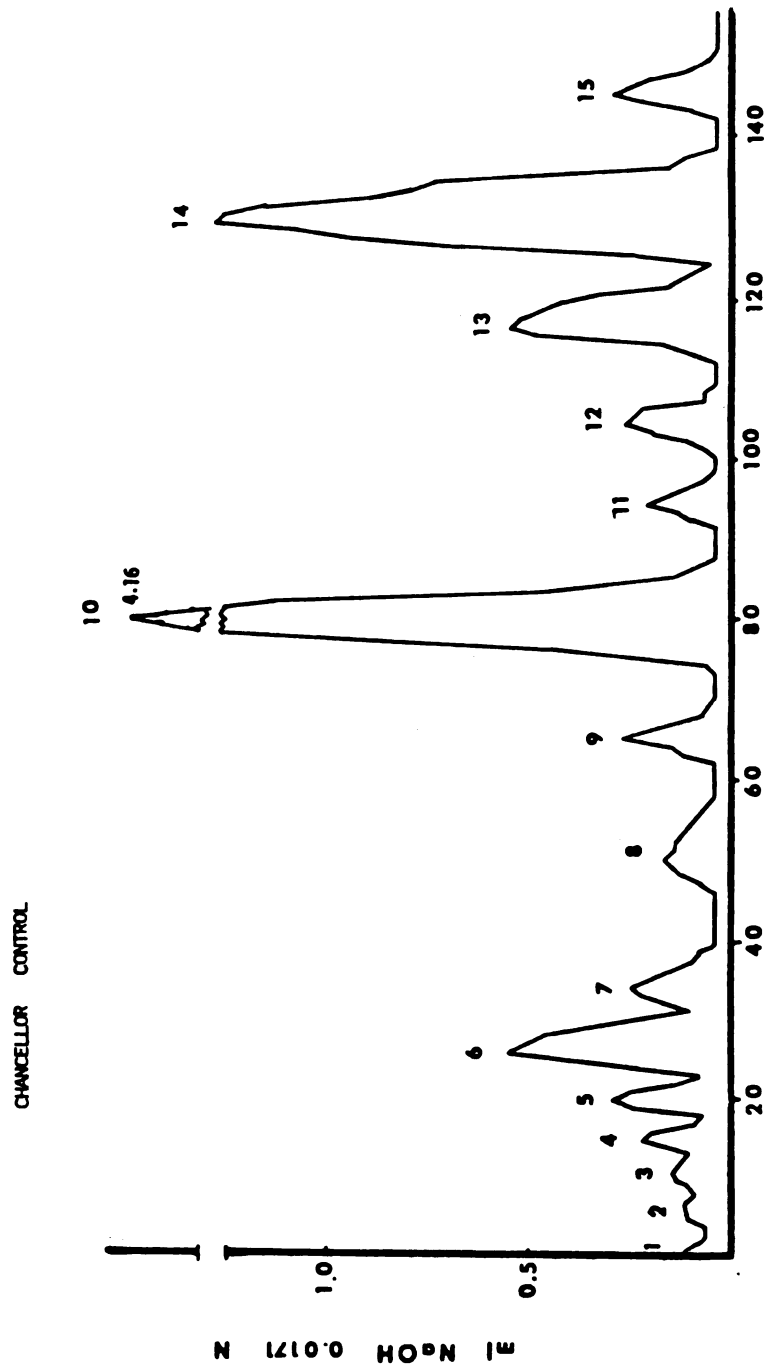


Figure 10. Silica gel column chromatography of the acids of Chancellor control wine (no malolactic cultures added).

