DETERMINATION AND REMOVAL OF GLUCONIC ACID IN REDUCED ALCOHOL WINE AND HIGH ACID GRAPE JUICE

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A rapid high performance liquid chromatography (HPLC) method incorporating the use of an Aminex HPX-87H organic acid column was developed for the separation, identification and quantitative analysis of gluconic acid and other major acids in grape juice and wine. This method was used to investigate the effectiveness of deacidification treatments for removing gluconic acid from high acid grape juice and reduced alcohol wine produced by using a glucose oxidase-catalase (GOD/CAT) juice treatment.

Müller Thurgau juice was subjected to a GOD/CAT treatment as a means of reducing the concentration of glucose in the grape juice before fermentation to produce a reduced alcohol wine. The enzyme is an aerobic dehydrogenase which catalyses the oxidation of glucose to gluconic acid. The juice was found to contain ~75g/L gluconic acid, and when a portion of this was fermented to dryness and cold stabilised, the gluconic acid had reduced in concentration to ~45g/L and the reduced alcohol wine contained 8.3% alcohol (v/v). The

corresponding increase in acidity in both the reduced sugar juice and reduced alcohol wine had to be neutralised to present a palatable product.

Three deacidification treatments were investigated in reduced alcohol wines and high acid grape juice and these included: neutralisation with $CaCO_3$ (calcium carbonate), seeding with gluconate salts and $CaCO_3$, and anion exchange with an Amberlite IRA-93 resin. Both the neutralisation and seeding treatments produced disappointing results, with a significant but minimal decrease in gluconic, tartaric and malic acids. Even though the solutions analysed would have been saturated with potassium tartrate and gluconate, there was obviously a stable equilibrium in force, and the addition of seed crystals and chilling to ~2^0C had no effect. The anion exchange treatment showed considerable promise though, with reduction in all the three major organic acids. A new technique was investigated, which involved charging the resin with tartaric acid, and then passing the wine/juice through. The weaker gluconic acid in solution exchanged with the stronger tartaric acid. This technique has the potential to selectively remove gluconic acid. A hindrance to this technique is the removal of colour from the wine/juice due to the resin matrix. The effect of resins on colour and flavour warrants further investigation.

KEYWORDS: High performance liquid chromatography (HPLC); gluconic acid; glucose oxidase-catalase; deacidification; reduced alcohol wine; neutralisation; seeding; anion exchange.

Wine is a chemical symphony composed of ethyl alcohol,
several other alcohols, sugars, other carbohydrates, polyphenols,
aldehydes, ketones, enzymes, pigments, at least half a dozen vitamins,
15 to 20 minerals, more than 22 organic acids, and other
grace notes that have not yet been identified.

Maynard Amerine, 1911-

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

There are two distinct climate types in which grapes are grown for winemaking. Wines from grapes grown in warm climates such as Australia, South Africa and California are generally "bland", "soft", higher in alcohol, and low in acidity. Cool climate regions e.g., New Zealand, often produce wines that are fruitier, lower in alcohol, higher in acidity, and more delicate and subtle in aroma and flavour. However this environment is marginal for attaining adequate ripeness in grapes and they often do not reach maturity before they are harvested. The sugar content of the grapes may be too low and/or the acidity may be too high.

The practice of adding sugar to accommodate this natural deficiency is used widely in cool viticultural areas, even if it is not altogether desirable (Amerinė and Ough, 1980). The addition of sugar has no adverse effects on the wine quality and is easily incorporated into winery operations. Reducing excess acidity in winemaking is a different matter. There are several alternative methods available to the winemaker for reducing acidity. Some require considerable skill and calculation, and some can have significant secondary effects on wine quality.

At veraison, malic and tartaric acids have been accumulated in the berry. During the phase of berry ripening as sugar is accumulating the malic acid concentration decreases due to dilution as a result of water uptake and conversion of acids to salts.

With tartaric acid, the concentration will change due to dilution but tartrate does not normally get metabolised within the berry. At veraison tartaric acid exists in the form of H_2T and its usual form HT^-. Boulton (1984), proposed that the protons of the organic acids (tartaric acid) in the berry cells are exchanged for potassium ions. Effectively it increases the conversion of tartaric acid to the bitartrate ion HT^- or KHT. This reaction can continue where ditartrate ions are formed T^{2-} or K_2T.

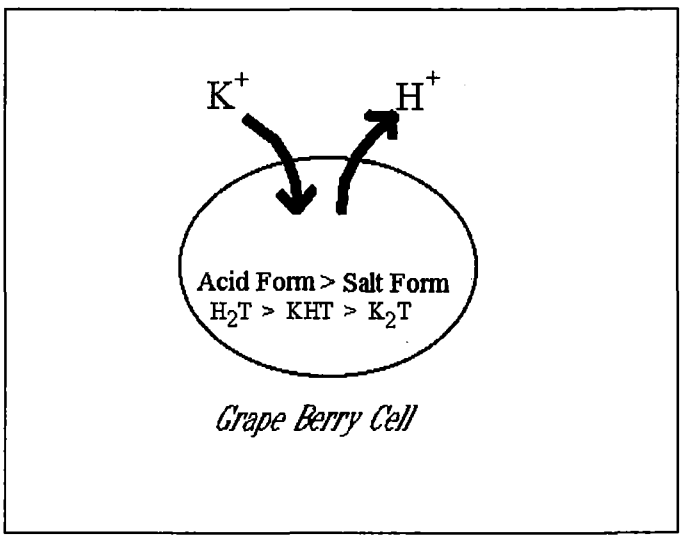


Figure 1.1. Exchange of protons for potassium ions in grape berry cell.

Therefore at maturity we have different combinations of the forms of tartaric acid, different amounts of malic acid and different amounts of potassium. The varying amounts of the forms of tartrate with the undissociated malic acid that enter the juice at crushing determine the juice pH and titratable acidity level (Iland, 1987).

The acidity measures most commonly used are the titratable acidity and pH. These are both dependent variables that basically tells the winemaker little about the independent variables of tartaric acid, malic acid and potassium concentrations. A simple measure of the organic

acid pool can be obtained by noting the quantity of base required to neutralise the must by one pH unit. The number of hydroxide ions (moles) required to raise the pH by one unit in a litre of must is defined as the buffer capacity. It is the numerical indication of the resistance of an acid mixture to a change of pH. More importantly it can be used to estimate the sum of tartaric and malic acid concentrations by solving the acidity relationships (Berg and Keefer, 1958) together with the exact buffer equations for tartaric and malic acid.

The third and major acid that will be examined in this thesis is gluconic acid. This acid is created from the conversion of glucose to gluconic acid by the glucose oxidase-catalase enzyme. The removal of glucose from the grape juice means that less sugar is available to be converted to alcohol by fermentation with *saccharomyces cerevisiae* yeast. With less alcohol being produced the resulting product can be termed as reduced alcohol wine.

There are several methods of acid reduction with the aim of improving wine quality through achieving better acid balance. These include neutralisation of wines with K_2CO_3 and $CaCO_3$ (McKinnon *et al.*, 1992; Nagel, Johnson and Carter, 1975; Munyon and Nagel, 1977); calcium double salt deacidification of musts (Steele and Kunkee, 1978; Abguéguen and Boulton, 1993; Clark, Fugelsang and Gump, 1988); malic acid fermentation to ethanol and CO_2 with *Schizosaccharomyces pombe* in musts (Gallander, 1977; Magyar and Panyik, 1989); *Leuconostocoenos* ML-34 in wines (reds) (Munyon and Nagel, 1977), and the use of anion exchange resins (Rankine, 1965; Bonorden, Nagel and Powers, 1986; Zubeckis, 1957).

1.2 ACIDITY

1.2.1 QUALITY ATTRIBUTES

Grape quality may be determined by parameters such as sugar concentration, pH, titratable acidity (TA), terpene concentration and by the intensity of varietal aroma and flavour in the juice. The final balance of these parameters determines overall grape quality. To produce good wine, the sugar, acid, and tannin content of the grapes should be properly balanced (Amerine, 1980).

However no component of the wine has such extensive and important functions as the acidity. The most important function is the tart taste imparted by the acids. Additionally, the acidity has an important influence on the colour, clarity and stability of the wine. The acids in wine have important secondary effects on quality, e.g., functioning as substrates for microbial metabolism and increase sensory complexity of wine. However the most readily apparent aspect of the acidity is its effect on taste. If too little acid is present in the juice, the resultant wine will cause it to taste sour rather than pleasantly tart (Rankine, 1991).

Wine tartness is influenced by the types and amounts of the various acids present, the buffering capacity of the wine, and the sugar and other components present. The reduction of excess acidity to a level providing appropriate tartness does not cause problems with wine colour or stability provided the method employed does not alter pH excessively. A dealcoholised wine is much more tart tasting than the same wine with its alcohol (Amerine and Ough, 1980). At low concentrations ethanol has only a slight odour, suggestion of sweetness and it moderates the taste of acids. Therefore with the production of reduced

alcohol wine by the glucose oxidase-catalase (GOD/CAT) enzyme process (refer to Chapter 2), not only is there an increase in tartness through the production of gluconic acid, but also there is an added effect through the reduction in alcohol.

1.2.2 CHEMISTRY

The acid taste is due to the hydrogen ion concentration and undissociated acid, hence there is no direct relationship between pH and acid taste (Amerine, Roessler and Ough, 1965). When discussing the interpretation of acidity parameters in grape juice it is necessary to consider pH, titratable acidity (TA), and total acidity:

- pH - relates to the concentration of free hydrogen ions in the solution, i.e., $[H^+]$ in

  $$H_2T \Leftrightarrow H^+ + HT^-.$$

- TA - gives the total available titratable hydrogen in solution,

  $$\mathbf{H_2T \Leftrightarrow H^+ + HT^-}.$$

- Total acidity - gives the total available amounts of organic acid anions in solution.

The pH of the grape juice or wine has been determined by the amounts of tartaric acid, bitartrate, and malic acid. The titratable acidity has only been determined by the amount of tartaric acid, bitartrate and malic acid because the di-potassium tartrate does not have any titratable protons (Iland, 1987).

The acid taste of must and wine is related to both the free hydrogen ion concentration (pH) and to the undissociated acid components (largely determined by the titratable acidity). Little is known concerning the optimum relationship between pH and total acidity in regard to

sensory quality of wine. Nagel and McElvain (1977), attempted to determine this relationship for table wines based on sensory scores of wines where pH and total acidity data were known. They found in white table wines with pH values in the range 3.05-3.20, 3.20-3.30, and 3.30-3.50 that optimum range of total acidities were 0.60-0.65%, 0.60-0.85%, and 0.85% respectively.

A net loss in titratable acidity is usually experienced when comparing the grape must to the finished wine. Kluba and Beelman (1975) reports that the greatest loss occurs with these varieties highest in initial titratable acidity and that tartrates decreased more than malates during vinification, due to the precipitation of potassium bitartrate. As the alcohol content increases during fermentation, the solubility of potassium bitartrate decreases, and a portion is precipitated from the wine. Since crystallisation of the excess potassium bitartrate is not immediate, wineries commonly employ low temperatures to accelerate the precipitation. Tartrate holding capacity of a particular wine depends upon its pH, alcohol, potassium, and tartrate contents (Berg and Keefer, 1958), among other things.

1.2.3 BENEFITS

Low pH (high acidity) has the following important advantages in processing and increasing quality:

- Increases the antimicrobial and antioxidant properties of SO_2.
- Inhibits microbial spoilage.
- encourages clarification of juices and wines.
- Generally accentuates the fruitiness and balance of wines.

Very little research has been done on the sensory attributes of the acid components in juice and wine. Amerine, Roessler and Ough (1965) employed a trained panel to rank the sourness of the different acids found in wine at the same total acidity and found malic>tartaric>citric>lactic. No research has been done on the sensory attributes of gluconic acid, although Gump and Kupina (1979), reported that gluconic acid and lactones had a sweet acid taste. This would be an attractive attribute, as this would compliment the relatively harsh tartaric and malic acids in the reduced alcohol wine produced from the GOD/CAT enzyme process (refer Chapter 2).

Organic acids play an important role in determining wine quality, with each acid contributing its own characteristic taste. Malic acid has a sour taste reminiscent of unripe apples or gooseberries (Fowles, 1992), while gluconic acid has a relatively sweet acid taste (Gump and Kupina, 1979). In addition, organic acids and their salts act as buffers, thus ensuring that the wine maintains a relatively low pH, approximately 3.0 to 3.7; this helps to protect the wine against bacterial attack and subsequent spoilage. These acids help to conserve wine colour and influence esterification with a consequent change to the bouquet.

1.3 OBJECTIVES

The main objectives of this thesis were therefore to develop methods for the determination and reduction of gluconic acid found in grape juice and wine after the GOD/CAT enzyme treatment. Three main deacidification treatments were investigated (Figure 1.2):

Neutralisation with $CaCO_3$ or Na_2CO_3.
Seeding with sodium gluconate or calcium gluconate, with $CaCO_3$.
Anion exchanger.

The main body of this thesis has been written in three parts. Chapter three investigates the development of an HPLC technique used for the identification and quantitation of gluconic acid, and the optimisation of this method. Chapter four investigates application of the chemical deacidification techniques that are currently practiced in the wine industry. Chapter five investigates the use of anion exchange, a deacidification technique that is not widely employed, but one which the author feels has merit in the selective removal of gluconic acid.

Each of these chapters have been designed to include a review of literature and conclusions specific to that chapter. An overall conclusion is presented (Chapter 6) which includes discussion on the suitability of the HPLC and deacidification methods for the determination and reduction of gluconic acid in juice and wine, and suggestions for further research.

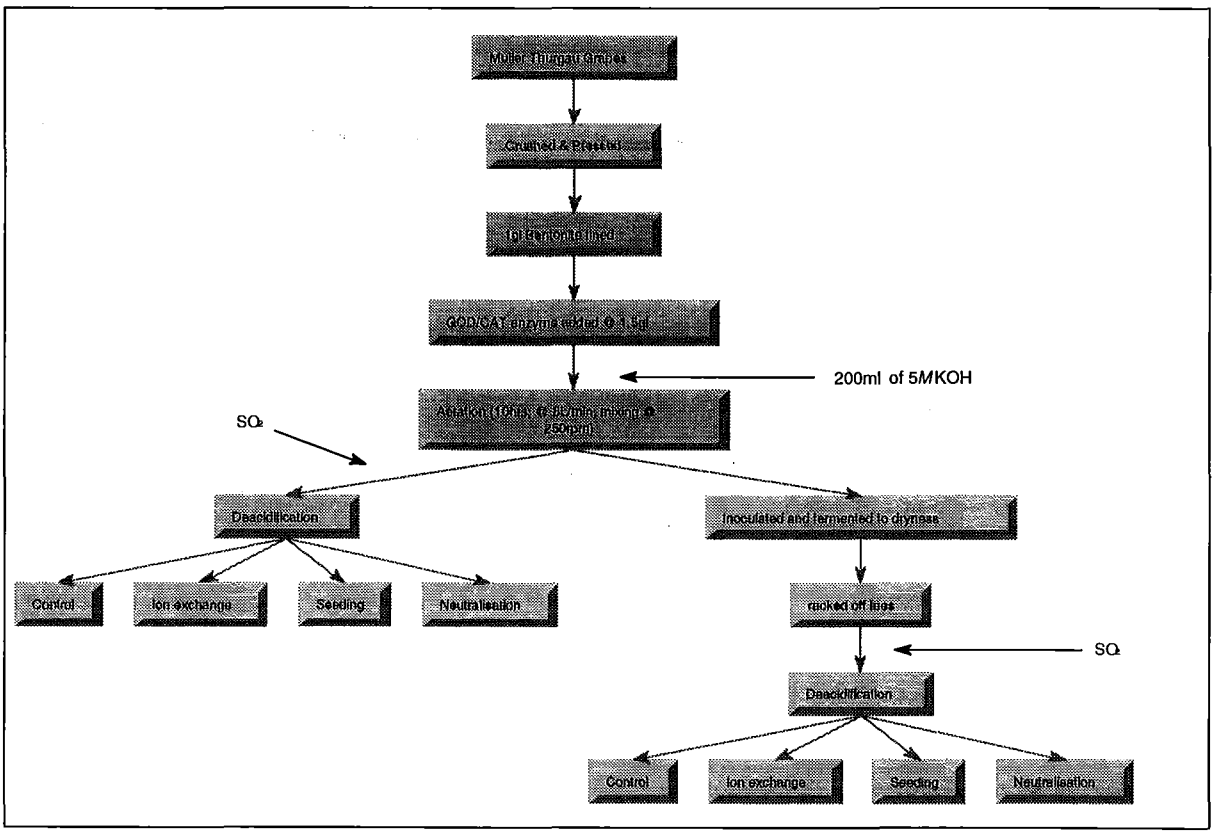


Figure 1.2. Flow diagram of the various deacidification treatments being applied to reduced alcohol wine and high acid juice.

CHAPTER TWO

GLUCONIC ACID

2.1 REVIEW OF LITERATURE

During normal vinification, gluconic acid is present in trace amounts in the grape juice and resulting wine. These amounts are so small that there is very little research done on this organic acid in relation to wine production.

Gump and Kupina (1979), and McLoskey (1974) have both determined the presence of gluconic acid in wines, produced by *Botrytis cinerea* mould. Gluconic acid is the oxidised product (aldonic acid) of glucose. The aldehyde group of glucose is oxidised to the carboxyl group by the action of the enzyme, glucose oxidase.

McLoskey (1974) reported levels of gluconic acid in red and white California table wines. The analysis was performed by an enzymatic procedure involving a coupled reaction with gluconate kinase (reaction 1) and 6-phosphogluconate dehydrogenase (reaction 2). The assay mechanism was based on the following reactions:

1) D-gluconate + ATP $\Leftrightarrow$ gluconate-6-phosphate + ADP

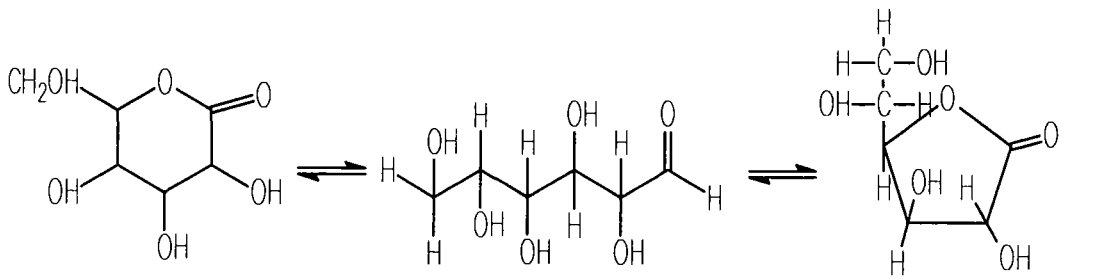
2) gluconate-6-phosphate + $NADP^+$ $\Leftrightarrow$ ribulose-5-phosphate + NADPH + H^+ + CO_2

Another method of quantitatively determining gluconic acid in wine has been reported by Gump and Kupina (1979). Following treatment with activated charcoal, filtration, acids were

absorbed on an ionite column. The acids were then eluted with formic acid and estimated colourimetrically by periodic acid oxidation in a fuchsin-sulfurous acid solution.

Blake, Clarke and Richards (1984) have also analysed gluconic acid in biological fluids, using high performance liquid chromatography (HPLC) on a column packed with Aminex A-28 anion exchange resin, using ammonium formate as the eluent. Rajakylä (1981) reported a method used for the determination of gluconic acid and sodium gluconate as well as other acids formed in biochemical or catalytic oxidations of glucose. The acids were separated on a column of cation exchange resin and eluted with dilute sulphuric acid. The effluent was monitored by an ultraviolet detector at 210nm.

The equilibrium between the two lactones and the parent gluconic acid has been studied by Sawyer (1959) and it was found that in the pH range of 3 to 5, the following equilibrium existed between the two lactones and gluconic acid.



D-Glucono-δ-lactone D-Gluconic acid D-Glucono-γ-lactone

This was also confirmed by McCloskey (1974), who used enzymatic techniques to determine gluconic acid content in *botrytis* infected wines.

Other time consuming methods that have been used to determine gluconic acid include: gas and paper chromatography, spectrophotometric and enzymatic analysis (Blake, Clarke and Richards, 1984). In comparison high performance liquid chromatography (HPLC) is an analytical technique that is ideally suited for this type of analysis.

2.2 GLUCOSE OXIDASE-CATALASE

2.2.1 INTRODUCTION

Current research in our laboratory (G.Pickering, PhD candidate) has demonstrated that commercially available glucose oxidase/catalase enzyme system can be used to remove glucose from fruit juice which can then be fermented to produce reduced alcohol wine. This research is an extension of earlier work by Villettez, (1986); Ough, (1975) and Heresztyn, (1987).

The enzyme system, Novozym 358 (Novo Nordisk Ferment Ltd, Switzerland) containing both glucose oxidase and catalase is obtained by a controlled fermentation of *Aspergillus niger var.* The glucose oxidase component catalyses the oxidation of D-glucose to D-gluconic acid with molecular oxygen being reduced to hydrogen peroxide. The catalase component catalyzes the reaction in which one molecule of hydrogen peroxide acts as a donor and a second molecule acts as an acceptor of hydrogen atoms yielding the products of water and molecular oxygen (Figure 2.1).

This appears to be a promising new technology for the production of

1) reduced alcohol wine
2) production of modified juice products in their own right. (e.g. reduced sugar/reduced calorie juices, juices with modified sweetness/acidity balance).

The demand for low and reduced alcohol beverages, including wine, has risen dramatically in recent years. However, traditional methods for producing low alcohol wines have been expensive and the resulting quality has generally not been satisfactory resulting in poor consumer acceptance. Concerning juice products, many consumers regard grape juice as too sweet (typically 20% sugar) and too high in calories. To a lesser extent this can also be true for some apple juices.

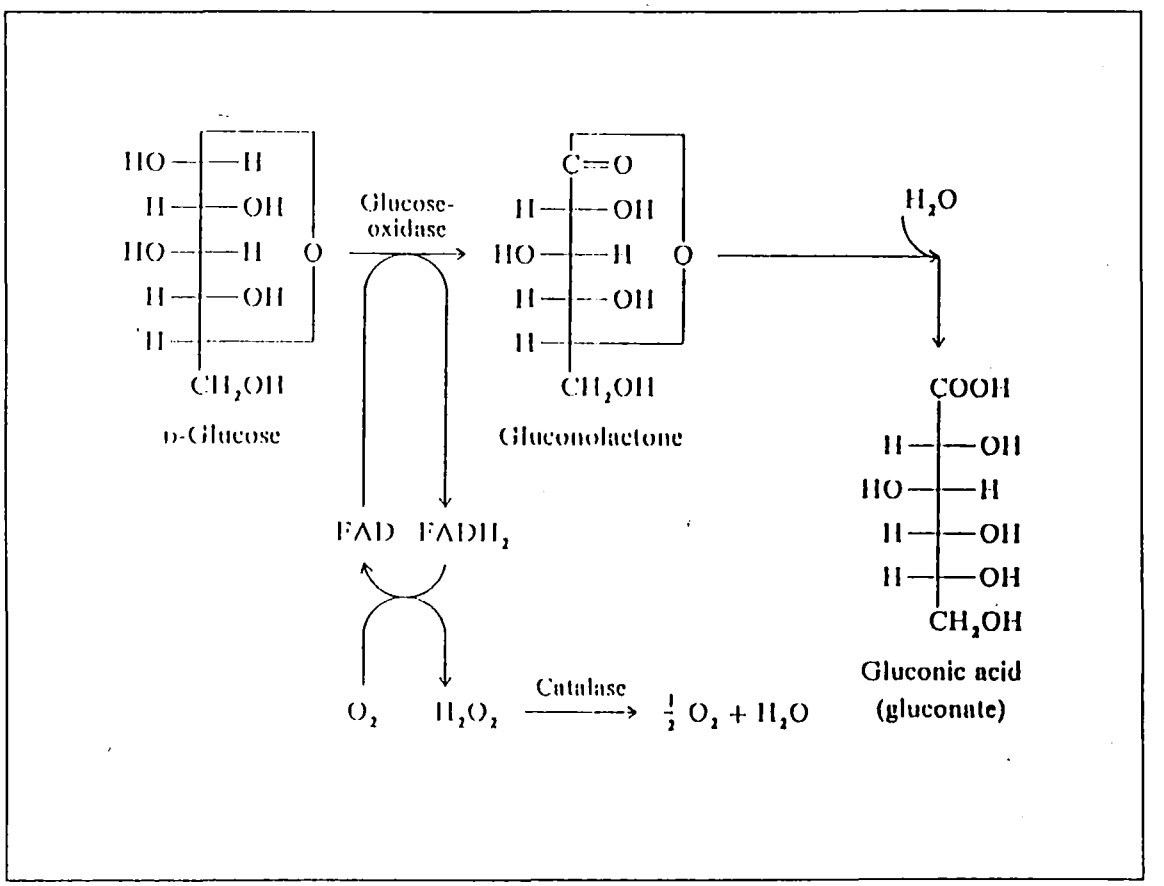


Figure 2.1. Conversion of glucose to gluconic acid

The sugar content in grape juice contains approximately 50% glucose and 50% fructose. Theoretically this enzymatic treatment is able to convert the glucose fraction to gluconic acid and thus leaving only half the sugar available for fermentation to alcohol. This corresponds to the highest potential alcohol reduction of 50%, or, approximately 6% (v/v).

