Comparison between Simultaneous and Traditional Consecutive Malolactic Fermentations in Wine

by

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ABSTRACT

COMPARISON BETWEEN SIMULTANEOUS AND TRADITIONAL CONSECUTIVE MALOLACTIC FERMENTATIONS IN WINE

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Successfully inducing malolactic fermentation in the production of grape wines can be challenging, especially in wines after finishing alcoholic fermentation with limited energy sources, low pH values and high ethanol concentrations. In this thesis, the kinetics of several chemicals of enological relevance were studied in a white wine (Chardonnay) and a red wine (Cab Franc) vinified by traditional, consecutive alcoholic (AF) and malolactic fermentations (MLF), and simultaneous AF/MLF, where bacteria were co-inoculated with yeast. The Chardonnay must was adjusted to four pH values (3.20, 3.35, 3.50 or 3.65), the cab Franc was kept as original pH value (3.56) and the concentrations of sugars, organic acids as well as acetaldehyde were followed throughout the fermentations. For Chardonnay the degradation of glucose and fructose was slower at the lowest must pH value (3.20) and independent from the time of bacterial inoculation. In all cases, malolactic conversion was faster after yeast-bacterial co-inoculation and was completed in simultaneous treatments at pH values of 3.35-3.65, and consecutive treatments at pH 3.50 and 3.65. No statistically significant difference was observed among the final acetic acid concentration, in all inoculation and pH treatments. For Cab Franc, it confirmed that co-inoculation shortened the fermentation periods while having minor effects on other parameters.

Overall, simultaneous AF/MLF allowed for greatly reduced fermentation times, while the must pH remained a strong factor for fermentation success and determined the final concentration of various wine components. The time of inoculation influenced formation and degradation kinetics of organic acids and acetaldehyde significantly.

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ABBREVIATIONS

Abbreviations and units not specified in this list are standard SI-units.

ADH ..... alcohol dehydrogenase

AF..... alcoholic fermentation

Ald-DH ..... aldehyde dehydrogenase

AMRS ..... apple MRS medium

ANOVA.... analysis of variance

ATP..... adenosine triphosphate

BSA ..... bovine serum albumin

CFU..... colony forming unit

CV ..... coefficient of variation

DNA ..... deoxyribonucleic acid

DO ..... dissolved oxygen

Dry wine.... wine with no or low sugar concentration (generally below 1 g l^{-1})

EDTA..... ethylenediaminetetraacetic acid

GC..... gas chromatography

HCl..... hydrochloric acid

HPLC..... high performance liquid chromatography

l..... litre

LAB..... lactic acid bacteria

Lb. ..... *Lactobacillus*

µl..... microlitre

µm ..... micrometer

mg..... milligram

ml..... millilitre

MLF ..... malolactic fermentation

N..... normality

NAD ..... β-nicotinamide adenine dinucleotide

NADP..... β-nicotinamide adenine dinucleotide phosphate

nm ..... nanometer

OCPP..... Organic Crop Producers & Processors

OD ..... optical density

O. oeni..... *Oenococcus oeni*

PVPP ..... polyvinylpolypyrrolidone

S. cerevisiae .. *Saccharomyces cerevisiae*

SE..... standard error

SO_2 ..... sulphur dioxide

SO_4 ..... sulphate

sp. ..... species

TFA..... trifluoroacetic acid

THF. ..... tetrahydrofuran

U ..... enzymatic unit (enzyme quantity leading to formation of 1 μmol of product min^{-1} at 25°C

v/v..... volume/volume

w/v..... weight/volume

Y_{max}..... maximum growth yield

CHAPTER 1. INTRODUCTION

1.1 THE DEVELOPMENT OF WINE MICROBIOLOGY AND WINE TECHNOLOGY

The history of winemaking runs parallel to civilization. The first archaeological evidence of wine production is from the Middle East and dates back to 6000 BC (Cavalieri et al. 2003). From there, wine production spread from Mesopotamia to Egypt and Phoenicia, then over the Mediterranean across all of Europe and Northern Africa through the Greek and Roman Empires (Johnson and Robinson 2001). During the Roman Empire, the production of wine and its distribution improved considerably as barrels and bottles were developed and wine could be shipped over large distances. Accordingly, many grape varieties also became well known beyond their regional significance (Phillips 2000). However, in ancient times, little was known about the scientific basis of wine production and wine quality was quite variable.

The advances in the scientific understanding of winemaking are more recent. Anton van Leeuwenhoek invented the microscope in the 17th century(Anton 1683). He first observed microscopic life existing in wine, but did not realize that he was observing yeast and bacteria, nor did he establish a relationship between these entities and alcoholic fermentation (Cano and Colome 1998). At the end of the 18th century, Antoine Lavoisier successfully proved that the sugar contained in the must was transformed into alcohol and carbon dioxide(Hunter 2000). Joseph Louis Gay-Lussac, who continued Lavoisier's research, formulated a mathematical relationship for the transformation of sugar into alcohol and carbon dioxide (Cano and Colome 1998). Certainly, the birth of wine microbiology goes

back to Louis Pasteur, who demonstrated that yeasts were responsible for the spontaneous alcoholic fermentation of grape must or crushed grapes, and isolated several genera and species (Pasteur 1876). From this point onwards, alcoholic fermentation became better understood, and therefore, winemaking could be better controlled.

Besides microbiological aspects, many other important vinification variables depend on grape quality, which in turn is determined by the climate and viticultural practices. The following section will provide a short overview of grape maturation and composition as related to winemaking.

1.2 GRAPE MATURATION, COMPOSITION AND HARVEST

During early stages of grape berry development, grapes of both red and white varieties are hard, acidic and green. After a stage called veraison, grapes will soften and red grapes will develop their characteristic colour while white grapes will develop a yellowish colour (Palomo et al. 2007).

During this maturation phase, the concentration of organic acids, which predominate among the soluble solids of pre-veraison grapes, is reduced which is specifically true for malic acid, while other organic acids, such as citric acid, are accumulated (Watson 2003). At the same time, sugars and aroma compounds will start to accumulate. For the harvest date, an optimum balance between sugar content, acidity and the aroma expression of the grape is sought. The specific optimum values for these parameters will depend on the time of harvest

and many other factors such as grape variety, climate, soil quality, and the wine type to be produced. Besides sugars, organic acids, and aroma compounds, other grape components and factors are also important in winemaking, e.g., the nutrition of yeast and bacteria used in winemaking which impacts on wine quality. This is the case for nitrogenous compounds, whose transport to the grape essentially occurs in the form of ammonium cations and amino acids. During maturation, the ammonium cation concentration decreases, while the concentration of free amino acids increases, representing up to 90% of the nitrogen in the final grape juice. At maturity, arginine is the predominant amino acid accumulated in grapes of most varieties. (Kliewer 1968). A rapid accumulation of phenolic pigments also occurs during berry maturation which is especially important for the colour and taste of red wines.

Grapes are organized in clusters of varying shapes according to grape variety, and are composed of stalks, seeds, skin and pulp. The stalk and seeds are high in phenolic compounds, carbohydrates and oil, and generally only have a small contribution to winemaking (Bird 2003). On the other hand, the skin contains significant quantities of phenolic compounds, aromas and aroma precursors, and thus, plays an important role in red winemaking (Amos 2007). The pulp makes up 70-80% of the weight of ripe berries and contains sugars (mainly glucose and fructose and minor amounts of saccharine, arabinose, xylose, rhamnose and raffinose), organic acids (mainly tartaric, malic and citric acids), cations, amino acids and pectic substances, most of which may be degraded by microorganisms during winemaking (Ribéreau-Gayon et al. 2000).

Besides grape composition, must quality also depends on the absence of damaged or diseased fruit. Accordingly, the protection of grapes from insects, moulds, birds and other pests in the vineyard before harvest is important (Ough and Berg 1979).

A suitable harvesting technique is required in order to comply with vinification and final product goals. In some vineyards, harvesting can be carried out very efficiently by automatic harvesting machines. While this minimizes labour costs, it may lead to vine damage and generally causes at least partial crushing of the grapes thus requiring efficient protection form oxidation. Thus, high quality wines are still often made from grapes that were hand-picked since this allows selecting grapes by quality in the vineyard and minimizing harvest damage (Jackson and Lombard 2007).

After harvest, grapes may be protected from oxidation and microbiological deterioration by addition of SO_2, and antioxidant and antimicrobial agent, or dry ice (CO_2) (Ough 1992). Reducing the delay between harvest and grape processing, e.g., by setting up fermentation facilities near the vineyard, is also beneficial with regards to avoiding exposure to oxygen.

Large variations exist among the different winemaking protocols according to wine style and region. In the following section, a standard protocol for the vinification of dry white and red table wines will be presented.

1.3 OVERVIEW OF BASIC WINEMAKING STEPS

Upon delivery to the winery, grapes may be sorted in order to separate grapes of varying maturation levels and to discard MOGs (material other than grape), i.e., leaves, shoots, insects, etc. Subsequently, grapes are destemmed to remove the berries from the stalks, and then crushed, in order to expose the pulp for juice extraction (Ough 1992). During this process, grape seeds should not be crushed in order to avoid the release of bitter compounds into the must.

In red wine making, skins and seeds are most often left in the grape must for a certain duration following destemming and crushing, in order to extract colour and aroma compounds in a process called skin or alcoholic maceration (Ough 1992). An alternative to this type of colour and aroma extraction is thermovinification, where the must with skins and pips is heated to 60-80°C for several hours (Ough 1992). In general, the release of compounds from the skins will depend on various parameters, including contact duration, temperature, the addition of hydrolytic enzymes and the concentration of alcohol. Different maceration methods can be used to optimize extraction (Ough 1992).

In white winemaking, maceration is not commonly applied and the grape juice is separated from pips and skins during pressing before the fermentation starts. Thus, compared with the grape must for making red wine, the grape juice for making white wine contains fewer phenolic compounds, and is more easily oxidized. Accordingly, white wine has to be protected from oxygen more carefully(Singleton et al. 1979). If the juice is too turbid after pressing, it may be clarified or filtered.

After destemming and crushing in red winemaking or destemming-crushing and pressing in white winemaking, generally the alcoholic fermentation takes place. The alcoholic

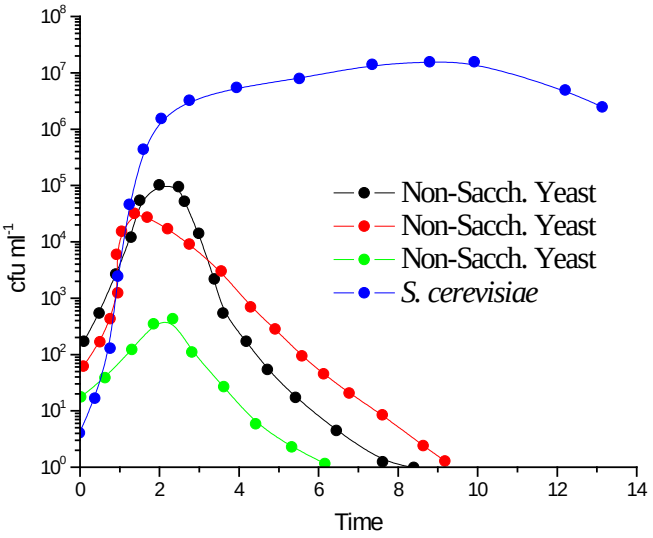
fermentation by yeast leads to the degradation of sugars and the formation of ethanol and smaller concentrations of many other compounds, which will influence the final organoleptic profile of the wine (Edwards et al. 1990; Malacrinò et al. 2005). After alcoholic fermentation, most red wines and some white wines undergo a secondary fermentation called malolactic fermentation. It is carried out by lactic acid bacteria and leads to deacidification by transformation of dicarboxylic L-malic acid to monocarboxylic L-lactic acid, and flavour modifications (Davis et al. 1986).

After these fermentation steps, wines may be aged in various containers, including stainless steel tanks and oak barrels, and racked (decanted) several times to remove sediments formed by sedimenting yeast and bacteria (lees). Further winemaking steps may include finings and stabilizations (Ough 1992). These are carried out to remove compounds that constitute or may lead to colour and aroma deviations or unsightly sediments or hazes (Ribéreau-Gayon et al. 2000). Among the findings, the removal of white wine proteins that can lead to hazes during storage by bentonite is important, as well as the softening of red wine phenolics by fining with proteins such as gelatin, casein or isinglass (Ough 1992). Wines may also be cold stabilized, i.e., stored at cool temperatures for a certain time to favour the precipitation of unstable potassium tartrate crystals which may otherwise precipitate during storage (Clark et al. 1998; Ough 1992; Pilone and Berg 1965). While not imperative, today many wines are also filtered before bottling in order to remove insoluble compounds and microorganisms that could lead to spoilage after bottling (Garza and Boulton 1984). Wine stability also relies heavily on the presence of the preservative sulphur dioxide (SO_2) which has antioxidant and antimicrobial activity (Romano and Suzzi 1993).

1.4 WINE MICROBIOLOGY AND FERMENTATIONS

1.4.1 Wine yeast and Alcoholic fermentation

Figure 1 Yeast dynamic during spontaneous fermentation (Mira de Orduña 2007).



A large number of microorganisms, mainly composed of acetic acid bacteria, lactic acid bacteria, yeasts and moulds ($1x10^6$ cfu/berry), are acquired during grape cultivation, in addition to post-harvest handling (James et al. 1972; Rosini et al. 1982). The obligate aerobes are suppressed under anaerobic conditions used in wine production although yeasts and lactic acid bacteria remain active to initiate fermentation. With regards to yeast, the majority

of populations are composed of non-*Saccharomyces* species that includes the genera *Brettanomyces*, *Zygosaccharomyces*, *Pichia* and *Schizosaccharomyces* (Fleet and Heard 1993).

Historically, wine fermentation relied on the endogenous yeast fermentation (spontaneous fermentation) and is still practiced in traditional European vineyards. In the course spontaneous alcoholic fermentations, *Saccharomyces cerevisiae* eventually dominates by virtue of high ethanol tolerance whilst those originally dominating dying away due to sensitivity towards the alcohol (Fleet and Heard 1993). Yet, the metabolic by-products derived from non-*Saccharomyces* contribute to the flavour notes of the final wine and can be modulated by adjusting the fermentation conditions (pH, temperature).

The balance of yeast in musts may be modified by different means. For example, yeast vary with regards to the temperature optimum and their tolerance to the preservative SO_2. Accordingly, the strains of *Saccharomyces cerevisiae* with tolerance of higher fermentation temperature and SO_2 additions will generally be favoured in spontaneous fermentations (Fleet and Heard 1993)

Certainly, the most efficient method of ensuring dominance of a *Saccharomyces cerevisiae* strains during AF is to inoculate a large amount of a suitable strain to induce a rapid onset of alcoholic fermentation. Today, a diverse range of freeze dried *Saccharomyces cerevisiae* strains are commercially available which only have to be rehydrated before their addition to musts. Usually, these yeasts have been selected from collections that were isolated from spontaneous fermentations, well physiologically characterized. Desirable properties for commercial yeast may include low foaming potential, tolerance for high alcohol concentrations and low temperatures (for white wine yeast), low production of fusel oils and

acetic acid, osmotolerance, flocculation ability, low nutrient requirements and the production of fruity fermentation esters.

1.4.2 Wine Lactic acid bacteria and Malolactic fermentation

Wine relevant lactic acid bacteria, which are responsible for the MLF, comprise the genera *Pediococcus*, *Lactobacillus* and *Oenococcus* with its only species, *Oenococcus oeni*. These bacteria, which equally occur as winery contaminants, are found in grape musts in low populations (10^3 cfu/ml), but generally most of them die off during alcoholic fermentation. Only after completion of AF, the bacterial population might increase and reach up to 10^6 cfu/ml when malolactic fermentation occurs (Fleet et al. 1984). As for yeast, the development of wine LAB during the vinification may be negatively affected by the low fermentation temperature, acidic pH, high SO_2 and ethanol concentration (Davis et al. 1988). To ensure the success of the Malolactic Fermentation (MLF) it is common practice to use freeze-dried cultures of *Oenococcus oeni*. The strains of *Oenococcus oeni* selected is based on low acetic acid formation, high alcohol tolerance and low biogenic amine formation.

In spite of the advances with regards to our understanding of the microbiological and chemical bases of oenology as well as the availability of selected yeast and bacteria starters to induce AF and MLF and specific nutrients to support their growth, fermentation problems are still widespread. In the following section, the reasons for fermentation problems as well as some consequences will be discussed.

1.4.3 Challenges of alcoholic fermentation and malolactic fermentation

While being necessary for the transformation of must into wine both *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast can also lead to wine spoilage. For example, *Saccharomyces cerevisiae*, itself, may lead to acetic acid concentrations which can make wines unfit for consumption, for example, in the vinification of very high sugar containing musts such as those intended for the production of ice wines (Pigeau and Inglis 2005). In the presence of elemental sulphur, e.g., from vineyard spray residues, or in nutrient deficient musts, yeast may also produce hydrogen sulphide and/or organomercaptans reminiscent of rotten eggs or similar aromas which can render wines unacceptable (Rauhut and Kürbel 1994).

Similarly, besides being responsible for the MLF, wine lactic acid bacteria may also lead to quality deterioration of wines (Lonvaud-Funel 1999). Excessive formation of acetic acid, acrolein, a bitter compound formed from the degradation of glycerol, ropiness caused by exopolysaccharides from residual sugar degradation, or the formation of biogenic amines could be cited (Lonvaud-Funel 1999; Zee et al. 1983).

Accordingly, it is important to control production as well as potential spoilage organisms in order to prevent quality deviations. Many fermentation problems are caused by sluggish or stuck fermentations (Bisson and Butzke 2000).

Stuck or sluggish alcoholic fermentations (AF) occur when yeasts consume sugar either slowly or incompletely resulting in wines with undesired sugar residues and lower than desired ethanol levels. In the case of a sluggish or stuck MLF, the degradation of malic acid may be overly protracted or stalled before malic acid is depleted. Stuck and sluggish fermentations remain a widespread problem in the wine industry which can lead to product

depreciation or complete loss. Specifically, wines with residual sugar and/or malic acid are more susceptible with regards to microbial spoilage because of higher substrate concentrations. At the same time, wines with residual sugar or acid may simply be perceived as being unbalanced with regards to their sugar acid concentrations, and thus, hard to sell in the competitive wine market. In the winery, sluggish and stuck fermentations may lead to time loss and organizational problems since product cannot be stabilized and bottled (Sablayrolles and Blateyron 2001).