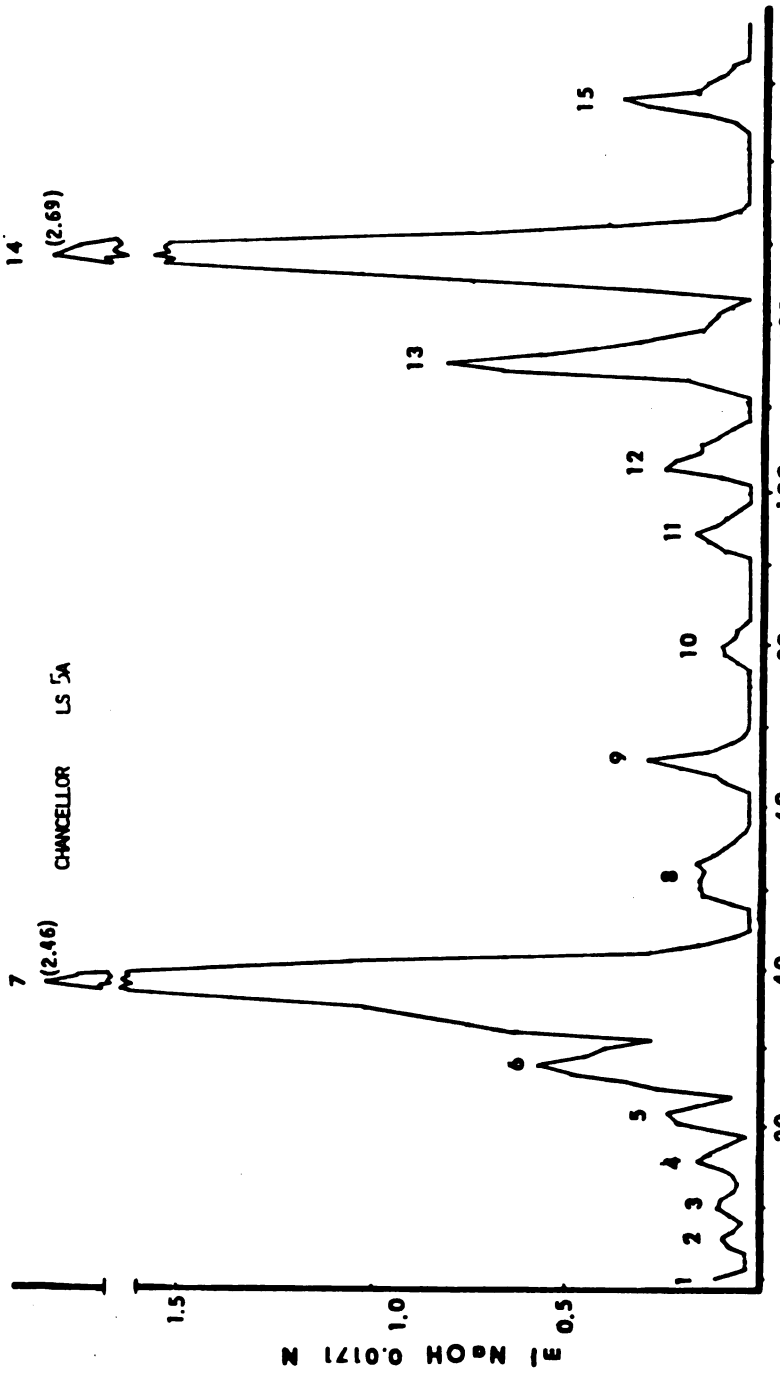


Figure 11. Silica gel column chromatography of the acids of Chancellor wine treated with L. oenos LS-5A.

Table 1. Identification code of the acids in the following chromatograms.

1. Unknown
2. Unknown
3. Glutamic
4. Aspartic
5. Shikimic and One Unknown
6. Succinic
7. Lactic
8. Pyruvic
9. Citramalic
10. Malic
11. Unknown
12. Phosphoric
13. Citric
14. Tartaric
15. Galacturonic

Table 2. Acid content in mg/100 ml of Foch must and wines, determined by Silica gel column chromatography.

ACID		TREATMENTS				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown					
2.	Unknown					
3.	Glutamic	5.21	3.41	2.70	2.52	3.05
4.	Aspartic	9.43	8.78	5.52	5.85	5.69
5.	Shikimic and Unknown[a]	33.45	45.20	28.90	26.35	36.12
6.	Succinic	3.61	43.56	38.90	38.08	39.95
7.	Lactic	6.16	11.88	204.20	202.12	206.85
8.	Pyruvic	4.30	15.91	14.63	14.63	16.78
9.	Citramalic	-	16.28	15.37	15.56	16.01
10.	Malic	313.95	304.94	5.24	4.42	4.84
11.	Unknown					
12.	Phosphoric	9.18	7.90	5.59	5.26	5.35
13.	Citric	64.53	34.41	35.20	34.42	34.88
14.	Tartaric	378.01	124.84	108.34	92.58	93.86
15.	Galacturonic	25.60	25.60	25.60	23.71	25.60
16.	Acetic[b]		81.00	95.10	94.40	90.00

[a] The whole fraction is expressed as shikimic acid.

[b] Volatile acidity expressed as mg acetic acid/100 ml sample.

