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The purposeful induction of malolactic fermentation (MLF) in wines such as Pinot Noir and Chardonnay is an established commercial wine making practice in Oregon. This induction is not always successful, especially with white wines, such as Chardonnay. A study was initiated to examine the compatibility of yeasts commonly used in Oregon winemaking with various strains of malolactic bacteria.

In preliminary and pilot plant scale experiments, the yeast strain found to be most conducive to malolactic fermentation by lactic acid bacteria was Montrachet (Red Star). The malolactic bacterial strains that were best able to complete malolactic fermentation in various wines, fermented by different yeast strains, were the two Oregon commercial strains, ER1A and Ey2d, and the Pinot Noir juice isolate, DAPN85A.

Sensory analysis of aroma by difference from control test was done on Chardonnay wine fermented by 4 different yeast strains and 3 different malolactic bacterial strains. In all cases, there was an overall significant difference in malolactic fermented wine aroma when compared to control wines.

Organic acid analyses by high pressure liquid chromatography (HPLC) and analyses of volatile compounds by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were done on selected Chardonnay wines. Propionic acid was found to diminish in malolactic fermented wines while acetic acid content increased. Isobutanol and

isobutyraldehyde increased significantly in MLF wines, compared to the controls. Chemical analyses of MLF and control wines suggested two possible chemical reactions resulting from the MLF. The first was the reduction of isobutyraldehyde to isobutanol, and the second was the hydrolysis of isobutyl acetate to isobutyraldehyde and acetate. On all GC chromatograms of wines, where MLF had occurred, there was an unidentified peak close to the retention time of isoamyl acetate. This peak was not evident in wines where MLF had not occurred.

Eight compounds were tentatively identified by GC-MS in malolactic fermented wines which were not found in the control wines. These were 4-methyl-3-pentanoic acid, methyl acetate, ethyl hexanoate, hexyl acetate, 1,12-tridecadiene, hexadecanoic acid, and a compound which was tentatively identified as farnesol, or 1,2-benzenedicarboxylic acid. The latter four compounds had identity fits of less than 900 from the mass spectral analysis. Whether any of these eight compounds match the unknown "ML peak" found in the GC chromatograms is unknown.

Roles of Yeast and Lactic Acid Bacteria in Malolactic Fermentation of Wines: A Chemical and Sensory Study

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BCG	Brom cresol green
CFU	Colony forming units
DMF	Dimethyl fumarate
GC	Gas chromatography
GLC	Gas liquid chromatography
GC-MS	Gas chromatography-mass spectroscopy
HPLC	High performance (also, high pressure) liquid chromatography
LA	Lactic acid
LSD	Least Significant Difference
MF	Membrane filtration
mg/l	Milligrams per liter
ML	Malolactic
MLF	Malolactic fermentation
ppm	Parts per million
psig	Pounds per square inch guage measurement
SAS	Statistical Analysis Systems
SD	Standard deviation

Continued ABBREVIATIONS

T.A.	Titratable acidity
μ	Micron (micrometer)
μg	Microgram
μl	Microliter
$\bar{X}$	Mean

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ROLES OF YEAST AND LACTIC ACID BACTERIA IN MALOLACTIC FERMENTATION OF WINES: A CHEMICAL AND SENSORY STUDY

CHAPTER I.
INTRODUCTION

In 1920, Ferre in Burgandy and Ribereau-Gayon in Bordeaux, France demonstrated the enological importance of converting malic acid to lactic acid in wine. This malolactic fermentation (MLF) has been encouraged to decrease the amount of malic acid, to make wine less acidic. In the Pacific Northwest, wine grapes and the juice prepared there from, are notorious for their high malic acid content and low pH. Therefore, natural MLF is encouraged in red and white wines, such as Pinot Noir and Chardonnay, respectively. Induction of MLF by inoculating juice or must with starter cultures of specific strains of LA bacteria also is encouraged (Watson *et al.*, 1984; Watson, 1986.)

MLF has two important effects upon the sensory quality of wine. These are deacidification by decarboxylating L-malic acid to produce L-lactic acid and carbon dioxide, and metabolism of other constituents to cause subtle changes in flavor (Wibowo *et al.*, 1988; Lafon-Lafourcade *et al.*, 1983). These beneficial properties of the MLF have been discussed by Beelman and Gallander (1979) and Davis *et al.* (1985). MLF may provide beneficial effects by causing a reduction of acidity in high acid wines and by flavor modification from endproduct compounds such as acetaldehyde, acetic acid,

ethanol, diacetyl, acetoin, 2,3-butanediol, as well as volatile acids, diethyl succinate, numerous volatile esters, ethyl acetate, n-propanol, 2-butanol, n-hexanol, and ethyl lactate (Davis *et al.*, 1985).

Another perceived effect of the MLF is obtaining bacteriological stability in the wine, once MLF is completed (Lafon-Lafourcade *et al.*, 1983). However, Wibowo *et al.* (1988) have shown that LA bacteria can still grow, even after the MLF has been completed.

Several studies have been initiated in this laboratory to isolate and characterize LA bacteria that are able to induce MLF in wines from grapes grown in cool climate viticultural areas such as the Pacific Northwest. Izuagbe (1982) isolated several strains of *Leuconostoc oenos* from the wines of Pinot Noir, Merlot and Chardonnay made in Oregon wineries. These strains were characterized to confirm their identity and to determine their ability to carry out MLF at various temperatures and pH's, similar to the enological conditions common in Oregon. Volatiles from one of these ML bacterial strains grown in artificial medium were examined. Compounds identified included acetoin, 2-propanol, isobutanol, butanol, isoamyl alcohol, 1-pentanol, and benzaldehyde.

In 1983, Henick-Kling compared some of these Oregon ML bacterial strains to additional isolates from Oregon wineries and evaluated their ML activity, pH and temperature tolerance as well as their sensitivity to SO_2, fumarate and alcohol. Strains performing best under enological conditions in

Oregon were then compared in pilot scale wine production using Pinot Noir, Chardonnay, and Pinot Blanc wines. The two strains that completed MLF the best were ER1a, an isolate from a 1979 Pinot Noir, and EY2d, isolated from a 1978 Merlot. ER1a was more low-pH tolerant while EY2d was more tolerant to cool temperatures.

Dohman (1983) also studied these two ML bacteria along with 16 other bacterial isolates. They were compared for growth on various carbohydrates, over different pH ranges, in varying amounts of ethanol and malate and for MLF activity, SO_2 tolerance, inhibition by fumaric acid and tolerance to freeze drying. The optimal pH range for ER1a and EY2d was between pH 4.5 and 5.8, and the practical limit of MLF was at pH 2.9 to 3.0. Optimal growth temperature was between 28 and 31 ^{o}C. Sulfur dioxide at 30 ppm (mg/l) was sufficient to completely inhibit growth of these strains in artificial medium, while at 20 ppm only minimal inhibition was noted. Ey2d strain was only slightly inhibited in 10% ethanol, but at 12% and 14% this strain was markedly inhibited in both growth and MLF.

Eschelbach (1984) examined bacteriophages that can infect *Leuconostoc oenos.*. Electron micrographs of wine samples which had failed to complete MLF in local wineries showed the presence of phage particles of the type which can infect *L. oenos* . An infective strain of phage from Austria was shown to be able to infect *L. oenos,* ER1a. He found that these phages

are inhibited by 1% ethanol and are non-infectious at 15% ethanol.

Michaels (1985) inoculated the ER1a and EY2d bacterial strains as starter cultures into commercial vats of Chardonnay and Pinot Noir musts in attempt to induce MLF. The fermentation was completed in 56 days by EY2d in a Chardonnay at temperatures of 8 to 13 ^{o}C, while ER1a completed MLF in the same juice in 101 days. In a Pinot Noir at 14^{o}C, ER1a completed MLF in 38 days. Storage methods for these bacterial strains were examined and it was deterrmined that glycerol greatly aided survival in frozen storage.

Extensive literature reviews concerning LA bacteria in the MLF of wines has been carried out by Michaels (1985), Eschelbach (1984), Dohman (1983), Henick-Kling (1983), and especially Izuagbe (1982). The review of literature on this subject offered here will be from 1985 to the present.

Numerous studies have indicated that the metabolic activities of LA bacteria during MLF can lead to changes in the concentration of compounds which affect the sensory quality of wines either by increasing complexity of the wine or by producing off-flavors and aromas (Beelman and Gallander, 1979; Kunkee, 1974; Lafon-Lafourcade and Ribereau-Gayon, 1984; Pilone *et al.*, 1966; Ribereau-Gayon *et al.*, 1975). Also it has been found that MLF is not dependent upon growth of ML bacteria and can be an independent event, once high numbers of cells ($\sim 10^{6}$ CFU/ml) are reached (Wibowo *et al.*, 1988.)

There are numerous reports suggesting that the strain of wine yeast used for primary alcoholic fementation can affect subsequent growth and MLF by LA bacteria, (Fornachon, 1968; Mayer, 1978; Beelman *et al.*, 1982; King and Beelman, 1986; Lemaresquier, 1987.) A portion of the present work deals with this problem, particularly to determine which yeast strains commonly used in Oregon winemaking allow the fastest and most complete MLF, and to match yeasts with specific strains of ML bacteria to optimize MLF. It has been noted that different strains of LA bacteria perform variably in the MLF. Two widely used industrial strains of *Leuconostoc oenos* , PSU-1 and ML-34, also have shown variable performances with different wines and yeasts, (King, 1984; Izuagbe *et al.*,1985.) Both these strains have been tested in our laboratory with different yeast strains in fermenting different types of wines; ML-34 did not do well for either growth or conduction of MLF when the juice, must, or wine pH was below 3.5. However, because these two strains are considered industry standards, they have been included in most of the MLF experiments described in the present study.

When inoculating grape juice, must, or wine with starter cultures of ML bacteria (usually *Leuconostoc oenos*), the best time to inoculate, with respect to the extent of alcoholic fermentation which has taken place, is debatable. In the Pacific Northwest, starter cultures are usually added to the must shortly after yeast inoculation, in order to avoid inhibition of ML bacterial growth by alcohol. Ribereau-Gayon *et al.* (1975), recommended inoculation

with ML bacteria but not before the sugars were completely exhausted. Gallender (1979) however, indicated that the LA bacteria could be added to wine before, during, or after the alcoholic fermentation. In preliminary experiments, described herein, ML bacterial cultures were added to finished wines after completion of alcoholic fermentation, followed by centrifugation and membrane filtration. The latter two steps were carried out to remove competitive microorganisms. In pilot plant scale experiments, the Chardonnay musts were inoculated with ML bacterial cultures two days after yeast inoculation, which is the industrial practice in Oregon.

Numerous studies have indicated that yeasts are the primary source of flavor and aroma in various wines, and that their contributions are influenced by the method(s) and style of winemaking. Flavor is contributed by a large number of different compounds, and most of them are formed by yeast cells during fermentation (Nykanen, 1986). There are also many minor compounds originating in the raw materials which contribute to the quality, flavor and aroma of wines (Nykanen, 1986). This all contributes to the extreme complexity that makes up any composite of flavor and aroma of wine. Parish and Carroll (1987) examined fermentation characteristics of *Saccharomyces cerevisiae* isolates from *Vitis rotundifolia* grapes and musts, and concluded that the yeast strain had a subtle but definite influence on the flavor and aroma of wines.

Millan and Ortega (1988) examined the Crabtree effect where the high

reducing sugar concentration of grape musts represses the oxidative metabolism of the yeast, thereby inducing the decarboxylation of pyruvate to acetaldehyde. Acetaldehyde is then partly excreted into the medium, reduced to ethanol, and oxidized to acetate; these events can affect the flavor and aroma of the resulting wine by increasing the bitterness and acetic acid aroma.

Strains of *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (*bayanus*) were examined by Nykanen (1986) for production of the volatile fatty acid esters. Strains of *S. cerevisiae* were found to produce more isopentyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and phenethyl acetate than strains of *S. uvarum.* These fatty acid and phenolic esters are primary components to the fruity, sweet and winey characters of wine aroma (Drawert and Christoph, 1984; Amerine *et al.*, 1965; Vock, 1981; Ribereau-Gayon, 1978).

In addition to flavor and aroma contributions made by yeasts, the ML bacteria also play a role in this process. Their contribution may either be desirable or undesirable. A desirable contribution is made to most dry, red wines and some white wines by reduction of acidity and contributing to the "complexity" of the wine; an undesirable contribution can be made when wine is spoiled due to an excess production of diacetyl (buttery off-flavor). Also, Edinger and Splittstoesser (1986) determined that *Leuconostoc oenos* strains are able to reduce sorbic acid to sorbic alcohol which is the

established precursor of the geranium off-odor. In view of these facts it was of interest to examine wines produced in this study for types and amounts of flavor compounds. This was done by gas liquid chromatography (GLC), to detect aldehydes, ketones, esters, and higher alcohols; by high performance liquid chromatography (HPLC) to detect organic acids; and by gas chromatography-mass spectrometry (GC-MS) to detect volatiles of all types.

Any study of a food product where flavor and/or aroma components are examined would be incomplete without a sensory evaluation. Therefore a pilot plant scale experiment, with an Oregon Chardonnay, was carried out in order to have sufficient quantity of each type of wine for sensory analyses. Some winemakers have questioned whether or not the MLF truly contributes anything more to the flavor and aroma of wines other than decreasing sourness by deacidification; sensory analyses addressed this question. Therefore, a Difference-From-Control type analysis was carried out to determine if a wine flavor panel could detect differences in aroma in the ML fermented Chardonnay wines when compared to non-MLF control wines.

McDaniel *et al.* (1986), carried out a descriptive analysis of the aroma of Pinot Noir wines femented by several strains of ML bacteria. The bacterial strains compared were ER1a, EY2d, ML-34, PSU-1 and MLT. ML-34 was high in berry notes, but often low in other aromas. MLT and ER1a were high in blackberry, vegetable and butterscotch aroma characters, while ER1a was highest in overall intensity and in intensity for fruity characters. EY2d was

rated the highest of the inoculated wines in spicy characters, while PSU-1 was highest in vegetative intensity. The conclusion of this study was that there was no question that the strain of ML bacteria selected definitely influenced the perceived aroma of Pinot Noir wine.

Cottrell and McLellan (1986) examined the chemical and sensory profiles of several varietal wines, including a Chardonnay and White Reisling, when processed at different fermentation temperatures. Little to no difference was evident in the Chardonnay wines produced at the highest and lowest temperature, but significant differences in the floral character were noted in the White Reisling. The volatiles detected were acetaldehyde, methanol, ethyl acetate, 1-propanol, isobutanol, active amyl alcohol, isoamyl alcohol, and ethyl lactate.

The objectives of the present research were as follows:

1. To develop a medium and methods for selectively isolating ML fermenting bacterial strains from grape juices, musts, and/or partially finished to finished wines.

2. To develop a rapid method to detect heterofermentative ability of ML isolates so as to differentiate between LA cocci of the *Pediococcus* and *Leuconostoc* genera.

3. To submit commercial yeasts being used in Oregon wineries to biochemical tests in order to identify them as *Saccharomyces cerevisiae, Saccharomyces bayanus,* or a wild type yeast.

4. To determine whether or not any commercial yeasts had appreciable MLF activity.

5. To determine which yeast strains best supported MLF with which LA bacterial strains and to identify those bacterial strains best able to complete MLF in the different varietal wines, musts, and juices.

6. To determine which LA bacterial strains best carry out the MLF in Chardonnay wines and contribute, if at all, to the aroma of these wines.

7. To determine whether or not the chemistry of MLF wines differed from that of the control wines, and if so, to identify the chemical changes in these wines other than the conversion of malic acid to lactic acid.

CHAPTER II.
SELECTIVE MEDIUM FOR ISOLATION OF MALOLACTIC BACTERIA

INTRODUCTION

Isolation of bacteria from fermenting must or finished wine is of interest as related to microbial sediment in bottled wine or monitoring product undergoing secondary malolactic fermentation. To maintain and study malolactic bacteria, especially strains of *Leuconostoc oenos*, our laboratory has been using a modified deMan-Rogosa-Sharp medium (deMan *et al.*, 1960) supplemented with commercial V-8 juice (MRS-V8) (Izuagbe, 1982; Rogosa and Sharpe, 1959). Both tomato juice and V-8 juice apparently provide nutritional requirements for the fastidious *Leuconostoc oenos* . A glycosyl derivative of pantothenic acid, found originally in tomato juice, was determined to be the important growth factor, (Amachi,*et al.* 1971; Imamoto *et al.*, 1973.)

In 1959, Luthi and Vetsch showed that malolactic bacterial growth was promoted by the addition of proteose peptone and yeast extract which are used today in MRS medium. These undefined additives provide vitamins, some carbohydrates, proteins and non-protein nitrogen compounds that contribute to growth of many organisms.

The objective of this study was to develop a medium and methods for

selecting and isolating bacterial strains. This medium should inhibit contaminating bacteria and yeast commonly found in wines, must, and juices. Since wine is a rather hostile environment to many microbes due to its alcoholic content, (8-14%), low pH (2.8 - 4.5), and low amounts of SO_2 produced by some wine yeasts, the number and types of bacteria are already somewhat limited.

Vancomycin is an antibiotic effective against the gram positive *Streptococcus* sp. to which *Leuconostoc* sp. have been shown to be resistant (Orberg and Sandine, 1984). Cycloheximide has been used in media to inhibit wine yeast growth, but it often only delays their growth, and over time many yeasts are able to grow on media containing this inhibitor. Preliminary experiments in our laboratory indicated that dimethyl fumarate has some anti-yeast activity. Therefore both cycloheximide and dimethyl fumarate along with vancomycin were tested as additives to a selective medium for isolating malolactic bacteria from wine, musts, and juices.

MATERIALS and METHODS

A. Basal Medium I

Tryptone (Difco)	2.0 %
Peptone (Difco)	0.5 %
Yeast Extract (Difco)	0.5 %
Glucose	0.5 %

Fructose	0.3 %
l-Malic Acid (Sigma)	0.2 %
Tween 80	0.1 %

Dissolve in distilled water. Adjust the pH to 4.5 for broth and to pH 5.5 for agar medium. Add agar to make a 2 % suspension and heat to melt before aliquoting and sterilizing.

B. Basal Medium II

Commercial (Difco) *Lactobacillus* MRS medium was used. No pH adjustment was made with this medium. Agar (2 %) was added and dissolved as done for basal medium I.

C. V-8 Juice

Commercial V-8 juice was centrifuged to remove the solids and filtered twice using Whatman GF/A Glass Microfilters, 11.0 cm. The juice was then autoclaved 15 minutes at 121^{o} C, cooled and added aseptically to either Basal Medium I or II to make a 25 % juice content. This was done before pH adjustment was made.

D. Antibiotics

Cycloheximide (Sigma)

Dissolve in 10 ml of distilled water to make a final concentration of 100 mg/l. Filter-sterilize and aseptically add to the sterile, cooled basal medium and mix thoroughly.

Vancomycin HCl (Sigma)

Dissolve in 10 ml of distilled water to make a final concentration of 50 to 100 mg/l. Filter-sterilize and aseptically add to sterile, cooled basal medium and mix thoroughly. This may be dissolved in the cycloheximide solution above so that both antibiotics may be added simultaneously to the same medium.

EXPERIMENT 1

TESTING FOR POSSIBLE PRESERVATION EFFECT OF TWO COMMERCIAL SOURCES OF V-8 JUICE

Two commercial namebrand V8 juices were purchased: Juice #1 was from a Northwest grocery chain with their own name brand, and juice # 2 was a national brand juice. Both V8 juices were processed identically. They were centrifuged at 10,000 X g for 25 minutes to remove the pulp, filtered twice with Whatman GF/A glass filters, and sterilized by autoclaving for 10 minutes at 121° C.

Each juice then was added aseptically to a final concentration of 25% to melted, sterile MRS agar containing either 100 mg/l cycloheximide or 100 mg/l cycloheximide + 50 mg/l vancomycin HCl. Various strains of *Leuconostoc* sp. and *Pediococcus* sp. were streak-plated onto the four types of agar media and incubated in GasPak (BBL) anaerobic jars for 4 - 7 days at

30° C and examined for growth.

The results may be seen in **Table 2.1**. Neither brand of V8 juice inhibited any of the test strains when used with MRS agar medium. One bacterial winery isolate, SB2-A, which exhibited a staphylococcal-like morphology, was sensitive to vancomycin.

EXPERIMENT 2

METHODS AND MATERIALS

The following media were used:

Medium A - MRS-V8

Medium B - MRS-V8 + 100 ppm cyclohexamide + 50 ppm vancomycin- HCl

Medium C - Difco LB MRS-V8

Medium D - Difco LB MRS-V8 + 100 ppm cyclohexamide

Medium E - Difco LB MRS-V8 + 100 ppm cyclohexamide + 50 PPM vancomycin HCl.

Medium F - Difco LB MRS-V8 + 50 ppm vancomycin HCl.

The organisms tested in the above media included 6 strains of *Leuconostoc oenos* from the OSU stock culture collection and 4 strains of *Pediococcus* sp. isolated from wine.

All cultures were grown in sterile MRS-V8 broth at pH 4.5. Test culture (0.1 ml) was applied to duplicate agar plates for spread plate culturing. This was done for each of the different test media, A - F. Inoculated spread plates

TABLE 2.1. Comparison of two commercial V-8 juices for preservation effect.

BACTERIUM	JUICE 1 MRSV8 ± CH	JUICE 1 MRSV8 +CH+VN	JUICE 2 MRSV8 +CH	JUICE 2 MRSV8 +CH+VN
ER1a	+	+	+	+
EY2d	+	+	+	+
ML34	+	+	+	+
MLTkli	+	+	+	+
LDE	+	+	+	+
E-26	+	+	+	+
DAR-1	+	+	+	+
Leuconostoc sp.	+	+	+	+
SB1-C *	+	+	+	+
SB2-A *	+	-	+	-
P. cerevisiae 992	+	+	+	+
Pediococcus sp. W-3	+	+	+	+
Pediococcus sp. W-8	+	+	+	+
Pediococcus sp. W-10	+	+	+	+
Pediococcus sp. W-11	+	+	+	+

* Winery isolates.

(+) = growth after 7 days. (-) = no growth after 7 days. CH = cycloheximide at 100 mg/l. VN = vancomycin HCL at 50 mg/l.

were incubated in an anaerobic chamber (GasPak) for 3 - 5 days at 25° C.

RESULTS

As seen in **Table 2.2,** good growth was evident on all media by all test strains.

Isolated wine yeast contaminants plus malolactic bacterial cultures were spread plated onto media B, D, and E (those containing 100 ppm cycloheximide) and incubated 3 days at 25° C. In all cases the malolactic bacterial cultures grew and no yeast growth was evident at any time.

EXPERIMENT 3

DIMETHYL FUMARATE VERSUS CYCLOHEXIMIDE AS A YEAST INHIBITOR IN SELECTIVE MEDIA

METHODS AND MATERIALS

MRS-V8 agar plates, pH 5.5, were made up containing the following additives: cycloheximide at final concentrations of 0.1, 1, 10, 50, or 100 mg/l; dimethyl fumarate (DMF) at final concentrations of 0.1, 0.5, 1, 5, 10, 50, or 100 mg/l; 10 % ethanol; and control plates with no additives. DMF is insoluble in water and not very soluble in ethanol. In order to make up a medium containing DMF at 100mg/l it was necessary to dissolve it in 95 % ethanol . As a consequence, agar media containing DMF at 100 mg/l also contained 10 % ethanol. If inhibition was seen in these plates, then it had to be determined if the organisms were inhibited by 10 % ethanol alone.

The test yeast strains included both commercial and wine/grape wild

TABLE 2.2. Effect of various media and antibiotics on growth of wine bacteria.

ORGANISM	MRSV8 TEST MEDIUM-A	MRSV8 ONLY MEDIUM-B	LB MRSV8 C + V MEDIUM-C	LB MRSV8 ONLY MEDIUM -D	LB MRSV8 + C + V MEDIUM-E	LBMRSV8 + V MEDIUM-F
ER1a	+3	+3	+1.5	+1.5	+1.5	+3
EY2d	+4	+4	+4	+4	+3.5	+4
ML-34	+4	+4	+4	+4	+4	+4
MLTkli	+4	+4	+4	+4	+4	+4
LDE	+4	+4	+4	+4	+3	+4
E-26	+4	+4	+4	+4	+4	+4
Pediococcus W-3	+3	+2	+4	+4	+4	+4
Pediococcus W-8	+3	+2	+4	+4	+4	+4
Pediococcus W-10	+4	+4	+4	+4	+4	+4

Above values are the means of paired plates. Growth on the agar plates was estimated as: +1 = slight or weak growth (<20 CFU), +2 = moderate to light growth, +3 = moderately heavy growth, and +4 = too numerous to count or confluent growth. C = cycloheximide at 100 mg/l, V = vancomycin at 50 mg/l

type isolates. See **Table 2.3** .

Each of five agar plates for each test medium/concentration was divided into 4 quadrants. Each yeast strain, pregrown in YM broth (Difco), was streak plated in the appropriate quadrant on the agar plate. All agar plates were incubated at 25^{0} C and were examined daily for evidence of growth for 10 days.

RESULTS:

The results are shown in **Tables 2.4a** and **2.4b.** As can be seen, DMF had little effect on yeast strains at concentrations less than 100 mg/l. When there was an effect at 100 mg/l, there may have been a combined effect from the DMF and 10 % ethanol. DAPN 85, a wild type yeast isolated from crushed Pinot Noir grapes, and the SPN wine isolates showed resistance to cycloheximide and were able to grow in the presence of 10 % ethanol. This suggests that wild type yeast, which can successfully compete with the inoculated strains, may be in the must. When sampled, these wild types will grow in the presence of cycloheximide on selective media and possibly interfere with the isolation of malolactic bacteria.

A SECOND TESTING OF SELECTED YEAST STRAINS AGAINST CYCLOHEXIMIDE AND DIMETHYL FUMERATE:

METHODS and MATERIALS

The same bank of test yeasts were used in a second experiment. All yeasts were first grown in sterile MRS-V8 broth at pH 4.5 for inoculation.

Table 2.3. Yeast strains used for determining yeast inhibition in selective media.

YEAST STRAIN	SOURCE	NOTES
A. Commercial Strains		
Pasteur Champagne	Red Star Co.	UCD 595
Epernay 2	Red Star Co.	
Montrachet	Red Star Co.	UCD 522
K1VIII6	Lalvin	
EC 1118	Lalvin	Pris de Mousse
71B1122	Lalvin	
R-92	J.P. New Zealand	From S. Africa
8A95	J.P. Australia	
10A81	J.P. Australia	
B. Winery Isolates		
DAPN 85 a	PN juice isolate	
DAPN 85 b	"	
DAPN 85 c	"	
DAPN 85 d	"	
DAR 1	"	
SPN 1	PN wine isolate	
SPN 2	"	
HC70-84, a3	PN wine isolate	
HC50-84	Chardonnay isolate	

TABLE 2.4a. Effect of dimethyl fumarate and ethanol concentration on growth of wine yeast.

(Concentration of DMF, mg/l)

YEAST STRAIN	0.1	0.5	1.0	5.0	10	50	100	10% ETOH
K1V1116	1	1	1	1	1	1	7	2
10A81	1	1	1	1	1	2	5	1
Montrachet	1	1	1	1	1	1	7	2
EC1118	1	1	1	1	1	2	7	2
71B1122	1	1	1	1	1	1	-	2
Epernay 2	1	1	1	1	1	1	-	2
8A95	1	1	1	1	1	2	7	2
R-92	1	1	1	1	1	2	-	2
Pasteur Champagne	1	1	1	1	1	2	-	2
DAPN85 a.	1	1	1	1	1	3	-	4
DAPN85 b.	1	1	1	1	1	3	-	10
DAPN85 c.	1	1	1	1	1	3	-	4
DAPN85 d.	1	1	1	1	1	3	-	5
DAR-1	1	1	1	1	1	2	10	2
SPN-1	1	1	1	1	1	3	5	4
SPN-2	1	1	1	1	3	3	-	2
HC70-84,a3	1	1	1	1	1	3	-	3
HC54-84	1	1	1	1	1	3	-	3

Values indicate the day of incubation that growth was evident at each concentration of DMF or 10% ethanol.
(-) = No growth after 10 days of incubation.

TABLE 2.4b. Effect of cycloheximide on growth of wine yeast.

YEAST STRAIN	Concentration of cycloheximide (mg/l) 0.1	1.0	10	50	100	CONTROL
K1V1116	-	-	-	-	-	1
10A81	-	-	-	-	-	1
Montrachet	-	-	-	-	-	1
EC1118	-	-	-	-	-	1
71B1122	7	-	-	-	-	1
Epernay 2	7	-	-	-	-	1
8A95	-	-	-	-	-	1
R-92	10	-	-	-	-	1
Past. Champagne	-	-	-	-	-	1
DAPN85 a	1	1	1	1	2	1
DAPN85 b	1	1	1	1	2	1
DAPN85 c	1	1	1	1	2	1
DAPN85 d	1	1	1	1	2	1
DAR 1	-	-	-	-	-	1
SPN 1	2	2	2	4	4	1
SPN 2	3	3	5	5	10	1
HC70-84,a3	-	-	-	-	-	1
HC54-84	-	-	-	-	-	1

Values indicate incubation day that growth became evident at the respective concentration of cycloheximide or control. (-) = no growth after 10 days incubation.

Multiple MRS-V8 agar plates at pH 5.5 were made containing various additions as follows: cycloheximide at final concentrations of 1, 10, 50, or 100 mg/l; dimethyl fumarate (DMF) at final concentrations of 25, 50, 75, or 100 mg/l; ethanol at 1, 5, or 10 % final concentration; and "controls" with no additions.

Plates were divided into four quadrants and representative yeast types were streaked onto an appropriate quadrant of each test medium and concentration as before.

RESULTS

Results are shown in **Tables 2.4c.** and **2.4d..** For all commercial strains, cycloheximide was inhibitory at all concentrations, while DMF was inhibitory only to the Epernay 2 yeast at 50 mg/l and greater. Of the winery yeast isolates, DAR 3 grew after ten days on one mg/l cycloheximide but was inhibited by DMF at 50 mg/l and above. SPN-1 and SPN-2 (which were isolated from a Pinot Noir wine that had been bottled and later developed microbial sediment) were able to grow at all concentrations of both cycloheximide and DMF. The DAPN85 yeasts (isolated from crushed Pinot Noir juice) were inhibited at 50 mg/l DMF but were also inhibited by 10 % ethanol. These yeasts however were able to grow in 100 mg/l cycloheximide in one day.

It was concluded that, in general, when examining most winery musts and wines for malolactic bacteria that cycloheximide is a better yeast inhibitor and, with the exception of some wild yeast, is most adequate. Also, wild yeast

TABLE 2.4c. Effect of cycloheximide on wine yeast growth.

YEAST STRAIN	Concentration of cycloheximide (mg/l) 1.0	10	50	100	CONTROL
K1V1116	-	-	-	-	1
10A81	-	-	-	-	1
Montrachet	-	-	-	-	1
EC1118	-	-	-	-	1
71B1122	-	-	-	-	1
Epernay 2	-	-	-	-	1
8A95	-	-	-	-	1
R-92	-	-	-	-	1
Pasteur Champagne	-	-	-	-	1
DAPN85 a	1	1	1	1	1
DAPN85 b	1	1	1	1	1
DAPN85 c	1	1	1	1	1
DAPN85 d	1	1	1	1	1
DAR 1	-	-	-	-	1
DAR 2	-	-	-	-	1
DAR 3	10	-	-	-	1
SPN 1	3	3	3	3	3
SPN 2	3	3	3	3	1
HC70-84, a3	-	-	-	-	1
HC54-84	-	-	-	-	1

Values indicate incubation day that growth became evident at the respective concentrations of cycloheximide or control. (-) = no growth after 10 days of incubation.

TABLE 2.4d. Effect of dimethyl fumarate on wine yeast growth.

YEAST STRAIN	(Concentration of dimethyl fumarate, mg/l) 25	50	75	100	(Concentration of ethanol, % v/v) 1%	5%	10%
K1V1116	1	1	3	4	1	1	3
10A81	1	1	3	4	1	1	1
Montrachet	1	1	1	4	1	1	1
EC1118	1	1	3	4	1	1	1
71B1122	1	1	3	4	1	1	1
Epernay 2	1	-	-	-	1	1	3
8A95	1	1	3	10	1	1	3
R-92	1	1	4	-	1	1	1
Pasteur Champagne	1	1	5	-	1	1	1
DAPN85 a.	3	-	-	-	1	3	-
DAPN85 b.	3	-	-	-	1	3	-
DAPN85 c.	3	-	-	-	1	3	-
DAPN 85 d.	3	-	-	-	1	3	-
DAR 1	1	1	3	7	1	1	1
DAR 2	1	1	3	4	1	1	4
DAR 3	1	-	-	-	1	1	1
SPN-1	3	3	3	7	3	3	4
SPN-2	1	3	3	10	1	3	3
HC70-84,a3	1	1	4	7	1	1	3
HC54-84	1	1	4	7	1	1	3

Values indicate incubation day that growth became evident at the respective concentrations of DMF and ethanol.
(-) = no growth after 10 days of incubation.

which grow in the presence of 100 mg/l cycloheximide may be inhibited by 10 % ethanol in the medium.

EXPERIMENT 4

MEMBRANE FILTER METHOD FOR ENUMERATING MALOLACTIC BACTERIA IN WINE AND MUST SAMPLES

INTRODUCTION

Official membrane filter techniques are used routinely by state and federal environmental agencies for examination of waters for microbial content. By using selective agar media, it was thought that perhaps wines, musts, and juices could be diluted, membrane filtered, and malolactic bacteria enumerated directly on the membrane filters. However, since these organisms characteristically produce minute punctiform colonies that are difficult to see, a dye system that would change color as lactic acid was produced would aid visualization. Bromocresol green is a dye with pH color changes within the pH range of the media and that of lactic acid.

METHODS and MATERIALS

MRS-V8 agar, pH 5.5, was prepared with the addition of 1.5 % bromocresol green (BCG) dye indicator, plus the antibiotics previously mentioned. The pH range of this dye indicator is yellow at pH 3.8 and blue at pH 5.4. First the following test strains were streak plated on the above media: ER1a EY2d, ML-34, LDE, MLT-kli, and a Pinot noir must isolate, DAR-4. Then membrane filters of the above organisms were placed on the above media

and incubated anaerobically (GasPak) for 10 days at 30° C and observed on day 4 and day 10.

MRS-V8 agar medium with 1.5 % BCG was buffered at different pH levels inoculated with the same bank of lactic acid bacterial strains cited above: The pH levels were 3.99, 4.53, 5.01, 5.51, and 5.99.

The following organisms were grown on MRS-V8 broth, pH 4.5 for inoculation and membrane filter (MF) trials: ER1a, EY2d, ML-34, MLT-kli, LDE, and *Pediococcus* sp. W-10. Using citrate-phosphate buffer, pH 5.75, each organism was diluted to 10^{-3} and 10^{-4}.

Using sterile Millipore Hydrosol stainless steel filter funnels, 10 to 100 ml of each dilution were filtered through sterile Millipore HAWG, 0.45 μ porosity membrane filters. The filters were then placed on the surface of medium A, B, D, or E as described earlier, and incubated for three to five days at 30° C in anaerobic GasPak jars.

RESULTS

All organisms exhibited growth as minute, clear, entire colonies on the membrane filters and were difficult to see without the use of a binocular dissecting microscope. Dye uptake could be seen only after incubation for ten days or more. There was good growth on all media by day 4, but dye uptake by the colonies was not seen until day 10. In some cases where enough lactic acid had been produced, the immediate surrounding of the colonies changed color to a greenish color, denoting the pH change. All strains grew at all pH values but dye color changes were not significant and

no uptake of the BCG dye was observed in four days.

DISCUSSION

The results of the above experiments indicate the following:

1. The two commercial V-8 juices used for our selective medium did not have an inhibitory effect on test strains of malolactic bacteria.

2. The MRS-V8 medium, either with or without antibiotics, supported growth of all our test strains including winery isolates from wines, musts, and juices from crushed grapes. The commercial medium, Lactobacilli MRS Broth (DIFCO), is a buffered medium with a final pH of 6.5. This allows good growth of *Lactobacillus* sp. However, in the Pacific Northwest, the wine grapes, resulting juices and wines are usually at pH levels too low to support growth of lactobacilli and therefore, a non-commercial MRS medium with a pH of 3.5 to 4.5 is preferred for broth media and pH 5.5 for agar media.

