Determination and Method Validation of the Major Organic Acids in Wine using HPLC and UV Detection

by

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ABSTRACT

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A method for the analysis of the major organic acids in wine was validated. The acids such as: tartaric (TA), malic (MA), lactic (LA), citric (CA), succinic (SA), and acetic acid (AA) were derivatized and detected at 254 nm. Detection at 254 nm was targeted to avoid interference from other wine components at 210 nm. The following validation parameters were studied: linearity, accuracy, precision, usable concentration range, limit of detection (LOD), limit of quantitation (LOQ), robustness, peak identification, and quantitation in wine.

ACKNOWLEDGMENT

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LIST OF ABBREVIATIONS

A	Eddy diffusion
AA	Acidic acid
ACN	Acetonitrile
APT	Absolute Pressure Transducer
ATP	Adenosine triphosphate
Avg	Average
B	Longitudinal diffusion
C	Mass transfer
CA	Citric acid
Co A	Coenzyme A
Conc.	Concentration
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography Mass Spectrometry
GTP	Guanosine triphosphate
g/L	Grams per liter
H	Theoretical plate height
HPLC	High Performance Liquid Chromatography
IR	Infra Red
IS	Internal standard
k'	Capacity factor

K_D	Distribution constant
L	Liter
LA	Lactic acid
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molarity
MA	Malic acid
MeOH	Methanol
mg	Milligram
mg/L	Milligrams per liter
min	Minute
mL	Milliliter
mL/min	Milliliters per minute
MLF	Malolactic fermentation
mm	Millimeter
N	Theoretical plate
NADH	Nicotinamide adenine dinucleotide
N_B	Theoretical plate at the base
$N_{1/2}$	Theoretical plate at the half height
nm	nanometer
PDA	Photodiode Array Detector
PDAM	1-pyrenyldiazomethane
ppm	Part per million

PTFE	Poly-1, 3-dioxole-co-tetraflurorthylene
R	Resolution
%R	Percent recovery
R^2	Correlation Coefficient
%RSD	Percent Relative Standard Deviation
RI	Refractive Index
SA	Succinic acid
Std	Standard
STD	Standard deviation
SI-FTIR	Sequential Injection Fourier Transform Infrared
TA	Tartaric acid
TCA	Tricarboxylic acid cycle
Temp	Temperature
t_o	Dead volume
t_R	Retention time
t_{RA}	Resolution time of component A
t_{RB}	Resolution time of component B
t_{Ro}	Adjusted retention time of an analyte
u	Linear velocity
UV	Ultraviolet
W_A	Peak width at the baseline A
W_B	Peak width at the baseline
W_B	Peak width

$W_{1/2}$	Peak width at half height of the peak
μL	Micro liter

CHAPTER ONE

INTRODUCTION

A. Chromatography

Chromatography is a technique in which components of a mixture are separated based on the rate they travel through a stationary phase with the help of a gaseous or liquid mobile phase. The stationary phase is in equilibrium with the mobile phase as shown below:

$$A_{\text{mobile phase}} \Leftrightarrow A_{\text{stat. phase}}$$

The partitioning between the stationary and mobile phase occurs based on intermolecular forces acting between solutes and the two phases. The equilibrium distribution K_D, constant, is defined as:

$$K_D = \frac{C_S}{C_m}$$

Equation 1. Distribution Constant

Where C_s is the concentration of solute in the stationary phase and C_m is the concentration of the solute in the mobile phase. In reversed-phase chromatography the stationary phase is non-polar and the mobile phase is polar. An example is seen with C_{18} or octadecylsilane phases that are composed of 18-carbon alkane chains covalently bonded to silica particles, where the mobile phase is typically composed of mixtures of water with a polar solvent such as an aliphatic alcohol. In these systems the more polar the analytes the longer they will stay in the stationary phase and the larger will be the K_D.

The following parameters, defined below, characterize liquid chromatography: peak retention, capacity factor, separation factor, number of theoretical plates, resolution, peak symmetry, and column dispersion mechanism.

Peak retention time of the analyte, t_R, is the time from injection to analyte peak elution. Dead time, t_0, is the time from injection to detection of an unretained analyte. The adjusted retention time of an analyte, t_{R0}, is t_R minus t_0 and is defined in Equation 2.

$$k' = \frac{t_R - t_0}{t_0}$$

Equation 2. Capacity Factor

The capacity factor describes the interaction between stationary phase and analyte. The higher the value of k' the more effective the stationary phase is in retaining the analyte.

The separation factor α is the ratio of two capacity factors (Equation 3). It is the measure of the spacing between two peaks, or the relative retention.

$$\alpha = \frac{k'_2}{k'_1}$$

Equation 3. Separation Factor

Number of theoretical plates, resolution, and peak symmetry define the column efficiency. Theoretical plate number is the ability of the analyte to flow through the column with minimum band broadening. This is usually expressed as the number of theoretical plates, *N*.

$$N_{1/2} = 5.45\left(\frac{t_R}{w_{1/2}}\right)^2 \quad \text{and} \quad N_B = 16\left(\frac{t_R}{w_B}\right)^2$$

Equation 4. Theoretical Plate

Where, N is the theoretical plate number, W_B the peak width at the base, $W_{1/2}$ is the peak width at the half height of the peak, and t_R is the peak retention time. The theoretical plate number indicates the quality of the packed bed within the column.

Peak resolution R is a measure of the separation between two adjacent peaks on a chromatogram. The resolution between two analyte peaks, A and B, is expressed as:

$$R = \frac{2(t_{RB} - t_{RA})}{w_A + w_B}$$

Equation 5. Peak Resolution

where, t_{RB} and t_{RA} are the retention times of the components A and B, and W_A, W_B are the peak widths at the baseline for those components. A resolution factor of 1.5 indicates that the two components are baseline separated. Peaks resolved at a resolution factor of less than 1.0 cannot be quantified reliably.

Peak symmetry is a measure of peak tailing and peak fronting. It is measured at 10% of peak height.

$$\text{Peak symmetry} = \frac{B}{A}$$

Equation 6. Peak Symmetry

Where A is the distance from peak front to peak maximum and B from peak max to peak end. A symmetrical peak has a peak symmetry of 1, a fronting peak is <1, and a tailing peak >1.

The column dispersion, or degree of band-broadening, is described by the Van Deemter equation as:

$$H = A + \frac{B}{u} + Cu$$

Equation 7. Column Dispersion Mechanism

Where, H is the theoretical plate height, A is the eddy diffusion coefficient, B is the longitudinal diffusion coefficient, C is the mass transfer coefficient, and u is the linear velocity of the mobile phase through the column. Band-broadening originates from the following three mechanisms. Eddy diffusion (A) results from the flow path (multiple path effect) of the solute molecules through the column packing material. Longitudinal diffusion (B) is due to simple diffusion, where high concentrations of solutes move spontaneously toward low concentrations. Mass transfer (C) affects band-broadening as solvent molecules cross the boundary between the stationary phase and mobile phase. The solute may be retained while other solvent molecules may travel with the stationary phase, causing unpredictable band-broadening that is dependent upon the speed of the phase transfer.

B. HPLC

High-performance liquid chromatography, HPLC is a type of chromatography that uses a liquid as the mobile phase and a solid as the stationary phase. Depending upon the nature of the stationary and mobile phases, different types of components can be separated. In this technique, a mixture of analyte solutes dissolved in a solvent matrix is injected onto a packed column of the stationary phase under high pressure. Within the column, the mixture of solutes is separated into its individual components based on the interaction between the stationary phase and the mobile phase. In reversed-phase chromatography the stationary phase is packed with non-polar material and the mobile phase is a polar liquid such as water or methanol. In reversed-phase chromatography non-polar components are retained longer than polar components. An HPLC system configuration is shown in Figure 1.

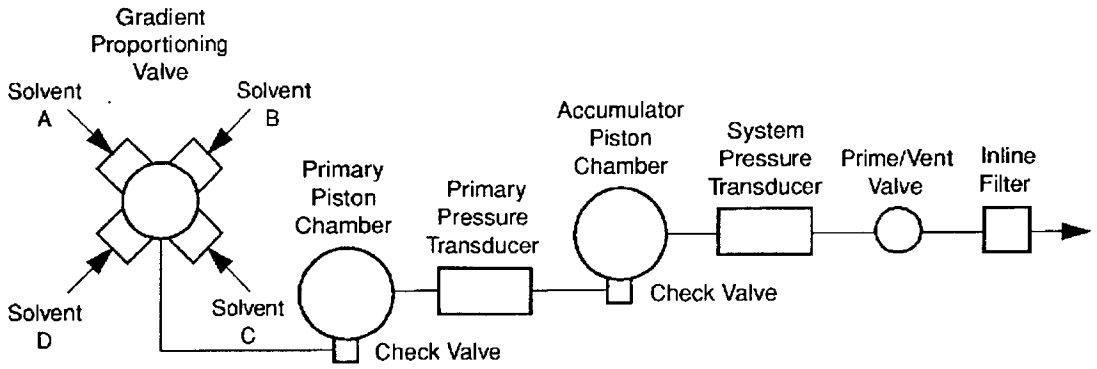


Figure 1. The System Configuration

C. Derivatization

Derivatization of analytes for HPLC analysis is used primarily to introduce a detector-oriented tag. In HPLC, common methods of derivatization are used to improve the detectability of analytes by ultraviolet absorption or fluorescence. Acylation is the conversion of compounds containing active hydrogens into esters, thioesters, and amides through the action of carboxylic acids [1].

D. Organic Acids in Wine

The separation and quantitation of organic acids in wine has been studied for many years. They play a major role in the taste and balance of a wine. Some are originally present in the grape while others appear during the alcoholic, or malolactic fermentation [2]. There are six major organic acids present in wine. They are acetic (AA), citric (CA), lactic (LA), malic (MA), tartaric (TA), and succinic (SA) acids. Currently, methods for determining carboxylic acids (organic acids) in wine include enzymatic analysis and HPLC.

LA occurs in fermented beverages but is generally not present in grape musts. It is produced from malic acid by bacterial action or by yeast during the alcoholic fermentation process. LA occurs as both L- and D- enantiomers, the origin of which depends upon the fermentation process. Alcoholic fermentation produces 180-400mg/L of D- (-)-LA. L (+)- LA is produced by lactic acid bacteria during the malolactic fermentation process (MLF), a desired process for certain wines because it decreases the acidity while increasing the biological stability and mouth feel [3]. It is present in the concentration range of (0-5g/L).

Malic acid (MA) is found in almost all fruits. In grapes it is an important indicator of the maturation process. In wines, its determination is required to monitor and control malolactic fermentation [4], where MA is converted to LA [2,3].

Citric acid (CA) is present in grape musts and wine in small amounts. It is sometimes added to the wine to avoid precipitation of the iron (III) salt. The evaluation of CA in wines is also of great interest for maintaining biological stability when it is present within certain concentration levels [3].

Succinic acid (SA) is produced in small amounts during alcoholic fermentation. The amount produced depends on the condition of fermentation and may range from (0 to 1.5g/L) [3].

Tartaric acid (TA) is one of the main organic acids in grapes and wine. Currently there is no enzymatic method available for its measurement [5,1]. The amount of TA in grapes depends on the grape variety, the region, and the growing season. The concentration stays relatively constant during the growing season, but decreases during

alcoholic fermentation and during cold stabilization. It may undergo degradation by lactic bacteria to lactic and acetic acids [3].

Acetic acid (AA) is another important acid that must be monitored. Nitrogen content and sugar concentration of the must influence AA levels [6]. Oxidation by acetic acid bacteria results in the formation of acetic acid. AA is formed in small amounts during alcoholic fermentation and by lactic acid bacteria during malolactic fermentation. LA bacteria and certain wild yeasts, like *Brettanomyces, Hansenula animala*, and *Kloeckera apiculata*, can also produce high levels of acetic acid [6]. If acetic acid is present in large amounts, a wine is considered defective [3].

E. Introduction of Biochemistry of Organic Acids

Some of the compounds of interest in wine analysis can be found in the various biochemical pathways (e.g., the citric acid cycle) associated with malolactic fermentation and alcoholic fermentation.

Alcoholic and malolactic fermentations are anaerobic processes that involve no net oxidation or reduction. The alcoholic fermentation pathway allows the yeast to make small amounts of adenosine triphosphate (ATP) from glycolysis by consuming pyruvate, which allows glycolysis to proceed. The net gain in ATP molecules by the glycolytic process is two moles for each mole of glucose utilized. This accounts for 24,000 calories of energy that is transferred to ATP [7]. Also, two molecules of the high-energy electron carrying compound, nicotinamide adenine dinucleotide (NADH) are produced. Glycolysis is the splitting of the six carbon sugars glucose and fructose to two molecules of the three-carbon molecule pyruvate. After glycolysis, pyruvate is converted into

ethanol by alcoholic fermentation or under aerobic conditions, it reacts with Coenzyme A (CoA) and enters the tricarboxylic acid cycle (TCA).

