A STUDY OF THE ISOMERISATION OF ACONITIC ACID WITH REFERENCE TO SUGARCANE PROCESSING

by

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ABSTRACT

Chromatographic methods were used to determine the absolute values of *cis*-aconitic acid and *trans*-aconitic acid present in sugar cane factory processing streams and to determine the rates of isomerisation from the *trans*-aconitic acid to the *cis*-aconitic acid isomer.

A reproducible, solid phase, ion exchange extraction method was developed to isolate the organic acids found in sugar factory process streams. The isolated acids were quantified using a dual ion-exclusion column, high performance liquid chromatographic method. To improve resolution of the acids, columns were maintained at different temperatures, whilst the combined use of both ultra-violet (UV) and refractive index detection proved useful in peak identification. Concentrations of the aconitic acid isomers were used to calculate the *cis/trans* aconitic acid isomer ratio occurring across the different processes found in a sugar cane factory. *trans*-Aconitic acid was found to be the predominant form present in the cane entering the factory. Analysis showed that isomerisation of the *trans*-aconitic acid to the *cis*-aconitic acid isomer occurred during processing.

To understand and model this reaction, a reproducible experimental isomerisation method was developed making use of buffers to maintain pH conditions during experiments. A chromatographic analysis method, using ion-exclusion chromatography and UV detection, was developed to analyse the isomerisation reaction mixture. Chromatography was used in both an on-line and off-line mode for quantitation of the isomers.

The method was used to study the isomerisation under conditions similar to those found in the factory. These included pH, temperature, ionic strength and the presence of monovalent and divalent cations found in sugar cane juices. It was shown that the isomerisation is a first order reversible reaction under the conditions studied. Temperature and pH were shown to be the important isomerisation variables. Temperature enhances the rate of isomerisation of the *trans*-aconitic isomer to the *cis*-aconitic acid isomer whilst pH affects the ultimate *cis/trans* aconitic acid ratio attained. Ionic strength was found to be a relatively unimportant factor.

The presence of divalent and monovalent cations, at concentrations usually found in cane juice, was shown to have little effect on the rate of isomerisation. Activation parameters, including the activation energy (E_a), pre-exponential factor (log A), enthalpy ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$), were calculated at each combination of buffer concentration and pH used in the experimental procedures. The values recorded are of a similar value to those reported for structurally similar compounds.

cis-Aconitic acid was shown to undergo decarboxylation to itaconic acid. This occurred at low pH values and high temperatures. A detailed study was not undertaken since the conditions under which it occurs are considered extreme from the viewpoint of a sugar technologist.

A model describing the equilibrium *cis/trans* aconitic acid isomer ratio was developed as a function of pH, temperature and time from the kinetic results. This was used to predict the equilibrium ratio for the aconitic acid isomers at the output of various processes in the sugar factory. Given the time, average pH and temperature the model can successfully predict the equilibrium ratio for the relevant process stream.

PREFACE

The work described in this thesis was carried out in the laboratories of the Sugar Milling Research Institute and is my own original and independent work unless otherwise acknowledged in the text.

Neither this thesis nor any part thereof has been submitted to any other University.

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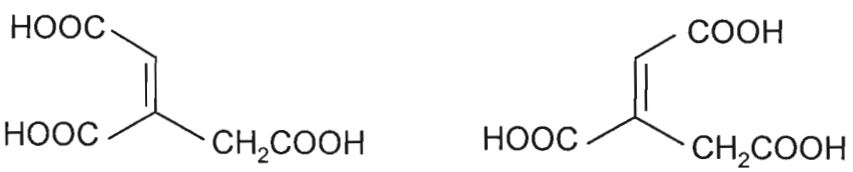
CHAPTER 1

INTRODUCTION

Organic acids constitute a variable but significant proportion of the total soluble nonsugars of sugar cane (*Saccharum Officinarum*), and are responsible for most of the titratable acidity and resultant buffering capacity of the expressed juice. Acids identified in cane juice include citric, malic, succinic, fumaric, lactic, acetic and aconitic acid (1-propene-1,2,3-tricarboxylic acid). The latter is by far the most abundant compound of this class (van der Poel, *et al.*, 1998).

Many investigations into optimisation of the numerous effects of sugar mill process parameters have been undertaken. (For reviews consult Lionnet, 1985, 1998; Morel du Boil, 1991.) However, the specific effects of aconitic acid have not been determined, due to the lack of simple, reliable analytical techniques for the determination of the acid. To a sugar technologist, aconitic acid has the further complication of being unsaturated and existing as two distinct geometrical forms *(cis* and *trans* isomers, see Figure 1.1). Furthermore, the technologist does not know the relative concentration of isomers present in a particular unit process.



(a) (b)

Figure 1.1 Geometric isomers of aconitic acid: (a) *cis*-aconitic acid; (Z)-1,2,3-propene-tricarboxylic acid and (b) *trans*-aconitic acid; (E)-1,2,3-propene-tricarboxylic acid.

Little quantitative data exists on the fate of the acid in sugar solutions under factory conditions. Furthermore, no reported study has been made on the effect

of isomerisation of the *trans*-aconitic and *cis*-aconitic acid isomers in factory juices. The aims of the present study are threefold:

- to develop a simple, reliable, quantitative method of analysis of the isomers in factory streams and for the isomerisation experiments.
- to investigate, in the laboratory, the rates of isomerisation of the *trans*-aconitic acid isomer to the *cis*-aconitic acid isomer form under conditions similar to those found in the factory. The variables studied include pH, temperature, ionic strength and the presence of different cations.
- to develop a model using the results obtained, to account for the ratio of the isomers as a function of the variables studied. Finally to use the model to explain the isomerisation of the *trans*-aconitic acid to the *cis*-aconitic acid isomer across the sugar mill unit operations.

In the following review, a historical and industrial perspective of aconitic acid is given, with emphasis placed on the analytical determination of the acid. Few of the analytical techniques described differentiate between the aconitic acid isomers resulting in little data on the concentrations of the isomers present in factory juices. Consequently, in this study, importance is placed on developing a simple, reliable quantitative method of analysis of the isomers in factory streams. This is necessary to compare the actual isomer ratios against the proposed model.

1.1 Historical and industrial perspective

Aconitic acid is also known as equisetic, citridic or achilleic acid from the plants from which it was first isolated (*Aconitum napellus*, *Achillea (Compositea)* and *Equisetum arvense* (Horsetail or Shavegrass))(Merck Index, 1976; Peschiser, 1822). It was first shown to be present in cane molasses by precipitation of the lead salt, and purification by crystallisation of the acid-ammonium salt (Behr, 1877). The acid was characterised by the preparation and analysis of other salts, comparison with dehydrated citric acid (see Figure 1.2) and known samples from other plant sources. Behr (1877) also succeeded in isolating the

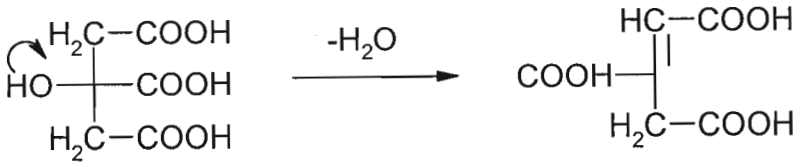


Figure 1.2 Preparation of aconitic acid by dehydration of citric acid.

acid from a sugar cane juice sample sent from the tropics that was preserved with phenol. This established the natural origin of aconitic acid in sugar cane.

Sugar technologists first showed an interest in the acid when it was described as the principle component of evaporator and refinery pan scale (see Chapter 3 for an explanation of these terms)(McCalip and Siebert, 1941). It was also found as a cream coloured sediment in Louisiana syrups and molasses storage tanks (McCalip and Siebert, 1941). During the Second World War and the period shortly after, aconitic acid offered a renewable, natural source of chemical feedstock. This resulted in the development of an extraction process with the recovered acid being used in the ester form as a plasticiser (Fox, *et al.,* 1949; Godchaux, 1949; Haines and Joyner, 1955). The most common ester manufactured was tributyl aconitate with the triamyl, triallyl and tri(2-ethylhexyl) aconitates also being produced. A novel feature of this extraction process was the removal of the aconitic acid from process streams in the sugar factory before crystallisation of the sugar. Removal of the acid improved sugar recovery since the aconitate is a melassigenic agent[1] (Martin, 1953; van Hook, 1946).

The success of this process resulted in great interest in determining the aconitic acid levels present in molasses in cane producing regions throughout the world. The values measured by various authors are shown in Table 1.1. Most used the decarboxylation method of analysis (see Section 1.3.4). Results are expressed as % Brix (Bx)[2].

[1] molasses forming, thereby reducing sugar recovery

[2]Brix is defined as the amount of soluble solids in a solution by mass expressed as a percentage. % Bx is the amount of a particular compound in a mixture (m/m) expressed as a percentage of the soluble solids content.)

Table 1.1

Aconitic acid content of cane molasses

Country	Content (% Bx)	Reference
Egypt	4.5 - 6.5	Lauer & Makar, 1951
USA (Louisiana)	2.9 - 6.3	Fort, *et al.*, 1952
Australia	0.9 - 3.1	Doolan, 1953
Taiwan	0.6 - 2.3	Chen & Tsai, 1957-58
India	1.4 - 4.2	Mukherjee & Chandra,1956
British West Indies	0.8 - 2.3	Drake, *et al.*, 1955
Philippines	2.2 - 3.6	Juliano, 1956
Puerto Rico	0.5 - 3.1	Blay, *et al.*, 1956
Brazil	2.2 - 6.1	Prates de Campos, 1967
South Africa	1.3 - 2.3	Macgillivary & Matic, 1970
Philippines	2.5 - 3.3	Solaiman & Sameniego,1971
Pakistan	2.7 - 3.7	Shahabaz & Qureshi, 1980
Egypt	4.5 - 6.7	Azzam & Radwan, 1986

Isolation of the acid in this process was based on the precipitation of a divalent cation salt. A multitude of patents were issued based on this process. These included the use of calcium and magnesium (Ventre, 1949; Ambler and Roberts, 1949), other alkaline earth salts (Reeves, 1950) and barium and strontium (Collier, 1950). Studies during this period showed that a minimum concentration of 3% on Brix of aconitic acid in molasses was required to justify economic recovery of the acid. This made isolation economically unviable in most countries other than the USA. The Louisiana values are higher due to milling of immature cane (Martin, 1953).

As a consequence of the constraint of high initial aconitic acid concentration and subsequent low yield in the precipitation process (Ambler, *et al.,* 1945), other possible recovery methods were studied. These included the use of the addition of an inert solvent (methanol) to decrease the solubility of the aconitate salt (Regna and Bruins, 1951), solvent extraction using ethyl acetate (Mukherjee and Srivastava, 1956; Malmary *et al.*, 1995), ion exchange from either diluted molasses or other sugar syrups (Ventre and Ventre, 1955; Liggett and Wimmer, 1953; Bryce, 1954) and vinasse - the solution remaining after

fermentation of molasses (N. V. Centrale Suiker Maatschappij, 1955). Hanine *et al.* (1991, 1992) studied the optimum precipitation conditions from regeneration effluents obtained during manufacture of liquid sugar from cane molasses by ion exchange. They concluded that excessive concentrations of sulphate or phosphate decrease the recovery of precipitated aconitate due to the formation of calcium phosphate and sulphate.

1.2 General and industrial chemistry

The two aconitate isomers are common constituents of the juice of many plants with the *cis*-aconitic acid isomer playing an important part in the Krebs cycle (Rafelson, *et al.,* 1971). The *trans*-aconitic acid isomer is widely distributed in nearly all plant species, but does not occur in appreciable quantities, whereas the closely related citric acid normally occurs in appreciable quantities. The sugarcane plant is unusual in the reversal of these acids; it contains large amounts of aconitic acid but very little citric (Martin, 1953). The actual function of the *trans*-aconitic acid in sugarcane physiology is not clearly understood. Some authors have suggested that it may be present to neutralise the basic alkaline earth elements adsorbed from the soil (Paturua, 1989).

The natural occurrence of two isomeric forms of aconitic acid was discovered by Beath (1926) due to variations found in the melting points of the acid. Malachowski and Maslowski (1928) confirmed the presence of geometrical isomerism and described the preparation of the *cis*-aconitic acid form by hydrolysis of the anhydride. They noted that in aqueous solution it was rapidly converted by heat into the *trans*-aconitic acid. Krebs and Eggleston (1944) studied the interconversion of the *trans*-aconitic acid and *cis*-aconitic acid isomers by using the aconitase enzyme to measure the quantity of *cis*-aconitic acid isomer formed. They concluded that heating aqueous solutions of the *trans*-aconitate salt at 100°C produced up to 30% of the *cis*-aconitic acid isomer and that the extent of the conversion depended upon the pH. The authors suggested that the conversion of the *trans*-aonitic acid isomer to *cis*-aconitic acid isomer as the former was heated, may have been responsible for the variation in reported melting points of the *trans*-aconitic acid isomer. A

study of the effect of pH on the stability of the *cis*-aconitic acid isomer was made by Ambler and Roberts (1948). Insoluble salts of the *cis*-aconitic acid isomer were used as an isolation technique. They found that decreasing pH gave decreasing recoveries of the *cis*-aconitic acid isomer. It was concluded that the *cis*-aconitate salts remained stable whilst the free acid underwent *cis-trans* isomerisation. The authors comment: *"Informative studies of these apparent equilibria are impossible until precise methods of determining each of the isomeric aconitic acids in the presence of the other have been found"*.

The tricalcium and dicalcium magnesium salts of aconitic acid were found to be a major component of the scale which forms on evaporator heating surfaces (see Section 3.1). This finding gave rise to an increased interest in the study of the solubility of these salts (Balch, *et al.* 1945). Formation constants of *trans*-aconitic acid with calcium and strontium were found to be 30 times less than those of citric acid with these cations under identical conditions (Schubert and Lindenbaum, 1952). No values for the *cis*-aconitic acid isomer were reported. Frias (1982) showed that the solubility of calcium aconitate (presumably the *trans*-aconitate isomer) increased with temperature but decreased with increasing sucrose concentration. Sodium and potassium chloride increased the solubility of the aconitate whilst calcium chloride and dipotassium hydrogen phosphate caused a decrease.

The crystallographic properties of some aconitate salts including tricalcium aconitate hexahydrate, calcium sodium aconitate dihydrate, dicalcium magnesium aconitate hexahydrate and magnesium acid aconitate tetrahydrate have been reported (Ambler *et al.*, 1945). The authors showed that the precipitated salts had the crystallographic properties of dicalcium magnesium aconitate hexahydrate, although they generally contained less than the theoretical proportion of magnesium. It was suggested that they are solid solutions of tricalcium aconitate hexahydrate with either trimagnesium aconitate or dicalcium magnesium aconitate hexahydrate. No distinction was made between the *cis*-aconitate or *trans*-aconitate isomers, although the authors used the *trans*-aconitic acid isomer to prepare these salts.

Aconitic acid has been associated with the flavour components of cane sugar and food products (Godshall, 1996a; Chu and Clydesdale, 1976), and has been implicated in the decomposition of stored, canned sugar syrups due to the decarboxylation of aconitic acid and subsequent formation of carbon dioxide (Henry and Clifcorn, 1949). In sugar refineries employing bone char, as a decolourising agent, aconitic acid present in raw sugars led to decreased decolourising capacity (Dietz and Rootare, 1957; Löwy, 1957). Japanese research showed that active carbon treatment lowered the pH of refinery sugar liquors. This was attributed to the presence of low concentrations of calcium aconitate, adsorption of calcium onto the carbon and subsequent release of the free acid (Otake *et al.*, 1961). Colour formation in sugar mill solutions has been linked to aconitic acid and reducing sugars when the pH of the solution is raised above 8 (Wolfrom *et al.*, 1955), conditions which could occur in parts of the diffuser and clarifier, in the raw house and parts of the carbonation/sulphitation refinery (see Section 3.1).

The few studies on the fate of aconitic acid in sugar factories and sugar solutions have given conflicting conclusions on its stability. In a study of organic acid formation associated with lime saccharate formation, Stringer *et al.* (1989) reported that aconitic acid did not vary during their experiments and used it as an internal standard for chromatographic analysis. Bruijn (1966) found no change in the aconitic acid concentration during deterioration of harvested cane before milling. The first study of the fate of the acid in molasses fermentation concluded that nearly all the acid was either consumed or reduced to tricarballylic acid (Nelson and Greenleaf, 1929). In apparent contradiction, Roberts *et al.* (1953-54) showed that more than 85% of the original acid in the molasses was still present in the stillage. Aconitic acid was still found in vinasse after undergoing anaerobic digestion but no figures were reported on recoveries (Celestine-Myrtil and Parfait, 1988). Chu and Clydesdale (1976) showed that on heating, the *cis*-aconitic acid isomer converted to the *trans*-aconitic acid isomer and decarboxylated to form itaconic acid. It has been reported both that there is (a) no loss of aconitic acid in the

defecation clarification process[3] (Balch, *et al.* 1945) and (b) a loss of 74% (Hanine, *et al,* 1990). It is claimed that precipitation of phosphate is adversely affected by aconitic acid (Fort and Smith, 1952), whilst it is also claimed by other authors that it does not affect this precipitation (Shephard, 1981). Oxalic acid is formed from aconitic acid when ozone is used as a decolourising agent in factory syrups, whilst sulphitation used for the same purpose appears to have little effect on the acid (Walford and Walthew, 1996). The dissociation constants of *trans*-aconitic acid in water and sugar solutions were reported in 1985 (Table 1.2).

Table 1.2

Dissociation constants for *trans*-aconitic acid (Guerra *et al.*, 1985)

	Water 25°C	15% sucrose 25°C	15% sucrose 90°C
K_1	1.02×10^{-3}	1.27×10^{-3}	8.02×10^{-4}
K_2	6.55×10^{-5}	5.11×10^{-5}	3.00×10^{-5}
K_3	7.80×10^{-7}	5.40×10^{-7}	2.50×10^{-7}

1.3 Analytical determination of aconitic acid

A variety of analytical techniques has been developed over the years to determine the acid both qualitatively and quantitatively. Most cannot differentiate between the isomers but are reported here for completeness. Chromatographic methods are the most useful for differentiation of the isomers.

1.3.1 Extraction and precipitation

The first analytical isolation of aconitic acid in sugar factory juices was achieved by extraction with ether and differentially precipitating the acid as the calcium salt using different concentrations of alcohol (Yoder, 1911). Analysis of evaporator scale by this method required correction for oxalic acid which was also extracted (Balch *et al.,* 1945).

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[3]A term used to describe the addition of lime to a sugar solution to effect pH adjustment and clarification of the solution for futher processing (see Chapter 3).

1.3.2 Titration

Extraction followed by titration was used for the first quantitative determinations of aconitic acid in the juices of sugar-producing plants and products of sugar manufacture (McCalip and Siebert, 1941). The method was based on the original method of Yoder (1911). Precipitates and evaporator scale were solubilised with hydrochloric acid and extracted with ether. Process streams (syrups and molasses) were treated with dry, lead acetate; the precipitated lead salts filtered off, re-suspended in water, the acids liberated with hydrogen sulphide and the solution extracted with ether. This process is accurate only in the absence of all other non-volatile acids which may be extracted from the acidified aqueous solution.

An empirical titration method used potassium permanganate to totally oxidise the aconitic acid to carbon dioxide and water (Lauer and Makar, 1951). The amount of oxygen used depended upon the concentration of permanganate solution used (between 15 and 9 atoms of oxygen per molecule of aconitic acid for 0.05 to 1 N permanganate solution). This required the permanganate solution to be standardised against a pure, known sample of aconitic acid. No suggestions were made as to why the oxidation was not stoichiometric.

1.3.3 Colorimetric

A qualitative colorimetric test for aconitic acid which used acetic anhydride to produce a pink colour (Taylor, 1919) was later modified by the addition of a drop of pyridine to the acid-anhydride mixture to produce a more stable claret colour (Fürth and Herrmann, 1935). A quantitative, liquid-liquid extraction based, spectrophotometric measurement at 550 nm for aconitic acid in sorgo using acetic anhydride (Poe and Barrentine, 1968) followed by colour formation using acetic anhydride was later modified for the determination of this aconitic acid in sugar cane juices (Fournier and Vidaurreta, 1971).

1.3.4 Decarboxylation

A more specific quantitative method involved boiling in potassium acetate - acetic acid solution to decarboxylate the aconitic acid to itaconic acid (Roberts and Ambler, 1947). Again the method was based on the initial precipitation of

the aconitate lead salt. The accuracy of the method was dependent on the accuracy of determining the carbon dioxide produced. No interference from a range of acids was reported (oxalic, succinic, maleic, tartaric, citric, fumaric, itaconic, mesaconic, malic and lactic). However, a later report described the addition of boric acid to the potassium acetate - acetic acid solution in order to overcome interference from citric acid (Ambler and Roberts, 1947). It was thought that the hydroxyl group on the citric acid formed a boric acid complex, inhibiting formation of acetylcitric acid and subsequent decarboxylation. It was shown that this decarboxylation method gave values 40-60% lower, for final molasses, than a polarographic method (see Section 1.3.5) due to incomplete precipitation of the lead aconitate at pH 6.5. Increasing the pH to 11 reduced the error.

1.3.5 Polarography

The shift in the polarographic reduction wave of aconitic acid to more negative potentials was suggested as a simple means of estimating the amount of molasses film on raw sugars (Drake *et al.*, 1955). Pre-treatment of the molasses consisted of dilution of the sample with HCl, and removal of impurities with decolourising charcoal. However, it was found that the aconitic acid was also adsorbed onto the charcoal necessitating determination with varying amounts of charcoal and extrapolation to zero mass of charcoal. This problem was overcome by adjusting the acidic dilute sample to pH 9-10 with 6 N NaOH before adding a mixture of activated carbon and kieselguhr and heating (100°C for 5 minutes) (Matubara and Kinoshita, 1960). The filtrate was made acidic again by addition of 1 N HCl before analysis. This method was used in the comparison of the decarboxylation and polarographic methods of aconitic acid estimation (Gupta and Chetal, 1968).

1.3.6 Chromatogaphy

Chromatography has been widely used as a separation technique for isolating and identifying acids present in sugar factory streams including aconitic acid. The chromatographic modes used include paper, thin layer, partition, ion exchange, gas and liquid chromatography.

Paper Chromatography

Paper chromatography of organic acids in the sugar industry was developed for the rapid identification of organic acids in sugar beet processing liquors (Stark, *et al.*, 1951). A variety of eluent mixtures was necessary to overcome coelution of groups of acids. Identification of aconitic acid in maple syrups and sap by paper chromatography soon followed (Buch, *et* al., 1952). The authors also described the use of four different spray solutions to produce colour reactions that identified and differentiated certain groups of acids. Obara and Iwakura (1958) qualitatively analysed Cuban raw sugars and affination syrups by paper chromatography using three different solvent systems in order to resolve different groups of the acids. Aconitic acid was found to be the most abundant acid present.

Partition Chromatography

A partition chromatographic method for the separation of non-nitrogenous organic acids of sugar cane was first described by Roberts and Martin (1954). A preliminary drying stage or extraction technique was needed in order to remove water in the samples which would have altered the equilibrium between the silica packing and the organic mobile phase. The acids were separated by using a gradient elution of chloroform and butanol and the eluted fractions were titrated with 0.01 N NaOH. The relative positions of each acid were known from elution curves of known organic acids that had been processed by the same procedure. Although esterification of the acids with the eluent (butanol) was not detected, substitution of ketones for alcohol in the eluent was advocated to avoid esterification of the untitrated acids (Scott, 1955).