From the point of view of flavour, gluconic acid has an acid-sweet taste, the wines gain in body and are less "hard" than the wines acidified with tartaric acid (Villettaz, 1986). Gluconic acid as well as its salts and lactones are mild, non-corrosive, non-toxic organic compounds. They are physiologically compatible and can therefore be used in foodstuffs without risk (Rajakylä, 1981).

However, a potential limitation of technologies using glucose oxidase is the production of gluconic acid itself, the net enzyme reaction being:

$$2 \text{ glucose} + O_2 \rightarrow 2 \text{ gluconic acid}$$

which can result in acidity imbalance in the juice or fermented juice product (wine). As a rough estimate one can say that 2g/L of gluconic acid will increase the total acidity by 1g/L (tartaric acid) (Villettaz, 1986). Gluconic acid is not metabolised by the yeasts during the alcohol fermentation. According to the winemakers, there was no noticeable reduction in quality as a result of the aeration of the juice during enzyme treatment (Villettaz, 1986).

The glucose oxidase/catalase (GOD/CAT) system has been researched involving the removal of oxygen of some table wines bottled with residual sugar (McLeod and Ough, 1970). Other

suggested applications of this enzyme system include the removal of glucose from solutions of mixed saccharides, O_2 scavenger to stabilise foods, colorimetric or UV assay of glucose in biological fluids, and as an antigen or antibody for use in ELISA procedures.

2.2.2 PREPARATION OF HIGH ACID JUICE AND REDUCED ALCOHOL WINE

For the investigation of methods for the determination and reduction of gluconic acid in juices and wines, it was necessary to produce a GOD/CAT treated juice and wine.

2.2.3 MATERIALS AND METHODS

The method developed by Villetaz, (1986) and modified in our laboratory by Pickering, (1993) was used. The enzyme used was "Novozym 358", batch OKN1003, a product of Novo Nordisk Ferment Ltd, Dittingen, Switzerland. It contained glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) prepared from the same original natural product. The product is available as a standardised and stabilised liquid with an activity of 2000 GOX/ml, where 1 Glucose Oxidase unit is the amount of enzyme which, at standard conditions (25^0C and pH 5.1), catalyses the formation of 1 µmole H_2O_2.

Bentonite fined (1g/L) Müller Thurgau (1994) was obtained from Geisen Wines Ltd, New Zealand. The composition of the juice was 18^0Brix, pH 3.15, titratable acidity 6.94 g/L, and free SO_2 of 40 mg/L.

A New Brunswick Fermentor (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey) was used for the GOD/CAT treatment of the juice. The enzyme treatments were

carried out in 4 x 10 L quantities of grape juice with continuous aeration. The pH and titratable of the juice were measured as a function of time. Batch parameters include:

25^0C water bath
8 L/min aeration
250 rpm (agitation)
1.5 g/L GOD/CAT enzyme

The enzyme producers recommend using 1g/L enzyme dosage, but 1.5 g/L was used in this case to compensate for enzyme degradation due to storage. Pickering, Heatherbell and Barnes (1993) found that from 0-10 hours there was conversion of glucose to gluconic acid, but after 10 hours of aeration, no significant conversion occurred. The juice pH was adjusted to pH 4.3 by addition of 100ml of 5M KOH. Another 100ml was added after one hour to help regulate the pH. Aeration of the 40 L was stopped to terminate the glucose oxidase reaction after 10 hours. 20 L of the treated juice was frozen to be used for deacidification trials later on. The other 20 L was inoculated with 200 ppm *saccharomyces cerevisiae* yeast (Fermivin SF, Gist-Brocades, France) and fermented at 12^0C. Upon completion of the primary fermentation, the wine had a pH of 3.21 and TA of 19.34 g/L. The alcohol content on completion of fermentation was 8.3% (v/v) with the residual sugar, 5.0 g/L. Both juice and wine stock solutions were frozen and held at -10^0C until analysed.

2.2.4 RESULTS AND DISCUSSION

The pH and TA of the juice was measured as a function of time (Figure 2.2). The enzyme activity decreased markedly after 8 hours of the reaction process, by which time approximately 75g/L of gluconic acid had been produced with an equivalent loss in glucose

concentration. The juice browned within the first two hours of enzyme addition, and it was assumed that the glucose oxidase had sufficient O_2 present for the oxidation of glucose to gluconic acid with the simultaneous formation of H_2O_2. During subsequent fermentation of the high acid juice a brown precipitate was noticed which was probably oxidised phenolic material settling, and consequently the resulting wines did not appear oxidised, but instead had a straw yellow colour. However for the purpose of producing reduced alcohol wines, the excess production of gluconic acid needs to be removed to minimise the negative effects on the quality of the wine.

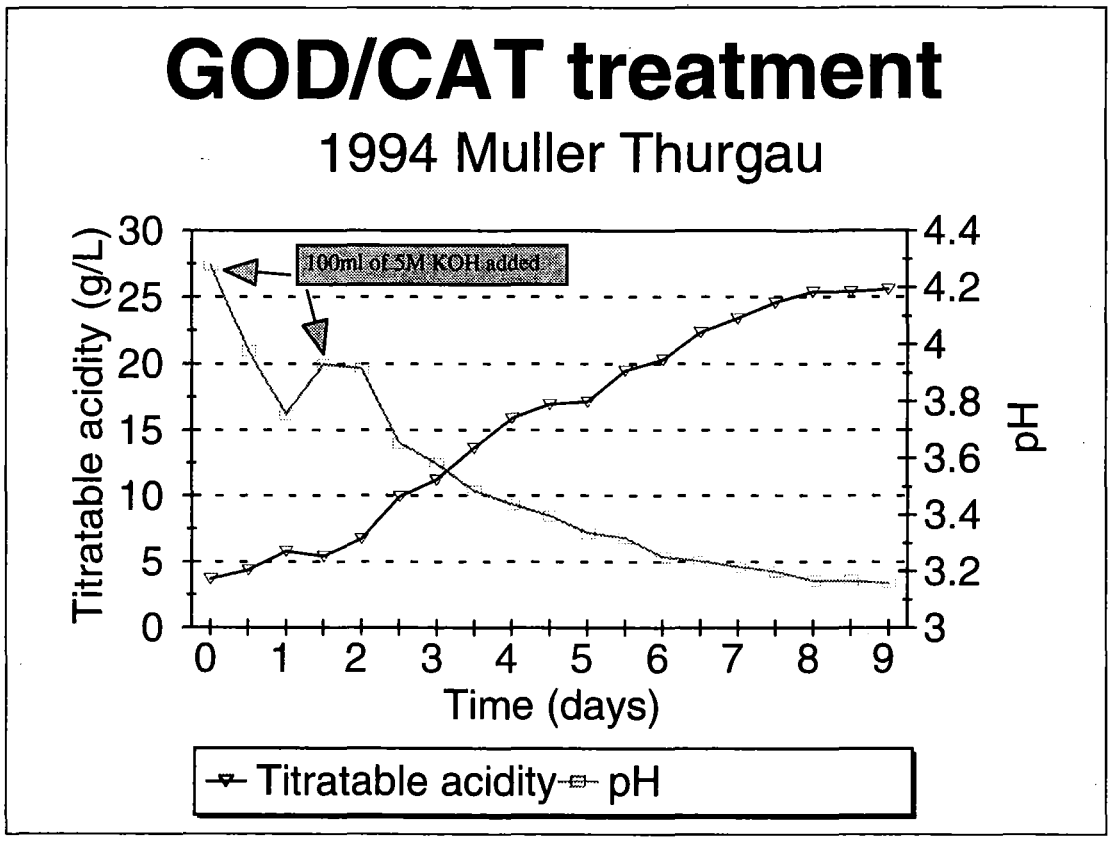


Figure 2.2. pH and titratable acidity of Müller Thurgau juice undergoing enzymatic treatment with glucose oxidase.

2.2.5 CONCLUSIONS

Theoretically ~96.5 g/L of glucose was available in the grape juice to be converted into gluconic acid. Of this only 74.7 g/L was actually converted, which equates to a 77% efficiency rate. The loss of enzymatic activity can be attributed to a number of factors that will be addressed by Pickering, Heatherbell and Barnes (1993). In particular the affect of SO_2 and pH need to be further researched, as these are two attributes that are present in grape juice that can inhibit the enzymes ability to convert glucose to gluconic acid.

CHAPTER THREE

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 REVIEW OF LITERATURE

3.1.1 INTRODUCTION

The HPLC technique that we will use to identify and quantitate gluconic acid in high acid grape juice and reduced alcohol wines, incorporates three of the basic mechanisms; partition, adsorption and exclusion chromatography. HPLC has recently become a regular and acceptable form of wine analysis and some of the bigger producers have included this analytical method as a standard laboratory tool. The technique has the advantage of being able to analyse for single or simultaneously for multiple wine components using only a minimal amount of sample and analysis time.

High performance liquid chromatography (HPLC) is unquestionably the fastest growing of all the analytical separation techniques. This is attributable to the sensitivity of the method, the ready adaptability to accurate quantitative determinations, its suitability for separating nonvolatile species or volatile species. There are four basic types of column chromatography where the mobile phase is a liquid. The four include:

- partition chromatography
- adsorption chromatography
- ion-exchange chromatography
- exclusion chromatography

Each of these types work better in certain applications. That is, for solutes having molecular weights > 10 000, exclusion chromatography is utilised. For lower molecular weight ionic species, ion-exchange chromatography is widely applied. Small polar but nonionic species are best handled by partition chromatography. Adsorption chromatography is often chosen for separating nonpolar species, and compound classes such as aliphatic hydrocarbons from aliphatic alcohols (Skoog, 1985; Lindsay, 1992).

Wrolstad and Spanos (1987) utilised HPLC for the determination of red raspberry anthocyanin pigments, nonvolatile acids, and sugars. He also applied this technique for the analysis of sugars and nonvolatile acids in blackberries (Wrolstad *et al.,* 1980). Sepúlveda and Kliewer (1986) used HPLC to monitor the effect of high temperature on grapevines with regards to the distribution of soluble solids. Takeda, Saunders and Saunders (1983) used HPLC to measure the sugar and acid change in Muscadine grapes during postharvest storage. The lyophilised samples were suspended in distilled water and heated to 60^0C to help dissolution, filtered through a 0.45μm membrane and eluted with 0.01*N* phosphoric acid and analysed using a UV detector at 210nm.

Other researchers have used HPLC systems to isolate and identify organic acids and their lactones (Hicks, Lim and Haas, 1985; Pecina *et al.,* 1984; Schwarzenbach, 1982; Palmer and List, 1973; Bennett and Bradey, 1984.) The standard HPLC method of analysis for wine acids involves using a polystyrene-divinylbenzene cation exchange resin (Aminex HPX-87H) with an acidic eluent for separation followed by short wavelength (210nm) UV and/or refractive index (RI) detection. There seems to be two different approaches regarding sample preparation. There are some researchers that use an anion exchange resin (e.g., Amberlite

IRA-93) to split the wine into acidic and neutral fractions. The neutral fraction contains sugars and ethanol, while the acid fraction; the wine acids (McCord, Trousdale and Ryu, 1984; Hunter, Visser and De Villiers, 1991; Sepúlveda and Kliewer, 1986; Wrolstad *et al.*, 1980, and Wrolstad and Spanos, 1987.) These authors believe that this enhances the resolution of the eluting acids and also conserves the analytical column.

However there are others that found that, except for filtration during sample preparation, they were able to analyse samples on HPLC columns without prior separation of compounds with different ionic character (Frayne, 1986; Schneider, Gerbi and Redoglia, 1987; Tusseau and Benoit, 1987; Takeda, Saunders and Saunders, 1983; Rajakylä, 1981; Gump and Kupina, 1979.) Separations were excellent and several hundred samples were run without deterioration of the column performance. Mentasti *et al.*, (1985) and Caccamo *et al.*, (1986) have both published an HPLC method for the identification, separation and determination of acids in wines and beverages. They incorporated derivatisation with phenacyl bromide and separation was accomplished on standard octadecylsilica columns using reversed phase chromatography.

3.1.2 ORGANIC ACID COLUMN

The usual column for sugar and acid analysis in wine products is the Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). Dilute sulphuric or phosphoric acid is used as the eluent, and filtering is the only sample preparation required. The Aminex organic acid column can also be used for rapid and non-destructive alcohol analysis. The column separates organic acids using initially ion exclusion and reversed phase mechanisms. When dilute sulphuric acid is used as the eluent, organic acids elute from the column in order of

increasing pKa. Partitioning also contributes to the separation of compounds not absorbed by the nonpolar column matrix (Skoog, 1985). The column separates neutral species, such as carbohydrates and alcohols, by reversed phase partitioning. The eluent is polar while the resin matrix is nonpolar, so the aliphatic nonpolar alcohols are adsorbed by the resin and are eluted after charged molecules (Bio-Rad, 1988). Pecina *et al.*, (1984) investigated the chromatographic behaviour of 63 substances on the Aminex HPX-87H column and the capability of this column for the separation of acids, aldehydes, ketones, alcohol and carbohydrates.

3.2 MATERIALS AND METHODS

3.2.1 STANDARD SOLUTIONS

Two separate standard solutions were used throughout this study. The first contained gluconic and tartaric acid in varying amounts, since these two acids elute very close together. Concentrations ranged from 50 to 10 g/L gluconic and 10 to 2 g/L tartaric. The second contained malic acid with a concentration range of 5 to 1 g/L. These acids were of analytical grade (standard purity 99%) and obtained from BDH (Great Britain) and Sigma (U.S.A).

3.2.2 SAMPLE PREPARATION

Three techniques, SEP-PAK C_{18}, ion exchange and membrane filtration were investigated for possible use in sample preparation.

SEP-PAK C_{18}

These cartridges are supplied by Waters (Waters Chromatography Division, Millipore Corp., U.S.A) for solid phase extraction. These columns are used generally for red wine and must to remove phenolic and lipophilic compounds. The cartridges were activated with 5mL methanol followed by 5mL deionised water prior to use.

Ion Exchange

A weakly basic anion exchange resin, Amberlite IRA-93 (Sigma Chemicals), was used. The wet mesh designation was 16-50 and the resin was hydrated prior to use. A 10cm long, 20mL burette was filled with approximately 5mL resin. The packed column was washed with 5 bed volumes of deionised water. Prior to sample application the water was drained to just above the resin bed. A 5mL aliquot of high acid juice or reduced alcohol wine was pipetted into the Amberlite IRA-93 resin bed and allowed to run through freely , followed by deionised water. This eluate contained the soluble sugars, while the organic acids were retained on the column. The organic acids were washed off with 5mL 10% (v/v) H_2SO_4 and analysed by HPLC.

Membrane

Disposable 0.45µm membrane filters (Millipore Corp.) were used for standard and sample preparation.

All three techniques were used separately and in combination and the chromatograms were compared. There was no noticeable increase in resolution from the ion exchange and SEP-PAK C_{18} treatments when compared to the membrane treated samples. So it was decided to prepare standards and samples for analysis by passing through the 0.45µm membrane filter.

This technique was recommended by Frayne, (1986); Schneider, Gerbi and Redoglia (1987); Takeda, Saunders and Saunders (1983) with the advantage being that it cuts down the sample preparation time from 15 minutes to 2 minutes per sample. The only concern about this technique is the effect on the HPLC column and componentry, however if a good conditioning and cleanup procedure is employed then the column life should not be compromised.

3.2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The standards and samples were filtered through a 0.45μm membrane and 2μL was analysed using a Sugar-Pak II guard insert (Millipore Corp.) and a 300mm x 7.8mm Aminex HPX-87H organic acid analysis cation exchange column (Bio-Rad Laboratories). The column was operated at room temperature and not at higher temperatures because of rapid deterioration of the column packing, presumably due to breakage of cross-linkages (Rajakylä, 1981). The mobile phase was 0.002N H_2SO_4 using distilled water with a resistivity of 15 megohms obtained from a Nanopure reagent water system. The solvent delivery system was a Waters 600-MS System Controller pump operating at a flow rate of 0.6mL/minute. The standards and samples were injected using a Waters 717_{plus} Autosampler.

The eluting compounds were monitored by a fixed wavelength ultraviolet (UV) detector (Waters 490E Programmable Multiwavelength Detector) at 210nm and 0.1 absorbance units full scale (AUFS). This wavelength was chosen as it is the best wavelength for absorbance of all acids analysed with the least interference.

The peaks were quantified using external standard calibration based on peak height estimation with integration on the baseline forced at valley point using negative peak logic using the Millenium 2010 Chromatography Manager (Waters Chromatography Division, Millipore Corp., U.S.A). The components were identified by a comparison of their retention times with those of the standards. There were three standard injections, the first to condition the column, the second for calibration, and the third at the end of each daily run to check column variability and stability.

3.3 RESULTS AND DISCUSSION

HPLC chromatograms of acid standard mixtures, and of acids present in high acid juice and reduced alcohol wine are shown in Figures 3.1-3.3. Note that fumaric acid is an impurity of the malic acid standard (Schneider, Gerbi and Redoglia, 1987).

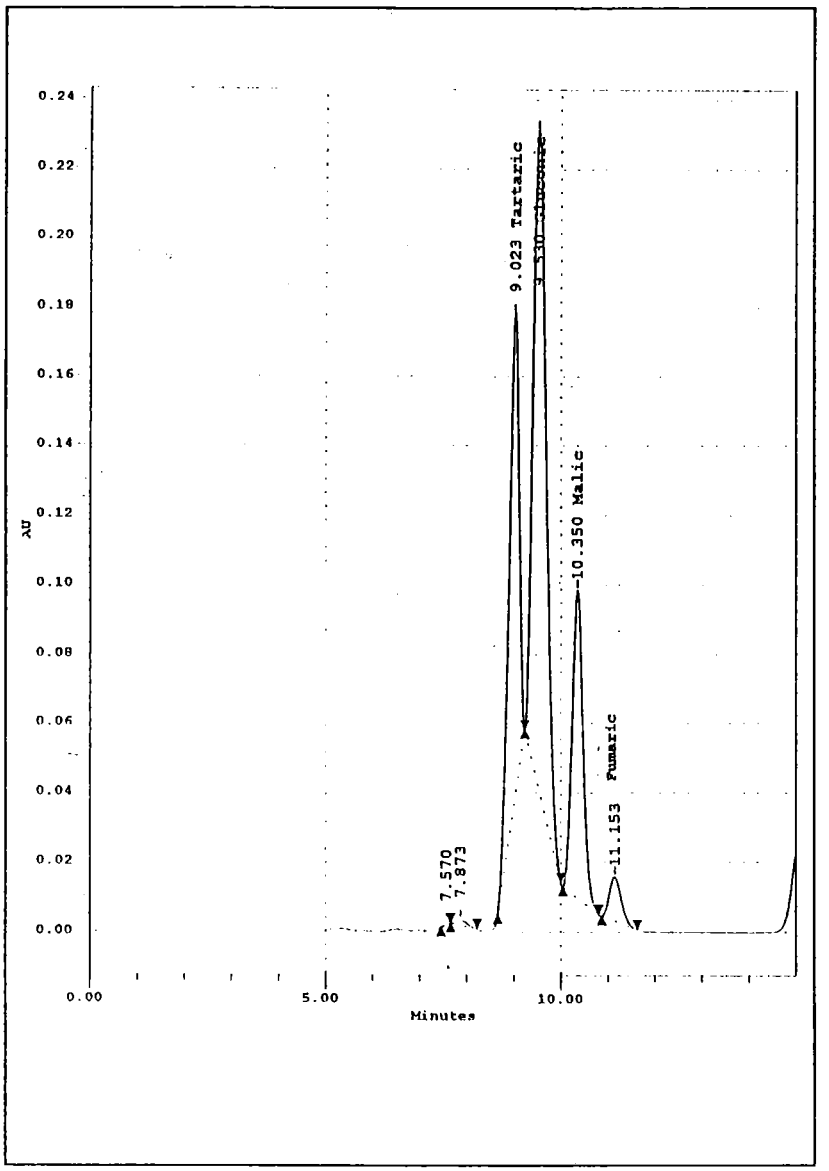


Figure 3.1. HPLC separation of acid standards.