3. Dimethyl fumarate as a yeast inhibitor at 100 mg/l concentration is ineffective for 5 out of 9 commercial strains of wine yeast but is more effective against wild yeast isolated from juices, musts, and wine sediments. DMF at 50 mg/l or less was not an effective yeast inhibitor. A major problem with dimethyl fumarate is that it is insoluble in water and has low solubility in ethanol. Our medium containing 100 mg/l DMF, also contained 10 % ethanol which may have worked synergistically with the DMF where yeast growth was evident at that concentration.

Cycloheximide at 1.0 mg/l concentration is a much better yeast

inhibitor for commercial strains of wine yeast. However, even at 100 mg/l cycloheximide, 6 out of 9 wild type yeast isolates were not inhibited. This suggests that there may be yeast overgrowth problems if wild type yeasts are predominant in crushed juice or musts. Since some of these wild types are inhibited by 10 % ethanol, it may be necessary to add 10 % ethanol to the selective medium.

5. Membrane filtering of diluted wine, musts, or juice on 0.45 μ membrane filters, which are then placed on selective agar medium, does work for enumerating numbers of colony forming units (CFU) per ml. However, after 4 days of anaerobic incubation at 30° C countable punctiform colonies are produced and visualized only with the aid of a dissecting microscope. The incorporation of bromocresol green dye indicator into the selective medium did not aid visualization of the colored colonies or cause medium color change until an extended period of incubation time, i.e. 10 days or more. The membrane filter technique may be subject to less error in enumeration than the spread plate method, but there seems little advantage to this method over the spread plate technique in terms of amount of preparation, incubation time and effort.

In conclusion, the suggested selective medium for isolating malolactic bacteria found in wines, musts, or crushed grape juices should contain the following: centrifuged and filtered V-8 juice or tomato juice to give a concentration of 25%, pH adjusted to 3.5 to 5.5 (depending on whether the

medium is a broth or an agar), the addition of the antibiotics cycloheximide and vancomycin to give the final concentrations of 50 to 100 ppm each, and an ethanol content of 5 to 10% to inhibit wild yeast and the bacteriophage of *Leuconostoc oenos* (Dohman, 1983).

CHAPTER III.
STUDY OF HETEROFERMENTATIVE WINE LACTIC ACID BACTERIA

INTRODUCTION

A large number of Gram (+), coccal type lactic acid bacterial isolates had been obtained from Oregon wineries and it was desirable, as a preliminary step, to determine if each of these isolates was homofermentative, such as *Pediococcus* sp., or heteroferementative as are *Leuconostoc* sp. The difference between these two types of fermentation is the pathway by which glucose is metabolized to form lactic acid. For the homofermentative microorganisms, glucose is degraded by the Embden-Meyerhof pathway to pyruvate which is reduced to lactate by lactate dehydrogenase. In the heterofermentative pathway glucose is degraded much the same way as the oxidative pentose phosphate cycle, where 6-phospho-gluconate is oxidized to ribulose-5- phosphate with CO_2 given off (Gottschalk,1980). This CO_2 is the component used to easily identify and differentiate heterofermentative organisms from homofermentative organisms.

The objective of this study was to develop a quick, reliable, consistent and easy means to determine heterofermentation by LA wine bacteria to help differentiate between *Pediococcus sp.* and *Leuconostoc* sp.

Study # 1

METHODS and MATERIALS

The test media used included the following:

Medium A

MRS-V8 broth, pH 5.5 (minus malate)

Medium B.

Modified Elliker's broth (Dr. D.B. Weddle, personal communication.)

2 % tryptone

0.5% yeast extract

0.25 % gelatin

0.50 % dextrose

0.50 % lactose

0.40 % sodium chloride

0.15 % sodium acetate

0.05 % ascorbic acid

Mix, aliquot and sterilize 15 minutes at 121^{o} C.

Medium C

Commercial Elliker's Medium (DIFCO). Same as above except that 0.5 % saccharose was added and the pH was adjusted to 6.8.

Medium D

Gibson's medium (Harrigan and Cance, 1976)

Ten ml sterile tubes of the above medium were prepared with an inverted Durham tube in each. Test strains were grown in MRS-V8 broth (complete) and inoculated into the above medium. After inoculation, melted vaspar (1:1, vaseline/parafin) was layered on top to seal the tubes. All tubes were incubated at 30^{o} for 5 to 10 days. Various test strains of known homofermentative and heterofermentative malolactic bacteria were used .

RESULTS

Results can be seen in **Table 3.1.** Bacterial strains, ER1a, EY2d, ML-34, MLT-kli, and LDE, are heterofermentative *Leuconostoc* and E-26, Ped. W-11, and *Pediococcus cerevisiae* # 992 are homofermentative pediococci. All heterofermentative test strains produced observable gas by day 5 in the MRS-V8 broth while the homofermentative pediococci did not. In both Elliker's media, the test strains did not show gas production. Gibson's medium did support gas production by the heterofermentative strains but to a lessor degree than the MRS-V8 medium.

Study 2

INTRODUCTION

Because MRS-V8 medium is an undefined medium, it was of interest to determine what components may contribute the carbohydrate component for heterofermentative gas production other than the added glucose. Therefore MRS-V8 broth, pH 5.5 was used as the base medium, and various additions or deletions were made and tested with various bacterial test

TABLE 3.1. Heterofermentation test media for malolactic bacteria.

(growth/gas production)

TEST ORGANISM	GIBSON'S MEDIUM	MRSV8 BROTH	MODIFIED ELLIKER'S BROTH	COMMERCIAL ELLIKER'S BROTH
EY2d	ND	(lost)	+/-	+/-
ER1a	+/+	+/+	ND	ND
ML-34	ND	+/+	+/-	+/-
MLTkli	ND	+/+	+/-	+/-
LDE	ND	+/+	+/-	+/-
E-26	ND	+/-	+/-	+/-
Pediococcus W-11	+/-	+/-	+/-	+/-
P. cerevisiae 992	+/-	+/-	ND	ND

Five day incubation at 30^{o} C. Strain E-26, from OSU culture collection, is a *Pediococcus* sp. (ND) = Not done.

strains.

METHODS and MATERIALS

The media used were as follows:

Medium A: MRS-V8 (no malate, no glucose) contains 0.2 % fructose.

Medium B: MRS-V8 (no malate, no glucose, no fructose)

Medium C: MRS-V8 (no malate, no fructose) contains 0.5 % glucose.

The test organisms are all heterofermentative *Leuconostoc* sp. and were first grown in complete MRS-V8 broth, ph 5.5 which was used as an inoculum into the test tubes of the above media. All tubes contained inverted Durham tubes and were inoculated and vaspar plugged as before. Incubation was for three days at 30^{o} C.

RESULTS

Results are shown in **Table 3.2.** Growth was obtained in all tubes but gas was only evident in tubes of Medium C where glucose was present. Fructose did support gas production by 3 of the 4 test strains. This could possibly have occurred by the phosphorylation of fructose to fructose-6-phosphate which could be isomerized to glucose-6-phosphate, and then fed directly into the heterofermentative pathway with release of CO_2.

Gas production by the winery strain, HC70-84, C-1 was evident in

TABLE 3.2. Effect of medium composition on growth and gas production by malolactic bacteria.

	(growth/gas production)		
TEST ORGANISM	MEDIUM A	MEDIUM B	MEDIUM C
ER1a	+/+	+/-	+/+
PSU-1	+/+	+/-	+/+
ML-34	+/-	+/-	+/-
HC70-84,C-1 *	+/+	+/+	+/+

* Winery isolate.

Medium A: MRSV8 medium with no malate or glucose, but contains 0.2% fructose. Medium B: MRSV8 medium with no malate, glucose, nor fructose. Medium C: MRSV8 medium with no malate or fructose, but contains 0.5% glucose.

medium B, by the space between the vaspar plug and the liquid medium (but not in the Durham tube.) This indicated that there was a small amount of carbohydrate in the yeast extract component of the medium.

Study # 3

INTRODUCTION

In this study, yeast extract was indirectly tested for contributing carbohydrates to the media in which heterofermentation was being tested.

MATERIALS and METHODS

Medium A: MRS-V8 (no malate, no sugars, no yeast extract)

Medium B: MRS-V8 (no malate, no sugars) contains 0.5 % yeast extract.

Medium C: MRS-V8 (no malate, no fructose, no yeast extract) contained 0.5 % glucose.

All tubes contained inverted tubes and were vaspar plugged upon inoculation as before. Incubation was for five days at 30^{o} C. The test strains included two *Leuconostoc* and two *Pediococcus* strains.

RESULTS

As seen from the results in **Table 3.3**, the two heterofermentative strains produced gas in all test media and the homofermentative pediococci did not. Medium C gave the best gas production indicated that the V-8 juice itself probably contributed fermentable carbohydrates. However, both homo- and hetero-fermentative organisms gave the expected responses in the above media.

Study # 4

TABLE 3.3. Effect of yeast extract on growth and gas production in heterofermentation by malolactic bacteria.

	(growth/gas production)		
BACTERIAL TEST STRAIN	MEDIUM A	MEDIUM B	MEDIUM C
ER1a	+/+	+/+	+/+
HC70-84,C1 *	+/+	+/+	+/+
P. cerevisiae 992	+/-	+/-	+/-
Pediococcus W-10	+/-	+/-	+/-

* Winery isolate of *Leuconostoc* sp.

Medium A: MRSV8 medium with no malate, sugars, nor yeast extract. Medium B: MRSV8 medium with no malate, nor sugars, but contains 0.5% yeast extract. Medium C: MRSV8 medium with no malate, fructose, nor yeast extract, but contains 0.5% glucose.

INTRODUCTION

In this last study, a number of commercial strains of *Leuconostoc* sp., OSU culture collection strains, and some winery isolates were tested for heterofermentation using the MRS-V8 medium which contained 0.5 % glucose but no malate, fructose, or yeast extract. The inoculations, treatments and incubations were as before.

RESULTS

The results as seen in **Table 3.4** show that heterofermentative and homofermentative strains gave the expected results in terms of gas production. This medium then became the test medium of choice for determining heterofermentation in winery bacterial isolates.

In conclusion, it is probable that yeast extract and V-8 juice both contributed some fermentable carbohydrate in MRS-V8 medium. However, the homofermentative and heterofermentative test strains all gave expected responses. Gas production from other sources than hexoses by homofermentative strains did not occur, i.e., no false positives. Therefore the recommendation for carrying out heterofermentation studies of wine malolactic bacteria is to use MRS-V8 broth with 0.5 % glucose, but with the deletion of fructose, yeast extract and malate from the original formula. Tubes should be vaspar plugged after inoculation with the test strain and incubated at 30 ^{o}C for 3 to 7 days. Gas production may be indicated either by bubbles trapped in the inverted Durham tubes and/or a gas space between the liquid medium and the vaspar plug.

TABLE 3.4. Effect of MRSV8 broth without malate, sugars, and yeast extract on growth and gas production by malolactic bacteria.

TEST STRAIN	GROWTH/GAS
ER1a	+/+
EY2d	+/+
ML-34	+/+
MLTkli	+/+
LDE	+/+
DAPN85 A	+/+
SB18	+/+
Pediococcus W-3	+/-
Pediococcus W-8	+/-
Pediococcus W-10	+/-
Pediococcus W-11	+/-
Lactobacillus casei 7469	+/-
Leuconostoc sp.	+/+
P. cerevisiae 10791	+/-

CHAPTER IV.
BIOCHEMICAL CHARACTERIZATION OF YEAST STRAINS

INTRODUCTION

Because many of the following studies use a variety of commercial and winery isolate yeast strains, the objective of this study was to differentiate commercial and winery isolate yeast strains by biochemical profile testing using API 20C and API YEAST IDENT test systems, (Analytab Products, Plainview, N.Y.). Yeast isolates could then be identified as *Saccharomyces cerevisiae, Saccharomyces bayanus,* or a wild type yeast, and possibly be matched with known commercial strains.

METHODS AND MATERIALS

For both API 20C and API YEAST IDENT tests, all cultures were first grown on YM agar (Difco) for 48 hours at room temperature before inoculation into the API systems. All other procedures were carried out according to the API brochure.

RESULTS

The results of the API-20C tests are seen in **Tables 4.1a** and **4.1b** Strains of *Saccharomyces cerevisiae* are differentiated from *Sacrcharomyces bayanus* primarily by the utilization of galactose by *S. cerevisiae*.. The winery isolates THPN85, SPN 1 & SPN 2, and HC70-84,a3 followed patterns of *S. cerevisiae* while the DAR group and HC54-84 followed the *S. bayanus* pattern.

TABLE 4.1a. Test results of API 20C characterization of yeast strains by carbohydrate utilization.

YEAST STRAIN	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	KNOWN STRAIN
Commercial Strains											
Pasteur Champ.	+	-	-	-	-	-	-	-	-	-	*S. bayanus*
Montrachet	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
Epernay 2	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
EC1118	+	-	-	-	-	-	-	-	-	-	*S. bayanus*
71B1122	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
K1V1116	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
10A81	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
8A95	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
R-92	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
Isolates											
UG-5	+	-	-	-	-	-	-	+	-	-	*S. cerevisiae*
THPN85	+	-	-	-	-	-	-	+	-	-	
DAPN85 a.	+	-	+	-	-	-	-	-	-	-	
DAPN85 b.	+	-	+	-	-	-	-	-	-	-	
DAPN85 c.	+	-	+	-	-	-	-	-	-	-	
DAPN85 d.	+	-	+	-	-	-	-	-	-	-	
DAR 1	+	-	-	-	-	-	-	-	-	-	
DAR 2	+	-	-	-	-	-	-	-	-	-	
DAR 3	+	-	-	-	-	-	-	-	-	-	
SPN-1	+	-	-	-	-	-	-	+	-	-	
SPN-2	+	-	-	-	-	-	-	+	-	-	
SB10	+	+	+	-	+	+	+	+	-	+	
SB16	+	+	+	-	+	+	+	+	-	+	
HC54-84	+	-	-	-	-	-	-	-	-	-	
HC70-84,a3	-	-	-	-	-	-	-	+	-	-	

(+) = growth, (-) = no growth. Key: GLU = glucose, GLY = glycerol, 2KG = 2-keto-D-gluconate, ARA = arabinose, XYL = xylose, ADO = adonitol, XLT = Xylitol, GAL = galactose, INO = inositol, SOR = sorbitol.

TABLE 4.1b. Continued test results of API 20C characterization of wine yeasts by carbohydrate utilization.

YEAST STRAIN	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	
Commercial Strains										
Pasteur Champagne	-	-	-	-	+	+	-	-	+	*S. bayanus*
Montrachet	-	-	-	-	+	+	+/-*	-	+	*S. cerevisiae*
Epernay 2	-	-	-	-	+	+	-	-	+	*S. cerevisiae*
EC1118	-	-	-	-	+	+	-	-	+	*S. bayanus*
71B1122	-	-	-	-	+	+	-	+	+	*S. cerevisiae*
K1V1116	-	-	-	-	+	+	-	-	+	*S. cerevisiae*
10A81	-	-	-	-	+	+	-	-	+	*S. cerevisiae*
8A95	-	-	-	-	+	+	-	-	+	*S.cerevisiae*
R-92	-	-	-	-	+	+	-	-	-	*S. cerevisiae*
Isolates										
UG5	-	-	-	-	+	+	+	+	+	*S. cerevisiae*
THPN85	-	-	-	-	+	+	-	-	+	
DAPN85 a	-	-	+	-	-	-	-	-	-	
DAPN85 b	-	-	+	-	-	-	-	-	-	
DAPN85 c	-	-	+	-	-	-	-	-	-	
DAPN85 d	-	-	+	-	-	-	-	-	-	
DAR-1	-	-	-	-	+	+	-	-	+	
DAR-2	-	-	-	-	+	+	-	-	+	
DAR-3	-	-	-	-	+	+	-	-	+	
SPN-1	-	-	-	-	+	+	-	-	+	
SPN-2	-	-	-	-	+	+	-	-	-	
SB10	-	+	-	-	+	+	+	+	-	
SB16	-	+	-	-	+	+	+	+	-	
HC54-84	-	-	-	-	+	+	-	-	+	
HC70-84,a3	-	-	-	-	+	+	-	-	+	

* Negative at 72 hours, but positive after 5 days.

KEY: MDG = methyl-D-glucoside, NAG = N-acetyl-D-glucosamine, CEL = Cellobiose, LAC = lactose, MAL = maltose, SAC = sucrose, TRE = trehalose, MLZ = melezitose, RAF = raffinose.

The DAPN85 group, isolated from Pinot Noir grapes, followed the pattern of *S. bayanus* but differed from commercial strains of *S. bayanus* by being negative for maltose, sucrose, and raffinose.

Identical patterns were exhibited by SB10, isolated from a fermenting White Reisling at a commercial winery, and SB16, isolated from a fermenting Pinot Noir at the same winery. This pattern followed quite precisely the pattern given by API for *Candida albicans* in the 20C series, and would have to be considered a wild yeast of the must.

The *S. cerevisiae* strain UG-5 is a research strain and was a gift from Dr. Paul Ritzenthaler of Institute for Applied Science at Toulouse, France. This strain was suspected of producing steroid-like substances that interfere with malolactic fermentation by LA bacteria. The pattern for this strain is identical to 71B1122 (Lalvin) in both API series.

The API 20C system did not make a distinction between the different strains of *S. cerevisiae* or between strains of *S. bayanus*; therefore additional tests were carried out on selected yeast strains using the API YEAST IDENT system.

Results for the API YEAST IDENT series may be seen in Tables **4.2a** and **4.2b.** This system allows differentiation between many strains of the same species of yeasts. Pasteur Champagne and Montrachet strains were differentiated by beta-fucosidase reactions and by testing for aminopeptidases of isoleucine and tyrosine. The phosphatase reaction

TABLE 4.2a. API YEAST-IDENT characterization of wine yeasts.

TEST YEAST	URE	PHS	BDF	BDG	NGL	ADG	BDX	PNA	INA	GLY
(Commercial Strains)										
K1V1116	-	+	-	-	-	-	-	-	-	+
10A81	-	-	-	-	-	-	-	-	-	+
8A95	-	+	-	-	-	-	-	-	-	+
R-92	-	+	-	-	-	-	-	-	-	+
Pas. Champ.	-	+	-	-	-	-	-	-	-	+
Montrachet	-	+	+	-	-	-	-	-	-	+
Epernay 2	-	-	-	-	-	-	-	-	-	+
EC1118	-	-	-	-	-	-	-	-	-	+
71B1122	-	-	-	-	-	-	-	-	-	+
(Winery Isolates)										
UG-5	-	-	-	-	-	-	-	-	-	+
SPN-1	-	-	-	-	-	-	-	-	-	+
SPN-2	-	-	-	-	-	-	-	-	-	-
SB10	-	+	+	-	-	-	-	+	+	-
SB16	-	+	+	-	-	+	-	+	-	-
DAPN85 A	-	+	-	-	-	-	+	-	+	-
DAPN85 B	-	-	-	-	-	-	-	-	-	-
DAPN85 C	-	-	-	-	-	-	-	-	-	-
DAPN85 D	-	-	-	-	-	-	-	-	-	-
DAR-1	-	+	-	-	-	-	-	-	-	+
DAR-2	-	-	-	-	-	-	-	-	-	-
DAR-3	-	-	-	-	-	-	-	-	-	-
THPN85	-	-	-	-	-	-	-	-	-	-
HC54-84	-	-	-	-	-	-	-	-	-	+
HC70-84,A3	-	-	-	-	-	-	-	-	-	+

Key: URE = urease, PHS = phosphatase, BDF = beta fucosidase, BDG = beta glucosidase, NGL = beta galactosaminidase, ADG = alpha glucosidase, BDX = beta xylosidase, PNA = proline aminopeptidase, INA = C2 esterase, GLY = glucine aminopeptidase. (+) or (-) indicate the presence or absence of the test enzyme.

TABLE 4.2b. Continued API YEAST-IDENT characterization of wine yeasts.

TEST YEAST	PRO	TRP	HPR	ILU	VAL	LUG	HIS	CYS	TYR	GLG
(Commercial Strains)										
K1V1116	-	+	-	-	+	+	+	+	+	+
10A81	-	+	-	-	+	+	+	+	+	+
8A95	-	+	-	-	+	+	+	+	+	+
R-92	-	+	-	-	+	+	+	+	+	-
Pas. Champ.	-	+	-	-	+	+	+	+	-	+
Montrachet	-	+	-	+	+	+	+	+	+	+
Epernay 2	-	+	-	-	+	+	+	+	+	+
EC1118	-	+	-	-	+	+	+	+	-	+
71B1122	-	+	-	-	+	+	+	+	-	-
(Winery Strains)										
UG-5	-	+	-	-	+	+	+	+	-	-
SPN-1	-	+	-	-	-	-	+	+	-	+
SPN-2	-	+	-	-	-	-	+	-	-	-
SB10	+	+	+	-	+	-	+	-	-	-
SB16	+	+	+	-	+	-	+	+	-	-
DAPN85 A	-	+	-	+	+	-	+	-	-	-
DAPN85 B	-	+	-	-	+	-	-	-	-	-
DAPN85 C	-	+	-	-	+	-	-	-	-	-
DAPN85 D	-	+	-	-	-	-	-	-	-	-
DAR-1	-	+	-	-	-	-	+	+	-	-
DAR-2	-	+	-	-	-	-	+	-	-	-
DAR-3	-	+	-	-	-	-	+	-	-	-
THPN85	-	+	-	-	+	-	+	-	-	-
HC54-84	-	+	-	-	+	+	+	+	-	+
HC70-84,A3	-	+	-	-	-	+	+	+	-	+

Key: For the above amino peptidases, PRO = proline, TRP = tryptophane, HPR = hydroxyproline, ILU = isoleucine, CYS = cystine, TYR = tyrosine, GLG = glycyl-glycine. (+) or (-) indicate the presence or absence of the test enzyme.

differentiated these strains from other commercial strains in the test group. The yeast strains Epernay 2, EC1118, and 71B1122 were separated by the tests for the aminopeptidases of tyrosine and glycyl-glycine. The winery isolate SPN1 most closely associates with EC1118, but was negative for the aminopeptidases of valine and leucyl-glycine.

Remaining winery isolates all differed from commercial strains by being negative for leucyl-glycine, valine, histidine and/or cystine aminopeptidases, while all comercial strains were positive for these tests.

In the API YEAST IDENT system, SPN-2 and the DAR group seem to be identical, except that DAR-1 differs from DAR-2 and -3 b in the phosphatase and glycine aminopeptidase tests. However, in the API 20C series, SPN-1 and -2 are positive for galactose and the DAR group strains are not. It is most likely that DAR-2 and DAR-3 are the same yeast.

Winery isolates HC54-84, DAR-2 and DAR-3 gave patterns identical to EC1118 (Prise de Mousse). The one difference was that DAR-2 and -3 were negative for glycine aminopeptidase whereas HC54-84 and EC1118 were positive.

Pasteur Champagne (*S. bayanus*) differed from EC1118 (*S. bayanus*) only in the phosphatase test of the API YEAST IDENT series. Both 10A81 and Epernay 2 gave identical patterns in both systems and were not separated by use of the two API test series.

DISCUSSION

The API-20C, carbohydrate utilization series perhaps is best for identifying a wine yeast strain as *S. cerevisiae* or S. *bayanus* or an unknown wild type. The biochemical profile that best differentiates between strains is the API YEAST IDENT system which is based on the ability to hydrolyze various test components. Even these profiles are not absolute, for there is evidence that overlapping between recognized strains exists, *e.g.* the Australian 10A81 strain and Red Star's Epernay 2. In general, however, most of these strains can be separated and tentatively identified for recognition as needed by research investigators. A better method may have been that of Tredoux *et al.*, (1987), where extracted fatty acids are made into ethyl esters and then examined by gas liquid chromatography and results are compared to known yeast strains.

CHAPTER V.
SCREENING YEASTS FOR MALOLACTIC ACTIVITY

INTRODUCTION

Twenty years ago, Rankine (1966) evaluated the decomposition of L-malic acid by different wine yeasts. He found that *Schizosaccharomyces* strains were able to metabolize all the malic acid with no detection of lactic acid. Malate degradation therefore occurred by some means other than the malolactic fermentation. Some *Saccharomyces* strains could metabolize up to 45% of the L-malic acid. However, these strains also produced much H_2S during fermentation.

Lalvin yeast strain 71B1122 is advertised as having malolactic activity. The objective of this study then is to screen a number of yeast strains and determine if malolactic activity occurred to any appreciable amount.

MATERIALS and METHODS

The following yeast strains were used:

R-92: From South Africa, and is used in New Zealand.

8A95: From Australia.

10A81: From Australia. (The above three strains complements of Mr. John Paul, McMinneville, Oregon.)

71B1122: Lalvin, Lallemand Inc, Montreal, Quebec, Canada.

Pasteur Champagne: Red Star, Milwaukee, Wisconsin, U.S.A.

Each yeast strain was allowed to grow 48 hours in YM Broth (Difco) on a shaker and at room temperature before used as an inoculum.

The frozen grape juice was a Gewurtztraminer juice donated by Mr. Barney Watson, Oregon State University, Corvallis, Oregon. The juice was rapidly thawed and divided into four equal treatment lots as follows:

A. Negative Control: No treatment and remained uninoculated and refrigerated until the test time.

B. Positive Control: Juice was untreated but inoculated with test yeast strains.

C. Autoclaved Juice for 15 minutes at 121^{o} C.. This was inoculated with test yeast strains.

D. Juice steamed for 30 minutes, cooled and inoculated with test yeast strains. Each lot was aliquoted to 9 ml per sterile tube with caps.

Malolactic fermentation was followed by paper chromatography according to the methods of Kunkee (1968) and Rankine (1969). Paper chromatographs were done with all juice lots before inoculation to establish a base chromatograph. Standards of both malic acid and lactic acid, each at a concentration of 100 ppm were spotted on all chromatographs at 1, 2, and 4 µl concentrations. The 2 µl juice spot corresponded in intensity to the 1 µl spot of malic acid standard. Upon inoculation, paper chromatography was followed every 2 to 3 days to monitor the cultures over a 20-day period.

RESULTS

Results can be seen in **Table 5.1.** An arbitrary value system of 1 to 10 was given to the lactic acid spot site with 10 = intense spot with no spot evident at the malic acid site, and 1 = intense malic acid spot with a very slight spot at the lactic acid site. A small amount of succinic acid, which is produced in yeast metabolism, also appeared at the lactic acid site.

After 20 days, in all cases, only a very light yellow spot could be seen at the lactic/succinic acid site. Of these, the yeast strain, R-92 in the positive control wine provided a spot of at least a +3 value. In all cases there was not an observable decrease in the malic acid spot. The very light yellow spots at the lactic acid site were probably succinic acid or very small amounts of lactic acid.

Quantitative enzymatic analyses or HPLC analyses of malic acid and lactic acid would have been preferred, especially if there were any indication by paper chromatography of malolactic fermentation activity. At least in Gewurtztraminer juice there did not seem to be any evidence of malolactic activity by the yeast strains tested, as seen by the paper chromatography method. It was assumed then that these strains probably do not contribute substantially to malolactic fermentation and subsequent deacidification of juice, must, or wine.

TABLE 5.1. Possible malolactic fermentation by selected yeast strains in Gewurtztraminer juice.

			(JUICE TREATMENT)	
YEAST STRAIN	A. NEGATIVE CONTROL	B. POSITIVE CONTROL	C. AUTOCLAVED	D. STEAMED
R-92	-	+3	+1	+2
8A95	-	+1	+1	+2
10A81	-	+1	+1	+1
71B1122	-	+1	+1	+1
Pasteur Champagne	-	+1	+1	+2

Values represent the approximate degree of malolactic fermentation as measured by paper chromatography on an arbitrary scale of 1 to 10: (-) = no MLF represented by no lactic acid spot; 1 = very weak lactic acid spot; 5 = equal intensity of the lactic acid and malic acid spots; 10 = most intense lactic acid spot with no trace of the malic acid spot

CHAPTER VI.
EVALUATION OF COMBINATIONS OF YEASTS AND LACTIC ACID BACTERIA IN MALOLACTIC FERMENTATION IN VARIOUS WINES OF *VITIS VINIFERA*

INTRODUCTION

The objectives of this study include the following: (1) to determine which yeast strains best support various malolactic bacterial strain(s) in terms of successfully carrying out the malolactic fermentation; (2) to determine which strain(s) of lactic acid bacteria undergo the most rapid and complete MLF; and (3) to determine if the chemical difference between the control and MLF wines, other than the conversion of malic acid to lactic acid, can be detected by volatile analysis (done by GLC) and organic acid analysis (done by HPLC).

Juices used included Pinot Noir, Chardonnay, Sauvignon Blanc, and White Reisling. Some of these juices are not commonly used in malolactic fermentation but were used in this study because of the availability of the juice for the study, and because they often present problems for the malolactic fermentation to proceed. The yeast strains used in these studies were from commercial sources; private individuals in the winemaking industry; winery isolates from juices, musts, and wines; and non-commercial sources of juices, musts, wines, and crushed grapes grown on the private property of the author. The latter have not been in a commercial winery

where contamination from inoculated commercial malolactic strains abound.

The malolactic bacterial strains are mostly *Leuconostoc oenos* and *Pediococcus* sp. and sources included the O.S.U. culture collection, commercial sources, and winery isolates from crushed grapes, juice, musts, and wines.

The juices were divided into several lots each of which was inoculated with a specific wine yeast. Primary fermentation was allowed to proceed to near completion. In all preliminary experiments, except for the last experiment dealing with an Oregon Chardonnay juice, the resulting wine was centrifuged and filtered to remove potential malolactic bacteria, since some of these wines are known to undergo natural malolactic fermentation, *e.g.* Pinot Noir, and Chardonnay. Processing the wine in this manner makes it more difficult for the inoculated bacteria to grow because sediments necessary for the adherence of the bacteria are removed. However the ecological significance of competition between strains of bacteria and yeasts strains is unknown, and therefore it seemed pertinent in this preliminary study to avoid this problem with potential unknown competitors.

Another important factor that makes it difficult for inoculated malolactic strains to grow and carry out malolactic fermentation is the removal of yeast lees. It appears that a close physical relationship to yeast is necessary for these bacteria to obtain growth. Guilloux-Benatier *et al.* (1985) demonstrated the stimulating effect of five different yeast autolysates with 4 strains of lactic

acid bacteria. In our laboratory, it has been observed in wines where the yeast lees were not immediately removed, that many bacteria appear to adhere to the surface of dead yeast cells. It is suspected that the dead yeasts, once they lose membrane integrity, leach out important nutrients for the malolactic fermentation process. Therefore, by removing the yeast lees, an important malolactic bacterial nutrient source in the wines may have been removed. Generally, wines are low in nutrients and carbohydrate energy sources for these malolactic organisms upon completion of alcoholic fermentation by yeast.

Another important factor to consider is the inhibitory effect, or at least a stress factor, that occurs from the effect of the high alcohol content (10% or greater) of the wine. King and Beelman (1986) determined that ethanol stimulated early bacterial growth when the concentration was less than 2%, but inhibition of growth occurred when levels exceeded 6%. The most severe bacterial inhibition resulted from actual yeast growth.

To examine the compatibility of yeast and malolactic bacterial strains, five preliminary juice fermentation experiments were done. The first of these was to examine a large number of malolactic bacterial strains from commercial, private, and winery sources against each of several different wine yeasts in a White Reisling juice; the other juices studied were Sauvignon Blanc, two different Oregon Chardonnay juices, and an Oregon Pinot Noir.

1. WHITE REISLING, WR 83-12

The frozen juice was obtained from Mr. Barney Watson, Department of Food Science and Technology, Oregon State University. The grapes which contained a fair amount of rot, were harvested in October of 1983, crushed, pressed and frozen. The original data on the juice prior to freezing was: 19.6^{o} Brix, titratable acidity (T.A.) of 1.00, pH of 2.99, and no SO_2 addition.

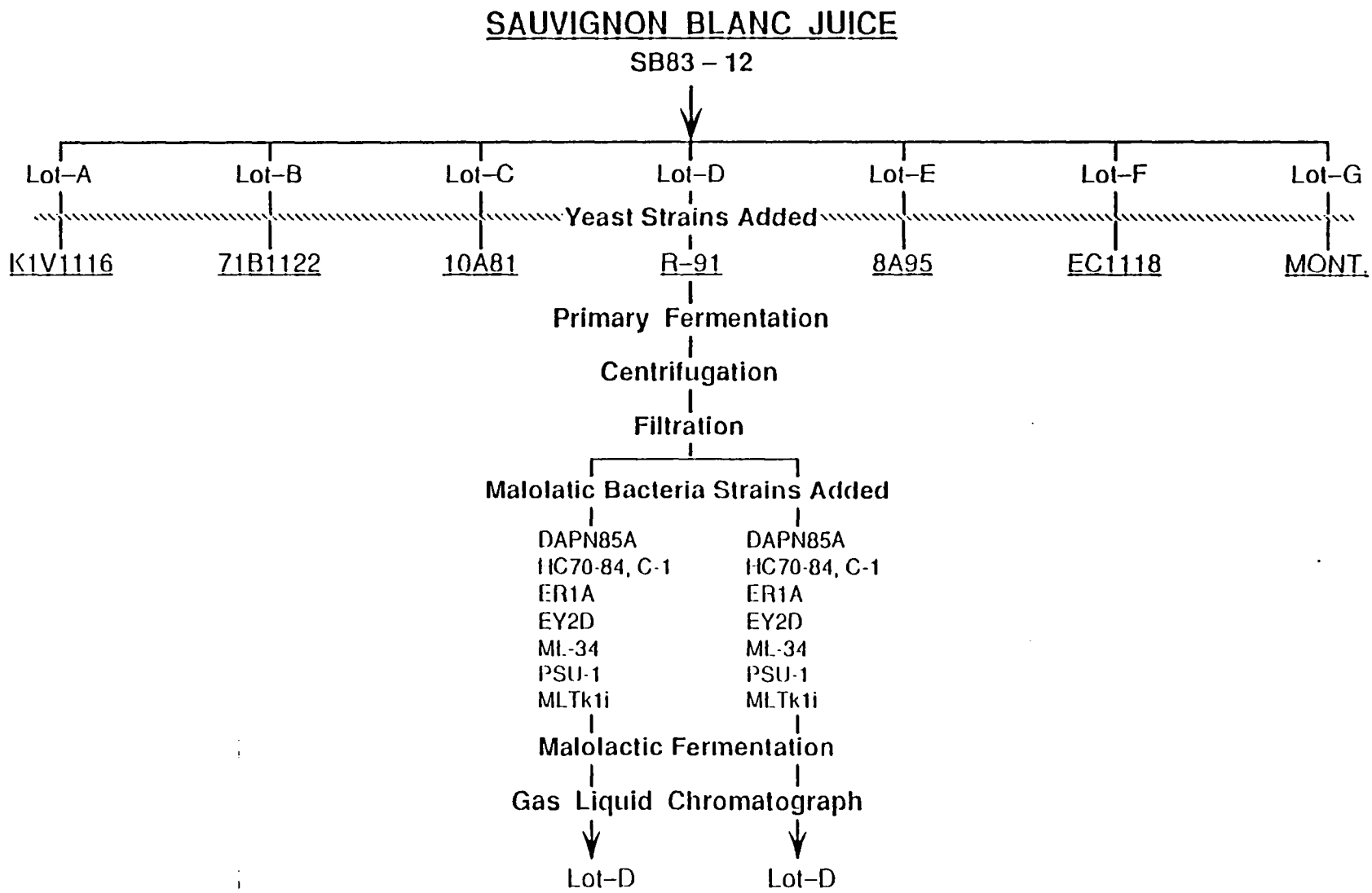
Fifteen yeast strains were used for primary fermentation, and each strain fermentation was subdivided and submitted to malolactic activity by a bank of 31 malolactic bacterial strains. The next set of preliminary experiments involved a Washington State Sauvignon Blanc, (SB83-12), an Oregon Pinot Noir, (OSU-PN85), and an Oregon Chardonnay, (CH85-47).