Extraction techniques for isolation of the acids before partition chromatography included ion exchange. Obara and Iwakura (1958) used anion (IRA 400 HCO_3-type) and cation (IRA 120 H-type) ion-exchange resins to isolate the acids from Cuban raw sugars and affination syrups. Bose and Datta (1962) used only an anion exchange resin before making use of a similar separation scheme as Roberts and Martin. Again aconitic acid was by far the most important acid detected.

Ion-Exchange Chromatography

Ion-exchange chromatography as an analytical technique for the analysis of organic acids in sugar products was first described for beet factories (Owens *et al.*, 1953). Acids in the collected fractions were quantitatively determined by specific tests, e.g. titration, ceric oxidation (lactic acid), permanganate oxidation (oxalic acid) and colourimetric (2,7-dihydroxynapthalene, glycolic acid). Quantitative ion exchange of organic acids (including aconitic acid) in cane refinery products (raw sugar, refined sugar and refinery molasses) is recorded by Borodkin and Berger (1964). The anion resin was equilibrated in the formate form and a non-linear gradient of increasing formic acid was used as eluent. Formic acid in the collected fractions was removed by addition of chloroform and volatilisation of the low boiling chloroform-formic acid azeotrope. The residue was dissolved in isopropanol and titrated coulometrically to pH 9. Identification and purity of collected fractions was checked by thin layer chromatography.

Gas Chromatography (GC)

Mehltretter and Otten (1971) first described the use of gas chromatography to determine aconitic acid in sugar processing solutions. Lead aconitate was precipitated from solution, washed, dried and silylated with excess trimethylchlorosilane and hexamethyldisilazane in pyridine. Less than 95% of the aconitic acid was silylated and tartaric acid was used as an internal standard. A packed SE30 column was used to separate the two acids. In another report an isolation procedure based on a cation (IR120 H^+) followed by an anion exchange resin (IRA400 CO_3^{2-}) was used before silylation with BSFTA (bis-trimethyl-silyl-trifluoro-acetimide) (Day-Lewis, 1979). The acids were separated on a packed OV17 column.

In a study of sugar colourants and precursors (including aconitic acid), Godshall (1996b) compared different extraction techniques, followed by GC analysis. These were solid phase extraction cartridges (SPE) containing strong anion exchange resin (SAX) or C18 packing, Empore-SBD membranes and liquid-liquid extraction (ethyl acetate and methanol/ethyl acetate). It was found

that the SAX SPE cartridge and methanol/ethyl acetate gave the most useful information. Eluents from these techniques were dried and derivatised with Tri-Sil concentrate to produce the trimethylsilyl derivatives. Separation was accomplished on a capillary column (5% phenyl methyl silicone phase). A quadrupole mass spectrometer was used as a detector. Compounds were identified on the basis of their mass spectral patterns and retention times. No GC method reported the separation of the derivatised aconitic acid isomers.

High Performance Liquid Chromatography (HPLC)

The qualitative, combined separation of sugars and organic acids by ion exchange HPLC was first achieved by using a refractive index detector (RI) and conductivity detector in series (Charles, 1981). Besides the naturally occurring acids, detection of formic, lactic, glycolic and glyceric acids produced by microbial and/or chemical degradation was reported. A novel method to analyse organic acids in sugar cane process juices used two HPX87H columns in series, equilibrated at different temperatures and a 5 mM H_2SO_4 solution as eluent (Blake, *et al.*, 1987). Refractive index detection was used necessitating prior isolation of the acids with DEAE-Sephadex resin in order to prevent carbohydrate interference. This system successfully separated the *cis*-aconitic and *trans*-aconitic acid isomers. Analysis and separation of the isomers in cane juice, molasses, vinasse and anaerobic digestion effluents was achieved on reverse phase C18 columns with UV detection at 214 nm (Celestine-Myrtil and Parfait, 1988).

Of all of the methods described in the literature, HPLC is the simplest and most direct method of analysing for the *cis*-aconitic acid and *trans*-aconitic acid isomers. Consequently, method development for isomer analysis concentrated on HPLC as the choice of analysis in factory streams and isomerisation experiments (Section 2.2 and 2.5 respectively).

1.4 Kinetic studies

The kinetics and mechanisms of the thermal, rotational *cis-trans* isomerisations about the carbon-carbon double bond, in the gas phase for a variety of

substituted ethylenes have been reviewed and reported (Table 1.3). It can be seen that the rate of isomerisation increases with increasing size of substitiuent on the double bond.

Table 1.3

Summary of Arrhenius parameters and the rate coefficients at 723 K for thermal *cis-trans* isomerisation of various substituted ethylenes (Bamford and Tipper, 1972).

Reactant	A (s^{-1})	E_a (kJ $mole^{-1}$)	k (s^{-1})
trans-CHD=CHD	10^{13}	271.9	$2.2x10^{-7}$
cis-CHCl=CHCl	$5,7x10^{12}$	234.2	$6.7x10^{-5}$
trans-CHCl=CHCl	$4,8x10^{12}$	231.3	$9.0x10^{-5}$
cis-CH_3CH=$CHCOOCH_3$	$1,6x10^{13}$	241.8±6.3	$5.4x10^{-5}$
cis-CH_3CH=CHCN	$1,0x10^{11}$	214.6±15.5	$3.1x10^{-5}$
cis-C_6H_5CH=CHC_6H_5	$6x10^{12}$	179.0	0.69
cis-C_6H_5CH=CHCN	$4x10^{11}$	192.5	$5.0x10^{-3}$
cis-C_6H_5CH=$CHCOOCH_3$	$3,5x10^{11}$	174.1	$9.3x10^{-3}$

Research into the thermal *cis-trans* isomerisation of the di- and tri-acid substituted ethylene type compounds have concentrated on the maleic-fumaric acid system. This system has been studied in both gaseous phase (Nelles and Kistiakowsky, 1932; Kistiakowsky and Smith, 1934) and in aqueous solution (Tamamushi and Akiyama, 1937; Davies and Evans, 1955). In a study of the catalytic effects of various compounds, it was found that isomerisation was accelerated by the presence of molecular oxygen, platinum black and palladium black (Tamamushi and Akiyama, 1937). Calculated activation energies showed that oxygen reduces the activation energy when compared with nitrogen (61.0 kJ $mole^{-1}$ versus 66.1 kJ $mole^{-1}$). Cundall (1964) reviewed the experimental and theoretical aspects of rotational *cis-trans* isomerisations in both the gas and solution phases.

The effect of catalysts on the rate of *cis-trans* isomerisation has been studied by a number of researchers. The results observed were found to depend on the substrate, the catalysts used and the solvent. In the maleic-fumaric system, increased rates were found to be proportional to the square of the concentration of the added catalyst (Cundall, 1964).

No study has been reported on the kinetics and equilibria of the *trans-cis* isomerisation of aconitic acid in aqueous media other than the qualitative remarks by Ambler and Roberts (1948) and the semi-quantitative values reported by Krebs and Eggleston (1944) and Chu (1976).

1.5 Conclusions and outline of the project

Research reported in the literature concentrates on methods of analysis after isolation of the aconitate salts. Little quantitative data appear on the fate of the acid in sugar solutions under factory conditions. This was due, in the main, to lack of rapid, quantitative, analytical techniques for isolation of the acids and subsequent analysis that could accurately measure both isomers of aconitic acid and possible breakdown products. These techniques now exist. A summary is best described by Clarke (1994-5): "An interesting work was published several decades ago in which the organic acid content of Louisiana cane was measured and high levels of aconitic acid were found ..... . They play a major role in the buffering capacity of cane juice. These materials also form calcium salts with complex solubility properties which affect clarification, evaporation and refinery operations. A re-evaluation of this subject is probably worthwhile, especially since the only comprehensive data is 40 years old." In addition there has been no reported study on the *trans-cis* isomerisation of aconitic acid in factory juices.

With this in mind, the aims of this study have been threefold:

- Firstly to develop a reliable, quantitative HPLC method to determine the levels of aconitate isomers found in factory streams.
- Secondly, on a laboratory scale, to develop an isomerisation method to investigate the effects pH, temperature, ionic strength and divalent cations on the rates of isomerisation of the *trans*-aconitic acid isomer to the *cis*-aconitic acid form. This includes the development of an HPLC method to measure the quantities of each isomer present in the isomerisation reaction mixtures.
- Finally, using the experimental data, obtained in the second part, to describe the isomer ratio as a function of the variables chosen. Values from

this model can be compared with the isomer ratio found in factory streams from the first part of the study. The aim is to explain the isomerisation of the *trans*-aconitic acid isomer to the *cis*-aconitic acid isomer across the sugar mill unit operations as a function of time, pH, temperature and ionic strength.

CHAPTER 2

EXPERIMENTAL PROCEDURE

This section describes the experimental procedures used to achieve the goals presented in the previous section .

2.1 Reagents

2.1.1 Factory product analysis

All buffer solutions and chromatographic eluents were prepared from Analar grade reagents and single distilled water. Organic acids (BDH Analar or Merck pro analysi unless otherwise specified) were used as received. Solid Phase Extraction (SPE) columns (Waters Accell Plus QMA SepPak) were used for isolation of the organic acids from sugar solutions.

2.1.2 Isomerisation experiments

All buffer solutions and chromatographic eluents were made from Analar grade reagents and single distilled water. Chromatographically pure *trans*-aconitic acid (Riedel-de-Haën) and *cis*-aconitic acid (Sigma, 96%) were used for HPLC calibration as received. The pH meter (Crison micropH 2002), used for buffer preparation and isomerisation reaction mixture pH monitoring, was calibrated using pH 7 and 4 buffers (Beckman) following the manufactures recommended procedure.

2.2 Aconitic acid analysis of factory products

Organic acids present in factory samples were isolated from the sugar matrix by SPE. Ion exclusion chromatographic separation of the isolated acids was used to quantitatively analyse the *cis*-aconitic and *trans*-aconitic acid isomer content.

2.2.1 Solid Phase Extraction

Samples were diluted as described below and the acids isolated on the SPE cartridge by using the scheme described in Table 2.1.

Mixed juice/clear juice/standard mixture: the pH of a 5.0 ml sample of juice was adjusted to approximately 8.5 with 0.1 N NaOH and made up to 10.0 ml volumetrically with water. A 2.0 ml aliquot was then used in the isolation scheme shown in Table 2.1.

Syrups: a weighed (1 g) sample of syrup was dissolved in approximately 5 ml of water, the pH adjusted to 8.5 with 0.1 N NaOH (typically 0.35 ml) and made up to 10.0 ml volumetrically with water. A 1.0 ml aliquot was then used in the isolation scheme shown in Table 2.1.

Molasses: a weighed (1 g) sample of molasses was dissolved in approximately 10 ml of water, 3 ml 0.1 N NaOH added, mixed and made up to 25.0 ml volumetrically with water. A 1.0 ml aliquot was then used in the isolation scheme shown in Table 2.1.

Raw sugar: a weighed (5 g) sample of sugar was dissolved in approximately 20 ml water and the total volume used in the isolation scheme shown in Table 2.1.

The development of this SPE method development is described in Appendix 1

Table 2.1

Solid phase extraction procedure for isolating organic acids from a sugar matrix

Step	Procedure on SPE	Purpose
1 2 3	5 ml 0.5N formic acid 5 ml H_2O 5 ml air (blow dry)	Equilibration of SPE
4 5	1 or 2 ml sample (20 ml for sugar) 20 ml H_2O	Isolation of acids Wash off sugars
6	10 ml air (blow dry)	Remove residual water
7 8	1.5 ml 0.2 N H_2SO_4 (into vial) 2 ml air (blow dry into vial)	Elution of acids

2.2.2 HPLC separation of isolated acids

The HPLC system consisted of a Spectra-Physics IsoChrom pump, either a Rheodyne 7125 syringe loading sample injector or a SGE LS3200 autosampler (both 20 µl loops), Linear 206 PHD UV detector (210 nm), Erma ERC-7512 refractive index detector and HP3396A integrator connected to a HP96-Peak workstation for data storage. Separation was achieved on a BioRad HPX87H column (7.8 mm x 300 mm) maintained at 30°C, followed by a Phenomenex Resex H^+ column (7.8 mm x 300 mm) maintained at 75°C. The eluent was 0.0075 M H_2SO_4, filtered (0,45 µm) before use and maintained at 65°C on a stirred hotplate. The acids were eluted at a flow rate of 0.5 ml/min.

Concentrations of the acids in the samples were calculated by an external standard calibration based on free acid. A mixed organic acid standard (Table 2.2), made up to resemble a factory stream, was prepared by dissolving the required quantity of acids in approximately 50 ml water, adjusting the pH to 7.5 with 0.1 N NaOH and volumetrically making up to 100.0 ml with water. This stock standard solution was transferred into 5 ml plastic sachets, sealed and immediately frozen. Standard solutions were prepared as required by thawing a sachet, removing a 1.0 ml aliquot and making up to 10.0 ml volumetrically with water. A 2.0 ml aliquot of this standard solution was treated in the same manner as a mixed juice sample except that no pH adjustment was necessary.

Table 2.2

Stock organic acid standard mixture for factory sample calibration (to be diluted 1:10 with water)

Acid	Salt weighed*	Mass (g)	Concentration in final dilution (g/ml)
Oxalic	$.2H_2O$	0.0275	2.006×10^{-5}
Citric	$.H_2O$	0.0340	3.109×10^{-5}
Phosphoric	KH_2	0.0306	2.205×10^{-5}
Tartaric		0.0196	1.960×10^{-5}
cis-Aconitic		0.1826	1.826×10^{-4}
trans-Aconitic		0.3759	3.759×10^{-4}
Succinic		0.0284	2.840×10^{-5}
Glycollic		0.0329	3.290×10^{-5}
Lactic	Li	0.0700	6.563×10^{-5}
Acetic	$Na.3H_2O$	0.0580	2.557×10^{-5}
Malic		0.0426	4.260×10^{-5}

* no entry = pure acid

2.2.3 Method development - effect of pH and temperature

The effect of eluent pH and column temperature on retention time of the organic acids and possible co-elution was studied by preparing five eluents of decreasing pH (2.10; 1.81; 1.64; 1.57; 1.51), equilibrating the columns and injecting individual acid solutions in duplicate. The standard organic mixture was also run to produce a composite chromatogram. The effect of different column temperature combinations was checked at 30/75°, 30/60° and 30/30°C. The chromatographic method development and results are discussed in Appendix 2. Concentrations of the individual isomers found in factory process streams are recorded in Appendix 3 and the results discussed in Section 3.2.

2.3 Isomerisation analysis

Isomerisations were carried out in one of two reaction vessels depending on the temperature of isomerisation. At temperatures below 100°C reactions were carried out at atmospheric pressure whilst at temperatures above 100°C a pressure vessel was used. For chromatographic quantitation of the isomers, stock standard solutions of separate *cis*-aconitic acid and *trans*-aconitic acid isomers (0.5 mg/ml in water) were prepared, stored in plastic sachets and kept frozen until required. These were thawed and diluted 1:10 with the buffer solution being used, for HPLC calibration.

2.3.1 Temperatures below 100°C

Reactions were carried out in a 500 ml Schott bottle which had been modified by the addition of four inlet/outlet screw ports (Schott GL25 fitting) and the cap modified for insertion of a condenser by using a gland fitting. Separate Pt100 (temperature) and pH probes connected to the pH meter utilised two of these inlets. A nitrogen sparging line, connected through the third port, was used to minimise oxidation and help mix the solution. The fourth inlet was used for continuous sampling through a Technicon peristaltic pump to an autosampler valve connected to the HPLC for *cis*-aconitic acid and *trans*-aconitic acid isomer analysis. The peristaltic tubing was replaced as necessary. Silicone tubing (0.5 mm ID) connected the reaction vessel to the peristaltic pump, autosampler injection valve and return line. Sample flow rate was maintained at 1.0 ml/min. The lengths of all transfer lines were kept as short as possible to

minimise dead volume and subsequent temperature loss as the reaction mixture circulated. Reaction temperature was maintained by placing the bottle in a constant temperature oil bath which was controlled by an Omron E5CW PID (Proportional, Integral, Derivative) temperature controller. Temperature and pH profiles during a run were collected on a PC using a Qbasic program written to accept the RS232 data output from the Crison pH meter.

Concentrations of the *trans*-aconitic acid and *cis*-aconitic acid isomers at pH 4, 5, 6, 7 and 8 at temperatures other than 70°C were measured on-line using a HPLC system consisting of a Spectra-Physics IsoChrom pump, SGE LS3200 Autosampler (20 µl injection loop), a Linear 206 PHD UV detector (210 nm) and HP3396A integrator connected to a HP96-Peak workstation for data storage. Separation was achieved on a BioRad HPX87H column maintained at 65°C using 0.02 N H_2SO_4 as eluent at a flow rate of 0.5 ml/min. The autosampler was programmed to inject a sample every 17, 30 or 60 minutes depending on the speed of isomerisation. Individual *trans*-aconitic acid and *cis*-aconitic acid calibration standards were run at the beginning of each isomerisation experiment. A schematic diagram of the system is shown in Figure 2.1.

Reactions at pH 6 and 7 at 70°C were sampled manually at approximately eight, twelve and twenty four hour intervals. A 0.5 ml sample was removed, placed in an autosampler vial, crimped and immediately frozen. These samples were analysed off-line using the HPLC system described above.

Procedure at temperatures below 100°C

The pH meter was calibrated (pH 7 and pH 4) at room temperature. Buffer solution at the required concentration and pH (380 ml) was placed in the Schott bottle with the pH and temperature probes, sparger and inlet and outlet tubes. This was placed in the oil bath and heated until equilibrium at the desired temperature was achieved, whereupon the peristaltic sampling pump was started. The HPLC was equilibrated simultaneously and calibration standards injected. A weighed sample of *trans*-aconitic acid (20 mg) was dissolved in the buffer (20 ml) and immediately added to the solution vessel. HPLC analysis was started five minutes later to allow mixing. The raw data results obtained

are recorded in Appendix 6 and calculated rate constants discussed in Section 3.7.

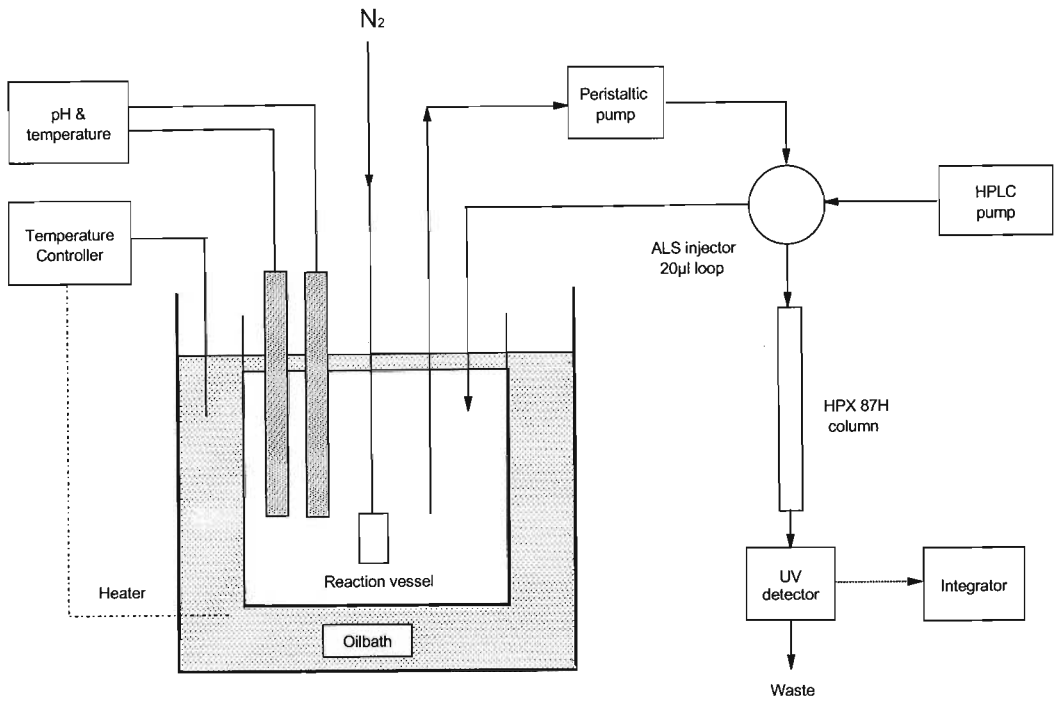

Figure 2.1 Schematic of reaction vessel used for temperatures less than 100°C and associated online HPLC analysis system.

2.3.2 Temperatures above 100°C

Reactions were carried out in a 400 ml teflon coated, brass pressure vessel. The vessel consisted of a well and top plate separated with an o-ring and held together by 10 mm Allen screws. Four threaded orifices were machined into the top plate into which a threaded male nut and gasket were placed. This allowed insertion of the temperature probe, removal of sample through a sampling port sealed with a GC septum and addition of a pressure relief valve (set to 150 kPa). The fourth port was blanked off for these experiments. Reaction temperature was maintained by a hotplate stirrer which was controlled by an Omron E5CW PID temperature controller connected to a Pt100 probe in the reaction vessel. A schematic diagram of the system is shown in Figure 2.2.

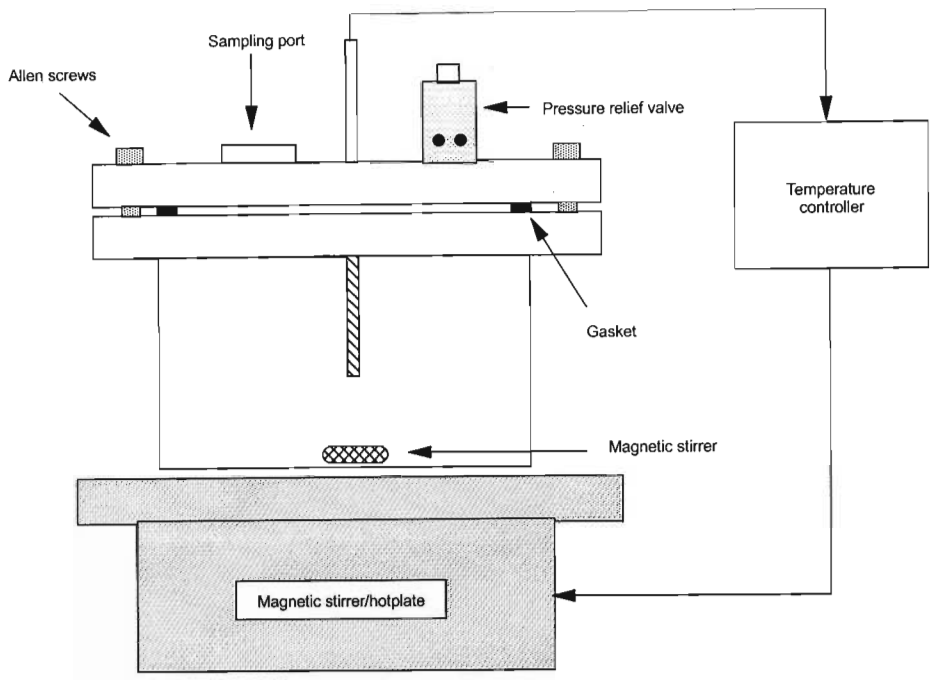

Figure 2.2 Schematic of reaction vessel used for temperatures greater than 100°C.