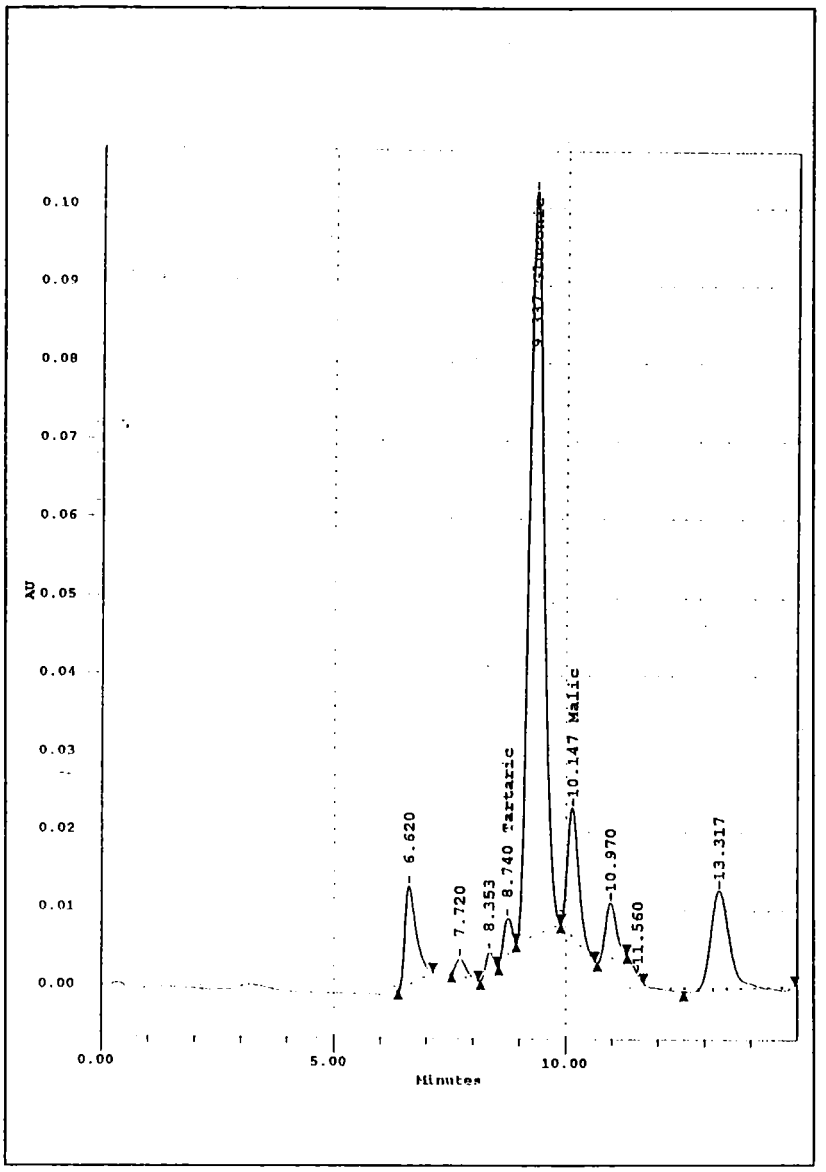


Figure 3.2. HPLC chromatogram of a high acid juice sample

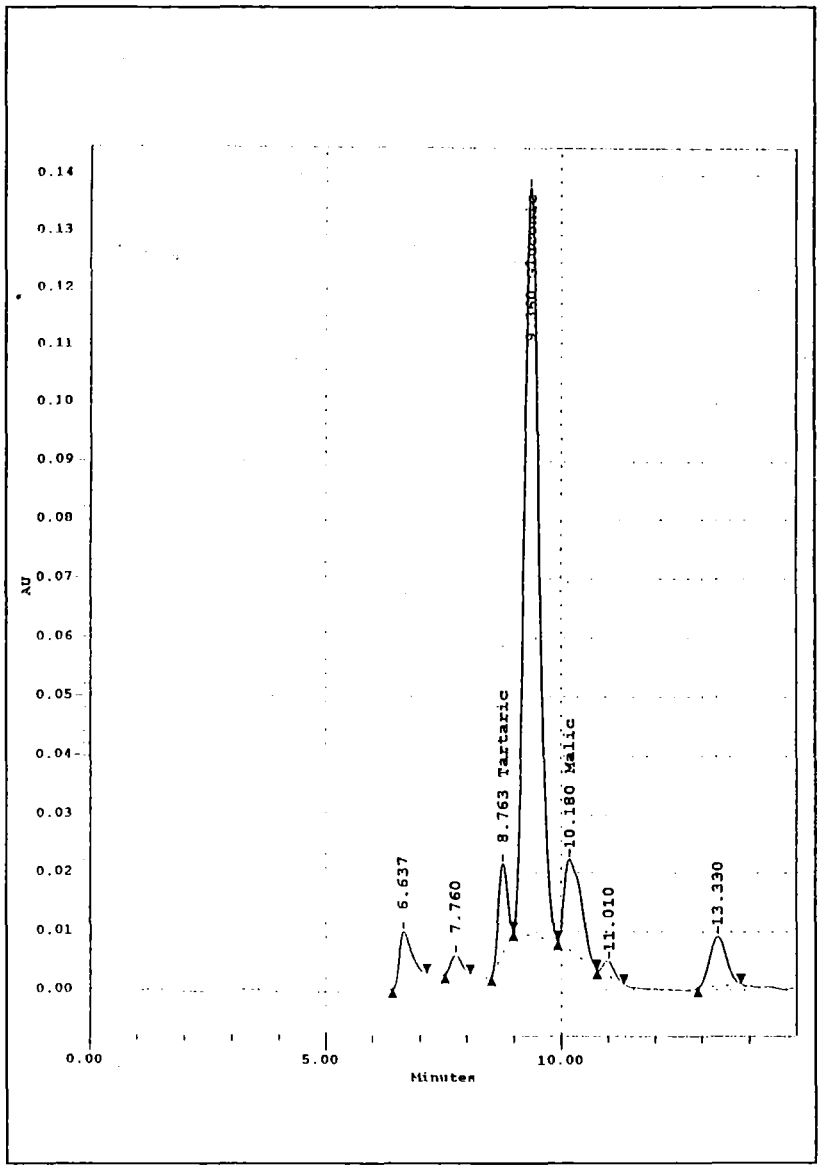


Figure 3.3. HPLC chromatogram of a reduced alcohol wine sample.

3.3.1 CALIBRATION

Detector response was measured as peak heights instead of peak area, since the gluconic and tartaric peaks eluted so close together. The peak heights were measured at different levels of standard solution concentration and the calibration curves (Figures 3.4, 3.5, and 3.6) were found to be linear. However the response factor was different from acid to acid so the quantitative analysis of the acids was carried out with the external standard method. Before and during the sample analyses, two different standard solutions were run to verify quantitation. Regression equations giving best fit of the data for each acid standard are:

Tartaric:	Y = 12922x - 818	(R^2 = 0.99%)
Gluconic:	Y = 4030x - 10000	(R^2 = 0.99%)
Malic:	Y = 8248x + 1060	(R^2 = 0.99%)

where Y = chromatogram peak height and x = acid standard concentration.

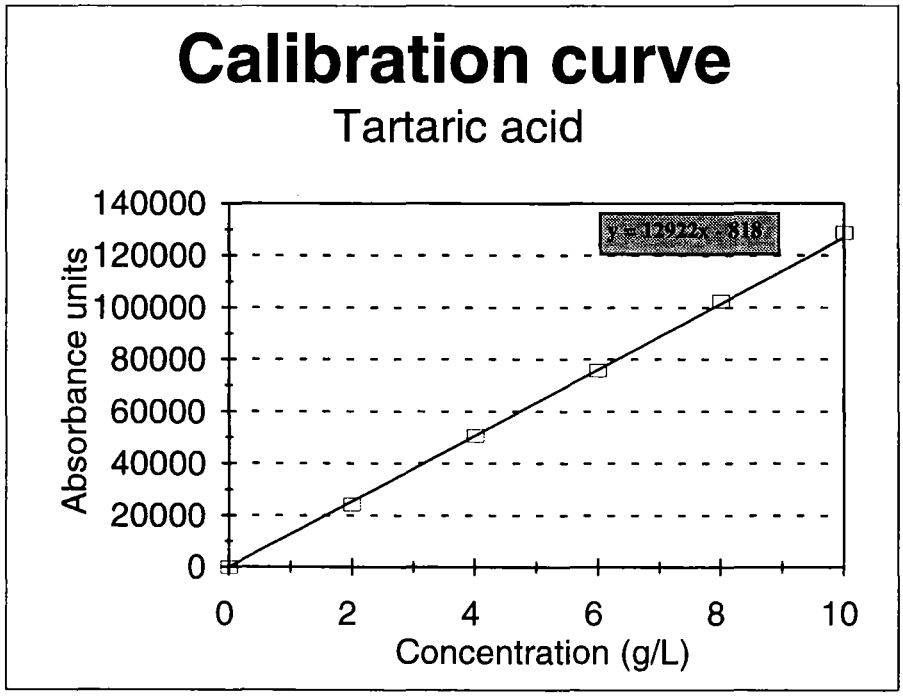


Figure 3.4. Calibration curve for tartaric acid.

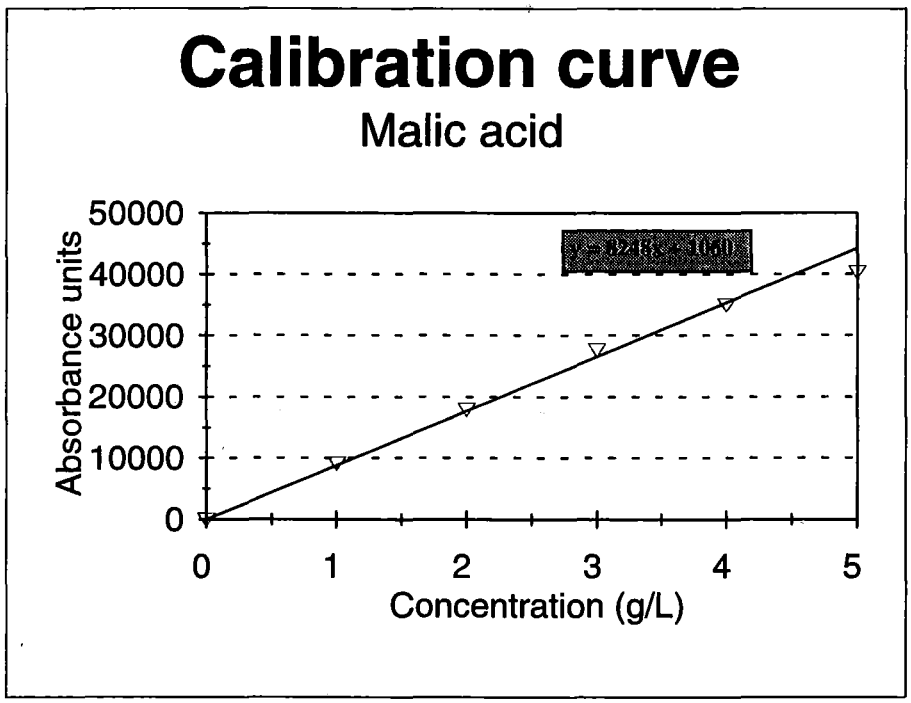


Figure 3.5. Calibration curve for malic acid.

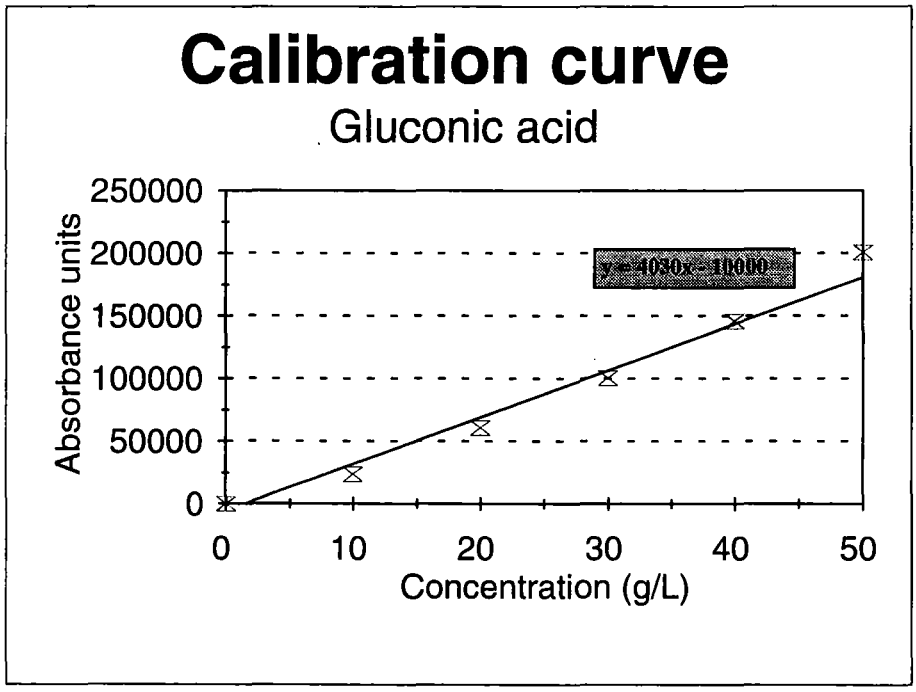


Figure 3.6. Calibration curve for gluconic acid.

Resolution of the tartaric and gluconic acid peaks were found to improve as the injection volume was reduced. 2μL gave the best resolution between the two acid peaks, and this is important as quantisation is only possible from adequate resolution. Any volume below 2μL further improved resolution, however, background noise became predominant and thus reducing the accuracy of the detector.

3.3.2 RECOVERY EFFICIENCY

The high acid juice and reduced alcohol wine were analysed for recovery percentages based on standard acid solutions (Table 3.1). This study was performed indirectly using juice and wine samples spiked with known amount of standard solution. The spiked samples were analysed and the results compared to non-spiked samples. The recovery of all the acids was better than 99%, demonstrating the viability of this procedure to analyse, quantitatively, high acid juice and reduced alcohol wine.

The recovery percentages of the HPLC results were calculated using the following formula:

$$\frac{A-B}{C}\times 100$$

where A = concentration of sample and standard solution
B = concentration of sample
C = concentration of standard solution

The standard solutions were 10 g/L tartaric acid, 50 g/L gluconic acid, and 5 g/L malic acid.

TABLE 3.1. Recovery efficiency of acids from high acid juice and reduced alcohol wine.		
Compound	High acid juice (%)	Reduced alcohol wine (%)
Tartaric acid	100.1	99.1
Gluconic acid	112.4	110.7
Malic acid	108.4	111.9
Values represent the mean of triplicate analysis.		

These results compare favourably to similar research in HPLC.

3.3.3 CAPACITY FACTORS

The effect of the concentration of the mineral acid in the mobile phase on the retention characteristics of the acid solutes were investigated. The capacity factor k' is a constant that is important in the evaluation of the migration rate of the solute in a column, and is defined as:

$$k' = \frac{t'_R}{t_M}$$

where t'_R, the adjusted retention time taken for the solute that is retained by the column packing, and t_M is the time for one molecule of the mobile phase to pass through the column. This was tested by injecting water into the mobile phase and observing the small signal from the RI detector (Skoog, 1985; Gump and Kupina, 1979).

The concentration of the eluent (H_2SO_4) seems to have only a slight affect on the retention time of gluconic acid (see Figure 3.7) on a column of strong cation exchange resin. A series of dilute sulphuric acid solutions were prepared for use with the Aminex cation exchange

column. These solutions were prepared by diluting 0.5*M* sulphuric acid (BDH 'Aristar' c.v.s) with distilled deionised water to form concentrations of 0.001, 0.002, 0.005, and 0.01*N* H_2SO_4.

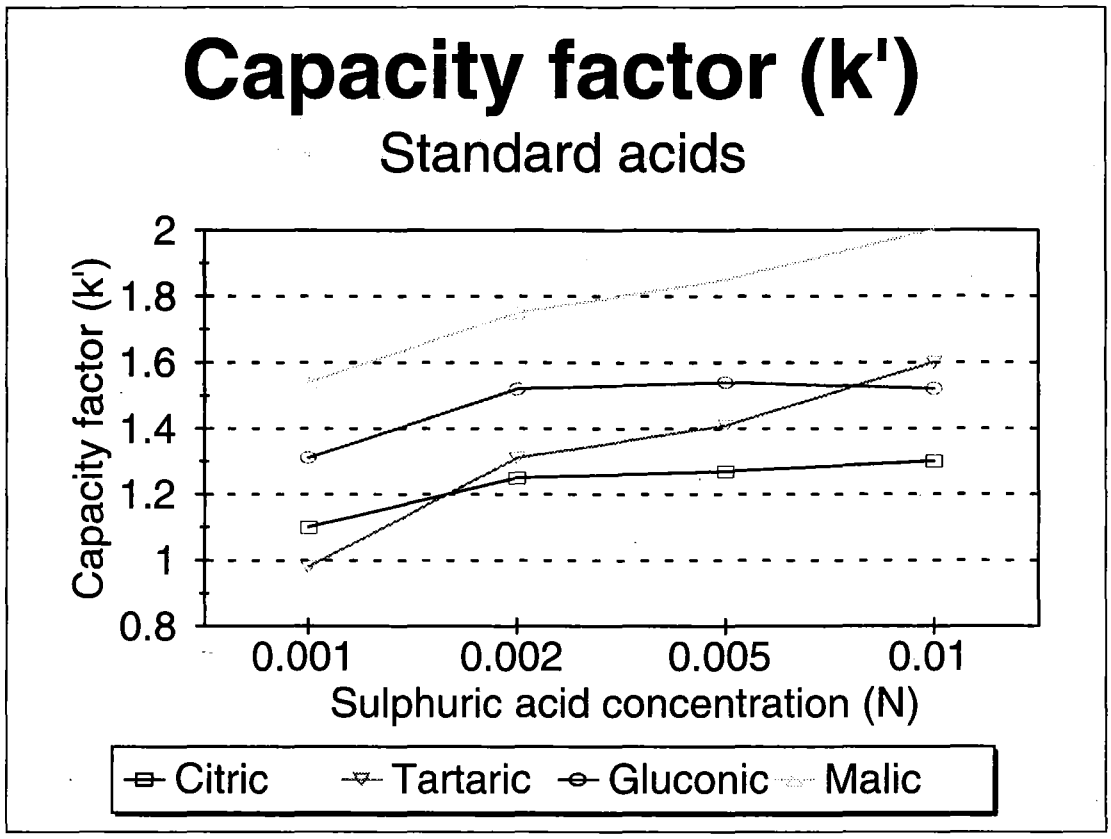


Figure 3.7. Effect of solvent concentration on capacity factor (k') of the major organic acids.

As observed from the graph, the capacity factors increased markedly as sulphuric acid is added to the mobile phase. This is due to the suppression of the ionisation of these acids, since it is in the molecular form that they can migrate into and partition into the internal resin volume.

The mixed chromatographic action of the column can be seen from Figure 3.7. If these acids eluted in the order of their pKa values (ion exclusion mechanism), tartaric acid would elute first followed by malic and then gluconic. This was not the case with the order of elution being, tartaric, gluconic, then malic acid. This indicates partition chromatography is also in action on these resin columns between the liquid held within the resin matrix and the mobile phase external to that matrix.

A concentration of sulphuric acid in the mobile phase can be selected to provide maximum separation of gluconic acid from the other wine acids. At a concentration of 0.002*N* H_2SO_4, gluconic acid is well resolved from tartaric and malic acid.

3.3.4 GLUCONIC ACID

As mentioned before in Chapter 2, gluconic acid exists in equilibrium with two lactone forms, and depending on the pH of the solution, the equilibrium will shift between the lactones to the free acid.

D-glucono-δ-lactone ⇔ D-gluconic acid ⇔ D-glucono-γ-lactone

An experiment was performed to measure the effects pH has on this equilibrium and standard solutions of gluconic acid were buffered with varying amounts of concentrated NaOH. Three solutions were analysed by HPLC:

gluconic acid 50g/L	pH 3	no lactone peaks
gluconic acid 50g/L	pH 6	one lactone peak (t_r=11.20 minutes)
gluconic acid 50g/L	pH 12	no lactone peaks

The gluconic acid standard at pH 3 (wine pH) was left for 48 hours and then reanalysed to determine if there was any shift in equilibrium with time. No change was evident. Therefore it was assumed that quantification of gluconic acid in high acid juice and reduced alcohol wine would be based on the free acid peak, since at wine pH the lactones had hydrolysed to the free acid.

3.4 CONCLUSIONS

With the little sample preparation time required, approximately 30 minutes for preparation, elution and quantitation, the proposed method can be considered to be a rapid procedure for carboxylic acid determination. Tartaric, gluconic and malic acids of the high acid grape juice and reduced alcohol wine eluted within 15 minutes.

The use of guard column cartridges and care with sample, standard, and solvent preparation increases the analytical column life; no loss of resolution or deterioration of separation capabilities was noticed after injection of more than 800 samples/standards. Different sample preparation techniques were trialed, however, no increase in resolution was found from any of them. Also there was no interference from fructose with the analysis of the organic acids.

The performance of the Bio-Rad cation exchange column operated in the ion exclusion-partition mode was investigated. Mobile phases containing various concentrations of sulphuric acid were used to evaluate the effect of acid strength on the capacity factors of the standard acids. With a mobile phase of 0.002 *N* H_2SO_4, gluconic acid was found to be well resolved from the tartaric and malic acids.

The results of the recovery efficiency and linear regression analysis show that this HPLC method can analyse quantitatively tartaric, gluconic and malic acid in high acid grape juice and reduced alcohol wine produced by the GOD/CAT treatment.

CHAPTER FOUR

CHEMICAL DEACIDIFICATION

4.1 REVIEW OF LITERATURE

4.1.1 COLD STABILISATION

Following fermentation, unfinished wines are frequently supersaturated with respect to the bitartrates of potassium, magnesium and calcium (Dickinson and Stoneman, 1958). If these excess salts are not removed, they tend to form unsightly haze and precipitates after bottling. The standard procedure for dealing with this type of instability has been prolonged chilling followed by racking or filtration.

Grape juice and wine contain a high concentration of potassium (0.1 - 1.2g/L) and tartaric acid (1 - 4g/L) (Rankine, 1991). Ionisation of tartaric acid produces bitartrate ions which, with potassium, form the solid potassium bitartrate salt $KC_4H_5O_6$.

$$K^+ + C_4H_5O_6 \Leftrightarrow KC_4H_5O_6$$

The amount and rate of potassium bitartrate formed is controlled by the solubility product

$$(SP) = [K^+][HT^-]$$

(where HT^- denotes the bitartrate anion, $C_4H_5O_6$) which depends on temperature, pH, and the ethanol content of the solution (Berg and Keefer, 1958).

Cold stabilisation is a process employed in the winery to ensure that the wine is tartrate stable. This need for stability is dictated by the market requirements of a clear product without sediment in the bottle. This sediment is composed mainly of KHT crystals. The solubility of KHT in wine is variable due to the complexity of wine composition; the kinetic processes taking place in wine are slower and complex (Rodriquez-Clemente and Correa-Gorospe, 1988). To ensure the wine is tartrate stable, it is usually chilled to between -2^0C to 2^0C. Temperature has an effect on the rates of nucleation and growth of potassium bitartrate crystallisation. KHT precipitation process does not always reach equilibrium in the amount of time allocated in the cold stabilisation phase of winery operations. The reasons for this uncertainty lies in the inhibitory effect on several wine components which adsorb on the growth sites of the crystal faces. Precipitation has an activation energy barrier that needs to be transcended by the system; this is a process that is enhanced with supersaturation, temperature, and agitation. Efficient stirring, high supersaturation, and seeding of the wine can ensure KHT precipitation in the stabilisation process (Roderiquez-Clemente and Correa-Gorospe, 1990).

4.1.2 NEUTRALISATION

Acidity adjustment and, more particularly, the reduction of acidity in grape juice and wine has received much attention from cool climate wine producers and researchers. Among the techniques suggested to achieve deacidification include: amelioration with sugar solutions, biological degradation of malic acid, neutralisation of a portion of the acid with carbonate

salts, precipitation of the double salt of tartrate and malate with calcium carbonate, and the use of anion exchange resins. Although experimental work has been performed on the use of anion exchange, the technique has not found wide acceptance for commercial use (Nagel, Johnson and Carter, 1975).