The malolactic fermentation pathway is an offshoot of the TCA cycle. Malic acid is converted to lactic acid and carbon dioxide. An overview of the process of glycolysis and alcoholic fermentation showing glucose or fructose being converted into ethanol is as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + CO_2$$

Glucose or Fructose $\rightarrow$ 2 Ethanol

Equation 8. Glucose Conversion to Ethanol

In yeast, the pyruvate that enters the alcoholic fermentation pathway is first decarboxylated and the resulting acetaldehyde is converted into ethanol in a step that also oxidizes NADH to NAD^+ [8]. This step replenishes NAD^+ in the cell so that glycolysis can continue to transform glucose and /or fructose into pyruvate.

The breakdown of glycerol from triacylglycerol degradation can be introduced in the glycolytic pathway by the formation of dihydroxyacetone phosphate, which can then enter the glycolytic pathway [7]. Glycerol $\rightarrow$ glycerol-3-phosphate $\rightarrow$ dihydroxyacetone phosphate $\rightarrow$ glycolysis.

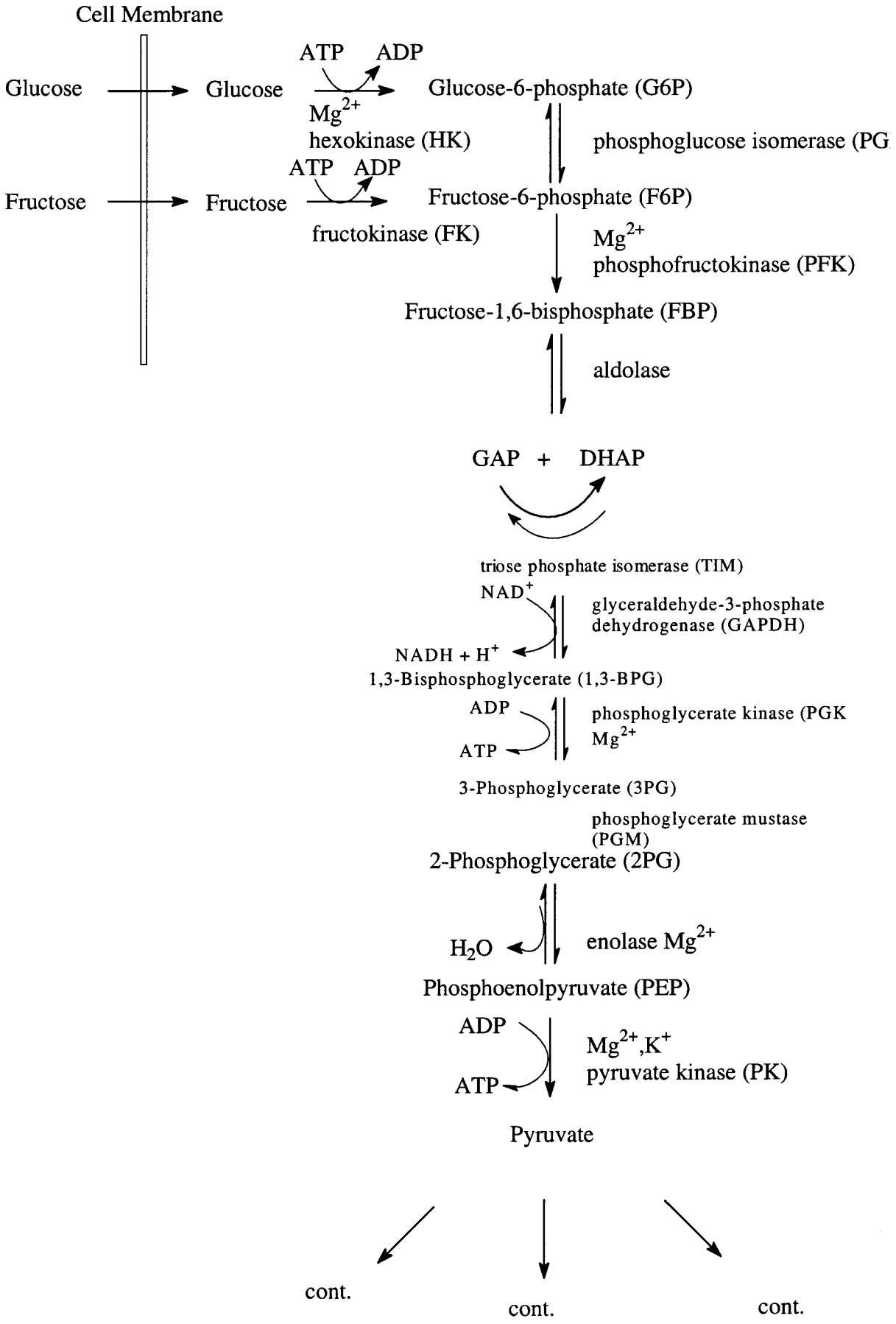
After pyruvate is combined with CoA it may enter the tricarboxylic acid cycle where citrate, malate, succinate, oxaloacetate and other products are formed. The tricarboxylic acid cycle (also called citric acid cycle or the Krebs cycle) starts after pyruvate is converted into acetyl-CoA. Through each turn of the TCA cycle, several molecules of high-energy electron carrying compounds are produced (NADH and $FADH_2$). These high-energy electron-carrying molecules transfer their electrons into the

electron transport chain, which generates a proton gradient across the inner mitochondrial membrane of the mitochondria that in turn drives ATP production through oxidative phosphorylation.

In the first reaction of the TCA cycle, acetyl-CoA reacts with oxaloacetate acid to produce citrate. The reaction is catalyzed by citrate synthase [7]. Succinic acid is also produced in the citric acid cycle. Succinyl-CoA contains a high-energy bond and uses this to synthesize a GTP rather than ATP. The reaction is catalyzed by succinyl-CoA synthetase and produces succinic acid and a free CoA [8].

Malic acid also is of importance in wine analysis. Its source can also be found in the citric acid cycle. After fumarate is formed in the cycle it accepts a water molecule in a reaction catalyzed by fumarase. The malic acid formed here is then oxidized in a reaction catalyzed by malate dehydrogenase resulting in the production of another NADH [8].

The biochemical pathways utilized by yeast during the process of fermentation of glucose to ethanol and CO_2 are illustrated in Figure 2.


Cell Membrane
Glucose
Glucose
ATP
ADP
Mg2+
hexokinase (HK)
Glucose-6-phosphate (G6P)
phosphoglucose isomerase (PG
Fructose
Fructose
ATP
ADP
fructokinase (FK)
Fructose-6-phosphate (F6P)
Mg2+
phosphofructokinase (PFK)
Fructose-1,6-bisphosphate (FBP)
aldolase
GAP + DHAP
triose phosphate isomerase (TIM)
NAD+
NADH + H+
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
1,3-Bisphosphoglycerate (1,3-BPG)
ADP
ATP
phosphoglycerate kinase (PGK
Mg2+
3-Phosphoglycerate (3PG)
phosphoglycerate mustase (PGM)
2-Phosphoglycerate (2PG)
H2O
enolase Mg2+
Phosphoenolpyruvate (PEP)
ADP
ATP
Mg2+,K+
pyruvate kinase (PK)
Pyruvate
cont.
cont.
cont.

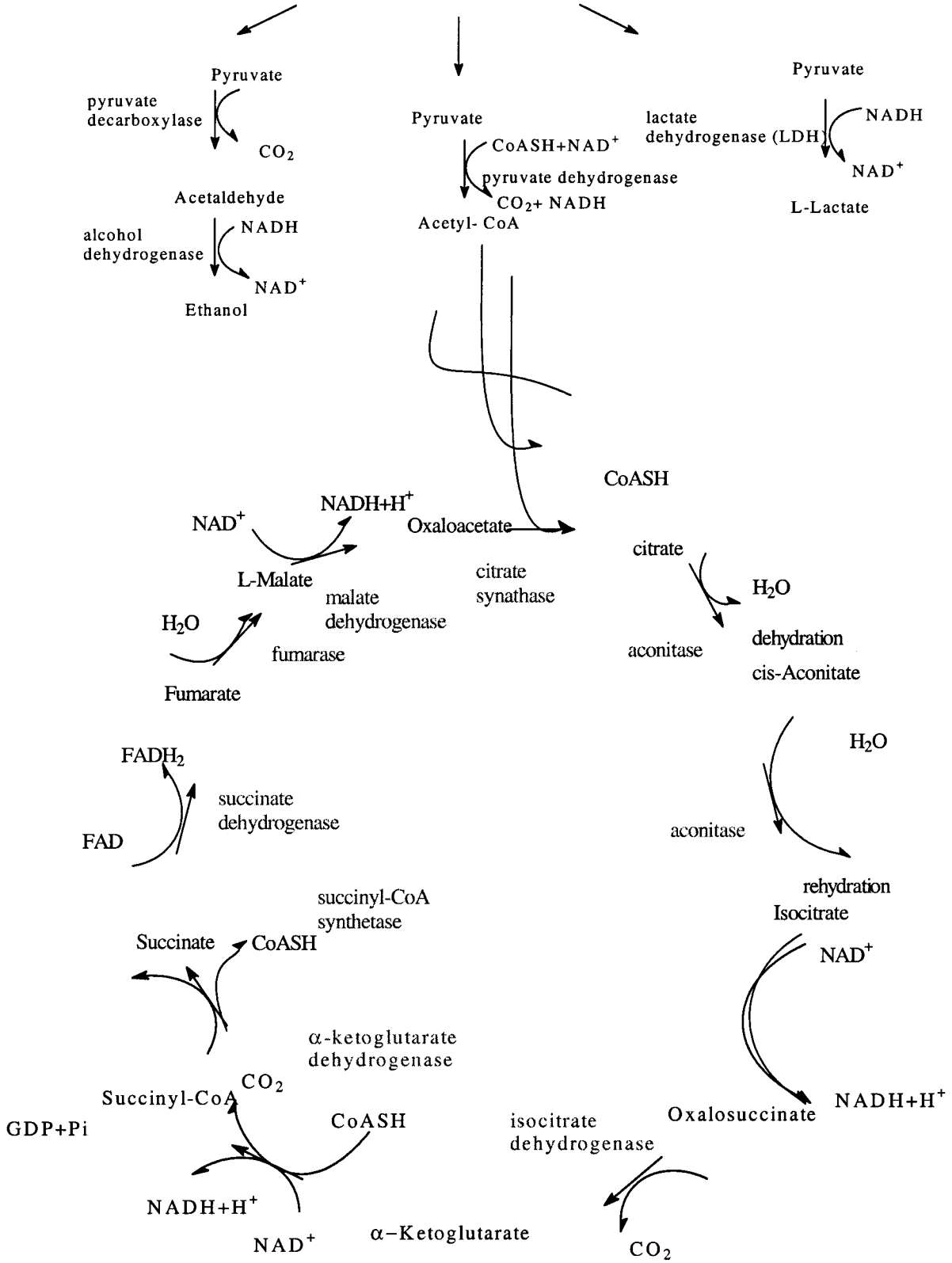


Figure 2. Biochemical Pathway of Organic Acids

CHAPTER TWO

HISTORICAL REVIEW

Several analytical methods have been used to separate and quantify the organic acids in wine, but to date complete validation has not been published. This chapter will outline a survey of some of the work done in the analysis and quantitation of organic acids and other components in wine using HPLC.

Frayne [9] used a dual cation exchange column on an HPLC with UV and refractive index (RI) detection to analyze the major organic acids, sugars, and alcohols in wine. No sample preparation was needed, and the separation was achieved in 40 minutes.

Schneider and coworkers [10] developed a method using a cation exchange resin to analyze citric, tartaric, malic, lactic, and acetic acid in wine. The acids eluted within 12 minutes. Although this was faster than Frayne's separation, succinic acid could not be determined due to coelution with shikimic acid.

Tusseau and coworkers [11] used reversed phase chromatography with UV detection to separate organic acids, but only five acids were analyzed. Therefore, acid peaks were resolved but further research was needed to include other acids.

Nimura and coworkers [12] prepared 1-pyrenyldiazomethane (PDAM), a new fluorescent labeling agent for carboxylic acids. PDAM readily reacted with carboxylic acids at room temperature without a catalyst to give an intensely fluorescent ester. Although this method was developed for the analysis of fatty acids, it may also be applied to wine acids.

Allenmark, [13] developed a method using N-(9-acridinyl)-bromoacetamide derivatives. A phase-transfer-catalyzed esterification was carried out with various carboxylic acids to give highly fluorescent esters. These were separated using reversed-phase liquid chromatography in 40 minutes.

Calull and coworkers, [14] used ion-exchange chromatography and refractive index detection to analyze sugars, lactic, succinic and acetic acid in wine. Because significant dilution of the samples was necessary the acids could not be quantified. Another ion-exchange method, developed by Lopez [15], was used to determine the major organic acids, sugar, glycerol, and ethanol in wine simultaneously in 45 minutes using UV detection, and refractive index (RI) for malic acid and fructose.

Vonach [16] reported a method using a resin-based ion-exchange stationary phase coupled with FTIR detection to determine organic acids. Complete separation was achieved in 20 minutes. The method took advantage of the IR absorption at different wave numbers by different functional groups. The organic acids absorbed at 1260 cm^{-1}, the carbohydrates at 1050 cm^{-1}.