Procedure at temperatures above 100°C

Buffer solution at the required concentration and pH (298 ml) was placed in the pressure vessel with the temperature probe and magnetic stirrer. This was placed on the hotplate stirrer and heated until equilibrium at the desired temperature was achieved. A weighed sample of *trans*-aconitic acid (15 mg) was dissolved in the buffer (2 ml) and immediately added to the solution vessel through the sampling port by means of a sampling syringe. After a period of half a minute for mixing, a 0.5 ml sample was removed, placed in a autosampler vial, crimped and immediately frozen. Samples were manually taken at 10 or 15 minute intervals and treated in the same manner. These samples were analysed off-line using the HPLC system as described in Section 2.3.1 connected to the SGE autosampler. The raw data results obtained are recorded in Appendix 6 and calculated rate constants discussed in Section 3.7.

2.4 Preparation of buffers

2.4.1 Preparation of buffers containing sodium

All buffers were prepared by weighing sufficient acid or salt (acetic and phthalic acid or sodium dihydrogen phosphate) to make 500 ml of the solution at the required concentration. This was achieved by dissolving the solids to approximately 400 ml, inserting a rinsed, calibrated pH probe and adding the necessary sodium hydroxide (1 M and 0.1 M solutions) with stirring to achieve the required pH. This was then made up volumetrically to 500 ml. The pH meter was calibrated daily (pH 7 and pH 4 buffers) before buffer preparation.

2.4.2 Preparation of buffers containing potassium, calcium and/or magnesium

The effect of potassium as the buffer counter-ion was studied at pH 5 and 90°C by adjusting the pH of the acetic acid with potassium hydroxide (Section 2.4.1). Calcium and magnesium were studied at pH 5 with the individual divalent ion (4.5 mM) in acetate buffer. The combined effect of calcium and magnesium ions (4.5 mM Ca + 4.5 mM Mg) was checked at pH 5 and 90°C in acetate buffer. Equivalent ionic strength solutions to 50 mM sodium acetate buffer were prepared by calculating the ionic strength of the calcium or magnesium acetate salt, subtracting from the ionic strength of the equivalent 50 mM acetate, and calculating the required acetate buffer concentration. These were made up as shown in Table 2.3 by weighing sufficient acetic acid to make 500 ml at the required concentration, adding the required mass of calcium or magnesium acetate, diluting to approximately 400 ml, inserting a rinsed, calibrated pH probe and adding the necessary sodium hydroxide (1 M and 0.1 M solutions) with stirring to achieve the required pH. This was then made up volumetrically to 500 ml. The raw data results obtained are recorded in Appendix 6 and calculated rate constants discussed in Section 3.11.

Table 2.3

Added divalent ions and required acetate buffer concentration for the preparation of equivalent ionic strength solutions

Divalent ion concentration (mM)	$Ca(OAc)_2$ g/l	$Mg(OAc)_2$ g/l	Ionic strength of divalent ions μ	Sodium acetate buffer (mM)
4.5	0.711	0.639	0.027	49.1
4.5 + 4.5	0.711	0.639	0.027	23.0

2.5 Method development for isomerisation studies

2.5.1 Effect of eluent pH and linearity of aconitic acid analysis

The effect of chromatographic eluent pH on the separation of the organic acids, including the aconitic acid isomers, was studied by preparing five eluents of decreasing pH (2.10, 1.81, 1.64, 1.57 and 1.51). A concentration of 7.5 mM sulphuric acid (pH 1.81) was chosen for the eluent. Possible coelution with the buffer used in the isomerisation experiment was also checked by injecting the blank buffer. The linearity of detection of the aconitic isomers and itaconic acid was checked across a range of concentrations (Table 2.4). (Itaconic acid was included in the calibration study due to it's appearance as a further reaction product later in the isomerisation studies). These effects are considered in Section 3.4.2.1

Table 2.4

Concentration of the *cis*-aconitic acid, *trans*-aconitic acid and itaconic acid used for checking the linearity of the HPLC method used for isomerisation experiments

cis -aconitic acid		*trans*-aconitic acid		Itaconic acid	
mM	g/ml	mM	g/ml	mM	g/ml
0.025	4.50×10^{-6}	0.028	4.84×10^{-6}	0.083	1.08×10^{-5}
0.050	9.00×10^{-6}	0.056	9.68×10^{-6}	0.166	2.16×10^{-5}
0.099	1.80×10^{-5}	0.111	1.94×10^{-5}	0.415	5.40×10^{-5}
0.248	4.50×10^{-5}	0.278	4.84×10^{-5}	0.831	1.08×10^{-4}
0.497	9.00×10^{-5}	0.556	9.68×10^{-5}	2.077	2.70×10^{-4}
1.242	2.25×10^{-4}	1.391	2.42×10^{-4}	4.154	5.40×10^{-4}

2.5.2 Effect of the initial *trans*-aconitic acid concentration on the isomerisation rate

The effect of the initial *trans*-aconitic acid concentration in the isomerisation solution was studied at 97°C and pH 5 (acetate buffer). The initial *trans*-aconitic acid concentration was varied between 0.1 mM and 1.2 mM (7.5 mg; 19.1 mg; 42.1 mg and 81.9 mg). Isomerisation of the acid was monitored in the isomerisation vessel described in Section 2.3.1. The raw data are found in Table 3.2 and discussed in Section 3.4.2.1

2.6 Treatment of data

The raw data from the isomerisation experiments are recorded in Appendix 6 whilst the calculated concentration data of the isomers is shown in Appendix 5. This data was treated in the following fashion to obtain the rate constants.

2.6.1 Calculation of rate constants

The isomerisation of the *trans*-aconitic acid to *cis*-aconitic acid can be described by:

$$trans \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cis$$

where k_1 is the forward rate constant and k_{-1} is the reverse rate constant. Let $[trans]^t$ be the concentration of the *trans*-aconitic acid isomer and $[cis]^t$ be the concentration of the *cis*-aconitic acid isomer at any time *t*. At the commencement of the experiment, there will be no *cis*-aconitic acid present; after time *t* the concentration of the *trans*-aconitic acid and *cis*-aconitic acid will be $([trans]^t - [cis]^t)$ and $[cis]^t$ respectively. The net rate of reaction at any instant will be given by:

$$-\frac{d[trans]}{dt} = k_1[trans]^t - k_{-1}[cis]^t \quad (2.1)$$

At time t_0

$$[trans]^t = [trans]^0 \text{ and } [cis]^0 = 0$$

Mass balance requires

$$[trans]^t + [cis]^t = [trans]^0 \quad (2.2)$$

(assuming no loss of either isomer due to further reactions). Substitution of equation (2.2) into equation (2.1) gives

$$-\frac{d[trans]}{dt} = (k_e)[trans]^t - k_{-1}[trans]^0 \quad (2.3)$$

where $k_e = (k_1 + k_{-1}) =$ equilibration rate constant.

At equilibrium $\frac{d[trans]}{dt} = 0$ and substitution into equation (2.3) gives

$$(k_e)[trans]^{eq} = k_{-1}[trans]^0 \quad (2.4)$$

where $[trans]^{eq}$ is the equilibrium concentration of the *trans*-aconitic acid isomer. Substitution of equation (2.4) back into equation (2.3) gives

$$-\frac{d[trans]}{dt} = (k_e)([trans]^t - [trans]^{eq}) \quad (2.5)$$

After separation of variables, equation (2.5) can be integrated to give equation (2.6), the integrated rate equation.

$$\ln\left[\frac{[trans]^t - [trans]^{eq}}{[trans]^0 - [trans]^{eq}}\right] = -(k_e)t \quad (2.6)$$

This can be written in the non-linear form (equation 2.7)

$$[trans]^t = [trans]^{eq} + ([trans]^0 - [trans]^{eq}) \exp[-(k_e)t] \quad (2.7)$$

A similar set of equations can be written to give the integrated rate equation as a function of the *cis*-aconitic acid concentration:

$$\ln\left[\frac{[cis]^{eq}}{[cis]^{eq}-[cis]^t}\right] = (k_e)t \quad (2.8)$$

and

$$[cis]^t = [cis]^{eq}(1 - \frac{1}{\exp(k_e)t}) \quad (2.9)$$

From equation (2.1) at equilibrium

$$k_1[trans]^{eq} = k_{-1}[cis]^{eq}$$

or

$$\frac{k_1}{k_{-1}} = \frac{[cis]^{eq}}{[trans]^{eq}} = K \quad (2.10)$$

where K is the equilibrium constant for the isomerisation.

The calculated concentration data from the HPLC integrator were used in a non-linear regression calculation (Statistica 5.1) [equations 2.7 and 2.9] to calculate the equilibrium values of the *cis*-aconitic and *trans*-aconitic acid isomers, and the equilibration rate constant, k_e. These values were then used to calculate the individual forward (k_1) and reverse (k_{-1}) rates from equation 2.10.

2.6.2 Calculation of non-buffered rates

The variation of activity coefficient γ_i with concentration in solution may be represented by the Debye-Hückel equation

$$-\log \gamma_i = \frac{Az_i^2 \sqrt{\mu}}{1+aB\sqrt{\mu}}$$

where μ is the ionic strength given by $\frac{1}{2}\Sigma c_i z_i^2$ (c is the molar concentration of the ion and z the charge on the ion).

A is defined as $$\frac{\sqrt{2000\pi N}\, e^3}{2.303(4\pi\varepsilon^0 k_b \varepsilon T)^{3/2}}$$

B is defined as $$\sqrt{\frac{8000\pi N e^2}{(4\pi\varepsilon^0)k_b \varepsilon T}}$$

N is Avogadro's number, e is the elementary charge, k_B is the Boltzmann constant, ε is the dielectric constant of the solvent, ε^0 the permittivity of free space and T the temperature in Kelvin. For water at 25°C this can be approximated (Glasstone,1960) by

$$-\log \gamma_i = 0.51 z_i^2 \sqrt{\mu}$$

Combining this with the transition state theory equation relating reaction rate to activity coefficients

$$k = k_0 \frac{\gamma_A \gamma_B}{\gamma^{\ddagger}}$$

yields

$$\log k = \log k_0 + 1.02 z_A z_B \sqrt{\mu} \qquad (2.11)$$

Thus the rate at zero ionic strength can be calculated by means of a least squares regression of log *k* against $\sqrt{\mu}$. These effects are discussed in Section 3.7.2.

2.6.3 Calculation of temperature effects - rate constant

The relationship between the absolute temperature T and the rate constant *k* of an elementary reaction is described by the Arrhenius equation

$$k = Ae^{-\frac{E_a}{RT}}$$

where *A* is the pre-exponential factor, E_a is the activation energy, *R* the gas constant and *T* the absolute temperature.

The traditional method of fitting data to this equation is to use the logarithmic form:

$$\log k = \log A - \frac{E_a}{2.303RT}$$

Thus log *k* will vary linearly with 1/T. This plot will yield *A* from the intercept and E_a from the slope. Section 3.8 reviews the results found in this work.

2.6.4 Calculation of temperature effects - thermodynamic functions

A study of the transition state theory can be used to derive an equation relating the thermodynamic properties to temperature (Espenson, 1995):

$$k = \kappa \frac{k_b T}{h} \exp\left(\frac{\triangle S^{\ddagger}}{R}\right) \exp\left(\frac{\triangle H^{\ddagger}}{RT}\right)$$

k is the rate constant, κ is the transmission factor (usually 1), k_b is Boltzmann's constant (1.381×10^{-23} J K^{-1} $molecule^{-1}$), *h* is Planck's constant (6.626×10^{-34} J s), *R* is the gas constant (8.314 J K^{-1} mol^{-1}), *T* is the absolute temperature (K), $\Delta S^{\ddagger}$ is the standard entropy of activation (J mol^{-1} K^{-1}) and $\Delta H^{\ddagger}$ is the standard enthalpy of activation (J mol^{-1}). After taking logarithms and assuming κ=1 this equation becomes:

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \frac{\triangle S^{\ddagger}}{R} - \frac{\triangle H^{\ddagger}}{RT} \qquad (2.12)$$

A plot of ln(k/T) will be a linear function of $1/T$. The slope will yield $\Delta H^{\ddagger}$ and the intercept the value of $\Delta S^{\ddagger}$, after allowance for ln(k_B/h) = 23.760. The thermodynamic functions are discussed in Section 3.8.

CHAPTER 3

RESULTS AND DISCUSSION

Reactions involving double bonds are amongst the most significant in chemistry. The presence of a double bond hinders rotation about the carbon-carbon axis leading to possible formation of *cis* and *trans* geometrical isomers. These differ from each other only in the way the atoms are orientated in space. Aconitic acid is an example of such a compound. Isomerisation of the isomers involves breaking of the double bond, rotation about the newly formed single bond and double bond reformation. Isomerisations can be brought about thermally, catalytically and photochemically by photosensitisation and by radiolysis. No study of the *cis-trans* isomerisation of aconitic acid has been reported. This work seeks to redress this with an emphasis on isomerisation in sugar factory streams. Only thermal isomerisation is considered here.

The study can be divided into three distinct areas.

- Initial development of a chromatographic method for the simultaneous determination of *cis*-aconitic acid and *trans*-aconitic acid in sugar mill factory streams was necessary. Use of this method allowed the levels of the *cis*-aconitic acid and *trans*-aconitic acid isomers and subsequent ratios to be determined as a function of sugar factory unit processes.

- The isomerisation was investigated in the laboratory over a temperature range of 70° to 110°C, a pH range of 4 to 8 and increasing ionic strength. The parameter values chosen fall within the range of typical factory conditions.

- The laboratory data was used to develop a model describing the rate of aconitic acid isomerisation in the sugar factory. Theoretical values from the model were compared to actual values obtained from factory streams.

3.1 Outline of the process used to manufacture raw cane sugar

To understand the nomenclature and processes referred to later in the text, a condensed description of raw sugar manufacture will be given. This should be read in conjunction with the flow diagram (Figure 3.1) representing the standard processes. In South Africa, the industry is located mainly on the KwaZulu-Natal coast within about 50 km of the Indian Ocean with two mills further north in the Mpumalanga lowveld. Both irrigated and rain-fed areas are included in the local industry. Harvesting begins in March and continues until January the following year. Harvesting practices vary and include both burning before cutting and green cane harvesting. Mechanical and manual cutting of the cane is practised.

Juice extraction

Preparation of the cane stalk for juice extraction consists of "knifing" (cutting) and shredding to expose and rupture the interior, sucrose containing cells. This prepared cane is exposed, in an enclosed carrier known as a diffuser, to a countercurrent washing process that removes more than 98.5% of available soluble material. This juice, known as **mixed juice** (MJ), has a pH of 5.5, contains approximately 15% dissolved solids and 13% sucrose. The discharged, washed fibre, known as bagasse, is used primarily as boiler fuel for steam generation. Other uses include chemical byproducts feedstock (primarily furfuraldehyde and diacetal), paper and particle board manufacture and cattle feed.

Juice clarification

Clarification is used to produce a juice which is neutral in pH, light in colour and free of suspended material. A neutral pH helps minimise sucrose inversion later in the process. Adjustment of pH is made by the addition of lime (calcium hydroxide solution). Reaction with either natural phosphate present in the juice or added phosphate produces insoluble calcium phosphate. This precipitate,

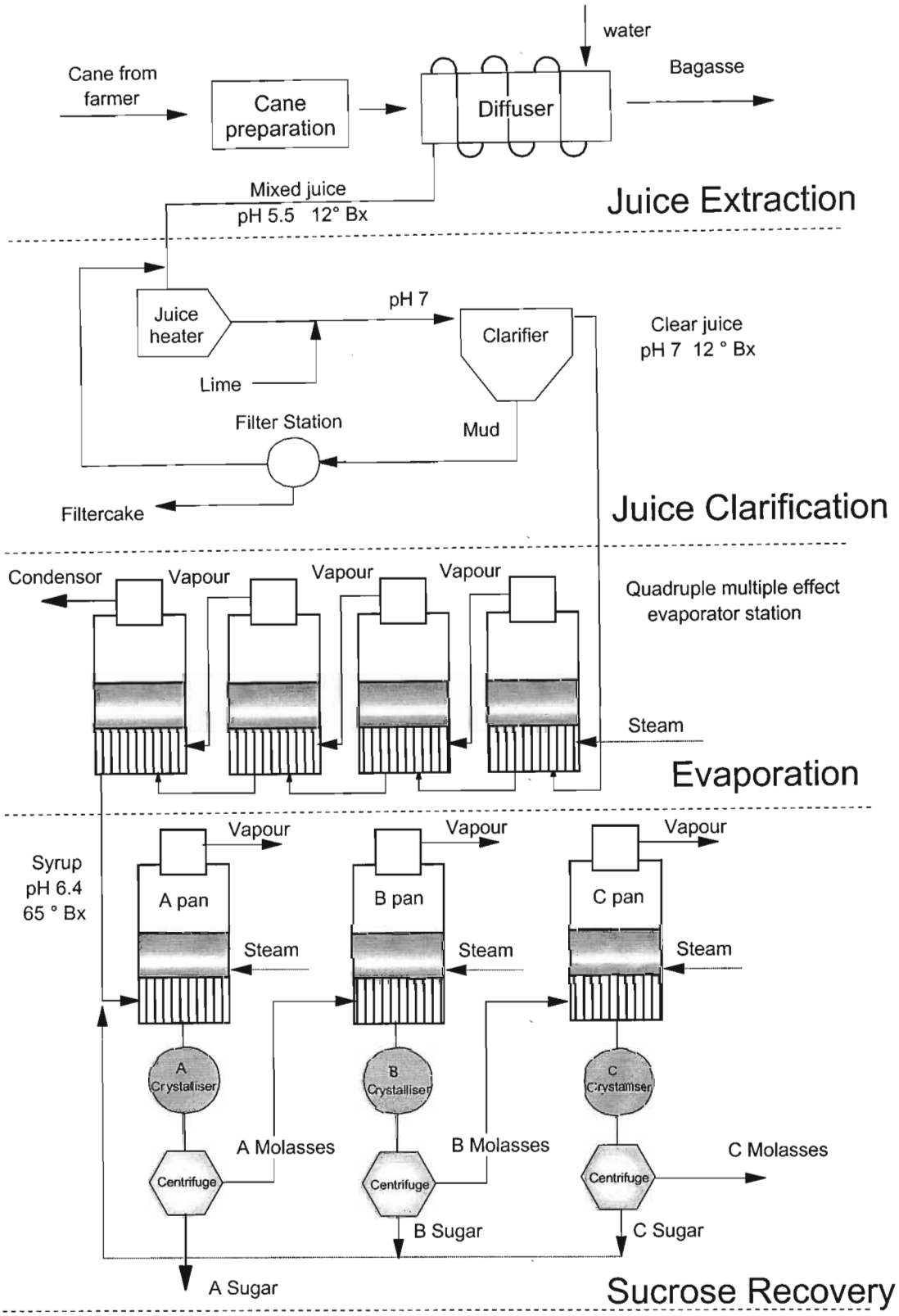

Figure 3.1 Schematic diagram of a South African sugar mill showing the four main unit processes and the three pan boiling system to produce VHP (Very High Pol) raw sugar.

combined with coagulated protein from the hot juice, encloses impurities which are removed in a settling process known as clarification. The resultant mud solution is filtered, washed and the filtrate returned to the MJ to maximise sucrose recovery whilst the filtercake is used as a fertilizer. The clear overflow from the clarification tank is neutral, has a similar composition as MJ and is known as **clear juice** (CJ).

Evaporation

Crystallisation of sucrose can only occur above 80-90% brix necessitating concentration of the CJ. Evaporation of this excess water takes place in a multiple effect evaporator which consists of a succession of vacuum vessels arranged in series. Each succeeding vessel has a higher vacuum and consequently the water boils at a lower temperature. The vapour from one vessel can thus be used to boil the juice in the next. Steam introduced into the first vessel can be used for "multiple-effect evaporation". The vapour from the final vessel is condensed thus producing the vacuum. Both four vessel ("quadruple") and five vessel ("quintuple") multiple effect evaporation are common. The particular scheme chosen depends on the desired steam usage in the factory. Multiple effect evaporation is used to help conserve steam, lower the boiling temperatures and consequent sucrose degradation. The concentrated juice at the exit of this stage is called **syrup**, has a pH of approximately 6.4 and a brix of 65%.

Sucrose recovery

Further concentration of the syrup to saturation (~ 95% brix) in a single effect batch or continuous vacuum pan, is followed by addition of seed material. Growth of the resultant crystals occurs by the simultaneous addition of syrup and evaporation of water. Once the pan is full, the massecuite (mixture of mother liquor and crystals) is placed in a crystalliser to allow for further crystal growth as some cooling occurs. The massecuite is placed in a centrifuge to separate the crystal and mother liquor, known as **molasses**. The sucrose crystals are known as A-sugar (from the A-pan or first pan), raw sugar or Very High Pol sugar (VHP sugar). The molasses from this pan is termed A-molasses. Since this molasses still contains considerable quantities of sugar,

it is processed in the same manner to produce a further two crops of crystals (B- and C-sugars from the B- and C-pans) and molasses (B and C molasses).

The B and C sugars have elevated levels of colour and ash. These are melted (dissolved) and added back to the syrup feeding the A pan. This scheme of sucrose crystallisation is known as the 3 pan boiling system and is used throughout the Southern African sugar region. Other boiling regimes (both 2 and 3 pan schemes) are used in different regions of the world.

The C-molasses (containing approximately 30% sucrose) is also known as final molasses as no further crystalisation of sucrose is possible. This is due to the increased levels of impurities including reducing sugars (6 to 10% each of glucose and fructose), ash (principally potassium, calcium, magnesium, chloride and sulphate) and organic compounds including aconitic acid (1.8 - 2.5%). This material is used as a feedstock for potable alcohol fermentation and cattle feed.

The VHP sugar produced by this process is light, golden brown in colour, contains more than 99.3% sucrose, less than 0.13% moisture and less than 0.2% conductivity ash. This is processed further either in a stand-alone or back-end (attached to the raw sugar mill) sugar refinery to remove the colour and ash by a combination of techniques. These include ion exchange, sulphitation, carbonatation, phosphitation, activated granular carbon and, more recently, ozonation. This produces common white sugar after crystallisation of the resultant decolourised syrups.

3.2 Analysis of factory streams

The initial investigation centered on determining the ratio of the aconitic acid isomers present in sugar factory process streams. The SPE/HPLC chromatographic method outlined in Section 2.2 was used to determine the concentrations of the isomers present. The study was divided into two phases. Firstly a survey of products from the main factory streams was undertaken. Samples analysed included clear juice, syrup, molasses and raw sugar over a

three year period. A second study focused on mixed juice. Table 3.1 summarises the findings of these surveys reporting only the *cis/trans* aconitic acid isomer ratio, not the absolute values. Individual absolute isomer values found in these surveys are recorded in Appendix 3. Typical pH values for each product are also recorded. The *cis/trans* aconitic acid isomer ratio can be seen to increase from the front to the back end of the factory. Furthermore there are only small differences across the unit operations in the front end of the factory but significant changes towards the rear. This work attempts to answer the question as to why this effect occurs.

Table 3.1

Average *cis/trans* aconitic acid isomer ratio and sugar mill product pH

Source	Fresh Cane	Mixed Juice	Clear Juice	Syrup	Raw Sugar	Molasses
cis/trans ratio	0.03	0.06	0.08	0.13	0.20	0.29
pH at 25°C	5.5	5.3	7.2	6.4	6.5	5.5

3.2.1 Mixed juice

Analysis of weekly composite mixed juice samples from the individual mills was undertaken to determine the levels of *cis*-aconitic acid and *trans*-aconitic acid entering the mill during the milling season. Mixed juice samples originate from a biological material (sugar cane juice). The absolute concentrations of *cis*-aconitic acid and *trans*-aconitic acid vary throughout the year according to the sugarcane's growth cycle. At the beginning (March/April) and end of the season (November/December), when immature and fast growing cane is harvested, the MJ *cis/trans* aconitic acid isomer ratio tends to increase (Figure 3.2). Warmer temperatures and water are conducive to cane growth (beginning and end of season). At this time the metabolism of the plant is at it's highest and sucrose storage at a low. This correlates with increased Kreb's cycle activity and consequently higher levels of *cis*-aconitic acid present in the cells, resulting in the higher *cis/trans* isomer ratios recorded here.