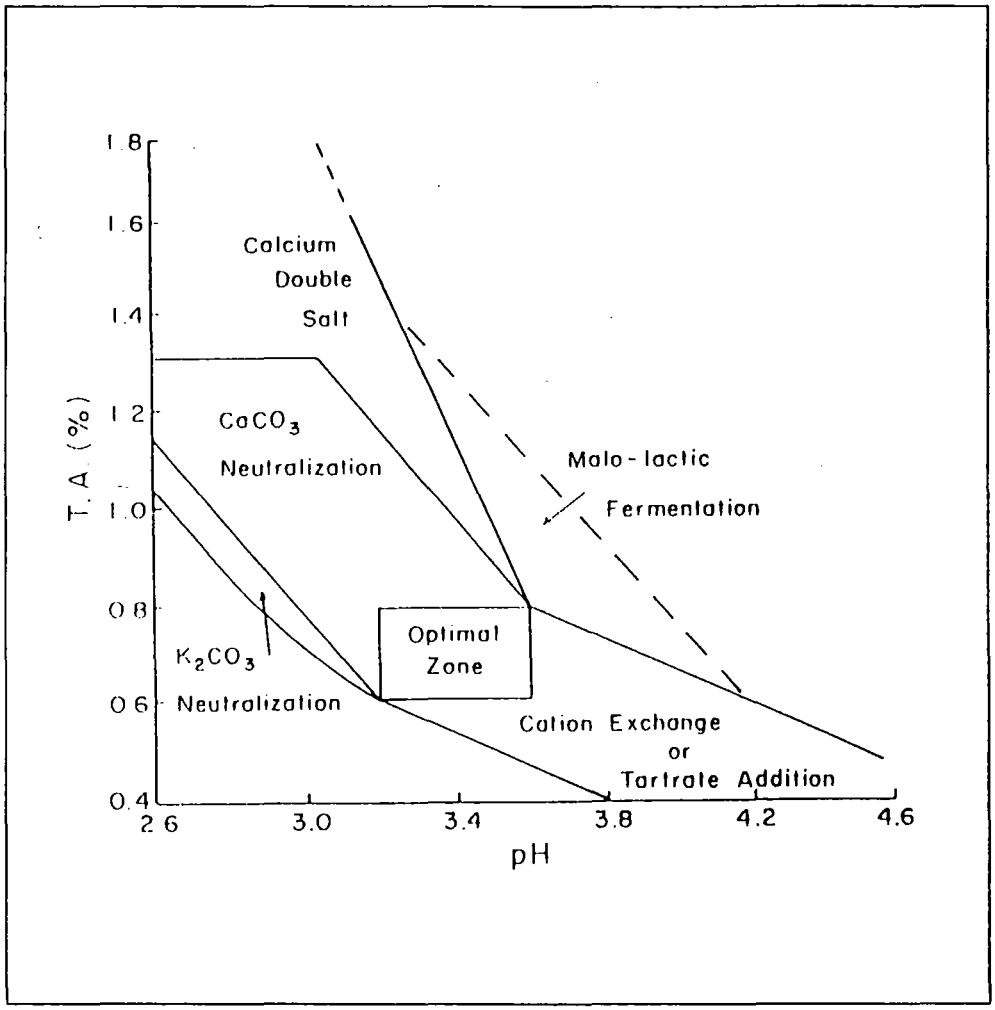


Figure 4.1. Limits of several deacidification methods in musts and wines (Munyon and Nagel, 1977)

Figure 4.1 shows how the different acidity modification techniques are affected by pH. With high acid/low pH juice and wine it can be seen that the several methods of chemical deacidification would be most effective.

Neutralisation of grape juice and wine by $CaCO_3$ or precipitation of the calcium double salt of tartrate and malate is the most commonly used method by the world's cool climate producers (Rankine, 1991; Clark Fugelsang and Gump, 1988). Acidity is neutralised by one of several mineral salts, with the main deacidification effect resulting from the precipitation of tartrate salts.

Nagel, Johnson and Carter, (1975) observed that deacidification was more effective when calcium carbonate was added to wine, rather than juice. They found that deacidification was only about half as efficient when calcium carbonate was added to grape juice compared to wines. They reasoned that the calcium added to the juice was competing for the same tartrate ions that normally would precipitate as potassium bitartrate after the fermentation. Neutralisation with $CaCO_3$ is most effective on cold stabilised and racked wines (Munyon and Nagel, 1977). However, Steele and Kunkee, (1979), reported that $CaCO_3$ deacidifications were best carried out on the grape juice rather than wine (which will have lost considerable amounts of tartrates), both in terms of resulting quality and stability.

4.1.2.1 CHEMISTRY

Calcium carbonate is the most common chemical employed for neutralisation of acids in juice and wine. In the presence of excess tartrate (H_2T), the reaction with calcium carbonate ($CaCO_3$) is:

$$CaCO_3 + H_2T \Leftrightarrow CaT\downarrow + H_2O + CO_2$$

Potassium carbonate can also be used:

$$K_2CO_3 + 2H_2T \Leftrightarrow 2KHT\downarrow + H_2O + CO_2$$

Most studies on calcium tartrate have attempted to develop models which would allow the prediction of precipitation. The use of concentration product (CP) has been suggested (Berg and Keefer, 1958; Pilone and Berg, 1964).

$$CP = [Ca^{2+}][T^{2-}]$$

where T^{2-} represents the tartrate anion. The ionised calcium is measured using a calcium ion selective electrode. A general analytical survey of wines indicated that an approach based simply on CP calculations could not be used to predict the possibility of calcium tartrate precipitation, since there was no logical relationship between the fraction of ionised calcium and the occurrence of precipitation (Scollary, 1990).

Calcium tartrate (CaT) precipitation continues to be an instability problem in wines, appearing most commonly in bottled products. Possible reasons are increased calcium content following deacidification with $CaCO_3$, use of calcium bentonites, pH conditions that cause tartaric acid to be in the form of tartrate ions, and the reduction of inhibitory substances at the final filtration and bottling stage (Abgueguen and Boulton, 1993).

Several studies have dealt with the solubility (Berg and Keefer, 1958; Pilone and Berg, 1964), stability values (De Soto and Yamada, 1963), precipitation (Pilone and Berg, 1964), and the reduction of CaT in unstable wines (Clark, Fugelsang and Gump, 1988).

McKinnon *et al.,* (1992), have shown that the onset, rate and extent of precipitation of CaT depends on the initial pH of the solution. The precipitation was found to be independent of the type of container, but dependent on the presence of foreign substances. Agitation of the solution was found to induce crystal formation at a much faster rate than for static solutions. Static solutions which had apparently reached equilibrium were found to undergo further precipitation when agitated.

Excess CaCO3 neutralisation will cause precipitation of Ca salt(s) other than calcium tartrate, as noted by the continued decrease in total anions, even after tartrate has been precipitated (Munyon and Nagel, 1977).

4.1.2.2 KINETICS

According to at least one theory of crystal growth, where nucleation is not limiting, there are two steps in the mass transfer from solution. The first is a diffusion process, whereby solute molecules are transported from the bulk of the fluid phase to the crystal surface, followed by a surface reaction, called surface integration, when solute molecules arrange themselves into the crystal lattice. Should either of these steps be much slower than the other, the overall growth rate will be limited by the slower step (Abgueguen and Boulton, 1993).

There are a number of different types of nucleation processes including: primary nucleation, which occurs due to high supersaturation in the absence of crystals. Secondary nucleation is caused by the presence of crystals of the material being crystallised. Heterogeneous nucleation is caused by the presence of particles of material other than that being crystallised, and contact nucleation, where new nuclei is formed from the parent crystal of the material being crystallised (Dunsford and Boulton, 1981).

As temperature decreases, the precipitation rate tends to increase, due to the supersaturation level and driving force increases, while the overall crystal growth co-efficient decreases according to an Arrhenius' type of relationship. The alcohol content has little effect due to little change of the dielectric of the medium and only slight changes in the dissociation constants of the acid (Abgueguen and Boulton, 1993).

Abgueguen and Berg, (1993), have studied the theory of calcium tartrate precipitation using seed crystals and have shown that the kinetics of the precipitation are controlled by crystal growth. McKinnon *et al.,* (1992), established a detailed mechanism of the precipitation process. They observed that the amount of bound calcium does not change during the induction period. For precipitation to occur critical nuclei of calcium tartrate have to form. Once a stable nucleus is formed the addition of further calcium tartrate molecules induces precipitation and both the ionised calcium and total calcium concentration will decrease.

Stage 1: Linking together of calcium tartrate molecules

$$CaT + CaT \leftrightarrow 2\{CaT\}$$

$$CaT + 2\{CaT\} \leftrightarrow 3\{CaT\}$$

$$CaT + (x\text{-}2)\{CaT\} \leftrightarrow (x\text{-}1)\{CaT\}$$

Stage 2: Formation of critical nucleus

$$CaT + (x\text{-}1)\{CaT\} \rightarrow x\{CaT\}$$

Stage 3: Precipitation

$$CaT + x\{CaT\} \rightarrow (x\text{-}1)\{CaT\}_{solid}$$

Stage 4: Crystal growth

$$CaT + (x+1)\{CaT\} \rightarrow \textbf{crystal growth}$$

For precipitation to occur, critical nuclei of calcium tartrate have to form and this can be achieved by stacking together molecules of CaT. This stacking together continues until a critical nucleus; that is x{CaT}, is achieved. Once a stable nucleus is formed, the addition of calcium tartrate molecules induces precipitation and both the ionised calcium and total calcium concentration will decrease (McKinnon *et al.*,, 1992).

4.1.3 SEEDING

A major problem resulting from neutralisation of musts or wines with calcium carbonate is that most of the deacidification is due to the precipitation of calcium tartrate. Most of the calcium malate formed remains in solution and can cause the wine to taste salty if the concentration is high enough. Neutralisation often increases the pH of the wine excessively which can lead to problems with colour and stability. Also, when tartrate concentration is reduced to a low level winemakers must be extremely careful to control malo-lactic

fermentation since it could reduce the acidity to dangerously low levels and result in completely "flat" tasting wine.

As malic acid is often present in concentrations greater than that of tartaric acid. Reducing acidity to a suitable level in grape juice and wine necessitates the removal of some malic acid as well as tartaric acid (Steele and Kunkee, 1979).

This type of deacidification is achieved with 'Acidex', a commercial preparation of calcium carbonate seeded with calcium double salt crystals (C.H. Boehringer Soln, Ingelheim am Rhein, West Germany) (see Figure 4.2).

Only a portion of the must or wine is treated with Acidex, and that portion is then blended back with the untreated portion to strike the desired amount of deacidification. The treated portion is made to reach pH 4.5-6.5 as quickly as possible. This maintenance of the pH level is essential, for both the tartaric acid and the malic acid must be dissociated into their dicarboxylate forms sufficiently to ensure a double salt formation. At pH above 4.5, the malate and tartrate rapidly combine with calcium to form crystals of the double salt. If the pH is not maintained above pH 4.5, the equilibrium will be shifted in favour of the single salt (calcium tartrate).

Steele and Kunkee (1978) found that the amount of acid removed from these musts did not show a reduction, in equal proportions, of tartaric and malic acids. Some malic acid was removed, but tartaric acid was the predominant acid removed in all cases.

While chilling does increase the level of calcium tartrate supersaturation in wine, this level is generally not high enough to induce formation of calcium tartrate crystals (Berg and Keefer, 1958; De Soto and Yamada, 1963). This can be attributed to a number of impurities present in the grape juice or wine. Pilone and Berg (1964) found that the inhibiting effect of colloidal pigments on self-nucleation was sufficient to prevent crystal growth during the initial refrigeration period. Tartaric acid could attach itself to the pigment molecule by means of hydrogen bonding between the oxygens of the carboxyl groups of the acid and the hydrogens of the phenolic hydroxyls of the pigment. Impurities can favour or impede the nucleation of a supersaturated solution. The ability of these impurities to form complexes can favour the formation of clusters and decrease the surface energy barrier for nucleation or growth. However, they can also act in the opposite way - blocking both processes (Rodriguez-Clemente, Correa-Gorospe and De Castro, 1988).

Another inhibitor to calcium tartrate precipitation is the presence of malic acid, this acid caused a marked increase in the induction time and also slowed the rate of precipitation (McKinnon, 1993).

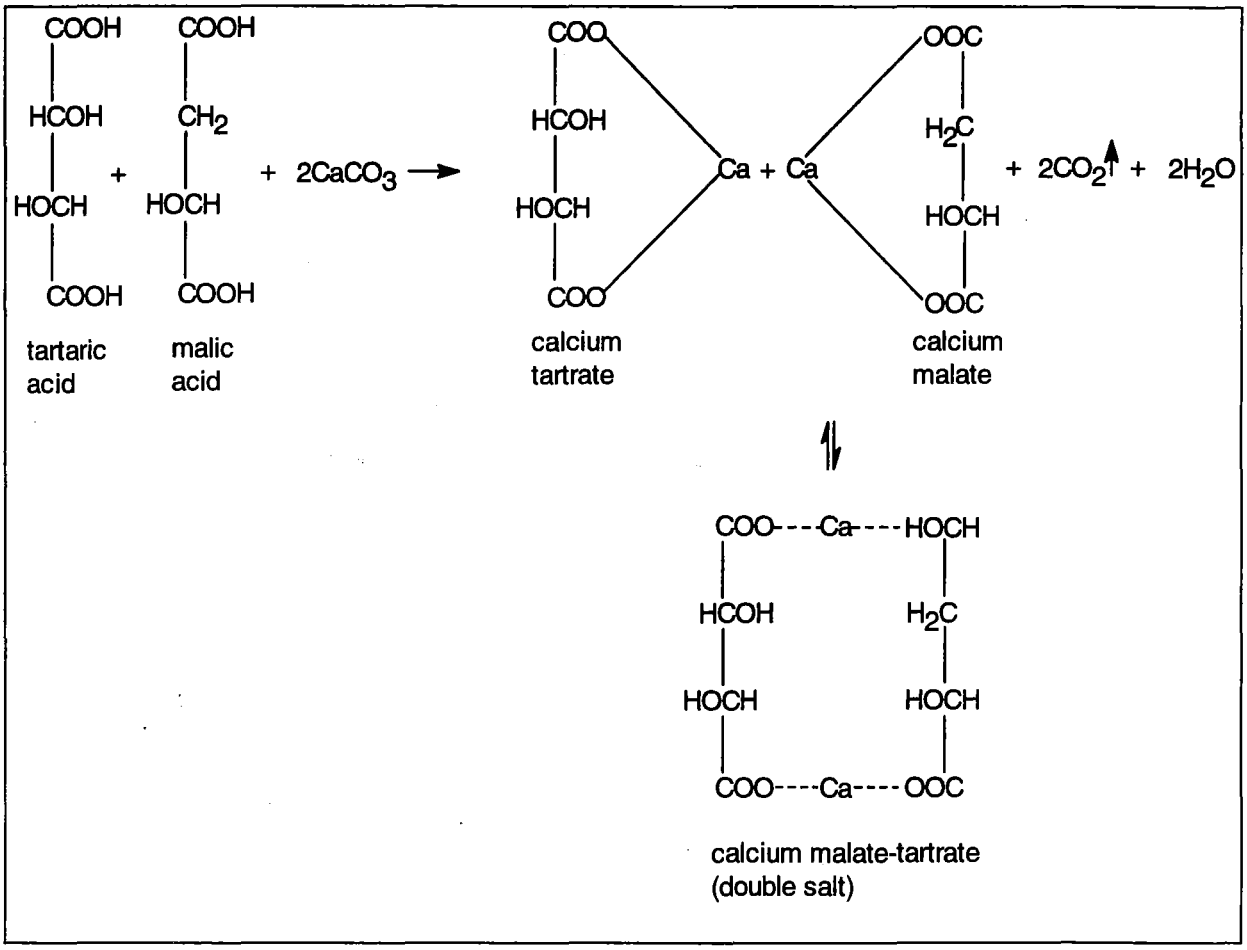


Figure 4.2. Formation of the double-salt crystals

Malic acid exerted a highly inhibitory effect on the rate of crystallisation by competing with tartrate ions or interacting with calcium either in solution or on the crystal (Abgueguen and Boulton, 1993). Thus malate acts as an inhibitor of calcium tartrate precipitation appearing to interfere with nucleation-controlled crystal growth. CaT precipitation rate increases with greater amounts of seed crystals added to the system. Thus, the inhibition by malate can be overcome, to a certain extent, by seeding and thereby providing more surface area for crystal growth (Clark, Fugelsang and Gump, 1988).

Recently a deacidification protocol has been proposed by Garcia-Ruiz, Alcantara and Martin (1991), using the following conditions: maximum cooling temperatures of 2-3°C; seed

concentration of 8g/L; particle size < 50μm and mechanical stirring (no ultrasounds). Except for the seeding concentration, all the other recommendations were incorporated in the methods used in this study.

4.2 MATERIALS AND METHODS

4.2.1 STANDARDS

Two model solutions were made for the preliminary investigation into the deacidification techniques. The first was made to imitate the high acid grape must and contained 10g/L tartaric, 50g/L gluconic, and 5g/L malic acid with 90g/L fructose (GOD/CAT treatment converts all the glucose to gluconic acid) and buffered to pH 3.1 with concentrated NaOH. The second model solution was made to imitate the reduced alcohol wine and contained 10g/L tartaric, 50g/L gluconic, and 5g/L malic acid with 7% (v/v) ethanol and buffered to pH 3.1 with concentrated NaOH. All chemicals were sourced from BDH Chemicals Ltd.

4.2.2 PREPARATION OF HIGH ACID JUICE AND REDUCED ALCOHOL WINE

The juice and wines were produced by the GOD/CAT enzyme treatment(as described in Chapter 2). Müller Thurgau juice was obtained from Geisen Wines of Canterbury, New Zealand. The juice had a pH of 3.15, TA of 6.94g/L, soluble solids of 18.0° Brix and a total SO_2 concentration of 45.6 ppm. The juice was then treated with 1.5g/L GOD/CAT enzyme, aerated with oxygen at 8g/L while being stirred for 10 hours. Upon completion the juice had a pH of 3.15 and TA of 26.46g/L. Half of this juice was then inoculated with *Saccharomyces cerevisiae str.* Fermivin® SF (Gist-brocades, Seclin cedex, France) and fermented at 12°C. Upon completion of the primary fermentation, the wine had a pH of 3.21 and TA of 19.34g/L.

The temperature of the wine was reduced to 2°C and cold stabilised at that temperature for 48 hours. The wine was then treated with 40 ppm SO_2 (5% Sulphurous acid, BDH Chemicals Ltd, Poole, U.K.). The analysis of these liquids before deacidification are presented in Table 4.1.

Table 4.1. Analysis of juice and wine produced by the glucose oxidase-catalase treatment.

Analysis[a]	High acid juice	Reduced alcohol wine
pH	3.0	3.2
T/A[b]	26.5	19.3
Ethanol (% v/v)	0	8.3
Tartaric acid (g/L)	1.2	1.0
Gluconic acid (g/L)	75.5	45.5
Malic acid (g/L)	4.1	3.2
Free SO_2 (mg/L)	40	40
Total SO_2 (mg/L)	92	86

a. Mean values of duplicate analysis
b. As g/L tartaric acid.

4.2.3 NEUTRALISATION

Neutralisation with $CaCO_3$ and K_2CO_3 (BDH Chemicals Ltd) was carried out with the model juice and wine solutions. With both liquids, $CaCO_3$ was more effective, while K_2CO_3 had a negligible affect on acid content, therefore only $CaCO_3$ was used for further investigation.

In preliminary trials using model solutions 1g/L $CaCO_3$ removed 1.58g/L gluconic acid. However it was decided to keep a commercial outlook on this experiment, and a range of 1 to 5g/L $CaCO_3$ was added to 200mL juice or wine that had been prechilled to 2°C, and agitated with an orbital shaker for 30 minutes in a 250mL conical flask. The samples were then cold

stabilised for two weeks at 2^0C. After cold stabilisation, samples were filtered through a 0.45μm membrane and analysed by HPLC for tartaric, gluconic and malic acid content. pH and titratable acidity (TA, as g/L tartaric acid) were also determined.

4.2.4 COLD STABILISATION

All the deacidification techniques being tested in this project involve a period of cold stabilisation of two weeks at 2^0C. Therefore 200mL of untreated juice and wine was stored at 2^0C and a sample of both was taken each day and analysed. The results of this served as a baseline for the other deacidification techniques.

4.2.5 SEEDING

The deacidification agent was created in the form of the commercial preparation of calcium carbonate seeded with calcium double-salt crystals called 'Acidex'. Two trials were performed on the juice and wine, one consisting of varying amounts of calcium carbonate and calcium gluconate, the other consisting of varying amounts of calcium carbonate and sodium gluconate.

This custom made deacidification agent followed the same method as for 'Acidex' (refer Chapter 4.1.3). Only a portion of the must or wine is treated with the agent and that portion is then blended back with the untreated portion to achieve the desired amount of deacidification.

The treated portion, in effect, becomes a slurry, and made to reach pH 4.5-6.5 as quickly as possible. This is needed to dissociate the gluconic acid into its carboxylate form to ensure a salt formation (pKa of gluconic acid is 3.60).

Of the 200 mL juice/wine sample, 100ml was stirred into the 1 g/L seeding agent, the pH was monitored to ensure that the solution remained above 4.5 pH. After 15 minutes stirring the suspension was filtered and the filtrate added to the untreated 100mL of juice/wine. The sample was then chilled to 2^0C, to ensure the best conditions for nucleation and therefore precipitation. After two weeks of cold stabilisation the samples were analysed for gluconic, tartaric and malic acid contents, pH and TA were also determined.

4.2.6 STATISTICAL ANALYSES

All results were analysed using a one-way analysis of variance or two-way analysis of variance (Minitab release 9.2 General Linear Model). Statistical analyses for the determination of significant differences between treatment means were conducted using a Scott-Knott analysis.

4.2.7 CHEMICAL ANALYSES

The major organic acids found in the high acid grape juice and reduced alcohol wine were determined by High Performance Liquid Chromatography (HPLC). This technique was optimised and reviewed in Chapter Three.

The pH and titratable acidity (TA) were simultaneously determined using the 670 Titroprocessor coupled with the 665 Dosimat, an intelligent burette (Metrohm Ltd, Switzerland).

4.3 RESULTS AND DISCUSSION

4.3.1 COLD STABILISATION

Tables 4.2 and 4.3 shows the effects of cold stabilisation over a 12 day period at 2^0C on a sample of high acid grape juice and reduced alcohol wine respectively. These results are also presented graphically in Appendices 2.1-2.4. These results were statistically analysed and there were some confusing results. On observation it would seem that there was no change in acid content throughout the stabilisation period. However statistical analysis of the reduced alcohol wine sample revealed a quadratic increase (R^2=89%) of gluconic acid by 9.46%, tartaric acid showed a quadratic decrease (R^2=46%) of 3.56% as with malic acid (R^2=69%) of 3.40%.

BLE 4.2: The Effect of Cold Stabilisation on Organic Acid Concentration in Reduced Alcohol Wine.[a]

	Cold Stabilisation time (days)											
	0	1	2	4	5	7	9	11	12	CV%	Δ%[b]	p value[c]
uconic acid	44.45	45.92	45.70	46.34	47.34	48.01	47.70	48.83	48.66	0.60	+9.46	<0.001
D	0.30	0.01	0.25	0.12	0.47	0.17	0.42	0.54	0.28			
rtaric acid	1.00	1.01	1.00	0.99	1.04	1.00	0.97	0.98	0.97	0.63	-3.56	<0.001
D	0.008	0.004	0.007	0.004	0.004	0.005	0.001	0.008	0.015			
lic acid	3.17	3.25	3.19	3.18	3.06	3.07	3.03	3.08	3.06	0.64	-3.40	<0.001
D	0.016	0.007	0.023	0.013	0.031	0.014	0.036	0.024	0.016			
	3.28	3.26	3.29	3.29	3.27	3.21	3.25	3.24	3.25			NS
[d]	19.7	19.6	19.7	19.8	19.6	19.3	19.5	19.4	19.4			NS

a. Figures quoted are in g/L and are the mean values of triplicate analysis.
b. Mean conc. day 12 minus mean conc. day 0.
c. Probability corresponding to significance of F ratio for selected model.
d. As g/L tartaric acid.