Schindler [17] developed a method based upon Sequential Injection Fourier Transform Infrared Spectrometry (SI-FTIR). This method allowed the simultaneous determination of all the major organic components in wine, such as: glucose, fructose, glycerol, citric, tartaric, malic, lactic, acetic acids and ethanol in 3 minutes. Succinic acid was not included in the study. Using this method the results for some organic acids (acetic acid and tartaric acid) were relatively poor, which was explained by the low concentration and the uncharacteristic absorbance of these acids in the investigated spectral range. The relative standard deviation of a single sample was smaller than 8%

for all components, except for acetic acid at 30%, and for ethanol, which was less than 2%. The major drawback of this method was that the accuracy for determination of concentration levels was diminished due to the FTIR analysis. The author concluded that FTIR spectroscopy was best suited for identification and screening purposes rather than validation. The calibration procedure was time-consuming because they used seventy-two calibration solutions, with more being desirable for accurate analysis. When analyzing synthetic samples, only six components were used for the calibration and nine samples were analyzed. In order to develop a complete method for all compounds of interest, an HPLC method was developed.

Another method was developed by Giumani et al.[18] for analyzing wine acids using GC-MS. The formation of phenacetyl esters through derivatization allowed the determination of 20 different acids, many of which had not been recorded previously, in 18 minutes.

In liquid chromatography, the only useful method to determine small amounts of carboxylic acids was based on pre-column derivatization with the formation of an ester group, which strongly absorbs UV radiation and / or fluoresces [13].

Borch [19] separated long fatty acids as phenacyl esters by HPLC. The method described allowed a rapid and convenient way to derivatize and subsequently analyze fatty acid mixtures on the microgram scale and gave a high degree of resolution in most cases. This method could be further applied to complex samples matrixes such as wine.

Caccamo and coworkers [20] developed a method by derivatizing the major organic acids in wine with phenacyl bromide and crown ether in acetone including lactic, acetic, tartaric, succinic, malic, and citric acids. The major organic acids were

determined as phenacyl esters using reversed phase HPLC in sixteen minutes. Recoveries were 95% and higher. The derivatizing agent worked best in a buffered solution, as 0.08 M phosphate buffer.

CHAPTER THREE

STATEMENT OF THE PROBLEM

Wineries need to monitor the concentrations of tartaric, malic, lactic, citric, and acetic acid during the winemaking process to ensure the quality of their wines. Tartaric, citric, and malic acid may all be added legally to wine. The quantitation of citric acid is important as wines destined for Europe must comply with regulations that specify that the citric acid level in wine must be below 1.0 g/L. Malic acid levels must also be monitored closely as many wines undergo a process known as malolactic fermentation, which "softens" the wine as malic acid is converted to lactic acid. Acetic acid is a by-product of the primary and secondary fermentation processes. Acetic acid can also be formed by acidic acid bacteria and other microorganisms. High levels of acetic acid are indicative of high volatile acid, which is considered a wine defect [13].

Many articles have been published on the separation and quantitation of organic acids in wine, but none has been fully validated. The primary purpose of this research was the modification and validation of the method by Caccamo et al. [20]. To accomplish this, it was necessary to optimize the mobile phase and the separation conditions. Methods from the literature were used to derivatize the major carboxylic acids to esters allowing detection at 254 nm. This approach avoids interference from other wine components, which may occur at the commonly used detection wavelength of 210 nm. The method validation includes studying standard curve linearity, accuracy and precision, usable concentration range, analyte detection and quantitation limits and robustness. After validation the method was used to analyze Pinot Gris and Chardonnay wine samples from YSU's Enology laboratory.

CHAPTER FOUR

MATERIALS AND EQUIPTMENT

A. Reagents

All reagents used in this research were of the highest grade available and are listed in Table 1 along with relevant purity and source data.

Standard	**Percent Purity**	**Source**
Lactic acid	85%	Fisher Scientific
Glacial Acetic acid	100%	Fisher Scientific
L-(+)-Tartaric acid	99.8%	Fisher Scientific
DL- Malic acid	99+%	Fisher Scientific
Succinic acid	99.5+%	Aldrich
Citric acid	99.5+%	Fisher Scientific
Methylmalonic acid	99%	Aldrich
Mobile Phase Reagents		
Ethyl alcohol	200 Proof	Pharmaco
Methanol	HPLC grade	Burdick and Jackson
Acetonitrile	Optima grade	Fisher Scientific
Water	Purified	In-house (Modulab)
Derivatizing Reagent		
Dicyclohexano-18-Crown 6	98%	Sigma
2 Bromoacetophenone	98%	Aldrich
Sodium phosphate anhydrous	A.C.S.	J.T. Baker
Sodium hydroxide	98.4%	Fisher Scientific
Acetone	Pesticide grade	Fisher Scientific
Hydrochloric acid	A.C.S.	Fisher Scientific
Potassium dihydrogen phosphate	99.14%	Fisher Scientific
Test Mixture Reagents		
Uracil		Eastman
Phenol		Mallinckrodt
N,N-Diethyl-m-toluamide	98%	Aldrich
Toluene	A.C.S.	Fisher Scientific
Sensitivity Reagents		
D(-) Fructose		Sigma
Dextrose anhydrous		Fisher Scientific

Table 1. Reagents Used in This Research

Source	Supplier
Aldrich	Aldrich Chemical Company Inc. (Milwaukee, WI, U.S.A.)
Alltech	Alltech Associates, Inc. (Deerfield, IL, U.S.A.)
Burdick and Jackson	Burdick and Jackson (Muskegon, MI, U.S.A.)
Eastman	Eastman Kodak Co. (Rochester, NY, U.S.A.)
Fisher	Fisher Scientific (Fairlawn, NJ, U.S.A.)
J.T. Baker	J.T. Backer (Phillipsburg, N.J, U.S.A.)
Mallinckrodt	Mallinckrodt Chemical Works (St. Louis, MO, U.S.A.)
Pharmaco	Pharmaco (Brookfield, CT, U.S.A.)
Sigma	Sigma Chemical Co. (St. Louis, MO, U.S.A.)

B. Equipment

The HPLC system used for this work was a Waters 2695 Separations module (Milford, MA, U.S.A.), equipped with an absolute pressure transducer (APT) and an online degasser. It included an LC-pump, an eluent mixing chamber, an auto sampler, a 100-μL loop injector, a column oven heater, a 996 Photodiode Array Detector (PDA), and Millennium 3.2 software.

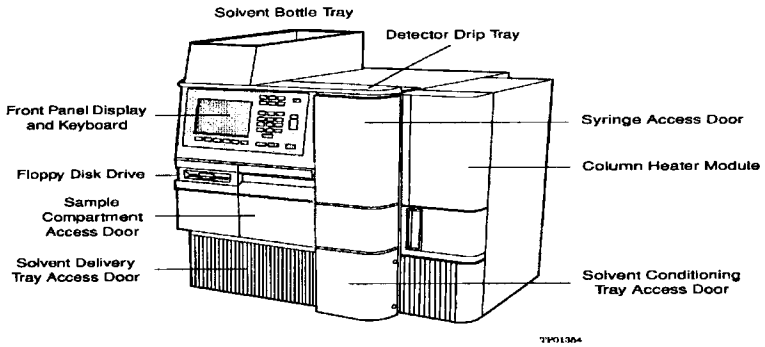


Figure 3. HPLC System

CHAPTER FIVE

METHODS

A. Grape Must

Grape musts were obtained from regional wineries. Twenty-gallons of Chardonnay must was provided by Markko Vineyards, Conneaut, Ohio, from their October 4th, 2001 harvest. Twenty-gallons of Pinot Gris must was provided by the Kingsville Grape Research Branch (OARDC), Kingsville, Ohio, from their October 1st, 2001 harvest.

B. Fermentation

Musts were divided into 12, 3-gallon glass fermentation vessels, 6 each for Chardonnay and Pinot Gris. Three each for Chardonnay and Pinot Gris were control fermentations (C-1, C-2, C-3), and three each were spontaneous fermentations (S-1, S-2, S-3). Fifty ppm of SO_2 was added to the control vessels to inhibit the growth of indigenous yeasts; none was added to the musts for the spontaneous fermentations. The musts were allowed to settle by gravitation for 24 hours at 38 °C. The clear supernatant was then separated from the sediments and controls were inoculated with commercial freeze-dried yeast (Prise de Mousse, EC 1118) at one gram per gallon. No additional yeast was added to the spontaneous fermentations. The vessels were kept at 22 °C during the fermentation. After the fermentations were completed the wine was racked off, the amount of free SO_2 was adjusted to 40 ppm, cold stabilized at 4 °C for 3 weeks, and bottled.

C. Wine Sampling

Each day 10 mL of wine was collected from the center of the vessel into a plastic vial with screw cap and stored frozen at -80 °C until the analysis was performed.

D. Wine Analysis

Wine samples from both the spontaneous and inoculated fermentations were analyzed every fourth day. The wine samples were thawed, thoroughly mixed, and centrifuged for ten minutes to remove yeast and other particulate matter. For the analysis one mL of wine was used and was spiked with an internal standard. The mixture was then diluted 1 to 10 with a mixture of 12% ethanol in water. The acid concentrations in each sample were calculated using Equation 9.

$$AcidConc. = \frac{\frac{(LASample)Area}{IS(Sample)}}{\frac{(LAStd.\#5)Area}{IS(Std.\#5)}} xStd.\#5LAconc.g/L$$

Equation 9. Acid Concentration Determination

E. Derivatization of Organic Acids

The purpose of derivatization of the major organic acids in the wine samples was to enable their detection using UV spectrophotometry at 254 nm. The acids were derivatized to form phenacyl esters, as shown in Figure 4.

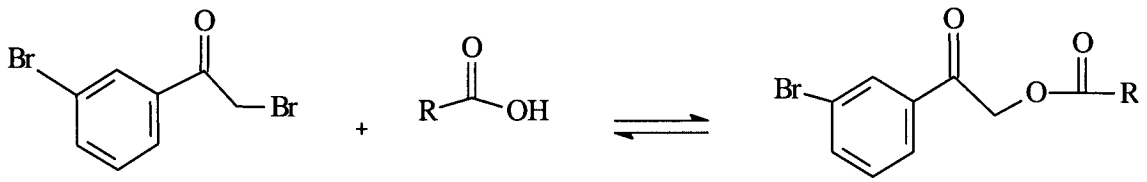


Figure 4. Phenacyl Ester Formation

The derivatization reaction was carried out in a PTFE (poly-1, 3-dioxole-co-tetrafluoroethylene) lined screw capped glass-tube containing 20 μL of wine sample, 80 μL of phosphate buffer, and 300 μL of derivatizing reagent. The derivatizing reagent was prepared by dissolving phenacyl bromide and dicyclohexane-18-crown-6 in acetone to give concentrations of 30 and 1.5 g/L, respectively [20]. The buffer was prepared by adding 2.7 g of KH_2PO_4 and 2.84 g Na_2HPO_4 in 1 L of deionized water to give a 0.08 M phosphate buffer of pH 6.8. Wine samples were diluted 1 to 10 using a mixture of 12 % ethanol in water. After thorough mixing the mixture was heated for 45 minutes at 95 °C. After cooling the mixture to room temperature, a 15.0 μL aliquot was taken and subjected to chromatographic analysis.

F. HPLC Method

The chromatographic separation was performed using an 250 mm x 4.6 mm Alltima C_{18} packed column with a 5-micron particle size. The analytical column was preceded by a 7.5 mm x 4.6 mm C_{18} guard column. Both the analytical and guard columns were supplied by Alltech Associates (Deerfield, IL, U.S.A.). The following mobile phases were used : solvent A: water, solvent B: acetonitrile-methanol (50:50), and solvent C: acetonitrile. A linear gradient was applied by increasing solvent B (50:50) (methanol: acetonitrile) from 45 to 90% in 25 min., followed by a 10-minute wash with

100% acetonitrile. The flow rate was 1.000 ml/min. The oven temperature was 30.0 °C and UV detection was set at 254 nm.

G. Standards Preparation

Standards were prepared by dissolving the seven acids of interest in 12 % ethanol in water to give concentrations as shown in Table 2. The standards were then diluted 1 to 10 with 12% ethanol in water. This dilution was applied to all samples to eliminate any potential problems due to the derivatizing agent becoming the limiting reagent in the derivatization reaction. All other standards were weighed individually into a volumetric flask and then diluted 1 to 10 with 12 % ethanol in water. All standards were adjusted to pH 3. For weights less than 80 mg a microbalance was used; otherwise a standard analytical balance was used for all weighings. All glassware was volumetric.

Standards	LA (g/L)	AA (g/L)	TA (g/L)	MA (g/L)	SA (g/L)	CA (g/L)	IS (g/L)
Std#1	0.2528	0.2510	0.6347	1.6166	0.3811	0.1805	1.0004
Std#2	0.4944	0.49664	1.2437	3.268	0.7334	0.2419	1.0004
Std#3	1.0300	1.0057	2.5763	6.488	1.5074	0.49940	1.0004
Std#4	1.5328	1.4112	4.292	9.944	2.2672	0.73792	1.0004
Std#5	1.9856	1.9729	5.428	13.01	3.0509	1.0047	1.0004
Std#6	2.4465	4.2110	5.216	14.43	3.0973	1.1828	1.0004

Table 2. Concentrations of Standards 1 to 6, Undiluted

H. Method Validation

Method validation is the process of testing an analytical method for specificity, linearity, accuracy, precision, usable concentration range, detection limit, quantitation limit, sensitivity and robustness.