Table 3. Acid content in meq/100 meq of Foch must and wines, determined by Silica gel column chromatography.

ACID		TREATMENTS				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown	0.32	0.24	0.29	0.27	0.36
2.	Unknown	0.48	0.29	0.36	0.34	0.42
3.	Glutamic	0.58	0.45	0.49	0.47	0.56
4.	Aspartic	1.17	1.28	1.11	1.22	1.15
5.	Shikimic and Unknown[a]	1.50	1.97	2.22	2.10	2.22
6.	Succinic	0.50	7.18	8.30	8.96	9.06
7.	Lactic	0.56	1.28	30.36	31.15	30.75
8.	Pyruvic	0.40	1.76	2.22	2.17	2.55
9.	Citramalic	-	2.14	2.78	2.92	2.90
10.	Malic	39.82	44.27	1.05	0.92	0.72
11.	Unknown	1.03	1.28	1.34	1.53	1.47
12.	Phosphoric	2.30	2.35	2.29	2.24	2.19
13.	Citric	8.86	5.23	7.36	7.47	7.29
14.	Tartaric	41.54	16.92	19.33	17.14	16.75
15.	Galacturonic	1.01	1.28	1.76	1.70	1.77
16.	Acetic[b]		10.50	16.97	17.48	16.06
TOTAL		100.07	97.72	98.78	98.08	96.24

[a] The whole fraction is expressed as shikimic acid.

[b] Volatile acidity expressed as meq acetic/100 meq of sample.

Table 4. Acid content in mg/100 ml of DeChaunac must and wine, determined by Silica gel column chromatography.

ACID		TREATMENTS				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown					
2.	Unknown					
3.	Glutamic	7.01	5.57	3.95	3.59	3.95
4.	Aspartic	8.45	7.80	4.88	5.36	5.69
5.	Shikimic and Unknown[a]	26.35	25.92	25.07	24.65	25.92
6.	Succinic	3.17	35.77	35.19	34.62	35.77
7.	Lactic	3.97	19.58	221.59	219.39	235.67
8.	Pyruvic	3.22	16.77	16.13	17.21	16.77
9.	Citramalic		12.84	10.85	10.85	11.22
10.	Malic	338.51	298.71	5.57	3.93	3.93
11.	Unknown					
12.	Phosphoric	9.58	7.34	6.47	6.79	6.55
13.	Citric	78.53	47.24	47.24	47.56	48.34
14.	Tartaric	396.71	168.84	171.41	153.63	117.82
15.	Galacturonic	49.32	47.42	47.42	47.42	47.42
16.	Acetic[b]		54.00	68.00	78.00	77.00

[a] The whole fraction is expressed as shikimic acid.

[b] Volatile acidity expressed as mg acetic acid/100 ml of sample.

Table 5. Acid content in meq/100 meq of DuChaunac must and wines, determined by Silica gel column chromatography.

ACID		TREATMENT				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown	0.53	0.28	0.32	0.32	0.53
2.	Unknown	0.60	0.67	0.75	0.70	0.67
3.	Glutamic	0.52	0.61	0.94	0.63	0.83
4.	Aspartic	1.39	1.65	1.79	1.32	1.84
5.	Shikimic and Unknown[a]	1.27	1.76	2.77	2.06	2.00
6.	Succinic	0.63	7.27	8.40	8.26	8.35
7.	Lactic	0.54	2.92	27.94	30.43	28.12
8.	Pyruvic	0.46	1.65	1.88	1.80	1.94
9.	Citramalic		1.95	1.98	1.77	1.94
10.	Malic	30.12	39.67	0.78	0.63	0.80
11.	Unknown	1.20	1.66	1.99	1.64	1.84
12.	Phosphoric	2.29	3.03	2.69	2.98	2.84
13.	Citric	9.00	6.33	7.33	6.84	7.35
14.	Tartaric	46.78	21.47	25.31	24.79	24.38
15.	Galacturonic	2.12	3.11	3.93	3.61	3.67
16.	Acetic[b]		5.40	8.82	7.43	11.29
TOTAL		97.45	99.41	97.57	95.41	98.38

[a] The whole fraction was expressed as shikimic acid.

[b] Volatile acidity expressed as meq acetic acid/100 meq sample.

Table 6. Acid content in mg/100 ml of Chancellor must and wines determined by Silica gel column chromatography.