The outliers shown at the extremes of the season are due to the three northern mills: Malalane, Komati and Pongola, which all practice irrigation and cane ripening. All three mills are found in the warmest areas of the sugar growing region. Possible post harvest/pre-milling delays and treatment has not been considered to eliminate outliers. The effect of delays can be seen when comparing the lower *cis/trans* isomer ratio found in freshly harvested, extracted cane against normal MJ from the mill (Table 3.1). Consequently, average values of the *cis/trans* isomer ratio for each product type have been used and seasonal effects ignored.

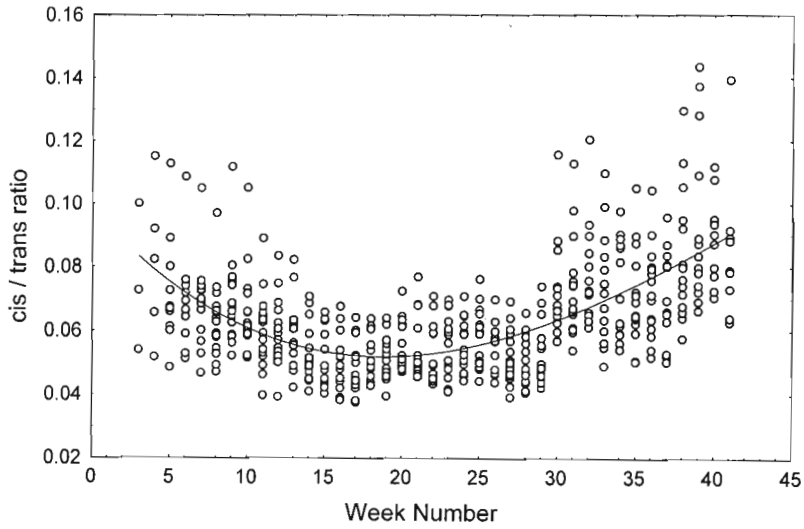

Figure 3.2 Graph showing the effect of seasonal trends on the *cis/trans* aconitic acid isomer ratio found in weekly composite mixed juice samples for 15 factories during the 1998/99 season. Data from Appendix 3.

3.2.2 Aconitic acid and "Ash"

It has been suggested that *trans*-aconitic acid may be present in sucrose storage cells to neutralise the basic alkaline earth elements adsorbed from the soil (Paturua, 1989). At the Sugar Milling Research Institute the weekly mixed juice composite samples from each mill undergo a variety of routine analyses including a sulphated ash analysis (Official Methods for South African Sugar Factories. South African Sugar Technologists' Laboratory Manual, 1985). The ash value obtained is indicative of the total levels of potassium, calcium and magnesium present in the juice and is used as a process control tool in the

factory. To test the hypothesis of Paturua the *trans*-aconitic acid results from the weekly mixed juice samples were plotted against the corresponding ash value for that sample (Figure 3.3). It can be seen that an increasing amount of *trans*-aconitic acid in the juice is associated with increasing ash levels. The correlation between these variables is significant at $p < 0.05$ (N=541) whilst the linear regression is defined as *trans*-aconitic acid = 139.09 + 1159.2 x ash. This would appear to some give credence to Paturua's suggestion.

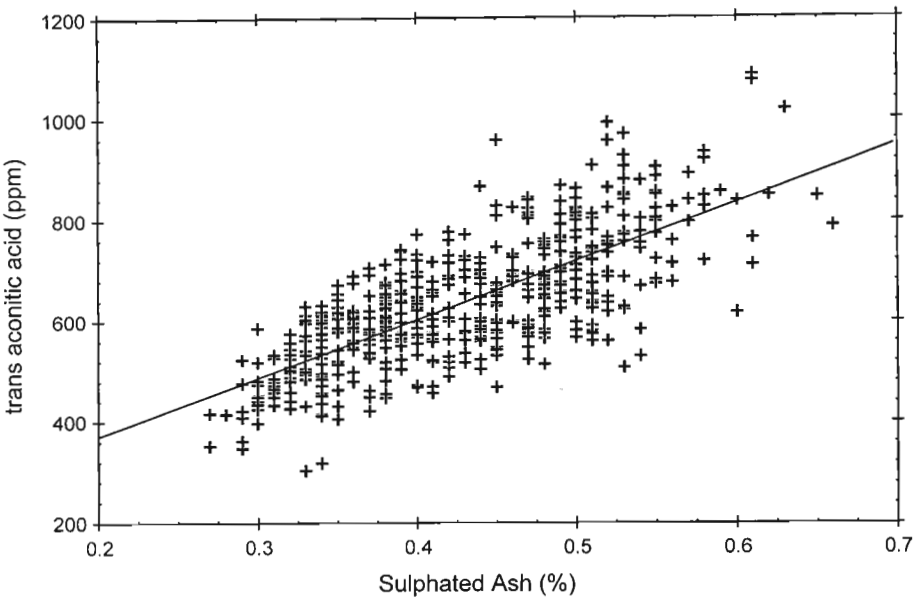

Figure 3.3 Graph showing the correlation between mixed juice sulphated ash and *trans*-aconitic acid concentration for the weekly MJ composite samples from all the mills for the 1998/99 season.

3.3 Factory Considerations

Having shown where the *trans*-aconitic acid originates and having quantified the levels and ratios entering the mill and present in mill process streams, lays the basis for the remainder of the study. The main thrust of this work therefore is the determination of the factors affecting the isomerisation of the *trans*-aconitic acid isomer present in the juice and the subsequent *cis/trans* isomer ratio found in the mill. It is proposed that the operational differences in the major unit operations could account for the increase in the *cis/trans* aconitic acid isomer ratio. Operational differences include:

Freshly harvested cane to the juice extracted by the mill (mixed juice):

- cane burnt or unburnt before harvesting
- varying harvest to crush delay between farm and mill
- temperature of storage between harvest and crushing
- time spent in the diffuser undergoing extraction from the cane (a residence time of approximately 15 minutes at 85°C)

Between mixed juice and clear juice:

- adjustment of pH to 7.0 with lime
- juice temperature maintained at greater then 95°C for an average of 30 minutes

Production of a syrup from the clear juice:

- consecutive temperatures of up to 115°, 103°, 98°, 82° and 60°C for between five and twenty minutes each in a quintuple multiple effect evaporator
- concentration of solids from 12% to 65%
- drop in pH from 7.0 to approximately 6.4

Crystallisation of the sugar from the syrup:

- pan boiling times of between 3 hours (A pans) and 10 hours (C pans) at temperatures of 65°C
- crystallisation times of between 8 hours (A crystallisers) and 50 hours (C crystallisers) at temperatures of between 65° and 45°C
- a drop in pH to approximately 5 to 5.5
- increase in ionic concentrations from approximately 0.03 M to 1 M

The major differences in these operations are changes in pH, temperature and/or ionic strength of the processing solutions due to removal of water with heat. It was therefore believed that these parameters should be studied with a view to their effect on the isomerisation of the *trans*-aconitic acid to *cis*-aconitic acid isomer.

Before the results of the changes in these parameters on the isomerisation reaction are discussed, an evaluation of the precision of the analytical methods used must be made. This will enable an assessment to be made whether differences are significant.

3.4 Precision of the experimental methods

An evaluation of the precision of the experimental methods used for the various aspects studied in this work will be reported. These include methods for both factory products and the isomerisation experiments.

3.4.1 Factory products

As some of the changes in the *cis/trans* aconitic acid isomer ratios found in the factory streams are small (Table 3.1), the quality of the analytical data must be considered before credence can be given to the values reported. Details of the development of the SPE method, including recoveries and precision obtained for individual acids, are given in Appendix 1. The chromatographic method development for factory products is detailed in Appendix 2.

3.4.2 Isomerisation experiments

Confidence in the results obtained from the kinetic studies to explain the factory findings are dependent on the analytical technique used to monitor the reaction and the actual isomerisation experimental factors. These are discussed in detail below.

3.4.2.1 Analytical Technique

In the present study, chromatography was used for quantitative analysis. Factors affecting the method include the detection technique, choice of eluent and method of calibration.

Chromatographic factors - *detection*

The use of instrumental methods in kinetic experiments allows any property directly proportional to concentration of one of the compounds of interest to be

used directly in data reduction, e.g. UV absorbance. However, the similar UV absorption spectra of the two isomers of aconitic acid precluded the use of this method in the study (see Figure 3.4). A chromatographic method allows the separation and quantitation of the individual isomers. In order to increase the sensitivity of the method a wavelength of 210 nm was chosen for detection of the acids on elution from the column.

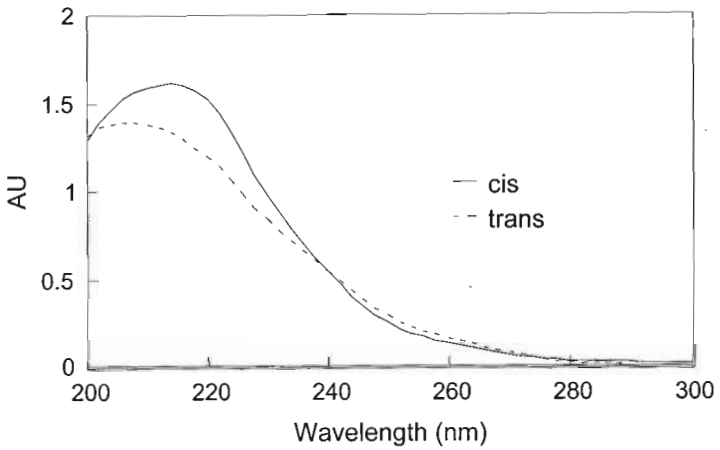

Figure 3.4 UV absorption spectra of *cis*-aconitic acid and *trans*-aconitic acid in water. Measured with a Philips PU8620 UV/VIS spectrophotometer, 1 cm cell. (0.238 mM *cis*-aconitic acid; 0.299 mM *trans*-aconitic acid, normalised at 200 nm)

For quantitation of the isomers direct use of the HPLC integrator counts was considered, but abandoned as the isomers have different chromatographic response factors due to unequal absorbances at 210 nm. The response factor is defined as the ratio of the concentration of a known standard to the area count of that standard. It is used to calculate the absolute concentration of the unknown sample from the relevant area counts. Since both isomer response factors are used in the calculation of the equilibrium constant *K*, this would have led to an incorrect value being reported.

Chromatographic factors - *eluent*

Separation of weakly ionised anionic solutes (such as aconitic acid isomers) using strong cation exchange resins was first introduced by Wheaton and Bauman (1953). It is commonly referred to as ion-exclusion chromatography. A BioRad HPX87H HPLC cation exchange column was used in the present study. The stationary phase consists of a sulphonated divinylbenzene-styrene copolymer resin with 8% crosslinking. With a hydrogen counter-ion it acts as an ion exclusion column when used with acidic eluents. The most significant factors affecting weakly ionised anionic solute retention on this column are the degree to which the solute ionises in the eluent and it's degree of unsaturation. As the solutes become more ionised, the Donnan exclusion effect (from the resin) increases in magnitude leading to decreased retention (Haddad and Jackson, 1990). The more unsaturated a solute, the greater the retention due to interaction with the divinylbenzene-styrene resin. Similarly, the more unionised a solute, the greater the retention. Solute retention time is thus dependent on the pK_a of the solute, eluent pH and unsaturation. A study was undertaken to determine the optimum pH for separation of the acids of interest. These included *cis*-aconitic acid, *trans*-aconitic acid and itaconic acid.

The HPLC system was equilibrated with a series of eluents of increasing pH. Three replicate injections of a mixture of aconitic acid isomers and itaconic acid were made and the retention times averaged. These were expressed in terms of the capacity factor k' and plotted as a function of pH (Figure 3.5) Capacity factor is defined as $k' = \frac{t_r}{t_r - t_0}$ where t_r and t_0 are the retention times of the peak of interest and unretained peaks respectively (Pryde and Gilbert, 1979).

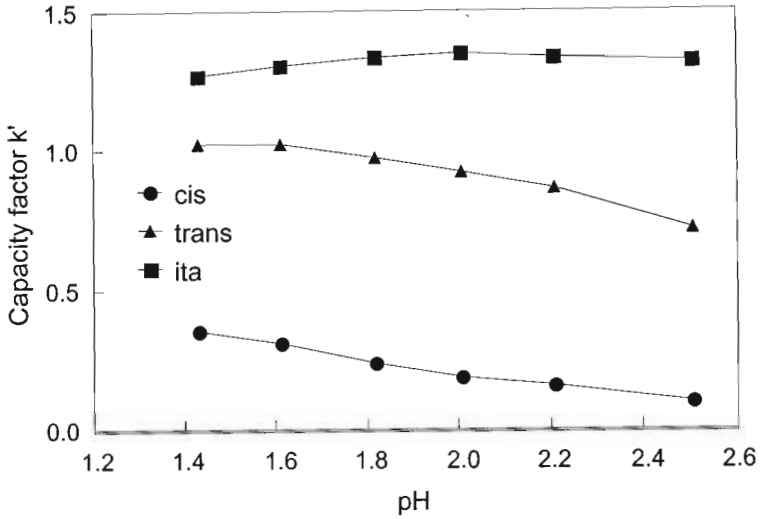

Figure 3.5 Effect of eluent pH on the separation of *cis*-aconitic acid, *trans*-aconitic acid and itaconic acid. Separation of the acids is measured as a function of capacity factor k'. Conditions : HPX87H column at 65°C; sulphuric acid eluent; flow rate 0.5 ml/min; UV detection at 210 nm; 20µl injection volume.

As pH increases the retention of the aconitic acid isomers decreases whilst that of itaconic acid remains approximately constant. Itaconic acid has pK_a values of 3.8 and 5.7 (Kirk-Othmer, 1967). Over the pH range studied here, both carboxylate groups remain unionised, and the acid is thus well retained. Conversely, at least one of the aconitic acid carboxylate groups can ionise in this range (see Section 3.10.1). From a chromatographic viewpoint, the *cis*-aconitic acid peak should be reasonably well resolved from the void or solvent peak for good reproducibility (Waters Publication, 1991). Reproducible integration from day to day of a peak close to the solvent void (the *cis*-aconitic acid isomer) is more difficult than one later in the chromatogram (the *trans*-aconitic acid isomer) where determination of the baseline is easier. Resolving the *cis*-aconitic acid peak from the void will also reduce possible co-elution with unknown compounds eluting in this area. Increased retention of the *trans*-aconitic acid isomer as the pH is reduced leads to a decrease of resolution with itaconic acid. An eluent pH of approximately 1.8 was therefore chosen as a compromise between these conflicting parameters.

Chromatographic factors - *calibration*

Calculation of the individual isomer concentrations in the isomerisation mixture required calibration of the HPLC. The following procedure was followed. The response factor from a calibration run (standards) at the beginning of the isomerisation experiment was used to calculate the concentration of the individual isomers at each time interval during the experiment. Each set of calibration data is reported with the associated experimental data in Appendix 6. Reporting of individual isomer concentrations had the advantage of allowing a mass balance check during the isomerisation. At each analysis time interval, the total aconitic acid concentration ([*cis*]+[*trans*]) was compared to the amount of *trans*-aconitic acid initially added to the vessel. This also had the advantage of giving an indication of possible further reactions (see Section 3.12).

A chromatographic method using one calibration standard for measurement of the isomer concentrations assumes the HPLC method is linear across the range of concentrations used. A calibration study (see Section 2.5.1) showed linearity across a range of 0.02 to 1.4 mM for each of the aconitic acid isomers and 0.083 to 4.154 mM for itaconic acid (see Table 3.2 and Figure 3.6 a, b and c). Itaconic acid was included in the calibration study due to it's appearance as a further reaction product later in the isomerisation studies (see Section 3.12). A 20 µl injection volume was used throughout as this is the same as was to be used in the isomerisation experiments. At concentrations above these shown for the *cis*-aconitic acid and *trans*-aconitic acid isomers, the detector was saturated. Consequently all isomerisation experiments used a starting concentration of 0.3 mM of *trans*-aconitic acid to ensure linearity.

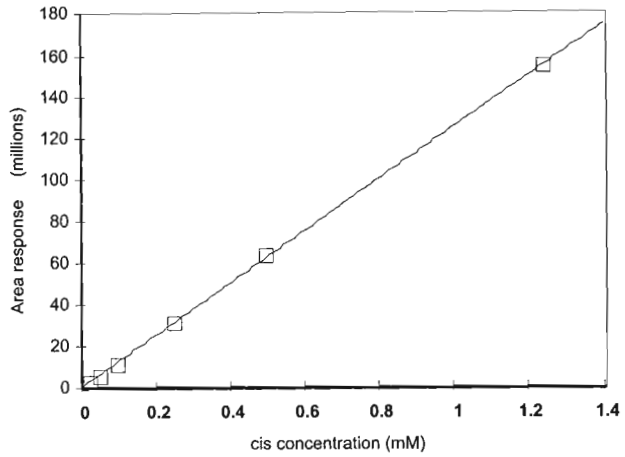

Figure 3.6a Graph showing linearity of HPLC detector response for the *cis*-aconitic acid isomer. (Data from Table 3.2).

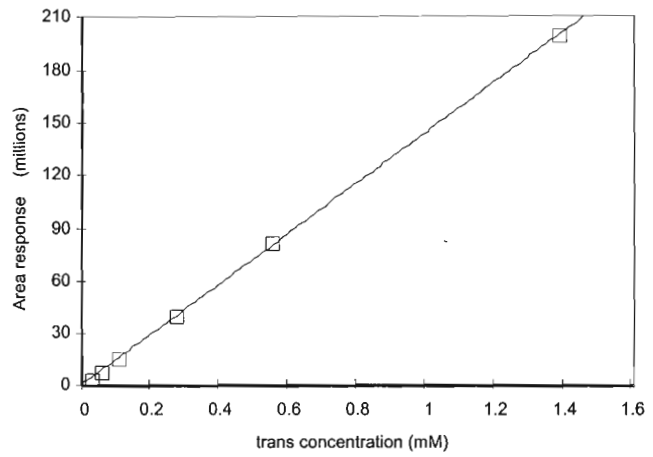

Figure 3.6b Graph showing linearity of HPLC detector response for the *trans*-aconitic acid isomer. (Data from Table 3.2).

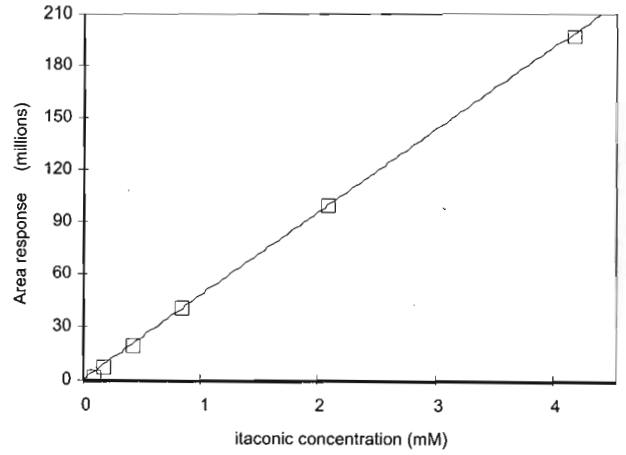

Figure 3.6c Graph showing linearity of HPLC detector response for itaconic acid. (Data from Table 3.2).

Table 3.2

Concentration and area counts of the *cis*-aconitic acid, *trans*-aconitic acid and itaconic acid used to determine the linearity range of the chromatographic method used for the isomerisation experiments

cis-aconitic acid		*trans*-aconitic acid		itaconic acid	
Concentration (mM)	HPLC area counts	Concentration (mM)	HPLC area counts	Concentration (mM)	HPLC area counts
0.025	3733562	0.028	4349184	0.083	3155469
0.050	6616803	0.056	8398822	0.166	8421165
0.099	11792448	0.111	16486712	0.415	20900656
0.248	31651024	0.278	40416992	0.831	41497312
0.497	63876500	0.556	81520896	2.077	100957660
1.242	155706320	1.391	199176560	4.154	198071280
R^2	0.9999		0.9999		0.9999
Std Err of Y	983747		731706		1203733
Slope	124339813		143029557		47689204
Constant	567988		703013		759458

3.4.2.2 Experimental Technique

Temperature and pH measurements are important variables in kinetic experiments. In the present study these were monitored during the isomerisation reactions carried out at temperatures lower than 100°C. These factors are now discussed.

Experimental factors - *temperature*

Temperature as a variable needs to be controlled precisely, since the rate constant of a reaction varies exponentially with temperature. Espenson (1995) suggests that control to within ±0.2°C is necessary to obtain reasonably precise data. At 300 K this error in temperature would result in an error of 1.4% in the calculated rate constant. In the experiments reported here a PID temperature controller was used to ensure precise control. A typical temperature profile during an isomerisation run at 90°C is shown in Figure 3.7. A small drop in temperature to 88.6°C was recorded when the *trans*-aconitic acid solution was added (indicated as "<< Start"). The solution temperature recovered to 89.8°C (within 0.2°C of 90.0°C) within 15 minutes. This time interval during which this decrease in temperature occurred is considered small compared to the overall run time of more than 10 hours. During this set of runs the average temperature throughout was 90.00 ± 0.15°C.

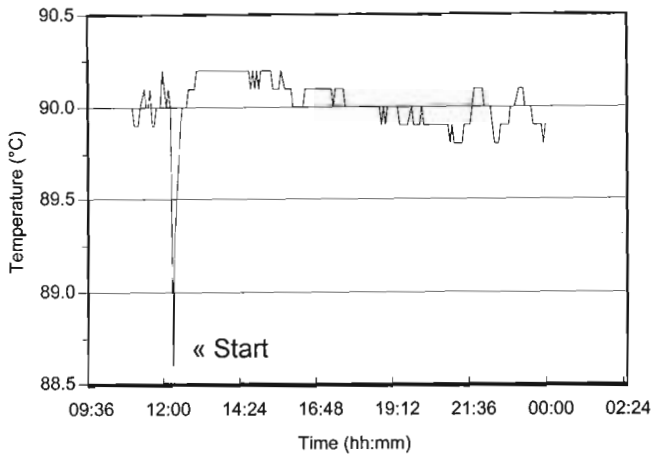

Figure 3.7 Typical temperature profile during the course of an isomerisation experiment at 90°C.

Experimental factors - *pH measurement*

No attempt was made to adjust the pH during any isomerisation experiment. The pH probe was calibrated at room temperature before each experiment at pH 7 and 4 with standard Beckmann buffers. The required pH of the isomerisation buffer was adjusted and reported at room temperature. All isomerisations, however, occurred at the pH of the buffer at the particular temperature of the experiment.

pH was monitored continuously during isomerisations below 100°C. No major change was found in any buffer used during a run, indicating that the pH remained essentially constant. A typical pH profile during a run is shown in Figure 3.8. A change of less than 0.15 pH units was recorded during this run. This could be attributed to drift in the pH probe response as much as a change in buffer pH during the 10 hour run.

Continuous measurement of the pH in isomerisation reactions above 100°C was impractical due to the unavailability of high temperature pH probes. A further problem with a pressurised vessel is the need to equalise the pressure in the reference electrode with that in the vessel. Measurement of buffer

solution pH was recorded before use (at room temperature) and in the solution at the end of the isomerisation run after being allowed to cool (to room temperature). No major changes in buffer pH were noted in these solutions.