2. SAUVIGNON BLANC, SB83-12

This 1983 Sauvignon Blanc, obtained as a frozen juice from Mr. Barney Watson of the Enology Laboratory, Department of Food Science and Technology, Oregon State University, was a 1983 Washington State product. The grapes were crushed and pressed in September, 1983 and the following data were determined at that time: ^{o}Brix = 24.2, T.A. = 0.80, pH = 3.15, specific gravity = 1.092, and $SO_{2\ (total)}$= 16 ppm. The juice was then frozen until used in July, 1986 when it was quick thawed, racked, and divided equally into seven carboys.

The general scheme (see **Figure 6.1**.) for this juice was to inoculate

FIGURE 6.1. General scheme for 1983 Sauvignon Blanc treatment in prelimary experiment of malolactic fermentation



each carboy with a specific wine yeast and allow primary yeast fermentation to proceed to completion. The resulting wine was then centrifuged, filtered, and subdivided into paired one-liter bottles. For each yeast lot there were fifteen, one-liter bottles of wine. Each of seven pairs was designated for inoculation from a specific malolactic bacterial culture, and one liter remained as an uninoculated control wine. Malolactic fermentation was followed and chemical analyses of finished wine volatiles were done by gas liquid chromatography.

3. CHARDONNAY CH85-47

Local Chardonnay grapes were obtained in late 1985 from Mr. Barney Watson, Department of Food Science and Technology, Oregon State University. The grapes were crushed, and 25 ppm SO_2 were added. After overnight storage at 5^{o} C, the crushed grapes and juice were pressed and ten gallons of juice were divided into two lots. Soluble solids was measured at 22.5^{o} Brix, pH 2.88, and titratable acidity of 1.080 percent as tartaric acid. The juice was then stored for five days at 5^{o} C, racked a second time, and yeasted by two different yeast strains, a *Saccharomyces bayanus* and a *Saccharomyces cerevisiae*. Primary yeast fermentation was allowed to go to completion and then the resulting wines were centrifuged and membrane filtered to remove any competitive bacteria.

Each lot was subdivided into six pairs of one-liter bottles, each pair being inoculated with a different, specified malolactic bacterial strain.

Malolactic fermentation was followed until termination of the experiment.

4. PINOT NOIR, OSU PN85

Local Pinot Noir grapes were harvested in mid-October, 1985, destemmed and crushed the following day to give about ten gallons of must, which was sulfited to give 25 ppm SO_2. Soluble solids measured at 22^{o} Brix, with pH 2.91 and a titratable acidity of 1.17 percent as tartaric acid. Only half of the grapes were available for this experiment, and so only one yeast strain was tested against a variety of malolactic bacterial strains.

Oregon Pinot Noir has the reputation of undergoing natural malolactic fermentation. Therefore it was decided to first inoculate with yeast and allow completion of alcoholic fermentation; and then carry out centrifugation and membrane filtration to remove all competitive malolactic bacteria before inoculation with test strains.

The last preliminary experimental juice involved a 1986 Oregon Chardonnay.

5. CHARDONNAY, HV86CH

In October, 1986, Willamette valley Chardonnay grapes were purchased and transported to Sokol Blosser Winery, Dundee, Oregon, where they were destemmed, crushed, drained overnight, and pressed. We obtained 120 gallons of unsulfited juice which was stored frozen in four-gallon pails with lids.

The juice, designated HV86CH, was measured at 20.16^{o} Brix, pH 3.15, malate concentration of 4.264 g/l, and a titratable acidity of 1.054 percent as tartaric acid.

This bench scale, preliminary experiment, involved using about 14 liters of juice. The juice was first racked and then subdivided into paired lots for yeast inoculation, (without any further clarification of the juice) in order to simulate commercial winery conditions. Each yeasted subdivision was malolactic inoculated during the early part of yeast fermentation so that the malolactic bacteria would not be initially subjected to high alcohol concentration and yet have grape derived nutrients available for both growth and malolactic fermentation. Again, this simulates commercial winery practice. MLF was followed and GLC was carried out to examine the wines for aldehydes, ketones, alcohols, and esters. HPLC analysis for organic acids was also done.

EXPERIMENT

1. WHITE REISLING, WR83-12

METHODS AND MATERIALS

Once the juice was quick thawed, it was divided into 15 one-liter lots. Each lot was then inoculated with a specific wine yeast, allowed to undergo and complete primary fermentation. The resulting wine was then centrifuged, filtered, and then subdivided into 38 sterile, capped tubes for each lot. Each

tube was inoculated with a specific malolactic bacterial strain and allowed to undergo and complete the malolactic fermentation.

The yeast strains used are listed in **Table 6.1.** Each yeast strain was obtained as a pure culture on YM agar slants and transferred to sterile YM broth (DIFCO), and allowed to grow for 48 hours at room temperature. The yeast cells were centrifuged and rinsed twice with sterile distilled water and then used as an inoculum to the respective juice lots.

The malolactic bacterial strains were commercially used industry standards, Oregon winery isolates, research strains, and culture collection strains from the laboratory of Dr. W. Sandine, Department of Microbiology, Oregon State University. Of the original 38 strains scheduled for this experiment, seven were eliminated due to inability to carry out malolactic fermentation (MLF) in grape juice. The strains used are listed in **Table 6.2.** The ML bacterial cultures were obtained as pure cultures on MRS-V8 agar slants and were first grown in MRS-V8 broth, pH 4.5, and then centrifuged and washed twice with citrate buffer, pH 5.5, before inoculation into the respective wines. The citrate-phosphate buffer was prepared according to Colowick and Kaplan (1955).

Malolactic fermentation was followed by paper chromatography using malic acid and lactic acid standards, as previously described. A comparison of the intensities of the developing lactic acid spot compared to the disappearance of the malic acid spot was made, using an arbitrary scale as

TABLE 6.1. Yeast strains used with White Reisling, WR83-12.

YEAST STRAINS	SOURCE	NOTES
(Commercial Strains)		
71B1122	Lalvin Lallemand	Advertised as a malate degrader.
EC1118	Lalvin Lallemand	Prise de Mousse
K1V1116	Lalvin Lallemand	Killer yeast strain
UG-5	Dr. P. Ritzenthaler	Possible LA bacteria inhibitor
R-92	Mr. J. Paul	New Zealand, originally from South Africa
(Winery Isolates)		
HC54-84	Oregon Chardonnay	
HC70-84,a3	Oregon Pinot Noir	
SB10	Oregon Pinot Blanc	
SB16	Oregon Pinot Noir	
THPN85	Oregon Pinot Noir	
SPN-1	Oregon Pinot Noir	
SPN-2	Oregon Pinot Noir	
DAPN85	Crushed Pinot Noir grapes - not winery associated	
DAR-1	Crushed Pinot Noir grapes - not winery associated	

TABLE 6.2. Malolactic bacterial strains used in White Reisling WR83-12 wines.

MALOLACTIC BACTERIAL STRAINS	SOURCE	TENTATIVE IDENTIFICATION TO GENUS
ER1a	OSU culture collection	*Leuconostoc oenos*
EY2d	OSU culture collection	" "
ML-34	U.C. Davis	" "
PSU-1	Tri Bio Laboratories	" "
MLTkli	Microlife Techniques	" "
E-26	OSU culture collection	*Pediococcus* sp.
LDE	OSU culture collection	*Leuconostoc* sp.
BH-1	Winery Isolate	"
BH-4	" "	"
SB3-J1	" "	"
SB3-J2	" "	"
SB3-J3	" "	"
SB3a	" "	"
SB3b	" "	"
SB3h	" "	"
SB3-6	" "	"
SB3-17	" "	"
SB-18A	" "	"
SB-18a	" "	"
HC54-84,12a	" "	"
HC54-84,21a	" "	*Pediococcus* sp.
HC54-84,A16b	" "	*Leuconostoc* sp.
HC70-84,A4a	" "	"
HC70-84,C-1	" "	"
HC70-84,C-6b	" "	"
DAR-2	Crushed Pinot Noir grapes	"
DAR-3	" " " "	"
DAR-4	" " " "	"
DAR-G	" " " "	"
DAPN85D	" " " "	"

Genera were tentatively identified by gram stain, morphology, malolactic fermentation activity in MRSV8 broth, sensitivity testing to vancomycin, and heterofermentation ability.

follows:

0% MLF = Strong intensity MA spot and no LA spot

25% MLF = Strong intensity MA spot and light intensity LA spot.

50% MLF = Both MA spot and LA spot at about the same intensity.

75% MLF = Light intensity MA spot and strong LA intensity spot.

100 % MLF = No MA spot and a strong intensity LA spot.

Malolactic fermentation was also examined periodically by the enzyme assay of McCloskey (1980) modified by Mr. Barney Watson, Department of Food Science and Technology, Oregon State University, as follows.

A) Standard Curve.

L-Malic acid (Sigma No. M-1000), prepare stock solution in distilled water: 2,500 mg/l (freeze to store).

Prepare standards in 50 ml volumetric flasks:

Ml Stock/50 ml	50	25	20	15	10	5	2	1
mg/l L-MA	2,500	1,250	1,000	750	500	250	100	50

B.) Sample Dilution.

For levels greater than 2,500 ppm (0.25%), dilute with water 1:1 or 1:2 as necessary.

C.) Reagents. Preparation and Source.

Reagent A

6 ml glycine buffer + 0.20 ml (200 microliters) NAD solution for each sample analyzed (for sample and sample blank).

1. Glycine Buffer:

11.4 g glycine (Calbiochem 3570)

3.0 g glutamate (Calbiochem 3510)

250 ml distilled water

Adjust to pH 9.8 with 5N NaOH or KOH, bring to 300 ml. Stable when stored at 2 to 10°C up to 120 days before use.

2. NAD:

1.0 g NAD free acid (Calbiochem 481911) + 6 ml distilled water. Stable at 4°C for 120 to 200 days.

Reagent B

500 microliters of GOT + 500 microliters of MDH = 1 ml with GOT activity of 500 µl/ml and MDH activity of 1250 µl/ml.

GOT - Glutamate-oxaloacetate transaminase (Calbiochem 3518) about 2,500 IU/mg at 30° C. Add 0.25 ml GOT to 0.25 ml distilled water to prepare 0.5 ml at about 1,000 µl.

MDH - Malate dehydrogenase: (Calbiochem 442610) about 10,000 IU/ml or 88.2 µg/mg Add 28.3 mg to 0.5 ml water to prepare 0.5 ml to give about 2,500 µg/mg.

D.) **Assay**

To each of two tubes add:

TUBE 1	TUBE 2 (Reference Blank)
3 ml Reagent A	3 ml Reagent A

25 µl sample	25 µl sample
25 µl Reagent. B	No Reagent B

Incubate 10 minutes at 30° C in a water bath. Read sample reaction tube #1 against the reference blank tube #2 at wavelength of 340 nm. Assays were read on a Beckman DU 50 spectrophotometer.

Soluble solids readings in °Brix were done either by a hand held Bausch & Lomb refractometer or by a Bausch & Lomb temperature regulated refractometer, Model 33.46.71. Ethanol concentrations were determined on a Salleron-Dujardin Eubulliometer at the O.S.U. Enology Laboratory of the Department of Food Science and Technology.

RESULTS

Titratable acidities, pH, ethanol concentrations, malic acid concentration after primary yeast fermentation are shown in **Table 6.3**.

The original malate concentration in the juice before frozen was 4.176 g/l. After 27 days of incubation for MLF at room temperature, the experiment was terminated and the results are shown in **Tables 6.4a** and **6.4b.** The approximate per cent completion of MLF as determined by spot intensity from the paper chromatographs are recorded. At the end of each row and each column is the mean value for the set. The yeast strains with the highest scores were SPN-2, SPN-1, HC54-84, THPN85, and EC1118. The winery from which THPN85 was isolated used the EC1118 commercial yeast for that particular wine, and that may be the identity of the THPN85 isolate. Also, the

TABLE 6.3. Titratable acidity, ethanol concentration, pH, and malate concentration upon completion of primary fermentation of White Reisling, WR83-12 wines.

YEAST STRAIN	SOURCE	(g/l) T.A.	(% v/v) ETHANOL	PH	(g/l) MALATE
71B1122	Lalvin	1.213	11.2	3.09	2.80
K1V1116	Lalvin	1.228	11.7		2.90
EC1118	Lalvin	1.190	11.6		3.20
UG-5	P.Ritzenthaler	1.267	11.6		3.15
10A81	J.P. Australia	1.213	11.7		2.78
R-92	J.P. New Zealand	1.167	11.7		2.72
SPN-1	Winery isolate	1.221	11.1		3.10
SPN-2	Winery isolate	1.228	11.5		3.20
HC54-84	Winery isolate	1.382	11.2	3.03	3.28
HC70-84,a3	Winery isolate	1.159	11.5	3.11	3.06
THPN85	Winery isolate	1.228	11.8	3.06	3.28
SB10 *	Winery isolate	1.328	11.5	3.07	3.20
SB16 *	Winery isolate	1.413	11.3	3.05	2.74
DAR-1	Grape isolate	1.236	11.7		3.08
DAPN85 a	Grape isolate	1.745	11.7	3.09	2.40

* Probable *Candida* sp. (see Chapter III. on Biochemical Characteristics of Yeast Strains).

TABLE 6.4a. Percent completion of malolactic fermentation results of various wine yeast strains versus various malolactic bacterial strains in White Reisling, WR83-12 wines.

MALOLACTIC BACTERIAL	(YEAST STRAINS)							
STRAINS	SB16	UG-5	SPN-1	K1V1116	EC1118	DAPN85	HC5484	SPN2
ER1a	100	100	100	100	100	100	100	100
EY2D	100	100	100	100	100	100	100	100
ML-34	0	0	0	0	0	0	50	0
PSU-1	0	0	0	0	0	0	0	50
MLT kli	50	50	50	50	50	50	50	50
LDE	50	75	50	50	50	50	90	50
E-26	50	50	50	50	50	50	50	50
BH-1	0	100	100	100	100	100	100	100
BH-4	75	100	100	100	100	100	100	100
SB3-J1	100	100	100	100	100	100	100	100
SB3-J2	25	25	25	25	25	25	25	25
SB3-J3	100	100	100	100	100	100	100	100
SB3-a	0	0	100	0	0	0	0	0
SB3-b	50	50	50	50	50	50	50	50
SB3-h	100	100	100	100	100	100	100	100
SB3-G	0	0	0	0	0	0	25	0
SB17	100	100	100	100	100	100	100	100
SB18A	100	0	90	50	100	25	25	100
SB18a	50	100	100	100	100	100	100	100
HC54-84,A16	50	50	75	75	75	50	50	75
HC54-84,12a	50	50	50	50	50	50	50	50
HC54-84,21a	25	50	75	50	75	50	90	100
HC70-84,A4a	50	25	50	50	50	75	50	75
HC70-84,C6b	50	0	50	25	50	50	50	50
HC70-84,C1	0	0	100	0	0	0	50	0
SPN-5b	0	100	100	100	100	100	100	100
DAR-2	25	100	100	50	50	50	50	100
DAR-3	0	0	0	0	0	0	100	75
DAR-4	0	0	0	0	25	25	25	100
DAR-G	0	75	100	0	100	0	100	75
DAPN85-D	100	100	100	100	100	100	100	100
YEAST $\bar{X}$ RANK	45.2	54.8	68.2 (#2)	54.0	61.3 (#4)	53.2	67.1 (#3)	72.6 (#1)
# 100 % MLF per Yeast Strain	8	12	15	11	13	11	13	15

Values represent approximate percent of malolactic fermentation according to the appearance or disappearance of the lactic acid and malic acid spots and their intensity on paper chromatographs. Rank refers to the yeast strains with the greatest mean values.

TABLE 6.4b. Continued results of malolactic fermentation of yeast strains vs. malolactic bacterial strains in White Reisling WR83-12 wines.

MALOLACTIC BACTERIAL STRAIN	A-3	SB10	R-92	10A81	71B1122	DAR-1	THPN85	$\bar{X}$ VALUE FOR MALOLACTIC ORGANISM
ER1a	100	100	100	100	100	100	100	100 *
EY2d	100	100	100	100	100	100	100	100 *
ML-34	0	0	0	0	0	0	0	3.3
PSU-1	0	0	0	0	0	0	0	3.3
MLTkli	50		50	50	50	50	50	50
LDE	50	50	50	50	50	25	50	52.7
E-26	50	50	50	50	50	50	50	50
BH-1	100	0	100	0	100	100	100	80
BH-4	100	100	100	100	100	100	50	95 *
SB3-J1	100	100	100	100	100	100	100	100 *
SB3-J2	0	0	0	0	0	0	0	13.3
SB3-J3	100	100	100	100	100	100	100	100 *
SB3-a	0	0	0	0	50	0	50	18.3
SB3-b	50	50	50	50	50	0	50	46.7
SB3-h	100	100	100	100	100	100	100	100 *
SB3-G	0	0	0	0	0	0	0	1.7
SB-17	100	25	100	100	100	100	100	95 *
SB18-A	0	0	25	0	25	0	100	42.7
SB-18a	100	0	100	100	100	100	100	90
HC54-84,A16	50	50	50	50	50	50	50	56.7
HC54-84,12a	50	50	50	50	50	25	50	48.3
HC54-84,21a	50	25	50	50	50	50	50	49.3
HC70-84,C6b	50	50	50	50	50	50	50	45
HC70-84,C1	25	0	25	25	25	25	25	20
SPN-5b	100	0	100	100	100	100	100	86.7
DAR-2	25	0	100	0	0	0	100	50
DAR-3	0	0	0	0	0	0	0	11.7
DAR-4	0	0	0	0	0	0	0	18.3
DAR-G	0	25	50	0	0	0	100	41.7
DAPN85D	100	100	100	100	100	100	100	98.3 *
YEAST $\bar{X}$ RANK	51.6	37.1	56.5	53.2	53.2	47.6	60.5 (#5)	
# 100% MLF per Yeast Strain		11	7	12	9	11	11	13

* = Mean values of 95% or better for lactic acid bacterial strains completing malolactic fermentation in wines fermented by the various strains of yeast.

Pinot Noir from which SPN 1 and SPN 2 were isolated were originally yeasted with Montrachet (Red Star) strain. The bacterial strains that did the best were EY2d, ER1a, SB3-h, SB3-J1, and SB3-J3. The two industrial standards, ML-34 and PSU-1 did not do well, probably due to the low pH of this juice. Other ML bacterial strains that did fairly well were the winery isolates BH-4, SPN-5b, SB18-(a), SB-17, and DAPN85D. The last strain was isolated from crushed Pinot Noir grapes and was not associated with any winery or commercial vineyard.

The yeast strains SB10, SB16, and 10A81 appeared to have had an inhibitory effect on many ML bacterial strains.

DISCUSSION

The bacterial strain, UG-5, is under investigation in France for possible steroid production which is inhibitory to MLF. However, in this study UG-5 did moderately well, though not outstanding in supporting MLF.

Although White Reisling is not a very hospitable wine/juice to test malolactic activity, it is interesting to note that the two strains of *Leuconostoc oenos*, ER1a and EY2d which were isolated in this laboratory and are now used commercially in the Pacific Northwest, did exceedingly well with all yeast strains for this varietal wine.

2. SAUVIGNON BLANC, SB83-12

MATERIALS AND METHODS

The seven commercial yeast strains used for this experiment were the

following:

YEAST STRAIN	SOURCE
K1V1116	Lalvin, Killer strain
71B1122	Lalvin (Advertised as a malate degrader)
EC1118	Lalvin (Prise de Mousse)
10A81	J.P. Originally from Australia
8A95	J.P. Originally from Australia
R-92	J.P. Used in New Zealand, from S. Africa.
Montrachet	Red Star

All yeast strains were grown at room temperature for 48 hours in YM Broth (DIFCO), centrifuged and washed twice, then used as an inoculum for the juice.

The seven strains of malolactic bacteria that were used for each lot included four commercial strains, one from our laboratory culture, and two *Leuconostoc* sp. isolates from wine/grapes. These are as follows:

ML STRAIN	SOURCE
ER1a	Isolated in our laboratory. Used commercially.
EY2d	Isolated in our laboratory. Used commercially.
ML-34	U.C., Davis. Industry standard
PSU-1	Pennsylvania State U., Industry standard
MLTkli	Microlife Technics, Sarasota, Florida
HC70-84,C-1	Winery Isolate, Pinot Noir
DAPN85 A	Pinot Noir crushed grape isolate

The malolactic bacteria were grown in one-liter sterile MRS-V8 broth, pH 4.5 for five days, centrifuged and washed twice with sterile citrate-phosphate buffer, pH 5.5.

The cells were resuspended in sterile distilled water and equally aliquoted to the respective one-liter, paired bottles of wine. Malolactic fementation was followed by both paper chromatography as before and malic acid enzyme assay kits (Boehringer Mannheim Biochemicals, Indianapolis, In.) Gas-liquid chromatographic analyses were accomplished on selected finished wines using a Hewlett Packard Gas Chromatograph 5710A with a FID detector and a Hewlett Packard 3390 Integrator for peak identification and quantification. The parameters for the GLC operation were as follows: temperature program was from 90 to 200 ^{o}C at a rate of 4 ^{o}C per minute. The carrier gas was nitrogen at 70 psig and a flow rate of 20 ml per minute. The first column used, labeled "Column B", was a two meter glass column, 1/4 inch OD with a packing of 80/120 Carbopack B AW / 5% Carbowax 20 M (SUPELCO). The second column, labeled "Column A", is the same as the first except the packing contained 3.35% Carbowax 20 M.

Standards were prepared in 12%, 50% or 95% ethanol depending on their solubility at 10,000 mg/l as stock standards. These were diluted 1/100 in 12% ethanol and were injected as a one µl injection into the G.C. The internal standard method was used for integration, and sec-butanol at 90 mg/l was used as the internal standard.

The protocol for GLC analyses of wine samples required one to first fine tune the GC with fresh standards each day, and to make up fresh stocks of standards each week, or earlier if deterioration appeared to occur. A 12% ethanol flush run was done after each injection of standards, and after each wine sample injection, from two to five, 12% ethanol flush injection-runs were done to ensure that the column had no remaining material to interfere with further sample runs.

The wine samples were first filtered through a Millipore 0.45µ membrane filter into sterile capped tubes. Two ml of filtered wine were pipetted into a small tube, and 20 µl of internal standard was added and mixed. One µl of this mixture was then injected into the GC. A minimum of two injections were quantified for each wine sample and the mean value for each recognized peak was determined.

RESULTS

The juices, just prior to yeast inoculation, read at 21.5 to 23^{o} Brix, pH of 3.09 to 3.13, and T.A. of 0.69 to 0.77 g/100 ml as tartaric acid. Upon completion of primary yeast fermentation the pH measured from 3.13 to 3.29, (see **Table 6.5**).

Malolactic fermentation was followed for 200 days. By day 36 MLF was complete in three of the malolactic inoculated wines of the yeast lot R-92. These were DAPN85 A (one bottle), ER1a (both bottles), and EY2d (both bottles).

TABLE 6.5. Measured parameters of Sauvignon Blanc SB83-12 wines after completion of alcoholic fementation.

YEAST STRAINS	((g/l) MALATE	(% v/v) ETHANOL	(mg/l) ACETIC ACID	(mg/l) ACETALDEHYDE	PH	(g/l) TITRATABLE ACIDITY
10A81	1.535	13.6	95.3	23.75	3.22	0.898
8A95	1.677	12.7	623.4 *	17.38	3.21	0.913
R-92	1.679	12.3	158.4	14.88	3.13	0.708
K1V1116	1.809	12.7	400.4	28.97	3.18	0.958
EC1118	2.056	14.8	314.8	15.45	3.29	0.915
71B1122	1.919	12.9	419.5	22.38	-	0.897
Montrachet	1.645	13.5	246.9	17.50	3.21	0.860
Control Juice	3.052	-	17.8	79.88	3.15	0.800

* This is at the detectable limit of taste for acetic acid in wine (lPeynaud, 1984).

On day 60, selected pairs of malolactic inoculated wines that did not show any evidence of having started MLF were selected for a reinoculation of the appropriate strain of malolactic bacteria. One bottle was reinoculated with the selected ML organism and the other bottle remained as a control which was not reinoculated. One mililiter of 0.1 % yeast extract was added to the one liter reinoculated wine.

On day 62, one more bottle of the R-92 wine, inoculated with MLTkli, completed MLF.

To determine if viable ML bacteria were still present in the wines which showed no MLF on day 82, five mililiters of sediment was taken from each wine and used as inoculum to streak plate for growth on pairs of MRS-V8 agar plates with 100 mg/l cyclohexamide (to inhibit yeast). Plates were incubated anaerobically at 30^{o} C for four to six days and examined. The results are seen on **Table 6.6.** All wines of K1V1116, EC1118, 8A95 and 10A81 were negative for growth by ML bacteria. No yeast lot had viable ML bacteria in all wines. The wine yeast that had the most viable organisms present was the R-92 lot which had viable organisms in all ML inoculated wines except for the HC70-84,C-1 and the PSU-1 lots.

Also on day 82, of the 71B1122 wines, one bottle each of the DAPN85 A (reinoculated) and EY2d wines completed MLF. On day 127 of the R-92 wines, the remaining bottle of DAPN85 A completed MLF. This was the last of the non-reinoculated wines to complete MLF. Between day 127 and day

TABLE 6.6. Viability study of malolactic bacteria in Sauvignon Blanc SB83-12 wines.

MALOLACTIC BACTERIAL STRAIN	BOTTLE NO.	K1V1116	71B1122	EC1118	8A95	10A81	R-92	MONTRACHET
DAPN85 A	2	-	-	-	-	-	+	+
DAPN85 A	3	-	+	-	-	-	+	+
HC70-84,C-1	4	-	-	-	-	-	-	-
HC70-84,C-1	5	-	-	-	-	-	-	-
ER1a	6	-	-	-	-	-	+	+
ER1a	7	-	-	-	-	-	+	-
EY2d	8	-	-	-	-	-	+	-
EY2d	9	-	+	-	-	-	+	-
MLTkli	10	-	-	-	-	-	-	-
MLTkli	11	-	-	-	-	-	+	-
ML-34	12	-	-	-	-	-	-	-
ML-34	13	-	-	-	-	-	+	-
PSU-1	14	-	-	-	-	-	-	-
PSU-1	15	-	-	-	-	-	-	-
Control	1	-	-	-	-	-	-	-

Key: (-) = no growth, (+) = growth on paired MRS-V8 agar plates after streak-plating with wine sediment samples.

200, nine more bottles of the reinoculated wines completed MLF. These included the following:

YEAST STRAIN	ML BACTERIAL STRAIN	NOTES ON THE COMPLETION OF MLF
71B1122	DAPN85A	Day 127, second bottle
71B1122	ER1a	Day 132, bottle # 1
71B1122	EY2d	Day 141, second bottle
Montrachet	DAPN85 A	Day 127, bottle # 1
Montrachet	DAPN85 A	Day 141, second bottle
K1V1116	DAPN85 A	Day 196, bottle # 1
R-92	HC70-84,C-1	Day 196, bottle # 1
R-92	ML-34	Day 196, bottle # 1
R-92	PSU-1	Day 196, bottle # 1

Gas liquid chromatography was done on those wines of the R-92 group that had completed malolactic fermentation along with the R-9s control wine. The mean values in mg/l for two or more runs are given in **Table 6.7** for peaks with the same retention times as the listed standards.

DISCUSSION

Acetic acid, often measured as volatile acidity, is normally found in wine at 0.15 to 0.30 g/l due to yeast metabolism (Peynaud, 1984). Aerobic metabolism by contaminants of *Acetobacter* sp. or *Gluconobacter* sp. can substantially increase the amount of acetic acid and resulting ethyl acetate levels in wine. The 8A95 wine upon completion of primary fermentation was tested enzymatically for acetic acid at 0.623 g/l (**Table 6.5**). This level affects

TABLE 6.7. Gas liquid chromatography of selected Sauvignon Blanc SB83-12 wines.

REFERENCE COMPOUNDS	R-92 CONTROL	R-92 MLTkli	R-92 EY2d	R-92 ER1a	R-92 DAPN85A
Acetaldehyde	13.8	25.4	-	7.2	-
Methanol	32.2	50.0	62.5	73.1	50.5
Acetone	68.1	52.1	67.3	68.3	-
Ethanol	15.5	11.1	10.7	12.7	12.2
Ethyl acetate	117.8	183.7	143.7	217.7	206.3
Diacetyl	-	-	8.8	-	14.1
1-Propanol	9.1	9.3	6.8	11.9	11.0
Isobutanol	93.5	88.2	70.2	101.5	94.6
Isoamyl alcohol	511.0	465.2	389.8	562.0	587.7
Acetic acid	309.0	919.8	483.2	455.4	418.6
Isobutyric acid	3.4	3.2	3.6	2.3	4.5

The listed values are in mg/l except for ethanol (% v/v).

the flavor and aroma of the wine adversely, and is probably due to excess aeration by incomplete topping of the wine with subsequent growth by acetic acid bacteria.

Malolactic fermentation proceeded slowly in the majority of the R-92 wines, and not at all in the K1V1116, EC1118, 8A95, nor 10A81 wines. There was only limited success with MLF in the 71B1122 wine with DAPN85 A and EY2d, and in the Montrachet wines with again DAPN85 A and ER1a. This probably exemplifies the problems referred to earlier concerning the late inoculation of the ML organisms, removal of the yeast lees and sediment, as well as the toxic effect of ethanol at > 10% and the inhospitable environment of Sauvignon Blanc juice for providing adequate nutritional requirements for bacterial malolactic fermentation. In spite of these possible negative factors, the R-92 yeasted wine was most successful in having MLF proceed by all ML strains except the industry standard PSU-1 and the winery isolate HC70-84,C-1.

Reinoculated wines of the yeast fermentations from 71B1122, Montrachet, R-92, and one K1V1116 wine (DAPN85 A) did finally achieve MLF by day 200 (6 1/2 months). This may verify the problems just mentioned above. Yeast extract was added to provide additional nutrients for the reinoculated ML bacteria, and apparently this did help.

Under the stressful conditions mentioned above, ML organisms that did the best were: DAPN85 A (7 bottles), EY2d (4 bottles), ER1a (3 bottles)

and one bottle each of MLTkli, ML-34, PSU-1, and HC70-84,C-1.

From GLC chemical analysis of the R-92 wines, those which completed MLF were compared to the uninoculated control wine. As seen in **Table 6.7** in the MLF wines there were averaged increases in methanol (83.3 %), ethyl acetate (59.5 %), and acetic acid (84.2 %) when compared to the control wines. A small amount of diacetyl was measured in two of the wines which indicate that there was a small amount of citrate available for the *Leuconostocs* to produce this compound.

Most other compounds identified were consistent with known products from yeast metabolism and were seemingly unaffected by the action of malolactic fermentation. However there was one large, unidentified peak that appeared consistently with wines that had undergone malolactic fermentation but was not found in the chromatograms of the control wines.

3. CHARDONNAY CH85-47

METHODS and MATERIALS

The thawed juice was stored for 5 days at 5^{o} C, and then racked, divided into two lots and yeasted as follows:

LOT A (4.5 gals)	LOT B (4.5 gals)
10 g Epernay 2 (Red Star)	10 g Pasteur Champagne (Red Star)

Primary yeast fermentation was completed by day 8. On day 28, both lots were centrifuged and membrane filtered with 0.45 micron filters to remove any competitive malolactic bacteria.

Both lots were then subdivided into 6 paired, one-liter bottles, with each pair being inoculated with one of the following malolactic bacterial strains:

DAPN85 A	Private grape/wine isolate.
HC70-84,C-1	Commercial winery isolate.
ER1a	Commercial Oregon strain.
EY2d	Commercial Oregon strain.
ML-34	Industry standard, Commercial strain
MLT-kli	O.S.U. culture collection, Dr. W. Sandine.

All strains were *Leuconostoc oenos* or *Leuconostoc* sp.

The malolactic bacteria were first grown in MRS-V8 broth, pH 5.5, and then centrifuged and washed twice with sterile citrate-phosphate buffer, pH 5.5, before used to inoculate wine.

Malolactic fermentation (MLF) was followed by paper chromatography and the enzymatic assay for malic acid was done as previously described. The experiment was terminated after 188 days (7 months).

On day 58, selected wines were subdivided into two 500 ml aliquots. The first aliquot was to remain as a control, while the second aliquot was reinoculated with the specified ML bacterial strain plus one ml of 0.1 % sterile yeast extract as a nutrient additive.

RESULTS

These results may be seen in **Table 6.8.** Initially, the Epernay

TABLE 6.8. Results of malolactic fermentation in Chardonnay CH85-47.

		(Day MLF completed)			
WINE A (EPERNAY)	BOTTLE NO.	NOT REINOCULATED	(DAY 58) REINOCULATED	(Day 41) VIABILITY STUDY	(mg/l) MALATE STUDY
DAPN85 A	#1	21	ND	ND	ND
DAPN85 A	#2	35	ND	ND	ND
HC70-84,C-1	#3	76	76	+	0.07
HC70-84,C-1	#4	-	-	+	ND
ER1a	#5	83	83	+	0.07
ER1a	#6	59	NR	+	ND
EY2d	#7	86	86	+	0.05
EY2d	#8	59	NR	+	ND
ML-34	#9	-	83	+	0.04
ML-34	#10	105		NR	+
ND					
MLTkli	#11	-	-	-	ND
MLTkli	#12	-	-	-	ND
WINE B (PAS.CH.)					
DAPN85 A	#1	105	76	+	0.05
DAPN85 A	#2	100	NR	+	ND
HC70-84,C-1	#3	-	-	-	ND
HC70-84,C-1	#4	-	-	-	ND
ER1a	#5	-	76	+	0.14
ER1a	#6	-	NR	+	ND
EY2d	#7	-	-	+	ND
EY2d	#8	-	-	-	ND
ML-34	#9	-	86	-	0.06
ML-34	#10	-	NR	-	ND
MLTkli	#11	-	-	-	ND
MLTkli	#12	-	NR	-	ND

ND = not done; NR = not reinoculated.

yeasted Chardonnay did considerably better than the Pasteur Champagne lot in supporting MLF. The bacterial strain that completed MLF the earliest was DAPN85 A, a strain completely unassociated with any commercial winery (obtained from the author's Pinot Noir grapes at his private residence.) In the Epernay lot, only MLT-kli apparently died out and had no MLF. However, in the Pasteur Champagne lot, three of the ML strains did not have viable organisms at day 41, and of those strains that did have viable organisms, only DAPN85 A was able to complete MLF in both bottles.

DISCUSSION

It appears that of the ML bacterial strains, DAPN85 A was best at completing MLF under the most adverse conditions of low pH, presence of ethanol, and minimal availability of an energy source in a clarified wine. The conditions to which the ML organisms were subjected were harsh but necessary to avoid the variable of unknown malolactic competitors.

Concerning the reinoculation of some wines with the specified ML organisms plus yeast extract, it seemed an unnecessary step in the Epernay wine. The exception is with ML-34, where one bottle underwent MLF only after reinoculation. However, in the Pasteur Champagne wines, reinoculation was more successful and necessary; but even after 147 days following reinoculation, four of the ML strains in the Pasteur Champagne did not undergo or complete MLF.

In summary then, the Epernay yeast seems to support malolactic

fermentation in Chardonnay better than the Pasteur Champagne yeast, and the ML bacterial strains, DAPN85 A, ER1a, and EY2d completed MLF in Epernay wines without requiring reinoculation. The DAPN85 A did best in both yeast types.

4. PINOT NOIR, OSU PN85

METHODS and MATERIALS

To the Pinot Noir must, 10 g of active dried yeast, Epernay 2 (Red Star) was added and mixed in a six-gallon glass carboy with a fermentation lock in place. This was placed in a water bath to maintain constant temperature of 21^{o} C. The cap was punched down and mixed daily for seven days, after which the fermented must was then pressed, centrifuged and membrane filtered. The clarified wine was then stored at 4^{o} C for one month.

The wine was then divided and aliquoted into seven pairs of one-liter bottles for inoculation with specified strains of malolactic bacteria. The bacterial strains used were as follows:

ML STRAIN	SOURCE
DAPN85 A	Pinot Noir isolate from a private vineyard
HC70-84,C-1	Winery isolate
ER1a	Oregon commercial strain
EY2d	Oregon commercial strain
ML-34	Industry standard, U.C., Davis, California
MLT-kli	O.S.U. culture collection
PSU-1	Industry standard, Pennsylvania State U.