ACID		TREATMENT				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown					
2.	Unknown					
3.	Glutamic	7.01	5.75	3.93	3.59	3.95
4.	Aspartic	8.45	7.80	4.88	5.36	5.69
5.	Shikimic and Unknown[a]	26.35	25.92	25.07	24.65	25.92
6.	Succinic	3.17	35.77	35.19	34.62	35.77
7.	Lactic	3.96	15.98	221.59	219.39	235.67
8.	Pyruvic	3.22	16.77	16.13	17.21	16.77
9.	Citramalic		12.84	10.85	10.85	11.22
10.	Malic	338.51	298.71	5.57	3.93	3.93
11.	Unknown					
12.	Phosphoric	9.58	7.34	6.47	6.79	6.55
13.	Citric	78.53	47.24	47.24	47.56	48.34
14.	Tartaric	396.71	168.84	171.41	153.63	117.82
15.	Galacturonic	49.52	49.52	47.42	47.42	47.42
16.	Acetic[b]		54.00	68.00	78.00	77.00

[a] The whole fraction is expressed as shikimic acid.

[b] Volatile acidity expressed as mg acetic acid/100 ml sample.

Table 7. Acid content in meq/100 meq of Chancellor must and wines, determined by Silica gel column chromatography.

ACID		TREATMENT				
			WINE			
		MUST	CONTROL	ML-34	PSU-1	LS-5A
1.	Unknown	0.34	0.26	0.35	0.26	0.25
2.	Unknown	0.56	0.56	0.46	0.35	0.38
3.	Glutamic	0.73	0.72	0.63	0.59	0.60
4.	Aspartic	0.98	1.12	0.86	0.97	0.96
5.	Shikimic and Unknown[a]	1.16	1.42	1.70	1.70	1.67
6.	Succinic	0.41	5.76	7.01	7.04	6.79
7.	Lactic	0.34	2.07	28.94	29.94	29.33
8.	Pyruvic	0.15	1.81	2.16	2.35	2.13
9.	Citramalic		1.65	1.72	1.76	1.70
10.	Malic	38.73	42.33	0.98	0.70	0.66
11.	Unknown	1.01	1.35	1.38	1.47	1.50
12.	Phosphoric	2.25	2.14	2.33	2.50	2.24
13.	Citric	9.41	7.01	8.68	24.58	26.54
14.	Tartaric	40.55	21.37	26.87	24.58	26.54
15.	Galacturonic	1.95	2.41	2.87	2.93	2.74
16.	Acetic[a]		6.84	10.66	12.48	11.49
TOTAL		98.45	98.77	97.58	97.83	97.46

[a] The whole fraction is expressed as shikimic acid.

[b] Volatile acidity expressed as meq acetic acid/100 meq wine.

Table 8. Rf x 100 values of acids chromatographed on Whatman No. 1 paper after separation by Silica gel column chromatography.

	SOLVENT I[a]		SOLVENT II[b]	
ACID	KNOWN	UNKNOWN	KNOWN	UNKNOWN
Unknown	-	83	-	63
Unknown	-	80	-	70
Glutamic	18	17	48	47
Aspartic	13	13	32	31
Shikimic	50	52	61	61
Unknown	-	39	-	50
Succinic	78	78	56	55
Lactic	75	75	74	74
Pyruvic	71	70	65	65
Citramalic	68	69	57	57
Malic	55	55	49	49
Unknown	-	49	-	78
Phosphoric	45	44	20	19
Citric	48	50	24	24
Tartaric	35	35	15	15
Galacturonic	28	28	44	44
Bromocresol front	89			

[a] n-butanol: formic acid: water (upper phase) 100:10.7:100 v/v, and 1% bromocresol green.

[b] Ethanol (96%): ammonium hydroxide: water, 20:1:4 v/v.

in a basic solvent. The unknown peak in this work had an Rf value of 0.78 in the basic solvent system, indicating that this acid may be identical with the dimethylglyceric acid of Carles.