Samples used in the *specificity* study were Standard #5 and Chardonnay diluted 1.4 to 10. Three runs of each sample were analyzed in triplicate for retention time, peak resolution, theoretical plate count, peak tailing factor and capacity factor (see Figure 5). To facilitate the identification of peaks, each acid was individually spiked into the sample at a relatively high concentration. Comparison of retention times between spiked and unspiked samples gave verification of peak identity. Peak resolution was calculated according to Equation 5 in Chapter 1.

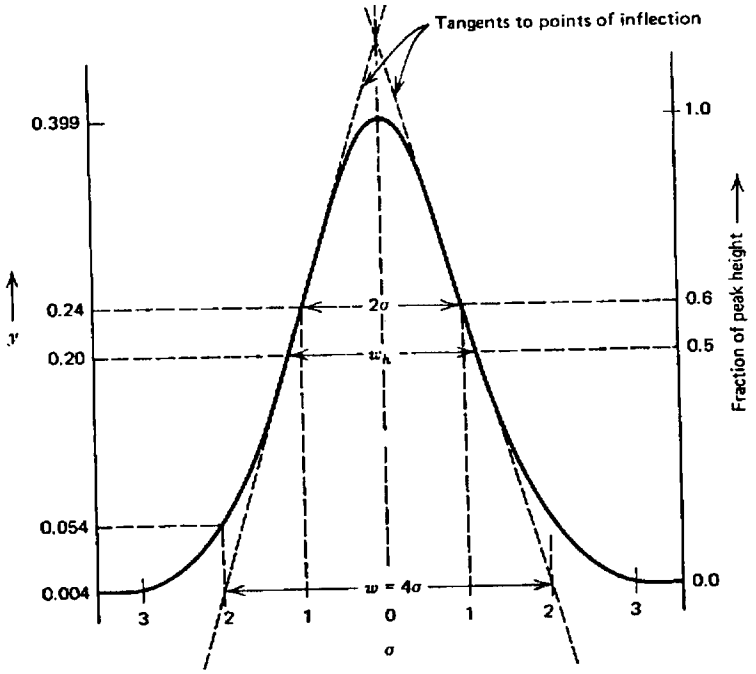


Figure 5. Calculation of Peak Symmetry, Theoretical Plate Count, and Resolution

Theoretical plate count is a measure of column performance. The number of plates is related to the ability of an analyte band to flow through the column with a minimum amount of band broadening (diffusion). The plate count was calculated according to Equation 4 in Chapter 1.

Asymmetry of a peak can occur in two different ways: fronting or tailing. Based upon considerations of the solute distribution coefficient, D, peak-tailing results from Langmuir type absorption isotherms, where the analyte is partitioned more into the stationary phase as the concentration of analyte in the migrating band of molecules increases. Fronting peaks result from anti-Langmuir isotherms, where the analyte partitions more into the mobile phase as concentrations within the band increases.

Capacity factor is related to the distribution coefficient, and may be thought of as the absorbing quality of stationary phase relative to the mobile phase. Capacity factor was calculated using Equation 2 in Chapter 1.

The evaluation of *linearity* was performed using six standard solutions ranging from 50% to 150% of the target analyte concentrations. Each concentration was analyzed a minimum of three times. The linearity range was determined by the correlation coefficient and the "y"-intercept of the linear regression line. A correlation coefficient of > 0.999 was generally considered as evidence of acceptable linearity. Along with these mathematical parameters a visual examination of the calibration curve was required.

The *accuracy* of a method can be measured in several ways. One way is based on recovery as determined by spiking analytes into a blank matrix. Another approach is the standard addition technique, which is used when a blank sample matrix is not appropriate.

In this project, accuracy was determined by calculating the recovery of each acid using Equation 10.

$$\%\,R = \frac{PA_{mix}}{(PA_{Std.\#5} + PA_{wine})/2} \times 100\%$$

Equation 10. Percent Recovery

The recovery study was set up as shown below:

► Vial #1. 0.50 ml wine plus 0.50 ml Std. #5; diluted 1:10 with 12% etOH in water

► Vial #2. 1.00 ml Std. #5 diluted 1:10 with 12% etOH in water

► Vial #3. 1.00 ml wine sample diluted 1:10 with 12% etOH in water

All vials contained equal concentrations of internal standard. Samples were analyzed on three different days in triplicate resulting in 9 injections. The peak areas were averaged.

The *standard addition* was performed with Pinot Gris and Chardonnay wines. To each variety seven different acids were added in increasing concentrations, (see Table 3 and 4). Each individual acid was extrapolated to the negative x-axis to determine the individual acid concentration as an absolute value for each variety of wine. The concentration of each acid in wine was determined by calculating the intercept with the x-axis.

Std. Add. Chardonnay	LA (g/L)	AA (g/L)	TA (g/L)	MA (g/L)	SA (g/L)	CA (g/L)	IS (g/L)
Std#1	0.331	0.1889	0.209	0.319	0.203	0.103	1.0004
Std#2	0.605	0.421	0.4038	0.611	0.4132	0.206	1.0004
Std#3	0.841	0.742	0.617	0.901	0.615	0.412	1.0004
Std#4	1.073	0.925	0.808	1.202	0.809	0.608	1.0004
Std#5	1.321	1.16	1.01	1.505	1.04	1.008	1.0004

Table 3. Standard Addition in Chardonnay

Std. Add. Pinot Gris	LA (g/L)	AA (g/L)	TA (g/L)	MA (g/L)	SA (g/L)	CA (g/L)	IS (g/L)
Std#1	0.347	0.200	0.217	0.299	0.210	0.104	1.0004
Std#2	0.589	0.423	0.435	0.605	0.411	0.215	1.0004
Std#3	0.866	0.655	0.6095	0.9114	0.617	0.404	1.0004
Std#4	1.075	0.937	0.8111	1.206	0.824	0.607	1.0004
Std#5	1.324	1.154	1.014	1.517	1.014	1.018	1.0004

Table 4. Standard Addition in Pinot Gris

The *range* of an analytical method is the concentration interval over which accuracy, linearity, and precision are valid. Generally, the range is determined by using the linearity and accuracy data. In this project, the range was determined to be from the limit of quantitation to the highest standard concentration (Standard # 6).

Precision is the amount of scatter in the results obtained from multiple analyses of the same sample. One type of precision examined was instrument precision, or injection repeatability. A minimum of 10 injections of one sample was required to test the performance of the chromatographic system. A second type of precision examined was the analyst precision, or the intra-assay repeatability. The analyst repeatedly analyzed

independently prepared samples. Ten samples were analyzed and the relative standard deviation calculated.

Limit of Detection (LOD) was the lowest analyte concentration that produced a response detectable at three times the noise level. The *limit of quantitation (LOQ)* was the lowest analyte concentration that could be precisely and accurately measured. LOQ is often calculated as the analyte concentration that gives a signal to noise ratio of ten. For the determination of LOD and LOQ the lowest standard concentration, Standard #1, was diluted to achieve appropriate levels, see Tables 5 and 6. AA and SA were diluted 1 to 10 while all other acids were diluted 1.4 to 10. The signal was calculated as peak areas of the analyte minus the peak area blank signal that may have been co-eluting with the peak of interest. The peak areas of the blank signals were averaged from 50 blank samples. The noise was averaged from different dilutions and on different days of analysis.

1.4-10	**LA dilution**	**TA dilution**	**MA dilution**	**CA dilution**
LOD	2-10	0.5-10	1-10	Std#1
LOQ	Std#1	1-10	3-10	Std#4

Table 5. LOD and LOQ Using Standards That Were Diluted 1.4 to 10

1.0-10	**AA dilution**	**SA dilution**
LOD	Std#2	3-10
LOQ	Std#3	Std#2

Table 6. LOD and LOQ Using Standards That Were Diluted 1 to 10

The analysis of the standard *stability* was performed using freshly prepared Standard #5 and comparing it to old standards, over a period of 43 days, both at room temperature and at refrigerated temperature (4 °C). Standards that were derivatized on day one were re-used and compared to standards that were derivatized fresh, daily. The stability was evaluated daily for the first 8 days, and thereafter twice a week until decomposition was observed.

Ruggedness tested the use of the analytical method by multiple analysts, multiple instrumentation, on multiple days in one laboratory to determine the method robustness. Different sources of reagents and multiple lots of columns should be used. The robustness of a method is the ability to remain unaffected by small changes in organic solvent, pH, mobile phase composition, buffer concentration, temperature, and injection volume. These factors can be evaluated one at a time or simultaneously as fractional experiments. In this project column temperatures ranging from 25°C to 45°C and various solvent compositions were evaluated.

Sensitivity tests consisted of ethanol and sugar additions to the standards and determining the effect on the slope. The ethanol concentrations studied ranged from 0 to 15 %. The second sensitivity test consisted of adding sugar (1:1) (fructose: glucose) in concentrations ranging from 0 to 250 g/L. All standards were diluted 1.4 to 10 with 12% ethanol water. The slopes of the lines in the ethanol and sugar addition study were analyzed for all six acids.

CHAPTER SIX

RESULTS AND DISCUSSIONS

A. Specificity

Specificity is the ability of an analytical method to accurately measure the analytes in the presence of all components. Three runs of each sample were analyzed in triplicates for retention time, resolution factor, theoretical plate count, tailing factor and capacity factor as shown in Table 7. A resolution factor of 1.15 or better was achieved for all acids meeting the minimum quantitation requirement of >1. For perfect resolution between adjacent Gaussian curves the resolution factor should be grater than or equal to 1.5.

The minimum theoretical plate count for an analytical column to be considered good should generally be in the range 1000 to 10,000 and depends upon the analytical requirements of the method. It was found that for N_B was greater than 14691 and $N_{1/2}$ greater than 12761 for all acids.

Asymmetry can occur in two different ways, fronting or tailing. Tailing is the analyte retaining more on the column and fronting is the analyte retaining less on the column.

Acids were added individually to wine to determine their retention times (see Figure 6).

	LA	AA	TA	MA	SA	CA
Rt. (min.)	7.727	9.810	12.573	8.742	17.530	21.026
Resolution	1.43	1.15	1.29	2.27	Not needed	1.33
N_B (plates/m)	14691	22431	37415	56458	67446	102263
$N_{1/2}$ (plates/m)	12761	14768	32167	29282	40510	91062
Symmetry	1.18	1.38	1.20	1.20	1.37	1.31
Capacity factor	4.15	5.54	7.38	8.742	10.68	13.017

Not needed: no other components were in the vicinity of succinic acid

Table 7. Specificity Test Using Wine Samples

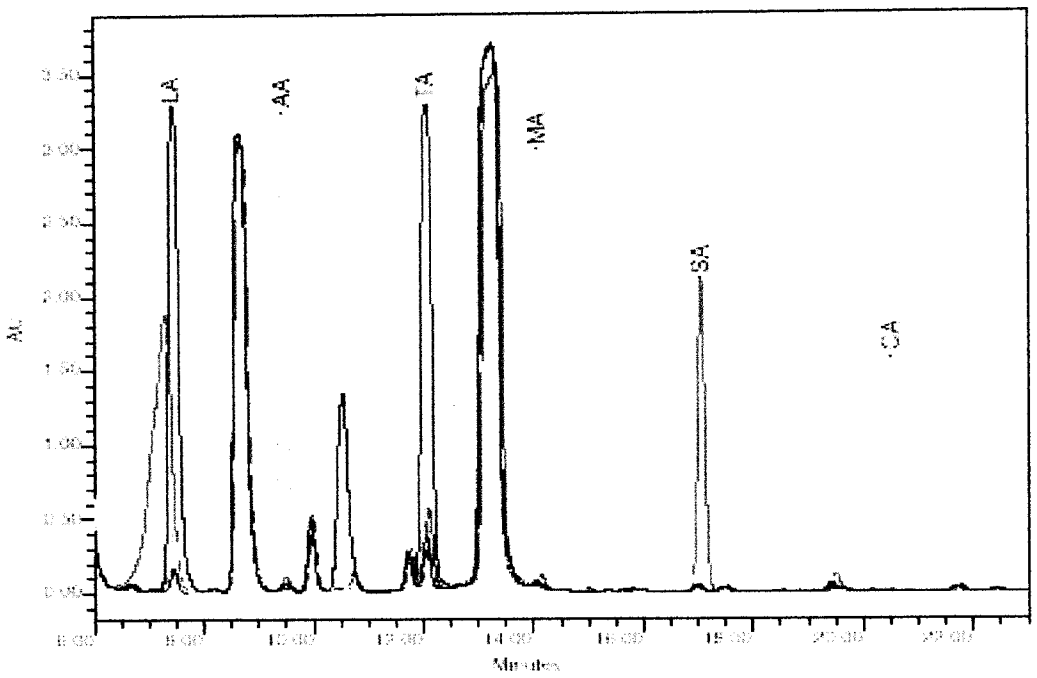


Figure 6. Overlaid Chromatogram of Wine Samples Spiked With Acids

In order to be valid a method needs to demonstrate *specificity*, which means that it will be able to accurately measure the analyte response, and only the analyte response, in the presence of a sample matrix containing the analyte. Once resolution is acceptable the chromatographic parameters, such as column type, mobile phase, composition, flow rate, and detection mode are considered to be valid.