All ML strains were *Leuconostoc oenos* or *Leuconostoc* sp.

The malolactic (ML) bacterial strains were first grown in buffer, pH 5.5, before used as an inoculum.

Upon inoculation of the wine, malolactic fermentation (MLF) was followed by paper chromatography and malic acid enzyme assay kits as previously outlined.

RESULTS

The results are shown in **Table 6.9.** The two ML bacterial strains that did best in completing MLF were DAPN85 A and ER1a. Also, one bottle of EY2d completed MLF within the same 2 1/2 week period. The remaining ML strains that completed MLF took from 40 days to about four months. HC70-84,C-1 was the only strain that did not complete MLF in either bottle. On day 132, a malic acid enzyme assay was done on the remaining test bottles of inoculated Pinot Noir wine and they all showed evidence of incomplete malolactic fermentation.

DISCUSSION

In spite of the adverse environmental condition of the wine for the bacteria to undergo MLF, strains DAPN85 A and ER1a both completed MLF within 2 1/2 weeks. This suggests that these two strains may have the least trouble in completing MLF in Pinot Noir musts under winery conditions, i.e. without the artificial stresses imposed by this study.

HC70-84,C-1 was isolated from a commercial Pinot Noir wine, but in

TABLE 6.9. Comparison of various strains of malolactic bacteria during malolactic fermentation in a Pinot Noir, OSU PN85.

BOTTLE NUMBER	ML BACTERIAL STRAIN	DAY MLF COMPLETED	(Day 17) MALATE (g/l)	(Day 132) MALATE (g/l)
#1.	DAPN85 A	17	0.04	-
#2.	DAPN85 A	17	0.05	-
#3.	HC70-84,C-1	-	-	0.43
#4.	HC70-84,C-1	-	-	0.67
#5.	ER1a	17	0.06	-
#6.	ER1a	17	0.08	-
#7.	EY2d	17	0.11	-
#8.	EY2d	-	-	0.78
#9.	ML-34	43	-	-
#10.	ML-34	-	-	0.67
#11.	MLTkli	43	-	-
#12.	MLTkli	91	-	-
#13.	PSU-1	121	-	-
#14.	PSU-1	-	-	0.50

(-) = did not complete MLF or, assay not done.

this study it did not do well, perhaps due to the initial low pH of the wine. However, results of malic acid assays indicated that MLF by HC70-84,C-1 had proceeded substantially but not to completion in all of the bottles that did not complete MLF in 132 days. Perhaps four months was not sufficient time for these organisms to complete malolactic fermentation.

5. CHARDONNAY, HV86CH

METHODS and MATERIALS

A four gallon pail of frozen HV86CH Chardonnay juice was quick thawed in warm water and allowed to settle for two days at 5^{o} C. The juice was then racked and subdivided into 14 one-liter bottles which were paired and yeasted. The yeast strains used were as follows:

MONTRACHET (Red Star)

PASTEUR CHAMPAGNE (Red Star)

EPERNAY 2 (Red Star)

SPN-2 (Winery isolate)

SB10 (Winery isolate)

SB16 (Winery isolate)

The yeast were grown in sterile YM Broth (DIFCO) for 48 hours at 21^{o} C on a shaker, then centrifuged and washed twice with sterile distilled water. The cells were resuspended in HV86CH juice and used as inoculum into paired one-liter juice bottles. The primary fermentation was maintained at 21^{o} C by means of a water bath. After day two the juices measured between

8.5 to 11.4 ^{o} Brix. Each one-liter lot was then subdivided into 14 sterile, screw capped, 50 ml Erlenmeyer flasks, i.e. six pair for inoculations by specified strains of ML bacteria, and one pair as an uninoculated control (**Figure 6.2**).

The folowing malolactic bacterial strains were inoculated into the above paired, yeasted juice for each yeast type:

ER1a (Oregon commercial strain, isolated in this laboratory.)

EY2d (Oregon commercial strain, isolated in this laboratory)

ML-34 (Industry standard, UC Davis)

PSU-1 (Industry standard, Pennsylvania State University)

MLT-kli (OSU Culture collection)

SB3-J1 (Oregon winery isolate)

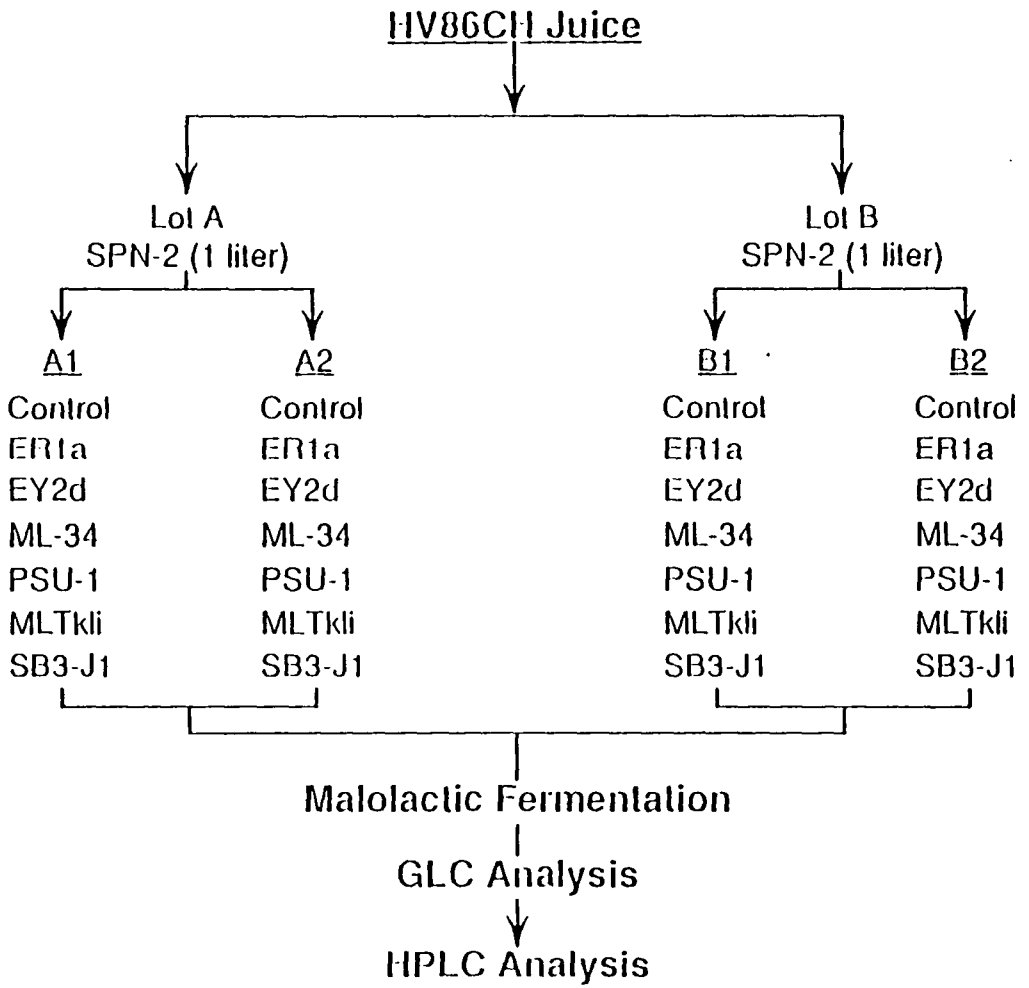
All oF the above strains were *Leuconostoc oenos* or *Leuconostoc* sp.

Each ML strain was first grown in MRS-V8 broth, pH 4.5, for seven days at 30^{o} C, then centrifuged and washed twice with sterile citrate-phosphate buffer, pH 5.5. The cells were then resuspended in sterile distilled water and used as inoculum into appropriate flasks.

Malolactic fermentation (MLF) was followed by paper chromatography and malic acid enzyme assay kits (Boehringer-Mannheim) as previously outlined.

Gas liquid chromatography was done as previously outlined.

FIGURE 6.2. General scheme for Chardonnay HV86CH juice yeasted with SPN-2 yeast.



Standards used for the A-column were the following:

Isobutanal	Pentanal
Ethyl propionate	2-Pentanol
Isobutyl acetate	Hexanal
2-Methyl butyraldehyde	1-Butanol
Ethyl isobutyraldehyde	1-Pentanol
Butyl acetate	Isobutyl acetate

Standards used for column B were the following:

Acetaldehyde	Propyl acetate
Methanol	Isobutanol
Propanal	3-Methyl-2-butanol
Acetone	Ethyl butyrate
Methyl acetate	Acetoin
Ethanol	Isoamyl alcohol
Isopropanol	n-Amyl alcohol
Ethyl acetate	Isoamyl acetate
Diacetyl	2-Furaldehyde
Isopentanal	Hexanol
1-Propanol	Isobutyric acid
Butyric acid.	

The internal standard for both columns was sec-butanol.

The protocol for GC analysis was the same as outlined before, using the

same temperature program, carrier gas, and instruments.

Wine samples were first filtered with a 0.45 µl membrane filter (Millipore). To two ml of filtered wine, 20 µl of internal standard was added and vortex mixed just prior to injection into the gas chromatograph.

HPLC ANALYSIS

The methodology used for examining organic acids by high performance liquid chromatography was essentially that of Schneider *et al.* (1987). A Beckman HPLC system, model 332, was used to analyze wine samples for organic acid composition. This system was composed of a Model 420 Controller, two Model 110B pumps, Model 210A sample injector, and a Model 163 Variable Wavelength Detector. The organic acid analysis column was an Aminex Ion Exclusion HPX-87H column (Bio-Rad, Richmond, Ca.) 300 X 7.8 mm. A Bio-Rad Micro-Guard cartridge column was placed just in front of the organic acid column. Both the guard column and organic acid column were fitted with a water jacket (Rainin, Emeryville, Ca.) for temperature maintenance of 60^{o} C for the preliminary experiments only. An isocratic system, i.e. a non-gradient system, using 0.010 N H_2SO_4 as the mobile phase, and with a flow rate of 0.8 ml/min was applied.

Standards were made up in 2X distilled water at 50 to 1000 mg/l concentration, with formic acid at 100 mg/l used as an internal standard. Quantification was done on a Hewlett Packard Integrator, Model 3390A, using the internal standard peak area method.

Wine samples were filtered with 0.45 µ Millipore HAWG membrane filters, and diluted 1:2 with 2X distilled water. Injections were ten µl of sample at 0 time. Each run was followed by two to three flushes of ten ml of 0.010 N H_2SO_4 through the injector and sample loop, and one or two runs of 0.010 N H_2SO_4 through the column, to ensure that no wine sample residues remained.

RESULTS and DISCUSSION

A. Malolactic Fermentation

The results may be seen in **Table 6.10.** The yeast strain that best supported malolactic fermentation was Montrachet. All ML organisms completed MLF in this yeasted wine within 35 days. Pasteur Champagne, SPN-2, and Epernay 2 wines completed MLF within 54 days by all ML test strains. The winery yeast isolates, SB10 and SB16, had successful MLF only with the two Oregon ML strains, ER1a and EY2d, and with the SB3-J1. The other malolactic bacterial strains MLT-kli, ML-34, and PSU-1 were unable to carry out MLF with these two yeast strains.

Both Oregon malolactic bacterial strains, ER1a and EY2d, completed MLF the earliest, i.e. within three weeks, in all yeasted musts. The remaining ML strains , MLT-kli, ML-34, PSU-1, and SB3-J1, all did about the same except that SB3-J1 was able to complete MLF in the SB16 yeasted must where the others could not. Some of the control wines of Montrachet, SB10, and SB16 did undergo natural malolactic fermentation by day 54. This is a common

Table 6.10. Completion day for malolactic fermentation in Chardonnay HV86CH musts by various strains of ML bacteria vs. various wine yeasts.

STRAIN OF MALOLACTIC BACTERIA	(mg/l) MONT.	PAS.CH.	EPER.	SPN-2	SB10	SB16
EY2d	17	17	20	17	53	53
ER1a	17	17	20	17	53	53
MLT-kli	35	51	48	32/53	(-)	(-)
ML-34	35	51	54	53	(-)	(-)
PSU-1	35	51	54	53	(-)	(-)
SB3-J1	35	51	54	32/53	46(1)	53
CONTROL	54(2)	(-)	(-)	(-)	83(3)	83(2)

Key: Number in parenthesis, ( ), indicates the number of flasks out of 4 flasks that completed malolactic fermentation. Numbers separated by a slash,/, indicate that some flasks finished MLF by the number of day indicated by the first number, and the remaining flasks finished by day indicated by the second number. (-) = did not complete MLF.

occurrence in wineries where inoculation with commercial stocks of MLF bacteria is not practiced; however, it usually takes much longer to occur than if the must is heavily inoculated early in the primary fermentation.

The pH also reflected the change in acidity from malolactic fermentation as seen in **Table 6.11.** There was an average pH shift from pH 3.17 to 3.31 between the non-MLF control wines and those wines which have completed MLF. A better reflection of the change of acidity would have been the titratable acidities measurements, which were not done at the time.

B. Gas Liquid Chromatography

The results of the gas liquid chromatographic, analyses of selected wines using B-column (5% carbowax 20 M) are seen in **Tables 6.12** to **6.14.** The values are in mg/l (parts per million), except ethanol which is in % (v/v), and are the means of multiple runs of each of the four wines for each wine yeast-ML strain. For some there were as many as 11 runs which have been averaged. For original chromatograms of control wine, and MLF wine see Appendix, pages 169 and 170.

B.1. Control Wines When comparing the control wines against each other, very little difference was seen between the different yeasts. The amount of isoamyl alcohol (3-methyl-1-butanol) varied the most from a low of 139.73 mg/l by the Epernay yeast to a high of 474.70 mg/l by the Pasteur Champagne yeast. Diacetyl was detected in small amounts in all of the control wines; yet acetoin, which is a single reduction product of diacetyl, was only detected in three of the control wines. Very small amounts of isobutyric acid were detected in all the control wines, and butyric acid, notorious for its malodorous aroma, was found in two of the control wines.

Table 6.11. pH of fermenting musts of Chardonnay HV86CH on day 48 of malolactic fermentation.

MALOLACTIC BACTERIAL STRAIN	MONT	PAS.CH.	EPER.	SPN-2	SB10	SB16
EY2d	3.31	3.34	3.30	3.28	N	3.32
ER1a	3.31	3.35	3.32	3.30	3.36	3.34
MLT-kli	3.30	N	3.34	3.29	N	N
ML-34	3.29	N	N	N	N	N
PSU-1	3.30	N	3.32	3.32	N	N
SB3-J1	3.29	3.35	3.33	3.32	3.34	3.32
CONTROLS	(a)3.16 (b)3.30*	3.20	3.15	3.16	3.18	3.17

*Two of the Montrachet controls, out of four flasks, underwent natural malolactic fermentation. N = not done.

TABLE 6.12. GLC column B results on preliminary tests of selected wines of an Oregon Chardonnay HV86CH.

(mg/l)

REF. NO.	CMPD	PAS.CH. CONTROL	EPERNAY CONTROL	MONTRA. CONTROL	SB10 CONTROL	SB16 CONTROL	SPN-2 CONTROL
1.	Acetaldehyde	305.0	213.2	277.8	252.2	288.5	199.1
2.	Methanol	66.4	84.0	44.1	45.1	216.7	86.0
3.	Propanal	28.4	8.6	43.6	-	39.9	31.1
4.	Acetone	13.6	24.7	14.5	-	12.5	12.7
5.	Methyl acetate	20.8 (1)	-	-	-	-	-
6.	Ethanol	-	8.8	13.2	13.9	13.9	13.8
7.	Isopropanol	-	14.0	-	-	-	-
8.	Ethyl acetate	19.2	6.3	10.8	-	10.3	15.6
9.	Diacetyl	11.8	14.0	7.9	5.2	18.2 (1)	7.6
10.	Isopentanal	24.9	-	23.2	18.5	18.1	31.2
11.	1-Propnol	-	18.6 (1)	-	-	15.8	-
13.	Propyl acetate	-	17.8 (1)	-	-	-	-
14.	Isobutanol	27.3	19.1	24.5	14.2	17.5	15.8
15.	3-Me-2-butanol	-	-	-	-	-	-
16.	Ethyl butyrate	27.6	95.9	-	-	-	-
17.	Acetoin	52.5	-	30.5	-	-	62.6
18.	Isoamyl alcohol	474.7	139.7	295.6	400.8	238.4	275.5
19.	n-Amyl alcohol	-	20.8	50.5	22.0	29.3	-
20.	Isoamyl acetate	7.6	-	82.0	-	-	12.9
21.	2-Furfural	8.2 (1)	-	-	-	-	-
22.	Hexanol	-	49.0	29.0	-	-	-
23.	Isobutyric acid	5.9	8.3	3.1	6.6	1.5	6.8
24.	Butyric acid	-	-	-	-	10.9	22.0

Numbers in ( ) indicate the number of flasks out of 4 examined.

TABLE 6.13. GLC column B results on preliminary tests of selected wines of an Oregon Chardonnay, HV86CH.

(mg/l)

REF. NO.	COMPOUND	MONT EY2D	MONT ER1A	MONT MLTkli	MONT SB3-J1	MONT ML-34
1.	Acetaldehyde	399.5	334.8	248.7	340.6	373.6
2.	Methanol	97.4	109.4	28.2	61.1	100.1
3.	Propanal	12.6 (2)	-	49.9	-	29.2 (2)
4.	Acetone	13.7	7.7	15.3	18.7	9.0
5.	Methyl acetate	12.0 (2)	-	-	-	9.5
6.	Ethanol (%)	14.5	12.6	10.0	13.3	12.6
7.	Isopropanol	-	-	44.5	58.3 (1)	158.2
8.	Ethyl acetate	19.9	10.3	9.9	7.9	8.6
9.	Diacetyl	-	13.8	-	7.7 (1)	4.9 (1)
10.	Isopentanal	32.9	25.6	21.9	27.1	27.7
11.	1-Propanol	7.4 (1)	7.9	-	-	4.9
13.	Propyl acetate	-	-	-	-	-
14.	Isobutanol	62.8	27.7	23.6	34.5	36.5
15.	Activeamyl alcohol	-	-	-	-	-
16.	Ethyl butyrate	-	-	-	-	-
17.	Acetoin	20.7	23.8	31.3	19.2	34.3
18.	Isoamyl alcohol	103.8	111.7	140.3	87.9	125.4
19.	n-Amyl alcohol	-	-	-	59.8 (1)	4.01 (1)
21.	2-Furfural	-	-	-	-	-
22.	1-Hexanol	11.5 (2)	-	7.2 (1)	8.1	14.8 (1)
23.	Isobutyric acid	16.6	42.6	7.2 (2)	24.0	3.4
24.	Butyric acid	157.2	-	-	6.1 (2)	17.9 (1)

( ) = number of flasks out of 4 that contained the compound identified. All other values are in mg/l.

TABLE 6.14. GLC column B results on preliminary tests of selected wines of an Oregon Chardonnay HV86CH.

(mg/l)

REF. NO.	COMPOUND	PAS.CH. ER1A	PAS.CH. EY2D	EPER. ER1A	EPER. EY2D	SPN-2 ER1A	SPN-2 EY2D
1.	Acetaldehyde	170.1	137.0	287.6	428.1	315.1	216.1
2.	Methanol	81.8	82.5	40.6	77.5	92.8	83.2
3.	Propanal	-	-	-	-	-	-
4.	Acetone	23.6	16.4	3.3	7.8	6.3	6.0
5.	Methyl acetate	12.5	13.9	-	-	-	-
6.	Ethanol (%)	14.4	14.7	10.3	10.8	11.5	15.4
7.	Isopropanol	58.5	49.5	-	51.1	-	-
8.	Ethyl acetate	16.2	15.6	12.5	19.4	9.3	5.4
9.	Diacetyl	-	-	-	-	-	-
10.	Isopentanal	27.9	28.2	18.5	26.6	53.0	48.0
11.	1-Propanol	4.6 (2)	4.4	5.2	4.3 (1)	3.9 (1)	-
13.	Propyl acetate	-	-	-	-	-	-
14.	Isobutanol	27.9	27.2	15.7	24.3	19.9	18.2
15.	active-Amyl alcohol	-	-	-	-	3.2 (1)	2.7 (1)
16.	Ethyl butyrate	-	-	20.0 (1)	15.2 (1)	83.3 (1)	77.8 (1)
17.	Acetoin	12.0	18.0	-	22.3	-	-
18.	Isoamyl alcohol	84.7	84.1	78.0	90.6	90.0	84.5
19.	n-Amyl alcohol	-	-	-	-	-	-
21.	2-Furfural	-	4.2 (2)	4.6	-	-	-
22.	1-Hexanol	-	-	-	-	-	-
23.	Isobutyric acid	-	5.2	3.9 (1)	19.2	21.2	23.3
24.	Butyric acid	9.7	-	-	-	-	-

Compound #12 = sec-Butanol (internal standard)

B.2. Malolactic Fermented Wines - Montrachet

These wines, for the most part, had increased amounts of acetaldehyde, methanol, isobutanol, isoamyl acetate, and isobutyrate. Methanol is not a fermentation product but is a cleavage product from the degradation of pectin in the fruit (Nykanen, 1986). The compounds that decreased in amounts included acetone, methyl acetate, ethyl acetate, ethyl butyrate, acetoin, and isoamyl alcohol.

B.3. Epernay 2

In the ER1a and EY2d wines, substantial increases occurred in acetaldehyde, ethyl acetate, isopentanal (isovaleraldehyde), and isoamyl acetate contents, while decreases occurred in the amounts of methanol, propanal, acetone, diacetyl, 1-propanol, ethyl butyrate, isoamyl alcohol, n-amyl alcohol, and hexanol.

B.4. Pasteur Champagne

For ER1a and EY2d in this wine, substantial increases were seen in the levels of methanol, acetone, isopropanol, and isoamyl acetate, while the following compounds decreased: acetaldehyde, methyl acetate, diacetyl, ethyl butyrate, acetoin, and isoamyl alcohol.

B.5. SPN-2

In the wines of ER1a and EY2d, the increases occurred in acetaldehyde, isopentanal, and the isoamyl acetate peak. The remaining compounds either remained essentially unchanged or decreased, as in isoamyl alcohol.

A second column, (Column A.) was used in gas liquid chromatography to examine a different set of compounds (**Table 6.15**). The only significant standout in these compounds was with isobutanal. The Epernay wines, control and MLF wines, had less isobutanal than did the other

TABLE 6.15. Results of GLC column A (3.5 % Carbowax 20 M) analysis of selected wines of an Oregon Chardonnay HV86CH.

REF. NO.	COMPOUND	EPERNAY CONTROL	EPERNAY ER1A	EPERNAY EY2D	PAS.CH. CONTROL	PAS.CH. ER1A	SPN-2 ER1A	SPN-2 EY2D
1.	Ethanol %	14.0	13.7	10.2	8.9	11.7	15.6	13.4
2.	Isobutanal	29.1	27.3	25.3	54.7	61.3	54.6	97.4
3.	Butanal	4.5	3.2	-	1.2	1.8	-	-
4.	1-Butanol	-	5.5	5.2	3.1	3.5	4.7	3.0
5.	2-Pentanol	-	-	-	1.0	-	-	-
6.	Isobutyl acetate	31.8	64.7	-	52.3	-	78.8	-
7.	Hexanal + ethyl-lactate *	27.5	-	-	-	-	13.3	-

* These compounds do not separate on this column. All values are in mg/l except ethanol.

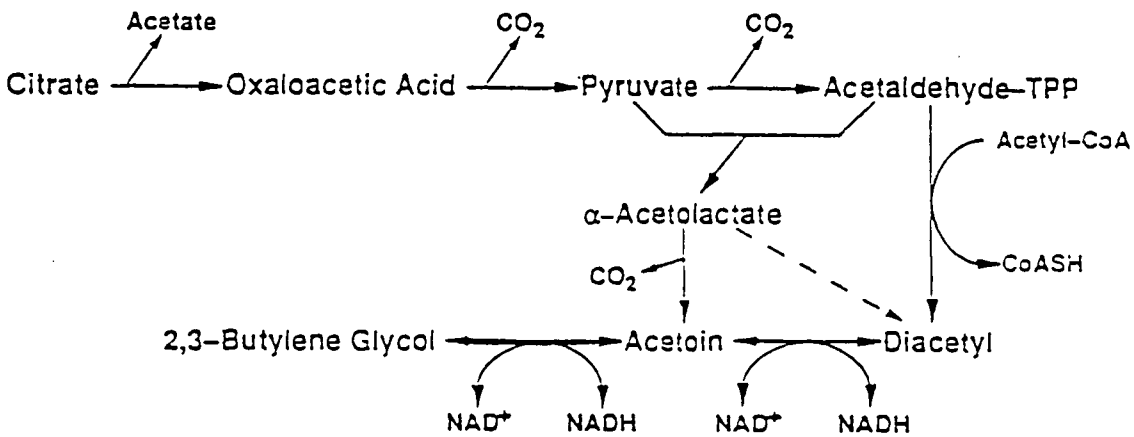
wines tested. Overall, there seemed little difference among the controls and among the MLF wines for the remaining compounds tested. Acetaldehyde increases in MLF wines may be due, in part, to the oxidation of lactic acid (Amerine, 1974).

In all of the malolactic fermented wines tested above, there appeared to be an increase in the esterification of isoamyl alcohol to isoamyl acetate as isoamyl alcohol decreased and isoamyl acetate appeared. However, in later tests, wine samples were spiked with 100 mg/l and 200 mg/l isoamyl acetate and two peaks appeared where the integrator had recognized isoamyl acetate. Therefore, the peak at the relative retention time of 13.55 minutes, on column B, is a mystery peak, and not isoamyl acetate as first thought.

It has been noted that greater diacetyl, acetoin, and acetic acid contents were associated with malolactic fermentation, (Bousbouras and Kunkee, 1971; Arlete-Mascarenhas, 1984; and Pilone *et al.* 1966). This was the case for most of the wines that underwent MLF.

Acetoin can be formed from pyruvate or pyruvate + acetaldehyde and then reduced to 2,3-butanediol (diacetyl), (Masuda and Murake, 1975). However, acetoin is also produced as a result of citrate utilization by *Leuconostoc* sp. as outlined in **Figure 6.3,** (Cogan *et al.*, 1981; Lonvaud-Funnel *et al.* 1984; and Speckman and Collins, 1968). Shimazu *et al.*. (1985) tested for acetic acid, acetoin and diacetyl production from citric acid by the wine malolactic bacterium, *Leuconostoc mesenteroides.* They confirmed the scheme outlined in **Figure 6.3.** However, their proposed pathway deviates from that of Cogan *et al.* (1981) by suggesting that α-acetolactate converts diacetyl directly without an acetaldehyde-TPP interaction, although they show that TPP is a required component.

Figure 6.3. Citrate utilization to acetoin, diacetyl and 2,3-butanediol by lactic acid bacteria.



α-Acetolactate can also give rise to α-ketoisovaleric acid which is precursor to valine, pantothenic acid (tomato juice factor for ML bacteria), and CoASH (Collins, 1972).

Acetaldehyde was found to increase in some MLF wines while in others it was fairly stable or decreased, possibly through the scheme in **Figure 6.3** Acetaldehyde is thought by many to be the most important carbonyl compound found in fermented products, such as wine. (Amerine, 1954; Amerine *et al.*, 1971). Autooxidation of wines can lead to the oxidation of ethanol to acetaldehyde. However, it is thought that this process takes time through complex reaction cascades involving phenolic compounds (Jones *et al.*,1986). Acetaldehyde, together with 2-keto acids are key compounds in the biochemical reaction when the yeast produces fusel alcohols from amino acids and sugars (Nykanen, 1986). Another aldehyde often found as a flavor component in wine is 2-hexenal (Nykanen, 1986).

C. High Performance Liquid Chromatography Analyses for Organic Acids

The results for HPLC analyses of selected wines for organic acids are shown in **Tables 6.16a** and **6.16b.** As seen by the malic acid and the inversely related lactic acid levels, several of the control wines underwent partial or complete natural malolactic fermentation. These were not averaged with control wines that did not undergo MLF, but were evaluated separately.

In general, there was a decrease in citric acid as wines underwent MLF. Citric acid is a precursor used by many *Leuconostoc* sp. to produce diacetyl, (2,3-butanedione), which can give rise to a "buttery" aroma .

The high acetic acid values of these wines gives evidence to the problems that occur when wines are not properly topped to occlude air. Oxidation can occur as well as allowing acetic acid bacteria to produce acetic

TABLE 6.16a. Results of HPLC analysis for organic acids in the preliminary study of selected wines of an Oregon Chardonnay HV86CH.

(mg/l)

REF. NO.	COMPOUND	EPERNAY* CONTROL	EPERNAY CONTROL	EPERNAY ER1A	EPERNAY EY2D	SPN-2 CONTROL	SPN-2 * CONTROL	SPN-2 ER1A	SPN-2 EY2D
1.	Citric acid	42.7	178.9	47.5	32.0	64.0	-	4.9	39.0
2.	Tartaric acid	1557.5	32.3	1810.1	285.0	364.8	834.4	479.4	477.7
3.	Malic acid	205.6	262.0	228.8	166.8	1924.0	33.3	106.6	45.4
4.	Pyruvic acid	-	-	32.6	7.0	-	-	-	-
5.	Succinic + shikimic acids	4487.0	2509.0	3660.0	4137.0	1765.2	1771.8	5717.2	3099.5
6.	Lactic acid	2782.0	1032.5	3207.0	2361.0	390.8	449.5	1353.6	748.1
7.	Acetic acid	1200.3	389.0	1301.2	1060.8	1011.6	719.4	1041.5	574.6
8.	Propionic acid	1181.3	2333.7	1942.7	2209.7	1341.8	462.8	1412.7	-
9.	Isobutyric acid	113.3	-	123.4	-	-	-	-	-
10.	Butyric acid	43.0	36.4	35.1	42.3	41.4	167.1	147.4	102.2
11.	Valeric acid	-	-	-	-	-	7.0	-	-

* Control wines that underwent natural malolactic fermentation.

TABLE 6.16b. Continued results of the preliminary study of HPLC analysis for organic acids in selected wines of an Oregon Chardonnay, HV86CH.

NO.	ORGANIC ACID	PAS.CH. CONTROL*	PAS.CH. CONTROL	PAS.CH. ER1A	PAS.CH. EY2D
1.	Citric acid	18.1	341.7	98.2	89.5
2.	Tartaric acid	1455.3	3847.1	925.3	955.6
3.	Malic acid	52.6	2874.4	108.0	79.6
4.	Pyruvic acid	-	-	-	-
5.	Succinic + Shikimic acid #	2543.1	6616.5	1803.2	1898.2
6.	Lactic acid	568.4	621.5	953.2	819.2
7.	Acetic acid	601.3	1130.9	351.3	110.9
8.	Propionic acid	-	-	62.2	30.9
9.	Isobutyric acid	-	-	-	-
10.	Butyric acid	42.8	193.1	51.5	42.4
11.	Valeric acid	-	-	-	-

* Control wine underwent natural malolactic fementation.
# These compounds could not be separated by this column.

NOTE: Only on these last runs was a large peak seen at 60.46 minutes. This peak had not been noticed before because the runs had originally been monitored for 45 minutes per run.

acid and ethyl acetate from the ethanol. The European EEC legal values for volatile acidity, which is mostly acetic acid, is generous at 0.92 to 0.98 g/l (Peynaud, 1984). This range value was exceeded for wines in most of these preliminary studies. For threshold values and aroma characteristics for various volatile aroma compounds, see Appendices pages 189-192.

CHAPTER VII.
EVALUATION OF COMBINATIONS OF YEASTS AND LACTIC ACID BACTERIA IN MALOLACTIC FERMENTATION BY CHEMICAL AND SENSORY ANALYSES IN AN OREGON CHARDONNAY WINE

INTRODUCTION

Results of the preliminary, bench scale experiments indicated that differences existed between various strains of wine yeast and malolactic bacteria as to time and rate of completion of malolactic fermentation and compounds produced as a result of fermentation. Therefore the objectives of this study included the following: to determine which of the wine yeast strains commonly used in Oregon for making Chardonnay wine, best supported malolactic fermentation using the usual Pacific Northwest winemaking methods; to determine which of the lactic acid bacterial strains commonly used in wine making best completes malolactic fermentation in Chardonnay wine; to determine if the aroma of MLF wines is different from the aroma of the non-MLF control wines of the same yeast type as determined by sensory analysis; to determine if chemical changes in volatiles and organic acids (other than the conversion of malic acid to lactic acid) can be detected in MLF wines when compared to control wines; and to make a tentative identification of the compound(s) responsible for the "ML" peak detected in the preliminary studies.

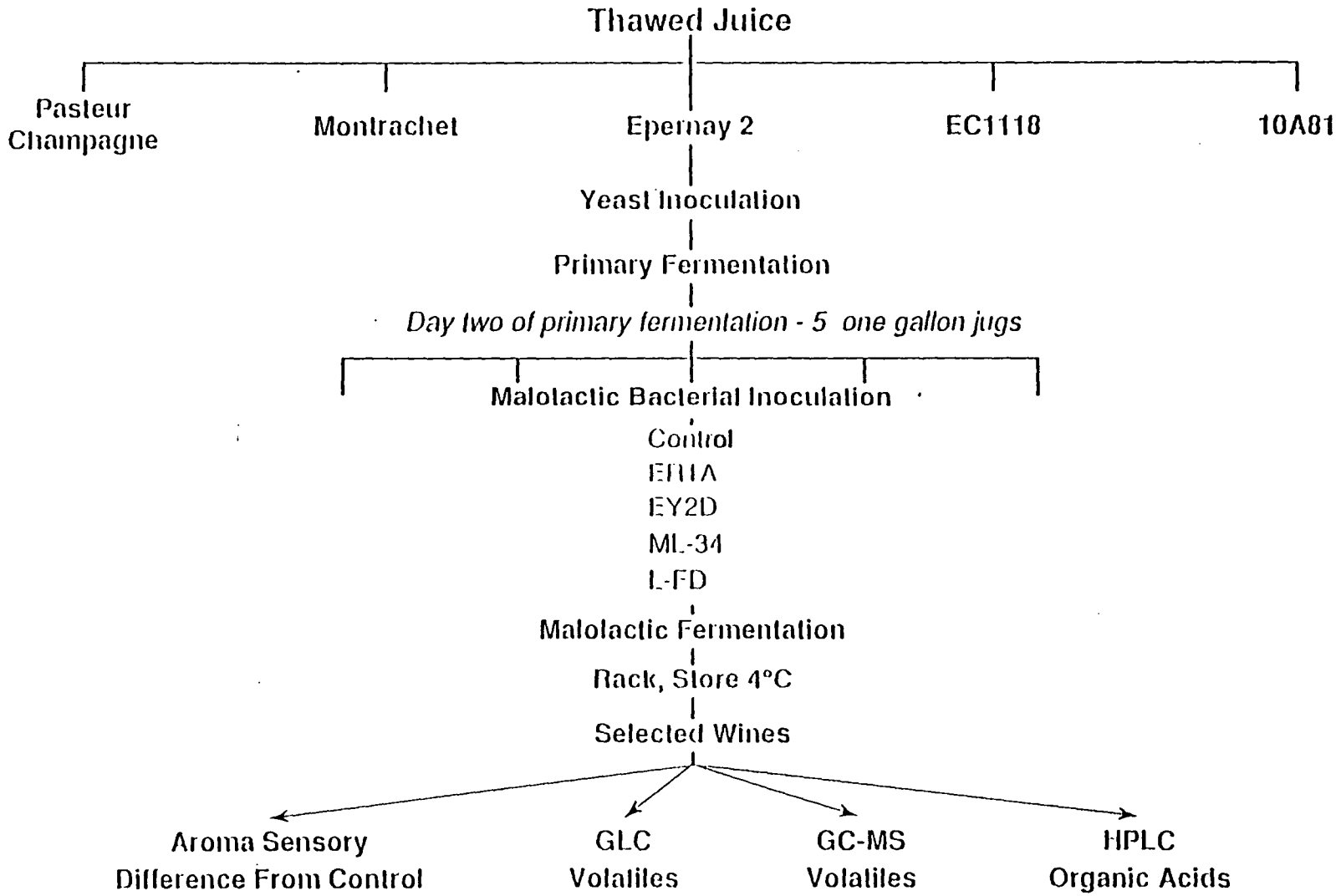
A pilot plant scale experiment, using a 1986 Oregon Chardonnay

HV86CH frozen juice, was initiated so that sufficient wine could be made for sensory evaluation and chemical analyses. Five yeast strains were chosen, based on their use by Oregon commercial wineries in Chardonnay wine production. These included Pasteur Champagne (Red Star, Universal Foods Corp., Milwaukee, Wis.), Montrachet (Red Star), Epernay 2 (Red Star), EC1118, also known as Prise de Mousse (Lalvin, Lallemand Inc., Montreal, Canada), and an Australian strain, 10A81, compliments of Mr. John Paul, McMinnville, Oregon (**Figure 7.1**).