Problematic AF may be related to nutrient limitation, the presence of toxic substances, extremes of temperature or pH, and poor ethanol tolerance by yeast (Bisson and Butzke 2000). Some studies have shown that production of medium chain fatty acids, which can arrest yeast growth, are also related to stuck or sluggish fermentations (Bardi et al. 1999).

Sluggish or stuck MLF is less problematic than stuck AF since chemical deacidification could be used as an alternative method to reduce acidity in wines (Krueck and Seckler 1990). Yet, from an aroma and sensory point of view, MLF cannot be replaced and essential to reduce the harshness of high malic acid wines. Also, delayed MLF may lead to spoilage since wines have to be kept under conditions that favour growth of other microorganisms such as low SO_2 concentrations and high temperature. Factors, such as nutritional deficiencies in the wine, low pH values, or unfavourable fermentation temperatures, contribute to this problem or the cause of it (Van der Westhuizen and Loos 1981). Antibacterial compounds such as fatty acids and SO_2 can be produced by yeast during AF as well (Bardi et al. 1999; Rozès et al. 1992).

Occurrences of sluggish and stuck MLF vary from winery to winery and also depend on the grape variety. However, anecdotal evidence suggests that over the last years fermentation problems have become more widespread mainly because of the effects of climate change and late harvest dates leading to musts with extremely high sugar concentrations, and thus, wines with high ethanol concentrations. In cool climates, high sugar and alcohol concentrations are less frequently encountered. However, here, lack of temperature control in wineries often lead to sluggish and stuck MLF if the temperatures drop too rapidly in autumn (Llaurado et al. 2002).

Given that the traditional MLF is undertaken under sub-optimal conditions following the alcoholic fermentation there is interest in performing both stages of fermentation simultaneously. The additional benefits of simultaneous fermentation would be to reduce the potential of spoilage or failed batches (Beelman and Kunkee 1987). Co-inoculation of yeast and bacteria into the must might prevent sluggish or stuck MLF since lower AF by-products in the must before AF such as lower alcohol, fatty acids concentrations in the must, could cause less inhibition of LAB growth (Capucho and San Romão 1994). In economic terms, an added benefit of simultaneous AF/MLF is to increase productivity through reduced fermentation times. However, simultaneous fermentations would require that bacteria and yeast are active at the same time potentially leading to conflicts between yeast and bacteria. Several studies have recently investigated the different types of microbial interactions in musts and wine.

1.5 MICROFLORA INTERACTIONS

Several interactive associations between microorganisms have been described in microbial ecology (Boddy and Wimpenny 1992). The most significant in wine involve

inhibition and enhancement (Fleet 2003). At the early stage of fermentation, some microorganisms mainly yeast deplete most of the nutrients from the must, decrease the pH and produce high ethanol concentrations that inhibits most microorganisms (Heard and Fleet 1985). In addition, yeast growth generates an array of metabolites, which could be toxic to other species. In general, the production of ethanol and fatty acids from yeast present the most inhibitory effects on other wine microorganisms (Bisson 1999). Inhibitory peptides, proteins or glycoproteins, and enzymes, produced by yeasts include *Saccharomyces cereuisiae* (Byrd et al. 1982), may also destroy other species by leading to cell wall lysis (Fleet 2003). However, other mechanisms lead to enhanced microbial growth. For instance, the free amino acids and vitamins released from the yeast lees, the yeast sediments formed after conclusion of the AF, may encourage the growth of other species later in the process (Alexandre et al. 2004).

1.5.1 Interaction between yeast and bacteria

With traditional vinification method, LAB are usually inoculated after a completed AF, thus many of the nutrients (including sugars, vitamins and amino acids) have been consumed by yeast before LAB inoculation which makes wine conditions physicochemically restricting for LAB growth. Besides lack or deficiency of nutrients, some products released by yeast during AF such as ethanol, SO_2, and medium chain fatty acids could also become inhibitory factors for MLF. LAB growth could be retarded when the concentration of ethanol reaches 4%(v/v) (Davis et al. 1985). SO_2 is another inhibitory factor for LAB produced through AF which is also added manually into juice or wines in order to prevent wine microflora contaminations. Recent research has shown that a clear correlation between SO_2 produced by yeast and LAB inhibition did not always exist (Larsen et al. 2003; Osborne

and Edwards 2006). Medium chain fatty acids are also responsible not only for LAB growth inhibition, but also for reducing the ability of LAB to catabolise malic acid (Guilloux-Benatier et al. 1988). Furthermore, certain yeast strains might release some proteins/peptides with antibacterial activity during AF such as the production of F63 extracellular compound(s) from *S. cerevisiae* F63 (Comitini et al. 2005; Dick et al. 1992; Osborne and Edwards 2006).Therefore, suitable strains of yeast and bacteria should be used if the wine is being prepared to undergo MLF. In addition, previous research demonstrated that the LAB tolerances for fatty acids, ethanol, and SO_2, mainly produced by yeast, are directly influenced by pH level (Britz and Tracey 1990; Gockowiak and Henschke 2004), therefore, greater LAB growth inhibition was observed from these yeast by-products at lower pH. Thus ironically wines that would benefit more from acid reduction by MLF, i.e., low pH wines, inhibit their activity most (Lonvaud-Funel 1999).

Based on these studies, wines undergoing MLF by traditional vinification, especially with low pH value, have the challenges of LAB growth and LAB malic acid degradation which could cause a sluggish or stuck MLF.

In wineries, spontaneous MLF is most observed when problematic fermentations occur (Fornachon 1968; Henick-Kling and Park 1994; Kunkee 1991). Therefore, in past decades, the interactions between LAB and yeast have not been comprehensively studied, especially, when inoculated or grow simultaneously. In recent years, greater effort has been placed on studying factors such as the yeast/bacteria strain combination involved, the uptake and release of nutrients, and the ability to produce metabolites that are either stimulatory or toxic. Furthermore, the inoculation time and fermentation period will also influence those

interaction types. Not only the inhibitions, but also the conditions which stimulate growth were found among different yeast strains and LAB strains under different physico-chemical fermentation conditions (Nancy et al. 2008). Thus, selecting suitable yeast and LAB strains, changing physico -chemical fermentation conditions such as changing LAB inoculation time (Rauhut et al. 2001), adding suitable nutrients when inoculation occurs would alleviate the difficulties of inducing and completing MLF. Previously, simultaneous inoculation yeast/LAB method have been studied in Chardonnay must at grape original pH and suitable yeast/ LAB combinations had been identified (Jussier et al. 2006).

Since fermentation conditions such as wine pH could considerably influence yeast and LAB growth and metabolic transformations (Ribéreau-Gayon et al. 1998), and additionally effect the LAB tolerance on antibacterial compounds (Britz and Tracey 1990; Gockowiak and Henschke 2004), more studies should be done on malic acid degradation during simultaneous AF/MLF under different pH levels, and its effects on a wider array of wine components, especially on acetic acid production.

1.6 COMPOUNDS RELEVANT TO WINE QUALITY

1.6.1 Residue Sugars

Residue sugar levels are critical to wine quality and wine stability. A high residue sugar level usually indicates an inadequate alcohol level or an unsuccessful alcoholic fermentation and increases the chance of microbial spoilage during wine making for table

wine. Therefore, in order to observe and control fermentation processing, sugar concentrations are monitored during the wine making process (Bisson 2004).

During alcoholic fermentation sugars are metabolized to ethanol through the glycolytic pathway by yeast (Boulton et al. 1996; Ough 1992) (details shown in Appendix 1 and 2). Although yeast can also metabolize sugar through the respiratory metabolic pathway during winemaking, in order to avoid depreciation from oxidation, respiration is seldom encouraged especially for white wine.. Since ethanol production is tied to sugar consumption during AF, sugar levels have been used as an important indicator to follow the course of fermentation were, therefore, followed during the experimental period.

During the fermentation period, compounds such as organic acids (e.g., acetic acid), carbonyls (e.g., glycerol, acetaldehyde) and carbon dioxide are also produced (Appendix 1) which could either influence the microbial stability (Schutz and Gafner 1993) or affect the flavour and aroma of wine (Matthews et al. 2007).

1.6.2 Ethanol

Ethanol is the major product of yeast sugar degradation. Ethanol concentration is an important indicator of successful AF. With a suitable sugar concentration in the grape must and a completed AF, a dry wine usually contains ethanol levels over 10% (v/v). Ethanol gives wine its essential character and prevents microbial spoilages after fermentation, however, some strains such as *S. cerevisiae* could still metabolize ethanol in the absence of glucose though respiratory pathway (Appendix 5), therefore, from fermenting to bottling,

oxygen should be avoided and ethanol concentrations should be continuously determined (Andrews 1984).

1.6.3 Malic acid and lactic acid

Malic acid is a dicarboxylic acid which brings a mellow, smooth persistent sourness to wine. Wines with a high malic acid concentration have a sharp, tart, and unpleasant taste. In order to deacidify, wines with higher acidity undergo the MLF. During MLF, malic acid is degraded to lactic acid, a monocarboxylic acid which is softer on the palate than malic acid resulting in a smoother, less acidic wine. Malate dehydrogenase (substrate oxaloacetate and $NADH_2$) and malic enzymes (L-malate: NAD+ oxidoreductase, oxalacetate-decarboxylating EC 1.1.1.38 or 1.1.1.39) are used for catalyzing this reaction (details are shown in Appendix 1) (Caspritz and Radler 1983).

Malic acid as one of the important parameters tested in the wine industry in order to control MLF. Occasionally, lactic acid is used as another indicator of a successful MLF, since during sugar metabolism, small amounts of lactic acid can also be produced. In this study, both malic and lactic acids were investigated through AF/MLF in order to follow malic acid degradation by LAB.

1.6.4 Citric acid

Citric acid, as an intermediate in the TCA circle(Appendix 3), plays an important role in grape growth and during the grape maturation period (Bird 2003). For wine, citric acid could bring a fresh flavour and boost wine acidity. However, citric acid is not added frequently to wine to increase total acidity since it could be degraded by yeast (Bartowsky and Henschke 2004; Ramos et al. 1995) and also by LAB to acetic acid (Lonvaud-Funel 1999)

causing problems of microbial stability . Thus, citric acid concentrations are usually observed during fermentation and the wine stabilization period.

1.6.5 Acetic acid

Acetic acid is present in small quantities in all the wines as the principal volatile acid (VA). As an important physicochemical parameter, acetic acid should be monitored in bottled wines even in the sample trials throughout the winemaking processing since high acetic acid concentrations in wines result in a thin and sharp sour unpleasant feeling. In addition, high acetic acid concentrations could lead to a programmed yeast cell death process (Guaragnella et al. 2007; Ludovico et al. 2001). In order to control the final quality of wine on the market, regulations are in place to control VA level worldwide. For example, in Canada, the maximum VA in the table wine is 0.13% w/v (1.3 g l^{-1}) calculated as acetic acid (Canada Gazatte 2004). In United State, the legal maximum VA of red table wine is 0.14 % w/v(1.4 g l^{-1}) and for white is 0.12 % w/v (1.2 g l^{-1}) (e-CFR Data 2003).

During the wine making processing, there are many reasons of producing excessive acetic acid. Previously, acetic acid bacteria and chemically oxidation were highly responsible for high acetic acid level in wines, but today, these are of less concern since oxidation and contamination are highly controlled. Today, unexpected high acetic acid level in the wine result primarily from yeast and LAB metabolism. Through sugar degradation by yeast, acetic acid is a secondary product from decarboxylation and hydrolyzation of pyruvic acid (in Appendix 1) (Casal et al. 2007). The quantity of acetic acid produced by yeast depends on yeast strains, sugar concentration, and the amount of nitrogen assimilated (Bely et al. 2003; Erasmus et al. 2004). The higher the initial sugar concentration present in must, the more acetic acid is produced. Therefore, sweet wines or ice wines with high sugar concentration

have elevated volatile acidities. Acetic acid could be produced by LAB through the degradation carbohydrates (hexoses, pentoses, and glycerol most of which are from yeast sugar degradation) and/or organic acids (citric, malic, tartaric, and gluconic acids). LAB strains and sugar concentration could affect acetic acid production level (Lonvaud-Funel 1999), and therefore, VA may increase when MLF occurs in wines with high sugar levels (Ribéreau-Gayon et al. 2000).

In addition to accumulating acetic acid, some yeast strains could use acetic acid as a sole carbon and energy resource. Those strains are used in some wineries for AF, but not used to control acetic acid concentration (Vilela-Moura et al. 2007).

1.6.6 Volatile esters

Esters are present in grape must prior to fermentation but are in small amounts since most wine esters are produced during fermentation by yeast (Hosono et al. 1974; Plata et al. 2002) and LAB (Matthews et al. 2007) , or formed slowly during ageing and bottling (Edwards et al. 1985). During AF, yeast strains (Plata et al. 2002; Soles et al. 1982) as well as fermentation conditions such as temperature, fermentation period, nitrogen levels (Lambrechts and Pretorius 2000) are relevant to the production of esters . Many studies have found ester production is increased in the wines undergoing MLF (Bartowsky and Henschke 1995; Diaz Maroto et al. 2001; Edwards et al. 1990; Matthews et al. 2007; Sergi et al. 1999). LAB strains and physicochemical conditions including pH level, temperature, ethanol concentration, and existing esters in both wine and/or must will affect quantities and the types of esters during MLF (Matthews et al. 2004; Matthews et al. 2007; Matthews et al. 2008). During storage, esters are formed from the reversible reaction of alcohols and acids.

A large number of possible esters might be found since different alcohols and acids exist (Matthews et al. 2007).

Volatile esters are of most concern due to their effect on the sensory attributes of wine. Ethyl acetate, as the main volatile ester, is suggested to be observed since it contributes to sweet, fruity, and vinegary smell depending on the quantity. At concentrations below 80mg l^{-1}, this compound has favourable effects on wine aroma. Once the concentration of ethyl acetate is above 200mg l^{-1}, an undesirable vinegary odor is found, which is detrimental to wine aroma and quality (Ribéreau-Gayon 1978). Other volatile esters (such as ethyl butanoate, caproate, caprylate, caprate, laurate) are also important since they contribute to the pleasing aroma at low concentrations (Rapp and Mandery 1986).

In order to monitor fermentations and control wine quality compounds including sugars, alcohols, organic acids and volatile esters, acetaldehyde, amino acids should be tested from final wine samples and glucose, fructose, malic acid, acetic acid, ethanol should be monitored during wine making. In the next two chapters, the measurement of the above compounds will be detailed.

1.7 AIM OF THIS STUDY

The objective of this study was to conduct a comparative study between a simultaneous AF/MLF vinification method with a traditional consecutive AF/MLF vinification method in order to provide a detailed kinetic analytical comparison at different wine pH levels. During this study, the sugars and malic/lactic acid concentrations which are

indicative of AF and MLF, respectively, degrees were followed as a function of time as well as acetic acid levels in order to determine whether excessive acetic acid could be produced by LAB if inoculated simultaneously. In addition, the concentrations of other compounds such as esters and amino acids relevant to final wine quality were monitored. Finally, a sensory discrimination test was used to distinguish if any differences existed among wines made using different fermentation protocols.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1 GENERAL

2.1.1 Water

Water was purified by Mill-Q water purification system (Cartridge 1: Model Milli-Q A 10; Cartridge 2 Model: Quantum ^{TM} EX Ultrapure Organex) with a resistance of 18.2 MΩ cm^{-1} at 25°C followed by purification though Milli Pak filters (0.22-μm pore size, 47-mm diameter; Sterile Millipore) and used for all the work related to HPLC.

Water purified by an E-pure reagent water system (Model no. D4641, Barnstead/Thermolyne, Dubuque, U.S.A.) with a resistance of 17 MΩ cm^{-1} was used for all other work.

2.1.2 Origin of chemicals, media and equipment

Unless otherwise stated, all media and chemicals were supplied by Fisher Scientific (Whitby, ON, Canada).

2.1.3 Glass or polypropylene containers

Unless otherwise stated, all glassware and disposable polypropylene tubes were obtained from Fisher Scientific (Whitby, ON, Canada). The disposable polypropylene tubes used throughout the work are identified by their capacity as 1.7 ml microcentrifuge tubes (sterile with a screw-cap, or non-sterile and capped), and sterile 15 ml or 50 ml tubes.

2.1.4 Solid media

Solid media, such as agar plates and deep agar vials were prepared by adding 15 g l^{-1} agar to the media before autoclaving (The media detailed information were listed in following section).

2.2 GROWTH MEDIA AND BUFFERS

2.2.1 Microorganisms

The commercial wine LAB strains were either provided by Lallemand, Inc. (Montréal, PQ, Canada) or by the Wine Microbiology Laboratory Culture Collection at the Department of Food Science, University of Guelph, Guelph, ON, Canada.

2.2.2 Growth media and buffers

2.2.2.1 Mineral solution

Mineral solution was prepared by dissolving $MgSO_4$ x 7 H_2O and $MnSO_4$ × 4 H_2O to a final concentration of 200 g l^{-1} and 50 g $l^{-1,}$ respectively, in water to a final 100 ml volume. The solution was stored at –30°C.

2.2.2.2 Tween 80 solution

Tween 80 solution (5% w/v) was prepared by dissolving 5 g Tween 80 in water to a final volume of 100 ml. The solution was stored at –30°C.

2.2.2.3 General apple lactic acid bacteria medium

Apple LAB medium (Liu *et al.* 1995) was made of 5 g tryptone, 5 g peptone, 5 g yeast extract and 5 g glucose dissolved in 200 ml of commercial pure apple juice (Rougemont, Canada), 1 ml of mineral solution (2.2.2.1), 1 ml of Tween 80 (2.2.2.2), and 600 ml of water. The apple LAB medium was adjusted to pH 3.34 with 5 M H_3PO_4 and the solution was completed to a 1 l final volume before autoclaving.

2.2.2.4 Apple MRS medium

Apple MRS medium (AMRS) is a modification of MRS medium (de Man *et al.* 1960) for the growth of wine LAB and contained 55 g of Lactobacilli MRS broth (Difco, Sparks, MD, U.S.A.) diluted in 200 ml of a commercial pure apple juice (Rougemont, Canada) and 600 ml of water. The pH was then adjusted to 4.5 with 2 M NaOH or 2 M H_3PO_4 solution and made up to 1 l with water before autoclaving.

2.2.2.5 Modified MRS medium

The modified MRS medium was used to differentiate *Lactobacillus* from *Oenococcus* wine LAB (Krieger *et al.* 1992), and as a pre-conservation medium. The following ingredients were dissolved in 800 ml of water: 55 g of Lactobacilli MRS broth, 10 g of fructose and 4 g of L-malic acid (Sigma-Aldrich, St-Louis, MO, USA). The pH was then adjusted to 5.00 with 2 M KOH and made up to 1 l with water before autoclaving.