Figures 7 and 8 show the separation of the acids in two different wines, Pinot Gris and Chardonnay. The chromatogram of a blank shown in Figure 9 illustrates a small interference with lactic, acetic, succinic, and metylmalonic acids.

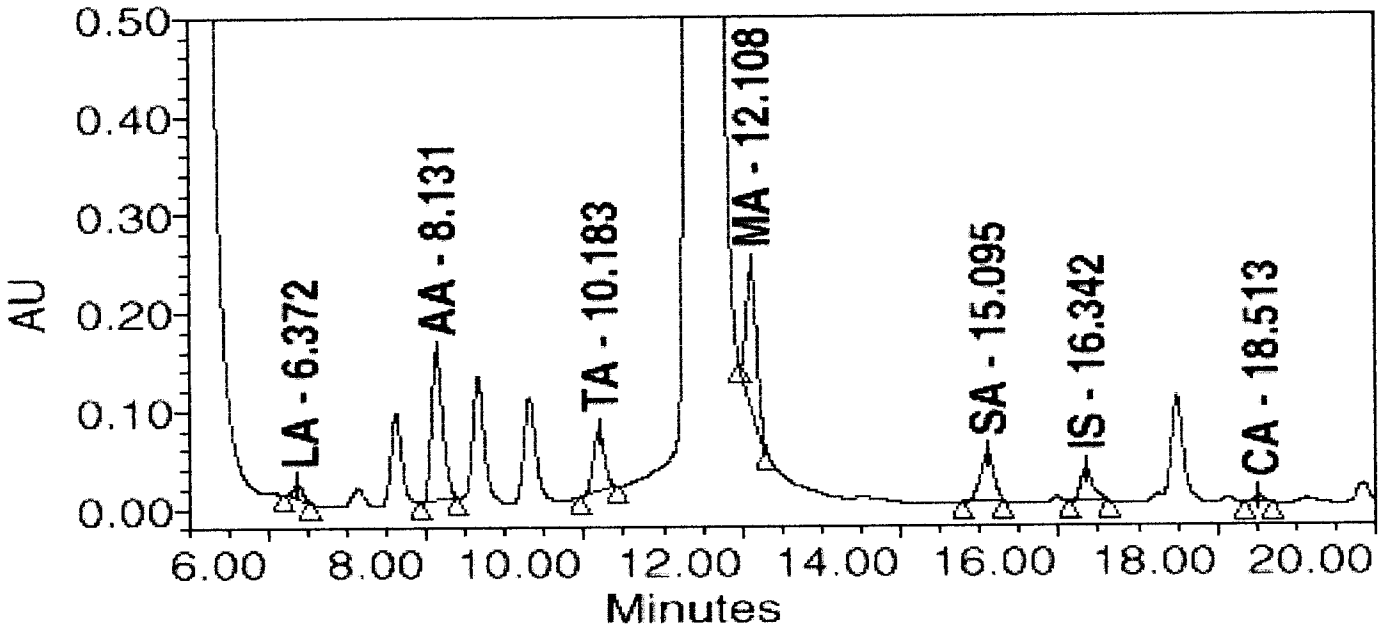


Figure 7. Chromatogram of Pinot Gris Wine

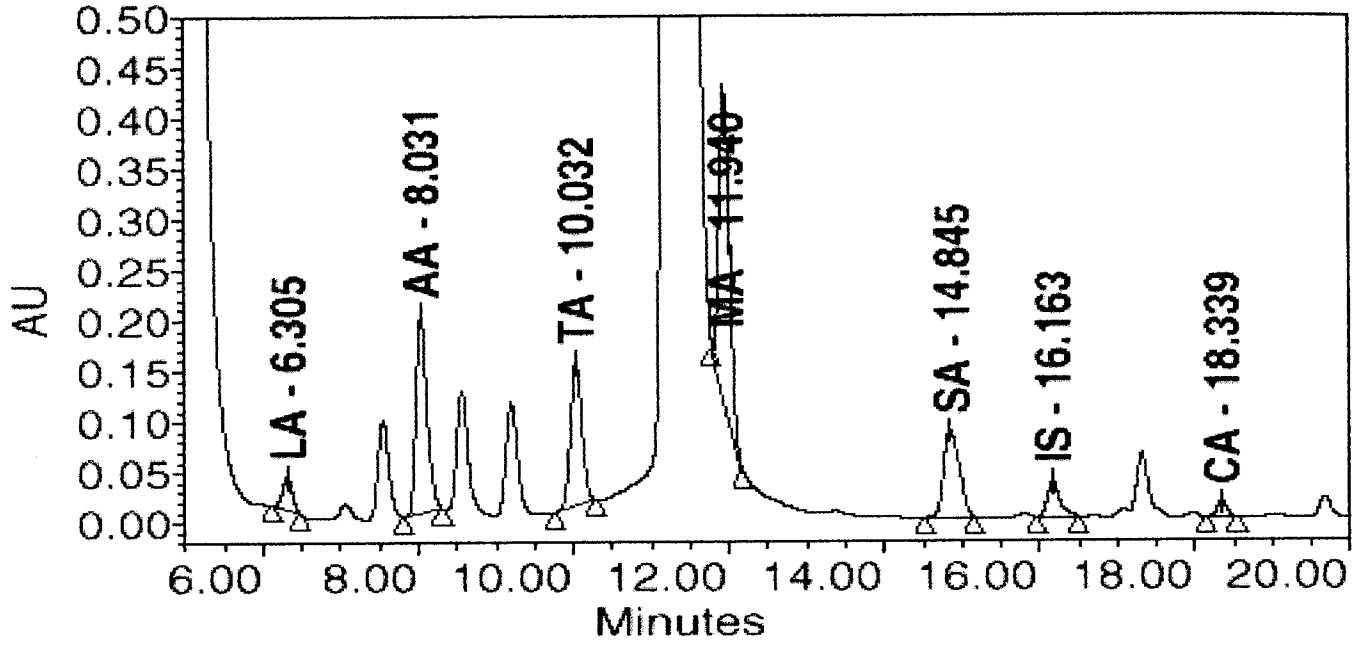


Figure 8. Chromatogram of Chardonnay Wine

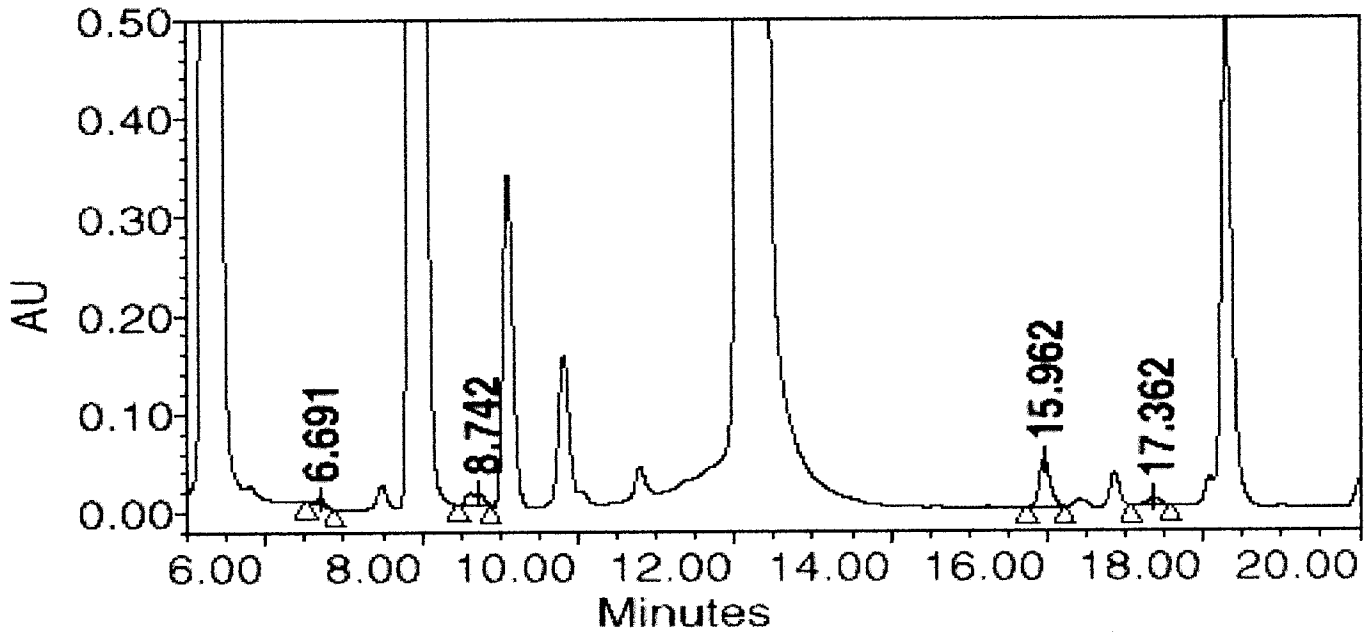


Figure 9. Chromatogram of a Blank

Several different solvent compositions and gradients, as shown in Table 8, were tested to overcome various interferences. The best composition was found to be solvent A water, solvent B (50:50) methanol: acetonitrile and solvent C acetonitrile, at 1.000 mL/min flow rate. The solvent B was increased from 45% to 90% in 25 minutes followed by a 10-minute was with 100% acetonitrile. This resulted in a 35-minute separation where all analyte peaks were resolved.

B. Derivatizing Agent Study

The time of derivatization and the concentration of derivatizing agent were studied using the parameters outlined below. Table 9 shows the effect of changing concentrations of phenacyl bromide and dicyclohexano-18-crown-6.

	Phenacyl bromide (g/L)	**Dicyclohexano-18-crown-6 (g/L)**
A	30.96	3.020
B	34.40	4.500
C	30.60	7.500
D	32.70	15.60
E	32.80	30.60

Table 9. Effect of Derivatizing Agent Concentrations on Peak Area and Shape

	Time start (min)	Gradient start A: H_20 B: MeOH/ACN C: ACN A/B/C	Time (min)	Gradient Proceeding A: H_20 B: MeOH/CAN C: ACN A/B/C	Time (min)	Gradient Proceeding A: H_20 B: MeOH/ACN C: ACN A/B/C	Flowrate (ml/min)	Temp (°C)
1.	0	80/20	10	90/10	34		1.000	30.0
2.	0	70/30	15	10/90	35		1.000	30.0
3.	0	60/40	22	10/90	40	10/90	1.000	30.0
4.	0	60/40	22	42/58	45	10/90	1.000	30.0
5.	0	60/40	30	20/80	32.5	10/90	1.000	30.0
6.	0	60/10	35	10/90			1.000	30.0
7.	0	60/40	14	49/51	18	47/53	1.000	30.0
8.	0	70/30	17	10/90			1.000	30.0
9.	0	70/30	34	10/90	38	10/90	1.000	30.0
10.	0	60/40	30	10/90			1.000	30.0
11.	0	55/45	30	10/90			1.000	30.0
12.	0	95/5	30	65/35			1.000	30.0
13.	0	100/0	30	70/30			1.000	30.0
14.	0	90/10	30	70/30			1.000	30.0
15.	0	95/5	8	95/5	30	65/35	1.000	30.0
16.	0	100/0	35	75/25			1.000	30.0
17.	0	98/2	30	85/15			1.000	30.0
18.	0	75/25	30	25/75			1.000	30.0
19.	0	70/30	30	10/90			1.000	30.0
*								
20.	0	65/35	30	10/90			1.000	30.0
21.	0	60/40	25	10/90	35	0/0/100	1.000	30.0
22.	0	50/50	25	10/90	35	0/0/100	1.000	30.0
23.	0	55/45	25	10/90	35	0/0/100	1.000	30.0

*Face seal wash, plunger seal was changed.

Table 8. Solvent Compositions and Gradients Tested to Optimize the HPLC Separation A: H_2O, B: MeOH/ACN, C: ACN

Based on this study it was concluded that the concentrations of these reagents had no effect on the reaction. The concentrations used in the article by Caccamo and coworkers [20] were applied.

Various derivatizing times at constant derivatizing agent concentrations were studied as shown in Table 10.

It was confirmed that the ideal derivatization time was 45 minutes, as stated in the article. Broad chromatographic peaks appeared when heating the sample for more than 45 minutes, indicating that an undesirable by-product was formed, or that the derivatives were decomposing.

Time (min)	**Derivatizing agent** **a, phenacyl bromide (g/L)** **b, dicyclohexano-18- crown 6 (g/L)**	**Temp. (°C)**	**Peak Observation**
30	a,30.32 , b,1.508	30.0	Good
45	a,30.32, b,1.508	30.0	Ideal
90	a,30.32, b,1.508	30.0	Wide peaks
120	a,30.32, b,1.508	30.0	Wide peaks
180	a,30.32L, b,1.508	30.0	Wide peaks
240	a,30.32 , b,1.508	30.0	Wide peaks

Table 10. Effect of Derivatizing Time on Peak Area and Shape

Various derivation times were studied with double the phenacyl bromide concentration while keeping the catalyst (dicyclohexano-18-crown-6) constant. It was concluded that the derivatization time was ideal at 45 minutes as seen in the previous experiment (see Table 11).