BLE 4.3: The Effect of Cold Stabilisation on Organic Acid Concentration in High Acid Juice.[a]

	Cold Stabilisation time (days)										
	0	2	4	6	8	10	12	14	CV%	Δ%[b]	p value[c]
uconic acid	74.78	74.73	74.91	74.94	74.86	74.85	75.00	74.91	0.16	+0.17	<0.001
D	0.086	0.030	0.213	0.111	0.106	0.155	0.140	0.129			
rtaric acid	1.23	1.23	1.18	1.19	1.19	1.17	1.17	1.15	1.28	-6.5	<0.001
D	0.004	0.002	0.014	0.002	0.042	0.023	0.014	0.023			
lic acid	4.03	4.01	4.00	4.04	4.03	4.04	4.03	4.01	0.80	-2.0	<0.001
D	0.018	0.029	0.057	0.063	0.033	0.013	0.017	0.035			
	3.03	3.02	3.09	3.10	3.07	3.05	3.01	3.02			NS
[d]	27.5	27.4	27.6	27.5	27.4	27.4	27.5	27.5			NS

a. Figures quoted are in g/L and are the mean values of triplicate analysis.
b. Mean conc. day 14 minus mean conc. day 0.
c. Probability corresponding to significance of F ratio for selected model.
d. As g/l tartaric acid.

It was alarming to find that there seemed to be some production of gluconic acid! Although this did level off by day 10. A possible explanation of this increase could be the shift in equilibrium between the free acid and the two lactones due to the reduction in temperature from the cold stabilisation process. Even though initial analysis of standard acid solutions showed no lactone peaks, let alone, interference. The quadratic increase of gluconic acid concentration would also support this theory, where the lactones would eventually create a new equilibrium with the acid. An experiment was set up to test this theory.

50g/L of gluconic acid standard was made and pH buffered to 3.1 with concentrated NaOH. Half of this solution was placed in a water bath at 60^0C for 4 hours. Both solutions were analysed by HPLC. The treated standard solution (60^0C) showed a ~5% increase in free gluconic acid, and the lactone peak did decrease in concentration with the treatment. This shows that there is a change in equilibrium through the cold stabilisation process and goes as

far as supporting the apparent increase in gluconic acid in the juice and wine samples. These results exhibit the unstable nature of gluconic acid with respect to lactone formation, and the frustration in trying to quantify this organic acid.

One should remember that cold stabilisation is a technique used in the winery to simply make the wine tartrate stable. When the wine is cooled the solubility limit of potassium tartrate, or hopefully in our case, potassium gluconate, is exceeded and some comes out of solution as a crystalline deposit. This should effectively reduce some of the acid content of the wine, however, not in amounts significant enough to be termed as a deacidification technique. To my knowledge there has been no work done on the effect cold stabilisation has on the acid concentration found in juice and wine. So I cannot comment on whether my findings are consistent with previous findings or not.

4.3.2 NEUTRALISATION

Tables 4.4 and 4.5 show the effects of neutralisation with $CaCO_3$ on organic acids in wine and juice samples respectively. These results are presented graphically in Appendices 3.1-3.4.

TABLE 4.4: The Effect of Neutralisation with Calcium Carbonate on Organic Acid Concentration in High Acid Juice[a].

	($CaCO_3$ dosage in g/L)								
JUICE	0	1	2	3	4	5	CV%	Δ%[b]	p value[c]
Gluconic acid	75.71	74.05	75.75	74.54	75.39	72.37	0.51	-4.4	<0.01
±SD	0.44	0.07	0.06	0.69	0.20	0.81			
Tartaric acid	1.26	1.23	1.22	1.22	1.20	1.18	0.41	-6.4	<0.001
±SD	0.01	0.002	0.002	0.01	0.001	0.005			
Malic acid	4.03	4.02	3.96	3.91	3.83	3.76	0.61	-6.7	<0.001
±SD	0.01	0.02	0.03	0.04	0.004	0.04			
pH	3.03	3.11	3.15	3.21	3.29	3.36			<0.001
TA[d]	27.8	25.8	23.8	22.6	21.4	20.5			<0.001

a. Figures quoted are in g/L and are the mean values of triplicate analysis.
b. Mean conc. at 5g/L minus mean conc. at 0g/L.
c. Probability corresponding to significance of F ratio for selected model.
d. As g/L tartaric acid

For the high acid grape juice treated with $CaCO_3$, tartaric acid exhibited a quadratic decrease (R^2=87%) in concentration with increasing $CaCO_3$ of 6.4%. Gluconic acid also conformed to a quadratic model (R^2=48%), even though it was weak, and the concentration decreased by 4.4%. However malic acid followed a linear decrease (R^2=91%) in concentration of 6.7%. For the reduced alcohol wine treated with $CaCO_3$, tartaric acid conformed to a decreasing linear model (R^2=70%) and the concentration was lowered by 5.9%. Gluconic acid also followed a linear decrease (R^2=54%) of 3.9%, while malic acid exhibited a weak decreasing linear model (R^2=42%) and reduced in concentration by 4.1%.

TABLE 4.5: The Effect of Neutralisation with Calcium Carbonate on Organic Acid Concentration in Reduced Alcohol Wine[a].

	($CaCO_3$ dosage in g/L)								
WINE	0	1	2	3	4	5	CV%	Δ%[b]	p value[c]
Gluconic acid	44.85	44.68	43.76	44.62	42.74	43.09	1.0	-3.9	<0.01
±SD	0.72	0.65	0.05	0.38	0.60	0.28			
Tartaric acid	1.01	0.99	1.00	0.99	0.94	0.95	0.92	-5.9	<0.001
±SD	0.02	0.01	0.004	0.009	0.01	0.001			
Malic acid	3.18	3.13	3.06	3.11	2.95	3.05	1.5	-4.1	<0.005
±SD	0.04	0.05	0.009	0.02	0.05	0.11			
pH	3.17	3.32	3.45	3.60	3.63	3.77			<0.001
TA[d]	20.3	18.6	16.9	15.3	14.5	13.4			<0.001

a. Figures quoted are in g/L and are the mean values of triplicate analysis.
b. Mean conc. at 5g/L minus mean conc. at 0g/L.
c. Probability corresponding to significance of F ratio for selected model.
d. As g/L tartaric acid.

4.3.3 SEEDING

Tables 4.6, 4.7, 4.8 and 4.9 show the effects of adding varying amounts of seed crystals on the concentration of organic acids in wine and juice samples respectively. These results are shown graphically in Appendices 3.5-3.8. For these trials two seeding crystals were used, sodium gluconate ($NaC_6H_{11}O_7$) and calcium gluconate ($Ca(C_6H_{11}O_7)_2 \cdot H_2O$). These were applied with varying amounts of $CaCO_3$ to make a total dosage application of 1g/L. The four treatments for each seeding trial were as follows:

Treatment	$CaCO_3$	NaGluconate or CaGluconate
1	20%	80%
2	40%	60%
3	60%	40%
4	80%	20%

TABLE 4.6. The Effect of Sodium Gluconate Seeding on Organic Acid Concentration in High Acid Juice.

	Treatment			
	1	2	3	4
Tartaric acid	1.41^a	1.16^b	1.07^c	1.04^d
% change	+16.5	-0.04	-11.6	-14.0
Gluconic acid	82.3^a	80.5^b	81.3^c	79.6^d
% change	+10.2	+7.8	+8.8	+6.6
Malic acid	3.85^a	4.59^b	4.93^c	4.93^c
% change	-8.3	+9.3	+17.4	+17.4

Figures quoted are in g/L and are the mean values of triplicate analysis.
Percentage change calculated on comparison with control.
Mean values within the same row designated by the same letter do not differ significantly ($p>0.05$) for each treatment. Values designated by different letters differ significantly at $p<0.01$.

For Table 4.6 tartaric acid followed a strong linear decrease (R^2=99.7%) in concentration with increasing sodium gluconate concentration. There was a weak linear relationship (R^2=64.9%) with gluconic acid, with an increase in concentration occurring for all treatments. As with the neutralisation, this can be accounted for by the equilibrium shift from the lactone to the free acid due to the cold stabilisation process. Malic acid exhibited a strong linear relationship (R^2=98.7%), however, with two of the treatments there was a significant increase in malic acid concentration.

TABLE 4.7. The Effect of Sodium Gluconate Seeding on Organic Acid Concentration in Reduced Alcohol Wine

	Treatment			
	1	2	3	4
Tartaric acid	1.08[a]	1.06[b]	1.03[c]	1.13[d]
% change	-3.6	-5.4	-8.0	+0.9
Gluconic acid	50.9[a]	51.8[b]	52.5[c]	52.7[c]
% change	+13.6	+15.6	+17.2	+17.6
Malic acid	3.18[a]	3.20[b]	3.28[c]	3.16[a]
% change	0	+0.6	+3.1	-0.6

Figures quoted are in g/L and are the mean of triplicate analysis.
Percentage change calculated on comparison with control
Mean values within the same row designated by the same letter do not differ significantly ($p>0.05$) for each treatment. Values designated by different letters differ significantly at $p<0.01$.

For Table 4.7 there was no predictable relationship for the change in tartaric and malic acid concentration with the different treatments. However, gluconic acid showed a strong linear relationship (R^2=93.5%) with a constant increase in concentration of ~15%. This can be attributed to the equilibrium shift from the lactones to the detected free acid. However this would only account for 5% of the increase (refer Chapter 4.3.1). The other 10% may have come from the sodium or calcium gluconate crystals which are both soluble in the juice and wine, and since they stayed in solution and did not initiate precipitation, then some of the gluconate could have contributed to the gluconic acid concentration.

TABLE 4.8. The Effect of Calcium Gluconate Seeding on Organic Acid Concentration in High Acid Juice.

	Treatment			
	1	2	3	4
Tartaric acid	1.12[a]	1.09[b]	1.01[c]	0.98[d]
% change	-7.4	-9.9	-16.5	-19.0
Gluconic acid	75.9	76.6	75.6	77.0
% change	+1.6	+2.5	+1.2	+3.1
Malic acid	4.71	4.80	4.74	4.71
% change	+12.1	+14.3	+12.9	+12.1

Figures quoted are in g/L and are the mean of triplicate analysis.
Percentage change calculated on comparison with control.
Mean values within the same row designated by the same letter do not differ significantly ($p>0.05$) for each treatment. Values designated by different letters differ significantly at $p<0.01$.

Table 4.8 shows that tartaric acid exhibited a strong linear relationship (R^2=98.5%) of decreasing concentration with increasing $CaCO_3$ concentration. Gluconic acid showed an unpredictable relationship with the change in concentration less than 4% for all treatments. Malic acid conformed to a weak linear relationship (R^2=42.7%), with all treatments showing a constant increase in concentration of ~13%. This was perhaps a result of a systematic error in the detection of the malic acid content.

TABLE 4.9. The Effect of Calcium Gluconate Seeding on Organic Acid Concentration in Reduced Alcohol Wine.

	Treatment			
	1	2	3	4
Tartaric acid	1.23[a]	1.19[b]	1.21[bc]	1.22[ac]
% change	+9.8	+6.3	+8.0	+8.9
Gluconic acid	53.3[a]	52.7[b]	52.3[c]	53.7[d]
% change	+18.9	+17.6	+16.7	+19.9
Malic acid	3.12[a]	3.09[b]	3.03[c]	3.15[d]
% change	-1.9	-2.8	-4.7	-0.9

Figures quoted are in g/L and are the mean of triplicate analysis.
Percentage change calculated on comparison with control.
Mean values within the same row designated by the same letter do not differ significantly ($p>0.05$) for each treatment. Values designated by different letters differ significantly at $p<0.01$.

With Table 4.9 both tartaric (R^2=91.5%) and gluconic acid (R^2=47.8%) exhibited quadratic gains in concentration. However both relationships looked similar, so there may have been some form of systematic error in the analysis that produced this unusual result. The gain in gluconic acid was in the same order of magnitude as for the sodium gluconate treated wines, which reinforces the equilibrium shift phenomenon. All the malic acid treatments were significantly lower than the control except for treatment 4, which was the same as the control.

4.4 CONCLUSIONS

4.4.1 NEUTRALISATION

All these results were very disappointing, as this technique was supposed to be the best for the reduction of acidity. A possible explanation for this could be the small amount of tartaric acid in the juice and wine to begin with. With such a small amount of acid to react with the $CaCO_3$, the precipitation of CaT may not have been possible. It was hoped that with such a small amount of tartaric acid, the calcium would then react with the abundant gluconic acid to form a precipitate of calcium gluconate. This was not the case. Of course, as referred in the introduction, one of the problems with neutralisation is that it can take time for the precipitation to occur. So perhaps the juice and wine samples had simply not precipitated completely by the time of analysis.

All the results were shown to be statistically significant, however in real terms the loss of acidity was negligible. The majority of acids decreased quadratically which is in accordance to what would be expected, as there would be a point where the loss in acid content would level off and become constant. It was decided to keep the dosage rates within a commercial range, so it was considered that 5g/L would be an absolute maximum in a winery. However unpublished preliminary studies by Pickering (1993) revealed that with model acid solutions, there was a significant decrease in all acids with dosages in the range of 30g/L. So perhaps the energy barrier for the precipitation of calcium gluconate that can only be surpassed by a very high dosage rates?

4.4.2 SEEDING

All the results were shown to be statistically significant, although, in real terms the loss of acidity was negligible. Most of the treatments showed either an increase or decrease in acid content under a linear relationship. Theoretically one would expect quadratic relationships to show a leveling in the effectiveness of the treatment. Of course, within the parameters used the linear relationship could be the initial reaction to the treatment, and if the dosages were to increase, then an overall quadratic relationship could be observed.

All treatments showed an increase in gluconic acid. Both juice treatments had an increase in acid content by <10%, while the wine treatments showed an increase by <20%. This confirms that the shift in equilibrium between the lactones and the free gluconic acid is a real affect, and one that could require further investigation. Also if the wine or juice is not saturated with respect to gluconate, then the addition of gluconate crystals could result in an increase in gluconic acid concentration. pH and titratable acidity of the treatments followed a trend in relation to the amount of $CaCO_3$ added, and seemed not to have been influenced by the seed crystals. That is, as the $CaCO_3$ content in the application increased compared to the amount of seed crystal, TA decreased, and pH increased.

Some results showed no predictable relationships, or followed a trend that was beyond explanation. These may have been due to a systematic error in the method of detection or merely due to the complex and uncertain nature of the product being dealt with.

CHAPTER FIVE

ANION EXCHANGE

5.1 REVIEW OF LITERATURE

5.1.1 GENERAL INTRODUCTION

Ion exchange in winemaking has been practised for about thirty years and its main use has been in preventing potassium bitartrate deposition. High pH reduces the quality of the wine by giving a "flat" unbalanced palate, dull colour and low resistance to chemical and microbiological spoilage. Ion exchange offers a practical means of achieving pH reduction and lowers pH further than does the addition of tartaric acid to give the same increase in titratable acidity. When tartaric acid is added both hydrogen ions and the weak base, the tartrate anion, are added, whilst with ion exchange, hydrogen ions alone are increased in the wine (Rankine, 1991).

Ion exchange has the promise of being able to stabilise wines quickly and cheaply. This can be performed without the sacrifice in quality that is associated with conventional cellar practices of chemical deacidification. Deacidification by ion exchange eliminates heavy capital investment in refrigeration equipment and allows flexibility in production scheduling.

Australia and California are the main winemaking regions using ion-exchange on an industry scale. There has been a mixed reception for this process in Germany and it is not so widely used (Rankine, 1965). In Australia, the main use for ion exchange has been for the

prevention of potassium bitartrate deposition. To my knowledge, ion exchange has not been used in the New Zealand wine industry, maybe due to the expense into the use of refrigeration. It is in Germany, where excess acidity is a problem, that ion exchange is used to reduce acidity. The basis of this process will be adopted in our objective to reduce gluconic acid in high acid grape juice and reduced alcohol wine.

5.1.2 CHEMISTRY

An ion exchange resin may be defined as an insoluble matrix containing labile ions capable of exchanging with ions in the surrounding liquid without physical change taking place in its structure. Ion exchange resins can be divided into two broad groups, cation and anion exchangers, and these can be further subdivided into weakly acid or basic and strongly acid or basic according to their chemical groupings (Rankine, 1965).

Ion exchange processes are based upon exchange equilibrium between ions in solution and ions of like sign on the surface of an insoluble, high molecular weight solid. Synthetic ion exchange resins were first produced in the 1930's for water softening, water deionisation and solid purification. The most common active sites for cation exchange resins are the sulphonic acid group $-SO_3^-H^+$ (strong acid) and the carboxylic acid group -COOH (weak acid). Anionic exchangers contain tertiary amine groups $-N(CH_3)_3^+OH$ (strong base) or primary amine groups $-NH_3OH$ (weak base).

Historically, ion exchange chromatography was performed on small, porous beads formed during emulsion copolymerisation of styrene and divinylbenzene. The presence of divinylbenzene (usually ~8%) results in cross-linking, which imparts mechanical stability to the beads. In order to make the polymer active towards ions, acidic or basic functional groups are then bonded chemically to the structure (Skoog, 1985).

Ion exchange resins will show a preference for a particular type of ion. This preference is often shown in terms of the 'selectivity coefficient' of the resin, which may be considered as the ion exchange resin equivalent of the 'equilibrium constant' of a chemical system. In a simple system where two ions A and B are exchanged:

$$A_r + B_l \Leftrightarrow A_l + B_r$$

Where r = resin phase, l = liquid phase,

Selectivity coefficient of the resin: $K_B^A = \dfrac{[A]r \bullet [B]l}{[B]r \bullet [A]l}$

That is: $$K_B^A = \frac{(\text{Conc. of ion A in resin}) \times (\text{Conc. of ion B in liquid})}{(\text{Conc. of ion B in resin}) \times (\text{Conc. of ion A in liquid})}$$

Therefore when $K_B^A > 1$ this shows a preference for A

$K_B^A < 1$ this shows a preference for B;

This applies to both anion and cation exchangers (Brady and Humiston, 1986).

The anion exchange resins derive their properties from the amino group and substituted amino groups in the resin structure (Figure 5.1). Weakly basic resins can only be used in neutral or acid solutions, having negligible exchange capacity under alkaline conditions (BDH, 1977).

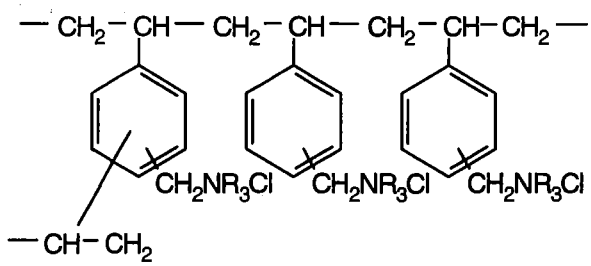


Figure 5.1. Structural formula for anion exchanger.

Weak base resins have a chemistry similar to that of ammonia, the free base form adsorbs strong acids. The application of ion exchange resins can be divided into a number of categories:

- Ion exchange - replacement of one ion in solution with another.
- Elimination - removal of unwanted ions from solution.
- Fractionation chromatography - ions captured on ion exchange column are selectively eluted.
- Neutralisation - addition of acid- or alkali-charged resin to solution (Amerine, 1980; Ough, 1975).

5.1.3 PRINCIPLES

In cool climates such as New Zealand and Germany excess acidity can be caused by incomplete ripening of the grape or insufficient sugar concentration, and reduction of acidity becomes necessary. This is usually carried out by adding calcium carbonate ($CaCO_3$) which precipitates some of the tartaric acid as calcium salt. This process is usually sufficient, but in some cases the addition of $CaCO_3$ can produce off-flavours and also subject the wine to slow precipitation, which inhibits early bottling. Furthermore it does not reduce the concentration of malic acid, which may contribute a considerable proportion of the acidity. Deacidification by anion exchange is an attractive alternative due to a number of reasons:

1. Reduction in acidity can be controlled.
2. Process is simple and does not require a precipitation reaction.
3. Technique can be used online for large scale wine production.

The wine is passed through a weakly basic anion exchange resin, usually in the hydroxyl form (Rankine, 1965). As the wine passes through the resin, the various anions are replaced with the hydroxyl ions thus reducing acidity.

Neutralisation of high acid in wines involves a weakly basic anion exchange resin. The weakly basic groups present in the resin neutralise the natural fruit acids which are commonly tartaric, malic and citric acids. This type of resin is easily regenerated with a solution of sodium hydroxide. The advantages of treating wine by ion exchange as opposed to conventional methods for stabilisation and acidity reduction lie in the unit-process nature of columnar ion exchange procedures (Percival, McGarvey and Sonneman, 1958).

In the past, most anion/cation exchange techniques used to adjust the acidity of wine, involved an anion exchange resin in the hydroxyl form. Bonorden, Nagel and Powers (1986) employed an anion/cation exchange treatment for the adjustment of high pH/high TA wines. The method involves charging the anion exchange with tartaric acid, placing it in the tartrate form. The tartrate would exchange with the malate anion. This would result in both pH and TA reduction because of the substitution of a stronger acid for a weak acid. This approach was applied to high gluconic acid juice and wine, where hopefully the tartrate would exchange with the gluconate anion.

There is no difficulty in the deacidification of wine in the normal way with calcium carbonate, when there is sufficient time. However, some wineries operate for the most rapid possible turnover of wine. It is not unusual to run short of old stock before the young wines of the new vintage must be used. Under such circumstances the main difficulty consists in that almost all wines have to be deacidified in a short time to make them suitable for consumption and to prevent tartrate precipitation in the bottle.

The anion exchanger has certain advantages over other methods of deacidification. It can be used repeatedly, thus reducing cost. The resin removes both malic and tartaric acid, while only tartrate is removed by chemical means (Moser, 1956). The use of anion exchange resin is currently less popular than cation exchangers.

There is conflicting information concerning the merits of treating wines with anion exchange resins. Rankine (1965) stated that wine deacidification by ion exchange was not comparable to the calcium carbonate procedure. In contrast, Moser (1956) reported that wines deacidified by anion exchange did not alter any sensory attributes and in fact, that the ion exchanged wines tasted better than the calcium carbonate treated wines. This finding is reinforced by Dickinson and Stoneman, (1958), who used cation exchange to stabilise wine with respect to tartrate. Most of the experts in his tasting panel were unable to select the wines subjected to the ion exchange treatment with a frequency sufficiently great to be of statistical significance.

Commercial anion exchange resins were evaluated in Canada for their influence on wine quality and degree of deacidification (Zubeckis, 1962). One of the resins tested included the Amberlite IR-45 which is now superseded by the resin we used; Amberlite IRA-93. Their characteristics are very similar, so it was promising to find that the results showed the treated wines had improved in flavour, although colour and bouquet was found to decrease. In another study, Zubeckis (1958) reported that the change in sensory quality of the treated wines was hardly detectable when the deacidified wines were mixed with the original wine to a desired acidity.

The GOD/CAT treatment, as mentioned before, converts glucose to gluconic acid, so the final juice and wine product has a very high acid content. The disadvantages of too high an acidity are noted by tartrate precipitation in the bottle and by too sour a taste (Moser, 1956). The objective of this study was to try and selectively reduce the gluconic acid content in the juice/wine.

5.2 MATERIALS AND METHODS

5.2.1 ANALYTICAL METHODS

The pH and TA were determined with a Metrohm 670 Titroprocessor coupled with the Metrohm 665 Dosimat, an intelligent burette (Metrohm Ltd, CH-9100 Herisau, Switzerland). Organic acid analysis was by a high performance liquid chromatography technique that was optimised for the detection of gluconic acid (refer Chapter 3). The samples were detected using a Waters 490E Multiwavelength Detector set at 210nm and the peak heights were determined with the Millennium 2010 Chromatography Manager (Millipore Corporation, Waters Chromatography Division, Milford, MA).