Succinic, lactic and pyruvic acids were present as minor peaks in the musts (Figures 3, 6 and 9), but their concentration increases in the control and the other treatments. Amerine (1980) stated that those acids are formed in large amounts during the first stages of the alcoholic fermentation. Citramalic acid was not found in the must (Figures 3, 6 and 9). Carles (1959) believes that this acid is a decarboxylation product of citric acid and Dimotaki-Kourakou (1962) believes that it is a product of the alcoholic fermentation. It can be also observed that malic acid is decreased during the alcoholic fermentation (Tables 4 and 6) in DeChaunac and Chancellor wines but not in Foch wines. In all wines can also be observed that the tartaric acid content was reduced due to the precipitation of tartrates. The content in the amino acids aspartic and glutamic also decreased during the alcoholic fermentation and even more during the malolactic fermentation. Probably they are assimilated by the yeasts and bacteria. A loss of phosphoric acid occurs, probably also due to its utilization by the microorganisms. Malic acid practically disappeared after the malolactic fermentation and lactic acid was formed. Tartaric acid and lactic acids were the dominant acids in wines subjected to the malolactic fermentation. Citric acid was slightly decreased during the alcoholic and malolactic fermentations.

From the results obtained from the analysis of musts and wines can be observed that there is a difference in acidity between the three replicates (Tables 9, 10 and 11) in all treatments. Generally

Table 9. Analysis of Foch must and wines.

TREATMENT	pH	TITRATABLE ACIDITY[a]	VOLATILE ACIDITY[b]	ALCOHOL[c]
MUST	3.03	0.910		
WINE REPLICATE I				
Control	3.71	0.606	0.086	12.25
ML-34	4.01	0.436	0.098	11.83
PSU-1	3.98	0.433	0.092	12.10
LS-5A	3.96	0.430	0.093	11.60
WINE REPLICATE II				
Control	3.96	0.644	0.080	11.90
ML-34	4.00	0.570	0.095	12.40
PSU-1	4.01	0.546	0.092	11.80
LS-5A	4.01	0.457	0.083	11.55
WINE REPLICATE III				
Control	3.79	0.771	0.081	11.80
ML-34	4.00	0.560	0.095	11.75
PSU-1	4.04	0.540	0.094	11.60
LS-5A	3.92	0.560	0.090	11.48

[a] Titratable acidity expressed as % tartaric acid.

[b] Volatile acidity expressed as % acetic acid.

[c] Determined by ebulliometer (% v/v).

Table 10. Analysis of DeChaunac must and wines.

TREATMENT	pH	TITRATABLE ACIDITY[a]	VOLATILE ACIDITY[b]	ALCOHOL[c]
MUST	2.97	0.984		
WINE REPLICATE I				
Control	3.73	0.760	0.051	11.98
ML-34	3.80	0.696	0.045	12.20
PSU-1	3.80	0.685	0.045	12.05
LS-5A	3.88	0.639	0.065	11.90
WINE REPLICATE II				
Control	3.81	0.666	0.036	12.10
ML-34	3.73	0.565	0.050	11.80
PSU-1	3.81	0.579	0.043	12.20
LS-5A	3.87	0.549	0.062	12.18
WINE REPLICATE III				
Control	3.88	0.712	0.036	12.20
ML-34	3.80	0.621	0.042	11.80
PSU-1	3.86	0.677	0.050	12.15
LS-5A	3.82	0.632	0.064	11.97

[a] Titratable acidity expressed as % tartaric acid.

[b] Volatile acidity expressed as % acetic acid.

[c] Determined by ebulliometer (% v/v).

Table 11. Analysis of Chancellor must and wines.

TREATMENT	pH	TITRATABLE ACIDITY[a]	VOLATILE ACIDITY[b]	ALCOHOL[c]
MUST	3.08	0.978		
WINE REPLICATE I				
Control	3.70	0.698	0.056	11.40
ML-34	3.75	0.632	0.076	12.00
PSU-1	3.77	0.510	0.087	11.60
LS-5A	3.80	0.670	0.076	11.40
WINE REPLICATE II				
Control	3.68	0.790	0.054	11.40
ML-34	3.70	0.638	0.068	12.00
PSU-1	3.75	0.625	0.078	11.60
LS-5A	3.82	0.670	0.077	11.40
WINE REPLICATE III				
Control	3.72	0.780	0.055	11.30
ML-34	3.78	0.690	0.074	11.90
PSU-1	3.81	0.630	0.080	11.60
LS-5A	3.80	0.626	0.073	11.40

[a] Titratable acidity expressed as % tartaric acid.

[b] Volatile acidity expressed as % acetic acid.

[c] Determined by ebulliometer (% v/v).

the acidity increases from the first to the third replicate. This is probably due to the distribution of the acids into the berry. (The replicates were obtained by pressing the crush at different pressures.) There is a loss in titratable acidity during the alcoholic fermentation and the subsequent cold stabilization and this is due to the precipitation of tartrates. In the controls this acidity is greater than the gain in acidity caused by the increase in succinic, lactic, pyruvic and other acids.