Time (min)	Derivatizing agent a, phenacyl bromide (g/L) b, dicyclohexano-18 - crown- 6 (g/L)	Temp. (°C)	Peak Observation
45	a,60.10, b,3.120	30.0	Ideal
90	a,60.10, b,3.120	30.0	Wide peaks
180	a,60.10, b,3.120	30.0	Wide peaks
135	a,60.10, b,3.120	30.0	Wide peaks

Table 11. Effect of Derivatizing Time at Double the Phenacyl Bromide Concentration on Peak Area and Shape

C. Linearity and Standard Addition

Linearity is the determination of the concentration range where the analyte is potted against concentration and it shows that the calibration curve is linear. Table 14, Figure 10 and Figure 11 show not only the linearity, but also matrix effects by comparing the standard addition curve of Pinot Gris and Chardonnay with the calibration curve. These three regression curves show no significant matrix effect in the samples when diluting the wine 1 to 10. Lactic acid was chosen as an example. In this project linearity was evaluated using six standard solutions varying from LOQ to the highest concentration where the curve was found to be linear. Each concentration was analyzed in triplicates, averaged, and plotted against the signal. Various dilutions were studied to determine which one yielded the best correlation coefficient (see Table 12).

Dilutions	LA Corr. Coeff.	AA Corr. Coeff.	TA Corr. Coeff.	MA Corr. Coeff.	SA Corr. Coeff.	CA Corr. Coeff.
1.4 to 10	0.969	0.995	0.990	0.998	0.943	0.999
5.0 to 10	0.997	0.000	0.988	0.996	0.973	0.968
3.0 to 10	0.995	0.965	0.994	0.957	0.983	0.939
1.0 to 10	0.999	1.000	1.000	0.999	0.998	0.998

Table 12. Dilution Studied for Determining and Analyzing the Correlation Coefficient

For various dilution injections, the acid peak areas were averaged to determine the derivatizing agent effect and were plotted against the dilutions. The relative standard deviation was high for some acids, indicating the necessity of dilution. (see Table 13).

Dilution: 0, 1.33x, 2x, 4x, 7.14x	LA	AA	TA	MA	SA	CA
Average	3090363	502211	12565028	4805016	633463	976730
%RSD	9.9	81.2	8.0	5.8	88.9	4.5

Table 13. Dilution Study and Relative Standard Deviations

Acids	Equations of linear regression, standards	R^2	Equation linear regression Pinot Gris Standard Addition	R^2	Equation linear regression Chardonnay Standard Addition	R^2
LA	Y=2538679x+9640	0.995	Y=2490900x+135080	0.998	Y=2377004x+83841	0.992
AA	Y=5719487x+417947	0.994	Y=6089000x+487340	0.990	Y=6322435x+658662	0.981
TA	Y=4902932x-63718	0.992	Y=5162300x+3364000	0.979	Y=4313847x+1177499	0.978
MA	Y=5035905x+74928	0.993	Y=3214900x+9185000	0.903	Y=4542232x+2190626	0.922
SA	Y=5466538x+603885	0.990	Y=3450800x+1093400	0.977	Y=5584189x+1131267	0.983
CA	Y=4313466x+7283	0.976	Y=3372100x+606050	0.997	Y=478838x+134351	0.996

Table 14. Linear Regression Equation for Standard Solutions, Pinot Gris and Chardonnay Standard Additions

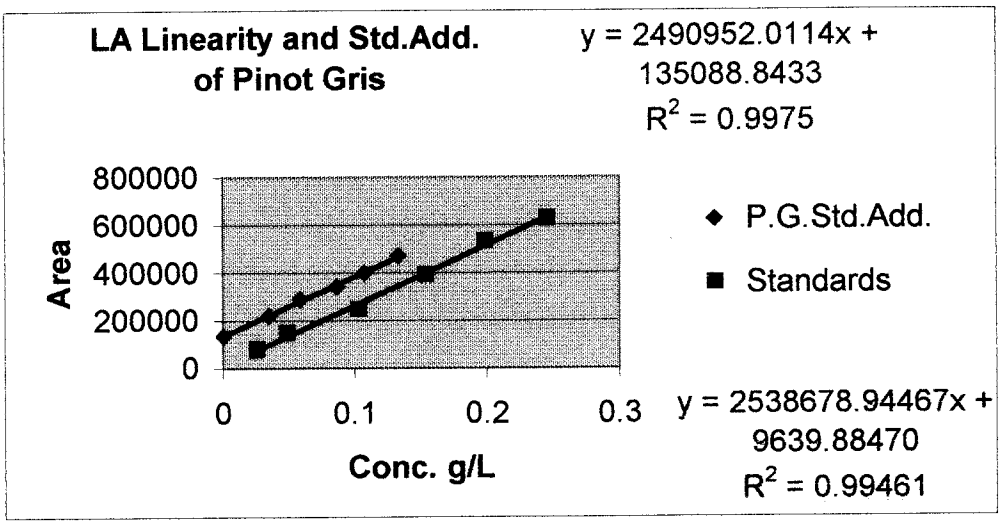


Figure 10. Lactic Acid Linearity Standards and Standard Addition in Pinot Gris

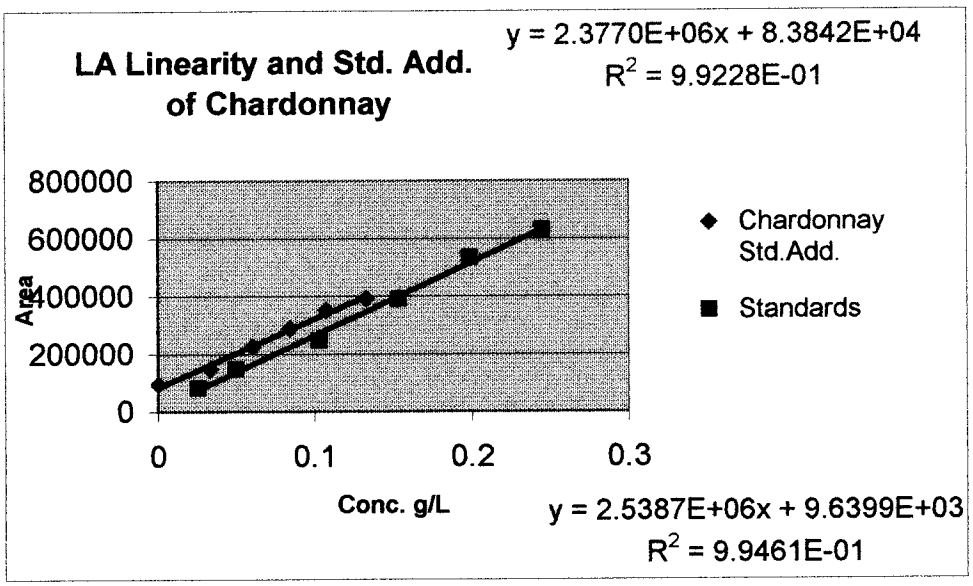


Figure 11. Lactic Acid Linearity Standards and Standard Addition in Chardonnay

D. Accuracy

Accuracy is the closeness between the value accepted as true value and the value found. The relative standard deviations in both Pinot Gris and Chardonnay were found to be 95.3-111.4% or better (see Tables 15, and 16). They were calculated from the recovery study according to Equation 1, in Chapter 5. The recoveries were performed with Pinot Gris and Chardonnay.

Chardonnay	LA	AA	TA	MA	SA	CA
Day 1	116.5	114.3	109.1	90.6	111.5	116.7
Day 2	101.5	101.6	112.5	100.9	105.5	108.7
Day 3	107.0	118.2	96.80	94.30	104.2	93.00
Avg. runs:	108.3%	111.4%	106.1%	95.3%	107.1%	106.1%
%RSD	7.0	7.8	7.8	5.5	3.6	11.4

Table 15. Acid Recoveries in Chardonnay

Pinot Gris	LA	AA	TA	MA	SA	CA
Day 1	104.4	105.6	107.2	107.4	107	109.5
Day 2	92.0	95.4	96.1	96.0	106.1	105.2
Day3	95.3	102.4	103.2	96.0	102.3	89.9
Avg. runs:	97.2%	101.1%	102.2%	99.8%	105.1%	101.5%
%RSD	6.6	5.2	5.5	6.6	2.4	10.1

Table 16. Acid Recoveries in Pinot Gris

E. Range

The range of an analytical procedure is the interval between the upper and lower concentrations of analytes in a sample for which precision, accuracy and linearity are valid. The range was determined to be from the LOQ to Standard #6 (see Table 17).

Range	LA (g/L)	AA (g/L)	TA (g/L)	MA (g/L)	SA (g/L)	CA (g/L)
	0.1700 to 2.446	0.1006 to 4.211	0.0427 to 5.216	0.0528 to 14.43	0.0733 to 3.097	0.0864 to 1.183

Table 17. Range

F. Precision

Precision is the amount of scatter between a series of measurements obtained from multiple samplings. For the injection repeatability the relative standard deviation of the retention times was < 0.80% and of the peak areas was < 5%, (Tables 18, 19 and Figure 12).

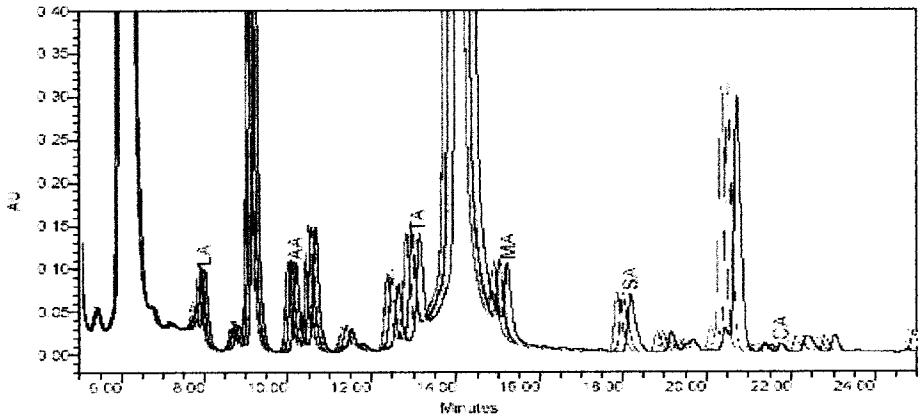


Figure 12. Overlaid Chromatogram from Injection Repeatability

	LA RT	AA RT	TA RT	MA RT	SA RT	CA RT
1.	7.787	9.899	12.741	14.824	17.770	21.315
2.	7.834	9.959	12.792	14.868	17.811	21.325
3.	7.849	9.973	12.797	14.870	17.814	21.331
4.	7.856	9.986	12.839	14.939	17.889	21.414
5.	7.907	10.050	12.889	15.003	17.926	21.420
6.	7.908	10.055	12.929	15.012	17.969	21.512
7.	7.908	10.055	12.932	15.019	17.969	21.512
8.	7.909	10.062	12.939	15.040	17.989	21.520
9.	7.971	10.123	12.949	15.076	17.993	21.530
10.	7.985	10.169	13.009	15.200	18.149	21.734
Avg.	7.894	10.0331	12.882	14.984	17.9279	21.461
STD	0.0610	0.08023	0.08542	0.11288	0.1122	0.1284
%RSD	0.77	0.80	0.66	0.75	0.63	0.60

Table 18. Injection Repeatability of Retention Time

	Area LA	Area AA	Area TA	Area MA	Area SA	Area CA
1.	553322	101016	1106823	706529	862530	90687
2.	531297	102138	1145954	705656	885454	87191
3.	555333	101282	1116771	715314	872718	95380
4.	544113	101069	1132580	704854	868731	92664
5.	581858	100013	116083	747974	897762	98030
6.	541488	103416	1115922	700996	872027	99048
7.	539473	103923	1168351	735641	898813	88526
8.	54814	103013	1169406	736264	873933	93999
9.	588907	99784	1172729	684378	850047	87976
10.	594003	98611	1124897	700605	861979	89510
Avg.	557961	101426.5	1136951.6	713821	874399.4	92301
STD	22207.23	1704.69	25.292.6	19873.8	15609.9	4220.3
%RSD	4.0	1.7	2.2	2.8	1.8	4.6

Table 19. Injection Repeatability of Peak Area

A second *precision* test, the intra-assay repeatability, indicated that the relative standard deviations for retention times were < 0.48 %, and for the peak areas < 7.9 %RSD (Tables 20, 21 and Figure 13).