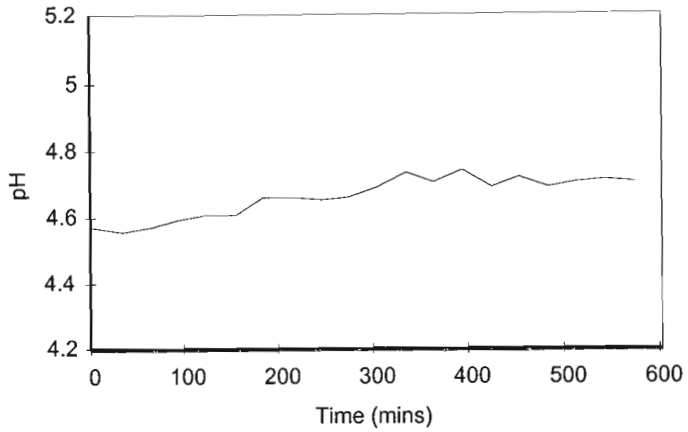

Figure 3.8 Plot of recorded pH (every 5 minutes) for a pH 5 acetate buffer containing *trans*-aconitic acid and *cis*-aconitic acid during a ten hour isomerisation experiment at 90°C.

Experimental factors - *selection of buffers*

The selection of buffers in which to carry out the isomerisation experiments was based on three criteria:

- their ability to buffer at a particular pH of interest
- buffer peaks on the HPLC chromatogram did not interfere with the separation of the *cis*-aconitic acid and *trans*-aconitic acid isomers
- on prolonged heating the buffer did not give spurious peaks on the chromatogram that eluted at the same time as the isomer peaks. This could cause overestimation of the *cis*-aconitic acid and *trans*-aconitic acid isomer concentrations.

Acetate was the buffer of choice at pH 5. In the chromatograms this eluted later than the isomers and was stable over the length of the isomerisations (Figure 3.9a). Buffering at pH 6, 7 and 8 was achieved with the phosphate anion. As this buffer does not absorb in the UV region at the detection wavelength of 210 nm, it was not considered as a possible source of error.

Citric acid was initially considered the buffer of choice for isomerisation at pH 4. However, from an experimental view it has two major disadvantages:

- chromatographically, elution of the citrate peak between the *cis*-aconitic acid and *trans*-aconitic acid isomers is undesirable
- a blank isomerisation procedure with no aconitic acid showed the increasing presence of both *cis*-aconitic acid and *trans*-aconitic acid with time (Figure 3.9b). This was attributed to dehydration of the citric to aconitic acid and subsequent isomerisation.

Phthalate buffer was therefore considered for this pH. Two disadvantages of this compound from a chromatographic viewpoint are:

- the late elution time of the phthalate peak causing extended chromatographic run times (35 minutes versus 20 to 25 minutes for the other buffers)
- the high absorbance of the phtahalate peak at 210 nm due to the presence of both the phenyl moiety and the two carboxylic acid groups.

These effects can be seen in a chromatogram of phthalate buffer (Figure 3.9c). The disadvantages were considered acceptable as only a small set of experiments were to be conducted at pH 4.

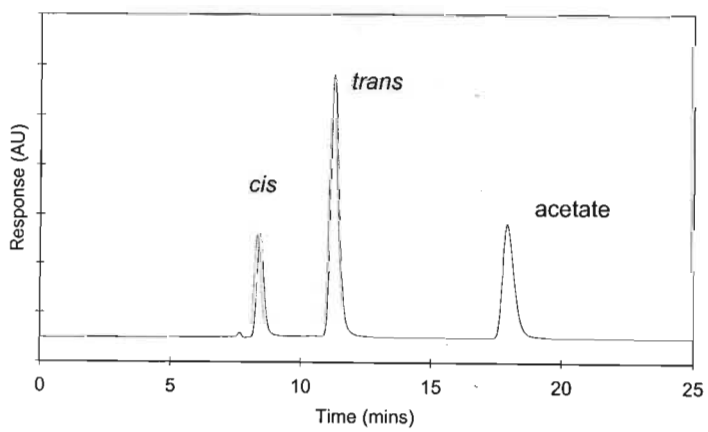

Figure 3.9a Chromatogram showing separation of the *cis*-aconitic acid and *trans*-aconitic acid isomers and the peak due to the acetate buffer.

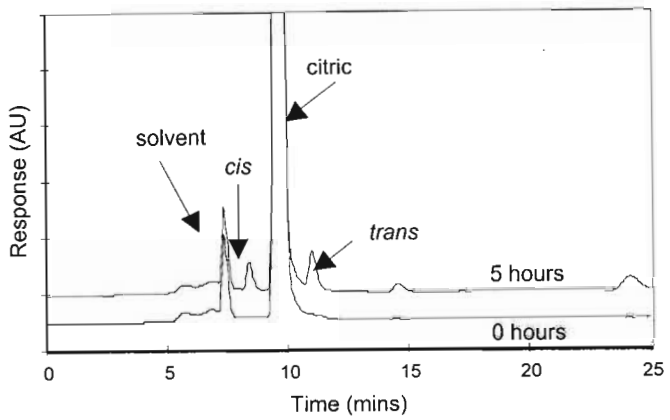

Figure 3.9b Chromatogram showing (bottom) absence of *cis*-aconitic acid and *trans*-aconitic acid at 0 hours; (top) presence of *cis*-aconitic acid and *trans*-aconitic acid and other compounds in citrate buffer after 5 hours at 97°C.

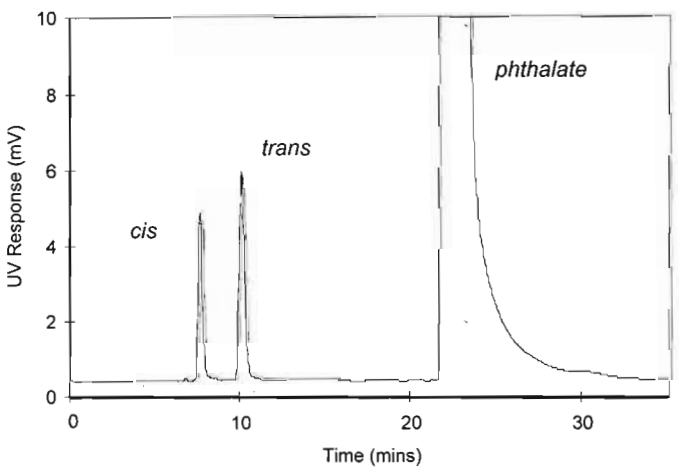

Figure 3.9c Chromatogram showing late elution and high absorbance of the peak due to the phthalate buffer, but well separated from the *cis*-aconitic acid and *trans*-aconitic acid isomer peaks.

3.5 Determination of the reaction order of the isomerisation

Isomerisation experiments were conducted in a buffered solution to ensure the pH remained effectively constant throughout the run. The presence of the

buffer could influence the order of reaction. Three possibilities exist which need to be considered:

- the buffer plays no role and the isomerisation reaction is first-order
- the buffer plays an active role in the isomerisation and the reaction is a second-order reaction
- the presence of high concentrations of the buffer compared to the *trans*-aconitic acid inadvertently makes the second-order reaction behave as a pseudo-first-order reaction.

Each of these possibilities will be considered in turn.

3.5.1 First-order reaction

Assuming the buffer plays no role in the isomerisation the reaction can be written

$$trans \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cis$$

where k_1 is the forward rate constant and k_{-1} is the reverse rate constant. At the beginning of the isomerisation the resultant *cis*-aconitic acid concentration from the forward reaction can be described by the first-order equation $\ln\left[\frac{[cis]^{eq}}{[cis]^{eq}-[cis]^{t}}\right] = k_1 t$ where k_1 is the first-order forward rate equation. A plot of $\ln\left[\frac{[cis]^{eq}}{[cis]^{eq}-[cis]^{t}}\right]$ against time ($\ln\frac{a}{(a-x)}$) would therefore be linear. The forward isomerisation data (*cis*-aconitic acid concentration) from an isomerisation reproducibility experiment at 90°C in 50 mM acetate buffer was plotted in this manner (Figure 3.10) and produced a good fit to the first-order equation.

3.5.2 Second-order reaction

If the buffer were to play a role in the isomerisation the reaction can be written

$$trans + \text{buffer} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cis + \text{buffer}$$

where k_1 is the forward rate constant and k_{-1} is the reverse rate constant. Note that the buffer appears on both sides of the equation since in the isomerisation

solutions analysed, no other reaction product is found other than *cis*-aconitic acid (other than at high temperatures and low pH - see Section 3.11). The buffer would then be acting as a catalyst. The resultant *cis*-aconitic acid concentration from the forward isomerisation reaction can be described by the second order equation $\left(\frac{[cis]^t}{[cis]^{eq}-[cis]^t}\right) = [cis]^{eq}k_2t$ where k_2 is the second-order forward rate constant. A plot of $\left(\frac{[cis]^t}{[cis]^{eq}-[cis]^t}\right)$ against time would therefore be linear. The same concentration data used in the first-order reaction plot is also shown in Figure 3.10 in this form. Obvious systematic departure from the second order equation is shown by the non-linearity of the graph. Similar plots were obtained for other buffers used.

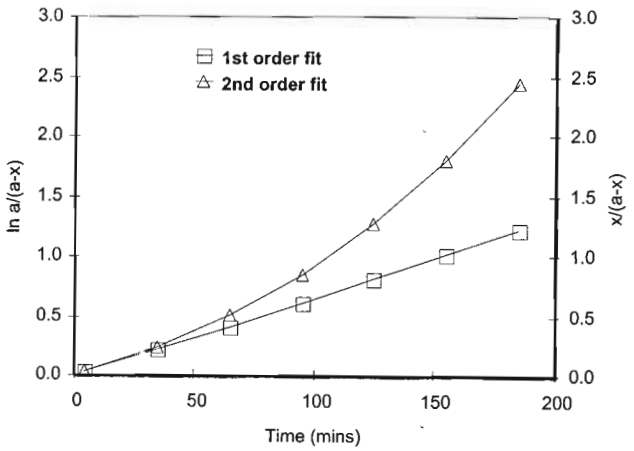

Figure 3.10 *cis*-Aconitic acid concentration isomerisation data in acetate buffer plotted to show both first-order and second-order forms. Noncompliance with second-order reaction kinetics is seen in the non-linearity of this form.

3.5.3 Pseudo first-order reaction

A possible complication in reaction order determination arises due to the use of a buffer. In the experimental procedure followed, the buffer concentration is greater than a 100-fold excess over the initial *trans*-aconitic acid concentration. If the buffer were to play a role in the isomerisation (second order reaction), the concentration of the buffer would remain effectively constant during the course of the experiment. Only the *trans*-aconitic and *cis*-aconitic acids would change

appreciably with time. In this case the reaction could follow pseudo-first-order kinetics.

Considering only the forward reaction, a second-order rate equation $v = kc_{trans}^{a} c_{buffer}^{b}$ can be written for which the reaction orders *a* and *b* should both be 1. In the isomerisation experiments reported here, $c_{buffer}^{0} \ggg c_{trans}^{0}$ establishing possible pseudo-first-order kinetics since the *trans*-aconitic acid has been "isolated" (Conners 1990). Normally, having determined the order for the *trans*-aconitic acid isomer *(a)*, the system would be reversed and the buffer isolated by setting $c_{trans}^{0} \ggg c_{buffer}^{0}$ and determining *b*, the order for the buffer. In this study it was not convenient analytically to use the isolation technique to determine the order with respect to the buffer due to the high concentrations of *trans*-aconitic acid that would be required. This problem can be overcome by repeating the initial experiment at several buffer concentrations and calculating the pseudo-first-order rate constants from the first-order plots. Pseudo-first-order reactions would give constant values for *b* under these conditions (Conners, 1990).

A set of experiments to determine if *b* was constant (pseudo-first-order) were conducted at 90° C in pH 5 acetate buffer by holding the *trans*-aconitic acid concentration approximately constant (0.3 mM) and increasing the buffer concentration (25 to 500 mM). From the first-order plots the pseudo-first-order rate constants were determined. (Table 3.3). The rate equation now becomes $-\frac{dc_{trans}}{dt} = k_e c_{trans}$ and it is anticipated that $k_e = kc_{buffer}$. If the reaction is second order, the quotient $\frac{k_e}{c_{buffer}^{0}}$ will be constant. This however is not the case (Table 3.3) showing the buffer does not take part in the isomerisation.

Table 3.3

Determination of reaction order constancy from pseudo-first-order rate constants (90°C, acetate buffer, pH 5)

Buffer concentration (mM)	Pseudo-first order rate constant k_e (s^{-1} x 10^{-3})	Constancy quotient $\frac{k_{obs}}{c^0_{buffer}}$ (M^{-1} s^{-1})
25	4.53	0.18
50	7.81	0.16
75	9.10	0.12
100	11.52	0.12
150	14.07	0.09
250	22.80	0.09
500	42.45	0.08

3.5.4 Effect of *trans*-aconitic acid concentration on isomerisation rate

A study of the effect of the *trans*-aconitic acid concentration on the rate of isomerisation can give further information on the order of reaction. A first-order rate constant is independent of the initial concentration of the starting material. A study of this effect was made at four different *trans*-aconitic acid concentrations in 50 mM acetate buffer at 97°C. The results are shown in Table 3.4. From the data presented there is no apparent difference within experimental error in the forward or reverse rate constants as the initial *trans*-aconitic acid isomer concentration increases.

Table 3.4

Rate constants (k_1, k_{-1}) and equilibrium constants (K) for isomerisation of increasing concentrations of *trans*-aconitic acid in 50 mM acetate buffer at 97°C

Initial concentration of *trans*-aconitic acid (mM)	Forward rate k_1 (s^{-1} x 10^{-5})	Reverse rate k_{-1} (s^{-1} x 10^{-5})	Equilibrium Constant K
0.107	4.55	10.05	0.45
0.297	3.99	9.55	0.42
0.605	4.20	9.10	0.46
1.176	4.28	9.36	0.46

All four tests described here, both negative and positive, confirm first-order kinetics for the isomerisation of the *trans*-aconitic acid to the *cis*-aconitic acid isomer. This has two implications. Firstly, it obviated the need for weighing exactly the same mass of *trans*-aconitic acid isomer for each experiment. Secondly, extrapolation of these experiments can be made to factory concentrations of aconitic acid.

3.6 Overall experimental precision

To obtain an estimate of the precision of the entire method, three replicate isomerisation experiments were carried out at 90°C in 50 mM pH 5 acetate buffer. In each case a plot of the residuals ($[trans]_{t,obs}$ - $[trans]_{t,calc}$) was examined for systematic deviations. It was found unnecessary to discard any data points. Table 3.5 summarises the results obtained. The rate and equilibrium constants were calculated for each replicate. The raw data are available in Appendix 4. The nonlinear equations 2.7 and 2.9 (Chapter 2) were used to fit the concentration data and calculate the rates in these and subsequent experiments. A typical concentration profile obtained during a run is shown in Figure 3.11.

Table 3.5

Calculated rate (k_1, k_{-1}) and equilibrium constants (K) for the reproducibility experiments at 90°C and pH 5 in 50 mM acetate buffer

Replicate	Forward rate k_1 (s^{-1} x 10^{-5})	Reverse rate k_{-1} (s^{-1} x 10^{-5})	Equilibrium constant K
1	3.17	7.43	0.43
2	3.51	7.74	0.45
3	3.33	7.65	0.44
Mean	3.34	7.61	0.44
SE*	0.10	0.09	0.01

* SE = standard error at 95% confidence level

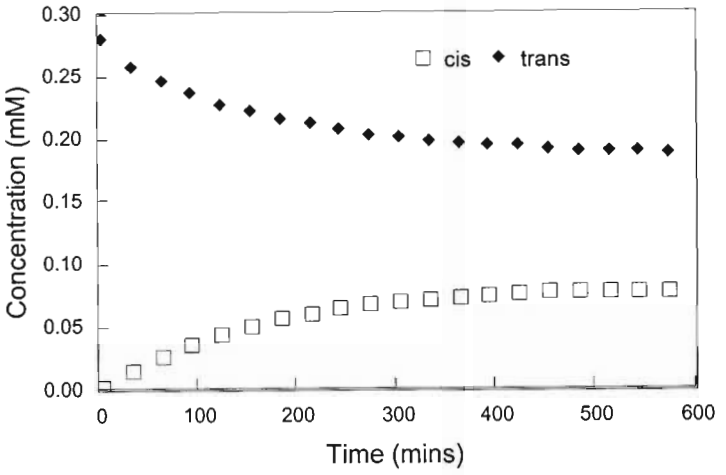

Figure 3.11 Typical concentration profile of the *cis*-aconitic acid and *trans*-aconitic acid isomers measured during the course of an isomerisation experiment. Conditions: 90°C, 50 mM acetate buffer, pH 5.0.

The reported standard error values (Table 3.5) (calculated at the 95% interval) include all aspects of experimental error. These include weighing, buffer preparation, temperature and chromatographic variability. The latter includes injection, eluent and standard preparation, and integration. The standard errors of approximately 0.1 x 10^{-5} s^{-1} (<2.8% for all aspects) are considered adequate for study of the isomerisation. All further isomerisations were run singly at a particular pH / buffer / temperature combination. This allowed as wide a range of combinations as possible. The rationale followed was that of Jencks (1969). He suggests that having five rate constants (with a 5% accuracy) exploring a variable are more valuable than a single constant with 1% accuracy.

3.7 Effect of buffer and pH on reaction rate at selected temperatures

3.7.1 Experimentally calculated rate constants

The forward and reverse isomerisation rate constants were measured as a function of buffer concentration and pH at selected temperatures (Tables 3.6 to 3.10). Each reported rate constant is determined from one isomerisation experiment at that particular temperature and buffer combination (as discussed in the previous section).

Table 3.6

Calculated forward (k_1), reverse (k_{-1}) and equilibration rate (k_e) constants ($s^{-1} \times 10^{-5}$) and the equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid at increasing buffer concentration (pH 4, phthalate buffer)

Temp °C	Buffer Conc (mM)	k_1	k_{-1}	k_e	K
97	10	5.30	7.92	13.21	0.67
	20	7.06	10.90	19.97	0.65
	25	7.16	10.62	17.78	0.67
	50	9.01	15.89	24.90	0.57

Table 3.7

Calculated forward (k_1), reverse (k_{-1}) and equilibration rate (k_e) constants ($s^{-1} \times 10^{-5}$) and the equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid at increasing buffer concentration (pH 5, acetate buffer)

Temp °C	Buffer Conc (mM)	k_1	k_{-1}	k_e	K
70	25	0.41	0.90	1.31	0.45
	50	0.50	1.24	1.74	0.41
	75	0.94	2.40	3.34	0.39
	150	1.68	4.32	6.00	0.39
80	25	0.91	2.19	3.11	0.42
	50	1.62	3.68	5.30	0.44
	75	2.41	5.72	8.12	0.42
	150	3.22	7.70	10.92	0.42
90	25	1.95	4.32	6.28	0.45
	50	3.17	7.23	10.40	0.44
	75	3.74	8.87	12.61	0.42
	150	6.11	10.51	16.61	0.41
97	25	2.33	5.29	7.62	0.44
	50	3.99	9.55	13.54	0.42
	75	6.19	14.13	20.32	0.44
	150	11.42	26.07	37.49	0.44
110	25	4.38	8.00	12.38	0.55
	50	9.73	19.50	29.23	0.50
	75	11.21	22.40	33.61	0.50
	150	42.18	83.89	126.00	0.50

Table 3.8

Calculated forward (k_1), reverse (k_{-1}) and equilibration rate (k_e) constants ($s^{-1} \times 10^{-5}$) and the equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid at increasing buffer concentration (pH 6, phosphate buffer)

Temp °C	Buffer Conc (mM)	k_1	k_{-1}	k_e	K
70	25	0.17	0.61	0.78	0.28
	50	0.31	1.19	1.49	0.26
	75	0.43	1.76	2.19	0.24
	150	0.64	3.05	3.67	0.21
80	25	0.30	1.07	1.37	0.28
	50	0.30	1.50	1.80	0.25
	75	0.78	3.24	4.02	0.24
	150	1.50	6.71	8.22	0.22
90	25	0.44	1.42	1.86	0.31
	50	1.29	4.65	5.94	0.28
	75	1.59	6.03	7.61	0.26
	150	2.99	12.64	15.63	0.24
97	25	1.02	2.97	3.99	0.35
	50	2.26	7.37	9.62	0.31
	75	2.59	9.25	11.84	0.28
	150	5.20	22.35	27.55	0.23
110	25	2.62	7.61	10.23	0.34
	50	6.56	21.44	28.00	0.31
	75	8.38	27.45	35.84	0.31
	150	13.46	47.96	61.41	0.28

Table 3.9

Calculated forward (k_1), reverse (k_{-1}) and equilibration rate (k_e) constants ($s^{-1} \times 10^{-5}$) and the equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid at increasing buffer concentration (pH 7, phosphate buffer)

Temp °C	Buffer Conc (mM)	k_1	k_{-1}	k_e	K
70	25	0.03	0.21	0.24	0.14
	50	0.03	0.19	0.22	0.14
	75	0.05	0.38	0.43	0.14
	150	0.11	0.83	0.94	0.14
80	25	0.06	0.44	0.50	0.14
	50	0.08	0.59	0.67	0.14
	75	0.15	1.10	1.25	0.14
	150	0.22	1.53	1.74	0.14
90	25	0.08	0.50	0.57	0.15
	50	0.16	1.09	1.25	0.15
	75	0.17	1.18	1.35	0.15
	150	0.31	2.17	2.48	0.15
97	25	0.20	1.22	1.43	0.17
	50	0.49	3.03	3.52	0.16
	75	0.52	3.24	3.76	0.16
	150	0.78	5.59	6.36	0.14
110	50	0.50	3.02	3.52	0.17
	75	1.07	6.04	7.10	0.18
	150	1.95	11.38	13.33	0.17

Table 3.10

Calculated forward (k_1), reverse (k_{-1}) and equilibration rate (k_e) constants ($s^{-1} \times 10^{-5}$) and the equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid at increasing buffer concentration (pH 8, phosphate buffer)

Temp °C	Buffer Conc (mM)	k_1	k_{-1}	k_e	K
97	25	0.07	0.51	0.58	0.13
	50	0.08	0.64	0.72	0.13
	75	0.09	0.64	0.73	0.13
	150	0.10	0.76	0.86	0.13

The most obvious effects that can be seen in this data are:

- a slightly increasing rate constant with increasing buffer concentration at any particular temperature
- an increasing rate constant with increasing temperature at any particular buffer concentration
- a decreasing rate constant with increasing pH at any particular temperature and buffer concentration
- an equilibrium constant (K) that is approximately constant at any particular pH, irrespective of temperature and buffer concentration.

The rate of isomerisation is seen to be dependent on the buffer concentration. This would appear more so at the higher temperatures and lower pH's. Comparison of the rates at any particular temperature or pH requires the effect due to the buffer to be eliminated. This is achieved by extrapolating the rate constants to zero buffer concentration from the rates determined in the individual buffers.

3.7.2 Calculation of isomerisation rates at 0 M buffer concentration

Equation 2.11 was used with the rate constants from the four increasing buffer concentrations at any particular pH and temperature to calculate the rate at zero buffer concentration. The natural log of the rate constant was plotted against the square root of the ionic strength of the buffer used (see Table 3.11).