5.2.2 PREPARATION OF HIGH ACID JUICE AND REDUCED ALCOHOL WINE

Müller Thurgau juice was obtained from Geisen Wines of Canterbury, New Zealand. The juice had a pH of 3.15, TA of 6.94g/L, soluble solids of 18.0° Brix and a total SO_2 concentration of 45.6 ppm. The juice was then treated with 1.5g/L GOD/CAT enzyme, aerated with oxygen at 8g/L while being stirred for 10 hours. Upon completion the juice had a pH of 3.15 and TA of 26.46g/L. Half of this juice was then inoculated with *Saccharomyces cerevisiae str.* Fermivin® SF (Gist-brocades, Seclin cedex, France) and fermented at 12°C.

Upon completion of the primary fermentation, the wine had a pH of 3.21 and TA of 19.34g/L. The temperature of the wine was reduced to 2°C and cold stabilised at that temperature for 48 hours. The wine was then treated with 40 ppm SO_2 (5% Sulphurous acid, BDH Chemicals Ltd, Poole, U.K.).

5.2.3 ANION EXCHANGE

Unused Amberlite IRA-93 (Sigma Chemicals Co., St Louis, MO) was washed and fined in distilled water. A glass column (2.5 cm i.d.) was packed with IRA-93 to a predetermined volume of 71.4cm^3, wet volume, necessary for the treatment of 500ml of juice/wine (see **Results and Discussion**). The column was washed in series with 3L of distilled water, 750ml of 10% (v/v) ethanol solution, and 1.25L of distilled water.

To prepare the column for treatment of juice/wine the following procedure was used: 250ml of 1N NaOH was passed through the column. The column was rinsed with distilled water to a pH of 8 to 9 and placed in the tartrate form by running 1N tartaric acid over the column. The column was then rinsed with 3 bed volumes of distilled water. Since aeration of wine and juice is possible due to the handling involved, the column and lines should be flushed with inert gas beforehand.

One bed volume of juice/wine was passed through the column and discarded. The treated wine/juice was refrigerated until a temperature of approximately 2^0C was reached, seeded with 1g/L potassium bitartrate, cold stabilised for a minimum of 48 hours, decanted and stored at 2^0C. The resulting solution was then backblended at various levels to produce a more balanced wine/juice.

5.3 RESULTS AND DISCUSSION

5.3.1 RESIN CAPACITY

An experiment was performed to determine the capacity of the tartrate anion exchange technique. The technique was used to reduce the gluconic acid in the wine/juice by replacing the weaker (gluconic) acid with the relatively stronger tartaric acid and by removal of tartaric acid from solution by the precipitation of potassium bitartrate. The reduction in acid content was due to the removal of the bitartrate anion as the insoluble potassium salt.

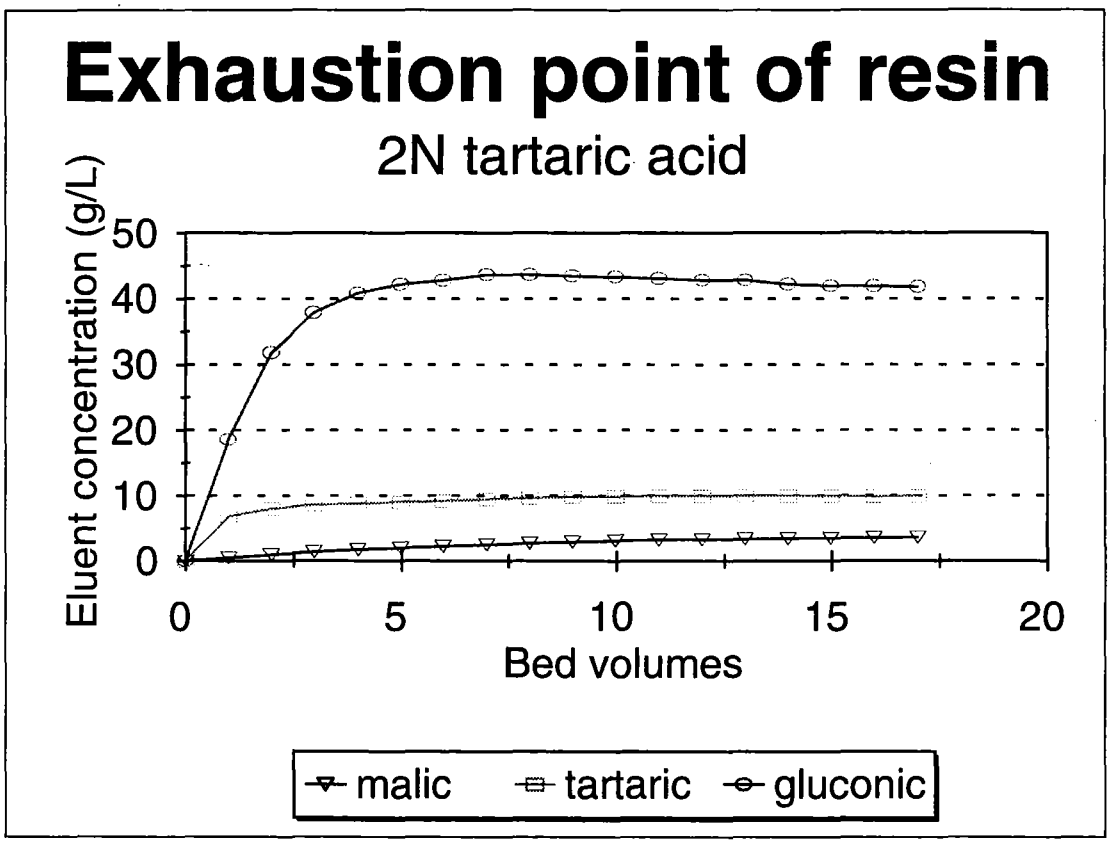


Figure 5.2. Capacity of resin

It was determined that 7 bed volumes was exchanged effectively by 2.61cm^3 of resin. This was the point where the slope of the reduction in acid concentration began to decrease (Figure 5.2). This allowed the estimation of the exhaustion point of the anion exchange column.

5.3.2 CHARGING CAPACITY

Another experiment was conducted to determine the capability of anion exchange by varying the charging capacity of the resin. An acid solution, pH adjusted, was eluted through the varyingly charged resin (Figure 5.3) and it was decided to use a 2*N* tartaric acid solution to charge the column prior to sample application.

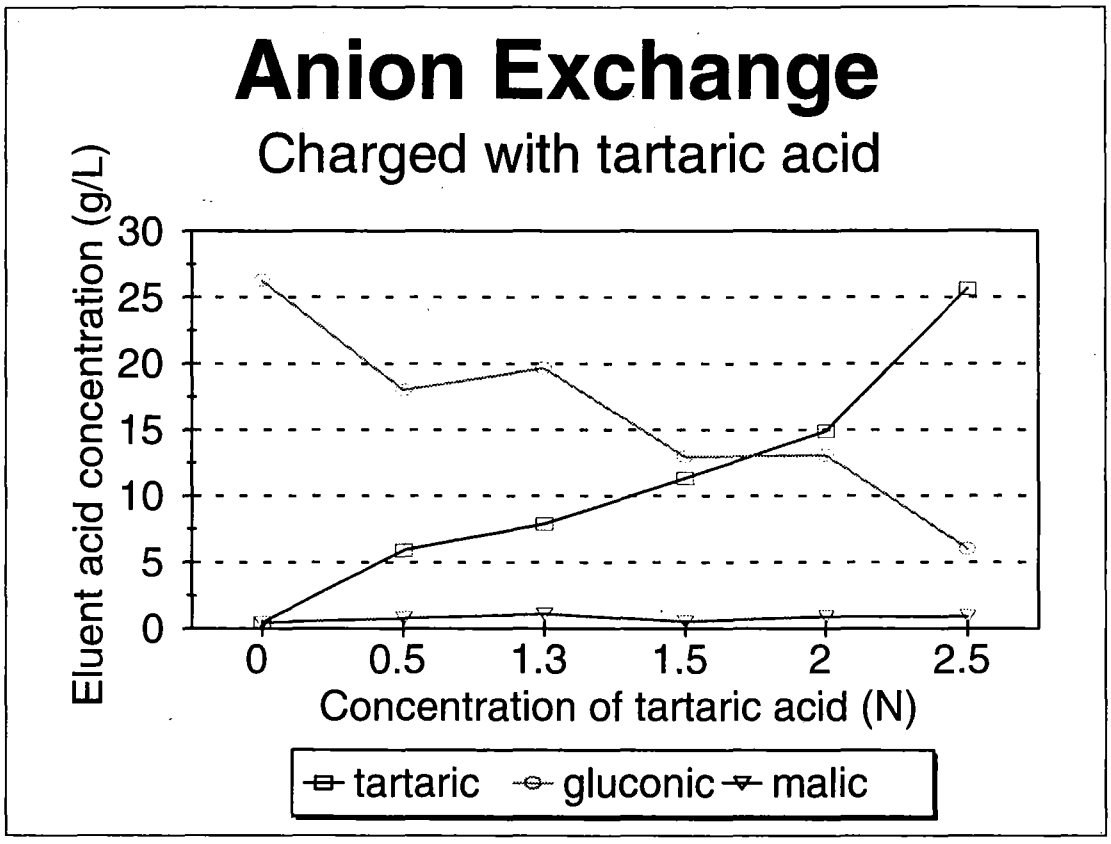


Figure 5.3. Charging capacity of resin

5.3.3 DEACIDIFICATION

Preliminary trials using model solutions were investigated. A model solution of 50g/L gluconic, 10g/L tartaric and 5g/L malic acid was buffered to pH 3.1 with concentrated NaOH. This model solution was then passed through the tartrate charged resin following the same conditions outlined above. After cold stabilisation the HPLC results showed that there was a significant reduction in all three acids. There was a 64% reduction in tartaric acid, 84% reduction in gluconic acid and 96% reduction in malic acid (Appendix 5.1). These results showed significant promise especially with regards to gluconic acid.

Figures 5.4 and 5.5, and Tables 5.2 and 5.3, show the changes in acid concentration for wine and high acid grape juice before and after the anion exchange treatment, and after seeding with potassium bitartrate and cold stabilisation for 48 hours. The data indicates that initially the weaker acids in the wine did exchange with the tartrate on the column. The pH and titratable acidity for the wine/juice samples are shown in Table 5.1.

TABLE 5.1. pH and TA of Wine and Juice, before and after Anion Exchange Treatment[a].

	Wine	Juice
pH before	3.2	3.1
pH after	3.3	2.8
TA before (g/L)	20	26
TA after (g/L)	17	28.5

a. Mean values of triplicate analysis of duplicate treatments.

Wine may be treated in two ways, either by treating a batch until the exhaustion point is reached, then blending this with untreated wine, or by treating the wine and passing it back

into the same vessel which is kept stirred. Both methods are in use commercially (Rankine 1986).

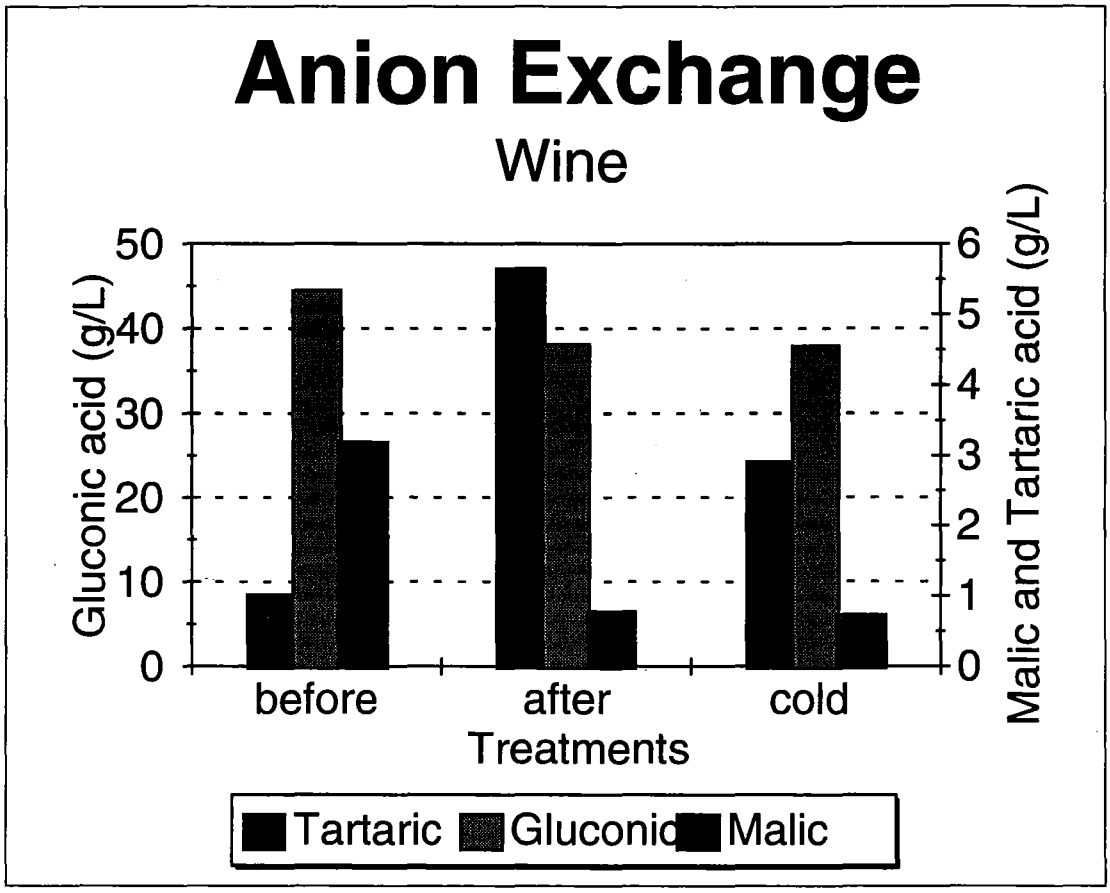


Figure 5.4. Effect of anion exchange on organic acid concentration in reduced alcohol wine.

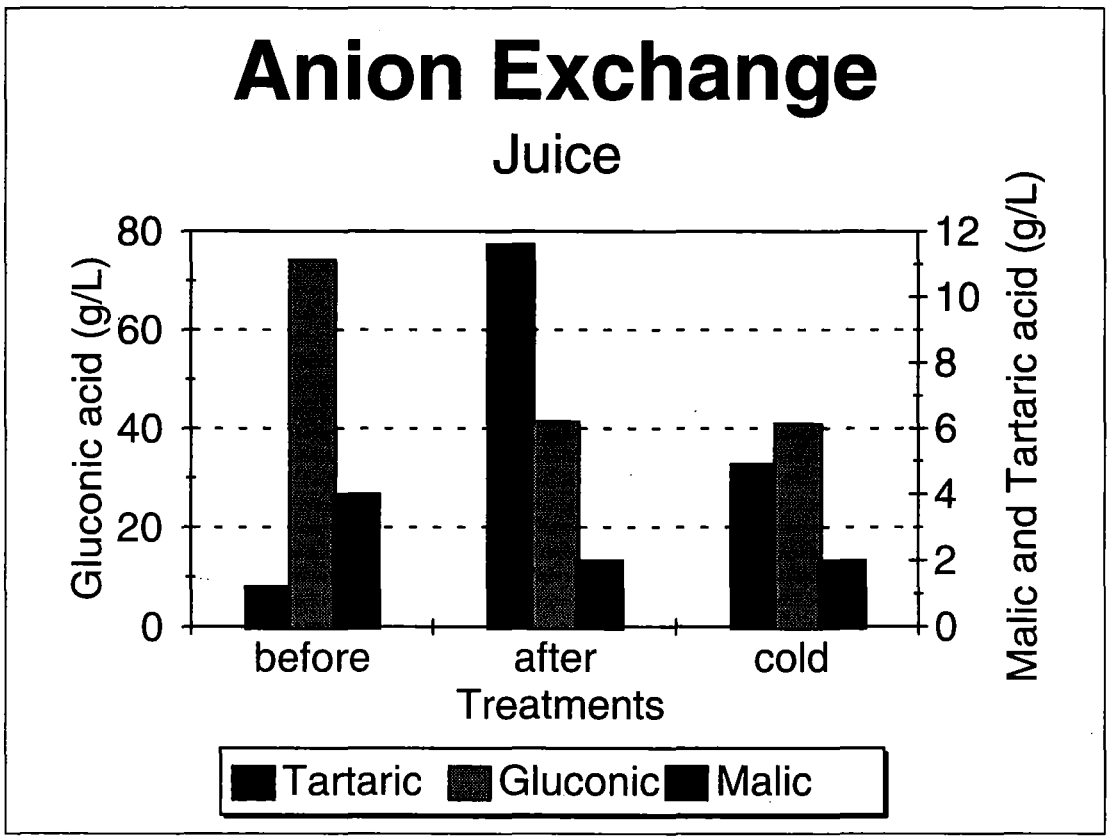


Figure 5.5. Effect of anion exchange on organic acid concentration in high acid juice.

The treated juice/wine produced here was backblended with untreated product. The ion exchange treatment seriously reduced the colour of the liquid and thus backblending was needed to compensate for this. This conforms with the observations made by Rankine (1965). Aroma loss did not seem to be a problem, and backblending would have also ensured that this was not a problem.

Table 5.2. Anion Exchange of High Acid Juice.

	Before anion exchange	After anion exchange	After cold stabilisation	CV%	Δ%
Gluconic acid	74.2[a]	41.3[b]	40.9[c]	1.92	-44.9
±SD	1.00	0.12	0.07		
Tartaric acid	1.2[a]	11.6[b]	4.9[b,c]	1.13	+308.3
±SD	0.028	0.17	0.09		
Malic acid	4.0[a]	2.0[b]	2.0[b,c]	2.01	-50.0
±SD	0.086	0.06	0.07		

Figures quoted are in g/L and are the mean values of triplicate analysis of duplicate treatments.
Values designated by the same letter do not differ significantly ($p>0.05$) for each acid. Values designated by different letters differ significantly at $p<0.001$.

Table 5.3. Anion Exchange of Reduced Alcohol Wine.

	Before anion exchange	After anion exchange	After cold stabilisation	CV%	Δ%
Gluconic acid	44.5[a]	38.1[b]	37.9[c]	1.68	-14.8
±SD	0.36	0.52	0.38		
Tartaric acid	1.02[a]	5.65[b]	2.91[b,c]	1.16	+185.3
±SD	0.018	0.09	0.017		
Malic acid	3.19[a]	0.78[b]	0.73[b,c]	1.58	-77.1
±SD	0.042	0.003	0.0035		

Figures quoted are in g/L and are the mean values of triplicate analysis of duplicate treatments
Values designated by the same letter do not differ significantly ($p>0.05$) for each acid. Values designated by different letters differ significantly at $p<0.001$.

5.4 CONCLUSIONS

It has been demonstrated that the tartrate anion exchange technique can be used for adjusting acidity of high acid juice and wine. Hypothetically, this procedure could reduce the total anion concentration of a wine by a factor equivalent to the amount of the total anions represented by gluconate, providing potassium does not become the limiting factor during the precipitation of KHT. This technique could be improved by decreasing this flow rate, however, this is probably economically unviable. Also, increasing the ratio of resin to wine/juice may not be practical because a given volume of wine would be exposed to such a large amount of resin it would be virtually stripped of its character , the achieved reduction in acid of the liquid would be offset by the loss of colour and aroma. This may be amended by backblending, but sensory evaluation of the final product would need to be conducted.

The anion exchanged grape juice decreased in gluconic acid concentration from ~75g/L to ~40g/L. If this juice was to be fermented, the acid content could drop another 20-30g/L (Table 4.1). Assuming no inhibitory effects, this concentration level of gluconic acid would then become well within a palatable level.

The efficiency of the technique depends on the total gluconate concentration and the ratio of gluconate to tartrate in the wine/juice since the ratio of gluconate to tartrate in the wine/juice determines the extent of exchange of gluconate for tartrate on the column.

Research is needed to identify a functional resin which will not affect the sensory quality of the wine/juice.

CHAPTER 6

OVERALL CONCLUSION

6.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The ion exclusion-partition mode of operation has been shown to be suitable for the separation of the three organic acids of interest. Due to the amount of gluconic acid present in the samples, it was necessary to inject as small a volume as possible, to obtain adequate resolution of the closely eluting gluconic and tartaric acid peaks. No elaborate sample preparation was required, although long term effects of injecting filtered wine and juice samples directly onto the column have not been examined. Sample preparation, injection and chromatography can be completed in 45 minutes. All the organic acids absorbed at 210nm, allowing the use of the sensitive variable wavelength UV detector.

The results from the recovery efficiency, linear regression analysis, reproducibility and column stability show that this HPLC method can quantitatively analyse gluconic as well as tartaric and malic acids in high acid grape juice and reduced alcohol wine. The results indicate the potential this HPLC method has for the monitoring of the acidity in wine products, and to determine whether a deacidification technique has been beneficial to the juice or wine.

One of the difficulties in the detection of gluconic acid was the development of lactones during the final stages of this experiment. The two glucono-lactones do have a sweet acid

taste and therefore would need to be quantified with the free acid peak (refer Chapter 1). As these lactone peaks were not present during the preliminary studies of gluconic acid, they were not accounted for during the course of this thesis.

6.2 CHEMICAL DEACIDIFICATION

6.2.1 NEUTRALISATION

Of the two chemical agents used $CaCO_3$ was found to be more effective than Na_2CO_3 in model solutions. However with the treatment of high acid juice and reduced alcohol wines, there was little change in the acid content after treatment with $CaCO_3$. With high acid juice, tartaric acid was reduced by 6%, malic acid by 7% and gluconic acid by a mere 4%. This trend was replicated with the reduced alcohol wine where tartaric acid was reduced by 6%, malic acid by 4% and gluconic acid by 4%.

Neutralisation and seeding trials involved a period of cold stabilisation and this was found to have a real effect on the gluconic acid content. With a shift in equilibrium from the lactone form to the free gluconic acid form, and the acid content was found to increase over time by ~5.0%. Also observed was the lack of precipitation of gluconate crystals, implying that the wine and juice samples were not saturated with respect to gluconate. The addition of crystals would increase the gluconic acid concentration due to the solubility of calcium and sodium gluconate in high acid juice and reduced alcohol wine.

As reported by McKinnon (1993), Abgueguen *et al.* (1993), and Clark *et al.* (1988), Malic acid is a serious inhibitor of the seeding process and interacts with the calcium. As there was

significantly more malic acid than tartaric acid in the wine and juice samples, this affect may have been very real in the treatment with $CaCO_3$ and seed crystals.

6.2.2 SEEDING

Calcium gluconate and sodium gluconate seed crystals were used with $CaCO_3$ to induce crystallisation in high acid juice and reduced alcohol wine. Both these trials were unsuccessful in achieving significant reduction in any of the organic acids. There are a number of reasons why this may have been the case:

1. The seed crystal and $CaCO_3$ mixture was made up to a concentration of 1g/L to keep within a commercial application. However this may not have been adequate enough to ensure formation of the critical nuclei or perhaps the stable nucleus was formed but there was not enough calcium or sodium gluconate molecules to induce precipitation.
2. As mentioned before, malic acid has been found to have an inhibitory effect on the precipitation of calcium tartrate. This would probably apply to calcium gluconate as the malic acid interacts with the calcium.

The trend of the equilibrium shift from the glucono-lactones to the free gluconic acid due to the temperature change in the cold stabilisation process, continued for the seeding trials.

6.3 ANION EXCHANGE

Of all the deacidification techniques employed, anion exchange appeared to have the most potential. With model solutions, tartaric acid was reduced by 84%, malic acid by 94%, and gluconic acid by 98%.