It can be also observed that the volatile acidity increases with the malolactic fermentation and in some cases this increase is large, e.g. in DeChaunac replicates II and III treated with LS-5A the increase in volatile acidity was almost 50% over the controls.

The three strains completed the malolactic fermentation in different times in a particular cultivar (Figure 12). ML-34 required much more time to complete the fermentation in all cultivars than the other two strains. On the other hand, all strains needed less time to complete the malolactic fermentation in Chancellor, more in Foch and even more in DeChaunac.

4.3. SENSORY EVALUATION

A twenty point grading system was used for a rough estimation of the quality changes with malolactic fermentation. There were twelve judges randomly selected. Though such a small number of untrained judges cannot provide significant sensory information, it was observed that the judges consistently scored the PSU-1 and LS-5A treatments much higher than the controls and ML-34 (Table 12). These results coincide with the following sensory evaluation tests.

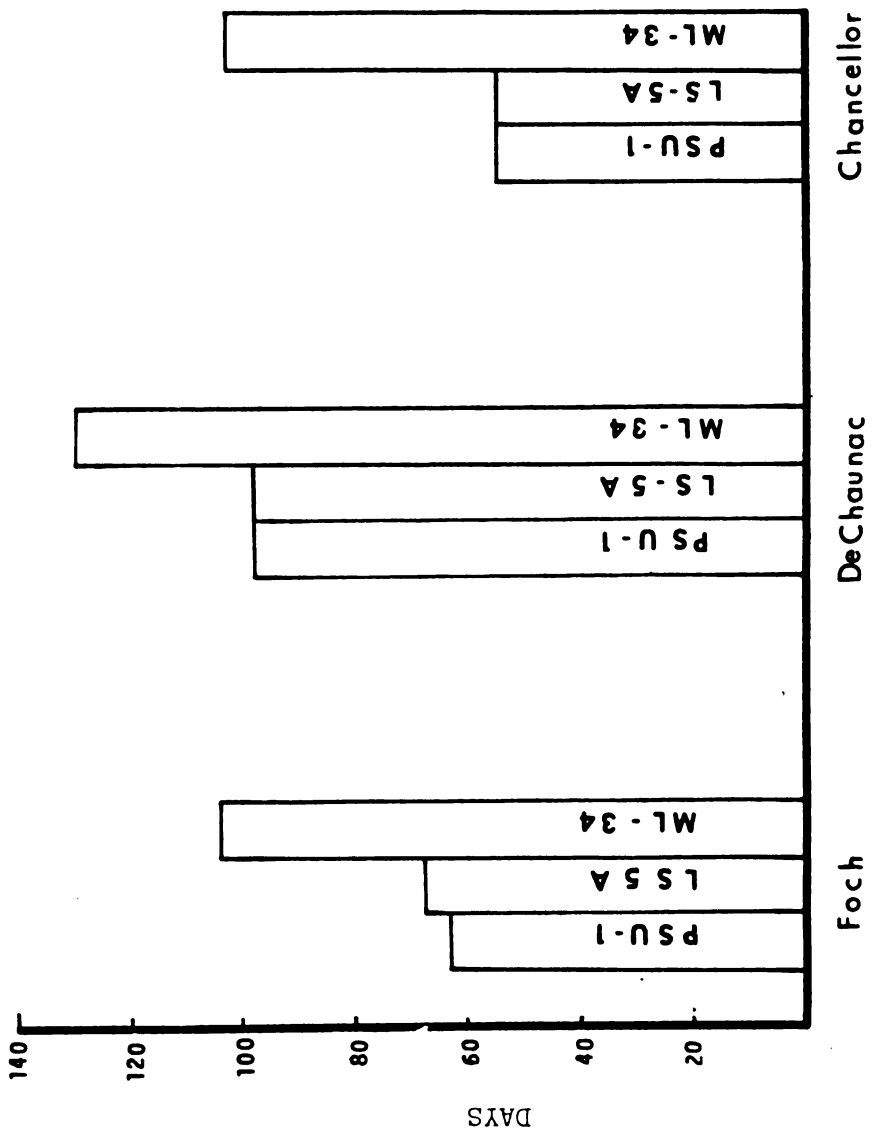


Figure 12. Time in Days required for the strains to complete malolactic fermentation in the different wines.