Table 3.11

Buffer concentration used and equivalent ionic strength

Buffer	Concentration (mM)	Ionic strength (M)
Phthalate	10	0.030
	20	0.060
	25	0.075
	50	0.150
Acetate	25	0.025
	50	0.050
	75	0.075
	150	0.150
Phosphate	25	0.150
	50	0.300
	75	0.450
	150	0.900

A regression was calculated and extrapolated to zero buffer strength (Figure 3.12 shows a typical plot). The intercept yields the rate constant at zero buffer strength (Table 3.12). The relative errors recorded across the range of experiments are reasonably consistent. Reported errors represent the accumulation of all errors at that particular pH, buffer and temperature combination. These include buffer preparation, analytical determination, temperature control and calculation uncertainties.

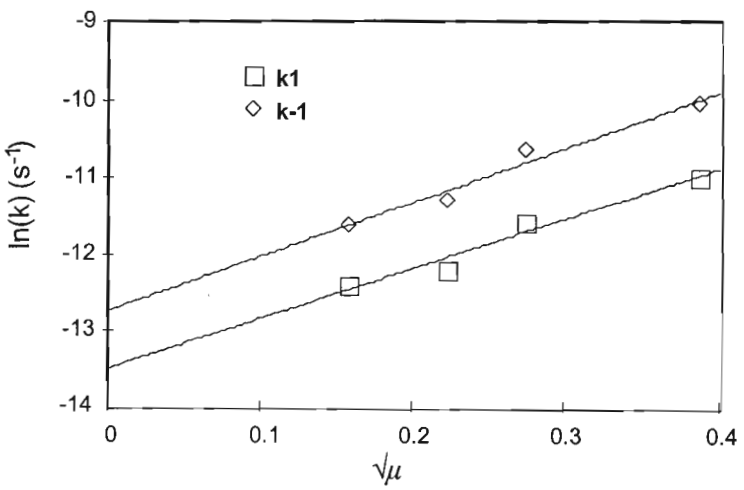

Figure 3.12 Typical plot of the natural log of the rate constant of the isomerisation of *trans*-aconitic acid to *cis*-aconitic acid against the square root of the ionic strength of the buffer solution used. This is used to calculate the rate at 0 M ionic strength. This plot shows the data at 70°C and pH 5.

Table 3.12

Calculated forward (k_1) and reverse (k_{-1}) kinetic rate constants ($s^{-1} \times 10^{-5}$) (with standard error of estimate) and the equilibrium constant (K) at 0 M ionic strength buffer for the *trans-cis* isomerisation of aconitic acid at increasing temperature and pH

pH	Temperature °C	k_1	k_{-1}	K
4	97	3.69 ± 0.40	4.68 ± 0.50	0.79
5	70	0.14 ± 0.03	0.29 ± 0.07	0.47
	80	0.45 ± 0.13	1.06 ± 0.28	0.43
	90	0.99 ± 0.14	2.03 ± 0.28	0.49
	97	0.83 ± 0.13	1.95 ± 0.33	0.43
	110	0.98 ± 0.24	1.77 ± 0.46	0.55
6	70	0.08 ± 0.02	0.23 ± 0.05	0.34
	80	0.09 ± 0.02	0.28 ± 0.07	0.31
	90	0.17 ± 0.03	0.46 ± 0.19	0.37
	97	0.41 ± 0.11	0.91 ± 0.24	0.45
	110	1.16 ± 0.42	3.03 ± 1.20	0.38
7	70	0.01 ± 0.00	0.06 ± 0.02	0.13
	80	0.03 ± 0.01	0.18 ± 0.05	0.14
	90	0.04 ± 0.01	0.23 ± 0.07	0.15
	97	0.11 ± 0.04	0.58 ± 0.21	0.19
	110	0.10 ± 0.05	0.62 ± 0.27	0.16
8	97	0.05 ± 0.01	0.42 ± 0.04	0.13

This data shows the normal effect of an increasing rate constant with increasing temperature for a fixed pH. It also shows decreasing rate with increasing pH. Between pH 5 and 7 experiments were undertaken at 70, 80, 90, 97 and 110°C whilst experiments at pH 4 and 8 were only conducted at 97° C. This temperature was chosen as a single temperature compromise for the temperature range found in the sugar factory (70 to 120°C) over which to study the pH effect (4 to 8).

3.8 Calculation of kinetic factors

The reaction rate increases with an increase in temperature due to an increase in the number of molecules having an energy in excess of the reaction energy barrier required to break the π bond. Numerical values for this barrier can be calculated using the Arrenhius equation. This describes the relationship between the rate constant and the temperature:

$$k = Ae^{-E_a/RT}$$

which can be placed in the linear form:

$$\log k = \log A - \frac{E_a}{2.303RT}$$

3.8.1 Temperature effects at 0 M buffer concentration

The pre-exponential factor log A and the activation energy E_a at 0 M ionic strength have been calculated at pH 5, 6 and 7 (Table 3.13) . It is assumed that both A and E_a are temperature independent over the temperature range considered. The thermodynamic terms enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) have been calculated by using equation 2.12 (Table 3.13). The standard error of estimate reported for these quantities appear quite large. However it should be borne in mind that the kinetic factors calculated are reported for 0 M buffer concentrations. Extrapolations across wide temperature and concentration ranges have been made and as such the errors reported are accumulated across all the rate constant calculations.

Table 3.13

Activation energy (E_a), pre-exponential factors (log A), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) for the forward and reverse *trans*/*cis* isomerisation of aconitic acid at 0 M ionic strength

	pH		
	5	6	7
	Forward (*trans* to *cis*)		
E_a (kJ mol^{-1})	52 ± 17	77 ± 13	71 ± 15
log A (s^{-1})	2 ± 2	5 ± 2	4 ± 2
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-213 ± 43	-134 ± 36	-164 ± 41
$\Delta H^{\ddagger}$ (kJ mol^{-1})	49 ± 17	74 ± 13	68 ± 15
	Reverse (*cis* to *trans*)		
E_a (kJ mol^{-1})	48 ± 19	71 ± 13	64 ± 12
log A (s^{-1})	2 ± 3	5 ± 2	4 ± 2
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-217 ± 19	-160 ± 35	-185 ± 34
$\Delta H^{\ddagger}$ (kJ mol^{-1})	45 ± 19	68 ± 13	61 ± 12

The thermodynamic term enthalpy of activation ($\Delta H^{\ddagger}$) represents the difference in heat content between the activated complex and the reactant (referred to their standard states). Similarly the entropy of activation ($\Delta S^{\ddagger}$) represents the difference in entropy between the activated complex and the reactant (referred to their standard states). $\Delta H^{\ddagger}$ is a quantity similar to the activation energy. Assuming $\Delta H^{\ddagger}$ is temperature independent it can be shown (Espenson, 1995) that

$$\Delta H^{\ddagger} = E_a - RT$$

and at a temperature of 90°C, E_a is approximately 3.0 kJ mol^{-1} larger than $\Delta H^{\ddagger}$. This agrees with the values found in this work (Table 3.13). The negative entropy values are indicative of bond breaking.

Kinetic data such as this have not been reported in the literature for the isomerisation of *trans*-aconitic acid to the *cis*-aconitic acid isomer. Maleic acid and its esters, which can isomerise to fumaric acid, have similar structural features to aconitic acid. Kinetic data for this isomerisation have been reported (Table 3.14).

Table 3.14

Activation energy (E_a) and pre-exponential factor (log A) for the forward reaction of the isomerisation of dimethyl maleate to dimethyl fumarate and maleic to fumaric acid

Reactant	Medium	Activation energy E_a (kJ mol^{-1})	Pre-exponential factor log A (s^{-1})	Reference
Dimethyl maleate	Vapour Liquid Anisole	110.9 ± 12.6 103.3 ± 2.9 99.6 ± 6.3	5.11 5.00 3.30	Davis and Evans, 1955
Maleic acid	Water	61.1 66.1	2.93 3.46	Tamamushi and Akiyama, 1937
Maleic acid	Fused	66.1	4.22	Højendahl, 1924

The activation energy of the maleate *ester* is greater than that recorded here for *trans*-aconitic acid. Possible reasons for this include:

- Measurement of the isomerisation was in the gas phase
- Measurement of the isomerisation were made in non-polar solvents
- The presence of the ester makes rotation about the double bond more difficult

Values reported for the isomerisation of maleic acid to fumaric acid in water at 80° to 100°C and in the fused state are similar to the values recorded here.

3.8.2 Buffer concentration and pH effects

The pre-exponential factor A, activation energy E_a, enthalpy and entropy of activation at each combination of buffer concentrations and pH have been calculated (Tables 3.15 to 3.17). The standard error of estimates for these values are also recorded.

Table 3.15

Activation energy (E_a), pre-exponential factor (log A), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) for the forward and reverse *trans-cis* isomerisation of aconitic acid at pH 5

	Buffer concentration (mM)			
	25	50	75	150
	Forward (*trans* to *cis*)			
E_a (kJ mol^{-1})	64 ± 5	77 ± 7	76 ± 5	87 ± 8
log A (s^{-1})	5 ± 1	7 ± 1	5 ± 1	8 ± 1
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-169 ± 15	-129 ± 20	-156 ± 13	-95 ± 21
$\Delta H^{\ddagger}$ (kJ mol^{-1})	61 ± 6	74 ± 7	63 ± 5	84 ± 8
	Reverse (*cis* to *trans*)			
E_a (kJ mol^{-1})	59 ± 8	73 ± 7	69 ± 12	80 ± 11
log A (s^{-1})	4 ± 1	6 ± 1	6 ± 2	8 ± 2
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-177 ± 21	-134 ± 18	-143 ± 34	-107 ± 31
$\Delta H^{\ddagger}$ (kJ mol^{-1})	56 ± 8	70 ± 7	66 ± 12	77 ± 11

Table 3.16

Activation energy (E_a), pre-exponential factor (log *A*), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) for the forward and reverse *trans-cis* isomerisation of aconitic acid at pH 6

	Buffer concentration (mM)			
	25	50	75	150
	Forward (*trans* to *cis*)			
E_a (kJ mol^{-1})	75 ± 8	88 ± 10	81 ± 6	82 ± 1
log A (s^{-1})	6 ± 1	8 ± 2	7 ± 1	7 ± 0
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-148 ± 23	-105 ± 28	-123 ± 15	-115 ± 4
$\Delta H^{\ddagger}$ (kJ mol^{-1})	72 ± 8	85 ± 10	78 ± 6	79 ± 1
	Reverse (*cis* to *trans*)			
E_a (kJ mol^{-1})	68 ± 8	83 ± 9	106 ± 15	111 ± 20
log A (s^{-1})	5 ± 1	8 ± 11	11 ± 2	12 ± 3
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-158 ± 23	-110 ± 25	-132 ± 14	-122 ± 4
$\Delta H^{\ddagger}$ (kJ mol^{-1})	65 ± 8	80 ± 9	71 ± 5	72 ± 2

Table 3.17

Activation energy (E_a), pre-exponential factor (log *A*), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) for the forward and reverse *trans-cis* isomerisation of aconitic acid at pH 7

	Buffer concentration (mM)			
	25	50	75	150
	Forward (*trans* to *cis*)			
E_a (kJ mol^{-1})	69 ± 15	85 ± 14	81 ± 10	78 ± 8
log A (s^{-1})	4 ± 2	7 ± 2	6 ± 2	6 ± 1
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-180 ± 43	-130 ± 40	-139 ± 28	-142 ± 23
$\Delta H^{\ddagger}$ (kJ mol^{-1})	66 ± 15	83 ± 14	78 ± 10	75 ± 8
	Reverse (*cis* to *trans*)			
E_a (kJ mol^{-1})	61 ± 14	79 ± 14	74 ± 10	73 ± 8
log A (s^{-1})	4 ± 2	7 ± 2	6 ± 2	6 ± 1
$\Delta S^{\ddagger}$ (J mol^{-1}K^{-1})	-185 ± 40	-131 ± 38	-143 ± 28	-141 ± 22
$\Delta H^{\ddagger}$ (kJ mol^{-1})	58 ± 14	76 ± 14	71 ± 10	70 ± 8

The calculated errors associated with the parameters are generally lower than for the zero buffer calculation (Section 3.8.1). The activation parameters show the forward reaction (*trans*-aconitic acid to *cis*-aconitic acid) to be marginally less favorable than the reverse *(cis*-aconitic acid to *trans*-aconitic acid). The isomerisation reaction is moderately slow as can be seen in the reasonably high activation energies. Accurate values of $\Delta S^{\ddagger}$ are not easy to obtain. The most precise calculated values of $\Delta H^{\ddagger}$ have an uncertainty of about ±RT or 2.5 kJ mol^{-1} whilst the uncertainty of the most precise values of $\Delta S^{\ddagger}$ is approximately 7-8 J mol K^{-1}. Reported uncertainties are often three times higher than these (Espenson, 1995) - approximately 25 J mol K^{-1}. Most of the data reported here falls within this range indicating confidence can be placed in the results reported here. An exception to this is the data shown for low buffer concentrations at pH 7. Higher uncertainties can be attributed to the long isomerisation times due to the slow isomerisation. The small change in aconitic acid isomer concentrations over long periods makes determination of the equilibrium ratio less accurate leading to consequent errors in rate constant calculations. The trends relating to pH, temperature and buffer concentration which can be seen here will be discussed later.

If it were assumed that the activation energy and pre-exponential factors are uninfluenced by the buffer and pH, an average value for the activation energy and pre-exponential factor can be calculated from Tables 3.15 to 3.17 (Table 3.18). This will be used in the development of an isomerisation model for use in the factory (see Section 3.13). There is no difference between the forward and reverse activation energies showing that it is relatively easy to convert between the two isomers. By comparison the conversion from fumaric acid (*trans)* to maleic acid (*cis*) requires 66.1 kJ mol^{-1} whilst the reverse requires 42 kJ mol^{-1}. The difference of 24 kJ mol^{-1} is greater than that for aconitic acid.

Table 3.18

Average activation energy (E_a) and pre-exponental factor (log *A*) from *trans-cis* isomerisation of aconitic acid at different pH's and buffer concentrations

	Forward (*trans* to *cis*)	Reverse (*cis* to *trans*)
E_a (kJ mol^{-1})	79	78
log *A*	6	7

3.9 Mechanism of isomerisation

To understand the process of isomerisation a quantum mechanical model of the carbon-carbon double bond can be considered. A carbon atom which is part of a double bond is attached to only three other atoms. Three sp^2 hybrid orbitals are used to accommodate the three equivalent bonding orbitals. In this mode of hybridisation the orbitals lie in one plane. As a consequence the angle between any pair of orbitals on the plane is 120°. This *trigonal* arrangement permits the hybrid orbitals to be as far apart as possible. Overlap of the carbon-carbon sp^2 orbitals results in a σ bond. In forming the sp^2 orbitals only two of the three *p* orbitals have been used. The remaining *p* orbital on each carbon consists of two equal lobes above and below the plane of the σ bond. Maximum overlap of the lobes is achieved when the molecule is planar resulting in a π bond. Thus a π electron cloud is found above and below the plane of the carbon-carbon bond. Because of less overlap, the π bond is weaker than the σ-bond. Rotation of one carbon with respect to the other requires the π bond to be broken. Distortion of the molecule from this planar configuration can only be achieved by supplying energy to decrease the overlap of the π-electons.

Most thermal isomerisation studies have been undertaken in the gas phase. The reactants are uninfluenced by the solvent molecule force fields making interpretation of results easier. Literature data can be arbitrarily divided into two classes (Cundell, 1964): those with low activation energies and pre-exponential factors, and a second class with normal activation energies and pre-exponential factors. Division of gas phase isomerisations into two classes was challenged in later studies (Laidler and Louckes, 1972). *Cis-trans*

isomerisations in solution also showed that both high and low *A* and E_a factors can occur. No challenge to this division for isomerisation in solution has been made.

Divergent views exist on the mechanism that applies for isomerisation under these conditions and will now be discussed. One follows the premise that the isomerisation proceeds from the singlet ground state via a triplet state (low pre-exponential factor). The second via a singlet mechanism (high pre-exponential factor). Magee *et al.* (1941) proposed two alternative mechanisms to account for the low and high factors. Figure 3.13 is a simplified energy diagram for the singlet and triplet states of an ethylenic compound (after Mulliken and Roothaan, 1947). The energy is shown as a function of the angle of twist. Rotation of one of the methylene groups of a substituted ethylene molecule in its ground state (I) through 180° degrees leads to an equivalent singlet state (II). The variation in potential energy is represented by the two overlapping parabolas. The energy of the first excited triplet state is shown as a straight line cutting the two curves.

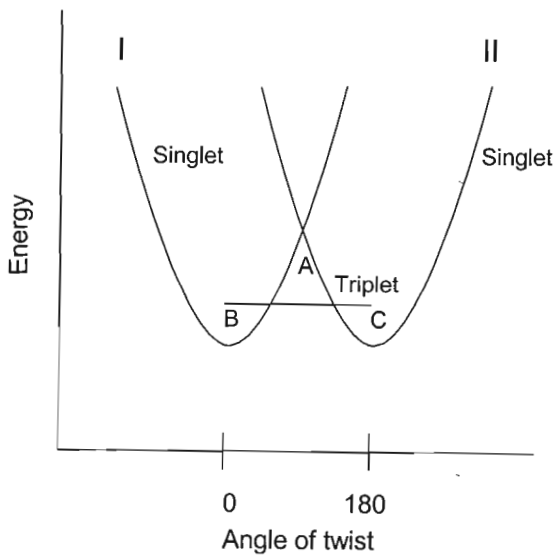

Figure 3.13 Simplified diagram of the potential energy curves for the rotation of ethylene about the double bond. Curves I and II are for the ground state singlet. The first triplet curve (shown as a straight line) cuts curves I and II at B and C. See text for details. (After Mulliken and Roothan, 1947.)

The higher energy singlet isomerisations involve a change of eigenfunction from ψ_1 to ψ_2. The activated complex occurs at A. For a low energy isomerisation, a transition to the triplet state is required. This has a lower energy requirement. The rate determining step would be passing over barrier B or C. The activation energy E_a is in this case the height of B or C above the minimum ground state energy. An unsatisfactory feature of the proposed mechanisms is the lack of any indication as to what decides the choice of reaction path.

In comparison, Lin and Laidler (1968), correlate the energies of activation for *cis-trans* isomerisations of ethylenic compounds with the π-bond energies of the double bond. The π-bond energy is defined as the difference between the dissociation energy for a molecule and its corresponding radical (Semenov, 1957). This provides support for π-bond breakage due to rotation through 90° in the activated states (Laidler and Loucks, 1972). The energy involved corresponds to the conversion of an sp^3 carbon atom to an sp^2 carbon. All work was undertaken in the gas phase. Consequently, the drawback with this proposal for the current study is the lack of results in aqueous solution. The values of the kinetic factors presented in this work would appear to fall close to the low activation energy values and agree with the singlet-triplet theory.

In this work no study was made of the effect the polarity of the solvent (water) may have on the isomerisation. The effect of pH was studied as a consequence of the processes occurring in a sugar mill (see Section 3.10). Aconitic acid is reasonably soluble in a variety of organic solvents, especially in the unionised form. This could be the scope of further research in understanding and explaining the isomerisation from a mechanistic viewpoint.

No difference is shown in the activation energies for the forward and reverse isomerisations. This could partially explain the accumulation of *trans*-aconitic acid isomer in the growing sugar cane stem. Freshly extracted cane juice has low levels of *cis*-aconitic acid and high levels of *trans*-aconitic acid. In the plant, *cis*-aconitic acid can easily isomerise to the *trans*-aconitic acid. If preferential

chelation of the *trans*-aconitic acid isomer occurs with calcium and magnesium from the soil (Paturua, 1989), this would prevent the reverse isomerisation leading to an accumulation of the *trans*-aconitic acid form which cannot be utilised in the Krebs cycle. In the sugar mill factory sufficient thermal energy is available to aid isomerisation of the *trans*-aconitic acid back to the *cis*-aconitic acid form.

3.10 Effect of pH

The effect of pH can be seen in two areas: on the actual isomerisation rates and the isomerisation equilibrium constant K. To overcome the effect of the buffer used, the rate constants at 0 mM buffer are used to study the effect of pH (see Section 3.7.2).

3.10.1 On the isomerisation

The relationship between pH and rate constants (pH-rate profiles) are best viewed graphically. Figure 3.14 shows the pH-rate profile at 0 mM buffer concentration at 97°C. The combined rate equation $k_e=k_1+k_{-1}$ is plotted against pH of isomerisation, not the pH of the buffer at room temperature (where it was made and measured). The pH at the temperature of isomerisation (97° C) was calculated for each buffer using the Nernst equation from the pH at room temperature.

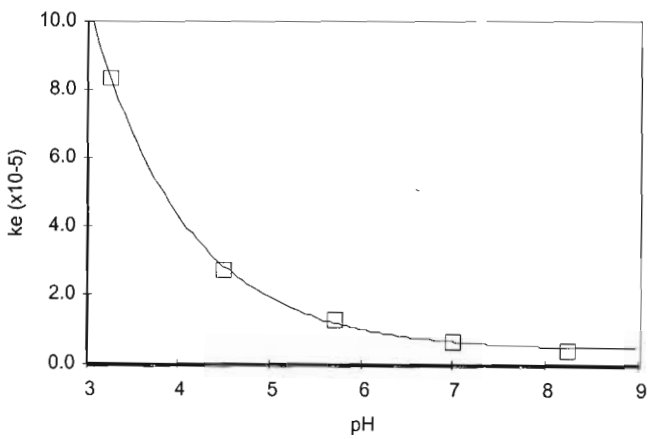

Figure 3.14 pH-rate profile for the isomerisation of *trans*-aconitic acid to *cis*-aconitic acid at 97° C and 0 mM buffer concentration.

It is obvious that the rate increases as the pH decreases. This would suggest a change in the nature of the aconitic acid due to the drop in pH. Aconitic acid contains ionisable groups in the pH range studied. A change in the degree of ionisation of the aconitic acid occurs with pH. This can be visualised by plotting a distribution of the aconitic acid - conjugate base species against pH.

Aconitic acid is a tricarboxylic acid with three protons available for dissociation. Given

H_3A	represents the un-ionised acid
H_2A^-	represents the mono-ionised acid
HA^{2-}	represents the di-ionised acid
A^{3-}	represents the totally ionised acid

and

$$K_1 = \frac{[H^+][H_2A^-]}{[H_3A]} = 1.02\times10^{-3}$$

$$K_2 = \frac{[H^+][HA^{2-}]}{[H_2A^-]} = 6.55\times10^{-5}$$

$$K_3 = \frac{[H^+][A^{3-}]}{[HA^{2-}]} = 7.80\times10^{-7}$$

(Guerra *et al.*, 1985)

The fractions of solute for each form are given by :

$$F_{H_3A} = \frac{[H_3A]}{\delta_t}$$

$$F_{H_2A^-} = \frac{[H_2A^-]}{\delta_t}$$

$$F_{HA^{2-}} = \frac{[HA^{2-}]}{\delta_t}$$

$$F_{A^{3-}} = \frac{[HA^{3-}]}{\delta_t}$$

Where δ_t , the total molar concentration of substrate is

$$[H_3A]+[H_2A^-]+[HA^{2-}]+[A^{3-}]$$

Combining these equations leads to expressions for the fractions of solute as a function of the dissociation constants and hydronium ion concentration:

$$F_{H_3A} = \frac{[H^+]^3}{[H^+]^3+K_1[H^+]^2+K_2[H^+]+K_1K_2K_3}$$

$$F_{H_2A^-} = \frac{K_1[H^+]^2}{[H^+]^3+K_1[H^+]^2+K_2[H^+]+K_1K_2K_3}$$

$$F_{HA^{2-}} = \frac{K_2[H^+]}{[H^+]^3+K_1[H^+]^2+K_2[H^+]+K_1K_2K_3}$$

$$F_{A^{3-}} = \frac{K_1K_2K_3}{[H^+]^3+K_1[H^+]^2+K_2[H^+]+K_1K_2K_3}$$

Figure 3.15 shows F_{H_3A}, $F_{H_2A^-}$, $F_{HA^{2-}}$ and $F_{A^{3-}}$ plotted as a function of pH. The pH-rate profile (Figure 3.14) is overlaid onto this plot. A good correlation is apparent between the rate profile and the mono-ionised form of the aconitic acid. Isomerisation would appear to be dependent on the presence of the singly charged form of the acid which is directly related to the decrease in pH.