2.2.2.6 Grape juices and wines

Base wines used as LAB growth medium were prepared from a 2001 Chardonnay grape juice with 20.4 Brix (1.0834 g ml^{-1} specific gravity). Fermaid K, a commercial yeast nutrient (Lallemand, Montréal, PQ, Canada), was mixed into the juice to a final concentration of 250 mg l^{-1} prior to alcoholic fermentation (AF) at 20°C by 300 mg l^{-1} *S. cerevisiae* strain CY3079 which was reactivated according to manufacturer's instructions with 300 mg l^{-1} of GoFerm (Lallemand, Montréal, PQ, Canada). After completion of AF, the wine was racked (decanted from the yeast sediments). The wine was fined with a bentonite/casein mixture at 9 g l^{-1} (Agrilact, Laffort Oenologie, France), and stored overnight at 4°C. The wine was then pre-filtered with cellulose filter pads (Nr. 2, Buon Vino MFG, Cambridge, ON, Canada), and sterile filtered (Millipore, USA) before inducing MLF.

2.2.3 Wine lactic acid bacteria maintenance conditions

For short-term preservation (up to two months), cultures were inoculated in deep AMRS agar vials, which were immediately stored at 4°C. For long-term conservation (up to one year), bacterial cultures grown in modified MRS medium (2.2.2.5) at 27°C for two days were mixed 1:1 with 60% (w/v) glycerol and immediately frozen at -80°C. For reactivation, samples were thawed, plated on an apple LAB medium (2.2.2.3) and incubated at 27°C.

2.2.4 Induction of malolactic fermentation in wine

To induce MLF, 200 mg l^{-1} of autoclaved Opti'Malo Plus, a commercial malolactic bacteria nutrient (Lallemand, Montréal, PQ, Canada) was mixed to a sterile filtered wine. Wine LAB were pre-grown in a complex general LAB medium (2.2.2.3) at pH3.34 and incubated at 20°C, leading to initial populations of 1×10^8 to 1×10^{11} CFU ml^{-1} before being inoculated at 2% (v/v). Unless otherwise stated, all MLF were carried out at 20°C in a temperature-controlled incubator (model MIR-153, Sanyo Electric Co. Ltd., Japan). Wines were protected from oxidation during inoculations and samplings by flushing the headspace with nitrogen. In order to ensure applied conditions, the absence of dissolved oxygen introduction during sampling was measured with a fluorescence lifetime quenching dissolved oxygen meter (FIBOX 3, Precision Sensing GmbH, Germany) with automatic temperature compensation.

2.3 ANALYTICAL METHODS

2.3.1 General analysis

2.3.1.1 Analysis of grape juices and wines

Soluble solids in grape juices were measured with a handheld refractometer (ATC-1125, Atago, Japan). The termination of AF and assessment of dryness were evaluated with a colorimetric test for reducing sugars (Clinitest, Bayer Inc., Etobicoke, ON, Canada).

2.3.1.2 pH determination

The pH was measured using a pH-meter (Accumet Basic AB15, Fisher Scientific, Whitby, ON, Canada) calibrated daily with standard buffer solutions (at pH values of 2.00, 4.00 and 7.00).

2.3.1.3 Spectrophotometric measurements

Spectrophotometric measurements were made with a standard laboratory spectrophotometer (Pharmacia LKB Novaspec II, Cambridge, UK). Disposable half-micro cuvettes (maximum 1.7 ml) were used throughout the work

2.3.2 Growth assessment

Viable bacterial cell counts were carried out by spreading 100 µl of diluted culture samples onto apple LAB (2.2.2.3) agar plates (2.1.4). The formation of colonies was quantified after 4-day incubation at 27°C. Sterile 0.1% (w/v) peptone water solution was used to dilute the samples. The cell numbers were calculated considering the average number of bacteria per chain as observed by light microscopy (Carl Zeiss, Standard 25 ICS, Germany).

Except for the initial determination of an inoculum, the evolution of the bacterial growth during incubations was measured as optical density (OD) throughout this thesis using a spectrophotometer at a wavelength of 645 nm.

2.3.3 Chemical or enzymatic analysis of metablites

Acetaldehyde glucose, fructose, acetic acid and L-malic acid

L-malic acid was determined using a Boehringer Mannheim (1989) L-malic acid enzymatic test kit at 25°C by UV detector at 340nm.

Organic acids by HPLC

Organic acids was quantified using a HPLC method described in Chapter 3

Sulphur dioxide

SO_2 was measured according to a modified Ripper method (Amerine and Ough 1974; Osborne 2000). Total SO_2 was determined by mixing 5 ml of 10% (w/v) NaOH in 25 ml of wine and incubated for 15 min. Free SO_2 was measured via the following: 5 ml of

25% (v/v) H_2SO_4 and a spatula of starch indicator were mixed in 25 ml of wine, and rapidly titrated with a 0.01 N standardized iodine solution until a blue end-point lasting 30 seconds was obtained. The SO_2 concentration was then calculated according to the following equation:

Equation 2-1
$$SO_2 = \frac{V_{\underset{Titrant}{Iodine}}(ml) \times C_{\underset{Normality}{Iodine}} \times 32 \times 1000}{V_{Wine}(ml)} \text{ in mg } l^{-1}.$$

2.3.1 Statistical analysis and precision of analytical methods

Where statistics are reported, the following standard error parameters are used: standard error (SE) and coefficient of variation (% CV).

Significance tests were carried out with the Student's t-test and F-test by excel and SPSS, the analysis of variance (ANOVA) or correlation at specified level ($p \leq 0.05$) of significance.

CHAPTER 3. DEVELOPMENT OF A HPLC METHOD FOR QUALITATIVE AND QUANTITANTIVE DETERMINATION OF ORGANIC ACID FROM GRAPE JUICE AND WINE

3.1 INTRODUCTION

Various existing methods can be used to assay organic acids in grape must and wine which include those to measure total acidity by titration (William et al. 1983), enzymatic testing to analyze every single acid, and GC/MS to measure volatile acids (Mato et al. 2005). However, every method has some disadvantages. Titration can quantify the total acidity but cannot qualitatively analyse single acids. Although enzymatic testing is a very accurate, rapid, and specific method for quantification of individual acids, but it can be expensive and time consuming. In this project a large number of samples were to be analysed, each with at least six different acids to be measured separately by different enzymatic tests. Furthermore, before enzymatic testing, wine or must samples usually need to have some preparation involving centrifugation, filtration, pH adjustment and decolorization, all of which could introduce errors. GC/MS is an efficient tool to identify and analyze volatile compounds, and is used in this project to measure the volatile acids, esters, alcohol and acetaldehyde. Nevertheless this method cannot measure all the organic acids in wine or grape must such as tartaric acid, malic acid and lactic acid (Mato et al. 2005). Therefore, a rapid, economic, and accurate method was required to be established and used for organic acid measurements in wine and must.

HPLC is an efficient tool for separation, identification, purification, and quantification of various compounds, based on the difference in the surface interaction of

the analyte and eluent molecules. Compared to the methods previously mentioned, HPLC is very sensitive not only for qualitative analysis but also for quantitative determination (Swadesh 1997). Furthermore it has no requirements for the compounds to be volatile. In a single injection, several organic acids can be analysed simultaneously. In addition, the HPLC system requires only some simple preparation of the wine or grape must sample (Barbieri et al. 1995; McCord et al. 1984).

Because HPLC has so many advantages for analysis of organic acids in wine and must, it has been thoroughly researched. Different types of column and several eluents have been tried, but present methods generally have at least one of the following drawbacks (Llorente et al. 2006). The seven major organic acids existing in grape must and wine cannot be simultaneously analysed during one injection. Some co-elutions, causing inefficient identification, might occur and the samples' recoveries are beyond acceptable ranges. Even though some methods have good separations, the run times are long, therefore, the method is not practical for measuring a large number of samples in a short period of time. Hence, there is a need to develop a simple, fast, and reliable HPLC method.

Different column types can also produce different separation results. This chapter discusses how two kinds of columns suitable for separating organic acids have been used and compared. One was Phenomenex Synergi 250 x 4.6 ID mm Hydro- RP 80A column filled with C_{18} with polar end capping. A 50 x 4.6 ID mm Hydro-security guard pre-column and a 0.5 μm in-line filter (Upchurch, Oak Harbour, WA, USA) were used. Unlike the usual C_{18} column which can only accurately retain or separate extremely polar analytes, this column method provides extreme retention of both hydrophobic as well as polar compounds via

polar interactions, hydrogen bonding or electrostatic interactions. Another column is the Supelcogel ^{TM} H which contains a polystyrene resin in the hydrogen form. It is ideal for separating mixtures of organic acids, fermentation products (e.g., alcohols), and carbohydrates. Such mixtures commonly occur in fruits, vegetables, and beverages. Larger and acidic analytes elute before smaller analytes. The column is stable between pH 2 and pH 7 (Sigma-Aldrich).

In order to improve the poor resolution between some compounds, one or several organic modifications were added to mobile phase, but this can only partially solve the problem. Adding an organic modification might change the pH of the mobile phase. Furthermore, for neutral or non-polar analytes, hydrophobic interactions may occur in the stationary phase. Some organic modifiers, such as THF, have ability in reducing peak tailing and improve peak shape because analyte diffusion is less hindered in the swollen polymer (Gooding and Regnier 1990). This chapter explains how different organic modifiers were tested and used in the above two columns.

3.2 METHOD DEVELOPMENT

3.2.1 Standard solution and sample preparation

Stock standard solutions of 8 organic acids were prepared in HPLC grade water based on the concentration range commonly found in grape must and wine (Table 1). The concentrations are listed in Table 1, at pH 1.9; the highest concentration was used as the standard (e.g., 8.0 g l^{-1} for tartaric acid), and others were derived by sequentially diluting the stock standard solution. The dilution factors are as follows: 0, 0.15, 0.25, 0.4, 0.55, 0.7, 0.85, and 1, e.g., tartaric acid concentrations are 0.0 g l^{-1}, 1.2 g l^{-1}, 2.0 g l^{-1}, 3.2 g l^{-1}, 4.4 g l^{-1}, 5.6 g l^{-1},

6.8 g l^{-1}, and 8.0 g l^{-1}. All the standard solutions were filtered through 0.2 μm nylon filters (Millipore, USA) and 20 μl were directly injected.

Table 1. Range of organic acids in wine or grape must and stock standard solution

Name	Range [g l^{-1}] (wine and grape must)	Stock standard solutions [g l^{-1}]
Tartaric acid	2-8	8
Malic acid	0-8	6
Citric acid	0.15-0.3	0.5
Acetic acid	0-1	1
Succinic acid	0.2-0.6	0.6
Pyruvic acid	<0.3	0.3
Lactic acid	0.2-5	5
Fumaric acid	<0.01	0.5
Formic acid	0	0.5

0.5 g ml^{-1} of PVPP was added to red wine or must to decolourize the solution. After mixing for 30 min, the sample was centrifuged at 1035g for 2 min. The suspensions were filtered through 0.2 μm nylon filters (Millipore, USA) and 20 μl were directly injected. White wine/must followed the same procedures without adding PVPP.

3.2.2 Apparatus and analysis conditions

3.2.2.1 Equipment

Organic acids were analysed on a Shimadzu Class UP HPLC system, consisting of a DGU-10 AVP degasser, a quaternary LC-10ADVP pump system, a SIL-20ACVP auto sample injector, a CTD-10AVP column oven, a SPD-10AVP UV-VIS-detector, and a CBM-20A system controller. This HPLC system was connected to a PC equipped with Class VP software (version 7.5), for data collection and analysis. UV-detection of organic acids was carried out at 210 nm.

3.2.2.2 Calibration, calculation and identification

Sample peaks were identified on the basis of retention times and spiking technique, while quantification was performed either through an external standard calibration curve or internal and external standard calibration curves. Linearity was obtained with peak areas of eight solutions.

Different parameters, such as linear least squares regression, were estimated for HPLC separation method evaluations, in order to calculate the slope, intercept and correlation coefficient. Capacity factor and separation factor (see below) were used to estimate the separation effect of the two different columns. Recoveries were calculated for each testing method application.

Capacity factor, K, also known as retention time factor, of a sample component is a measure of the degree to which a component is retained by the column relative to an unretained component, which is expressed as

$$K=(V_A-V_O)/V_O$$

V_O: First peak retention time

V_A: the retention time of the analysed peak (Gooding and Regnier 1990)

Changing the retention factor of different compounds in one column requires increasing or decreasing the polarity difference between the stationary and the mobile phase. Also the peak shape can be changed by changing the polarity. If a compound is retained on the column, the peak for this compound will be broadened through diffusion. In one single run every peak should have a different retention factor; otherwise co-elution will be found among those peaks. The bigger the differences among the peaks, the better the separations (Swadesh 1997).

The separation factor, also known as selectivity, measures the space between two peaks, expressed as:

$$\alpha=(V_A-V_O)/(V_B-V_O)$$

V_B: the retention time of the peak just behind the analysed peak

As with the capacity factor, when retention with solvent polarity is changed, all peaks show an equivalent shifting in the same direction which causes the separation factor to change. The bigger the separation factor, the better will be the separation between the relevant peak and the front peak (McMaster 1994; Swadesh 1997).

Recovery is another important parameter to evaluate the HPLC separation method. Recovery of the method was determined by spiking 10ml aliquots of wine / grape must sample with 10ml of standards of the 8 organic acid mixtures. The calculation formula is as follows

$$RECOVERY = \frac{2 \times C_{MIXED}}{C_{STD} + C_{SAMPLE}}$$

C_{MIX}: the concentration of 50% sample and 50% Standard (STD) mixed together measured by HPLC

C_{STD}: the concentration of 100% STD measured by HPLC

C_{SAMPLE}: the concentration of 100% sample measured by HPLC

Samples from wine and grape must are usually not comparable with standards which have good recoveries (Castellari et al. 2000; Herbert et al. 2006; Zotou et al. 2003). The sample's recoveries from wine or grape must are usually in the range of 80% to 120%. Compared to other methods and studies, if the recoveries of each peak can be in the range

of 95% to 105%, the method is considered to be relatively good. The method is still acceptable if the recovery of each peak is in the range of 85% to 115% (McMaster 1994).

3.2.2.3 Columns and column conditions

Table 2. Column abilities, suitable conditions, and isocratic separation conditions

Column name		Supelcogel ™ H	Synergi Hydro-RP
Limited column conditions	The max pressure [psi]	<1150	<3000
	pH range	1-9	1-13
	Polar resolution range (aqueous mobile phases)	0-10%	0-100%
Isocratic separation conditions	Flow rate [ml min^{-1}]	0.22	1.0
	Mobile phase [%, v/v]	16 H_3PO_4 9 Isopropanol	0.14 trifluoracetic acid
	Column separation temperature [° C]	30	30
	External standard	Formic acid 5mg l^{-1}	None

3.3 RESULTS AND DISCUSSION

3.3.1 Effects of mobile phase composition

For the Supelcogel ™ H column, in order to gain a better separation among organic acids from grape must or wine, different concentrations of H_3PO_4 were tested, with different concentrations of isopropanol, and ANC as an organic modifier. Formic acid was used as an external standard. The capacity factor and separation factor, which are shown in Tables 3, 4, and 5 were measured to compare separation results.

Table 3. Separation factor and capacity factor using different concentrations of H_3PO_4

Parameter	K					
H_3PO_4 (v/v%)	16.0000	10.0000	6.5000	2.6000	0.1750	0.0200
Citric	0.4364	0.2007	0.3454	0.5420	0.4249	0.3345
Tartaric	0.5186	0.2735	0.4366	0.6725	0.5432	0.5415
Malic	0.6991	0.4181	0.5871	0.8208	0.6909	0.7035
Pyruvic	0.8186	0.5161	0.6841	0.8836	0.6776	0.4294
Succinic	1.1161	0.7459	0.9559	1.2477	1.1157	1.1082

Lactic	1.1443	0.7876	1.0033	1.2976	1.3159	1.1893
Formic	1.3293	0.9170	1.1520	1.4861	1.3371	1.3262
Acetic	1.5051	1.0833	1.3393	1.6860	1.5332	1.5420
Fumaric	1.8451	1.3181	1.5180	2.0002	1.7449	1.0772
Parameter	α					
Citric	2.4099	1.5475	1.4570	1.3904	1.3146	1.2713
Tartaric	1.1881	1.3626	1.2639	1.2408	1.2786	1.2611
Malic	1.3482	1.5290	1.3447	1.2205	1.0196	1.2992
Pyruvic	1.1710	1.2344	1.1652	1.0766	1.2473	1.2838
Succinic	1.3634	1.4451	1.3974	1.4120	1.6150	1.0288
Lactic	1.0252	1.0559	1.0496	1.0400	1.1795	1.0732
Formic	1.1617	1.1643	1.1482	1.1453	1.0161	1.1152
Acetic	1.1322	1.1814	1.1626	1.1345	1.1467	1.1627
Fumaric	1.2259	1.2167	1.1334	1.1864	1.1381	1.5312

The results of separation factors and capacity factors of 6 different concentrations of H_3PO_4 are presented in Table 3. K shows the order of peaks retention time, and α indicates the separation effect. 0.02 %(v/v) H_3PO_4 in the mobile phase could not increase the poor resolution between succinic acid and fumaric acid sufficiently with a separation factor of 1.0288 (Table 3). This value is far from the required minimum separation factor of 1.05 (McMaster 1994; Swadesh 1997). Increasing the H_3PO_4 concentration to 0.175% (v/v) improved the resolution between succinic acid and fumaric acid, but at the same time other separation problems appeared, e.g., between malic acid and pyruvic acid, and also between succinic acid and lactic acid. Further increasing the H_3PO_4 concentration did not improve the poor resolution between succinic acid and lactic acid. At 16 %(v/v) H_3PO_4 all the other compounds had good resolution, except for lactic acid and succinic acid.