A bank of three strains and one commercial preparation of malolactic bacteria, which are used commercially in the wine industry, were chosen. These included ER1a and EY2d, two patented Oregon State University strains isolated in this laboratory, ML-34, an industry standard from University of California at Davis, and Lalvin's freeze dried pack, designated LFD. The LFD preparation consists of four different strains of malolactic bacteria, one, of which, is PSU-1, an industry standard from Pennsylvania State University, (Cone, 1987).

During the instrumental and chemical analyses of the wine samples, a "mystery malolactic (ML) peak" appeared in all cases where MLF occurred. This was first noticed in the GC chromatographs of the preliminary study done with the Sauvignon Blanc wine, SB83-12. This peak, it was concluded, can be used as an indicator to determine whether or not a commercial wine has undergone MLF without examining the malate content. Pilone *et al.*, (1966),

FIGURE 7.1. General scheme for treating and analyzing Chardonnay HV86CH juice, must and wines.



also noted an unidentified peak in their GC-MS analysis of malolactic fermented Grenache and Gamay Beaujolais wines. Whether or not this is the same compound observed in the present study is unknown. GC-MS analysis was conducted to identify this "mystery peak", which was only found in MLF wines. It was a large peak, indicating that it is a major component of wines having undergone MLF.

METHODS and MATERIALS

1. Winemaking: The HV86CH Chardonnay juice obtained for the preliminary experiments was also used for this pilot scale study. The juice had the following properties: soluble solids, 20.16^{o} Brix; pH 3.15; T.A., 1.054 percent as tartaric acid; and malic acid concentration, 4.264 g/l.

Approximately 120 gallons of frozen juice was divided into 5 lots. Each lot was separately processed. This involved quick thawing the frozen juice in circulating warm water at 38^{o} C and then placing it into a freshly cleaned dairy mixer with a slow circulating mixing paddle to blend the juice. A 500 ml sample-flask was filled, capped and chilled for tests on the juice prior to yeast addition.

One liter of a specific wine yeast strain pure culture was added to approximately 92 liters (24 gallons) of thawed, mixed juice. Yeasts were first grown in sterile Chardonnay, HV86CH juice for 7 days at room temperature (23^{o} C) then added as an inoculum into the test juice, and thoroughly mixed before dispensing into one gallon jugs. Thirty one-gallon, glass jugs were

sterilized for each yeast type. Three liters of mixed, yeasted juice was then dispensed into each jug, and loosely capped with presterilized screw caps. The jugs were put into 5 groups of 4 to 6 jugs per group, labeled, placed on pallets and allowed to undergo primary fermentation. Each yeast lot was started on a different day. The sequence of lot processing was as follows:

LOT	YEAST STRAIN	SOURCE	DATE YEAST ADDED	DATE ML BACTERIA INOCULATED
1.	Epernay-2	Red Star	12/16/87	12/18/87
2.	Pasteur Champagne	Red Star	12/21/87	12/23/87
3.	Montrachet	Red Star	12/28/87	12/30/87
4.	10A81	J.P. *	1/4/88	1/6/88
5.	EC1118	Lalvin	1/6/88	1/12/88

*Australian commercial strain with limited use in Oregon; private source.

With the exception of EC1118, all lots were inoculated with cultures of malolactic bacteria two days after yeast inoculation. This early primary fermentation inoculation of ML strains followed commercial practice in the Pacific Northwest, which allows maximum exposure to grape nutrients for ML growth, without inhibitory contact with alcohol or yeast-produced SO_2.

Each malolactic bacterial culture was first grown in one liter of MRS-V8 broth, pH 4.5, for 7 to 10 days, centrifuged and washed twice with sterile, citrate-phosphate buffer, pH 5.5, and then again with sterile distilled

water. The pellet was then resuspended in one liter of sterile HV86CH Chardonnay juice and incubated 7 days at 30° C, which was then used as an inoculum. Each gallon jug of must was inoculated with 33 ml of the appropriate malolactic culture.

The yeasted juice measured 19.9 °Brix on the day of yeast inoculation. After two days of primary fermenation, and just before malolactic inoculation, the soluble solids dropped to an average of 17.2 °Brix. Due to a contamination problem, malolactic inoculation of EC1118 was delayed 6 days. The starter culture was contaminated with yeast, and so was discarded and a new starter culture was started. In that time, (6 days), the soluble solids dropped to 10.2 °Brix before ML cultures could be inoculated into the must. According to Gallander (1979) and to Ribereau-Gayon *et al.* (1975) this delayed inoculation of ML bacteria should not cause problems. In general this seemed to be true.

On January 8, 1988, the temperature of the room used for primary fermentation dropped to about 14° C and consequently the fermentation slowed considerably, or stopped. A small space heater was placed in the room, but was not adequate to bring the temperature up until the outside temperature warmed up around the 1st of February.

Once vigorous fermentation ceased and the jugs began to clarify, the jugs were topped with well mixed must of the same yeast/malolactic bacterial type, to prevent aeration/oxidation, and contamination.

Malolactic fermentation was allowed to proceed for about 6 months, and after each jug completed MLF, or malic acid concentration reached 0.050 g/l or less, the wine was racked, SO_2 was added to give 20 ppm per jug or bottle, and it was stored at 4^{o} C.

The completion of alcoholic fermentation was followed by residual sugar analysis using the Rebelein Rapid Sugar Test method (Amerine and Ough, 1980).

2. Malolactic Fermentation: Malolactic fermentation was followed by assaying for malic acid concentration using malic acid enzyme assay kits, (Boerhinger-Mannheim Biochemicals, Indianapolis, In.). All wine/must samples were first membrane filtered with Millipore, HAWG, 0.45 μ filters, and appropriately diluted prior to assay. Malic acid and lactic acid standards were made for standard curve assessment as done previously in the preliminary experiments. Malic acid standards were not made since enzyme kits (Boehringer-Mannheim) included calibrated malic acid standards to use with each run.

3. Gas Liquid Chromatography (GLC): Upon completion of MLF, the musts were cold stabilized for approximately 30 days at 3 to 4^{o} C, racked, centrifuged, membrane filtered and stored at 4^{o} C prior to GLC analysis.

The GC instrumentation, program, columns, and protocol were the same as those used in the preliminary experiments with SB83-12 Sauvignon Blanc wine samples. The standards for "A" Column were as follows:

1. Ethanol
2. Isobutyraldehyde
3. Butyraldehyde
4. sec.-Butanol(IS)
5. Isobutanol
6. Isovaleraldehyde
7. Pentanal
8. 2,3-Pentanedione
9. Ethyl propionate
10. 2-Methyl-1-butanol
11. Ethyl Isobutyrate + 1-pentanol
12. Isobutyl acetate
13. Hexanal + ethyl lactate
14. Butyl acetate

Standards used for "B" Column included:

1. Acetaldehyde
2. Methanol
3. Propanal
4. Acetone
5. Ethanol
6. Isopropanol
7. Ethyl acetate
8. Diacetyl
9. 1-Propanol
10. sec.-Butanol (IS)
11. Isobutanol
12. 3-Methyl-2-butanol
13. Ethyl butyrate
14. Acetoin
15. 2-Methyl-1-butanol+acetic acid
16. Iso amyl alcohol
17. n-Amyl alcohol
18. Isoamyl acetate
19. 2-Furfural
20. Hexanol
21. Isobutyric acid
22. Butyric acid
23. Unknown (ML-peak)

The "Unknown-ML peak" showed up on the chromatograms for all wines that underwent MLF, but were either very small peaks or non-existent in control wines and wines that did not undergo MLF.

4. High Performance Liquid Chromatography: HPLC analysis for organic acids was conducted on selected controls and MLF wines. Due to time, costs, and the very large number of samples, HPLC analysis was

limited to the controls and MLF complete wines of ER1a, EY2d and L-FD for each yeast type. The instrumentation, column and guard column, and integrator method were the same as used for Sauvignon Blanc, SB83-12, preliminary experiments. The standards and protocol were the same as before, except that the column temperature was maintained at room temperature (23^{o} C) instead of 60^{o} C. The standards included the following:

1. Citric acid
2. Tartaric acid
3. Malic acid
4. Succinic + shikimic acids
5. Lactic acid
6. Formic acid, (IS)
7. Acetic acid
8. Propionic acid
9. Isobutyric acid
10. n-Butyric acid
11. Isovaleric acid
12. Valeric acid

5. Gas Chromatography-Mass Spectroscopy (GC-MS): GC-MS analyses were carried out on HV86CH control wine and its corresponding MLF wine. The first was done on a Montrachet Control and MLF wine (ER1a). This was carried out by Mr. Dan Hickman of Portland, Oregon. The instrumentation was a Finnigan, Model 1050, GC-MS-DS with a quadrapole EI Mass Spectrometer. The column was a 1/4 inch X 8 feet glass, SP1000, Carbopak B column (Supelco). A Tekmar LSC-1 purge and trap system was used for the purge method of analysis. No special sample preparation was done for this type of analysis. The trap was packed with Tenex and activated carbon.

The second analysis was done on Epernay 2 wines using the method of Lukes (1988). The two wine samples used for GC-MS analysis were an Epernay, control wine, and an Epernay, ER1a wine. Both wines were processed the same way. Celite, which had been previously purified by heating at 700 °C for 16 hours, was mixed with the wine sample in the amount of 450 g celite to 450 ml wine. This was then tamped into a 55 mm X 1 M glass column fitted with a Teflon stopcock and coarse porosity sintered glass bottom plate. Freon 114, (Racon), used to extract volatiles from wine samples, was first passed through a column of alumina acid, Brockman activity 1, 80-200 mesh (Fisher Scientific). The alumina was previously fired in an oven for 1 hour at 600 °C. Approximately 1300 ml of Freon 114 was applied to the column. The solvent flow rate was about 7 ml/minute. The column was allowed to run dry (ca. 5 to 5.5 hours) and 900 ml of solvent extract was recovered from the column. The extraction was done in a cold room maintained at 3° C. The recovered solvent extract was then stored in a freezer room at -10° C overnight.

Kudurna-Danish concentrators were assembled at room temperature and then stored in a freezer room overnight. The concentration/solvent recovery apparatus was set up under a chemical hood with a water bath set at 18° C. The round bottom, concentrator flasks containing the extract were fitted to the system and immersed in the 18° C water bath. Samples were concentrated to 10 ml and removed from the water bath to a dry ice-acetone

solution to quick chill. While the concentrated extract was maintained in a dry-ice/acetone solution, the final concentration was done under a gentle stream of nitrogen gas, to a volume of 2-3 ml. Samples were sealed and stored at - 40^{o} C until taken to be analyzed by GC-MS.

GC-MS was done on these samples at the Department of Agricultural Chemistry, Oregon State University, using a Finnigan Automated GC-EI-CI Mass Spectrometer System, Model 4023, connected to a Varian 3400 Gas Chromatograph. The column was a 30 meter, 0.32 silica capillary, Supelco SPB10 - 0.25µ. The temperature program was set to run at 30^{o} C to 300^{o} C, at a rate of 5^{o} C per minute. It was thought that using a different column and a different system of volatile extraction, a better identification of the "ML" peak might be made.

6. Sensory Analysis: Because of the amount of time involved, the size of the overall experimental design, and the limited availability of the wine panel at the close of the school year, it was decided to limit sensory analysis to analyzing wine aroma, using the Difference From Control method as outlined in Larmond (1977) under "Multiple Comparison Tests".

The purpose was to establish whether or not malolactic fermented wines contributed significantly different aromas (volatiles) from that of the control wines. Wine lot 10A81 was eliminated from this study because MLF was not complete in any of the musts by the time that the sensory study was being initiated.

A. Training: Training of the 12 member wine panel was conducted by Ms. Nancy Michaels, Food Science & Technology Sensory Laboratory, Oregon State University. The training period consisted of two meetings with the wine panel as a group. The panel was introduced to the test methodology and ballot, and was then familiarized with the aroma of 3 different Chardonnay wines. The HV86CH Chardonnay training wines (which had not undergone MLF) included 10A81 Control, Montrachet ML-34, and Pasteur Champagne ML-34. The types of aroma attributes that might be detected were discussed.

B. Testing Sequence: The sequence of the days for sensory testing were as follows:

INITIAL TEST	PANEL REPLICATION TEST
Day #1. Montrachet	Day #5. Pasteur Champagne.
Day #2. Epernay 2	Day #6. Montrachet
Day #3. EC1118	Day #7. EC1118
Day #4. Pasteur Champagne	Day #8. Epernay 2

For each yeast type, the control, ER1a, EY2d, and L-FD wines were matched as close by completeness of alcoholic and MLF as possible.

C. Sample Preparation and Presentation: Test wines were racked from the tartrate crystals, and allowed to equilibrate to room temperature the night before the sensory test. Sample volumes, 40 ml per glass, were

measured out about 40 minutes before the beginning of the sensory analysis. MLF wines were put in black painted wine glasses with glass covers, and the control wine samples were in clear, unpainted wine glasses with glass covers. Each wine was tagged with a 3 digit, random number, obtained from a random number table. Each tray contained one "CONTROL" glass and 4 test glasses, one of which was a hidden control. The glasses were placed on a tray in the pattern shown in **Figure 7.2.** A separate test ballot was placed on each tray. For original test ballot see Appendix, page 171.

The presentation patterns for each tray were standardized according to **Table 7.1.** These pattern sequences were followed for each wine test lot, for each day.

D. Testing Procedure: During a 45 minute period each day, for 10 days, panelists came to the sensory laboratory testing booths and completed the Difference-From-Control, test for aroma. In a closed booth, provided with normal incandescent lights, the panelist was presented with the first of 2 trays. After completing the first tray test, a second tray was presented from the second set of trays (# 13 - 24) listed in **Table 7.1.** The OSU Wine Panel consisted of both students and staff who had participated together in wine panel activities for a year or more.

E. Test Evaluation: From each panelist's completed ballot for each day's testing, values of 0 to 9 were given for the appropriately marked scale for each sample. "No Difference" was given a value of 0, and "Extreme

FIGURE 7.2. Sample glass position for each tray presentation: Aroma, Difference from Control Sensory Test.

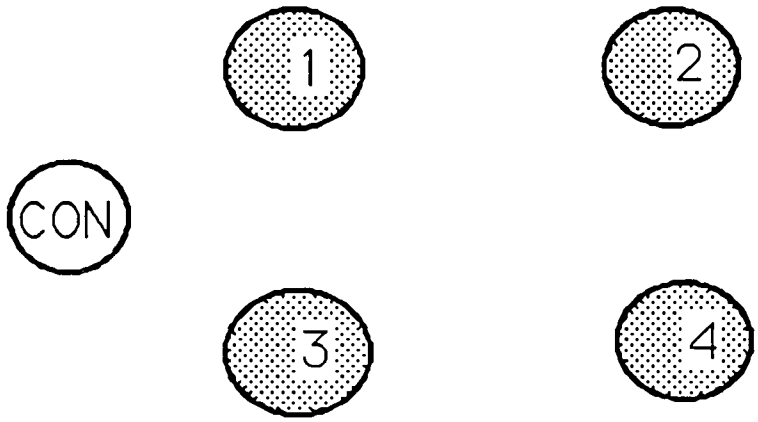


TABLE 7.1. Sample glass presentation positions for "Difference From Control" aroma sensory analysis of Chardonnay HV86CH wines.

	INITIAL TEST Tray Position					REPLICATION TEST Tray Position			
TRAY NO.	#1	#2	#3	#4	TRAY NO.	#1	#2	#3	#4
1.	1	2	3	4	13.	3	4	1	2
2.	1	3	4	2	14.	3	2	4	1
3.	1	4	2	3	15.	3	1	2	4
4.	2	1	3	4	16.	4	1	3	2
5.	2	3	4	1	17.	4	2	3	1
6.	2	4	1	3	18.	4	1	2	3
7.	3	1	2	4	19.	1	2	3	4
8.	3	2	4	1	20.	1	3	4	2
9.	3	4	1	2	21.	1	4	2	3
10.	4	1	2	3	22.	2	1	3	4
11.	4	3	1	2	23.	2	3	4	1
12.	4	2	3	1	24.	2	4	1	3

Key: 1 = control, 2 = ER1a, 3 = EY2d, 4 = L-FD.

Difference" was given a value of 9. This data was transferred to master data sheets which were used for computer analysis. For original master data sheets, see Appendix, pages 172 - 175.

Statistical analysis was carried out on the sensory data obtained from the Difference-From-Control tests for aroma of Chardonnay HV86CH wines. A SAS (Statistical Analysis Systems, Cary, N.C.) program was used on an IBM-XT personal computer. Variation and differences were examined as follows: treatments; panelists; panelists by treatments; sessions; and panelists by sessions. The error term used was the mean square of panelists by sessions. Session (Ses) refers to either the initial two tests given each panelist for a particular yeast type on the first day (session A), or the two replication tests given on the following week for the same yeast type (session B). Treatments (Trts) refers to the four sample glasses of wine that each panelist tested against the control wine to determine aroma difference.

The LSD (Least Significant Difference) test was done using SAS computer program (Statistical Analysis Systems, Cary, N.C.). This test determines which of the treatment wine(s) differ.

RESULTS and DISCUSSION

A. Primary Fermentation, Titratable Acidity, pH, and Malate Content

A summary of the means of residual sugar content, malate concentration, titratable acidity, and pH of the HV86CH wines by early May,

TABLE 7.2. Summary of residual sugar, malic acid, titratable acidity, and pH of Chardonnay, HV86CH musts after five months of fermentation.

TEST MUST	pH	(g/l) TITRATABLE ACIDITY	(g/l) MALIC ACID	(%) RESIDUAL SUGAR
Pasteur Champagne				
Control	3.37	1.04	4.59	0.28
ER1a	3.29	0.96	0.14	0.32
EY2Dd	3.25	0.98	0.12	0.30
ML-34	3.41	1.04	4.66	0.29
L-FD	3.51	0.94	0.05	0.27
Montrachet				
Control	3.31	1.05	4.93	0.29
ER1a	3.45	0.66	0.05	0.47
EY2d	3.47	0.67	0.03	0.45
ML-34	3.31	1.05	5.08	0.36
L-FD	3.47	0.70	0.03	0.41
Epernay 2				
Control	3.35	-	5.10	0.70
ER1a	3.47	0.72	0.03	0.93
EY2d	3.45	0.80	0.05	0.83
ML-34	3.34	1.01	4.72	0.92
L-FD	3.48	0.797	0.03	0.92
EC1118				
Control	3.31	1.13	4.90	0.29
ER1a	3.26	0.90	0.06	0.34
EY2d	3.27	0.87	0.09	0.42
ML-34	3.31	1.04	4.32	0.33
L-FD	3.39	0.89	0.06	0.26
10A81				
Control	3.33	1.92	4.59	0.31
ER1a	3.46	0.99	1.35*	0.30
EY2d	3.36	0.69	1.46**	0.29
ML-34	3.36	1.11	4.55	0.43
L-FD	3.25	0.97	1.25	ND

* Values ranged from 0.260 to 4.687 g/l malic acid.
** This is the mean of three jugs that had undergone partial MLF. The fourth jug, not included in this value, had 4.687 g/l malic acid. All values are the measurement means of two to four jugs or bottles for the specific yeast-MLF must or finished wine.
ND = Not done.

1987 (5-months of fermentation), is shown in **Table 7.2.** As seen by the higher residual sugar values, Epernay 2 had the greatest difficulty in completing primary fermentation. Incomplete alcoholic fermentation can be due to yeast produced substances, such as decanoic and octanoic acids and their corresponding ethyl esters, (Lafon-Lafourcade *et al.,* 1984), or due to nutrient free α-amino nitrogen (Wahlstron and Fugelsang, 1987).

The wines of Pasteur Champagne had the lowest amount of residual sugars remaining after 5 months fermentation. The titratable acidity consistently remained above 1.00 g/l as tartaric acid for controls and wines that did not undergo malolactic fermentation. For wines that underwent MLF, the T.A. ranged from 0.66 to about 0.98 g/l as tartaric acid for a partial malolactic fermented wine. This drop in T.A. is due to the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid plus carbon dioxide.

The malic acid values range from 0.03 g/l, which is about the lower limit of the assay system, to greater than 5 g/l for non-MLF or control wines. The wines inoculated with ML-34 did not undergo MLF in any of the wines. This organism has difficulty surviving in low pH, high acid wines and musts. The culture was flourishing at the time of inoculation but died out early in the fermentation. Other workers have had problems with ML-34 as well. Wibowo *et al.* (1988) using sterilized wines and the wine yeast strain, R-92 for

fermentation of Pinot Noir and Shiraz juices, tested various ML bacterial strains. As with our wines, ML-34 did not grow in their test wines, but slowly died off. As the musts approached completion of MLF, the pH of the resulting wine approached 3.50.

B. Malolactic Fermentation

The progress of malolactic fermentation for each of the yeast types is seen in **Tables 7.3a** to **7.3e.** The comparison of the control wine to each of the malolactic inoculated wines for each yeast type is recorded in **Figures 7.3a to 7.3e.**

B.1. Epernay: As seen in **Figure 7.3a** and **Table 7.3a**, the L-FD group completed MLF first, in 67 days, followed by ER1a in 99 days. In EY2d, three of the four jugs finished MLF in 126 days, but by 160 days the fourth jug still assayed at 0.10 g/100 g/ml malic acid, which is where MLF appeared to stop.

B.2. Pasteur Champagne: In 133 days, only L-FD was able to complete MLF whereas both ER1a and EY2d underwent MLF but stopped at about 0.10 g/l malic acid (**Table 7.3b**, and **Figure 7.3b.**) Given more time, these fermentations probably would have finished.

B.3. Montrachet: This yeast strain allowed the most rapid malolactic fermentation by ER1a, EY2d, and L-FD, compared to all other tested yeast strains (**Table 7.3c** and **Figure 7.3c**). Malolactic fermentation was completed by ER1a in 66 days, by L-FD in 76 days and by EY2d in 87 days.

TABLE 7.3a. Means and standard deviations (in parentheses) of malic acid concentration (g/l) in HV86CH Epernay control wine and wines inoculated with different malolactic bacteria.

DATES	CONTROL	ER1A	EY2D	ML-34	L-FD	FERM. DAY
12/22/87	4.64*	5.08*	5.08*	5.22*	4.99*	4
12/30	4.54 (0.21)	4.09 (0.71)	4.551 (0.32)	4.83 (0.31)	4.40 (0.06)	12
2/4	4.62 (0.07)	4.36 (0.14)	4.17 (0.12)	4.02 (0.21)	3.68 (0.18)	48
2/11	4.80 (0.16)	4.73 (0.13)	4.41 (0.34)	4.80 (0.08)	1.23 (0.81)	55
2/18	4.46 (0.10)	4.35 (0.83)	4.25 (1.26)	5.21 (0.16)	0.08 (0.01)	62
2/23	4.27 (0.06)	2.87 (1.58)	3.13 (1.51)	4.60 (0.21)	0.04 (0.01)	67
2/27	4.89 (0.04)	1.77 (1.68)	2.73 (0.84)	4.64 (0.19)	0.03 (0.01)	71
3/5	4.79 (0.149)	0.67 (1.14)	1.67 (0.43)	4.77 (0.02)	0.03 (0.00)	78
3/10	-	0.42 (0.68)	-	-	-	83
3/15-17	4.74 (0.10)	0.11 (0.15)	1.05 (1.05)	4.54 (0.07)	0.03 (0.01)	88
3/26	4.85 (0.14)	0.03 (0.00)	0.72 (0.58)	4.72 ((0.16)	0.03 (0.00)	99
4/5	-	-	0.28	-	-	109
4/12	-	-	0.18	-	-	116
4/22	-	-	0.12	-	-	126
5/4	-	-	0.07*	-	-	138
5/18	-	-	0.12**	-	-	152
5/26	-	-	0.10**	-	-	160

* Single value only.
** Indicates that only the single, high valued jug was assayed; all other jugs had completed MLF.

FIGURE 7.3a. Graph of malate utilization in Chardonnay HV86CH Epernay control and MLF wines.

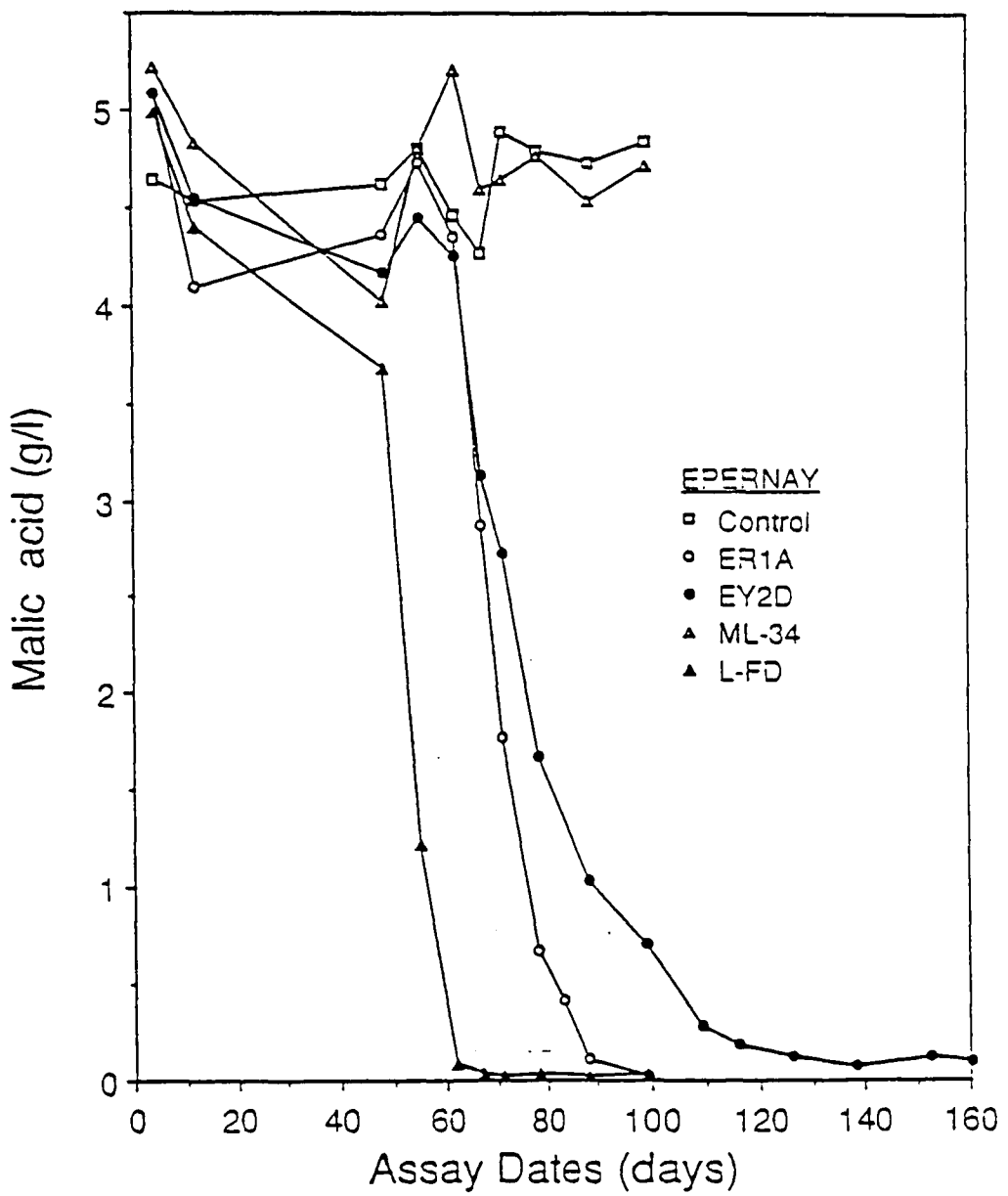


TABLE 7.3b. Means and standard deviations (in parentheses) of malic acid content (g/l) in HV86CH, Pasteur Champagne control wine and wines inoculated with different malolactic bacteria.

DATE	CONTROL	ER1A	EY2D	ML-34	L-FD	FERM. DAY
12/29/87	4.49 (0.18)	4.14 (0.43)	4.79 (0.65)	4.33 (0.11)	4.22 (0.82)	6
1/11/88	4.63 (0.06)	3.99 (0.21)	4.14 (0.05)	4.07 (0.18)	3.99 (0.13)	19
1/15/88	4.44 (0.77)	-	-	4.19 (0.18)	-	23
1/20/88	4.71 (0.15)	3.35 (0.08)	3.38 (0.10)	-	3.10 (0.08)	28
2/4/88	4.82 (0.05)	3.56 (0.24)	3.80 (0.18)	4.85 (0.24)	2.91 (0.22)	43
2/11/88	4.65 (0.15)	2.99 (0.23)	3.14 (0.26)	4.68 (0.11)	2.15 (0.22)	50
2/18/88	4.54 (0.14)	2.24 (0.30)	2.41 (0.25)	5.34 (0.16)	1.36 (0.24)	57
2/23/88	4.25 (0.12)	1.80 (0.27)	1.89 (0.33)	4.78 (0.21)	0.98 (0.24)	62
2/27/88	4.64 (0.24)	1.45 (0.23)	1.43 (0.27)	5.02 (0.46)	0.74 (0.21)	66
3/5/88	4.71 (0.08)	0.98 (0.17)	1.11 (0.31)	4.83*	0.29 (0.13)	73
3/15-17/88	4.82 (0.12)	0.58 (0.28)	0.67 (0.20)	4.74 (0.02)	0.22 (0.02)	83
3/26/88	4.91 (0.18)	0.42 0.11)	0.43 (0.15)	4.83 (0.35)	0.20 (0.01)	94
4/5/88	4.60 (0.07)	0.27 (0.09)	0.24 (0.10)	4.84 (0.06)	0.09 (0.03)	104
4/12/88	4.72 (0.06)	0.23 (0.07)	0.18 (0.03)	4.04 (0.06)	0.09 (0.01)	111
4/22/88	-	0.18 (0.06)	0.16 (0.06)	5.05 (0.25)	0.07 (0.00)	121
5/4/88	4.59 (0.12)	0.12 (0.04)	0.12 (0.04)	4.66 (0.06)	0.05 (0.00)	133
5/26/88	-	0.10 (0.01)	0.12 (0.03)	-	-	155

* Single value only.

FIGURE 7.3b Graph of malate utilization in Chardonnay HV86CH Pasteur Champagne control and MLF wines.

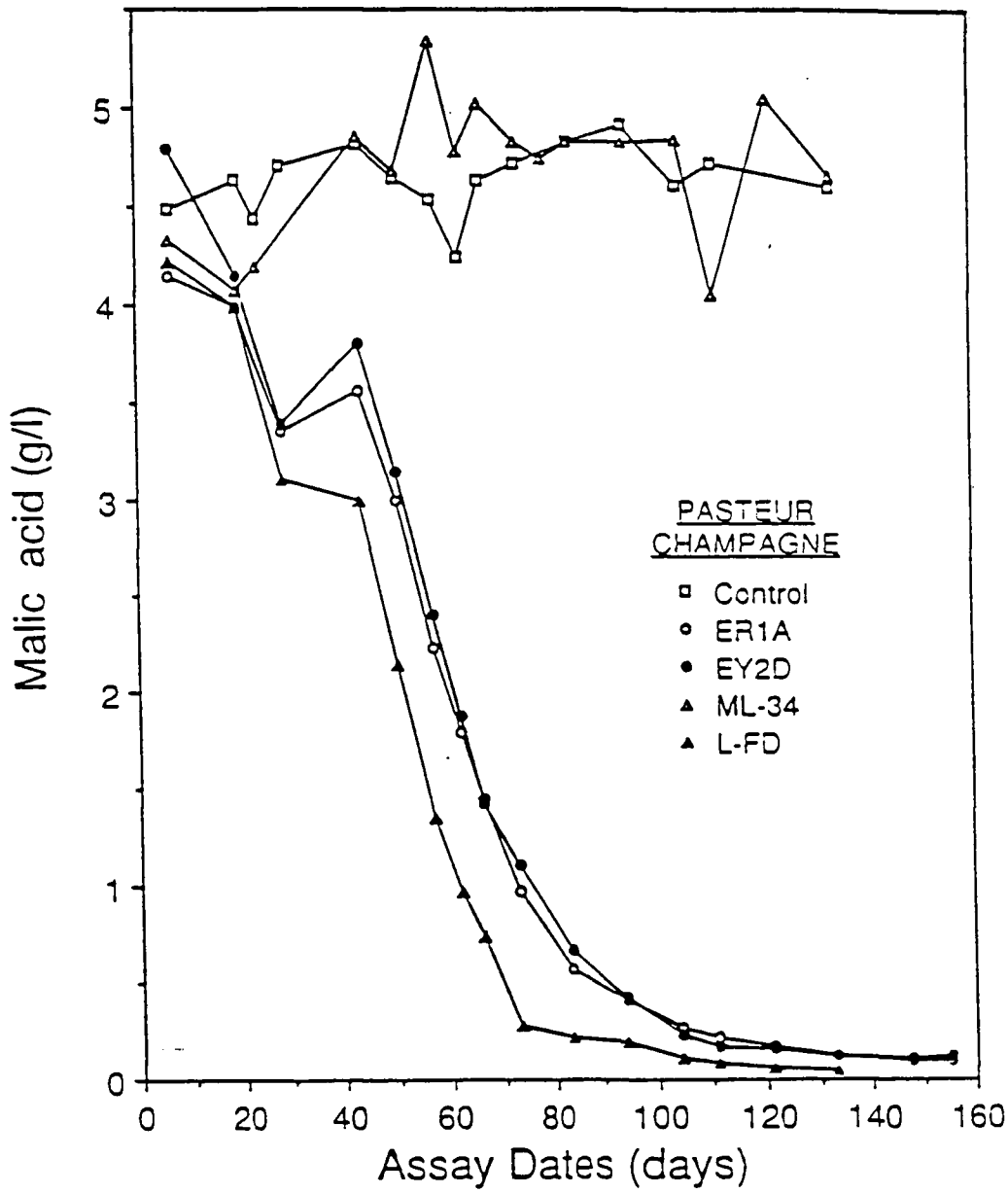


TABLE 7.3c. Means and standard deviations (in parentheses) of malic acid content (g/l) of HV86CH, Montrachet control wine and wines inoculated with different malolactic bacteria.

DATES	CONTROL	ER1A	EY2D	ML-34	L-FD	FERM. DAY
12/30/87	4.85 (0.19)	5.28 (0.21)	5.29 (0.09)	5.40 (0.30)	5.31 (0.30)	0
1/11/88	-	3.76 (0.20)	3.79 (0.17)	-	-	12
1/15/88	4.34 (0.33)	2.31 (0.35)	-	-	-	16
2/18/88	4.64 (0.04)	1.49 (0.94)	2.74 (0.51)	5.25 (0.27)	3.76 (0.27)	50
2/23/88	4.31 (0.06)	0.39 (0.38)	1.49 (0.38)	4.62 (0.18)	2.34 (0.19)	55
2/27/88	4.87 (0.19)	0.17 (0.15)	0.98 (0.23)	4.88 (0.15)	1.53 (0.17)	59
3/5/88	4.81 (0.08)	0.034 (0.00)	0.337 (0.11)	4.573 (0.11)	0.563 (0.34)	66
3/10/88	-	0.04 (0.01)	-	4.47 (0.00)	-	71
3/15-17	4.75 (0.11)	0.05 (0.01)	0.08 (0.04)	4.87 (0.25)	0.07 (0.02)	76
3/26/88	4.93 (0.43)	0.04 (0.01)	0.03 (0.01)	5.08 (0.22)	0.03 (0.00)	87

FIGURE 7.3c. Graph of malate utilization in Chardonnay HV86CH Montrachet control and MLF wines.