With reduced alcohol wine gluconic acid was decreased by 15%, and malic acid, by 77%. In contrast tartaric acid increased by 185% from 1.02g/L to 2.91g/L, which is still well within acceptable tartaric acid content levels. The anion exchanged grape juice decreased in gluconic acid content from ~75g/L to ~40g/L. If this juice was to be further fermented, the acid content could drop another 20-30g/L as seen in Table 4.1, and the concentration of gluconic acid could then become well within a palatable level.

However one drawback of this deacidification method is the effect the resin matrix of the anion exchange column has on the composition of the grape juice or wine. In this experiment, the resin seriously affected the colour of the liquid. On a commercial scale, and as long as the wine was not of high quality, then backblending with untreated wine could compensate for this.

6.4 FURTHER RESEARCH

Research into the deacidification of gluconic acid in high acid grape juice and reduced alcohol wines could focus more on anion exchange technology.

Another procedure used for the adjustment of acidity is the addition of sugar or sweet reserve. This method does not alter the acidity, but creates a more harmonious relationship between the high acid and sugar already present, therefore producing a more palatable wine. However there are some limitations with this method; firstly there could be no dry-style wine produced by this method. Also there is the risk of refermentation if the wine has not been membrane filtered adequately enough to remove all the yeast cells.

In conclusion, this technology used for the production of reduced alcohol wine through the enzymatic conversion of glucose to gluconic acid can only have market potential if the final product is palatable and retains all the characters found in standard wine. At present this technology produces a reduced alcohol wine of ~8% alc.(v/v) while retaining aromatic and colour components of the varietal. However, the excess acidity makes the wine unpalatable, therefore more research is needed in the reduction or masking of this acid, for any hope of the commercial production of this reduced alcohol wine.

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APPENDICES

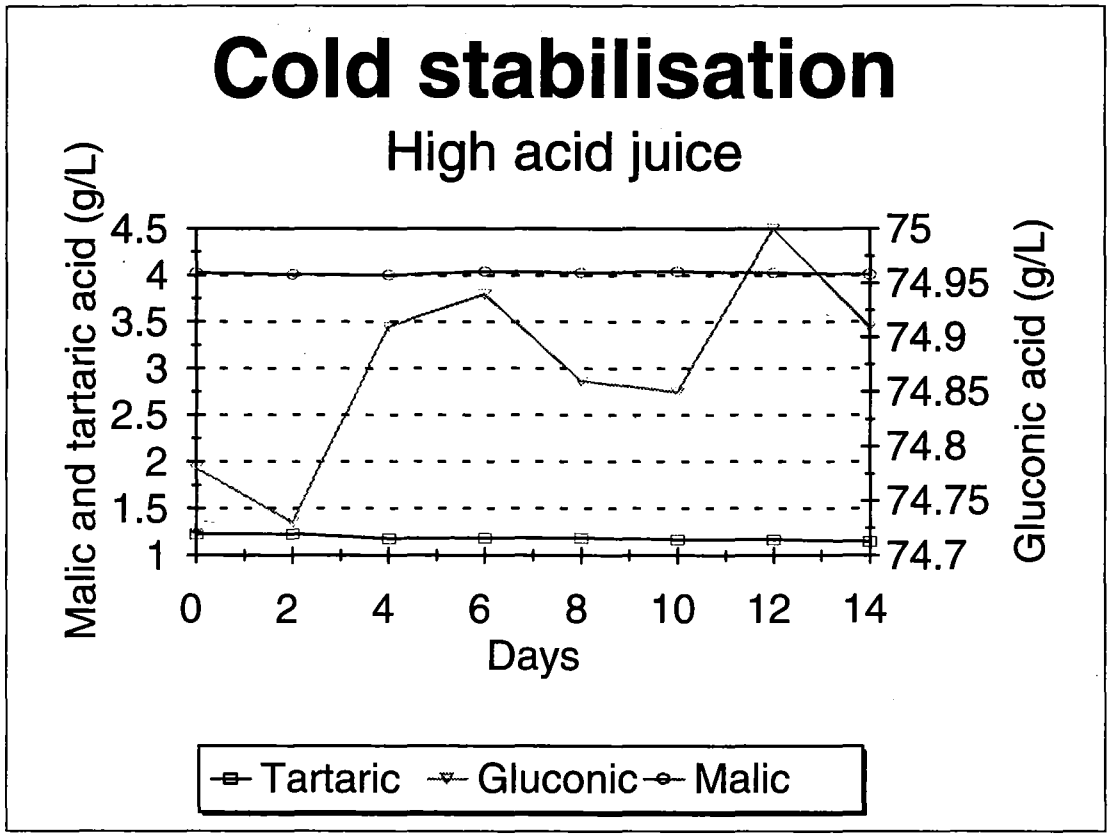

Figure 2.1. Behaviour of organic acids in high acid juice during cold stabilisation.

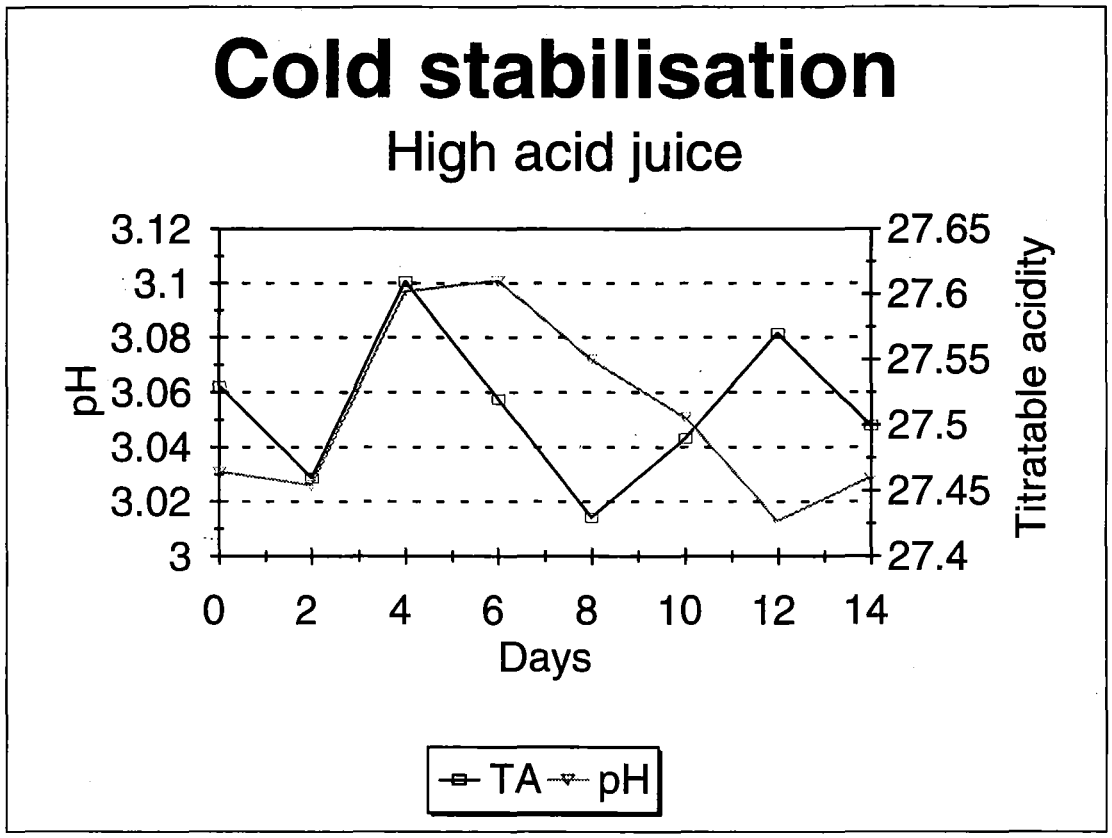

Figure 2.2. pH and titratable acidity during cold stabilisation of high acid juice.

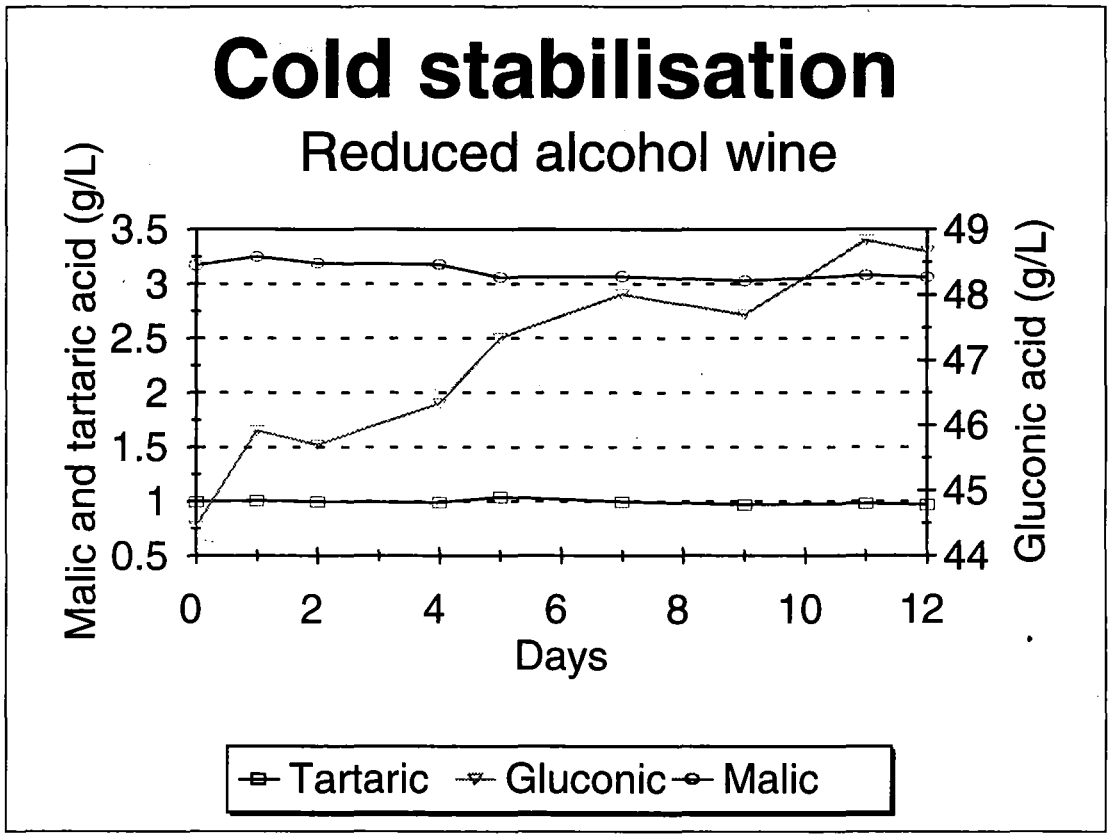

Figure 2.3. Behaviour of organic acids in reduced alcohol wine during cold stabilisation.

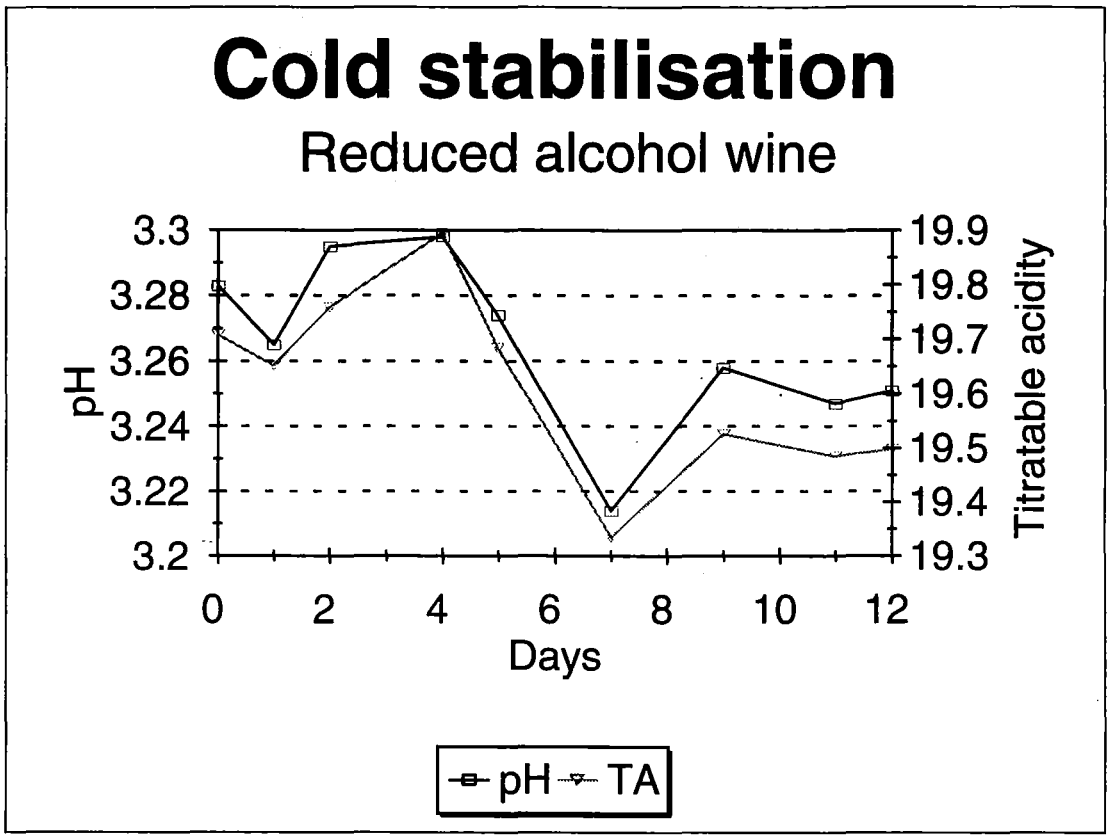

Figure 2.4. pH and titratable acidity during cold stabilisation of reduced alcohol wine.

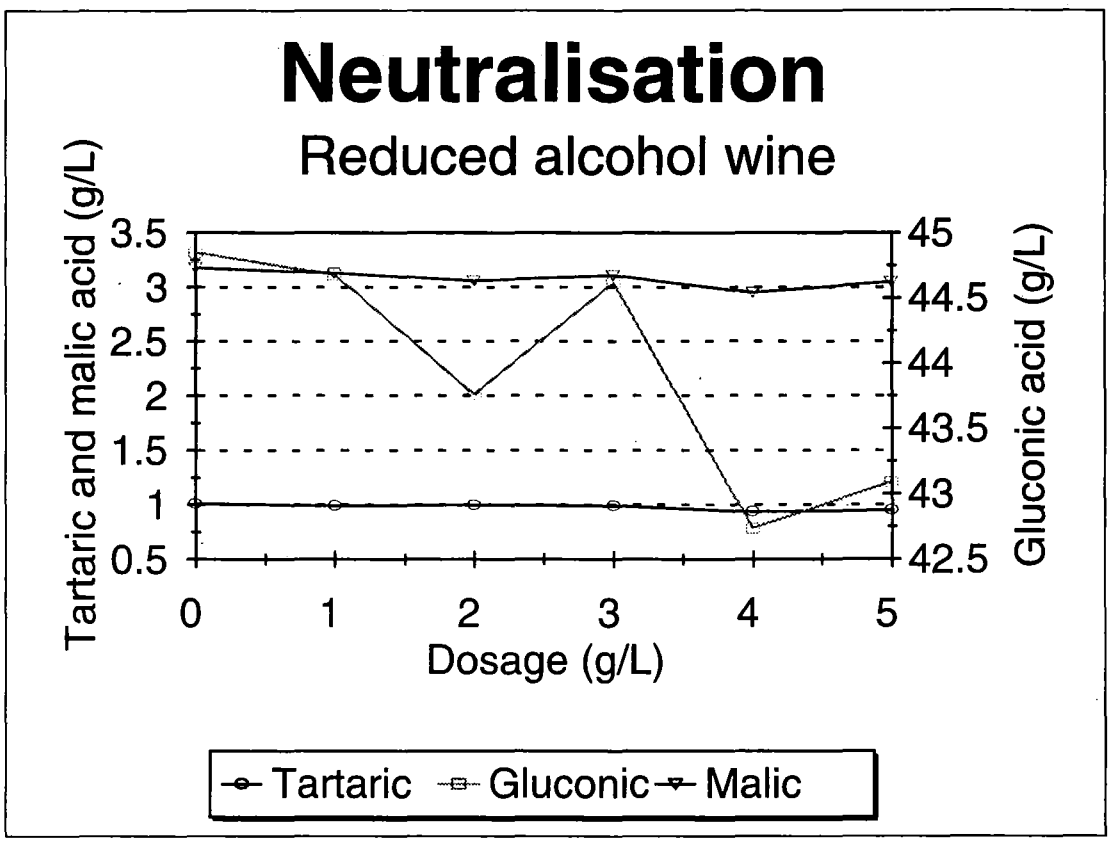

Figure 3.1. Behaviour of organic acids during neutralisation of reduced alcohol wine with increasing dosage of calcium carbonate.

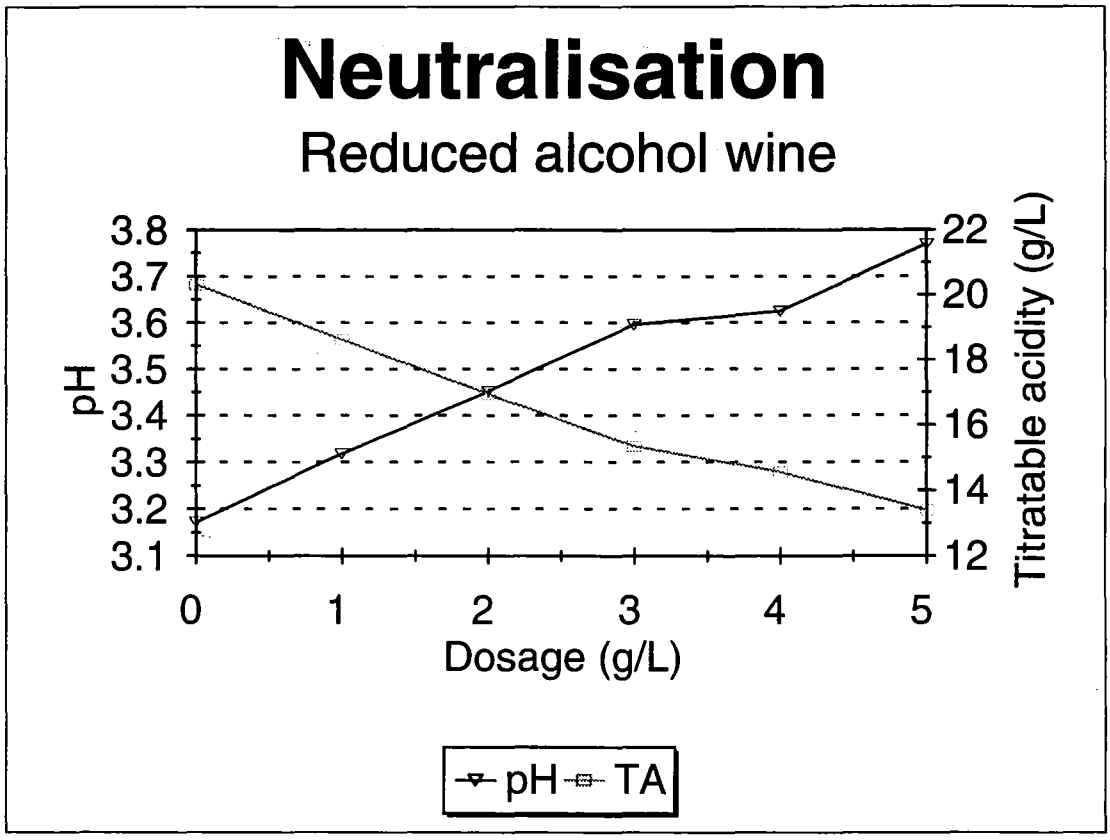

Figure 3.2. pH and titratable acidity during neutralisation of reduced alcohol wine.

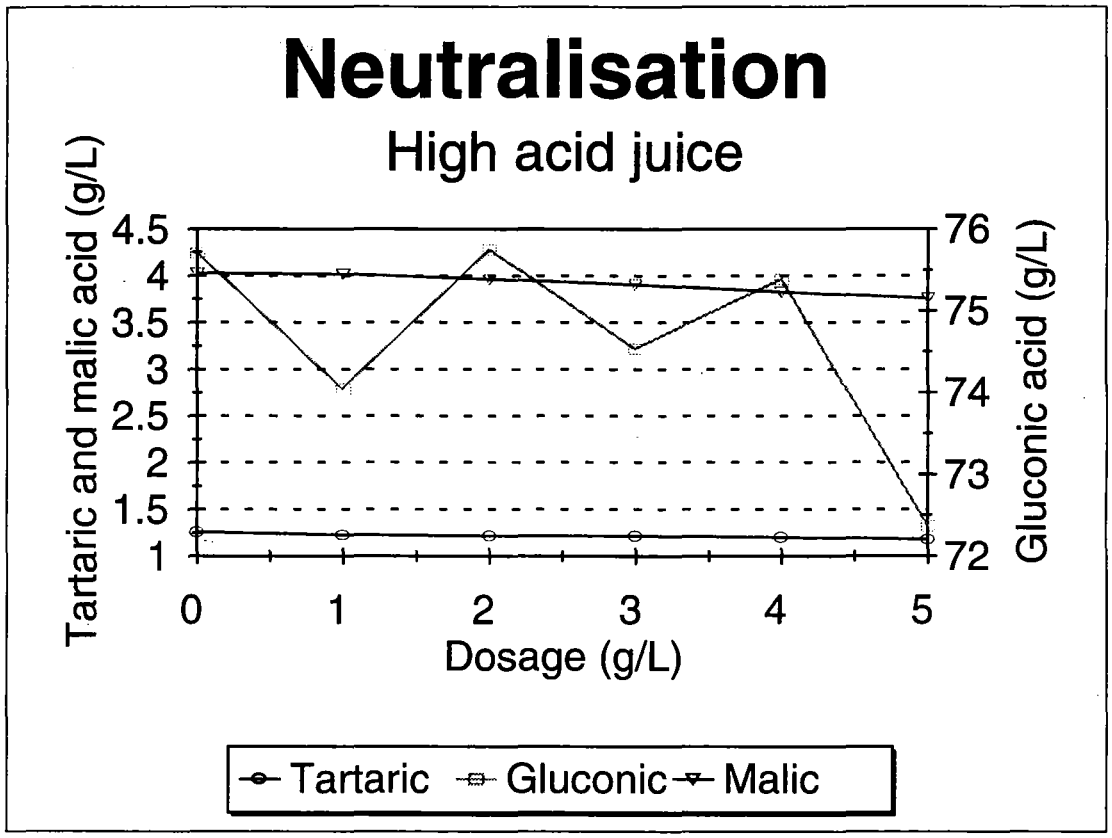

Figure 3.3. Behaviour of organic acids during neutralisation of high acid juice with increasing dosage of calcium carbonate.