Table 12. Twenty point grading of wines (Davis) evaluated by twelve judges.

CULTIVAR	MALOLACTIC TREATMENT	SCORE
FOCH	Control	12.80
	ML-34	13.50
	PSU-1	12.80
	LS-5A	11.90
DECHAUNAC	Control	12.70
	ML-34	11.10
	PSU-1	15.30
	LS-5A	13.35
CHANCELLOR	Control	11.00
	ML-34	13.15
	PSU-1	11.95
	LS-5A	13.90

Preference test. This test was done to reveal whether there are any differences among the replicates and whether there are any treatments that are significantly different than the others.

There were five judges in the first evaluation of Foch. The scores revealed that one of the judges could not select the best and the worst wine as determined by the rest four judges, who ranked them with unanimous decision. For example, the wine that the four judges selected as best, the fifth judge selected as the worst and vise versa. Since the test requires trained judges (Amerine, 1976), this judge was rejected for the remaining evaluations. Among the Foch wines those of the LS-5A treatment in the first replicate are significantly better than the others, since the LS-5A treatment in the third replicate is on the lower limit. The controls in the first and the second replicate were significantly worse than the other treatments. As a general observation, LS-5A was ranked as best and all wines treated with malolactic bacteria were significantly better than the controls.

DeChaunac controls in the second and third replicate and ML-34 in the first replicate were significantly worse than the other treatments. The LS-5A and PSU-1 treatments result in a better quality than the control and the ML-34. Both LS-5A and PSU-1 were very good and it was difficult to judge which was the best of the two. There is no doubt that at a significance level of 0.1 these wines would be judged as significantly better than the others.

In Chancellor there was one wine (LS-5A in the second replicate) that was significantly better than all of the others and the overall quality of LS-5A and PSU-1 were better than the control. ML-34

treatment in all replicates ranked significantly worse than the other treatments. These wines had a rubbery, leesy taste which probably was not due to malolactic fermentation, but due to oxidation. The results of this test are shown in Table 13.

Paired comparison test. The preference test revealed the most and the least preferred wines in each replicate. The next step was to examine the consistency of the judges in selecting the most preferred over the least preferred wine. By this test both the wine difference and the consistency of the judges are evaluated. Table 14 shows the results of this test. The fraction 6/6 means that this particular judge selected six correct wines out of six pairs; 5/6 means that 5 correct wines were selected out of six pairs, etc. There were three pairs in each cultivar (three replicates), and this test was done twice for each cultivar.

From Table 14 it can be observed that in DeChaunac 21 correct wines out of 24 were selected, since in Foch and Chancellor 19 out of 24 were selected.

By testing the hypothesis that H_0: The probability of selecting the correct wine is 0.5 and H_1: The probability of selecting the correct wine is more than 0.5, at a significance level of 0.05, there are 16 or more correct selections out of 24 trials required in order to reject the null hypothesis. In this test there were more than sixteen correct selections out of 24 trials (19, 21, 19), so the null hypothesis is rejected in favor of H_1; that means that the judges did not select the best wine by chance, at a significance level of 0.05.

This test can also be applied for evaluating the judges. At a significance level of 0.05, if a judge selects 13 correct or more out

Table 13. Sums of scores of wines subjected to duplicate ranking preference test by 5 judges (Foch) and 4 judges (Chancellor and DeChaunac).

	FOCH			
REPLICATE I	33	24	22	11
REPLICATE II	17	22	30	20
REPLICATE III	31	24	20	15
	DECHAUNAC			
REPLICATE I	22	27	19	14
REPLICATE II	24	23	18	15
REPLICATE III	32	18	15	15
	CHANCELLOR			
REPLICATE I	24	27	16	13
REPLICATE II	21	32	17	11
REPLICATE III	22	28	15	14

Table 14. Sum of the number of the best wines chosen out of 6 pairs (best-worse) of wines of each replicate, by 4 judges in the paired comparison test.

	FOCH	DECHAUNAC	CHANCELLOR	TOTAL
JUDGE 1	5/6	6/6	4/6	15/18
JUDGE 2	5/6	5/6	6/6	16/18
JUDGE 3	5/6	6/6	3/6	14/18
JUDGE 4	4/6	4/6	6/6	14/18
TOTAL	19	21	19	

of 18 wines, it is sufficient to state that the judge is discriminating and acceptable for evaluating wine quality. All judges selected more than 13 (one selected 16, one 15 and two 14 correct wines).

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