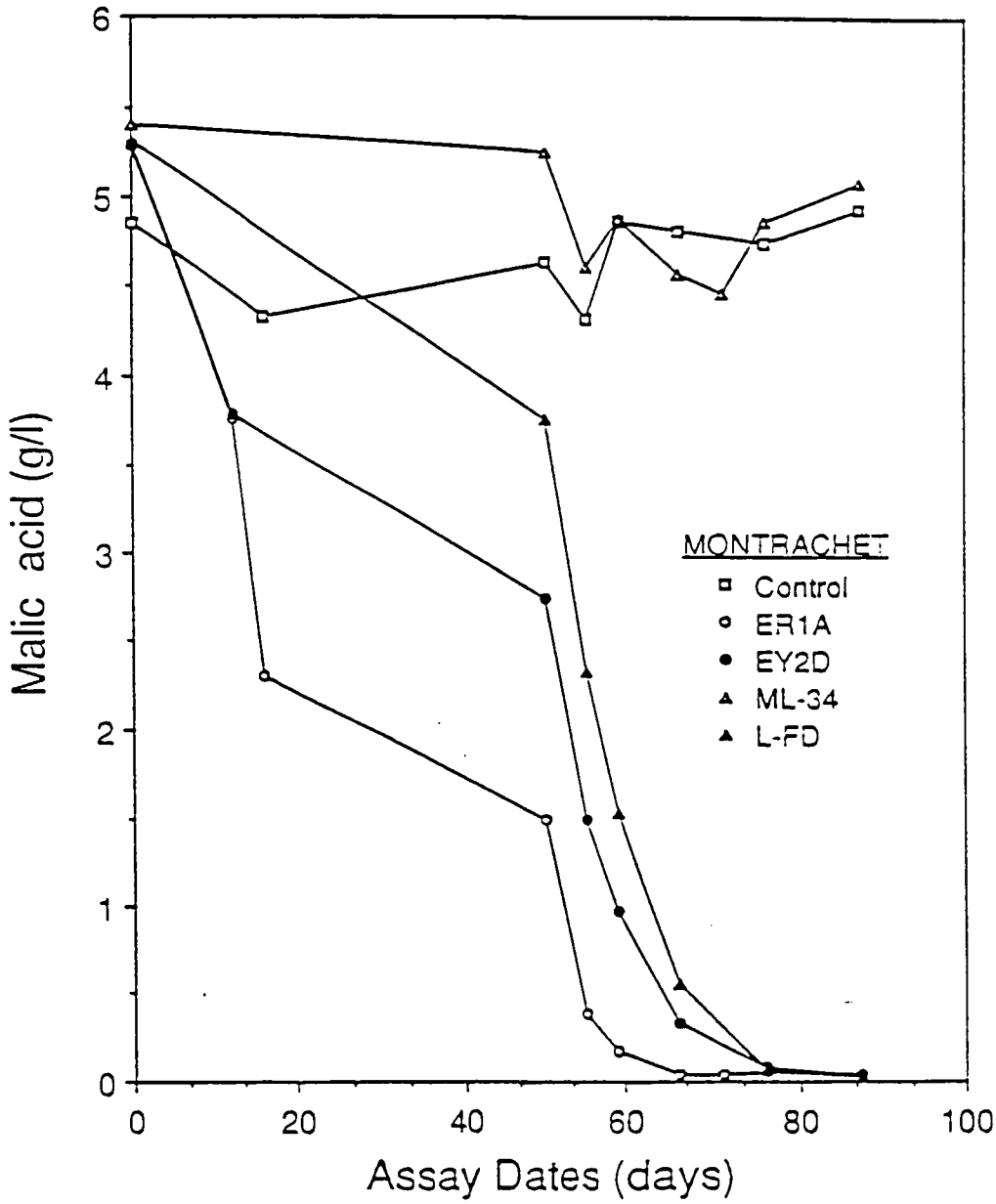


TABLE 7.3d. Means and standard deviations (in parentheses) of malic acid content (g/l) in HV86CH 10A81 control wine and wines inoculated with different malolactic bacteria.

DATE	CONTROL	ER1A	EY2D	ML-34	L-FD	FERM. DAY
2/4/88	4.61 (0.22)	4.39 (0.11)	4.56 (0.12)	4.62 (0.37)	4.65 (0.06)	29
2/11/88	5.00 (0.19)	4.78 (0.09)	5.03 (0.37)	4.91 (0.29)	5.02 (0.29)	36
2/18/88	4.87 (0.18)	4.78 (0.48)	4.82 (0.31)	5.04 (0.20)	5.09 (0.31)	43
2/23/88	4.32 (0.23)	4.13 (0.64)	4.25 (0.33)	4.69 (0.17)	4.63 (0.25)	48
2/27/88	4.54 (0.25)	3.55 (0.62)	3.61 (0.76)	4.27 (0.06)	4.17 (0.08)	52
3/10/88	4.58 (0.21)	3.11 (0.82)	3.57 (0.69)	4.42 (0.08)	3.53 (0.33)	64
3/15/88	4.73 (0.15)	2.18 (1.28)	3.40 (0.19)	4.72 (0.10)	3.54 (0.26)	69
3/26/88	4.84 (0.23)	2.01 (1.44)	2.93 (1.20)	4.77 (0.14)	3.18 (0.34)	80
4/5/88	4.98 (0.28)	2.17 (1.40)	2.71 (1.15)	4.13*	2.35 (0.40)	90
4/12/88	4.61 (0.10)	1.45 (1.38)	2.52 (1.14)	4.46 (0.19)	2.05 (0.35)	97
4/22/88	4.98 (0.09)	1.34 (1.55)	2.52 (1.58)	4.77 (0.25)	1.62 (0.30)	107
5/3/88	4.59 (0.23)	1.35 (1.13)	2.27 (1.41)	4.55 (0.26)	1.25 (0.39)	118
5/26/88	-	-	-	-	0.88 (0.12)	133

* Single value only

FIGURE 7.3d. Malate utilization in CHardonnay HV86CH 10A81 control and MLF wines.

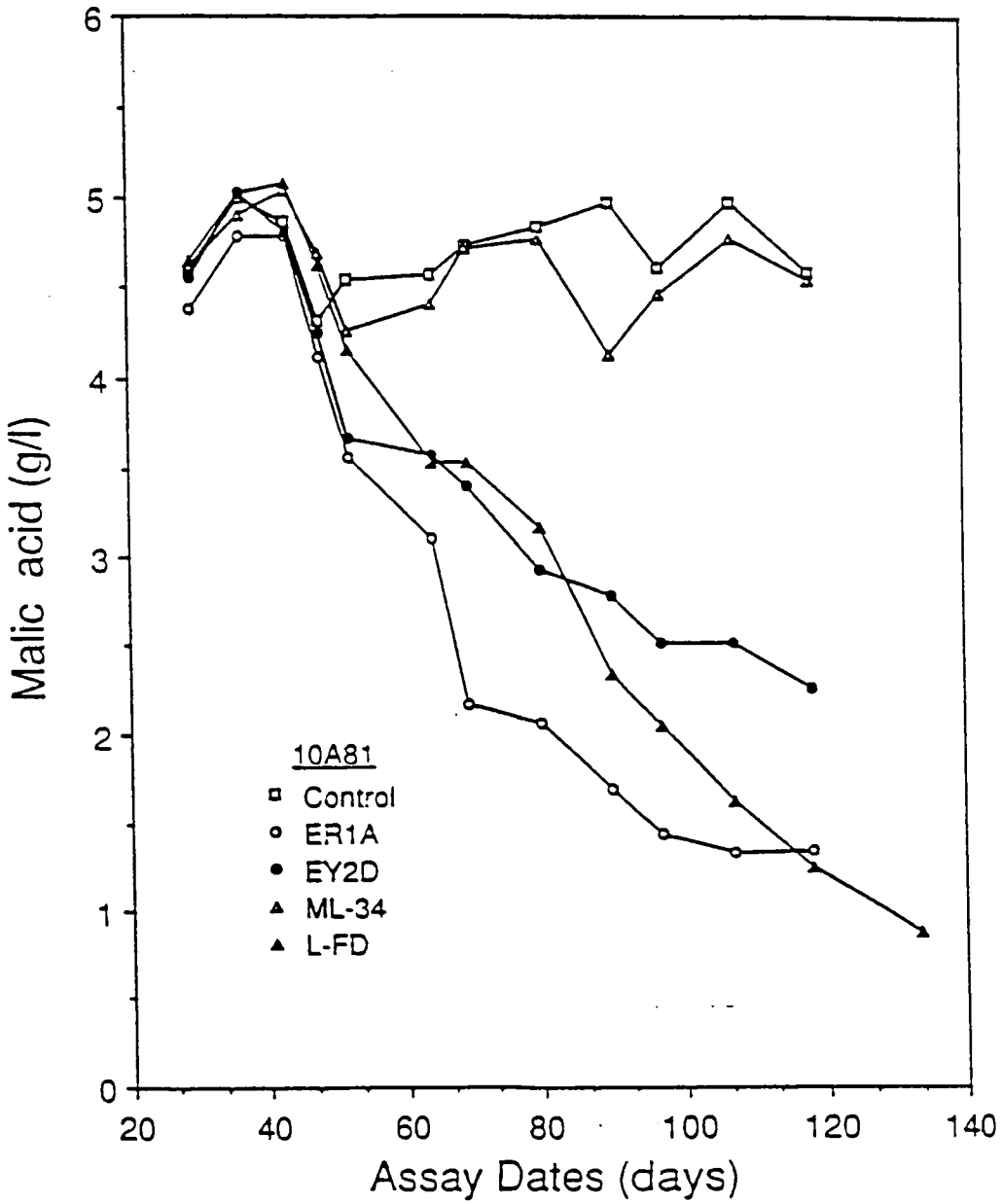
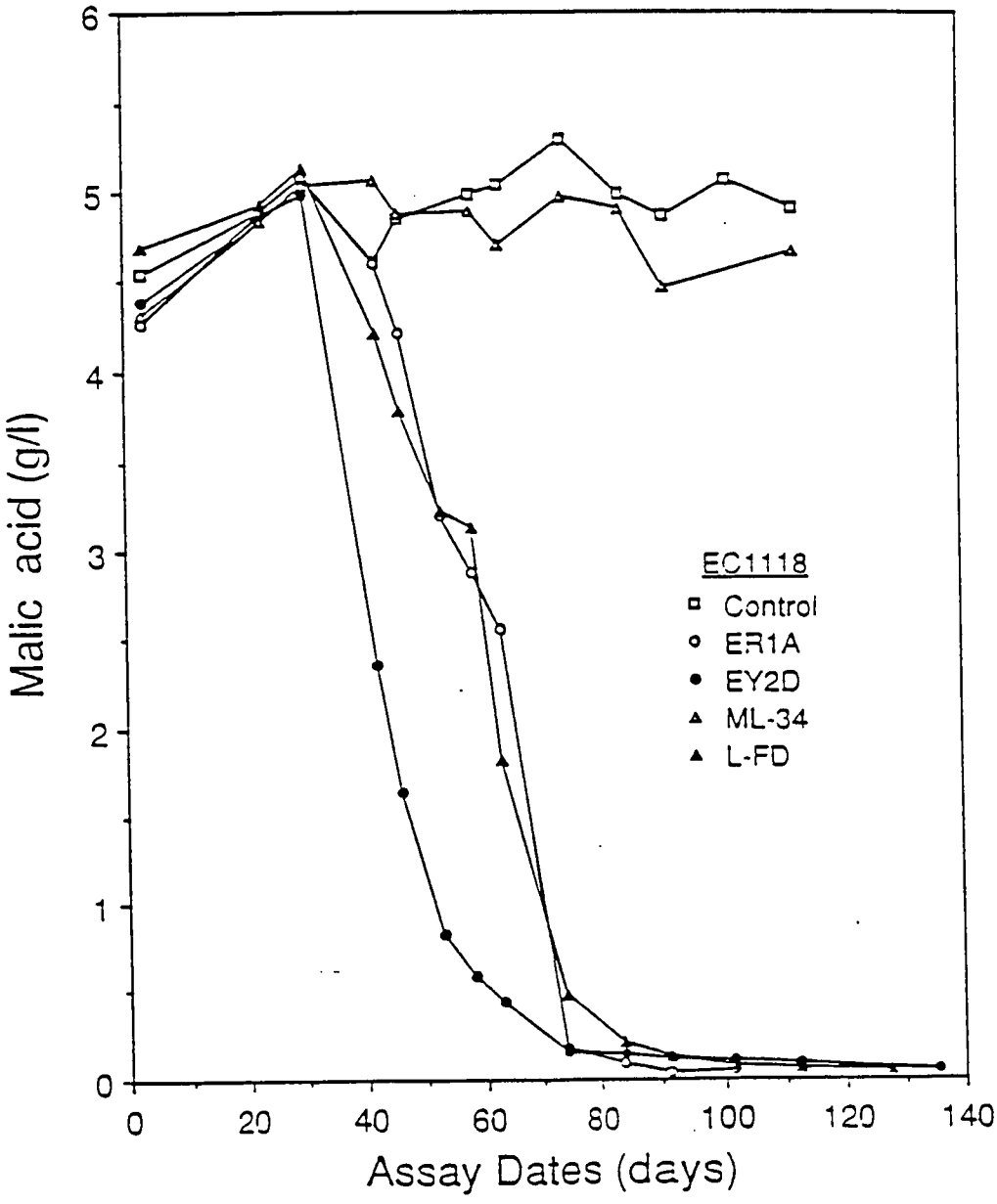


TABLE 7.3e. Means and standard deviations (in parentheses) of malic acid content (g/l) of HV86CH, EC1118 control wine and wines inoculated with different malolactic bacteria.

DATES	CONTROL	ER1A	EY2D	ML-34	L-FD	FERM. DAY
1/15/88	4.55 (0.20)	4.27 (0.31)	4.39 (0.37)	4.32 (0.48)	4.70 (1.36)	3
2/4/88	4.89 (0.10)	4.89 (0.14)	4.87 (0.16)	4.84 (0.03)	4.93 (0.13)	23
2/11/88	5.08 (0.10)	5.08 (0.34)	4.99 (0.19)	5.04 (0.11)	5.14 (0.20)	30
2/23/88	4.62 (0.04)	4.61 (0.43)	2.36 (0.76)	5.07 (0.30)	4.21 (0.40)	42
2/27/88	4.85 (0.15)	4.21 (1.09)	1.64 (0.75)	4.88 (0.12)	3.79 (0.71)	46
3/5/88	-	3.20 (1.88)	0.82 (0.55)	-	3.22 (1.47)	53
3/10/88	4.99 (0.13)	2.88 (1.82)	0..59 (0.45)	4.89 (0.06)	3.14 (1.69)	58
3/15/88	5.04 (0.15)	2.56 (1.87)	0.44 (0.40)	4.71 (0.15)	1.83 (1.38)	63
3/26/88	5.29 (0.18)	0.17 (0.16)	0.16 (0.14)	4.98 (0.04)	0.98 (0.77)	74
4/5/88	4.98 (0.22)	0.10 (0.10)	0.14 (0.16)	4.90 (0.18)	0.21 (0.24)	84
4/12/88	4.86 (0.15)	0.04 (0.01)	0.12 (0.13)	4.46 (0.13)	0.14 (0.18)	91
4/22/88	5.06 (0.27)	0.05 (0.01)	0.11 (0.10)	-	0.08 (0.10)	101
5/3/88	4.90 (0.20)	-	0.09 (0.07)	4.66 (0.11)	0.06 (0.05)	112
5/18/88	-	-	-	-	0.05 (0.04)	127
5/26/88	-	-	0.05 (0.01)	-	-	135

FIGURE 7.3e. Graph of malate utilization in Chardonnay HV86CH EC1118 control and MLF wines.



B.4. 10A81: No malolactic strain did very well with this yeast, (**Figure 6.3d**) By day 133, L-FD was the furthest ahead in malolactic fermentation, and yet still had more than 0.80 g/l malic acid (**Table 7.3d**). However, MLF proceeded slowly with the three active ML strains, ER1a, EY2d, and L-FD.

B.5. EC1118, Prise de Mousse: As seen in **Table 7.3e** and **Figure 7.3e**, MLF was completed for ER1a by day 91, and by day 112 for L-FD. Although EY2d lagged behind, three of the four wines completed MLF at about the same time as ER1a. This wine may have done better if the ML cultures had been inoculated two days after yeast inoculation, as was done for the other wines. However, because of a culture contamination problem, ML inoculation was delayed to day 12 of the primary yeast fermentation, at which point, the residual sugar level had dropped to about 10 ^{o}Brix. With one-half of the soluble solids utilized at this point, the ethanol content may have been about 5 to 6% at the time of ML inoculation. However, King and Beelman (1986) found that when grape juice was diluted from 20 ^{o}Brix to 10 ^{o}Brix with water, there was a reduced antagonistic effect between yeasts and malolactic bacteria making it possible to obtain both organisms in stationary phase simultaneously. This was important because they found that in the juice/wine system they used, the most severe bacterial inhibition resulted from actual yeast growth. If this is the case, then EC1118 should have allowed better growth and MLF from the bacterial inoculations than it did. Obviously other factors are involved in this matter, such as the possible

production of decanoic acid which is inhibitory to MLF (Edwards and Beelman, 1987).

C. Sensory Analysis: The results of the ANOVA statistical analyses are seen in **Table 7.4**. In all cases, with all yeasts, the differences in the treatments of the wines were very significant, i.e., at least at p=0.01. There was no significant variation between any of the sessions, nor was there significant variation caused by the panelists by treatments interactions, nor with the panelists by session interaction. Variations among the panelists did occur twice, which is to be expected. However, this did not affect the determination of differences in the treatment wines when compared to the control wine.

The LSD test for each of the yeast types (**Table 7.5**) clearly shows that the aroma of malolactic treated wines significantly differ from the aroma of the control wine at the p = 0.05 level. Only with the Epernay wine was a malolactic treated wine (EY2d) judged not significantly different from the control wine.

Overall, ML treated wines differed significantly from the control wines in aroma. However, the way in which the wines differed was not determined.

Additional sensory analysis in the form of descriptive analysis, plus an industrial panel for preference testing, would have been desirable, but time and resource constraints did not make this a practical consideration. Significant difference among panelists only occurred once out of 16 sets of sensory analyses. This does not effect the results of the determination of

TABLE 7.4. F-Values from the ANOVA for Difference From Control sensory analysis for aroma of Chardonnay HV86CH wines.

	MONT	EPER	EC1118	PAS.CH.
Treatments	11.47***	11.55***	6.09**	11.03***
Panelists	2.56	2.59	3.17*	3.30*
Pan. X Trts.	1.13	0.91	1.01	0.77
Sessions	0.26	1.28	1.05	2.33
Pan. X Ses.	1.11	0.92	0.57	0.49

*, **, ***, Significant at $p \leq 0.05$, 0.01, and 0.001 levels, respectively.

Table 7.5. Means and standard deviations of the Least Significant Difference tests of sensory analysis for aroma of Chardonnay HV86CH wines.

YEASTS	CONTROL	ER1A	EY2D	L-FD	LSD #
MONT	1.39^{c*}	4.25^{a}	3.48^{a}	2.84^{b}	1.04
	(1.17)	(1.96)	((2.30)	(1.50)	
EPERN	1.92^{b}	3.65^{a}	2.67^{b}	3.88^{a}	0.77
	(1.23)	(1.56)	(0.95)	(1.89)	
EC1118	2.19^{b}	3.56^{a}	3.56^{a}	4.38^{a}	1.06
	(1.23)	(2.06)	(1.67)	(2.20)	
PAS. CH.	1.67^{c}	3.94^{a}	3.15^{b}	2.85^{b}	0.86
	(1.22)	(1.79)	(2.30)	(1.27)	

* = Means in the same row with common superscripts are not significantly different at $p \geq 0.05$. Standard deviations are in parentheses below the given mean value.
# = Least Significant Difference.

aroma differences in the Difference From Control tests.

D. Organic Acid Analysis by High Performance (Pressure) Liquid Chromatography: For original chromatograms of the standards, and sample run, see Appendix pages 176 and 1717 Results of organic acid analysis of selected Chardonnay HV86CH wines are seen in **Tables 7.6a** and **b.** As indicated by the amount of malic acid in the MLF wines, samples were taken and analyzed before the wines had completed MLF.

There are two aspects to note from these tables. First, high acetic acid concentration greater than 600 mg/l was found only in the MLF wines. All controls had less than 600 mg/l acetic acid. Peynaud (1984) indicated that volatile acidity, which is primarily acetic acid, is noted in wines at about 600 ppm (mg/l). Acetic acid can be produced from carbohydrate or organic acid utilization by heterotrophic, lactic acid bacteria such as *Leuconostoc oenos* (Gottschalk, 1986). This may be one of the trade-offs when the must, which is high in glucose, is inoculated early with malolactic cultures. Davis *et al.* (1985) demonstrated that malolactic fermentation by *Leuconostoc oenos* was accompanied by degradation of malic, citric and fumaric acids with the production of lactic and acetic acids.

The second aspect to note is that propionic acid was found only in the control wines and not in the MLF wines. However, propanol, (GC data) was found in all wines. Propionic acid could possibly be reduced, in a two step pathway, to propanol, by way of propanal. In any case, propionic acid is

TABLE 7.6a. HPLC analyses for organic acids in selected Chardonnay HV86CH wines.

(mg/l)

NO.	ORGANIC ACID	PAS.CH. CONTROL	PAS.CH. ERIa	EPERNAY CONTROL	EPERNAY ER1a
1.	Citric acid	3.9	-	-	-
2.	Tartaric acid	2250.0	2809.8	2162.2	2951.9
3.	Malic acid	3141.8	1013.8	3426.2	679.7
4.	Succinic acid + shikimic acid *	5419.5	5919.5	5017.7	5025.1
5.	Lactic acid	2426.2	-	1973.1	-
6.	Acetic acid	571.8	900.5	556.7	693.3
7.	Propionic acid	737.2	-	109.9	-
8.	Isobutyric acid	28.6	-	-	-
9.	n-Butyric acid	-	-	55.1	-
10.	Isovaleric acid	31.9	-	21.4	1726.0

* Organic acids not separated by this column.

TABLE 7.6b. Continued HPLC analyses of Chardonnay HV86CH wines for organic acids.

(mg/l)

NO.	ORGANIC ACID	MONTRA. CONTROL	MONTRA. ER1a	EC1118 CONTROL	EC1118 ER1a
1.	Citric acid	-	-	-	-
2.	Tartaric acid	2250.8	1821.3	239.0	1928.4
3.	Malic acid	4544.5	872.3	3593.9	551.1
4.	Succinic acid + shikimic acid	5556.5	4261.9	4599.2	4593.3
5.	Lactic acid	-	1869.1	-	1958.8
6.	Acetic acid	307.1	740.0	394.0	7623.9
7.	Propionic acid	100.1	-	95.3	-
8.	Isobutyric acid	-	-	-	-
9.	n-Butyric acid	-	-	-	-
10.	Isovaleric acid	-	34.8	-	-

apparently utilized or changed by malolactic fermentation.

E. Gas Liquid Chromatography

Results of GLC (column B, 5% carbowax 20 M) on Chardonnay wines, which had completed MLF, are presented in **Tables 7.7(a-c)**. For the original chromatogram of GC standards and of sample wines, see Appendix, pages 178 and 179.

E.1. Control Wines: The control wines showed considerable variation between themselves in most compounds. This is probably indicative of the yeast strain. The Montrachet wine, which was most conducive to MLF, was low in ethyl butyrate, 2-methyl-1-butanol, and 2-furaldehyde, and highest in isoamyl alcohol, as compared to the other yeast strains. On the other hand, 10A81, which was least able to support MLF, was highest in acetaldehyde, methanol, propionaldehyde, ethyl acetate, and 2-methyl-1-butanol, while low in isobutanol, ethyl butyrate, and butyric acid.

Whether or not the above differences affected bacterial MLF is mostly conjecture at this point. Other unmeasured variables, *e.g.,* amino acid and vitamin balances, residual pentoses available, presence or absence of yeast-produced SO_2 and yeast produced steroids and/or fatty acids could play an important role in the yeast/malolactic bacteria interrelationship during malolactic fermentation.

E.2. Montrachet wines: The MLF wines inoculated with ER1a, EY2d, and L-FD were the most consistent in the types and amounts of each

TABLE 7.7a. Results of GLC, column B (5% Carbowax 20 M) of selected Chardonnay HV86CH wines.

REFERENCE COMPOUND	MONTRACHET CONTROL	EPERNAY CONTROL	PAS.CH. CONTRO	EC1118 CONTROL	10A81 CONTROL	EC1118 ER1A
1. Acetaldehyde	16.9	52.6	5.8	-	73.2	-
2. Methanol	68.0	48.5	61.3	58.2	74.7	50.6
3. Propanal	4.6	-	-	-	70.0	-
4. Acetone	-	-	-	8.7	-	9.2
5. Ethanol (%)	12.4	13.1	11.1	12.9	12.5	11.2
6. Ethyl acetate	51.3	77.1	103.3	72.3	125.9	102.8
7. 1-Propanol	19.1	19.0	16.0	41.2	35.8	42.4
8. Isobutanol	18.4	13.7	20.9	14.7	9.9	16.4
9. Ethyl butyrate	2.3	12.6	0.9	4.3	-	23.6
10. 2-Methyl-1-butanol	22.5	109.5	119.6	65.2	154.9	106.3
11. Isoamyl alcohol	131.7	96.8	128.7	130.0	95.6	140.7
12. Isoamyl acetate	1.8	-	0.7	-	-	-
13. 2-Furfural	0.7	1.4	7.0	11.5	2.6	12.2
14. 1-Hexanol	5.3	3.9	3.7	-	4.0	17.9
15. Isobutyric acid	21.3	8.8	-	25.2	15.0	154.6
16. Butyric acid	48.1	-	-	117.13	2.7	-

Values in mg/l.

TABLE 7.7b. Continued results of GLC (column B) analyses of selected Chardonnay HV86CH wines.

	COMPOUND	EC1118 EY2D	EC1118 L-FD	PAS.CH. ER1A	PAS.CH. EY2D	PAS.CH. L-FD	EPER. ER1A	EPER. EY2D
1.	Acetaldehyde	-	14.0	18.7	-	-	20.1	0.85
2.	Methanol	52.1	47.9	64.3	73.5	45.5	49.6	46.3
3.	Propanal	-	7.3	-	-	-	-	-
4.	Acetone	-	10.0	-	13.1	-	-	-
5.	Ethanol (%)	12.4	11.3	14.3	16.3	10.3	12.6	13.3
6.	Ethyl acetate	58.4	95.2	140.3	146.8	149.8	99.2	149.1
7.	1-Propanol	36.6	45.6	18.1	16.2	17.3	15.3	14.8
8.	Isobutanol	14.1	16.9	22.9	23.5	24.4	13.4	13.5
9.	Ethyl butyrate	22.2	4.1	1.2	2.0	3.3	-	4.4
10.	2-Methyl-1-butanol + acetic acid *	84.0	94.9	172.1	192.3	138.5	102.3	102.7
11.	Isoamyl alcohol	125.2	129.1	128.0	126.9	135.6	89.6	88.5
12.	Isoamyl acetate	-	-	9.0	-	-	1.2	1.4
13.	2-Furfural	10.7	13.2	8.0	7.6	4.2	-	2.8
14.	1-Hexanol	2.6	15.9	4.2	5.7	3.3	2.2	2.8
15.	Isobutyric acid	20.4	-	-	38.9	28.8	17.6	4.9
16.	Butyric acid	100.3	-	457.8	41.4	126.7	158.7	-
17.	ML Peak: RT	13.69	13.66	13.59	13.71	13.73	13.86	13.69
	AREA	1545650	1239400	1794900	2173050	1670233	1020925	1001300

* These compounds cannot be separated by this column. All values in mg/l.

TABLE 7.7c. Continued results of GLC (column B) analyses of selected Chardonnay HV86CH wines.

(mg/l)

COMPOUND	EPER L-FD	MONT ER1A	MONT EY2D	MONT L-FD
Acetaldehyde	42.9	10.8	12.8	7.6
Methanol	46.4	61.0	51.5	50.4
Propanal	-	-	-	-
Acetone	-	-	-	-
Ethanol (%)	11.2	12.0	12.2	11.9
Ethyl acetate	77.4	69.5	55.0	60.1
Diacetyl	-	-	58.3	-
1-Propanol	15.0	15.6	15.4	14.9
Isobutanol	18.5	25.0	25.3	24.8
Ethyl butyrate	1.7	1.6	6.8	0.6
2-me-1-Butanol + acetic acid *	30.1	46.2	36.2	33.9
Isoamyl alcohol	82.4	96.5	103.6	102.9
Isoamyl acetate	-	1.5	1.6	1.8
2-Furfural	-	-	-	-
1-Hexanol	1.6	1.4	1.7	1.5
Isobutyric acid	-	-	19.5	10.6
Butyric acid	-	-	8.5	-
"ML Peak" - RT *	13.94	13.99	13.82	13.90
Area **	45985	1087150	748303	1020405

* Relative retention time in minutes on column B. ** Integrator area.

compound measured. However, when compared to the control wine, the MLF wines showed greater amounts of isobutanol, but less hexanol and isoamyl alcohol. The "ML Peak" was found in all wines that underwent malolactic fermentation regardless of yeast strain.

E.3. Epernay 2: Compared to ER1a and EY2d, wines of L-FD had less ethyl acetate, ethyl butyrate and no isobutyrate, but more acetaldehyde and isobutanol. The MLF wines, in general, had considerably less acetaldehyde and ethyl butyrate, but more ethyl acetate than the control.

E.4. Pasteur Champagne: Two of the three MLF wines did not contain acetaldehyde, acetone, or isoamyl acetate. All other values were consistent with each other, except for the questionable high value of butyric acid in the ER1a wines. The MLF wines were higher in ethyl butyrate and hexanol than the control wine. Neither butyric acid nor isobutyric acid were found in the control wine, whereas both compounds were measured in the MLF wines.

E.5. EC1118: Acetaldehyde was present only in L-FD and ER1a wines, and was absent in both EY2d and control wines. Both ER1a and EY2d inoculated wines had significantly more ethyl butyrate than the L-FD and control wines. 1-Hexanol was not found in the control wine, but was found in small amounts in the MLF wines. Butyric and isobutyric acids were inconsistent among the MLF wines.

In general, the compounds of column B (80/100 B AW, 5% Carbowax 20M) analysis did not show a consistent pattern of increasing or decreasing

amounts of compounds measured by MLF when compared to the controls. For example, where Montrachet MLF wines decreased in 1-hexanol, as compared to the control, Pasteur Champagne and EC1118 MLF wines increased their 1-hexanol content. In the MLF wines of Epernay 2, ethyl butyrate decreased while in Pasteur Champagne MLF wines, it increased. Ducret *et al.* (1983) noted that 1-hexanol was found in wines from crushed grapes, but not in wines from carbonic maceration type of processing.

Simpson and Miller (1984), determined aroma compounds of a Chardonnay wine and identified 150 components by GC-MS. They found that 90 of 140 compounds could be grouped into 4 categories of alcohols, acetals, esters and others. The latter was composed of aldehydes, ketones, aromatic hydrocarbons and monoterpene alcohols and oxides. The ethyl esters of C_4 to C_{12} fatty acids were important contributors to aroma of Chardonnay wines. They found that the flavor threshold of ethyl-n-hexanoate was exceeded in all wines, and that the ethyl esters of butanoate, octanoate, and decanoate probably enhanced estery and wine-like aromas.

Ethyl lactate, which could not be separated from other compounds on our columns, occurs from esterification of lactic acid as influenced by malolactic fermentation (Meunier and Bott, 1979; Shinohara *et al.*, 1979). Pilone *et al.* (1966) found that the one compound that increased significantly in MLF wines was ethyl lactate.

Isoamyl alcohol was a major peak in our control and MLF wines. Kunkee (1984) reported that some wild type yeasts decarboxylate and reduce alpha-keto acids to isoamyl alcohol. Webb and Ingraham (1962) stated that isoamyl alcohol was produced from leucine by the Ehrlich reaction. Amerine *et al.* (1980) found that active amyl and isoamyl alcohols were major contributors to wine aroma. Other fusel alcohols found, which contributed to aroma and flavor, included 2-methyl-1-propanol (isobutanol), and 1-hexanol. See Appendices, pages 189-192, for tables of aroma characters and thresholds values of volatile compounds.

Pilone *et al.* (1966) identified hexanol, ethyl caprylate, isoamyl caproate, diethyl succinate, ethyl caprate, isoamyl caprylate, and 2-phenethyl alcohol in red wines that had undergone malolactic fermentation.

Goranov (1983) in examining volatiles of Bulgarian wines, determined that the ratio of esters to aldehydes, and the ratio of isobutanol to isoamyl alcohol could be used as an indicator to good sensory quality. He noted that wine storage in casks seem to cause an increase in the ester content of wines.

The one consistent expression of malolactic fermentation seen in both columns' chromatographs, was the "ML Peak", found in all wines that underwent MLF. Pilone *et al.* (1986) also found an unidentified peak, in malolactic fermented wines, but not in the controls, and determined that it was not isobutyl caproate.

TABLE 7.8a. Results of GLC, column A (3.5% Carbowax 20 M) analysis of selected Chardonnay HV86CH wines.

	COMPOUND	MONT. CONTROL	MONT. ER1A	EPER. CONTROL	EPER. ER1A	EPER. EY2D	PAS.CH. CONTROL	PAS.CH. ER1A
1.	Ethanol	13.2	13.9	13.3	12.8	12.9	13.5	12.8
2.	Isobutanal	51.0	26.0	26.7	26.9	25.5	30.1	25.0
3.	Butanal	-	-	7.9	-	-	-	-
4.	Isobutanol	17.4	48.0	36.3	-	-	22.7	-
5.	Isopentanal	12.1	15.1	8.4	6.0	6.3	9.7	9.7
6.	Pentanal	-	-	5.9	-	-	-	-
7.	2,3-Pentanedione	-	-	5.4	-	-	-	-
8.	Ethyl propionate	-	-	6.2	-	-	-	-
9.	2-Methyl-1-butanol	13.1	16.4	8.7	9.9	8.4	14.2	10.3
10.	Ethyl isobutyrate	-	-	-	1.0	-	-	-
11.	Isobutyl acetate	19.4	-	22.8	-	-	28.9	-
12.	Butyl acetate	-	-	5.9	5.0	-	-	-
13.	ML peak: RT		21.21		20.08		21.29	20.85
	AREA		434687		771327		963680	1146900

All values are in mg/l.

TABLE 7.8b. Continued GLC (column A) analyses of selected Chardonnay HV86CH wines.

(values in mg/l)

COMPOUND	EC1118 CONTROL	EC1118 ER1A
1. Ethanol (%)	12.9	12.5
2. Isobutanol	25.0	55.0
3. Butanal	-	-
4. Isobutanol	11.2	12.1
5. Isopentanal (isovaleraldehyde)	8.1	7.1
6. Pentanal	-	-
7. 2,3-Pentanedione	-	-
8. Ethyl propionate	-	-
9. 2-Methyle-1-Butanol	10.0	10.5
10. Ethyl isobutyrate + 1-pentanol *	-	-
11. Isobutyl acetate	24.7	-
12. Butyl acetate	-	-
13. "ML peak" RT:	-	20.80
Area:	-	804530

* These compounds could not be separated by this column.
RT = relative retention time. Area refers to the integrator peak area as relates to this column since there is no standard for comparison for quantitation.

E.6. Column A (3.35% Carbowax 20 M) The results of gas liquid chromatography on selected Chardonnay, HV86CH wines, analyzed on column A, can be followed in **Tables 7.8a** and **7.8b.** In the Montrachet wines, ER1a had about one-half the amount of isobutyraldehyde than the control wine, but concurrently increased by about the same amount in isobutanol, which was not found in the control wine. This suggests a reduction of isobutyraldehyde to isobutanol by the malolactic bacteria. These reactions are usually linked to the oxidation of some other compound such as $NADH^{+}+H^{+}$ to NAD^{+}. The proposed reaction is seen in **Figure 7.4.**

In both Epernay 2 and Pasteur Champagne wines, the control wine contained isobutanol and isobutyl acetate, which were not found in the MLF wines.