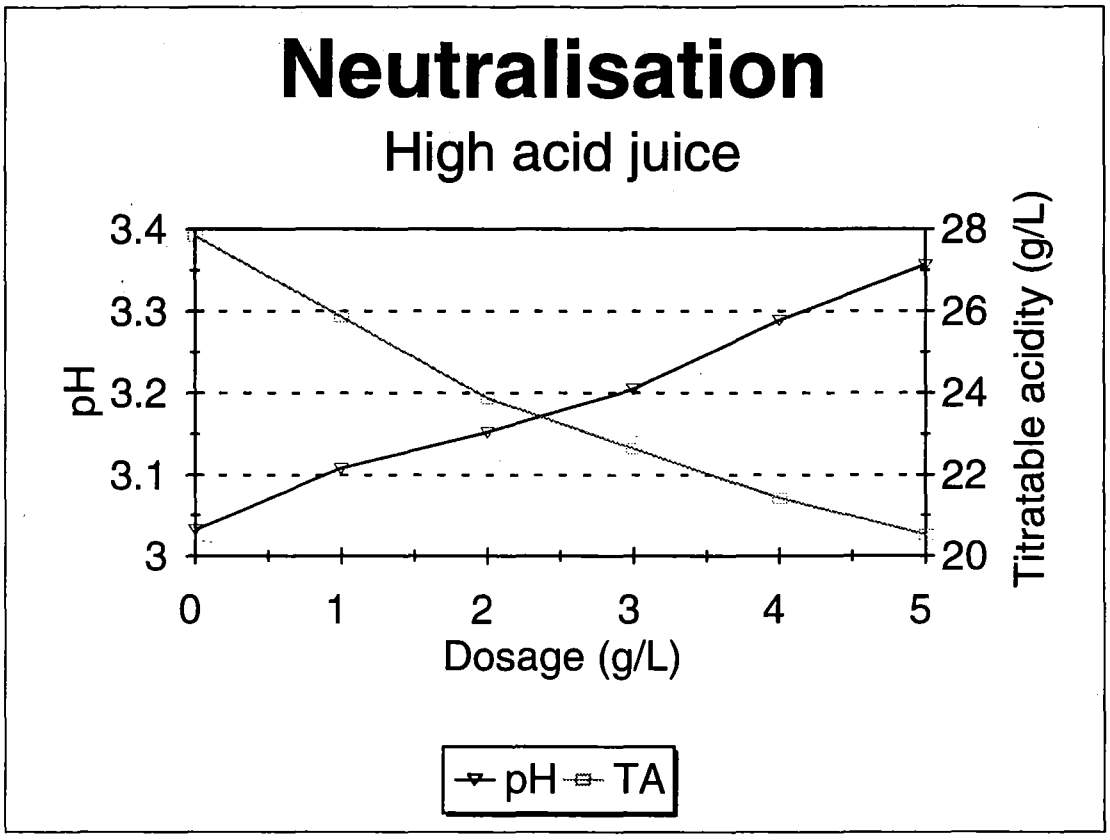

Figure 3.4. pH and titratable acidity during neutralisation of high acid juice.

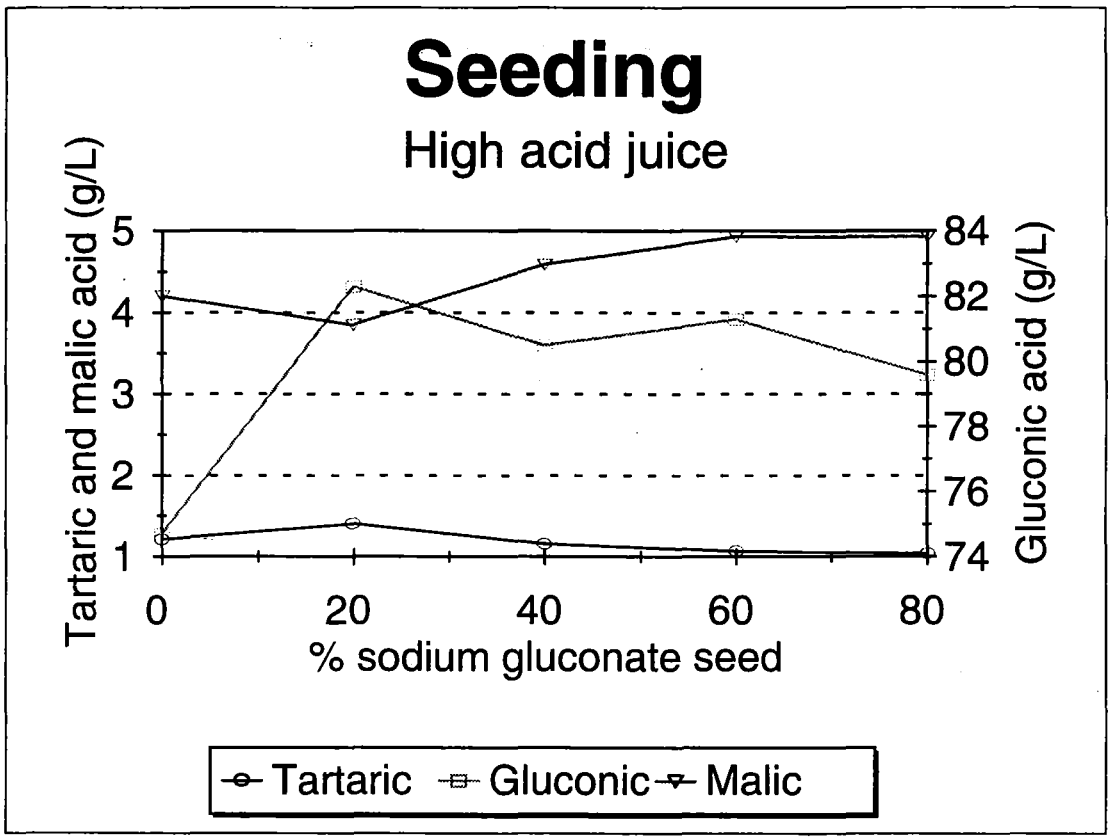

Figure 3.5. Sodium gluconate seeding of high acid juice. Calcium carbonate was added proportionally to make up a 1g/L dosage application.

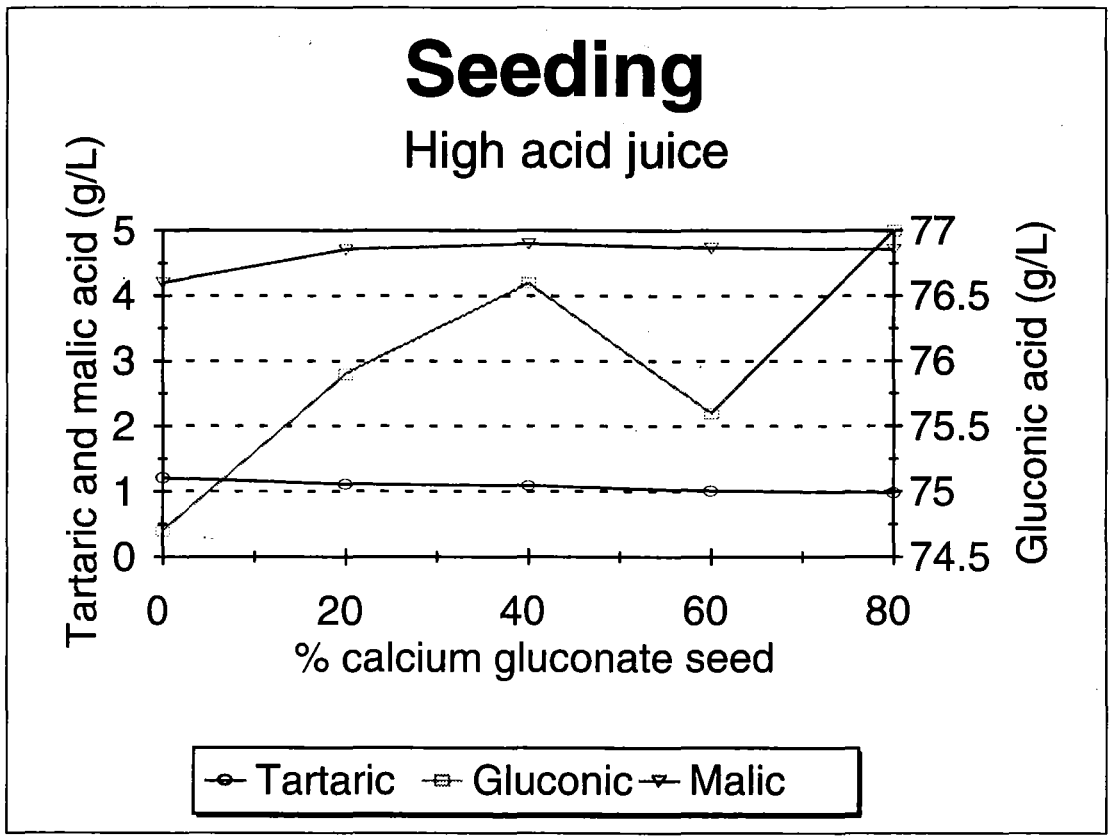

Figure 3.6. Calcium gluconate seeding of high acid juice. Calcium carbonate was added proportionally to make up a 1g/L dosage application.

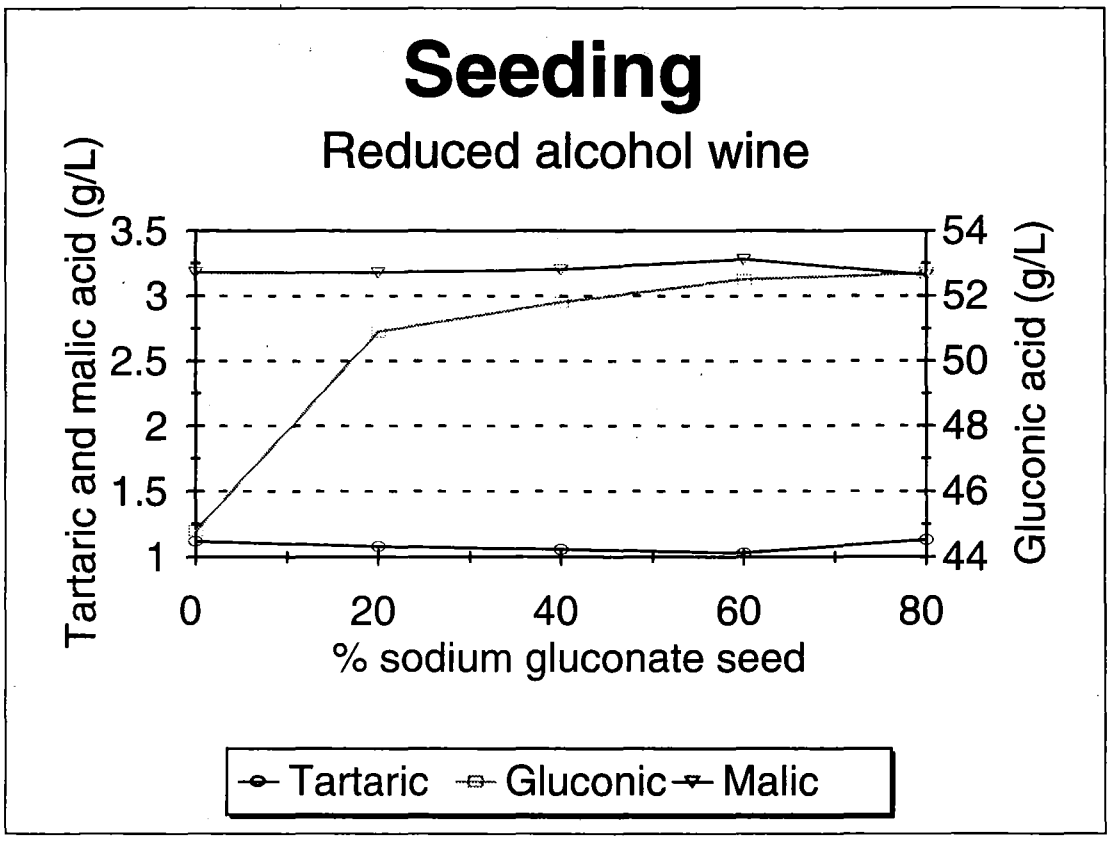

Figure 3.7. Sodium gluconate seeding of reduced alcohol wine. Calcium carbonate was added proportionally to make up a 1g/L dosage application.

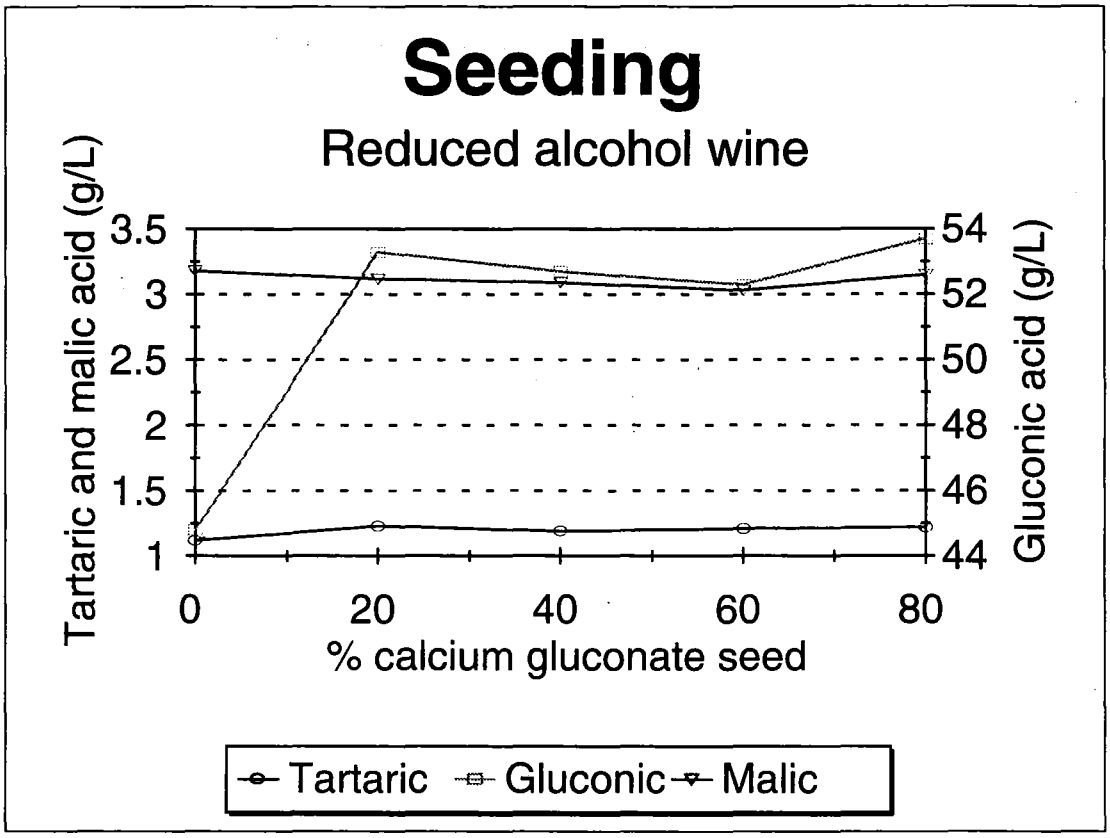

Figure 3.8. Calcium gluconate seeding of reduced alcohol wine. Calcium carbonate was added proportionally to make up a 1g/L dosage application.

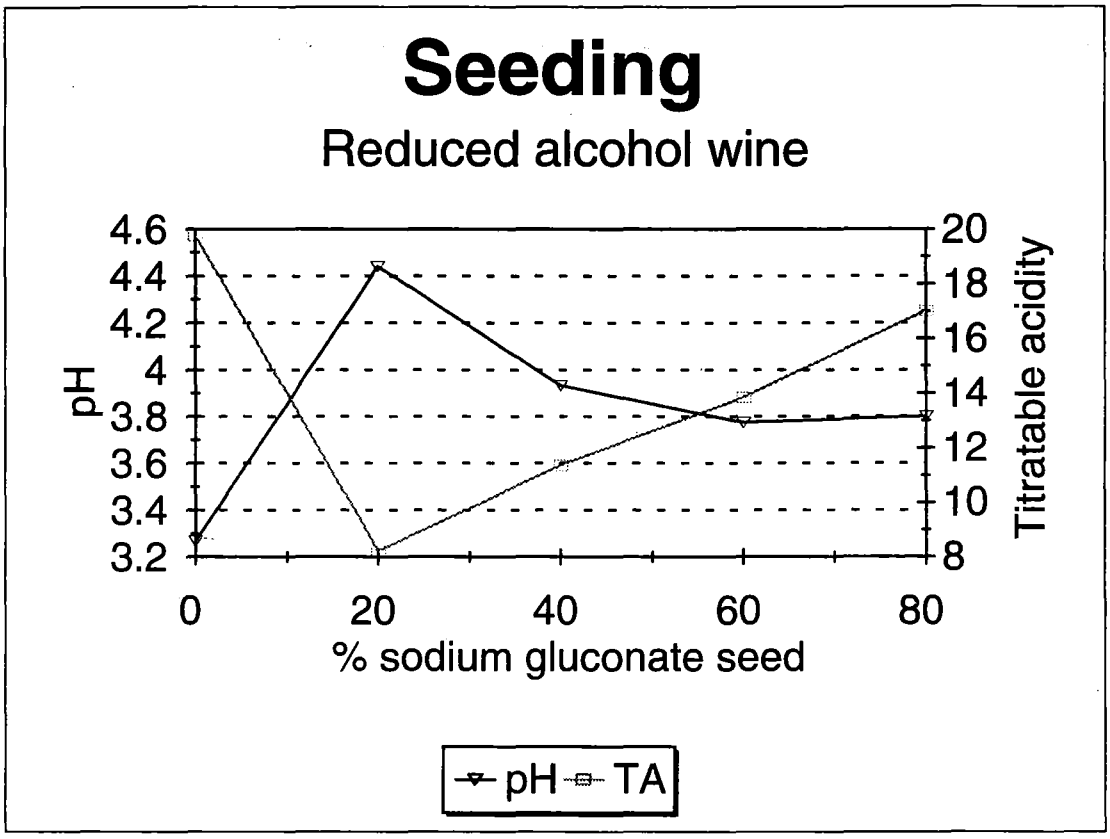

Figure 3.9. pH and titratable acidity of sodium gluconate seeded reduced alcohol wine.

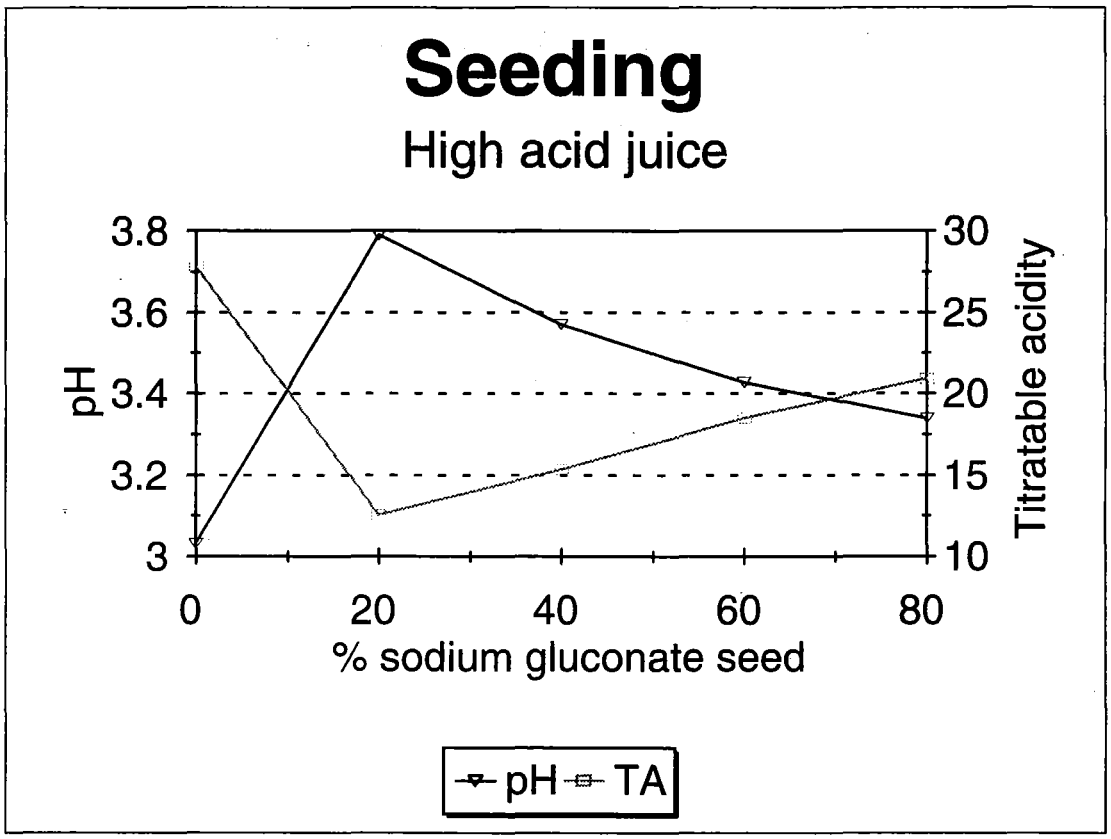

Figure 4.0. pH and titratable acidity of sodium gluconate seeded high acid juice.

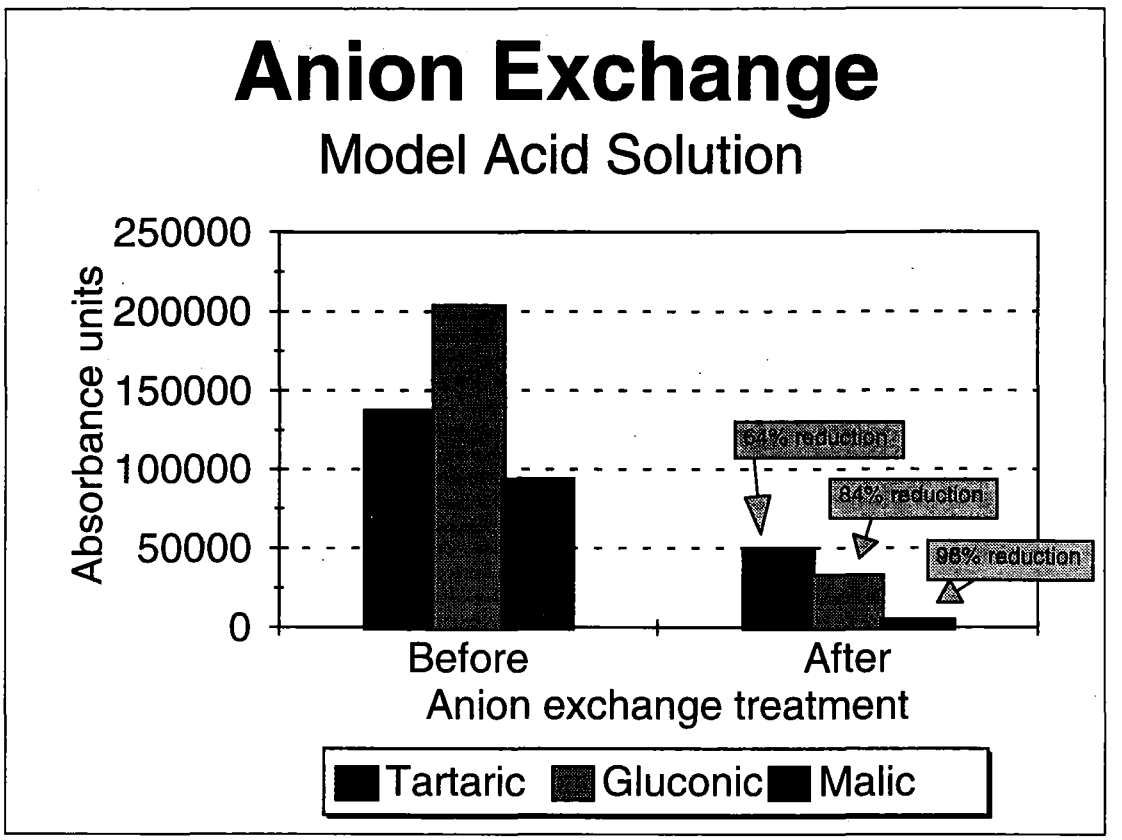

Figure 5.1. Anion exchange treatment of model acid solution buffered to pH 3.1.