Table 4. Separation factor and capacity factor using different concentrations of ACN

Parameter	K			
ACN (v/v%)	0	5	6.5	8
Citric	0.4364	0.5175	0.3881	0.3914
Tartaric	0.5186	0.6474	0.4551	0.5077
Malic	0.6991	0.6474	0.6313	0.6340
Pyruvic	0.8186	0.8359	0.6482	0.6523
Succinic	1.1161	1.0872	0.7945	0.8216
Lactic	1.1443	1.1419	0.9833	0.9437
Formic	1.3293	1.2960	1.1026	1.1047
Acetic	1.5051	1.4869	1.2976	1.2907
Fumaric	1.8451	1.7373	1.5421	1.4263
Parameter	α			
Citric	2.4099	4.9412	5.0376	3.6039
Tartaric	1.1881	1.2510	1.1728	1.2971
Malic	1.3482	1.0000	1.3871	1.2487
Pyruvic	1.1710	1.2912	1.0267	1.0290
Succinic	1.3634	1.3007	1.2257	1.2595
Lactic	1.0252	1.0503	1.2377	1.1486
Formic	1.1617	1.1349	1.1213	1.1705
Acetic	1.1322	1.1474	1.1769	1.1684
Fumaric	1.2259	1.1684	1.1884	1.1050

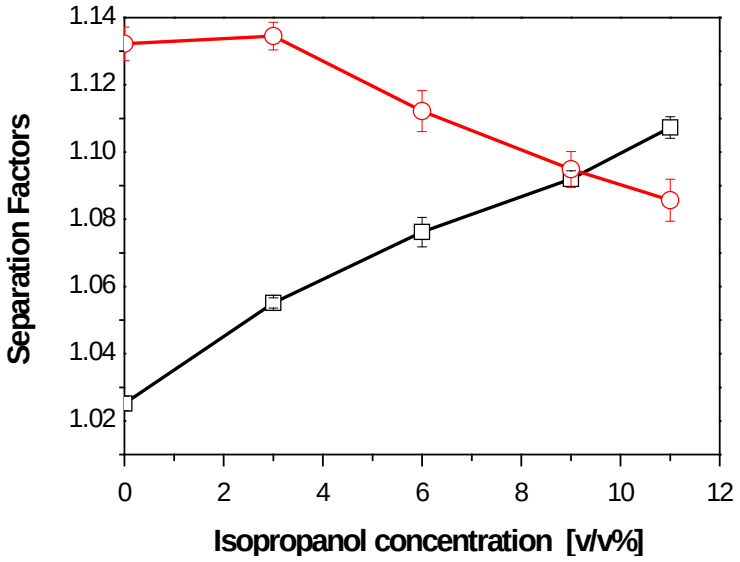
In order to solve the poor resolution between lactic acid and succinic acid, different concentrations of ACN were mixed with H3PO4 16% (v/v). ACN is one of the most important organic modifiers used in the reverse phase column (Lee 1982; Llorente et al. 2006). Separation and capacity factors are shown in Table 4. Although adding 6.5 %(v/v) ACN increased the separation between succinic acid and lactic acid, poor resolution between malic acid and pyruvic acid still occurred even after increasing the ACN to 6.5 % (v/v) (Table 4).

Table 5. Separation factor and capacity factor using different concentrations of isopropanol

Parameters	K				
Isopropanol(v/v%)	0	3	6	9	11
Citric	0.4364	0.4132	0.4049	0.3707	0.3945
Tartaric	0.5186	0.4964	0.4958	0.4713	0.4994
Malic	0.6991	0.6752	0.6556	0.6034	0.6322
Pyruvic	0.8186	0.8353	0.8020	0.8161	0.7769
succinic	1.1161	1.0687	1.0341	0.9684	0.9784
Lactic	1.1443	1.1277	1.1129	1.0575	1.0834
Formic	1.3293	1.3034	1.3203	1.2874	1.3246
Acetic	1.5051	1.4787	1.4684	1.4095	1.4380
Fumaric	1.8451	1.7609	1.7028	1.6164	1.6213
Parameters	α				
Citric	2.4099	5.1787	4.9906	7.1667	5.3536
Tartaric	1.1881	1.2013	1.2245	1.2713	1.2661
Malic	1.3482	1.3602	1.3223	1.2805	1.2658
Pyruvic	1.1710	1.2372	1.2233	1.3524	1.2290
succinic	1.3634	1.2794	1.2894	1.1866	1.2594
Lactic	1.0252	1.0551	1.0762	1.0920	1.1073
Formic	1.1617	1.1558	1.1864	1.2174	1.2226
Acetic	1.1322	1.1345	1.1122	1.0949	1.0857
Fumaric	1.2259	1.1908	1.1596	1.1468	1.1274

Since ACN addition did not totally solve the problem of poor resolution, other organic modifiers such as methanol, ethanol, and isopropanol, were tested. Table 5 shows the capacity (K) and separation factors (α) when different concentrations of isopropanol were added to 16 %(v/v) H_3PO_4. By increasing isopropanol concentration from 0 to 16 %(v/v) in the mobile phase, the separation factor of succinic acid and lactic acid, α_1, increased, therefore, the separation improved. However, at the same time, by increasing isopropanol, the separation factor of formic acid and acetic acid, α_2, was reduced. This relation between separation factors α_1, α_2, and isopropanol concentration is shown in Figure 2.

Figure 2. Separation factors: α_2 of formic acid and acetic acid (—O —); α_1 of succinic acid and lactic acid (—O —) as affected by changes in isopropanol percentages. (Chromatographic Conditions: Column Supelcogel ^{TM} H, at 30°C Flow 0.22 ml min^{-1}, Eluent 0.16% H_3PO_4, Detection at210 nm)



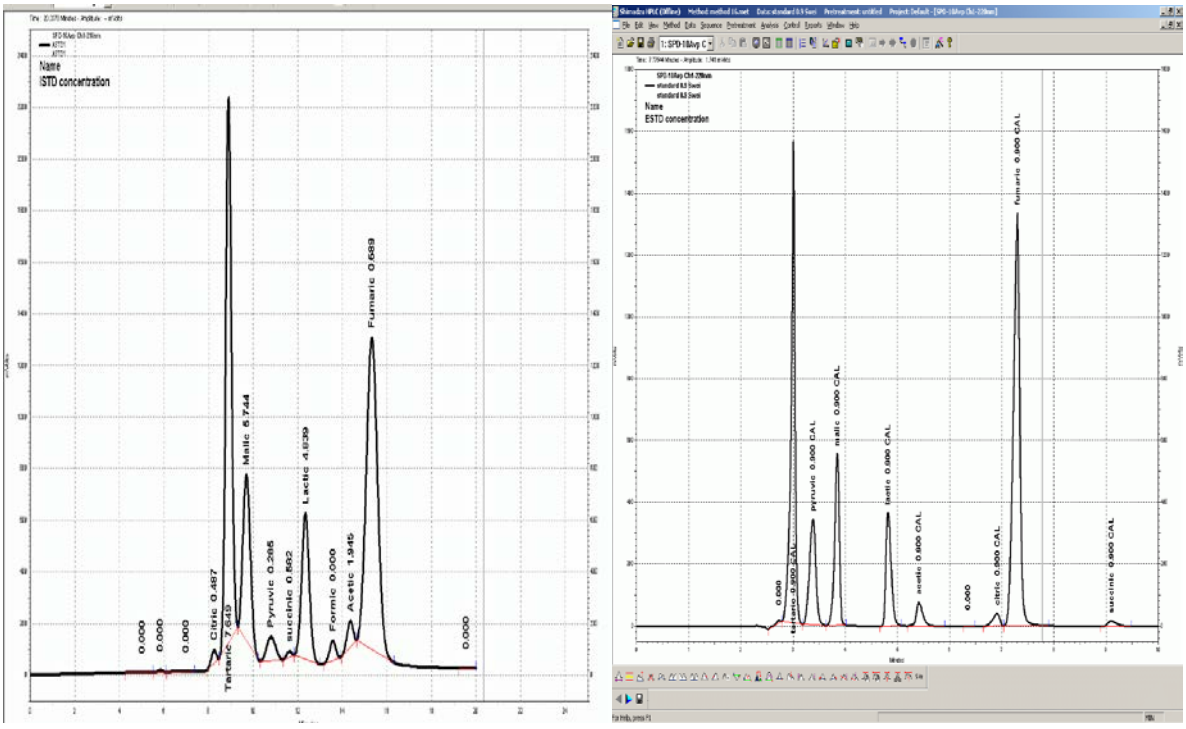
In order to obtain a good separation both between formic acid/acetic acid and between succinic acid/lactic acid, the optimal concentration of isopropanol was identified as the cross point of the two lines, which is the concentration at 9% (v/v). So for the Supelcogel ^{TM} H column, the mobile phase using 9% (v/v) of isopropanol and 16% H_3PO_4 was selected to separate organic acids in grape must/ wine.

For the Synergi Hydro-RP column, the mobile phase selection procedure was similar to the Supelcogel ^{TM} H. In order to gain good separation, H_3PO_4, H_2SO_4, TFA, ethanol, methanol, ACN and isopropanol were added as organic modifiers in a wide range of concentrations. All the separation factors and capacity factors are shown in the Table 5. TFA

at 0.1 %(v/v) was selected for the optimum mobile phase from the above listed organic modifiers.

The final separation results are shown in Figure 3

Figure 3. Separation of the organic acids present in wine with HPLC. Left panel Synergi Hydro-RP column, right panel Supelcogel ^{TM} H column.



3.3.2 Method validation

3.3.2.1 Linearity

The results are presented in Table 6. For both columns, good correlation coefficients were obtained for all eight organic acids.

Table 6. Calibration, calculation and identification of 8 organic acids (regression equations determined using Origin)

Name	Supelcogel ™ H			
	Linear	Retention	Regression equation	R^2
Tartaric acid	0-8	7.950	$y=1.65917x^2+10.4227x+0.0151242$	0.999
Malic acid	0-6	8.533	$y=0.821724x^2+2.11236x+0.139756$	0.996
Citric acid	0-0.5	9.300	$y=6.59741x^2+3.57793x+0.0019812$	0.999
Acetic acid	0-1	10.533	$y=0.4298x^2+3.128x-0.02775$	0.999
Succinic acid	0-0.6	11.417	$y=2.144x^2+2.152x+0.00514$	0.993
Pyruvic acid	0-0.3	11.933	$y=109.596x^2+12.8797x+0.0394$	0.998
Fumaric acid	0-0.5	13.975	$y=354.214x^2+121.227x-0.0230181$	0.999
Name	Synergi Hydro-RP			
	Linear	Retention	Regression equation	R^2
Tartaric acid	0-8	2.817	y = 1122180x + 796431	0.992
Malic acid	0-6	3.667	y = 104220x + 10946.2	0.998
Citric acid	0-0.5	6.442	y = 991546x + 59665.7	0.993
Acetic acid	0-1	4.867	y = 542246x + 85290.7	0.973
Succinic acid	0-0.6	7.800	y = 416074x + 21337.0	0.987
Pyruvic acid	0-0.3	3.150	y = 8882700x + 239739	0.993
Lactic acid	0-5	4.400	y = 354378x + 114380	0.994
Fumaric acid	0-0.5	6.942	y = 110047000x + 660398	0.991

3.3.2.2 Recovery

The results for wine and grape must recovery are shown in Table 7. All the peak recoveries of organic acids from grape must and wine were in an acceptable range, from 85% to 115% with only one exception greater than 115%. For the Supelcogel ™ H column, the recovery of tartaric acid from wine samples was 125%, possibly caused by co-elution with some compounds containing the C=O double bond (Castellari et al. 2000). Tartaric is one of the eight main organic acids in the wine and grape must, however, this result was not reliable since it could not be degraded or produced by microorganisms during fermentation and it

only changed physically, e.g., crystallization at due to various temperatures during different fermentation stages. Thus as long as the tartaric acid can be separated from other seven main organic acids, the method is acceptable.

Compared to recoveries using the Supelcogel ™ H column, the protocol for the Synergi Hydro-RP column was much better. All recoveries were in the range from 80% to 120%. In particular, the recoveries of the five most important organic acids during fermentation were in a small range, between 95% and 105%. Thus, the HPLC method is very accurate for separating organic acids.

Table 7 Recoveries of the eight main organic acids out of wine and grape must. The acids were analyzed by HPLC with a Supelcogel™ H (at 30 °C flow rate 0.22 ml min^{-1}, eluent 16% (v/v) H_3PO_4 +9% (v/v) isopropanol, detection @210 nm) and Synergi Hydro-RP (at 30°C, flow rate 1.0 ml min^{-1}, eluent 0.14 (v/v) trifluoracetic acid, detection @210 nm) column.

Name	Supelcogel ™ H						
	STD [g l^{-1}]	Wine [g l^{-1}]	50%STD & 50%wine [g l^{-1}]	Must [g l^{-1}]	50% STD & 50% must [g l^{-1}]	Recovery of wine	Recovery of must
Tartaric acid	6.072	0.656	4.235	1.595	4.183	1.25	1.09
Malic acid	2.401	2.818	2.488	4.001	3.578	0.95	1.11
Citric acid	0.374	0.122	0.219	0.247	0.315	0.88	1.01
Acetic acid	2.263	0.008	0.99	0	1.071	0.87	0.95
Succinic acid	1.747	4.596	3.038	2.941	2.71	0.96	1.15
Pyruvic acid	0.216	0	0.098	0	0.113	0.91	1.04
Lactic acid	4.915	0.098	2.408	0	2.346	0.96	0.95
Fumaric acid	0.608	0.046	0.32	0.027	0.33	0.98	1.03
Name	Synergi Hydro-RP						
	STD [g l^{-1}]	Wine [g l^{-1}]	50%STD & 50%wine [g l^{-1}]	STD [g l^{-1}]	Must [g l^{-1}]	50% STD & 50% must [g l^{-1}]	Recovery of wine[%]	Recovery of must[%]

Tartaric acid	2.888	0.916	2.235	7.145	2.47	5.701	1.17	1.18
Malic acid	2.458	1.542	2.319	5.486	0.795	3.281	1.05	1.04
Citric acid	0.07	0.207	0.14	0.384	0.509	0.452	1.01	1.01
Acetic acid	0.464	0.235	0.351	1.399	0.041	0.822	1.00	1.10
Succinic acid	0.277	0.71	0.498	0.472	0	0.245	1.01	1.03
Pyruvic acid	0.121	0.033	0.083	0.319	0.057	0.191	1.07	1.02
Lactic acid	3.518	0.697	2.099	7.558	0	3.907	0.99	1.03
Fumaric acid	0.047	0	0.022	0.096	0	0.049	0.93	1.02

3.3.2.3 Application

The method developed herein was applied to determine the content of organic acids in white or red wine and grape must and the results are reported in Chapters 4 and 5. The chromatograms of a white and a red wine sample, respectively, were treated and analyzed as described under Experimental. Peak identification was based on retention times and spiking technique.

3.4 CONCLUSION

The two methods described in this chapter were found to be suitable for the determination of the eight main organic acids in wine and grape must and involved the same efficient sample pre-treatment with PVPP. Good linearity, satisfactory recovery results for all acids and the high level of sensitivity, precision and selectivity along with the method's simplicity are recommended whenever there is a need for results of high analytical quality. Comparing the two separation methods, and considering the efficiency and economy of each, the method using the Synergi Hydro-RP was preferable due to a shorter sample separation time (i.e., less than 10 min), a rapid mobile phase preparation, and accurate analysis results as reflected by Tables 6, and 7 and Figure 3.

CHAPTER 4. SIMULTANEOUS AF/MLF IN WHITE WINE AT FOUR DIFFERENT PH VALUES

4.1 INTRODUCTION

The first chapter discussed the importance of MLF for most red and some white wines, but due to the interaction(s) between yeast and LAB, the wines undergoing MLF under the traditional vinification method have the challenge of LAB growth and LAB malic acid degradation, especially at low pH values. An alternative vinification method via simultaneous AF/MLF might overcome the stuck or sluggish MLD. However, due to the possibility of high acetic acid production and wine quality depletion and the lack of research, simultaneous AF/MLF has rarely been applied.

In this chapter, simultaneous AF/ MLF was compared with consecutive AF/MLF in Chardonnay must at four pH values within the range of must qualities obtained in cool and warm climates. The time course of the concentrations of the hexoses, glucose and fructose, as well as several organic acids and other compounds important to wine quality were investigated. While final values for several compounds were similar, significant differences among their kinetics during fermentation were observed.

4.2 MATERIALS AND METHODS

Flash pasteurized (72°C for 30 s) Chardonnay must (Cave des Corbières, Rivesaltes, France) was obtained from Kamil EX-IM International Trading Company, Guelph, ON, Canada. The must had a soluble solid content of 21.6 Brix (20°C), pH 3.00, a total acidity of 7.5 g l^{-1} as tartaric acid and contained 3.11 g l^{-1} of malic acid. After must filtration to remove sediments (10 μm Claris Series filter, Pall Corporation, New York, NY, USA), 500 mg l^{-1} of

diammonium hydrogen phosphate (Fisher Scientific, Nepean, ON, Canada) and 300 mg l^{-1} of Fermaid K (Lallemand, Montreal, PQ, Canada) were added as nutrients. Subsequently, the must was separated into 4 batches, which were adjusted to pH 3.20, 3.35, 3.50 and 3.65, respectively, by addition of appropriate amounts of a 300 g l^{-1} $KHCO_3$ solution followed by cold stabilization of the must. Four 1-gallon (3.79 l) glass carboys were filled with must of each pH value (16 in total). The eight treatments consisted of carrying out vivifications at the above four different pH values. All treatments were run in duplicate. The yeast *S. cerevisiae* CY3079 and the malolactic LAB *O. oeni* MBR 31 were inoculated either together (i.e., simultaneous AF/MLF), or LAB was added after completion of AF (consecutive AF/MLF). Specifically, the yeast was added at 300 mg l^{-1} and the MLB at 10 mg l^{-1}. The fermentation temperature was maintained at 19-20°C in a temperature-controlled room where the carboys with fermentation locks were placed in random order. After completion of AF and MLF, as assessed by stable sugar and malic acid concentrations, 30 mg l^{-1} of SO_2 were added to the wines, the carboys were then racked of the primary yeast residues lees(dead cells and sediments) The samples were then cold stabilized (2°C, for one week), and racked again. The concentration of free SO_2 was adjusted to 30 mg l^{-1} in three rounds over the period of two weeks. Finally, the wines underwent depth filtration with filter sheets (EK grade, Seitz-Schenk, Germany) followed by 0.22 μm membrane filtration (nylon disc filter, Whatman Internal Ltd. Maidston, England) before bottling. CO_2 was used for insertion of headspaces throughout the entire winemaking process. The wines were stored in 750 ml bottles for 12 months for further chemical analyses.