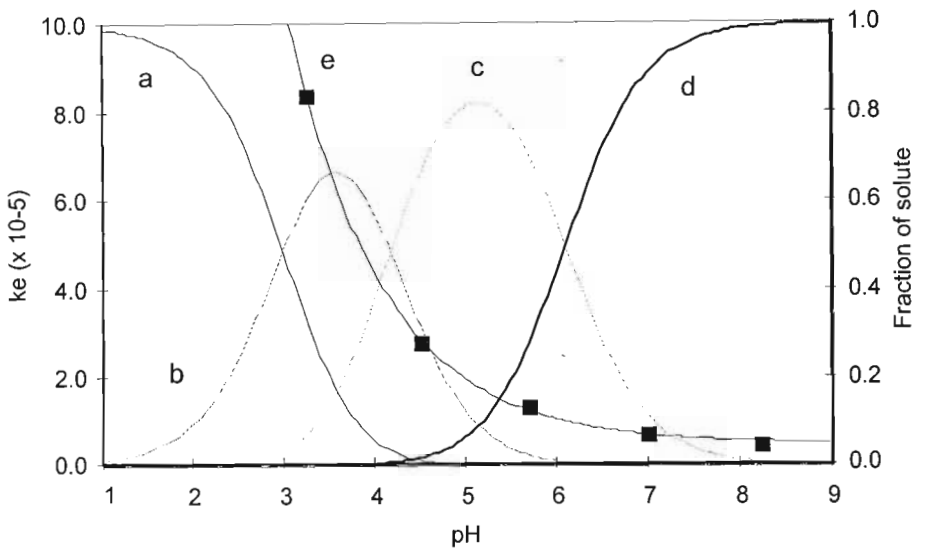

Figure 3.15 Composition of aconitic acid as a function of pH, overlaid with the *cis-trans* aconitic acid isomerisation rate k_e. a = H_3A; b = H_2A^-; c = HA^{2-}; d = A^{3-}; e = k_e.

The rate of isomerisation appears to be pH dependent. However, the hydronium ion does not take part in the isomerisation since the data presented here shows that both the *overall* isomerisation *(k_e)* and individual forward and reverse reactions are first order. It is evidently a thermal isomerisation. In many compounds containing the C=C-C=O group (as in aconitic acid), the *cis* form is

readily transformed into the *trans* in acid solution. Noyce *et al.* (1963) used *cis*-cinnamic acid to show that the rate determining step in non-aqueous media is the addition of a proton to the double bond. Davis and Evans (1955) found the maleic acid isomerisation in non-aqueous media to be a catalysed second order reaction. In contrast Horrex (1937) showed that in *aqueous media* using DCl as catalyst, no addition of the deuterium to the double bond occurred. Tamamushi and Akyama (1937) also showed the isomerisation of maleic acid in aqueous media to be first order.

The increase in rate with a drop in pH could be explained as follows. Isomerisation requires breaking of the π bond. Consider the di- and tri-valent ionised form of aconitic acid. Two or three of the carboxylate groups will be ionised. These carboxylate groups have a positive inductive effect meaning it is electron repelling. The π electrons from the double bond will tend to be repelled toward the carbon-carbon double bond. An increase in the overlap of the π electrons will occur resulting in a marginally stronger bond. A higher amount of energy will be required to break the bond before rotation can occur. In the mono-dissociated form the two undissociated groups (-COOH) have a negative inductive effect. The π electrons from these groups can conjugate with the π electrons from the double bond. This will reduce the amount of π electron overlap between the two carbon atoms in the double bond. The energy required to break the bond will therefore be less. Thus, as the pH drops, the degree of dissociation will decrease and isomerisation will therefore be easier, as evidenced in Figure 3.15.

3.10.2 On the equilibrium constant *K*

The increase in the equilibrium constant *K* with decreasing pH is a further consequence of the hydronium ion concentration. Average equilibrium constants at each temperature have been calculated from the values calculated and shown in Tables 3.6 to 3.10 (Table 3.19). The increase in the equilibrium constant as the pH drops is directly related to the degree of ionisation of the *trans*-aconitic acid isomer. Subsequent isomerisation is easier and the concentration of the *cis*-aconitic acid isomer increases.

Table 3.19

Summary of the average equilibrium constant (*K*) for the isomerisation of *trans*-aconitic acid to *cis*-aconitic acid as a function of pH

pH	Average *K*
4	0.64
5	0.43
6	0.27
7	0.15
8	0.13

The data from Table 3.19 can be summarised in an exponential form as :

$$K = 3.47 \exp(-0.4225 * pH) \qquad (R^2=0.977)$$

This empirical fit (Figure 3.16) will be used in the model describing isomerisation in the sugar factory (Section 3.13), but the equation is only valid for a pH range of 4 to 8.

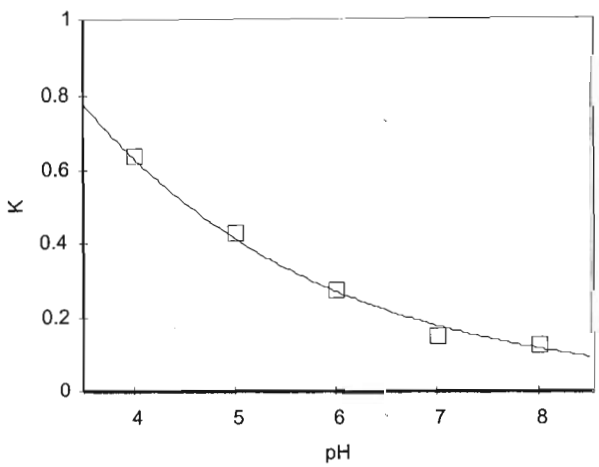

Figure 3.16 Average equilibrium ratio data as a function of pH with the solid line showing the empirical exponential fit to the data points.

3.11 Effect of cations on the rate of isomerisation

In all the work presented, sodium salts were used to prepare the buffers. In a sugar factory, many cations are present. These are predominately potassium, calcium and magnesium. The effect of these cations on the isomerisation was

studied at pH 5 and 90°C. Acetate buffer was used and the sodium substituted with the appropriate cation.

3.11.1 Effect of potassium ions on the rate of isomerisation

The potassium ion is by far the most abundant cation present in sugar juice samples, found at levels in excess of 1% in the juice. It is therefore important to know of any possible effects this cation may have on the isomerisation. Direct substitution of sodium acetate with potassium acetate in a pH 5 buffer was used as an indicator of possible effects. The results of the experiment are shown in Table 3.20.

Table 3.20
Comparison of the forward (k_1) and reverse (k_{-1}) isomerisation rates ($s^{-1} \times 10^{-5}$) and equilibrium constant (K) for the *trans-cis* isomerisation of aconitic acid in 50 mM sodium and 50 mM potassium acetate buffer (pH 5, 90°C)

Anion	k_1	k_{-1}	K
Sodium	3.17	7.23	0.44
Potassium	2.79	6.08	0.46

The isomerisation rate constants obtained for the potassium salt were similar to those obtained in the presence of sodium. It can therefore be concluded that potassium does not markedly affect the rate of isomerisation.

3.11.2 Effect of calcium and magnesium ions on the rate of isomerisation

The effects of the divalent cations, calcium and magnesium, on the isomerisation rate are important. Calcium and magnesium ions are naturally present in the juice extracted from the cane. Extra calcium ions are added in the factory at the clarification stage in the form of lime to effect neutralisation of the mixed juice to pH 7 and help form insoluble calcium phosphate. This precipitates, thereby helping to clarify the juice. This lime also contains some magnesium. In addition, aconitic acid may chelate these cations through its three carboxylate donor groups.

Effects due to the individual cations were measured by adding calcium or magnesium in increasing amounts to an acetate buffer. The amounts added were at the ratio of aconitate to calcium and magnesium found in typical factory streams (see Table 3.21). Due to the divalent charge on the alkali earth metals, the ionic strength of a 50 mM acetate buffer containing added calcium or magnesium would not be equivalent to a buffer without the added ions. To make comparisons with the previously calculated rate constants in acetate buffer, equivalent ionic strength solutions were prepared. Possible synergistic effects between calcium and magnesium were also considered. A mixed calcium/magnesium acetate buffer was prepared in a similar manner. The results obtained are shown in Table 3.22.

Table 3.21

Concentration of the divalent ion added to acetate buffer to determine the effect on the *trans-cis* aconitic acid isomerisation rates at pH 5 and 90°C

Concentration (mM)	$Ca(OAc)_2$ (g/l)	$Mg(OAc)_2$ (g/l)
4.5	0.711	0.639
4.5 + 4.5	0.711	0.639

Table 3.22

Comparison of the forward (k_1) and reverse rates (k_{-1}) ($s^{-1} \times 10^{-5}$) and equilibrium ratio (K) for the *trans-cis* isomerisation of aconitic acid in equivalent ionic strength sodium acetate buffer (pH 5, 90°C) containing calcium and magnesium

Added cation	k_1	k_{-1}	K
None	3.17	7.23	0.43
Ca^{2+}	3.56	8.76	0.41
Mg^{2+}	3.51	8.40	0.42
$Ca^{2+} + Mg^{2+}$	3.47	8.68	0.40

These data show that the presence of the two cations makes virtually no difference to the overall rate of the isomerisation of the *trans*-aconitic acid to the *cis*-aconitic acid isomer.

3.12 Decomposition of aconitic acid

Scrutiny of the raw data obtained at 110°C and pH 5, and 97°C and pH 4 shows that the isomerisation did not reach a final equilibrium point. The total aconitic acid value decreased with time, indicating a further reaction was taking place. Another compound appeared on the chromatograms with a concomitant decrease in the amounts of the *cis*-aconitic acid and *trans*-aconitic acid isomers. This peak eluted at the same retention time as itaconic acid (propylenedicarboxylic acid) and when checked using the UV_{210}/RI height ratio (see Section A2.3) gave a value indicative of itaconic acid. Calibration of the chromatograph with itaconic acid allowed calculation of the concentration present in solution. Addition of the molar concentrations of the two aconitic acid isomers and the itaconic acid gave reasonably accurate total mass balances (Table 3.23), especially at the earlier reaction times. A concentration profile for this data is shown in Figure 3.17.

Table 3.23

Mass balance of *trans-cis* aconitic acid isomerisation and subsequent decarboxylation to itaconic acid at pH 4 and 97°C

Time (mins)	*cis* (mM)	*trans* (mM)	Itaconic (mM)	Total Conc (mM)
0	0.002	0.312	0.001	0.315
15	0.024	0.289	0.001	0.314
30	0.042	0.267	0.001	0.310
45	0.057	0.252	0.002	0.311
60	0.068	0.240	0.003	0.311
80	0.080	0.224	0.005	0.309
100	0.087	0.212	0.006	0.305
120	0.093	0.201	0.008	0.302
150	0.099	0.191	0.011	0.301
180	0.101	0.180	0.014	0.295
240	0.103	0.167	0.020	0.290
300	0.099	0.156	0.025	0.280
360	0.096	0.148	0.031	0.275
420	0.092	0.140	0.036	0.268
510	0.084	0.128	0.043	0.255
1455	0.031	0.047	0.091	0.169
1815	0.019	0.033	0.098	0.150
1940	0.016	0.030	0.100	0.146

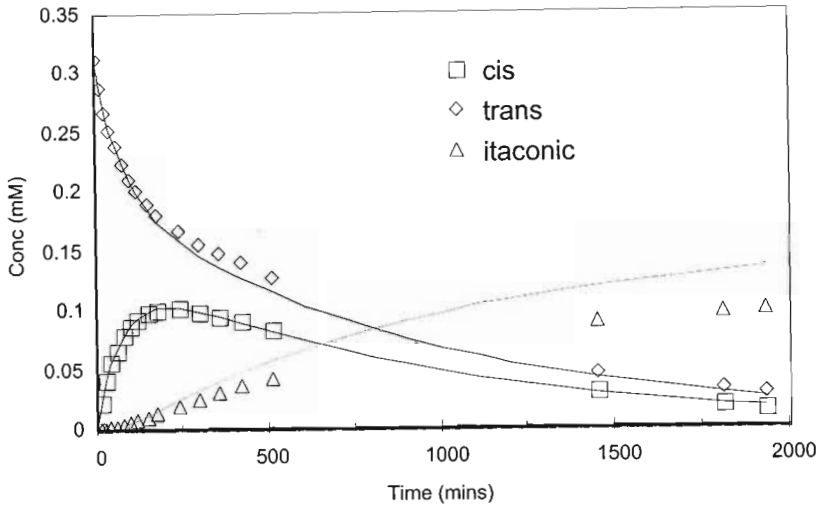

Figure 3.17 Concentration profile of the *trans-cis* aconitic acid isomerisation with subsequent itaconic acid formation. Data from Table 3.23. Drawn lines are estimated from calculated rate constants.

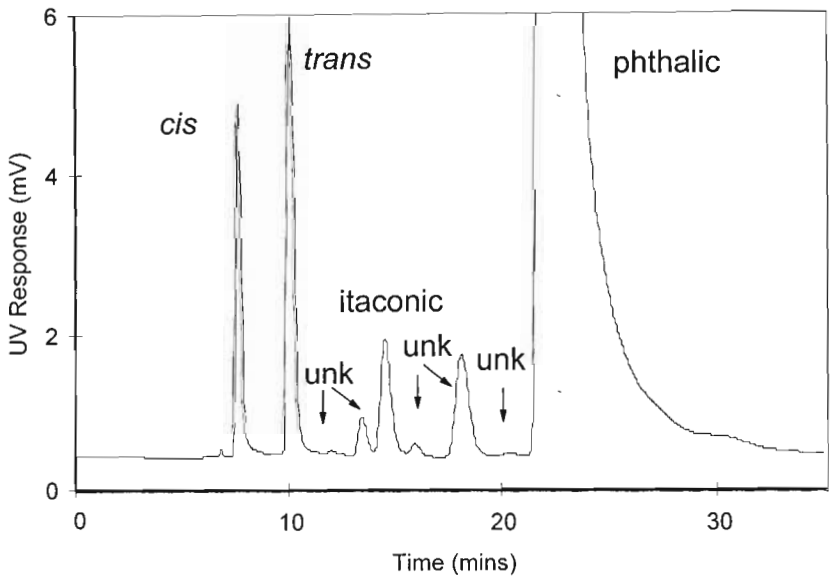

Figure 3.18 Chromatogram of *trans-cis* aconitic acid isomerisation after 510 minutes at 97°C and pH 4 showing formation of itaconic acid and unknown (unk) compounds. Also shown are the *cis*-aconitic acid, *trans*-aconitic acid and phthalic acid peaks (from phthalate buffer).

It can be seen that with extended times the mass balance is inaccurate. Unknown peaks appear in the chromatogram indicating that further reactions begin to occur (Figure 3.18). Also, if other saturated compounds form they may not be detected by the UV chromatographic system used in this work. The refractive index detector system used for analysis of factory samples would be a useful adjunct to the UV system if further work were to be considered in this field. Similar chromatograms were obtained in acetate buffer showing the compounds formed were from the aconitic acid and not the buffer.

In all instances when itaconic acid was found, there appeared to be a time lag between the start of the isomerisation and the appearance of itaconic acid. This paralleled the increase in *cis*-aconitic acid (Figure 3.19).

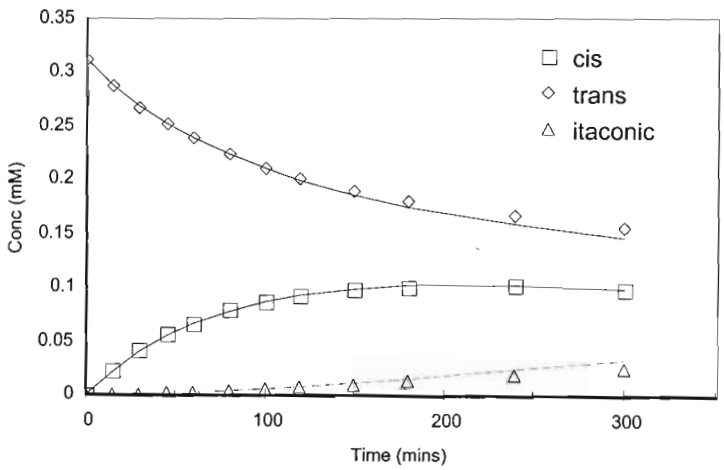

Figure 3.19 Concentration profile of the *trans-cis* aconitic acid isomerisation for the first 300 minutes showing appearance of itaconic acid with the increase in *cis*-aconitic acid. Data from Table 3.23. Drawn lines are estimated from calculated rate constants.

It was therefore assumed that the itaconic acid originated from decarboxylation of the *cis*-aconitic acid isomer and hence the following reaction scheme can be written:

$$trans \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cis \xrightarrow{k_2} \text{itaconic} + CO_2$$

The rate law for each of the species can be written as follows:

$$-\frac{d[trans]}{dt} = k_1[trans] - k_{-1}[cis]$$

$$\frac{d[cis]}{dt} = k_1[trans] - k_{-1}[cis] - k_2[cis]$$

$$\frac{d[itaconic]}{dt} = k_2[cis]$$

where [ ] is the concentration of the individual species. For the case where the initial concentration $[cis]^0$ = [itaconic]^0 = 0 (the case studied here), the concentrations at any time t for the three species are given by (Fersht *et al.*, 1970):

$$[trans]_t = \frac{k_1[trans]^0}{\lambda_2-\lambda_3}\left\{\frac{\lambda_2-k_2}{\lambda_2}\exp(-\lambda_2 t) - \frac{\lambda_3-k_2}{\lambda_3}\exp(-\lambda_3 t)\right\}$$

$$[cis]_t = \frac{k_1[trans]^0}{\lambda_2-\lambda_3}\{\exp(-\lambda_3 t) - \exp(-\lambda_2 t)\} \qquad (3.1)$$

$$[itaconic]_t = [trans]^0\left\{1 + \frac{\lambda_3}{\lambda_2-\lambda_3}\exp(-\lambda_2 t) - \frac{\lambda_2}{\lambda_2-\lambda_3}\exp(-\lambda_3 t)\right\}$$

where

$$\lambda_2 = \tfrac{1}{2}(p+q)$$

$$\lambda_3 = \tfrac{1}{2}(p-q)$$

$$p = k_1 + k_{-1} + k_2$$

$$q = \sqrt{(p^2 - 4k_1k_2)}$$

Any one of the concentrations measured can be fitted to the equation. From the calculated parameters (k_1, λ_2, λ_3), the constituent rate constants can be obtained. The *cis*-aconitic acid concentration data were fitted to equation 3.1. Calculated rate constants for this scheme at 97° and pH 4 are :

k_1 = 5.3×10^{-3} s^{-1}
k_{-1} = 6.0×10^{-3} s^{-1}
k_2 = 2.6×10^{-3} s^{-1}

These rate constants were used to obtain the calculated curves which are compared with the observed concentrations shown in Figures 3.17 and 3.19.

The apparent difference between calculated and observed values for itaconic acid at the later times can be attributed to further reactions of this acid which have not been taken into account in the calculated values. The itaconic acid will therefore not accumulate as the rate equation assumes, but reach some plateau and then tail off again. Although of interest from a kinetic viewpoint, this line of enquiry was not pursued further. In the sugar mill high temperatures and low pH's are not common. Sugar process engineers aim to minimise sucrose inversion which would occur at these pH's and temperatures. In the initial determination of aconitic acid concentrations present in factory streams, no itaconic acid was found. However, later studies have measured itaconic acid and associated carbon dioxide in final molasses which has undergone thermal degradation (85°C at pH 4.5 for extended periods) (Anon.; 2000)

3.13 Development of the isomerisation model

A model is required that can be used to predict the equilibrium value of the isomerisation of aconitic acid which can be applied to the major factory processes to determine the relative concentration of the isomers. Factory parameters that are easily measured are time, pH and temperature. The model must therefore express the equilibrium ratio as a function of these variables.

Given that the relationship between equilibrium constant K and pH is (Section 3.10.2):

$$K = 3.47 \exp(-0.4225 * pH)$$

and for the average activation energy E_a and pre-exponential factor A (Section 3.8.2), the rate equations can be written:

$$k_1 = A \exp(-E_a/RT) = 10^{6.2} \exp(-79000/8.314 * T)$$

$$k_{-1} = A \exp(-E_a/RT) = 10^{6.8} \exp(-78000/8.314 * T)$$

$$k_e = k_1 + k_{-1}$$

for any particular value of T (Kelvin).

Given $K = \frac{[cis]^{eq}}{[trans]^{eq}}$ and $[cis]^{eq} = [trans]^0 - [trans]^{eq}$, and assuming no further reaction, these can be combined to give:

$$[trans]^{eq} = \frac{[trans]^0}{(1+K)} \tag{3.2}$$

These can be combined to give the value of the *trans*-aconitic acid concentration at any time t (in seconds)

$$[trans]^t = [trans]^{eq} + ([trans]^0 - [trans]^{eq})\exp[-(k_e)t] \tag{3.3}$$

Assuming no other aconitic reactions occur the *cis*-aconitic acid isomer concentration can be calculated from:

$$[cis]^t = [trans]^0 - [trans]^t \tag{3.4}$$

and the *cis/trans* aconitic acid isomer ratio at time *t* as

$$K_t = \frac{[cis]^t}{[trans]^t} \tag{3.5}$$

By substitution of 3.2 into 3.3 and 3.4, and 3.3 and 3.4 into 3.5, the equilibrium ratio, at any time *t*, can be expressed as a function of pH, temperature and time. This can be written:

$$K_t = \frac{1-(\frac{1}{1+K})-(1-\frac{1}{1+K})\exp(-k_e t)}{(\frac{1}{1+K})+(1-\frac{1}{1+K})\exp(-k_e t)} + K_{initial}$$

If the initial equilibrium constant ($K_{initial}$) is known for any particular solution before further isomerisation occurs, this can be added to the calculated *K*. This gives an additive effect to predict the correct ratio for any particular part of the factory. These equations are only valid for the isomerisation reaction. At higher

temperatures the decarboxylation of the aconitic acid to itaconic acid may introduce errors. Using this model the *cis/trans* aconitic acid isomer ratios were calculated and compared to that found in the factory (Table 3.24). Reference to Figure 3.1 will aid understanding of the processes referred to in Table 3.24.