	LA	AA	TA	MA	SA	CA	IS
1.	6.840	8.889	11.247	13.249	16.235	19.664	17.540
2.	6.851	8.890	11.256	13.265	16.256	19.670	17.552
3.	6.820	8.836	11.186	13.196	16.195	19.625	17.499
4.	6.835	8.836	11.234	13.285	16.312	19.784	17.637
5.	6.888	8.921	11.337	13.347	16.339	19.775	17.650
6.	6.902	8.942	11.361	13.380	16.364	19.810	17.671
7.	6.837	8.867	11.227	13.232	16.255	19.720	17.576
8.	6.840	8.875	11.240	13.239	16.218	19.624	17.516
9.	6.817	8.840	11.209	13.215	16.198	19.614	17.497
10.	6.839	8.879	11.250	13.260	16.245	19.648	17.540
Avg:	6.847	8.8775	11.2547	13.2668	16.2617	19.6934	17.568
Std. Dev.	0.027	0.035	0.054	0.057	0.058172	0.073365	0.063672
%RSD	0.40	0.40	0.48	0.43	0.36	0.37	0.36

Table 20. Intra-Assay Repeatability of Retention Time

	LA	AA	TA	MA	SA	CA	IS
1.	141691	211828	1164243	2121611	777423	140677	668813
2.	146639	196659	1103760	1865760	723593	124290	586293
3.	163413	211868	1174891	2046940	769230	149572	643218
4.	140533	196601	1085597	1839777	702020	117540	578681
5.	164553	216181	1105794	2039862	787298	144980	642555
6.	133198	223764	1196932	2072466	764942	140599	634651
7.	150032	209568	1140578	1940531	733903	137572	613090
8.	128439	184564	1051680	1756580	749011	131985	544700
9.	141666	194314	1144003	1894986	762329	140164	571509
10.	144347	210241	1115085	1881469	741770	135782	601101
Avg:	145451.1	205558.8	1128256	1945998	751151.9	136316.1	608461.1
Std. Dev.	11563.02	11963.1	44156.07	118429.6	26251.32	9549.515	38849.44
%RSD	7.9	5.8	3.9	6.1	3.5	7.0	6.4

Table 21. Intra-Assay Repeatability of Peak Area

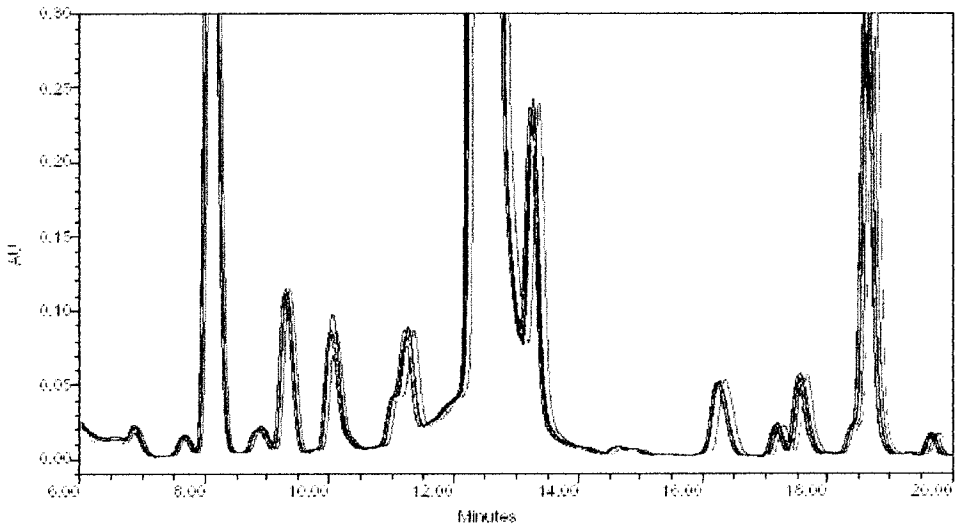


Figure 13. Overlaid Chromatogram for Intra-Assay Repeatability

G. Limit of detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection was the lowest amount of an analyte in a sample which could be *detected* reliably. The limit of quantitation is the lowest amount of an analyte in a sample which can be *quantitated* reliably. The LODs and LOQs are listed in Tables 22 and 23.

	LA	AA	TA	MA	SA	CA
S/N	2.9	2.4	4.4	5.0	4.0	3.2
Conc. (g/L)	0.0340	0.0497	0.0213	0.0176	0.0114	0.0216

Table 22. LOD of Acids

	LA	AA	TA	MA	SA	CA
S/N	12	10.	8.9	11	11	10.
Conc. (g/L)	0.1700	0.1006	0.0427	0.0528	0.0733	0.0864

Table 23. LOQ of Acids

H. Stability

Stability was the consistency of a response over a specified period of time. The stability of the standards was analyzed for 43 days both at refrigerated and room temperature, as shown in Figures 14 and 15.

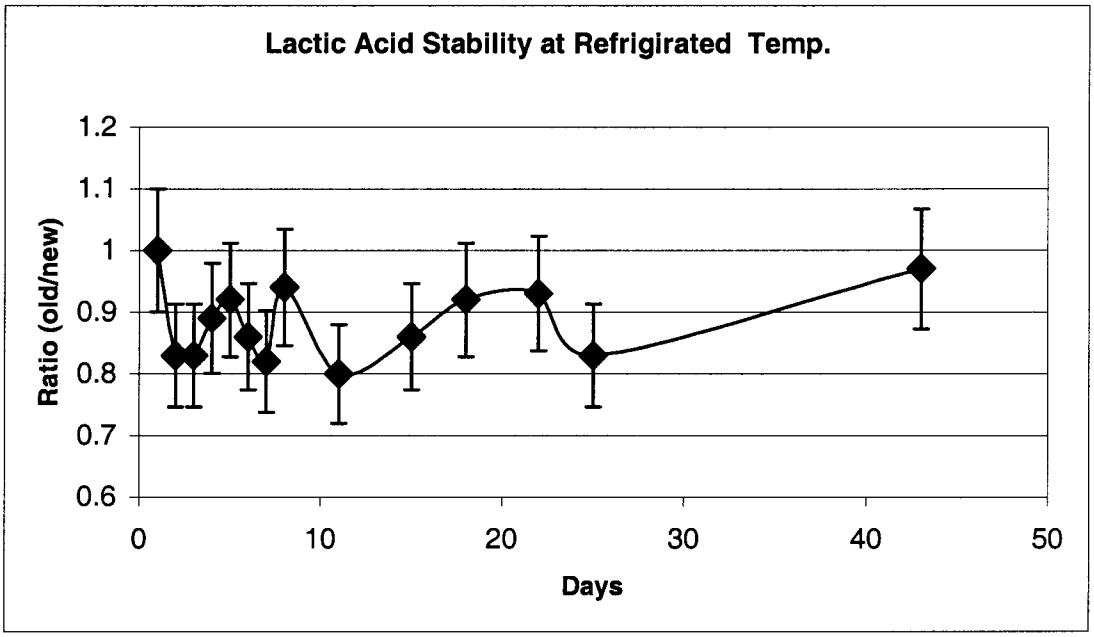


Figure 14. Stability of Lactic Acid at Refrigerated Temperature Using Std#5

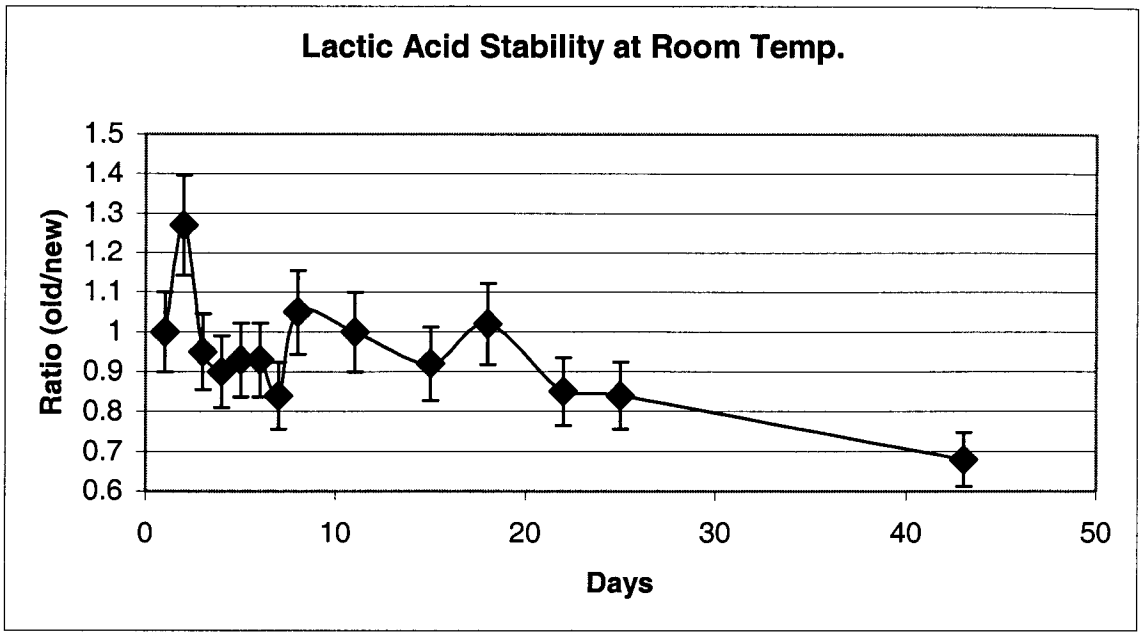


Figure 15. Stability of Lactic Acid at Room Temperature Using Std#5

I. Robustness

Robustness was the capability of the analysis to remain unaffected by small, but deliberate variations in the methods parameters. Studying the variation of the column temperature it was found that resolution was getting wore at 45 °C and higher. All acids peaks are shifted to the left and merge into other peaks (see Figure 16).

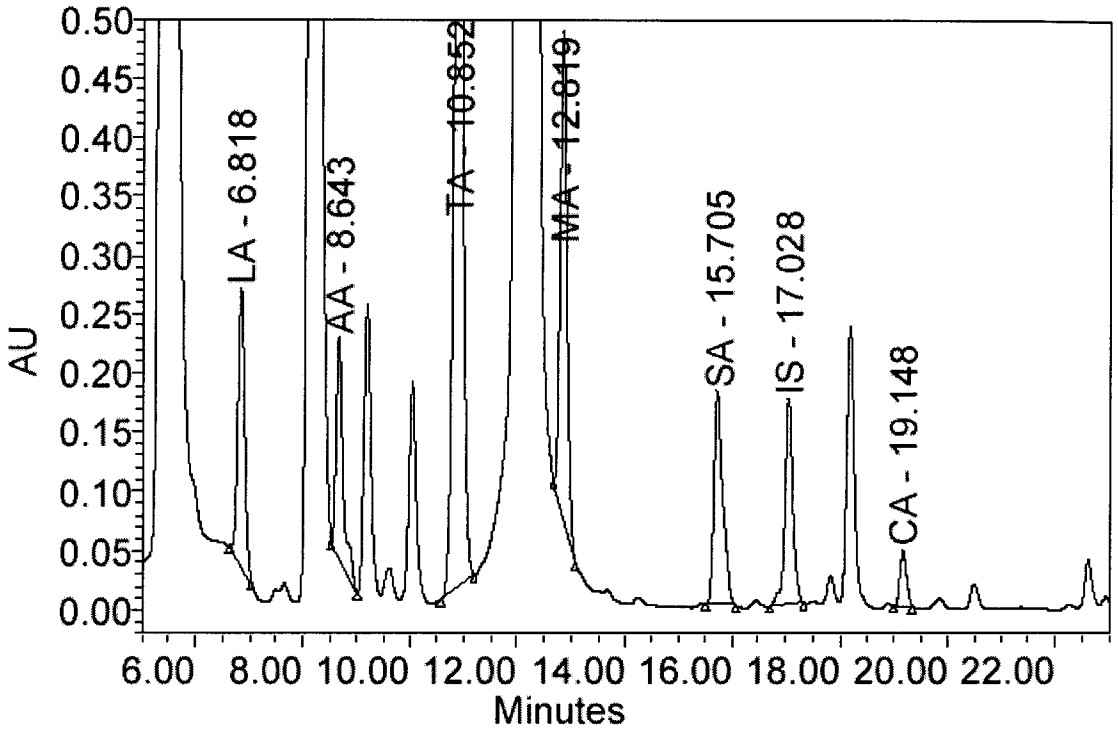


Figure 16. Chromatogram of Robustness Test at 45 °C Column Temperature

J. Sensitivity

To determine the sensitivity, the slopes of the calibration curves were analyzed by varying ethanol and sugar levels. The slopes obtained at various alcohol levels are shown in Table 24 and Figure 17. The slopes obtained at various sugar levels are shown in Table 25 and Figure 18. It was concluded that varying ethanol and sugar concentrations did not affect the acid.