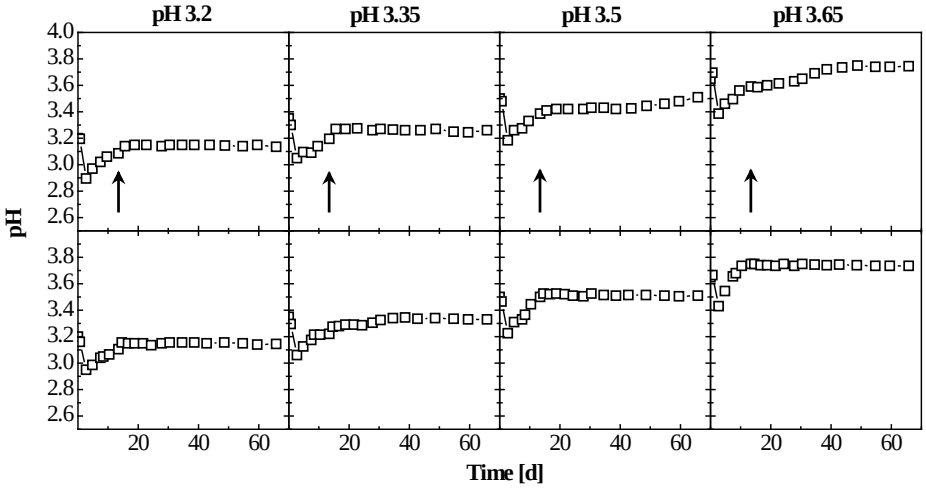
Inoculation of MLB in sequential treatments

Wines in consecutive treatments were inoculated with bacteria once the combined glucose and fructose concentrations fell below 1.5 g l^{-1}which was the case after 13.5 days in consecutive AF/MLF treatments.

4.3 RESULTS

4.3.1 pH values during fermentation

Figure 4. Time course of pH values during vinification of Chardonnay with *S. cerevisiae* CY3079 and *O. oeni* MBR31. Upper row, consecutive yeast bacterial inoculations (arrow indicates time of bacterial inoculation). Lower row, simultaneous yeast bacterial inoculations. Average results from duplicate incubations shown.

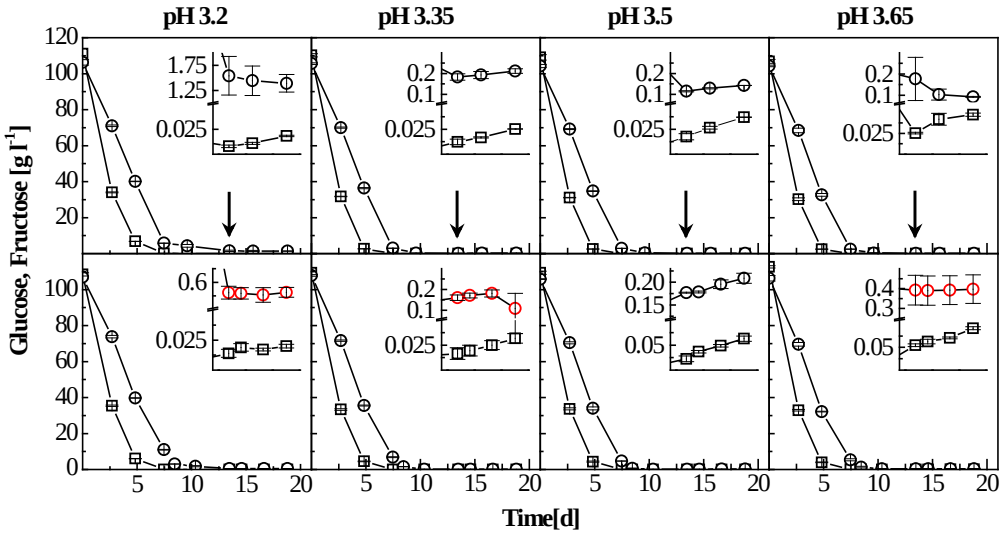


Regardless of the initial pH and vinification differences, during the first 5 days the trend for pH values in all fermentations decreased. However, this initial decrease was

slightly more pronounced in consecutive treatments (0.01 – 0.05 pH units). Between days 5 and 18, the pH values increased continuously. Only in the consecutive AF/MLF treatment, at initial pH 3.50 and pH 3.65, did the inoculation with LAB lead to a slight increase in pH which was not observed in the simultaneous AF/MLF treatments. There was no statistically significant difference ($p > 0.05$) among the average final pH values of treatments with similar initial pH values, except for pH 3.35 where malic acid was depleted in simultaneous, but not in the consecutive treatment

4.3.2 Sugar metabolism during fermentation

Figure 5. Time course of glucose and fructose concentrations during vinification of Chardonnay with *S. cerevisiae* CY3079 and *O. oeni* MBR31. Upper row, consecutive yeast bacterial inoculations (arrow indicates time of bacterial inoculation). Lower row, simultaneous yeast bacterial inoculations. ∀, glucose; –, fructose. Inserts magnify period from 12 to 20 days. Data points represent the mean from duplicate incubations ±SE.



The overall rate of sugar degradation during vinification appeared to be relatively homogeneous across the different initial wine pH values and different inoculation treatments (Figure 5). No stuck or sluggish alcoholic fermentation was found, and no substantial inhibition to yeast growth was recorded when LAB was inoculated along with yeast.

However, at the lowest initial pH (3.20), the sugar degradation was slightly delayed, and a statistically significant amount of fructose remained, especially with consecutive fermentation. The highest residue was recorded at the lowest initial pH (3.20) for both vinification methods with the same yeast-LAB strain. The results at the remaining pH values showed that the effects of the initial wine pH on residual hexose concentrations were opposite for consecutive and simultaneous AF/MF. After reaching minimum values of 2 – 13 mg l^{-1} at t=13.5 days, glucose concentrations increased in all treatments during the late

fermentation phase (inserts in Figure 5). Final glucose residues were higher in treatments produced by co-inoculation and at higher pH values, but remained below 100 mg l^{-1} in all treatments (Table 11).

4.3.3 Malic acid and lactic acid

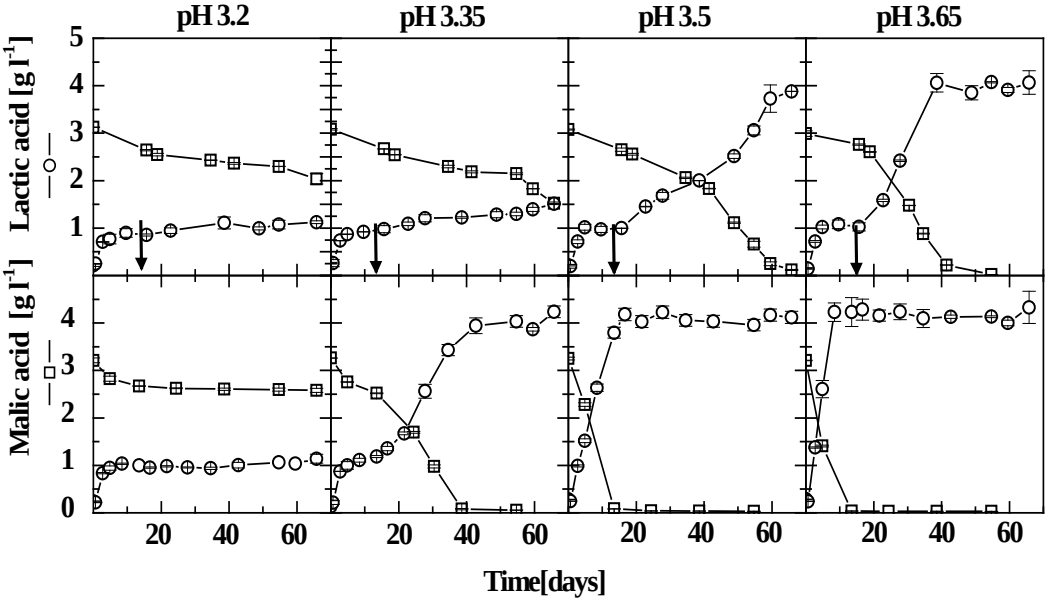
Table 8. Time required to reach dryness (combined glucose and fructose levels below 1 g l^{-1}), and to reach L-malic acid concentrations below 150 mg l^{-1} during fermentations of Chardonnay must with *S. cerevisiae* CY3079 combined with *O. oeni* strains MBR31 (consecutive treatments were inoculated with bacteria after reaching dryness at 13.5 days; L–malic acid residue in parentheses, where applicable).

	Simultaneous	Consecutive AF/MLF	
	treatment, time since yeast/bacterial	Time since bacterial	Total time since yeast
pH value	inoculation (d)	inoculation (d)	inoculation (d)
3.20	NR (2.58 g l^{-1})	NR (2.04 g l^{-1})	NR (2.04 g l^{-1})
3.35	38.5	NR (1.52 g l^{-1})	NR (1.52 g l^{-1})
3.50	13.5	41.5	65
3.65	13.5	31	54.5

N.R.: Not reach the detection level. (L-malic acid levels <150 mg l^{-1})

Figure 6. Time course of malic and lactic acid concentrations during vinification of Chardonnay by *S. cerevisiae* CY3079 and *O. oeni* MBR31. Upper row shows results from consecutive yeast and bacteria inoculations (arrow indicates inoculation with bacteria). The lower row shows results from the simultaneous inoculation of yeast

and bacteria. ∀, malic acid; −, lactic acid. Data points represent the mean from duplicate incubations ±SE.



Malic acid was not depleted within 65 days duration of the experiment in consecutive treatments at pH 3.20 and pH 3.35, and the simultaneous treatment at pH 3.20 (Figure 6), while in all other treatments MLF was successful. Where MLF was successful, malic acid degradation rates were statistically significant higher and MLF durations (after LAB inoculation) were statistically significant shorter in simultaneous treatments for any given pH value (Figure 6).

Lactic acid production tends to be correlated with malic acid degradation during malolactic fermentation (Bartowsky 2005), as was demonstrated in this study, with the

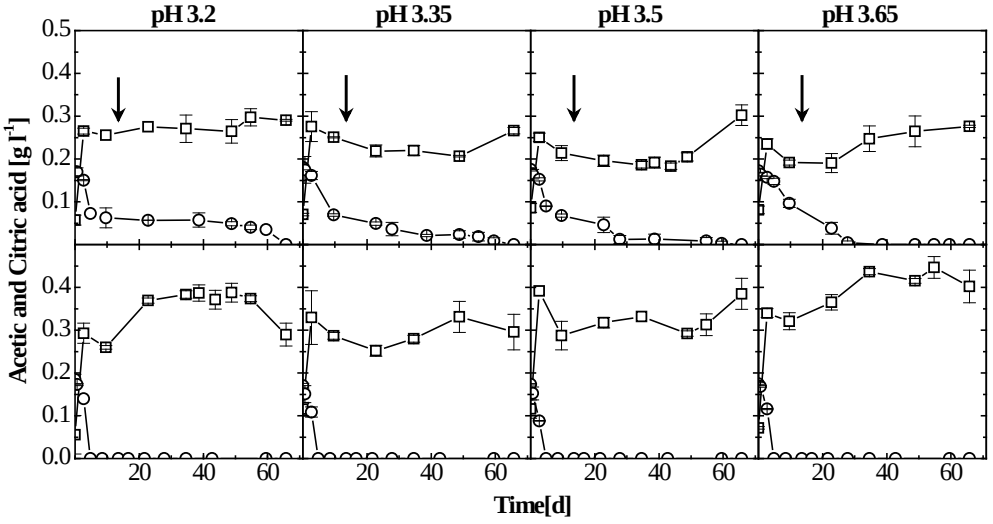
exception that early production of lactic acid was recorded in the consecutive vinification treatments without malic acid depletion (Figure 6). In all treatments with complete malic acid degradation, the molar malic to lactic acid conversion ratio was similar (average±SE = 1.87±0.07).

4.3.4 Citric and acetic acids

A substantial amount of citric acid (175 ±5 mg l^{-1}) was found in the co-inoculated juice. In simultaneous treatments, citric acid was rapidly and completely depleted within 5 days of inoculation with yeast and bacteria. Citric acid was also depleted in all consecutive treatments, but the complete degradation was slower and pH dependent, taking between 22 (pH 3.65) and 60 days (pH 3.20). No citric acid residues were detected in any of the wines after 65 days (Table 11).

Acetic acid levels increased sharply during the first 2 days of fermentation, but the increases were always more pronounced in simultaneous AF/MLF. After the initial rise, acetic acid concentrations continued to increase slightly, but were not clearly related with citric or malic acid degradation or with the depletion of sugars. Residual acetic acid concentrations correlated well with volatile acidities at any given pH levels. There was a clear trend towards higher final acetic acid concentration in wines produced by simultaneous AF/MLF, but the differences were not statistically significant ($p > 0.05$) across all treatments (Table 11).

Figure 7. Time course of acetic and citric acid concentrations during vinification of Chardonnay with *S. cerevisiae* CY3079 and *O. oeni* MBR31. Upper row, consecutive yeast bacterial inoculations (arrow indicates time point of bacterial inoculation). Lower row, simultaneous yeast bacterial inoculations. ∀, acetic acid; −, citric acid. Data points represent the mean from duplicate incubations ±SE.



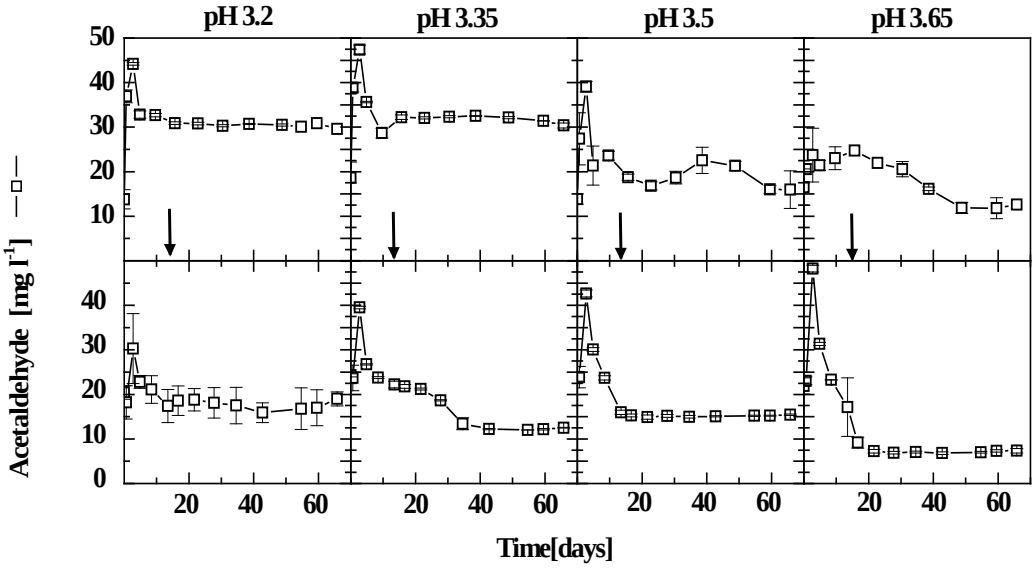
4.3.5 Acetaldehyde

Acetaldehyde concentrations also peaked during early phases of fermentation, regardless of the fermentation technique (Figure 8). However, the initial pH had a different effect on the maximum reached depending on the inoculation technique. In consecutive trials, the acetaldehyde peak was highest (44-47 mg l^{-1}) at the two lowest pH values pH 3.20 and steadily decreased to the lowest peak at pH 3.65. In simultaneous trials, on the other

hand, the lowest maxima were attained at pH 3.20 (30 mg l^{-1}) and the values increased with pH (48 mg l^{-1} at pH 3.65).

After this peak, acetaldehyde levels decreased in all fermentations. The trend of decreasing from simultaneous trails was more rapid towards the lower residual concentrations (7.3-19 mg l^{-1}) than those from consecutive trials, all of which were malic acid degradation.

Figure 8. Time course of acetaldehyde concentrations during vinification of Chardonnay with *S. cerevisiae* CY3079 and *O. oeni* MBR31. Upper row, consecutive yeast bacterial inoculations (arrow indicates time point of bacterial inoculation). Lower row, simultaneous yeast bacterial inoculations. Data points represent the mean of duplicate incubations ±SE.



4.3.6 Esters

Table 9. Values of several esters after stabilization with sulphur dioxide. Wines from simultaneous and consecutive alcoholic and malolactic fermentations (with malolactic strain MBR31) at four different initial pH values. F-test and Student's t-test were used to ascertain statistically significant differences at the 0.05 confidence interval unless otherwise stated. All values are expressed in mg l^{-1} ± SE.

Parameter	Treatment	Consecutive	Simultaneous
Diethyl Succinate	pH3.20	9.01±0.15	9.33±0.06
	pH 3.35	8.92±0.05	9.19±0.19
	pH 3.50	9.07±0.05	9.15±0.03
	pH 3.65	9.06±0.24	9.02±0.00
Ethyl Lactate	pH3.20	65.18±7.71	79.76±3.66
	pH 3.35	65.45±5,24	110.91±29.73
	pH 3.50	81.55±11.80*	163.2±6.04*
	pH 3.65	93.41±13.91*	127.28±1.93*
Isoamyl Acetate	pH3.20	8.09±0.05*	6.60±0.15*
	pH 3.35	7.07±0.35	6.63±0.05

	pH 3.50	6.93±0.23	6.57±0.08
	pH 3.65	7.21±0.25	6.20±0.02
Ethyl Isovalerate	pH3.20	0.15±0.00	0.17±0.00
	pH 3.35	0.18±0.00	0.17±0.00
	pH 3.50	0.18±0.00	0.17±0.00
	pH 3.65	0.17±0.00	0.17±0.00
Ethyl Acetate	pH3.20	708.47±43.47	538.28±22.20
	pH 3.35	359.57±109.49	576.3±32.70
	pH 3.50	437.72±133.30	551.3±15.94
	pH 3.65	612.22±14.10*	449.21±23.95*
Ethyl Caprylate	pH3.20	n.d.	n.d.
	pH 3.35	n.,d.	n.d.
	pH 3.50	n.d.	n.d.
	pH 3.65	n.d.	n.d.
Total amount of esters	pH3.20	790.90±51.40	634.10±26
	pH 3.35	441.20±115.00	703.20±62.70
	pH 3.50	535.40±145.00	730.40±22.10
	pH 3.65	722.10±18.00	591.90±25.90

* Statistically significant difference at 0.05 confidence interval.

n.d. not detected, i.e., below 0.01.

The ethyl lactate concentrations was higher with the simultaneous AF/MLF treatments than with consecutive AF/MLF treatments, especially statistically significant higher at pH 3.50 (163.2±6.04 mg l^{-1}) and pH 3.65 (127.28±1.93 mg l^{-1}).

In contrast, the end values of isoamyl acetate were slightly higher with consecutive inoculation than with simultaneous inoculation, especially at pH 3.20 where the statistically significant excessive isoamyl acetate was found (8.09±0.05 mg l^{-1}).

No statistically significant difference ($p > 0.05$) was found in the end values of other esters (diethyl succinate, ethyl caprylate and ethyl acetate) and total esters between two different trials at the same pH level. Only one exception, at pH 3.65, ethyl acetate

concentration was statistically significantly lower with the simultaneous treatment (449.21±23.95 mg l^{-1}) than with the consecutive treatment (612.22±14.10 mg l^{-1}).