In EC1118 (Prise de Mousse) wine, the MLF wines had double the amount of isobutyraldehyde while isobutyl acetate was not found when compared to the control. This is nearly stoichiometric, and suggests that the 25 mg/l isobutyl acetate of the control was converted to isobutyraldehyde by the malolactic organism, ER1a, during malolactic fermentation. This hydrolytic reaction, as shown in **Figure 7.5**, is suggested in both the Montrachet, and EC1118 wines.

Figure 7.4. Possible reaction by malolactic bacteria during malolactic fermentation of Chardonnay wine, HV86CH.

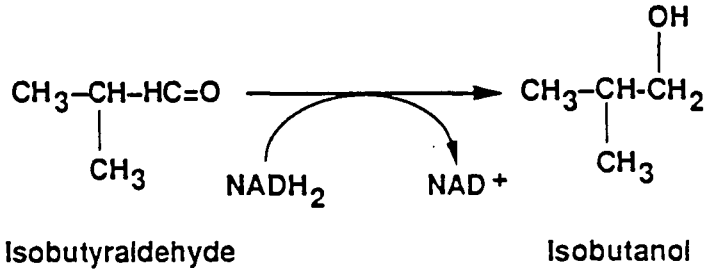
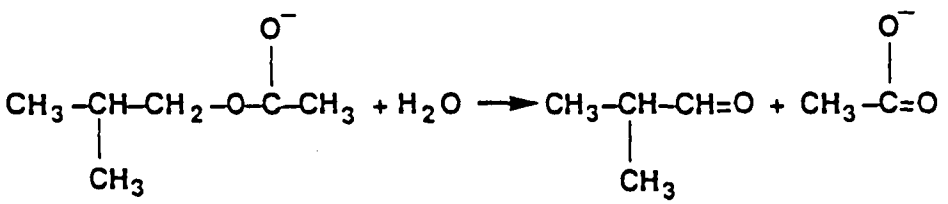


FIGURE 7.5. Proposed reaction during malolactic fermentation of Chardonnay wine by malolactic bacteria.



Isobutyl Acetate

Isobutyraldehyde + Acetate

Other compounds for which standards were made and retention times determined for GLC analysis included: butyraldehyde, hexanal, ethyl lactate, isopropanol, diacetyl, 3-methyl-2-butanol, acetoin and n-amyl alcohol.

F. Gas Chromatography-Mass Spectroscopy

On two Montrachet wine samples, GC/MS analysis was done by a purge and trap system as opposed to volatile, chemical extraction. Control wine compounds, identified by mass spectroscopy, with a fit of 900 or better, included: ethyl acetate, 3-methyl-1-butanol, (isoamyl alcohol), ethyl butyrate, 3-methyl-1-butanol-acetate, (isoamyl acetate), and 2,3-butanediol.

In the Montrachet, ER1a, malolactic fermented wine, the following compounds were identified and confirmed: ethyl acetate, 3-methyl-1-butanol, ethyl butyrate, acetaldehyde, ethanol, 4-methyl-3-pentenoic acid, methyl acetate, ethyl-hexanoic acid, and hexyl-acetate. For original chromatograms, see Appendix, pages 179 and 180. For original mass spectrum computer read-out, see Appendix, page 181 and 182. It was thought that the compounds, 3-methyl-1-butyl acetate or 2-methyl-1-butyl acetate, (active amyl acetate), might be the mystery "ML peak". These compounds were obtained (Fluka Chemical Corp., Ronkonkoma, New York), and 100 mg/l (ppm) standards in 12% ethanol were made up and added to a Montrachet, ER1a wine sample, as a spike. In both cases, a double peak on the chromatogram appeared and the ML peak did not increase in area or peak height. Therefore, these compound were ruled out as the "ML peak".

GC-MS analyses, through the Department of Agricultural Chemistry, Oregon State University, were done on Chardonnay, Epernay wine samples, which had been Freon-extracted for volatiles.

Chromatograms for the control wine can be seen in Appendix, pages 183 and 184, and the chromatograms for the ER1a, MLF wine are seen in Appendix, pages 185 and 186. For original mass spectrum with computer readout see Appendix pages187 and 188. A summary of the compounds identified are seen in **Table 7.9.** The malolactic fermented wine had four, late, additional peaks on the chromatogram. The spectra for these compounds were subjected to two different computer library searches for identification; yet the best computer fit for any of these compounds was 833 (83%), which is still less than 900. Therefore these identifications are considered tentative. Most of the compounds identified have been associated with wine yeast fermentation. Our GC-MS analyses recognized many of the compounds Suomalainen (1967) identified as those formed by yeast in alcoholic beverages, as seen in **Table 7.10.** Altogether, eight compounds were identified in malolactic fermented wines, but not found in the control wines. These include:

1. 4-methyl-3-pentenoic acid
2. methyl acetate (sweet, solvent-like aroma)
3. ethyl hexanoate (fruity, rum-like aroma)
4. hexyl acetate (fruity aroma)
5. 1,12-tridecadiene

Table 7.9. Compounds found in Freon 114 extractions of Chardonnay, Epernay wines by GC/MS.

CONTROL WINE	ER1A (MLF) WINE
Decanoic acid	Decanoic acid
2-Phenethanol	2-Phenethanol
Hexanoic acid	Hexanoic acid
Ethyl octanoate	Ethyl octanoate
n-Hexanol	
Octanoic acid	Octanoic acid
Ethyl lactate/2,3-butanediol	Ethyl lactate/ethanol,2,2,1-oxy bis
Isoamyl alcohol	Isoamyl alcohol
acetic acid	
1H-Imidazole	1H-Imidazole *
	1,12-Tridecadiene *
	Hexadecanoic acid *
	1,2-Benzene dicarboxylic acid *
	Farnesol * (or)
	2,6,10,18,22-Tetracosahexae *

* Spectral fit less than 900.

Table 7.10. Yeast produced compounds in alcoholic beverages, identified by Suomalainen (1967) by GC-MS.

1. Aldehydes.

Acetaldehyde *

2-Methyl butyraldehyde

Glyoxal

Isovaleraldehyde * (Isopentanal)

Propionaldehyde * (Propanal)

2-hexanal *

Butyraldehyde * (Butanal)

Isobutyraldehyde * (Isobutanal)

2. Diketones.

Diacetyl *

2,3-Pentanedione

3. Fusel alcohols

1-Propanol *

2-Methyl-1-propanol

2-Methyl-1-butanol*

3-Methyl-1-butanol *

Phenethyl alcohol *

4. Fatty acid esters

Diethyl succinate

Ethyl lactate *

* Compounds identified in this study.

6. hexadecanoic acid
7. 1,2-benzendicarboxylic acid * or farnesol * #
8. 2,6,10,14,18,22-tetracosahexae *

* Spectral fit less than 900.

This is a cyclic terpene which is a type of compound commonly found in the essences of the perfume industry.

It is not known at present if these compounds are involved with the unique "ML" peak in the chromatograms of malolactic fermented wines. Further research is needed to determine this, as well as the threshold levels, and the contribution to flavor and/or aroma of wines. A compilation of compounds associated with wine flavors and aroma, threshold values and aroma characteristics can be seen in the Appendix, pages 189-191.

SUMMARY

In summary, HV86CH Chardonnay Epernay-yeasted wines had the greatest difficulty in completing alcoholic fermentation; and Pasteur Champagne had the best success in completing alcoholic fermentation.

Montrachet yeast allowed the fastest and most complete MLF by all fermenting strains; and 10A81 was the most inhibitive to successful completion of MLF.

The malolactic bacterial strains that did the best were ER1a, L-FD, and EY2d. ML-34 died out in all wines and did not grow or carry out any MLF.

Lalvin freeze dried starter culture (L-FD) is really a mixture of four different strains of *Leuconostoc oenos*.

Sensory analysis of Chardonnay wine aroma, by "Difference From Control" test, indicated that there was a significant difference between wines. The Least Significance Difference Test confirmed that the differences occurred between the control and malolactic fermented wines indicating that malolactic fermentation is contributing to the aroma of Chardonnay wine.

Organic acid analysis indicated that MLF wines increased in acetic acid while propionic acid disappeared.

Volatiles analyses, by GC and GC-MS, of MLF wines, identified eight compounds not found in the control wines. These included four compounds by a head space purge and trap system, that have a mass spectral fit greater than 900; and 4 compounds from a Freon extraction of volatiles, with a mass spectral fit less than 900. Whether any of these compounds are associated with the "ML" peak of MLF wines as seen by GLC is not known at this time.

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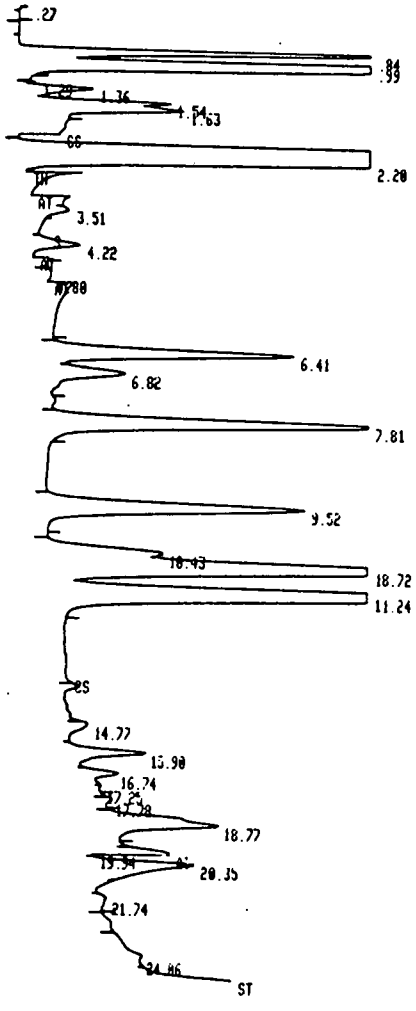
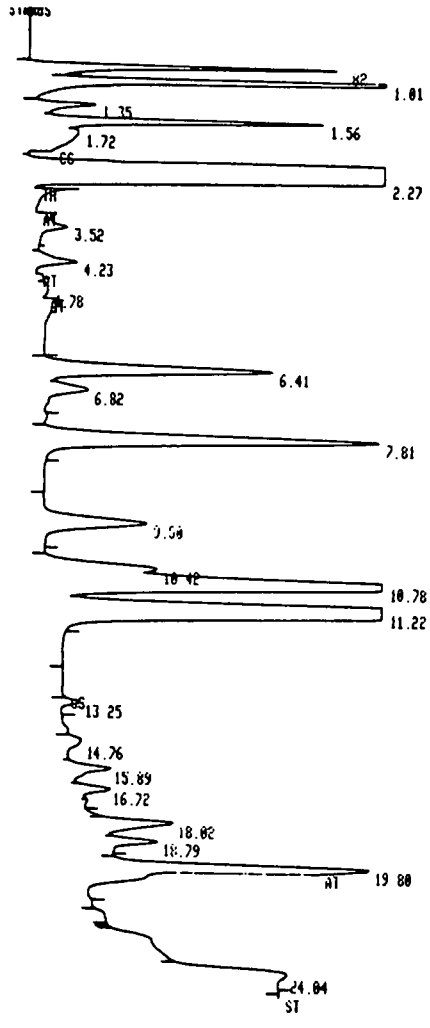
APPENDICES

Appendix A. GLC chromatograms of column B of Chardonnay HV86CH control wines.





RUN # 112 JUL/02/07 15:44:21

ISTD

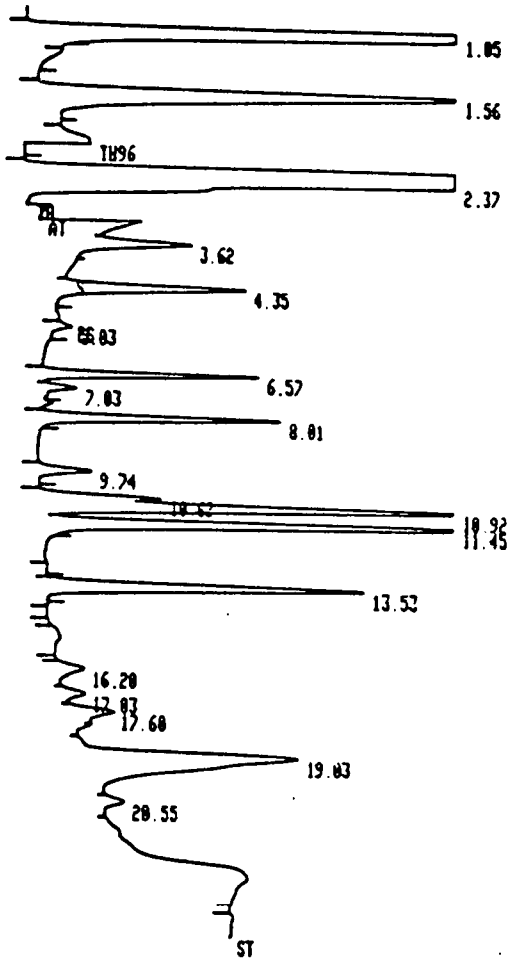
RT	AREA	TYPE	CAL#	AMOUNT
0.84	651980	PV		0.000
0.99	2387800	VB	1R	318.748
1.36	87269	VP		0.000
1.54	158660	PV	2R	24.076
1.63	240680	VB	3R	31.975
2.20	2.1014E+00	†SP8	5R	20.010
3.51	60252	TB8	7R	11.795
4.22	300250	00	9R	24.532
6.41	506020	0V	12R	27.700
6.82	179860	V0		0.000
7.81	770880	BB	14&	49.000
9.52	658640	00		0.000
10.43	221260	PV		0.008
10.72	1350200	VV		0.000
11.24	1670400	V0	16R	402.050
14.77	60139	0P		0.000
15.90	239640	PP		0.000
16.74	97397	PV		0.000
17.25	20197	V0		0.000
17.70	15857	0B	20R	1.747
18.77	5739[illegible]	BB		0.000

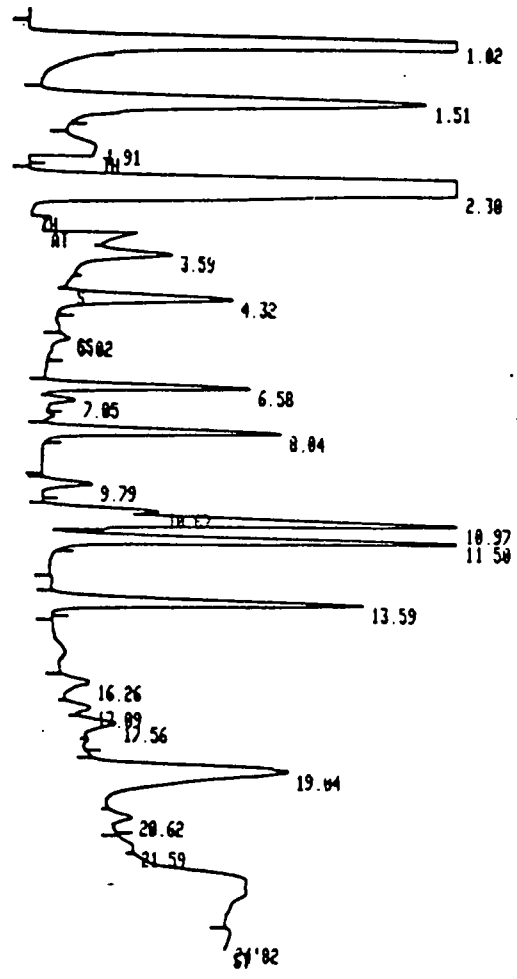
RUN # 113 JUL/02/07 16:17:31

ISTD

RT	AREA	TYPE	CAL#	AMOUNT
0.82	353620	PB		0.000
1.01	1208000	PB	1R	166.890
1.35	115160	PV		0.000
1.56	459510	VV	2R	71.777
1.72	237710	VN	4R	22.013
2.27	2.3814E+00	†SH0	5R	23.306
3.52	121450	TBP	7R	21.605
4.23	272490	TPP	9R	22.910
4.70	44494	TPP		0.000
6.41	470050	PV	12R	26.407
6.82	105340	V0		0.000
7.81	748898	0B	14&	49.000
9.58	259040	P0		0.000
10.42	211230	PV		0.000
10.70	1286900	VV		0.000
11.22	2470800	VB	16R	609.230
13.25	47843	0B	18R	4.450
14.76	63215	BP		0.000
15.89	146430	PP		0.000
16.72	98947	PV		0.000

Appendix B. GLC chromatograms of column B of Chardonnay HV86CH malolactic fermented wines. A : Montrachet ER1a; B : Montrachet EY2d.





RUN # 109 JUL/28/87 09:09:15

ISTD

RT	AREA	TYPE	CAL#	AMOUNT
1.05	2408900	PB	1R	462.650
1.56	748300	PB	2R	110.320
2.37	1.8235E+08	†SBB	6R	12.772
3.62	209690	TBB	BR	23.411
4.35	422700	BB	10R	30.761
6.57	665280	PV	14R	36.477
7.03	113540	VB		0.000
8.01	844120	BB	15R	49.000
9.74	210170	BB	17R	17.007
10.62	396690	PV		0.000
10.92	1967880	VV	18R	104.190
11.45	1952500	VB		0.000
13.53	1427580	BB	20R	105.990
16.20	161290	BP	22R	11.310
17.03	106470	PV		0.000
17.60	267300	VV		0.000
19.03	2313480	PV		0.000
20.55	142870	VB		0.000

RUN # 112 JUL/28/87 10:51:12

ISTD

RT	AREA	TYPE	CAL#	AMOUNT
1.02	2067900	PB	1R	390.460
1.51	707420	PB	2R	102.530
2.30	2.0197E+08	†SPB	6R	13.908
3.59	180240	TBB	BR	19.783
4.32	413730	BB	10R	29.600
6.58	652330	PV	14R	35.163
7.05	106900	VB		0.000
8.04	858610	BB	15R	49.000
9.79	196530	BB	17R	15.635
10.67	391380	BV		0.000
10.97	1935000	VV	18R	100.720
11.50	1935400	VB		0.000
13.59	1431700	BB		0.000
16.26	169160	BP	22R	11.661
17.09	106640	PV		0.000
17.56	268290	VV		0.000
19.04	2001200	BV		0.000
20.62	109830	VB		0.000

Appendix C. Ballot for aroma, Difference-From-Control sensory test of Chardonnay Wines.

No.____ Name _____
Date _____

CHARDONNAY

AROMA SENSORY TEST

Instructions:

You will be given a tray with 5 glasses of wine. One will be labeled "CONTROL" and the remaining 4 will be designated by a random 3-digit code number.

Record on the sheet the sample code number and then smell the CONTROL wine first. Replace the glass cover after smelling each wine. Refer back to the control wine as needed.

Rate each coded sample wine to indicate perceived difference in aroma if any, from the control wine by placing a checkmark at the appropiate place on the scale.

Please feel free to make any comments. Enjoy!

	Sample Number (_____)	Sample Number (_____)	Sample Number (_____)	Sample Number (_____)
No Difference				
Extreme Difference				

COMMENTS:

Thankyou for your participation! You may taste the wines after you have completed the AROMA sensory testing.

Dick Avedovech

Appendix D. Master sheets for data for aroma Difference From Control Sensory test of Chardonnay wines.

MASTER SHEET

HV86CH CHARDONNAY WINE: WINE YEAST/MALOLACTIC BACTERIAL CONBINATIONS
DIFFERENCE FROM CONTROL: DATA RECORD OF HV86CH WINE SENSORY AROMA TEST

INITIAL_____, (OR), REPLICATION_____.

Yeast Type:_____.

Date:_____Day #:_____

KEY: 1=_____
2=_____
3=_____
4=_____

PANELIST NAME	TRAY NUMBER	SEQUENCE NUMBER	CODE NUMBER	DIFFERENCE VALUE
	1.	1		
		2		
		3		
		4		
	2.	1		
		3		
		4		
		2		
	3.	1		
		4		
		2		
		3		
	4.	2		
		1		
		3		
		4		
	5.	2		
		3		
		4		
		1		
	6.	2		
		4		
		1		
		3		

Notes or Comments:

Continued master sheets

Initial___ or Replication__

Yeast Type:_____

Date:_____ Day #:_____

Key: 1=_____
2=_____
3=_____
4=_____

PANELIST NAME	TRAY NUMBER	SEQUENCE NUMBER	CODE NUMBER	DIFFERENCE VALUE
	7.	3		
		1		
		2		
		4		
	8.	3		
		2		
		4		
		1		
	9.	3		
		4		
		1		
		2		
	10.	4		
		1		
		2		
		3		
	11.	4		
		3		
		1		
		2		
	12.	4		
		2		
		3		
		1		

NOTES OR COMMENTS:

Continued master sheets

Initial___ or Replication__

Yeast Type:_____

Date:_____ Day #:_____

Key: 1=_____
2=_____
3=_____
4=_____

PANELIST NAME	TRAY NUMBER	SEQUENCE NUMBER	CODE NUMBER	DIFFERENCE VALUE
	13.	3		
		4		
		1		
		2		
	14.	3		
		2		
		4		
		1		
	15.	3		
		1		
		2		
		4		
	16.	4		
		3		
		1		
		2		
	17.	4		
		2		
		3		
		1		
	18.	4		
		1		
		2		
		3		

NOTES OR COMMENTS:

Continued master sheets

Initial___ or Replication__

Yeast Type:_____

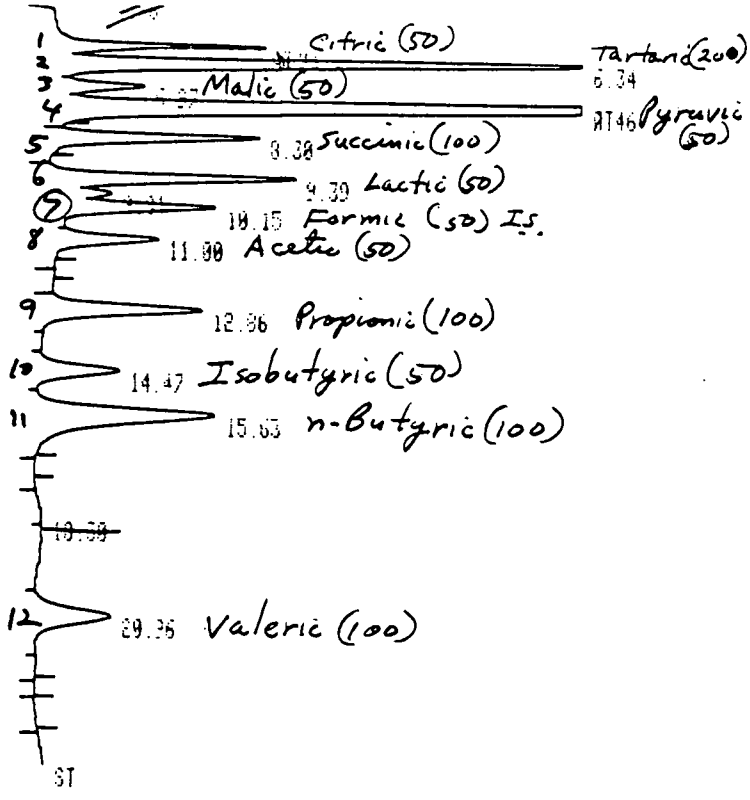
Date:_____ Day #:_____

Key: 1=_____
2=_____
3=_____
4=_____

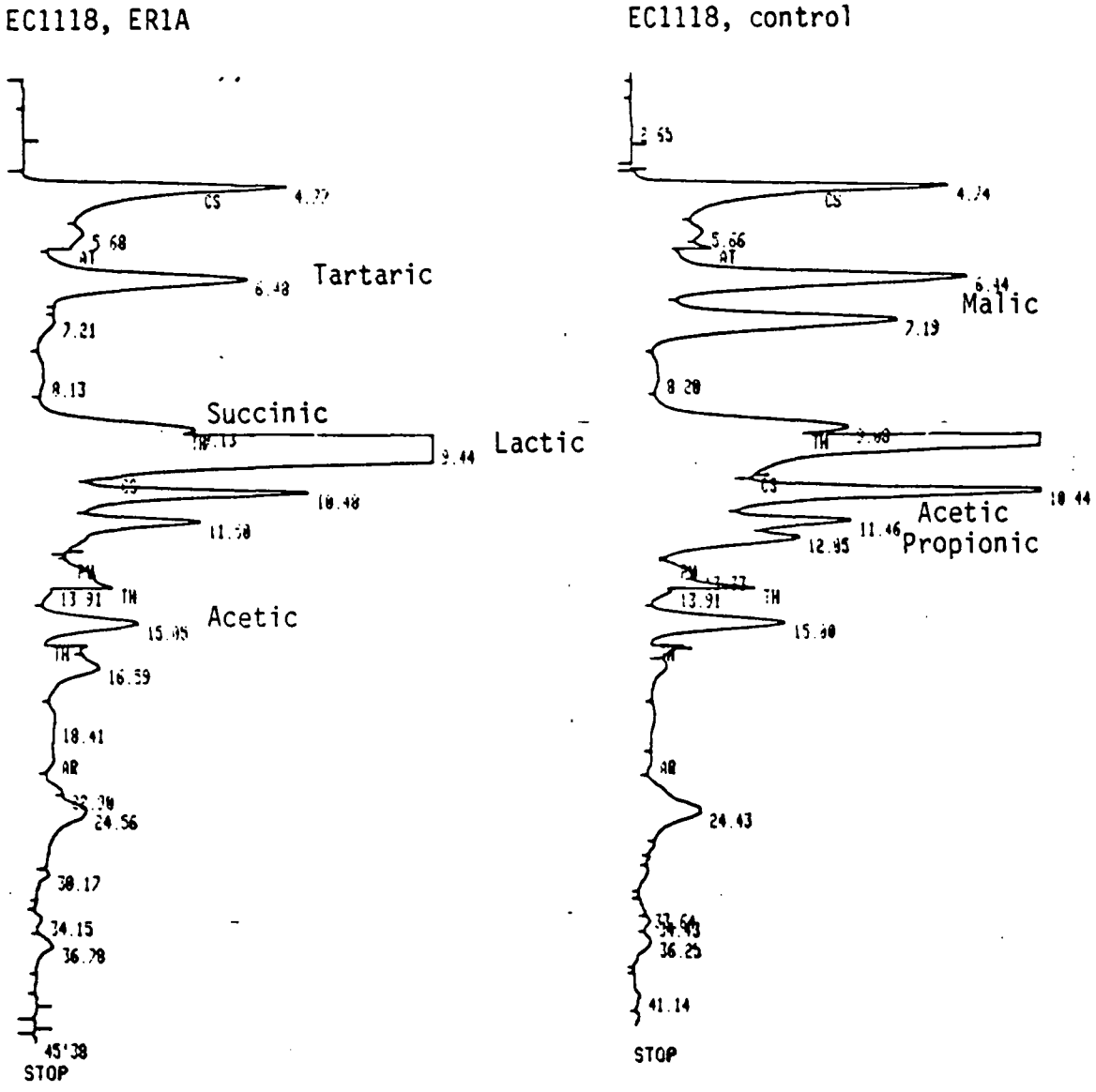
PANELIST NAME	TRAY NUMBER	SEQUENCE NUMBER	CODE NUMBER	DIFFERENCE VALUE
	19.	1		
		2		
		3		
		4		
	20.	1		
		3		
		4		
		2		
	21.	1		
		4		
		2		
		3		
	22.	2		
		1		
		3		
		4		
	23.	2		
		3		
		4		
		1		
	24.	2		
		4		
		1		
		3		

NOTES OR COMMENTS:

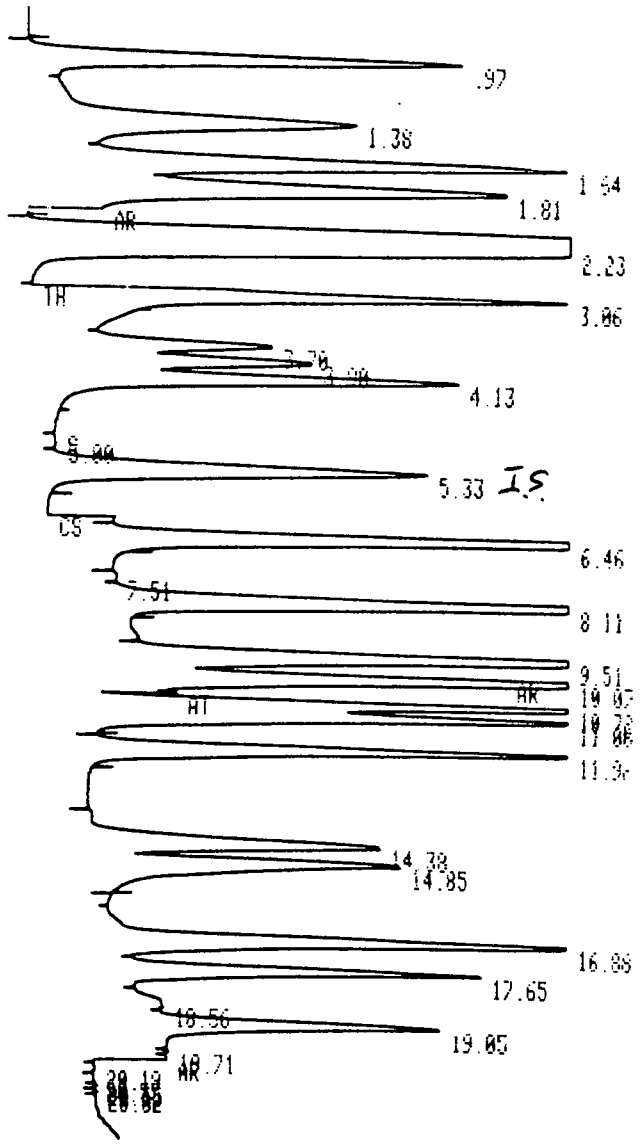
Appendix E. Chromatogram standards used in high performance liquid chromatography analysis of HV86CH Chardonnay wines for organic acids.



Appendix F. Chromatogram of organic acids in HV86CH Chardonnay wine samples determined by HPLC. A : EC1118 Control, B : Montrachet ER1a.

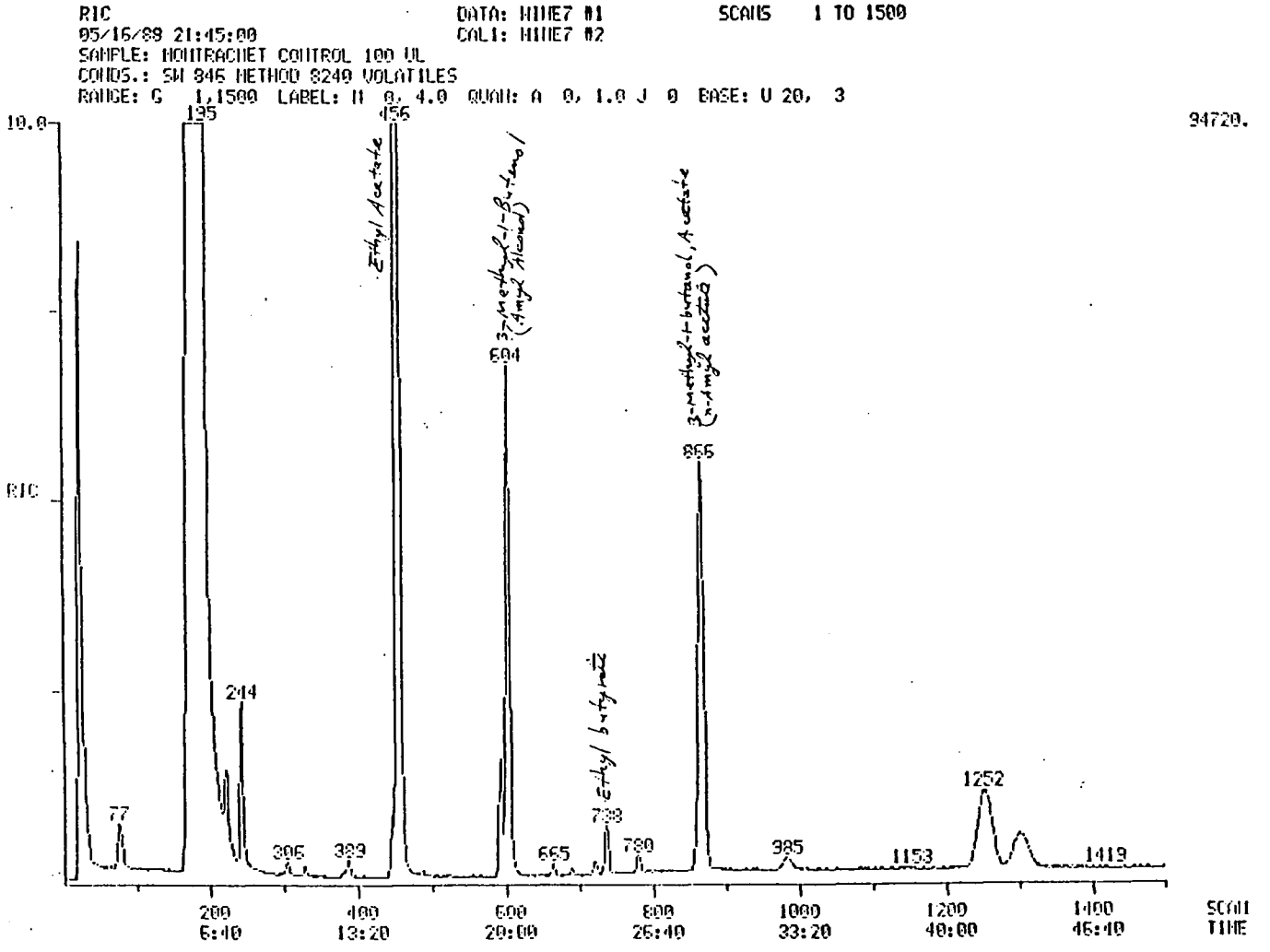


Appendix G. Chromatogram of volatiles standards used in gas liquid chromatography analysis of HV86CH Chardonnay wines.