Ethanol Conc.	LA slope	AA slope	TA slope	MA slope	SA slope	CA slope
0 %	$2.842x10^6$	$6.735x10^6$	$5.989x10^6$	$5.082x10^6$	$5.243x10^6$	$4.490x10^6$
5 %	$3.253x10^6$	$6.428x10^6$	$5.769x10^6$	$5.049x10^6$	$4.959x10^6$	$4.497x10^6$
10 %	$3.874x10^6$	$7.807x10^6$	$6.820x10^6$	$5.518x10^6$	$5.366x10^6$	$4.966x10^6$
12 %	$3.168x10^6$	$7.106x10^6$	$6.670x10^6$	$5.724x10^6$	$5.476x10^6$	$5.578x10^6$
15 %	$2.872x10^6$	$4.874x10^6$	$5.603x10^6$	$4.366x10^6$	$3.450x10^6$	$3.464x10^6$

Table 24. Linear Regression Equations at Varying Ethanol Concentrations

Sugar* conc.	LA slope	AA slope	TA slope	MA slope	SA slope	CA slope
0 g/L	$2.741x10^6$	$6.114x10^6$	$5.793x10^6$	$4.768x10^6$	$3.815x10^6$	$3.965x10^6$
50 g/L	$2.885x10^6$	$6.617x10^6$	$5.484x10^6$	$4.931x10^6$	$5.047x10^6$	$4.539x10^6$
100 g/L	$3.003x10^6$	$6.748x10^6$	5.50410^6	$4.930x10^6$	$5.047x10^6$	$3.951x10^6$
150 g/L	$2.917x10^6$	$6.956x10^6$	$5.445x10^6$	$4.835x10^6$	$1.995x10^6$	$1.323x10^6$
200 g/L	$3.033x10^6$	$6.729x10^6$	$5.457x10^6$	$4.814x10^6$	$5.929x10^6$	$4.286x10^6$
250 g/L	$3.197x10^6$	$5.972x10^6$	$5.566x10^6$	$4.792x10^6$	$4.069x10^6$	$3.904x10^6$

* (fructose: glucose) (1:1)

Table 25. Linear Regression Equations at Varying Sugar Concentrations

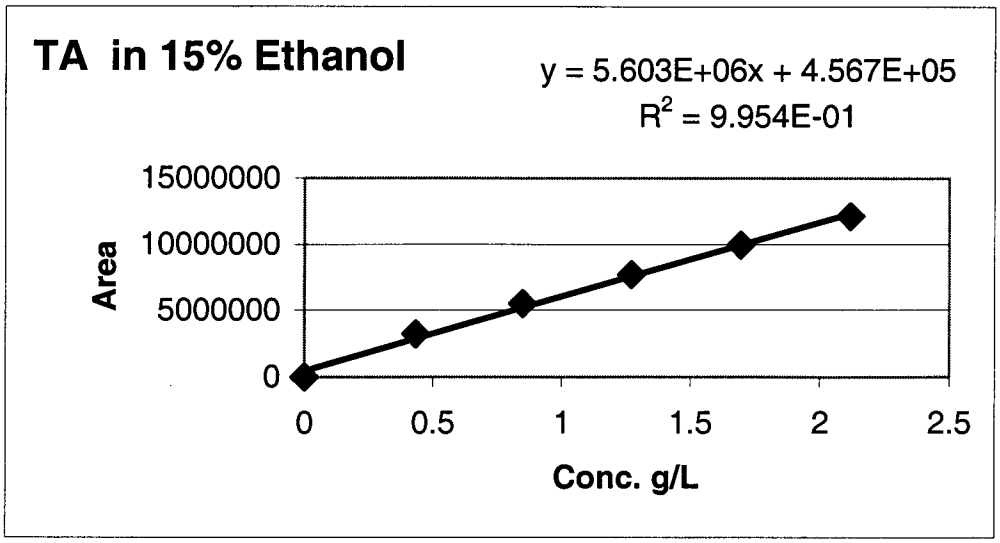


Figure 17. Calibration Curve of Tartaric Acid in 15% Ethanol

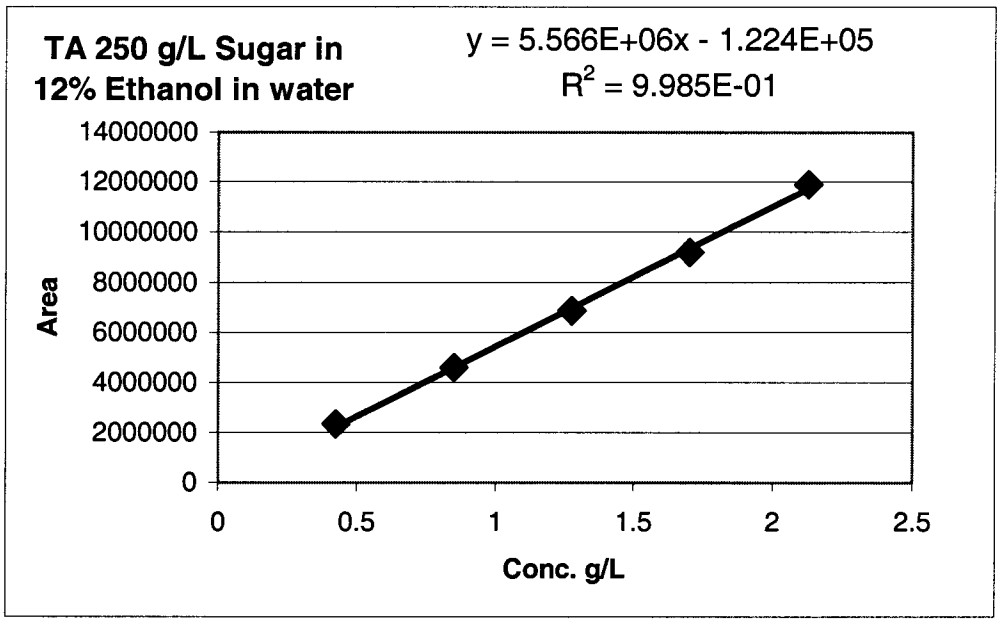


Figure 18. Calibration Curve of Tartaric Acid in 12% Ethanol / Water containing 250 g/L Sugar

K. Pinot Gris Wine Analysis

The acid concentrations determined in the Pinot Gris fermentation are shown in Table 26.

Fermentation Date	LA (g/L)	AA (g/L)	TA (g/L)	MA (g/L)	SA (g/L)	CA (g/L)
C: inoculated						
10.04.01	0.6030	1.332	4.343	3.500	0.8159	0.2435
10.08.01	0.6790	0.6298	4.568	4.069	1.006	0.3162
10.12.01	0.4887	0.3711	4.209	3.934	0.9503	0.3173
10.16.01	0.5498	0.4682	3.612	3.937	0.9413	0.3220
11.05.01	0.3654	0.7272	4.469	4.658	1.574	0.3128
11.09.01	0.3212	0.6451	3.933	4.196	1.3821	0.3078
11.13.01	0.2329	0.5515	3.455	6.612	1.110	0.2350
S: spontaneous						
10.04.01	0.6782	1.369	4.281	3.832	0.8385	0.2564
10.08.01	0.6787	1.392	5.143	2.854	0.8916	0.4559
10.12.01	0.4722	0.6121	4.922	4.131	0.8306	0.2906
10.16.01	0.4538	0.6269	4.728	3.911	0.8748	0.3088
10.20.01	0.5901	0.5695	3.641	3.118	0.9456	0.3070
10.24.01	1.1793	0.6047	4.388	2.344	0.9640	0.2955
10.28.01	3.582	0.6520	3.528	0.066	1.037	0.2030
11.01.01	4.060	0.7730	3.869	0.000	1.050	0.0260
11.05.01	3.812	0.9000	3.423	0.000	0.912	0.0000
11.09.01	4.027	1.0140	3.542	0.000	1.069	0.0000
11.13.01	4.123	0.9691	3.334	0.000	1.002	0.0000
11.16.01	4.205	1.013	3.308	0.000	1.007	0.0000

Table 26. Acid Concentrations in Pinot Gris During Fermentation

The spontaneous fermentations did go through malolactic fermentation but the inoculated fermentations did not. The lactic acid concentration was low and the malic acid concentration was high at the beginning of the spontaneous fermentation. They were high and low, respectively, at the end due to the conversion of malic to lactic acid.

Evidence of the malolactic fermentation is shown in, Figures 19 and 20, depicting the analysis in the early (Day 6) and late (Day 38) of fermentation.

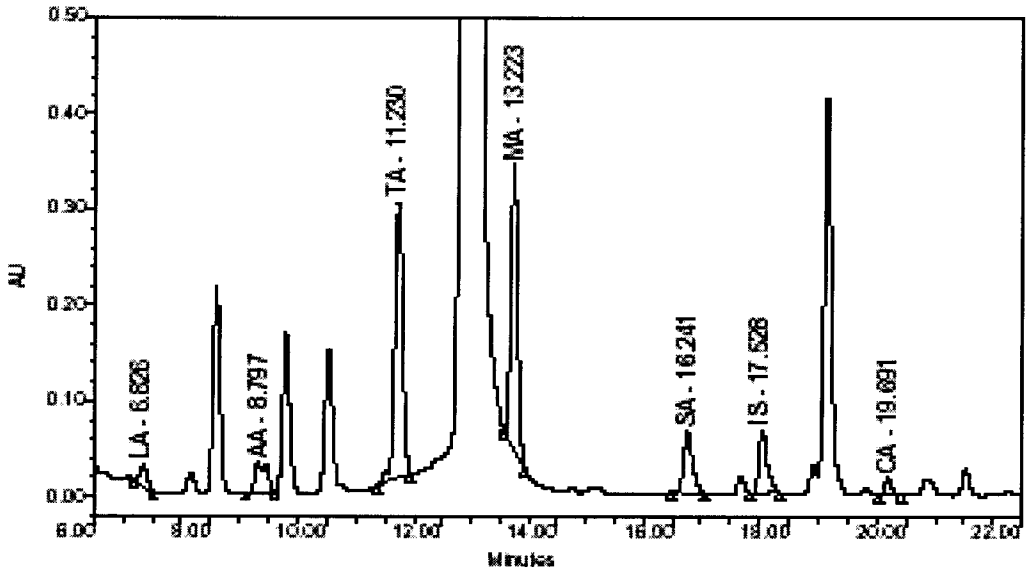


Figure 19. Spontaneous Fermentation Day 6

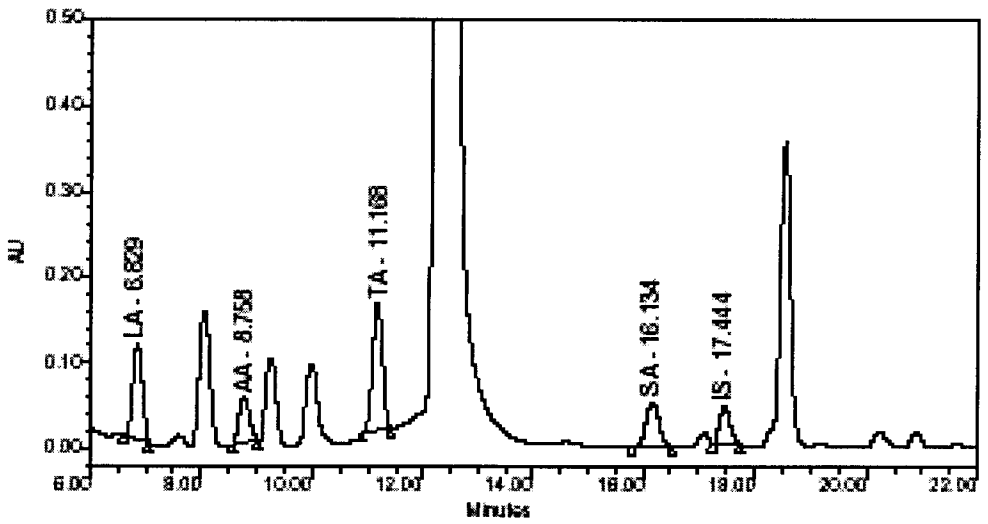


Figure 20. Spontaneous Fermentation Day 38

CHAPTER SEVEN

CONCLUSION

The method validated in this project can be used to analyze lactic, acetic, tartaric, malic, succinic, and citric acid. The original method from Caccamo et al. was modified and validated.

The method is specific for analyzing the organic acids as phenacyl esters at 254 nm. Most methods used today analyze non-derivatized acids and use detection at 210 nm, which can cause interference from the wine components. It is necessary to dilute the wine samples 1 to 10 to achieve the highest acid concentrations.

The range of an analytical procedure is the interval between the upper and lower concentrations of an analyte in the sample for which linearity, accuracy and precision are valid. The lowest acid concentrations that are quantifiable are: lactic acid 0.1700g/L, acetic acid 0.1006g/L, tartaric acid 0.0427g/L, malic acid 0.0528g/L, succinic acid 0.0733g/L and for citric acid 0.0864g/L. The highest concentrations up to which the range is linear are: lactic acid 0.2446g/L, acetic acid 0.4215g/L, tartaric acid 0.5216g/L, malic acid 1.443g/L, succinic acid 0.3097g/L and citric acid 0.1185g/L.

Taking the sample dilution into account, the highest quantifiable concentrations in wine are: lactic acid 2.4465g/l, acetic acid 4.2110g/L, tartaric acid 5.216g/L, malic acid 14.43g/L, succinic acid 3.0973g/L and citric acid 1.1828 g/L.

The recovery study indicates that with this method recovery was found to be 95.3-111.4 % or better. The measured acid concentrations in Chardonnay and Pinot Gris wine are consistent with the published data [20] from Caccamo at al. The reproducibility of

the analytical data indicates that the quantitation of the organic acids using this method can be performed with confidence.

Other validation parameters such as linearity, precision, specificity, stability, robustness and sensitivity are discussed in Chapter 6. Overall this method was found to be robust and repeatable in the reported range.

The modified and validated method can be used during fermentation to monitor acid concentrations.

CHAPTER EIGHT

FUTURE WORK

This project has not studied the sample collection parameters. It is assumed that by collecting samples from the center of the fermentation vessel the acid concentrations are distributed evenly.

Another suggestion for further research is the use of different derivatizing agents, if lower acid concentrations need to be analyzed.

Comparison with current enzymatic and HPLC methods can be also studied.

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