4.3.7 Amino acids

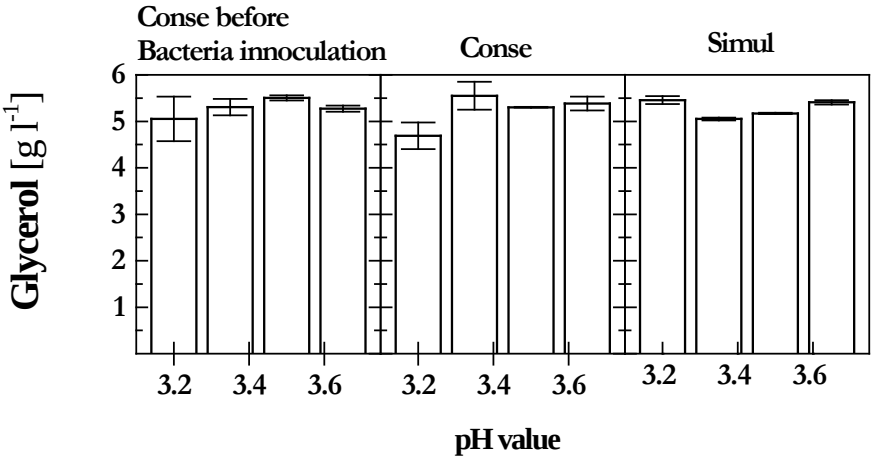
Table 10 Values of several amino acids after stabilization with sulphur dioxide. Wines from simultaneous and consecutive alcoholic and malolactic fermentations at 4 different initial pH values with malolactic strains MBR31 are compared. F-test and Student's t-test were used to ascertain statistically significant differences at the 0.05 confidence interval unless otherwise stated. All values are expressed in mg l^{-1} ± SE.

Parameters	Treatment	Consecutive	Simultaneous
Cit	pH3.20	9.00±1.00	1.00±1.00
	pH 3.35	71.00±62.00	9.00±9.00
	pH 3.50	1.00±0.00	11.00±1.00
	pH 3.65	2.00±0.00	50.00±35.00
Arg	pH3.20	9.00±0.00	9.00±0.00
	pH 3.35	7.00±1.00	31.00±20.00
	pH 3.50	8.00±0.00	4.00±1.00
	pH 3.65	9.00±0.00	15.00±6.00
Orn	pH3.20	0.00±0.00	0.00±0.00
	pH 3.35	0.00±0.00	0.00±0.00
	pH 3.50	0.00±0.00	0.00±0.00
	pH 3.65	0.00±0.00	0.00±0.00

Three main amino acids were measured, all of which were degraded completely during fermentations. No statistically significant differences in amino acid concentrations after 65-day experiment were found between the two different vinification methods at all pH levels.

4.3.8 Glycerol

Figure 9. Glycerol concentrations during vinification of Chardonnay by *S. cerevisiae* CY3079 and *O. oeni* MBR31. Error bars represent the ±SE of duplicate incubations.



No statistically significant differences ($p > 0.05$) were found for any combination of treatments for the concentrations of glycerol (average of all treatments = 5.27±0.05 g l^{-1})

4.3.9 Other wine parameters

Table 11. Values of several parameters in wines produced by consecutive and simultaneous alcoholic and malolactic fermentations at 4 different initial pH values after stabilization with sulphur dioxide. Values are averages from duplicate incubations expressed in mg l^{-1} ± SE. Different letters indicate statistically significant differences ($p \leq 0.05$) among values obtained for a specific parameter unless otherwise stated.

Parameter	pH	Consecutive	Simultaneous
Glucose	3.20	18 ± 1 e	20 ± 2 e
	3.35	25 ± 0 cd	32 ± 5 cde
	3.50	37 ± 0 cd	63 ± 5 b
	3.65	47 ± 2 bc	92 ± 3 a
Fructose	3.20	1393 ± 175 a	526 ± 36 b
	3.35	212 ±10 bc	109 ± 73 c
	3.50	143 ± 0 bc	209 ± 11 bc
	3.65	92 ± 3 c	398 ± 72 bc
Glucose and	3.20	1411 ± 176 a	546 ± 34 b
Fructose	3.35	237 ± 10 bc	140 ± 68 c
	3.50	180 ± 1 bc	272 ± 16 bc
	3.65	139 ± 1 c	490 ± 68 bc
Ethanol	3.20	12.3 ± 0.1 a	12.8 ± 0.3 a
[% vol.]	3.35	12.8 ± 0.1 a	12.7 ± 0.2 a
	3.50	12.9 ± 0.2 a	12.6 ± 0.1 a
	3.65	12.7 ± 0.2 a	12.6 ± 0.1 a
Malic acid	3.20	2038 ± 108 b	2595 ± 33 a
	3.35	1813 ± 27 b	55 ± 3 c
	3.50	105 ± 12 c	33 ± 1 c
	3.65	22 ± 1 c	30 ± 2 c
Lactic acid	3.20	1107 ± 67 b	1184 ± 12 b
	3.35	1512 ± 6 b	4105 ± 7 a
	3.50	3883 ± 10 a	4251 ± 211 a
	3.65	4068 ± 247 a	4340 ± 50 a
Citric acid	3.20	n.d.	n.d.
	3.35	n.d.	n.d.
	3.50	n.d.	n.d.
	3.65	n.d.	n.d.
Acetic acid	3.20	291 ± 2 a	290 ± 27 a
	3.35	267 ± 8 a	296 ± 42 a
	3.50	302 ± 24 a	385 ± 36 a
	3.65	276 ± 3 a	402 ± 38 a
Acetaldehyde	3.20	30 ± 0 a	19 ± 2 b
	3.35	30± 1 a	13 ± 0 bc
	3.50	16 ± 4 bc	15 ± 0 bc
	3.65	11 ± 2 bc	7 ± 1 c
Bound SO_2	3.20	75 ± 13 ab	62 ± 5 ab
	3.35	86 ± 9 a	50 ± 4 ab
	3.50	68 ± 2 ab	69 ± 5 ab
	3.65	64 ± 2 ab	39 ± 6 b
Glycerol	3.20	5121 ± 430 a	5344 ± 41 a
	3.35	5253 ± 203 a	5289 ± 120 a
	3.50	5181 ± 126 a	5389 ± 116 a
	3.65	5344 ± 41 a	5290 ± 120 a
pH	3.20	3.13 a	3.14 a
	3.35	3.26 b	3.33 c
	3.50	3.51 d	3.51 d
	3.65	3.74 e	3.73 e

n.d. not detected, i.e., below 0.01mg l^{-1}.

No statistically significant differences ($p > 0.05$) were found (Table 11) for any combination of treatments for the concentrations of ethanol (average of all

treatments = 12.7±0.2 % vol.), bound SO_2 (average of all treatments = 64.13±5.12 mg l^{-1}) and final pH value (average of all treatments = 3.53) in the final wines.

4.3.10 Overall fermentation durations

Defining a maximum of 1 g l^{-1} of combined glucose and fructose and 150mg l^{-1} of L-malic acid as required to establish wines as having undergone complete alcoholic and malolactic fermentations, only wines from simultaneous AF/MLF at pH 3.35, 3.50 and 3.65, and those produced by consecutive treatments at pH 3.50 and 3.65 could be considered complete. At pH 3.50, this status was reached over 50 days later in consecutive AF/MLF treatments, while and at pH 3.65, the delay was 41 days. If the delay to reach 100 mg l^{-1} of L-malic acid was only considered from the time of bacterial inoculation, simultaneous AF/MF still led to the completion of malolactic conversion which was 28 and 17.5 days faster than in consecutive treatments at pH 3.50 and pH 3.65, respectively.

4.4 DISCUSSION

This chapter examined the effect of simultaneous and consecutive AF/MLF on various wine quality relevant parameters during winemaking of Chardonnay at 4 pH values representative of conditions found in cool and warm winemaking climates. The main concern of co-inoculation is inhibition of yeast growth by LAB which might cause sluggish or stuck AF (Huang et al. 1996). Various studies have been undertaken to establish suitable yeast bacterial combinations in order to limit the inhibitory effects of LAB to yeast (Grossmann et al. 2002; Henick-Kling and Park 1994; Rauhut et al. 2001; Rosi et al. 2003). In this work, suitable yeast-bacteria combination (Jussier et al. 2006) was chosen which did

not result in sluggish or stuck AF. AF was completed in less than 14 days regardless of vinification methods and initial pH levels.

Simultaneous vinification method has little influence on the sugar degradation rate or final alcohol production, although LAB might take up some sugars for growth and metabolism when it was inoculated into high sugar concentration must. The sugar residues are associated with initial pH values (Charoenchai et al. 1998), thus the lowest pH value (3.20) led to statistically significant fructose content. In late fermentation phases, regardless of different inoculation treatments the consistent increase of glucose levels was indicative of hydrolysis of wine glycosides by LAB (D'Incecco et al. 2004; Grimaldi et al. 2000). Especially at high initial pH values, the stronger increase of glucose in co-inoculated wines may suggest that enzymatic hydrolysis prevailed over chemical hydrolysis.

Compared with consecutive inoculations, co-inoculations statistically significantly reduced the fermentation period and notably increased malic acid degradation rate. Once LAB inoculated after AF complete, nutrients were depleted and inhibition compounds were produced through alcoholic fermentation, all of which might delay or restrict the malic acid degradation (Nancy et al. 2008). With the exception of the lowest initial wine pH (3.20) stuck MLF were recorded and no significant amount ($p > 0.05$) of residual malic acid was reduced during the time of the experiment (65days) with both vinifications. These results confirmed that initial pH also had a large effect on the success of MLF (Bauer and Dicks 2004). The lower initial pH that exists in the wine, the more stress will be placed on LAB growth and metabolism (Davis et al. 1986; Fleet 1985). The overall success of MLF was associated to must and wine pH values rather than the time point of bacterial inoculation.

In addition to sluggish or stuck AF, the other main concern is producing excessive acetic acid (Henick-Kling 1993) through LAB heterofermentation when inoculated into a wine/must with sugar (Gottschalk 1986). In this study, there was no statistically significant difference ($p > 0.05$) among the final acetic acid levels across all treatments in spite of a trend towards higher levels in wines produced by co-inoculation. The highest acetic acid concentration recorded (simultaneous treatment at pH 3.65) was 402 mg l^{-1}, considerably lower than commonly encountered legal limits and sensory perception thresholds (Canada Gazatte 2004).

Yeast produces acetaldehyde during AF and LAB which is degraded to ethanol and/ or acetic acid during MLF (Osborne et al. 2000; Osborne et al. 2006), thus kinetics of acetaldehyde is dependent on not only inoculation technique but also on the degree of completion of AF and MLF which is highly associated with initial pH levels. Accordingly, the results also confirmed that the final acetaldehyde concentrations were mainly determined by the initial must pH value, although lower acetaldehyde concentrations were found as a result of simultaneous inoculation.

From a sensory perspective, major esters were detected which affected the final wine quality. The production of esters is associated to yeast and LAB strains (Plata et al. 2002; Soles et al. 1982) as well as fermentation conditions such as initial pH values, fermentation temperatures, fermentation periods, nitrogen levels, storage conditions (Lambrechts and Pretorius 2000). Although, a detailed study on esters formulation and degradation during AF/ MLF is beyond scope of this study, the results still indicated that there was a minor effect on the production of esters when LAB inoculation time was changed. Despite having

different inoculation techniques, all wines maintained relatively high ethyl acetate and relatively low isoamyl acetate concentrations. Co-inoculation of yeast and bacteria led to higher ethyl lactate levels at all pH values, however, the concentrations of ethyl lactate were still lower than the value reported as potentially affecting the overall aroma of wine (200mg l^{-1} (Amerine et al. 1980), the value reported as potentially affecting the overall aroma of wine.

This study demonstrated that during white wine fermentation, the time of LAB inoculation with determined the speed of MLF and the entire fermentation period. Changing LAB inoculation time led to different kinetics of several compounds. In the following chapter, the effects of simultaneous AF/MLF on various wine quality relevant parameters during the vinification of red wine were studied and compared to traditional consecutive fermentation.

CHAPTER 5. SIMULTANEOUS AF/MLF APPLIED IN RED WINE AT NATURAL PH VALUE

5.1 INTRODUCTION

In the previous chapter, it was shown that simultaneous AF/ MLF provided an interesting alternative to traditional consecutive vinification for the fermentation of white musts. Compared to white wine, red wine has more colour and aroma compounds from alcoholic maceration. In addition, more aroma compounds could also be the result of different LAB inoculation times. Sensory evaluations, using the triangle test (Roessler et al. 1978), were used in the present study to predict quality differences among various red wine samples to aid in the control of the final quality.

Triangle tests were conducted on wines not only from Cab Franc grape with two different vinification methods, but also on wines from Chardonnay with 8 different inoculation methods. However, after a long period storage, some Chardonnay wine samples were oxidized, therefore, only the samples from Cab Franc wine were subjected to the triangle test.

5.2 MATERIAL AND METHODS

Cab Franc grape (Cave des Corbières, Rivesaltes, France) from Kamil EX-IM International Trading Company, Guelph, ON, Canada was destemmed and crushed into grape must. The must had a soluble solid content of 22.5 Brix (20°C), pH 3.56, a total acidity of 5.70 g l^{-1} as tartaric acid and contained 4.76 g l^{-1} of malic acid. After must filtration to remove sediments (10 μm Claris Series filter, Pall Corporation, New York, NY, USA) 500 mg l^{-1} of diammonium hydrogen phosphate (Fisher Scientific, Nepean, ON, Canada) and 300 mg l^{-1} of Fermaid K (Lallemand, Montreal, PQ, Canada) were added as nutrients.

The two treatments (in duplicate) consisted of carrying out vivifications with the yeast *S. cerevisiae* D254 and LAB *O. oeni* LALVIN, where the bacteria were inoculated either together with the yeast (simultaneous AF/MLF), or after completion of AF (consecutive AF/MLF). Specifically, the yeast was added at 300 mg l^{-1} and the MLB at 10 mg l^{-1}. The fermentation temperature was maintained at 19-20°C in a temperature-controlled room where the carboys with fermentation locks were placed. After completion of AF and MLF as assessed by stable sugar and malic acid concentrations, 30 mg l^{-1} of SO_2 were added to the wines the carboys were then racked of the primary yeast lees. Cold stabilized (2°C, 1 week), and racked again. The concentration of free SO_2 was adjusted to 30 mg l^{-1} in three rounds over the period of two weeks. Finally, the wines underwent depth filtration with filter sheets (EK grade, Seitz-Schenk, Germany) followed by 0.22 μm membrane filtration (nylon disc filter, Whatman Internal ltd. Maidston, England) before bottling. CO_2 was used for insertion (filling headspaces) of headspaces throughout the entire winemaking process. The wines were stored in 750 ml bottles for 12 months for further chemical analyses and sensory analyses.

Triangle test was used to determine differences among treatments.

Triangle test was conducted by an untrained panel to determine any possible differences between wine samples produced via simultaneous versus consecutive inoculation. 63 testers (26 males and 37 females) were presented with three glasses of wine and asked which one is different from other two. Three random digital numbers were put on the glasses. Statistically analysis using the Student's t-test was conducted.

5.3 RESULTS

5.3.1 Microorganisms plate check

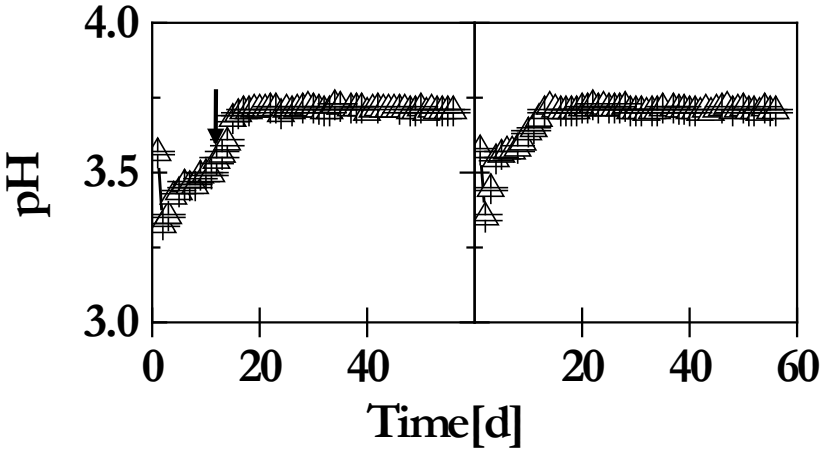
Table 12. Results of yeast and LAB plate check for consecutive and simultaneous AF/MLF immediately after yeast and bacteria inoculations.

Consecutive (after yeast inoculation)		Consecutive (after LAB inoculation)		Simultaneous (after yeast and LAB co-inoculation)	
Yeast(cfu/ml)	Bacteria(cfu/ml)	Yeast(cfu/ml)	Bacteria(cfu/ml)	Yeast(cfu/ml)	Bacteria(cfu/ml)
$5.2*10^4$	0	$2.9*10^6$	$8.4*10^6$	$2.5*10^5$	$3.7*10^3$

With consecutive inoculation technique, right after yeast inoculation, no LAB were detected in the must.

5.3.2 pH values

Figure 10. Time course of pH values during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Left figure, consecutive yeast bacterial inoculations (arrow indicates time of bacterial inoculation). Right figure, simultaneous yeast bacterial inoculations. Results represent the average from duplicate incubations.



The time course of pH values during experiment period is similar between two different vinification treatments. After day 2, pH values reached the minimum, pH 3.33 with consecutive vinification, and pH 3.35 with simultaneous vinification. After reaching the minimum point, the pH values began to increase; there were however, slight differences in the increases. With the simultaneous fermentation method, pH value rose relatively quickly from day 2 to 5, accordingly the malic acid was reduced rapidly by LAB (Figure 12) at the same time after which the values slowly increased until day 13 when the values plateaued reaching a pH of approximately 3.7. With consecutive inoculation, pH values increased relatively slowly until the pH stabilized at day 17.