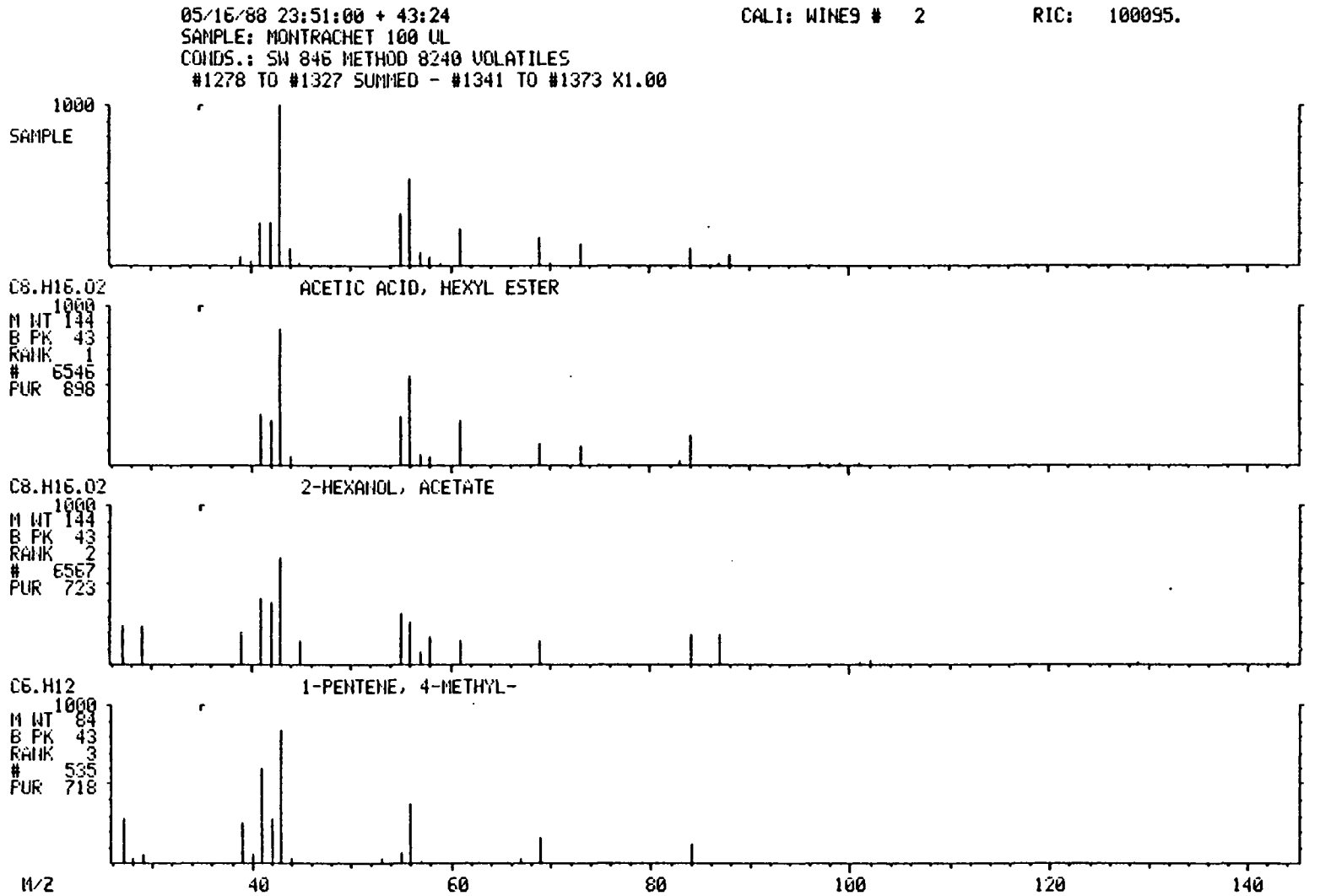


ISTD RT	AREA	TYPE	CAL#
0.42	11414	D BB	
0.97	918250	D PV	1R
1.38	1146900	VV	2R
1.64	1357400	D VV	3R
1.81	1382700	D VB	4R
2.23	1.9847E+08	†SBB	5R
3.06	1173300	TBB	6R
3.70	707340	TBV	7R
3.90	939780	TVV	8R
4.13	1623800	TVB	9R
5.00	7759	BV	
5.33	1783200	VB	10R
6.46	2243400	PB	11R
8.11	2080700	PB	12R
9.51	1620100	PV	13R
10.07	1390800	VV	14R
10.72	2451800	VV	15R
11.06	1906400	VB	16R
11.92	2007000	BB	17R
14.38	1285000	PV	18R
14.85	1539100	VB	19R
16.88	2074700	PV	20R
17.65	1315300	VP	21R
18.56	123840	PV	
19.05	1271500	VV	22R
19.71	27335	D VV	

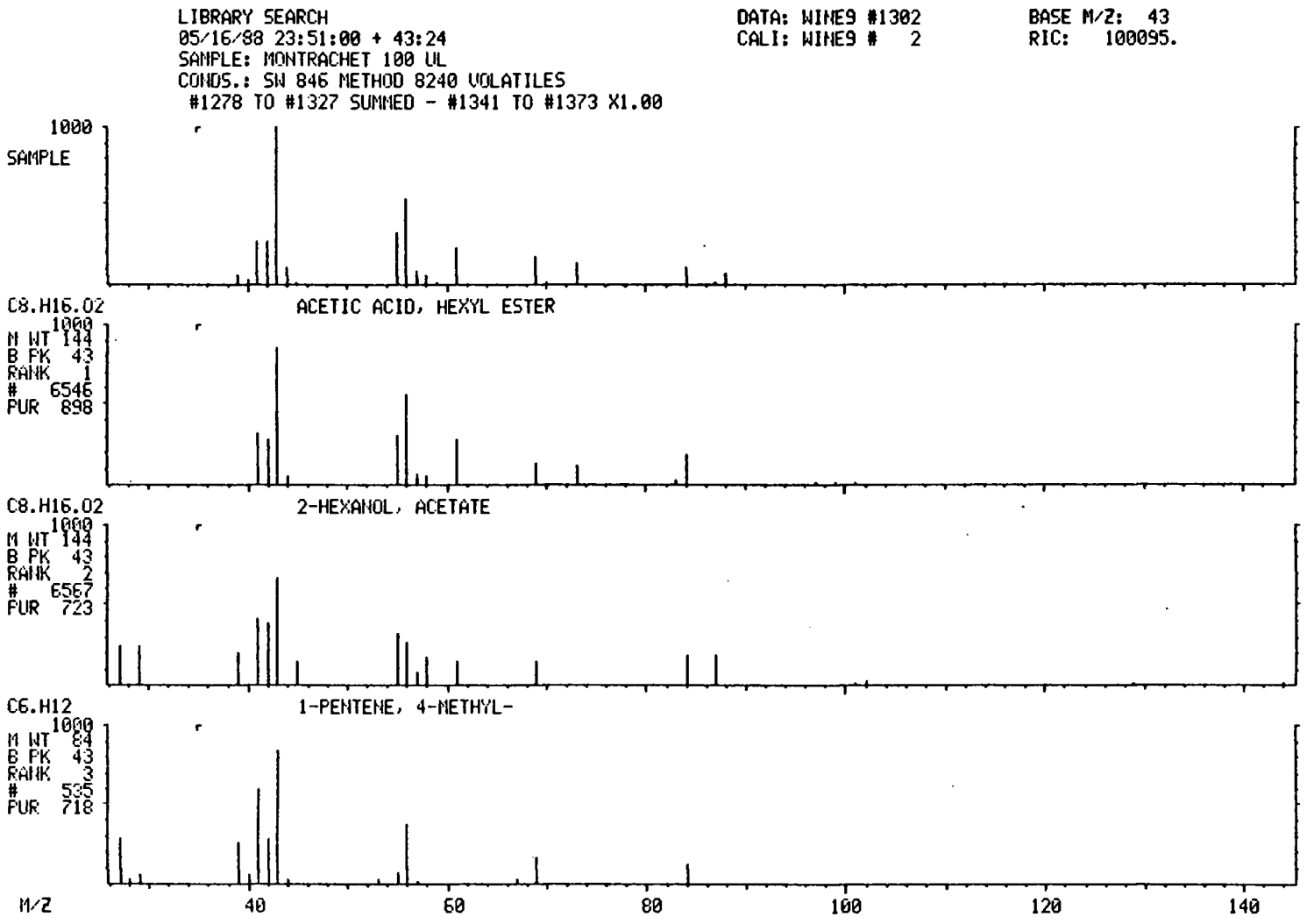
Appendix H. Chromatogram of HV86CH Chardonnay Montrachet control wine for GC-MS analysis for volatiles.



Appendix I. Chromatogram of HV86CH Chardonnay Montrachet ER1a (MLF) wine for GC-MS analysis for volatiles.



Appendix J. Mass spectrum of HV86CH Chardonnay Montrachet ER1a (MLF) wine for hexyl acetate.



Appendix K. Computer read-out of mass spectrum of HV86CH Chardonnay, Montrachet ER1a (MLF) wine for hexyl acetate.

```
Library Search                    Data:. WINE9 #1302          Base m/z:.   43
05/16/88 23:51:00 + 43:.24        Cali:. WINE9 #   2          RIC:     100095..
Sample: MONTRACHET 100 UL
Conds.:. SW 846 METHOD 8240 VOLATILES
 #1278 to #1327 summed - #1341 to #1373 X1.00

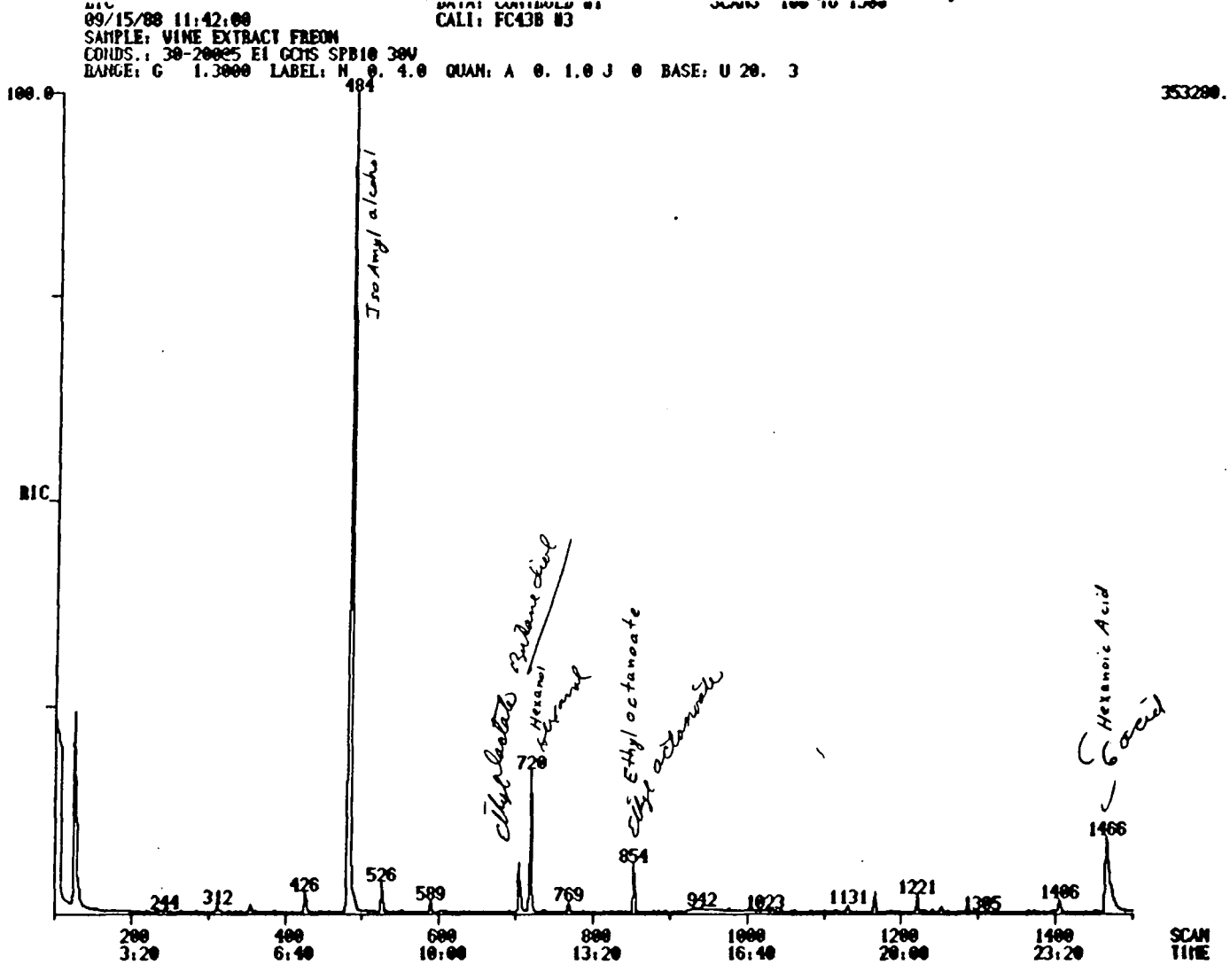
42223 spectra in LIBRARYNB searched for maximum PURITY
  140 matched at least 7 of the 16 largest peaks in the unknown

Rank In.       Name
1   6546 ACETIC ACID, HEXYL ESTER
2   6567 2-HEXANOL,. ACETATE
3    535 1-PENTENE,. 4-METHYL-
4   1515 1-PENTANOL,. 4-METHYL-
5   6648 HEXANE, 1-PROPOXY-

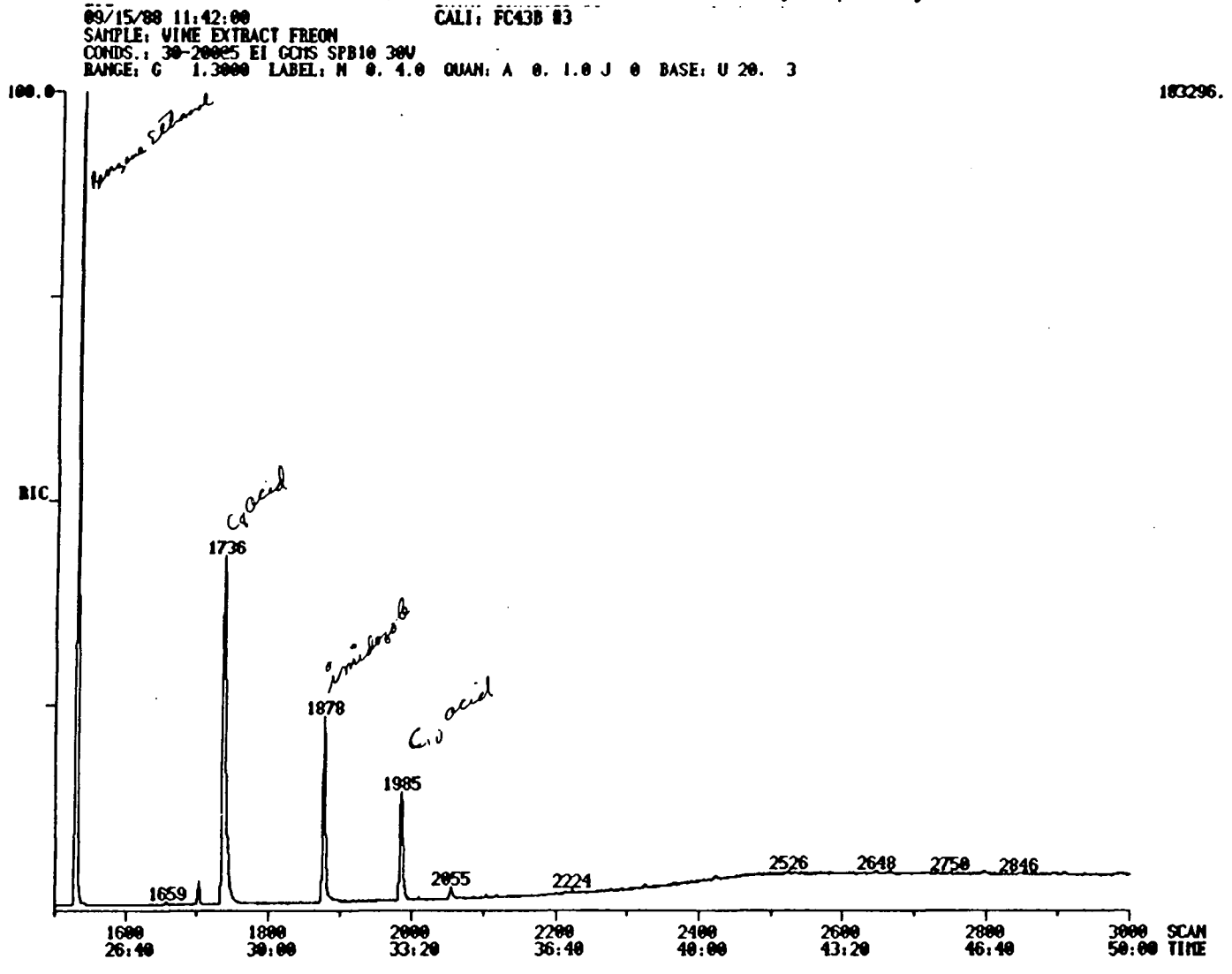
Rank  Formula                          M.Wt  B.Pk   Purity   Fit   RFit
1     C8.H16.O2                         144    43      898   950    934
2     C8.H16.O2                         144    43      723   822    746
3     C6.H12                             84    43      718   881    735
4     C6.H14.O                          102    56      709   846    733
5     C9.H20.O                          144    43      693   735    867

Mass   Inten      1      2      3      4      5
  27                   246    286     76
  28                          31
  29                   236     56     92
  31                                 111
  39      54         204    257     63    134
  40      21                 48
  41     273   325.   418    604    361    355
  42     271   285.   387    276    314    135.
  43    1000   848    661    834    549.  1481.
  44     109.   53.          29           61.
  45      13          141                 31
  53                          31.
  55     316   312    319.    70    171    133
  56     534   557.   265.   379    647    266
  57      82    61    80     17     167    103
  58      51    52    179
  59       0                              115
  61     221   275    144                 119
  67.                        22
  69     169   135    153    165    292     64
  70       0                  68
  71.                         72
  73     133   119                47     278
  83           33                        86
  84     111   184    184    124     53    139
  85                                     381
  86.                                     17
  87       2          183
  88      70
  97             15
  99             10
 101             16     14
 102                    24
 129                    14
 144                     0
```

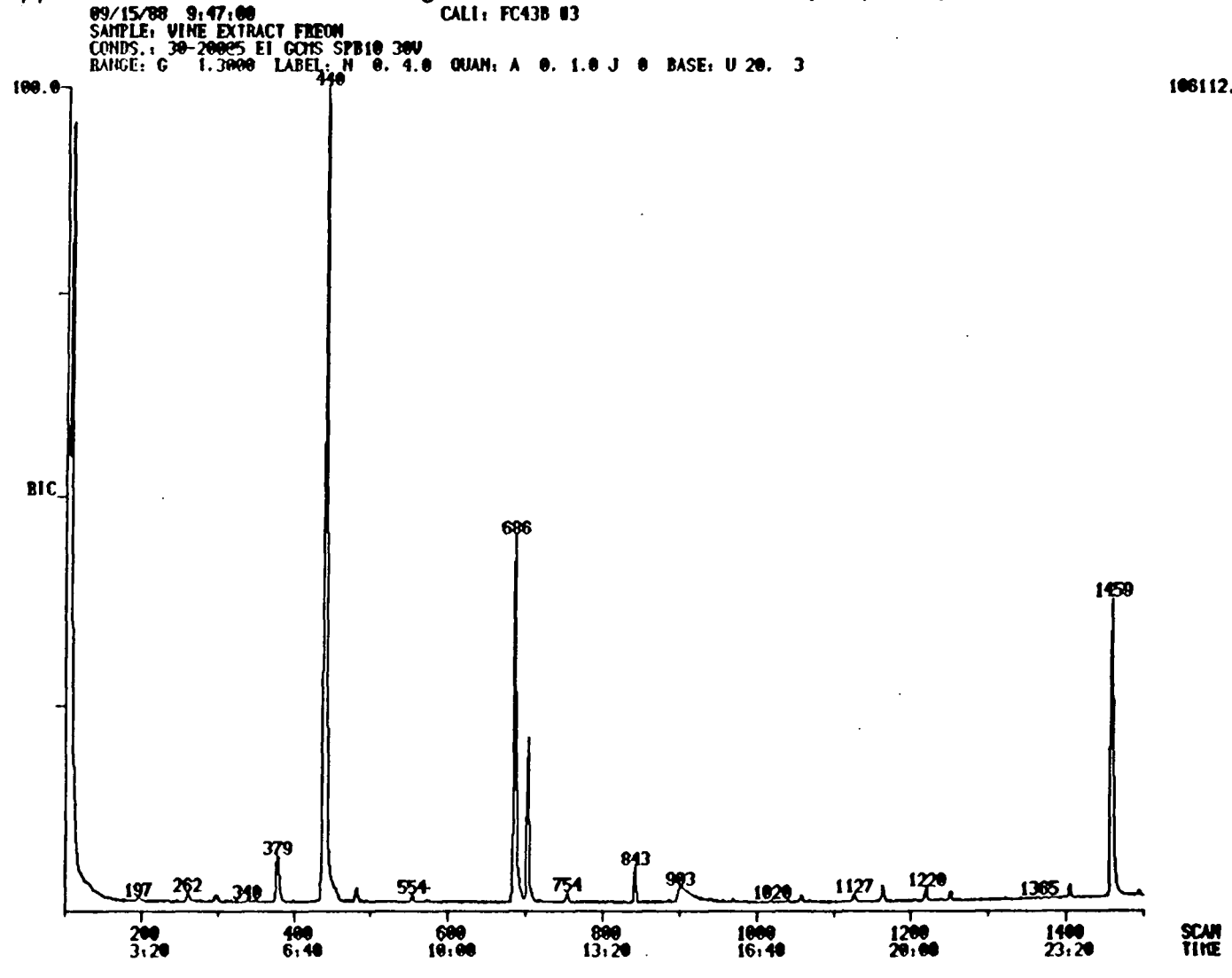
Appendix L. GC-MS chromatogram of HV86CH Chardonnay, Epernay control wine.



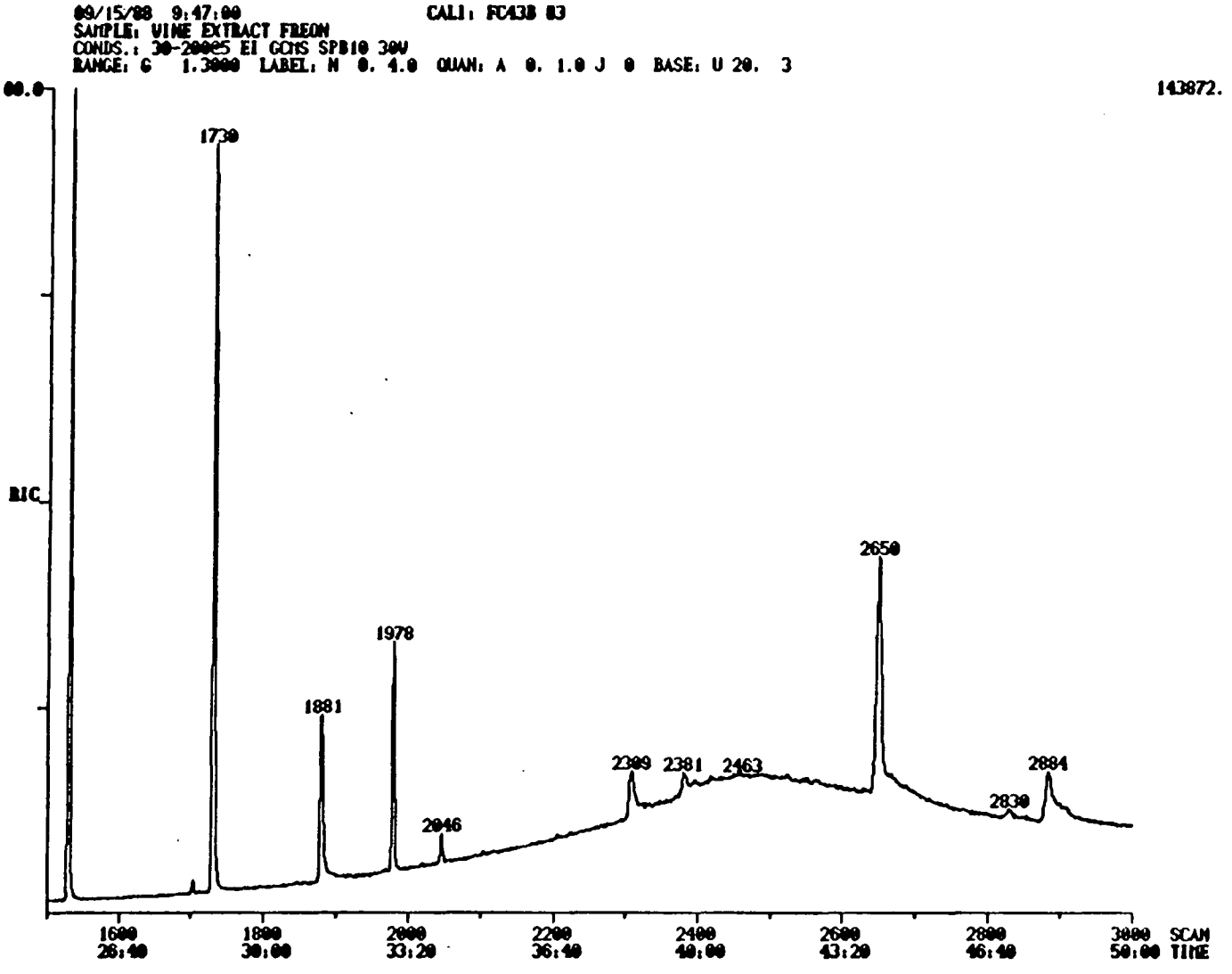
Continued GC-MS chromatogram of HV86CH Chardonnay, Epernay control wine.



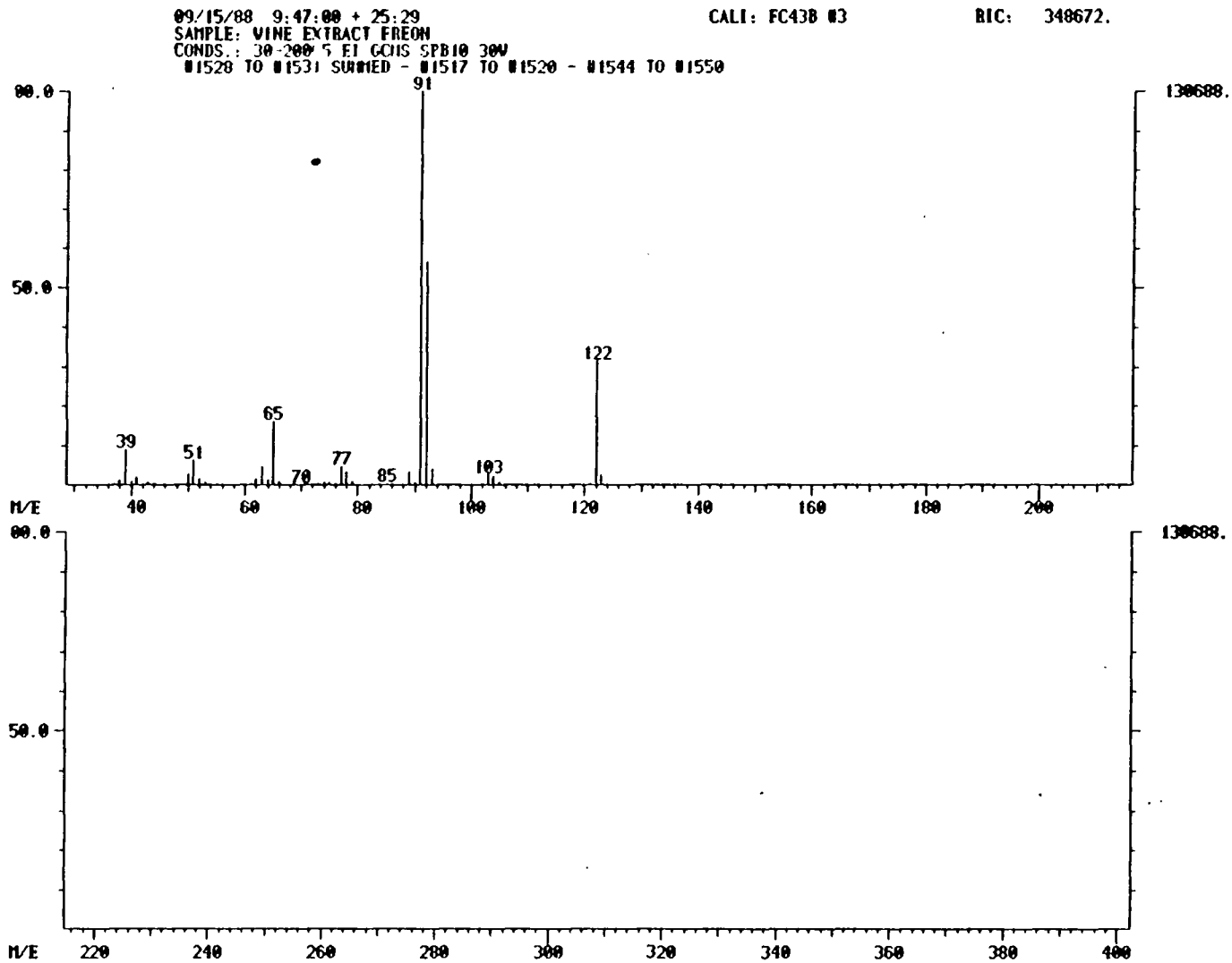
Appendix M. GC-MS chromatogram of HV86CH Chardonnay, Epernay ER1a (MLF) wine.



Continued GC-MS chromatogram of HV86CH Chardonnay, Epernay ER1a (MLF) wine.



Appendix N. Mass spectrum of HV86CH Chardonnay, Epernay ER1a (MLF) wine for 2-phenethanol.



Appendix O. Computer read-out of mass spectrum analysis of HV86CH Chardonnay, Epernay ER1a (MLF) wine for 2-phenethanol.

```
LIBRARY SEARCH                  DATA: SAMPLEC #1529        BASE M/E:   91
09/15/88  9:47:00 + 25:29       CAL1: FC438 #   3          RIC:    345599.
SAMPLE: WINE EXTRACT FREON
CONDS.: 30-20005 E1 GCMS SP810 30W
 #1520 TO #1531 SUMMED - #1517 TO #1520 - #1544 TO #1550

  421 SPECTRA IN LIBRARYHF SEARCHED FOR MAXIMUM PURITY
   94 MATCHED AT LEAST 2 OF THE 16 LARGEST PEAKS IN THE UNKNOWN

RANK  IN NAME
1     42 2-PHENETHANOL
2     23 TOLUENE
3    322 PHENYLACETALDEHYDE
4    198 PENTYL BENZENE
5     84 ETHYL BENZENE

RANK  FORMULA                          M.WT B.PK   PURITY   FIT   RFIT
1 C8.H10.O                              122   91      966   999    966
2 C7.H8                                  92   91      735   983    745
3 C8.H8.O                               120   91      634   845    634
4 C11.H16                               148   91      592   776    722
5 C8.H10                                106   91      521   681    644

MASS   INTEN     1     2     3     4     5
  38      12           20    16
  39      89   100   117   126   117    75
  40       8           15     7
  41      19    23     15    16   151
  45                   39
  46                    9
  50      29    36     37    28          45
  51      62    66     58    47    61   111
  52      15           7     4          28
  53       6
  57                                   30
  61       3
  62      15    16     19    14
  63      46    43     54    50    24    29
  64      12
  65     159   155    107   160   124    73
  66       9
  74       7
  75       5
  76       4
  77      46    57                   65    90
  78      35    42                   67    69
  79       8                         29    33
  89      33    30     29    30          17
  90       8
  91    1000  1004   1000  1225   929   945
  92     566   540    583   260   565    61
  93      41    30     27     4    46
 102       4
 103      26    20                   19    22
 104      21    21
 105       7                         100    37
 106                                  14   202
 107                                       16
 115                                  14
 117                                   6
 119                                  25
 120       1                206
 121       4                  3
 122     314   302
 123      26    24
 133                                  12
 140                                 194
 149                                  10
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Appendix P. Partial compilation of published aroma threshold values and descriptions of compounds.

COMPOUND	THRESHOLD VALUE	AROMA DESCRIPTION OR NOTES	REFERENCE
Acetaldehyde	0.12 ppm	(In water) Oxidized flavor/aroma	Mulders; Amerine (1980)
Acetaldehyde	100-125 ppm	(Red and White table wines)	Margalith
Acetic acid	54 ppb	(In water)	Ternashi et. al.
Acetic acid	26 mg/l	(In 9.4% ethanol)	Nykanen & Suomalainen
Acetoin		Buttery, creamy	Vock
Amyl acetate	0.039 mg/l	(Air)	Amerine, Pang. et. al.
n-Amyl acetate	5 µg/l	(In water)	Ternashi et. al.
Butanal		Sweet cocoa, malt	Vock
Butyric acid	0.009 mg/l (air)	Goaty odor	Amerine, Pang. et. al.
Butyric acid	240 µg/l	(In water)	Ternashi et. al.
Butyric acid	6.8 ppb	(In water)	Ternashi et. al.
n-Decanal	0.1 µg/l	(In water)	Ternashi et. al.
Diacetyl	1-10 ppm	(Dry red wine) Buttery	Rankine et. al.; Vock
Diacetyl	0.0065 ppm (v/v)	(In water)	Mulders
Diacetyl + Ethyl-Lactate	150 ml/l	(Beer & cider)	Shinahara et. al.
Diethyl succinate	100 mg/l		Ribereau-Gayon
Ethanol	100,000 µg/l	(In water)	Ternashi et. al.
Ethanol	100 ppm (v/v)	(Olfactory threshold)	Ternashi et. al.
Ethanol	0.55% (v/v)	(For odor only)	Amerine & Roessler
Ethanol	120 mg/l	(As 9.4% in distilled water)	Nykanen & Suomalainen
Ethyl acetate	0.0036 mg/l (air)	Fruity	Amerine, Pang. et. al.

Ethyl acetate	5.0 ppm	(Olfactory threshold)	Ternashi et. al.
Ethyl acetate	160 mg/l	(In wine)	Ribereau-Gayon
Ethyl acetate	25 mg/l	(In distilled water)	Ribereau-Gayon
Ethyl butyrate	0.2-0.3 nanograms	Fruity, rancid note	Drawert & Christoph
Ethyl butyrate	0.001 ppm (v/v)	(Olfactory threshold)	Ternashi et. al.
Ethyl decanoate		Fermented, winey yeasty	Vock
Ethyl hexanoate	0.6-1.0 nanograms	Fruity, rum like	Drawert & Christoph
Ethyl hexanoate		Fruity, fermented, winey	Vock
Ethyl hexanoate	0.08 mg/l		Nykanen & Suomalainen
Ethyl isobutanoate	0.2-0.3 nanograms	Fruity, orange like	Drawert & Christoph
Ethyl Isopentanoate	0.007-0.01 nanograms	Medicinal	Drawert & Christoph
Ethyl lactate	14-17 ppm	(in 9.4% ethanol)	Margalith
Ethyl 2-methyl-butanoate	.006-.012 nanograms	Golden Delicious apple	Drawert & Christoph
Ethyl pentanoate	0.03-0.04 nanograms	Fruity, pineapple like	Drawert & Christoph
Ethyl (and Methyl) phenyl acetate		Sweet, honey, fruity	Vock
Furfural		Woody, bready, carmel	Vock
Furfurol		Sweet, carmel, woody	Vock
Fusel alcohols	300 mg/l	(Odor only)	Amerine & Roessler
n-Heptanal	10-12 ppb	(In water)	Ternashi et. al.
Hexanal	2-4 nanograms	Green	Drawert & Christoph
n-Hexanal	0.005 ppm (v/v)	(Olfactory threshold)	Ternashi et. al.
Hexanoic acid		Fatty, sweaty	Vock
Hexanoic acid	5.4 ppb	(In water)	Ternashi et. al.

Hexanol	0.5 ppm (v/v)	(Olfactory threshold) Green	Ternashi et. al.
n-Hexanol	5.2 ppm	(In 9.4% ethanol) Woody	Margalith
Hexyl acetate	100 mg/l		Ribereau-Gayon
Isoamyl acetate	0.007 ppm	(In water) Bananna	Mulders; Amerine (1980)
Isoamyl acetate	0.20 ppm	(In 9.4% ethanol)	Margalith
Isoamyl alcohol	0.77 ppm	(In water) Fusel, pungent	Mulders; Amerine (1980)
Isoamyl alcohol	7.0 ppm	(In 9.4% ethanol)	Margalith
Isoamyl alcohol	6.5 mg/l		Nykanen & Suomalainen
Isobutanal	0.01 ppm	(In water) Sweet, cocoa, malt	Mulders; Vock
Isobutanol	3.2 ppm	(In water) Fusel, pungent	Mulders; Amerine (1980)
Isobutanol	75.0 ppm	(In 9.4% ethanol)	Margalith
Isopentanal		Sweet, cocoa, malt	Vock
Isovaleric acid	0.7 ppm	(In 9.4% ethanol)	Margalith
Isovaleric acid	0.75 mg/l		Nykanen & Suomalainen
Lactic acid		Mild sauerkraut	Amerine (1980)
2-methyl propanal	2-8 ppb	(In water)	Ternashi et. al.
1-Octanal	0.5-0.9 nanograms	Lemon, fruity, aldehyde	Drawert & Christoph
Octanoic acid		Fatty, sweaty	Vock
Phenethyl acetate	7.5 ppm	(In 9.4% ethanol)	Margalith
Phenethyl acetate		Sweet, honey, rosey	Vock
Phenethyl alcohol	30-200ppm	Flowery, rosey character	Margalith
Phenethyl alcohol	7.5 ppm	(In 9.4 % ethanol w/w)	Margalith
Phenyl-acetaldehyde + acetaldehyde		Sweet, rosey, cocoa, bitter	Vock
Phenyl-acetaldehyde + Isobutyraldehyde		Sweet, nutty, hazelnut, vanilla	Vock

Phenyl-acetaldehyde + Isovaleraldehyde		Rosey, cocoa, bitter aroma	Vock
Phenyl acetate		Sweet, honey	Vock
Propanol	9,000 µg/l	(In water)	Ternashi, et. al.
1-Propanol	40 ppm	(In water)	Mulders
Propionic acid	20.0 ppm	(In 9.4% ethanol)	Margalith