5.3.3 Sugar degradation

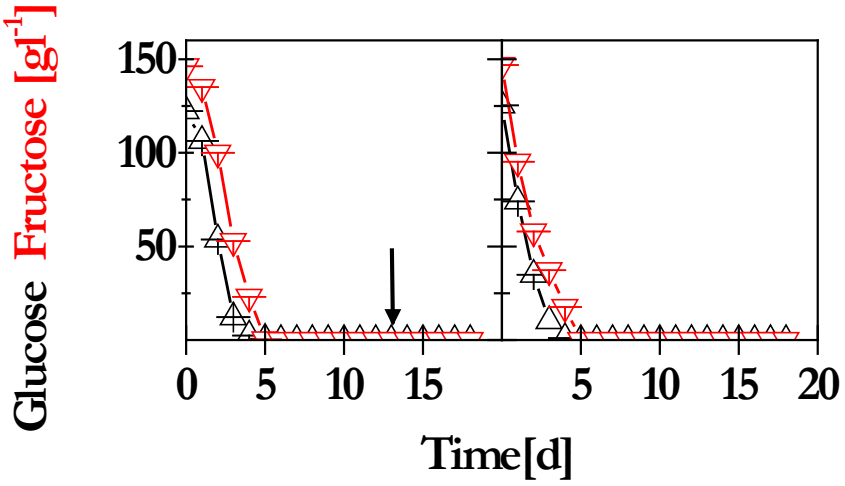


Figure 11. Time course of glucose and fructose concentrations during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Left figure, consecutive yeast bacterial inoculations (arrow indicates time of bacterial inoculation). Right figure, simultaneous yeast bacterial inoculations. ∀, glucose; –, fructose. Data points represent the mean of duplicate incubations ±SE.

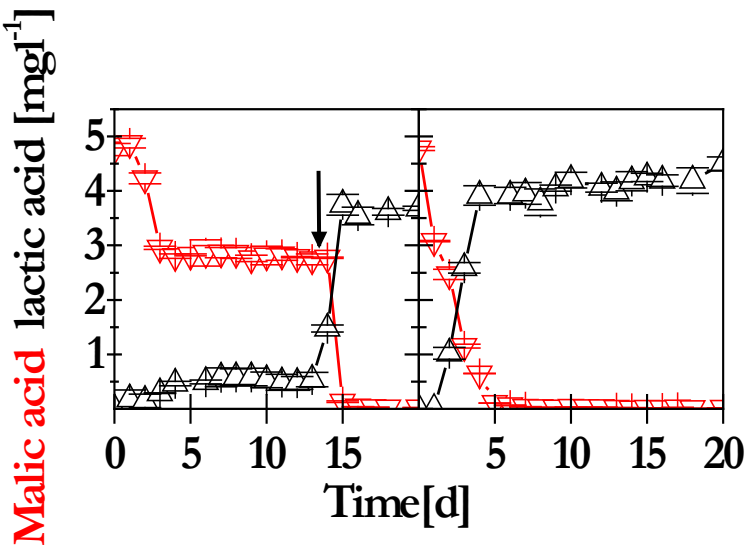
In the grape must, the concentration of fructose (146.48 g l^{-1}) was higher than the concentration of glucose (123.71 g l^{-1}). The time course of sugar degradation during experiment time was similar between simultaneous and consecutive fermentations. The sugar degradation was faster with simultaneous inoculation than with consecutive inoculation. Less than 0.05 g l^{-1} residue sugar was detected after 5 days of simultaneous AF/MLF, whereas it took 7 days to degrade sugar to lower than0.05 g l^{-1} after yeast was inoculated alone.

5.3.4 Malic acid and lactic acid

Table 13. Time required to reach L-malic acid concentrations below 150 mg l^{-1} during fermentations of Cab Franc must with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41 (consecutive treatments were inoculated with bacteria after reaching dryness at 13.5 days; L–malic acid residue in parentheses.

Simultaneous	Consecutive AF/MLF	
treatment, time since yeast/bacterial inoculation (d)	Time since bacterial inoculation (d)	Total time since yeast inoculation (d)
5	3	16

Figure 12. Time course of malic and lactic acid concentrations during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Left figure shows results from consecutive yeast and bacteria inoculations (arrow indicates inoculation with bacteria). Right figure shows results from the simultaneous inoculation of yeast and bacteria. ∀, malic acid; –, lactic acid. Error bars represent the standard error of duplicate incubations.

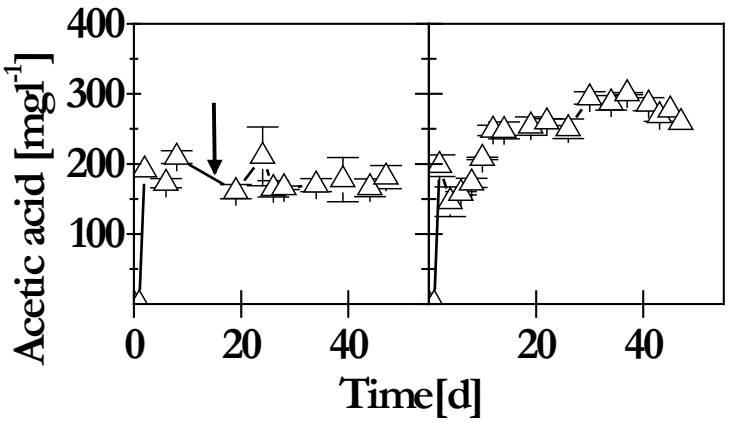


Malic acid degradation was simultaneous with lactic acid production during the beginning phase of fermentation regardless of different inoculation treatments. Malic acid degradation was halted at 3 days and malic acid concentration (3.00 g l^{-1}) became stable whereas lactic acid production (0.58 g l^{-1}) was halted at 7 days after yeast inoculation. However, when yeast and LAB were co-inoculated into grape must, malic acid was continuously degraded until completely depleted. Regardless of when LAB was inoculated, malic acid was reduced to lower than 0.05g l-1 which is ideal since below this level malic acid will not influence the microbial stability. The malic acid concentration (24.00*±0.59 mg l^{-1}) of consecutive fermentation was statistically significant higher than the concentration (17.8*±0.20 mg l^{-1}) from simultaneous fermentation. Accordingly, with consecutive inoculation, the end value of lactic acid (3.68*±0.04 g l^{-1}) is statistically significant lower than with simultaneous technique (4.49*±0.06 g l^{-1}) (Table 14). Table 13 indicated that the whole

fermentation time was statistically significantly reduced if yeast and LAB were inoculated at the same time.

5.3.5 Acetic acid

Figure 13. Time course of acetic concentrations during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Left figure shows results from consecutive yeast and bacteria inoculations (arrow indicates inoculation with bacteria). Right figure shows results from the simultaneous inoculation of yeast and bacteria. Error bars represent the standard error of duplicate incubations.

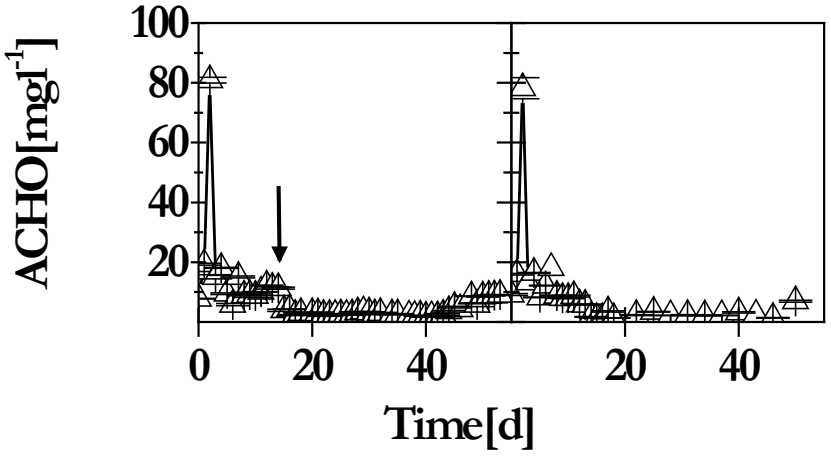


After the fermentation started, acetic acid levels increased sharply during the early phase. Acetic acid values were always found slightly higher in simultaneous fermentation; however, no statistically significant difference ($p > 0.05$) was found between the end values of acetic acid in fermentations with different LAB inoculation times. (Table 14)

5.3.6 Acetaldehyde

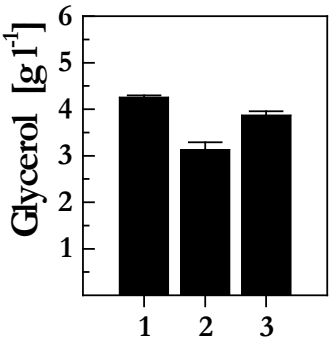
Regardless of different vinification methods, during the early phase of fermentation, acetaldehyde concentration peaked as approximately 80 mg l^{-1} after which acetaldehyde was continuously degraded to concentrations lower than 10 mg l^{-1}. The residue acetaldehyde concentration (9.5mg l^{-1}) from consecutive AF/MLF was statistically significant higher than that obtained (6.9 mg l^{-1}) from the simultaneous treatment.

Figure 14. Time course of acetaldehyde concentrations during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Left figure shows results from consecutive yeast and bacteria inoculations (arrow indicates inoculation with bacteria). Right figure shows results from the simultaneous inoculation of yeast and bacteria. Error bars represent the standard error of duplicate incubations.



5.3.7 Glycerol

Figure 15. Glycerol concentrations during vinification of Cab Franc with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41. Error bars represent the standard error of duplicate incubations. The left column shows result from wine samples after finished AF and MLF with simultaneous inoculation. The middle column shows results from wine samples after finished AF before LAB inoculation with consecutive technique. The right column shows results from wine samples after finished AF and MLF with consecutive technique.



No statistically significant differences ($p > 0.05$) of glycerol concentration were found among different inoculation treatments.

5.3.8 Other parameters

Table 14. Values of several parameters in wines produced by consecutive and simultaneous alcoholic and malolactic fermentations after stabilization with sulphur dioxide. Values represent the means of duplicate incubations unless otherwise stated expressed in mg l^{-1} ± SE. Stars indicate statistically significant differences ($p \leq 0.05$) among values obtained for a specific parameter.

	Consecutive	Simultaneous
Citrulline	0.01	0.019
Arginine	0.009	0.012
Ornithine	n.d.	n.d.
Free SO2, mg/L	<6	<6
Tot $_{SO2}$, mg/L	13	15
TRS, g/L	2.3	2.9
Ethanol, %v	12.9	12.3
Sorbic Acid, mg/L	<20	<20
VA, g/L	0.43	0.47
KFeCN, ug/L	<20	<20
Glucose mg/L	10.30±0.14	10.40±2.00
Fructose mg/L	10.00±0.40	10.20±1.40
Glucose& fructose mg/L	20.30±1.80	20.60±3.60
malic acid mg/L	24.00*±0.59	17.8*±0.20
lactic acid g/L	3.68*±0.04	4.49*±0.06
Glycerol g/L	3.13±0.16	3.87±0.09
Acetaldehyde mg/L	9.50*±0.20	6.9*±0.30
pH	3.71±0.01	3.71±0.01

n.d. not detected, i.e., below 0.01g l^{-1}.

No statistically significant difference ($p > 0.05$) was found between all other parameters, VA (0.47 g l^{-1}) and sulphur dioxide concentration (15mg l^{-1}) from the

simultaneous fermentation was slightly higher than the VA (0.43 g l^{-1}) and sulphur dioxide concentration (13mg l^{-1}) from the consecutive fermentation.

5.3.9 Sensory analysis

No statistically significant differences ($p > 0.05$) were found between wines with simultaneous and consecutive AF/MLF using the triangle test.

5.3.10 Over all fermentation duration

Table 15. Time required to reach dryness (combined glucose and fructose levels below 1 g l^{-1}) during fermentations of Cab Franc must with *S. cerevisiae* D254 and *O. oeni* LALVIN VP41

Simultaneous treatment, time since yeast/bacterial inoculation (d)	Consecutive treatment time since yeast inoculation(d)
5	7

With both vinification methods, alcoholic and malolactic fermentation were completed during the experiment period. However, with consecutive inoculation the AF time was 2 days longer and the whole fermentation time was 11 days longer than with co-inoculation.

5.4 DISCUSSION

This chapter's work demonstrated the feasibility of yeast and LAB co-inoculation vinification technique in red wine. This method was compared with traditional consecutive inoculation at the initial pH level in Cab Franc. A time course of various parameters relevant to wine quality were studied. Triangle test was used to see if there was any change in wine

flavour depreciation as a function of LAB inoculation time. Results indicated no statistically significant ($p > 0.05$) wine flavour differences were found between wines with simultaneous and consecutive AF/MLF.

No stuck or sluggish AF/MLF was observed during the experiment, therefore, no apparent yeast and LAB inhibition occurred as a result of inoculation time. Despite similar sugar degradation time courses, fermentations had slightly faster sugar degradation (Table15) with simultaneous treatment as compared to the consecutive treatment, which could be caused by either the faster yeast sugar degradation or by sugar degradation caused by LAB. However, according to the final ethanol concentrations (no statistically significant difference ($p > 0.05$) was found between the ethanol concentrations with different inoculation treatments) the sugars were mostly degraded by yeast not by LAB. Although the co-inoculation with suitable yeast and LAB strains had a minor adverse effect on the yeast growth, metabolism, and the final ethanol concentration, but led to faster yeast sugar degradation.

Residue malic acid concentration was consistent with residue sugar concentration. Statistically significant higher malic acid levels were found in the consecutive inoculation treatment, even though it was still below the ideal residue malic level which indicates that changing LAB inoculation time does not affect AF negatively, but may result in a more complete malic acid degradation.

In comparison to the results of Chardonnay wine in last chapter, Figure 12 demonstrated that malic acid was reduced in the consecutive fermentation without LAB

inoculation at the beginning of the experiment which may have been caused by the wild LAB malic acid degradation or by the yeast malic acid degradation. Lactic acid was simultaneously increased when malic acid was reduced, which indicated that the wild LAB was partly responsible for the malic acid degradation. A comparison of the lactic acid concentrations at the end of the experiment with those from the simultaneous inoculation indicated that the lactic acid concentration was statistically significant higher in samples from consecutive inoculation demonstrating that in consecutive treatments malic acid was not completely degraded by LAB. Therefore, yeast was also responsible for decreasing the malic acid content.

In agreement with the results from last chapter, a clear trend of higher acetic acid concentrations was found in the wine samples with simultaneous AF/MLF. However, the highest acetic acid concentration recorded was 276 mg l^{-1}, considerably lower than commonly encountered legal limits and sensory perception thresholds (Canada Gazatte 2004).

Results of acetaldehyde levels from this chapter also indicated that co-inoculation could lead to lower acetaldehyde concentration. For red wine, acetaldehyde is important for color stabilization. Thus, for a weakly colored wine, color development may suffer from reduced acetaldehyde concentrations (Ribéreau-Gayon et al. 1983).

In this chapter, the study of co-inoculation yeast and LAB method in Cab Franc must confirmed that as long as a suitable combination of yeast and LAB strains (non-inhibitory to one anther) was selected, changing the time point of LAB inoculation could reduce fermentation periods. Although this vinification method had varying effects on the

concentrations of various wine chemical compounds, no major differences in their final values, no negative effects on wine sensory and no depreciation of wine final quality were found.

CHAPTER 6. GENERAL DISCUSSION

This thesis examined simultaneous vinification methods where alcoholic and malolactic fermentations were occurring simultaneously in comparison with the traditional consecutive vinification method where lactic acid bacteria is inoculated after alcoholic fermentation (Chapters 4 and 5).In addition, HPLC methods were developed for analysis organic acids in grape must and wine (Chapter 3).

By studying the co-inoculation of yeast and LAB in white and red wine must, shortening the whole fermentation periods was found to be the single most advantage of successful simultaneous AF/MLF, in agreement with previously published studies (Jussier et al. 2006). The time gained from carrying out yeast bacterial co-inoculations was considerable allowing for more rapid stabilization, which would be beneficial in industrial settings, especially if malolactic stability was desired before winter, e.g., in non-temperature controlled cellars.

The other important benefit from successful simultaneous AF/MLF observed was the ability to increase microbial stability. The shortened fermentation period and the less sugar and malic acid residues in the wines after fermentation could decrease the potential for contamination by spoilage LAB or other microorganisms.

Recently, there has been a trend of higher sugar and organic acid concentrations possibly caused by global warming (Jones et al. 2005). Accordingly, in next few decades lower initial pH level will be found in the grape/must resulting in higher alcohol levels in

wine after AF. Such changes will make MLF more challenging when LAB is inoculated into the wines after undergoing AF with higher alcohol level and lower pH level. Therefore, co-inoculation of yeast and LAB will possibly become more feasible in the future years.

In order to achieve a successful simultaneous AF/MLF, using suitable yeast and LAB strains is very important. Generally, the yeast strains compatible for co-inoculation with LAB should produce low amounts of compounds which inhibit LAB growth such as medium chain fatty acids, and acetic acid. Beside those factors, a suitable yeast strain is still dependent on grape must qualities and composition which will not be discussed in this thesis since once wineries have their must composition they would receive suggestions from Commercial yeast companies. A suitable LAB strain for co-inoculation should not have inhibit yeast growth and should have less acetic acid production especially in high sugar concentration must. In addition, suitable LAB strains are required to have specific characters for some wines, e.g., low acetaldehyde consuming LAB strains are encouraged to induce to light color red wine and maintain a certain level of acetaldehyde as an antioxidant to prevent losing color; , high acidic tolerance LAB strains are required for lower pH wines

Currently, numerous strains of LAB and yeast with known characteristics are on the market. However, stuck/sluggish MLF occurs occasionally in different wineries caused mainly by low initial pH values. Higher acetic concentrations in the wines undergoing MLF are not unusual, although this depreciates wine quality. Therefore, more research on adapting some LAB strains with high acidic tolerance and low acetic acid production are needed. Since there is a large variance of must compositions depending on grape types, therefore, it is unlikely that a single e yeast and LAB strain combination could not satisfy all grape types.

Further studies on possible yeast and LAB strains as suitable co-inoculation combinations for different grape varieties should be encouraged. Besides selecting and adapting suitable strains, improving the viticulture techniques is another effective way to decrease the possibility of stuck/ sluggish MLF. With global warming increasing, grape composition will also be continuously changing. Therefore, it is important to keep sugar at an ideal level and to avoid high acidity along with a suitable viticulture technique.

In conclusion, simultaneous AF/MLF lab-scale experiments were conducted and identified the need to further conduct investigations under larger experimental/industrial scale.

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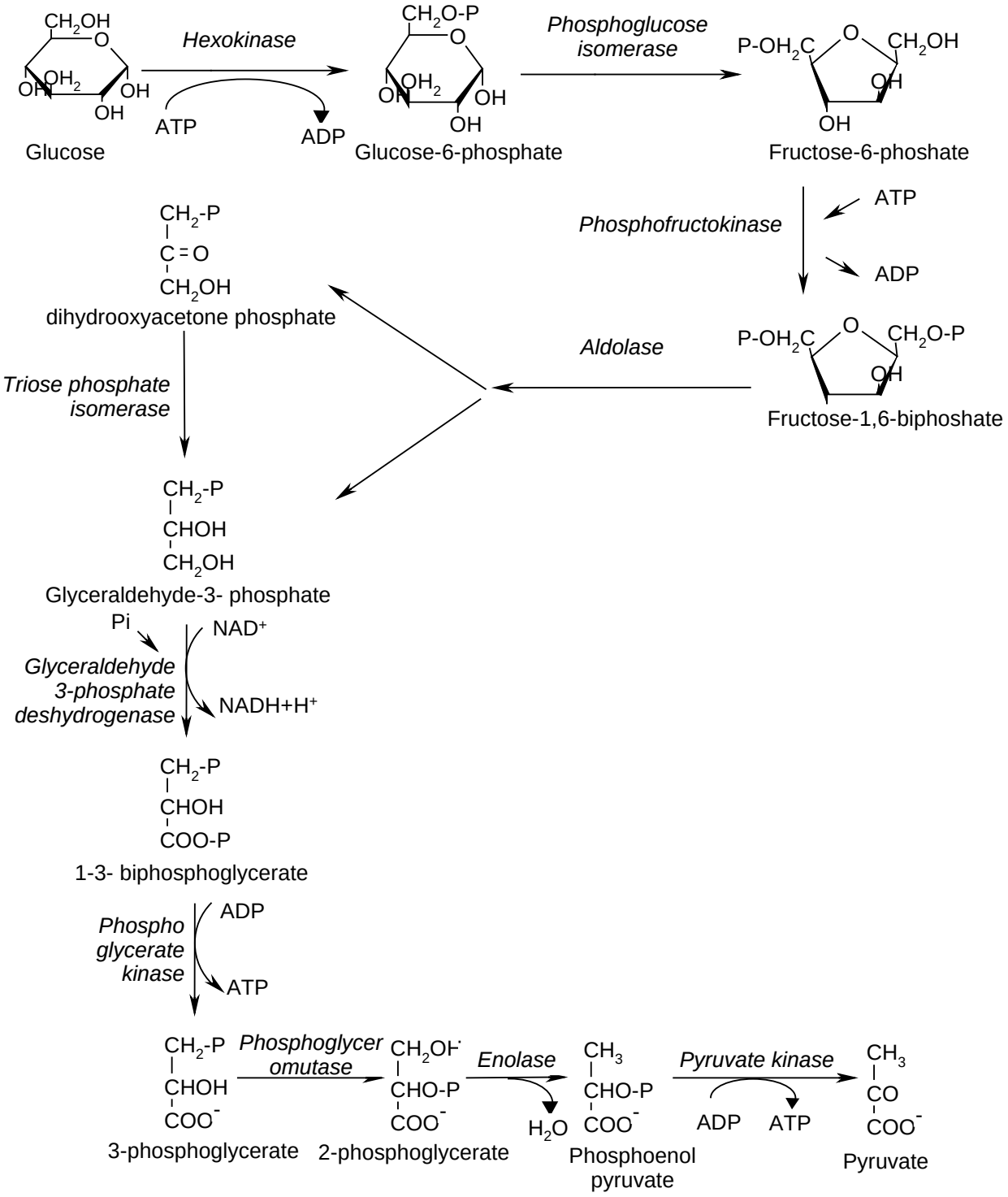
APPENDIX

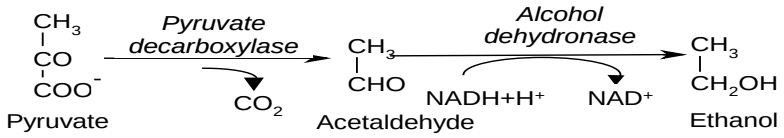
METABOLIC PATHWAYS

(Ribéreau-Gayon et al. 2000)

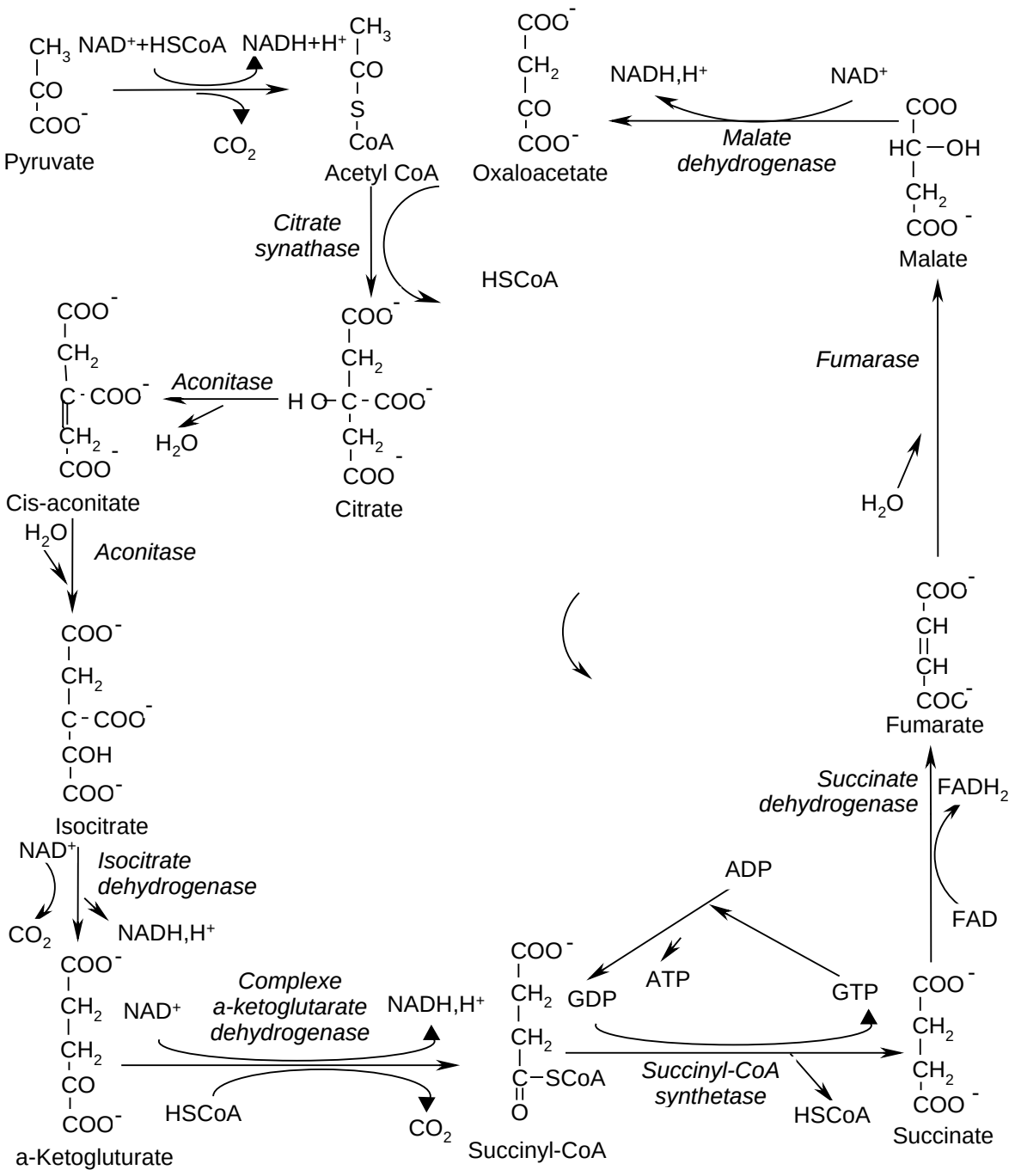
Appendix 1. Glycolysis



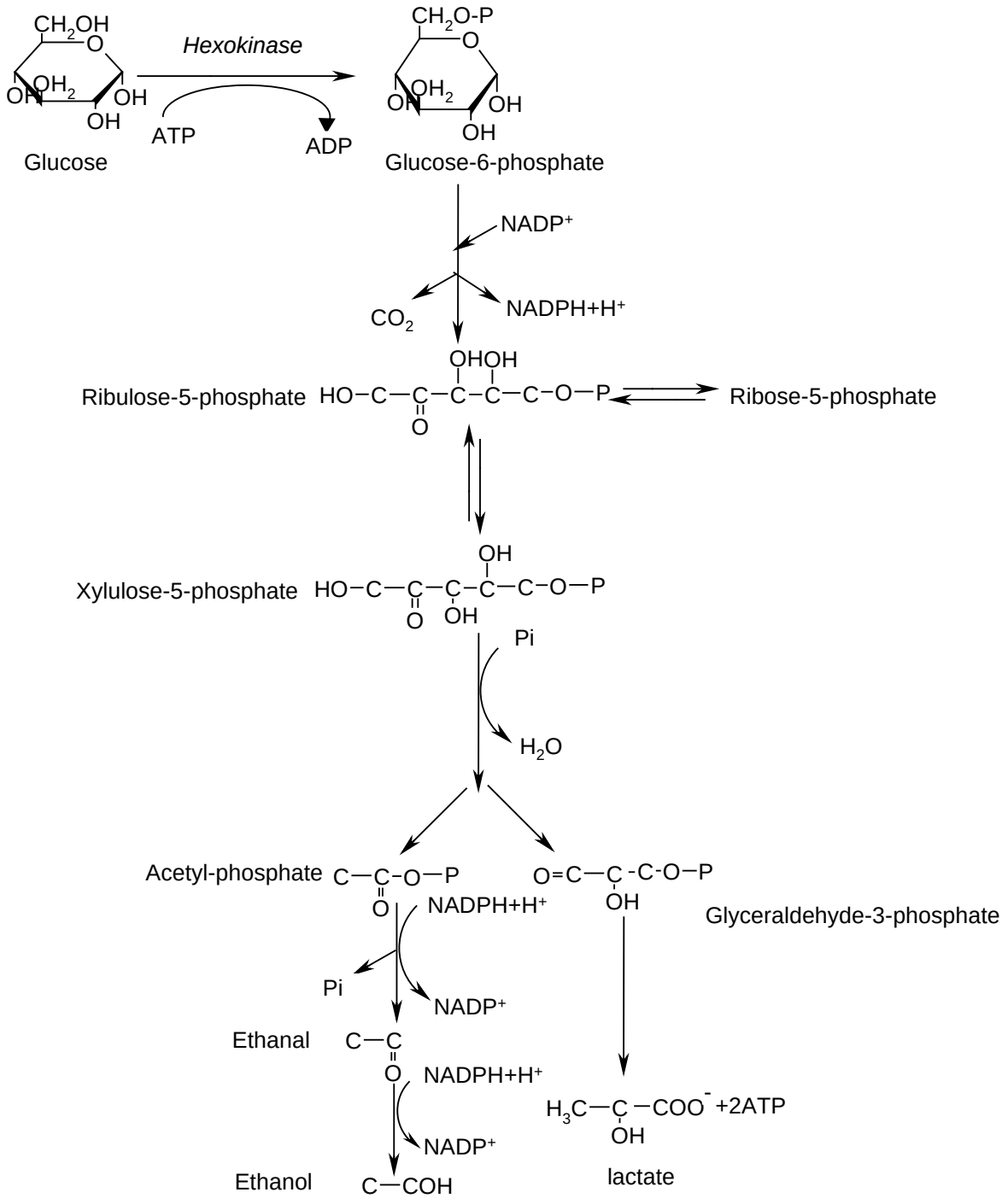
Appendix 2. Alcoholic fermentation pathway



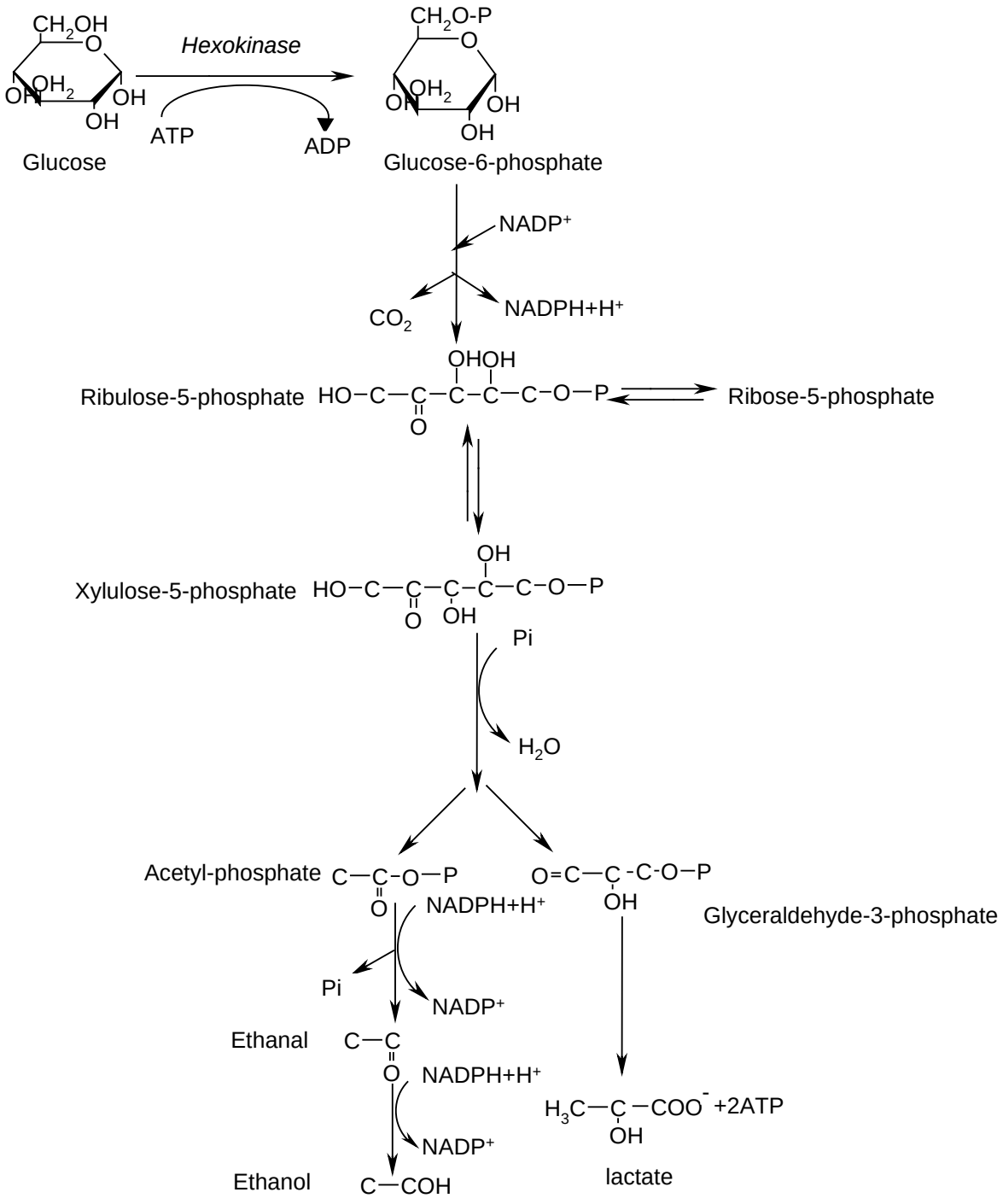
Appendix 3. Tricarboxylic acid or Krebs cycle



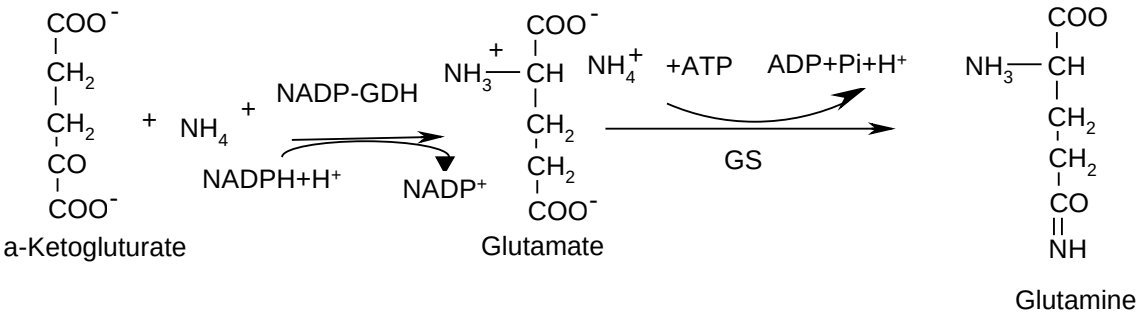
Appendix 4. Metabolic pathway of glucose fermentation by homolactic bacteria

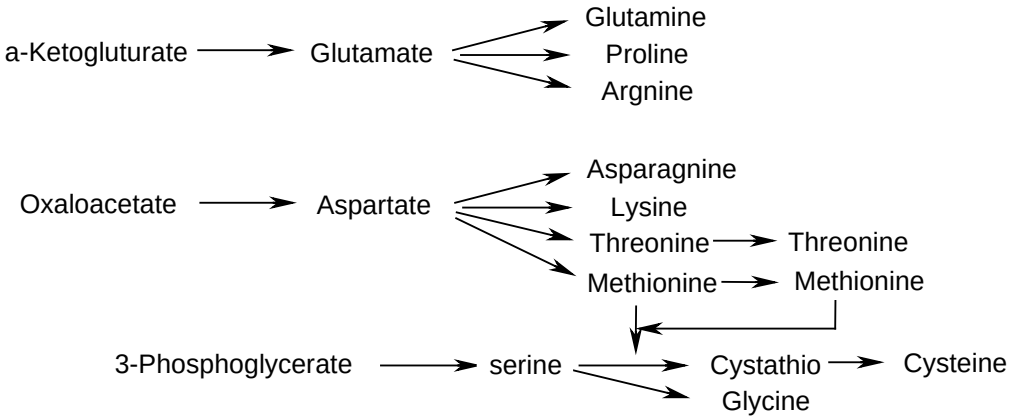


Appendix 5. Pentose phosphate pathway



Appendix 6. General biosynthesis pathways of amino acids


NADP-GDH
NADPH+H+
NADP+
+ATP
ADP+Pi+H+
GS
a-Ketogluturate
Glutamate
Glutamine


a-Ketogluturate
Glutamate
Glutamine
Proline
Argnine
Oxaloacetate
Aspartate
Asparagnine
Lysine
Threonine
Threonine
Methionine
Methionine
3-Phosphoglycerate
serine
Cystathio
Cysteine
Glycine
Ribose 5-phosphate
Histidine
Pyruvate
Alanine
Valine
Leucine
Phosphoenolpyruvate
Erythrose 4-phosphate
Phenylalanine
Tyrosine
Tryptophane