Table 3.24

Comparison of measured and calculated isomerisation equilibrium ratios for different stages in the sugar factory

Stage	Initial K	Time in stage (mins)	Average pH of stage	Average temperature of stage (°C)	Increase across stage	Calculated K at output	Actual K at output
Extraction							
MJ	0.03	15	6	85	0.01	0.04	0.06
Clarification							
CJ	0.05	30	7	95	0.02	0.07	0.08
Evaporation							
1st effect	0.07	5	7.0	115	0.01	0.08	
2nd effect	0.08	20	6.8	103	0.02	0.10	
3rd effect	0.10	10	6.6	98	0.01	0.11	
4th effect	0.11	16	6.4	82	0.01	0.12	
5th effect	0.12	20	6.4	60	0.00	0.12	
Syrup							0.13
Pan boiling (syrup to sugar)							
A pan	0.11	180	6.1	65	0.02	0.13	
A xtlers	0.13	480	6.1	55	0.03	0.16	
A-sugar							0.2
Final molasses							
B+C pans	0.16	900	5.9	60	0.07	0.23	
B+C xtlers	0.24	2700	5.8	50	0.08	0.31	
C mol							0.28

MJ = Mixed juice
CJ = Clear juice
C-mol = final molasses
Xtlers = crystallisers

The model describes the actual factory values very well. The expected MJ value is slightly lower than found in the process streams. In a sugar factory, many return streams are used to maximise the recovery of sugar. In the case of the MJ this includes filtrate return from the clarifier station. None of these returns have been accounted for in the model described above. Similarly, the figure for A-sugar is slightly less than the actual value (0.16 versus 0.20 found in A-sugar). This can be attributed to the recycling of B and C-sugars in the

factory back to the A pans. These sugars will contain *cis*-aconitic acid and *trans*-aconitic acid isomers from further in the factory which have not been accounted for in the model. A slight overestimation occurs toward the back-end of the factory. The times and pH values used in the model are average values and overestimation, especially of crystalliser retention times, are possible. It is pleasing to confirm the adequate explanation of the equilibrium increase found across the evaporator stage in earlier studies (Walford, 1998). Knowing the ratios of isomer present in a particular process stream could help in further investigation into calcium-magnesium aconitate and oxalate scales.

CHAPTER 4

CONCLUSIONS

This study has shown that aconitic acid present in sugar cane entering the sugar cane factory is present predominantly in the *trans*-aconitic acid form. A solid phase extraction technique using an anion exchange packing was used for isolation of the organic acids from factory process sugar solutions. The acids were analysed using an ion-exclusion, high performance liquid chromatography method. The absolute amounts of each aconitic acid isomer found in the process streams were used to determine the *cis-trans* aconitic acid ratio as a function of each sugar mill unit operation. It was found that isomerisation of the trans-aconitic acid to the *cis*-aconitic acid isomer occurs during processing.

The *trans-cis* aconitic acid isomerisation was found to be a reversible first-order process. An ion-exclusion chromatographic technique was used to measure the individual isomers as a function of time. The rates of isomerisation were measured as a function of pH, temperature, ionic strength and monovalent and divalent cation concentration. Values chosen were comparable to those typically found in sugar factories. Ionic strength and cation concentration were found to be minor factors. Major contributors to the rate of isomerisation are the temperature and the pH. It has further been shown that isomerisation is not the only reaction aconitic acid undergoes under these conditions. Decarboxylation to itaconic acid occurs with high temperatures and low pH's. A model has been developed from the isomerisation rates expressing the equilibrium ratio (K, the *cis/trans* aconitic isomer ratio) as a function of time, temperature and pH. The rate of isomerisation can be related to conditions present in the different factory processes. This has successfully been used to predict the observed equilibrium ratios found in the factory.

Aconitic acid salts have been implicated in recalcitrant evaporator scale. Knowing the equilibrium ratio as a function of factory control parameters would allow an in-depth study of this subject. Possible further investigations include

the possible precipitation of *cis*-aconitic acid and *trans*-aconitic acid salts, as a function of their equilibrium ratio, and a study of the decarboxylation of aconitic acid to itaconic acid. The latter could be related to the seasonal foaming found in massecuites, which is often attributed to the Maillard reaction.

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APPENDIX 1

SOLID PHASE EXTRACTION METHOD DEVELOPMENT

A chromatographic study of the organic acid composition of sugar factory streams requires a method to isolates the acids from other constituents. Sucrose, glucose and fructose co-elute with citric, tartaric, malic and *trans*-aconitic acids. Several isolation methods based on ion-exchange are described in the literature (Blake, *et al*, 1987; Oldfield, *et al*, 1973; De Bruijn, *et al*, 1984). A preliminary study of IR120H Amberlite ion-exchange resin packed in glass columns (15 x 125 mm) running under gravity was undertaken. This showed that large volumes of wash water were required to elute sugars from the column, resulting in long sample preparation times. This method was therefore abandoned.

A Waters QMA SepPak Plus SPE (solid phase extraction) column (a silica based quarternary methyl ammonium anion exchange resin) was chosen for acid isolation. The silica base material allows the use of higher pressures and flow rates than can be achieved on a resin-based packing, resulting in shorter sample preparation times. Connection to sample syringes and vacuum manifolds can be made by the use of Luer-lock inlet and outlet fittings. The packing is supplied equilibrated in the chloride form. To ensure that the ion-exchanger is in the formate form at the beginning of each isolation, the equilibration method described in Table 2.1 is followed. This is necessary since the packing has a higher affinity for chloride than for some of the organic acids which would otherwise not be retained. The formate counter-ion is not held as strongly and can be displaced by the organic acids of interest. Elution of a formic acid counter-ion peak in the chromatogram is a valuable marker. Its absence from the chromatogram indicates that the resin capacity had been exceeded (all the ion-exchange sites have been loaded with anions). This would mean that there are too many anions in the sample for the amount of ion-exchange material. Some of the acids will not have been retained. The sample would require further dilution.

A1.1 Determination of wash volume

A wash study was undertaken to determine the minimum volume of wash water required to rinse the SPE column free of residual sugar. The study was performed by passing 2 ml of a 10% sucrose solution through the SPE column, washing with 10 ml water and thereafter collecting 1 ml fractions. These were analysed for sucrose by means of cation exchange HPLC. This consisted of a Spectra-Physics IsoChrom pump, a Rheodyne 7125 syringe loading sample injector, a Erma ERC-7512 refractive index detector and HP 3396A intregrator. Separation was achieved on a Shodex IonPak KS-801 cation exchange column (8 x 300 mm) maintained at 80°C using 0.01 M sodium sulphate as eluent, filtered (0.45 µm) before use and maintained at 65°C on a stirred hotplate. The sugars were eluted at 0.6 ml/min. Table A1.1 records the sugar concentration in the collected fractions.

Table A1.1

Sucrose concentration in eluted SPE fractions

Total wash volume (ml)	Sucrose (%)
11	0.20
12	0.02
13	0.02
14	0.01
15	0.00
20	0.00

It can be seen that after 14 millilitres of wash water there is no sucrose left. A value of 20 ml was chosen to standardise the method.

A1.2 Determination of ion exchange capacity

The ion exchange capacity of the QMA SepPak determines the quantity of factory solution that can be passed through the SPE column to isolate the acids before no further retention occurs. A solution of potassium oxalate (21.5 mg/100 ml) was prepared and 25 ml passed through the SPE. Collected

fractions (1 ml) were analysed for the presence of oxalic acid on the HPLC system used for *cis/trans* isomerisation (see Section 2.2.2) .

It was found that the SepPak retained 2.9 mg oxalate ion which corresponds to a binding capacity of 0.18 milliequivalents/g (meq/g) QMA packing compared with the manufacturers claim of approximately 0.19 meq/g. South African sugar mill process streams contain on average 0.16 meq acid/ml of juice, 0.30 meq acid/g of syrup and 0.40 meq acid/g molasses (Walford, 1995). This limits the volume of sample to approximately 1 ml of juice, 0.5 g syrup and 0.4 g molasses before dilution if the ion exchange capacity of the SPE column is not to be exceeded.

A1.3 Recoveries and precision

To determine the recoveries of individual acids, a synthetic sample was prepared from the stock organic acid solution and diluted 1:10 with a 10% (m/v) sucrose solution. The SPE procedure was followed using 2.0 ml aliquots and the isolated acids analysed by HPLC with refractive index detection. Sulphuric acid was chosen as the SPE elution solvent as it was compatible with the HPLC eluent. It would also cause the least disturbance to the refractive index response at the void of the chromatogram. The effect of increasing concentration of the sulphuric acid used for elution was also studied. Sulphuric acid concentrations of 1.0 N, 0.5 N and 0.2 N were tested. Recoveries were calculated relative to the stock organic acid solution diluted 1:10 with water and injected directly into the HPLC (Table A1.2).

The three closely eluting acids (citric, phosphoric and tartaric acid) are not resolved when high concentrations of sulphuric acid are used to elute the acids from the SPE. As these acids elute near the void, it is believed that the strength of the injected sulphuric acid disturbs the equilibrium pH on the column, so causing co-elution of the acids. All experiments used an elution eluent of 0.2 N sulphuric acid.

Table A1.2

Recovery of acids through a QMA SepPak

Acid	Recovery (%)		
	1.0 N H_2SO_4	0.5 N H_2SO_4	0.2 N H_2SO_4
Oxalic	95	97	97
cis-aconitic	97	98	99
Citric	NR	NR	101
Phosphoric	NR	NR	97
Tartaric	NR	NR	98
Malic	98	101	100
trans-aconitic	99	100	97
Succinic	93	92	92
Glycollic	92	90	89
Lactic	91	89	89
Acetic	94	92	88

NR = not resolved

The precision of the method was checked by randomly taking a frozen Sezela sugar mill mixed juice sample and making five replicate isolations, elutions and injections on one day (Table A1.3).

The high RSD values for lactic and acetic acid reflect the limitations of integrating small, broad peaks of low concentrations. Oxalic acid integration is difficult as it appears as a shoulder on the void. Overall these results are acceptable for a solid phase extraction technique where values less than 10% RSD are considered good (Waters Sep-Pak Cartridge Applications Bibliography, 1991).

Table A1.3

Precision of the isolation method for selected acids

Acid	Peak Height (RSD %)
Oxalic	7.3
Citric	3.8
Malic	3.8
trans-Aconitic	3.7
Lactic	8.0
Acetic	11.2

APPENDIX 2

CHROMATOGRAPHIC METHOD DEVELOPMENT

Ion exclusion chromatography (IEX) or ion-moderated partition liquid chromatography (IMPC) is an alternative to GC analysis for the separation of mixtures of carboxylic acids and monosaccharides in a variety of matrices. A cation exchange resin column is eluted isocratically with dilute acid and combined with either UV or RI detection. The separation is based on the Donnan effect. Ionised compounds are more rapidly eluted than the non-ionic which are retained by electrostatic partition forces. Weakly ionic compounds are then eluted more or less quickly depending on their pK_a value and hydrophobicity. Thus factors affecting particularly the pK_a (temperature) and degree of ionisation (pH) can change elution order of the acids.

A2.1 Effect of eluent pH on k´

Chromatographically the retention time of a peak eluting from a column can be defined as:

$$k' = \frac{t_r}{t_r - V_0}$$

where k' is the capacity factor, t_r the retention time of the peak of interest and V_0 is the retention time of the void volume peak.

This value will be independent of eluent flow rate and column dimensions. Eluents with increasing sulphuric acid concentrations were prepared (Table A2.1), the columns equilibrated and solutions of individual organic acids injected in duplicate. The capacity factor k', was calculated from the refractive index chromatogram (to enable detection of phosphoric acid) and plotted against pH (Figure A2.1). The greatest effect on k' can be seen on the polyprotic acids due to their varying charge as a function of pH. A concentration of 7.5 mM H_2SO_4 was chosen as the eluent as a compromise between baseline separation of oxalic, *cis*-aconitic and citric acids while at the same time

ensuring separation between acetic and fumaric acids. Lower pH could result in the citric and phosphoric acids coeluting.

Table A2.1

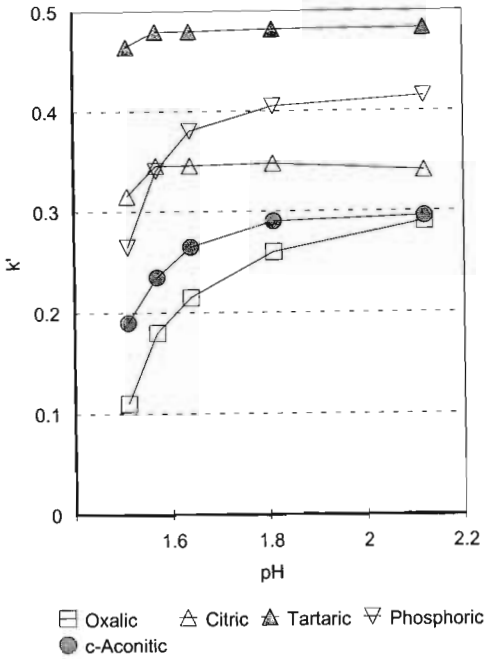
Sulphuric acid eluent concentration and measured pH for determining effect of pH on *k'*

Concentration (mM)	Measured pH
3.75	2.10
7.50	1.81
10.00	1.67
12.50	1.57
15.00	1.51

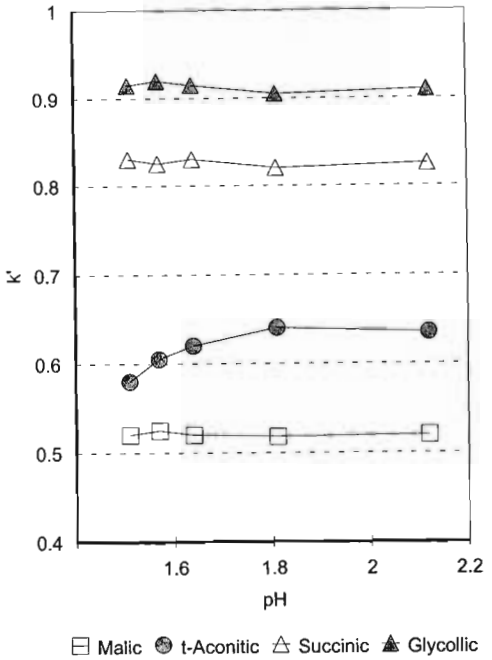
A2.2 Effect of column temperatures on *k'*

Satisfactory resolution of all the acids could not be achieved on one column. For example, oxalic and *cis*-aconitic acids co-elute no matter what combination of temperature or eluent concentration are used. Two columns in series were used to try to increase the separating power of the system. This allows another variable to be considered: the use of different individual column temperatures to aid the separation. The capacity factors of the acids generally increased with increasing difference between the two column temperatures (Figure A2.2).

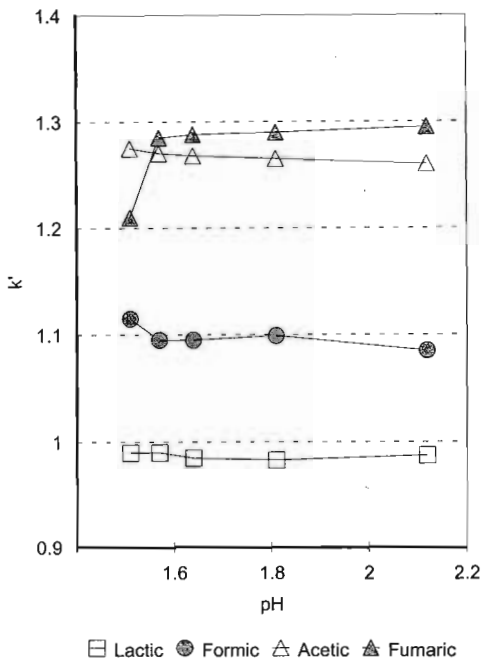
The greatest effect was on the resolution of the phosphate/citrate peaks and the succinic/glycollic/lactic separation. Reversal of the column temperatures made little difference to the separation shown here. All factory sample separations were carried out at 30/75°C.



(a)



(b)



(c)

Figure A2.1 Effect of pH on the retention time (expressed as k') of the individual organic acids ; (a) oxalic, *cis*-aconitic, citric, phosphoric and tartaric acids; (b) malic, *trans*-aconitic, succinic and glycollic acids; (c) lactic, formic, acetic and fumaric acids.

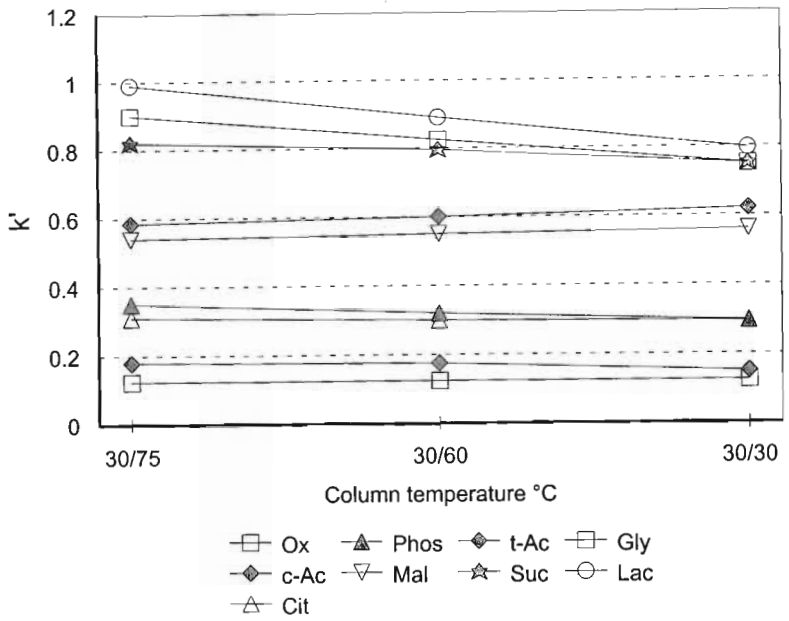


Figure A2.2 Effect of individual column temperature on the capacity factors (*k'*) of the organic acids (Ox=oxalate, c-Ac=*cis*-aconitic, Cit=citric, Phos=phosphoric, Mal=malic, t-Ac=*trans*-aconitic, Suc=succinic, Gly=glycollic, Lac=lactic)

A2.3 UV/RI height ratio as a peak identifier

As a separation technique, HPLC does not have the resolving power of capillary GC. Co-elution of compounds is possible. However, the less complicated sample preparation allows quicker sample preparation and more samples to be analysed. When analysing a complex mixture such as factory samples, the possibility exists of misreporting the presence of a compound. In order to highlight possible problem peaks, a method was developed to compare the response of the UV and RI detectors. For a standard solution of any particular acid the ratio of the UV to the RI response (height or area) will be constant, irrespective of compound concentration, at a fixed UV wavelength (in this case 210 nm). The ratios of some of the acids are shown in Table A2.2. It can be seen that the unsaturated compounds give a larger ratio than the saturated due to increased UV absorption. The saturated tricarboxylic citric acid gives a larger ratio than saturated monocarboxylic acids such as acetic and succinic.

Table A2.2

Ratio of UV_{210} to RI response for selected organic acid standards

Acid	UV_{210}/RI (height)
Oxalic	38.0
cis-Aconitic	69.9
Citric	6.0
Tartaric	9.0
Malic	5.0
trans-Aconitic	39.9
Succinic	3.0
Glycollic	3.0
Lactic	5.0
Itaconic	26.2
Formic	12.0
Acetic	4.0
Fumaric	56.6

When analysing an unknown sample, peak identification is generally based on retention time. Comparing the calculated ratio for any particular unknown peak with that of the corresponding standard (Table A2.2), gives an indication of possible coelution. A chromatogram comparing the UV and RI response is shown in Figure A2.3.

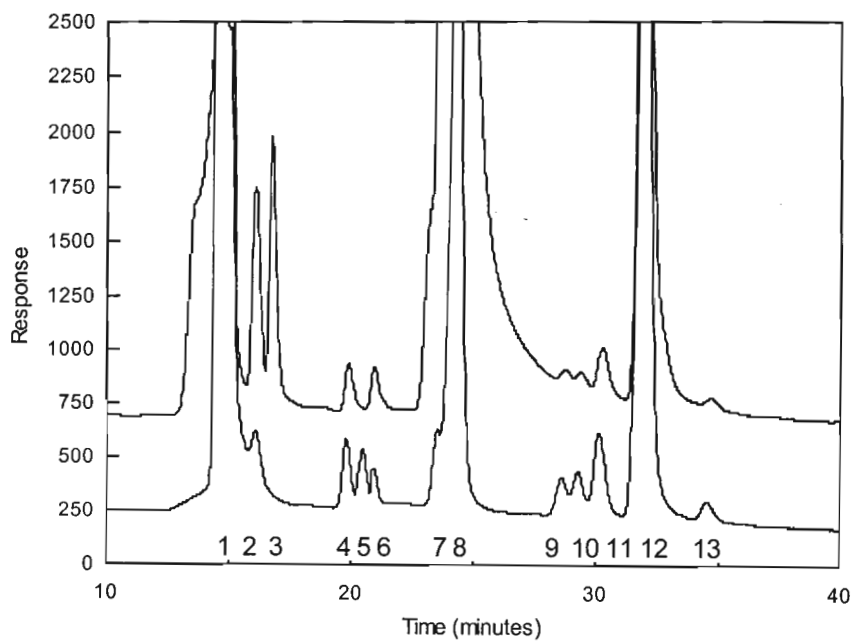


Figure A2.3 Chromatogram of a standard acid mixture showing UV (top trace) and RI (lower trace) response. Peaks : 1 solvent; 2 oxalic; 3 *cis*-aconitic; 4 citric; 5 phosphoric; 6 tartaric; 7 malic; 8 *trans*-aconitic; 9 succinic; 10 glycollic; 11 lactic; 12 formic; 13 acetic

APPENDIX 3

Cis/trans ACONITIC ACID RATIOS IN FACTORY PRODUCTS

Table A3.1 Mixed Juice

Week	ML			KM			PG			UF			EN		
	cis	*trans*	*cis/trans*	*cis*	*trans*	cis/trans	*cis*	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*
3							76	757	0.100						
4							78	678	0.115						
5				47	702	0.068	73	644	0.113						
6	62	823	0.075	63	908	0.069	77	710	0.109				25	353	0.072
7	61	845	0.073	66	992	0.067	77	735	0.105	46	629	0.073	24	347	0.069
8	44	647	0.068	57	959	0.059	80	825	0.097	49	677	0.072	24	432	0.055
9	53	697	0.076	62	957	0.065	89	797	0.112	51	630	0.080	22	359	0.062
10	60	820	0.073	55	840	0.066	91	868	0.105	48	663	0.072	23	362	0.062
11	59	904	0.065	71	1086	0.066	71	801	0.089	37	597	0.063	20	397	0.049
12	41	623	0.066	57	969	0.059	69	825	0.084	39	619	0.063	23	415	0.055
13	46	742	0.062	57	920	0.062	69	844	0.082	36	566	0.063	22	424	0.053
14	39	764	0.051	55	928	0.059	58	839	0.069	37	683	0.055	22	473	0.047
15	35	735	0.048	68	1074	0.063	54	852	0.064	36	667	0.054	20	463	0.043
16	37	763	0.048	45	878	0.052	53	837	0.064	35	671	0.052	20	447	0.044
17	26	582	0.045	41	776	0.052	50	824	0.060	34	676	0.050	20	472	0.042
18	28	575	0.049	48	891	0.054	53	824	0.064	34	676	0.050	22	501	0.044
19	33	658	0.051	44	828	0.053	53	850	0.062	34	644	0.053	23	489	0.047
20	33	663	0.050	38	719	0.052	55	880	0.062	39	686	0.056	26	520	0.050
21	34	696	0.049	43	855	0.051	56	812	0.068	35	682	0.051	26	488	0.052
22	28	599	0.046	37	758	0.049	48	811	0.059	29	657	0.044	26	527	0.050
23	33	567	0.059	37	616	0.060	51	747	0.068	41	645	0.063	22	485	0.045
24	36	660	0.054	50	850	0.059	47	725	0.065	37	623	0.060	26	514	0.050
25	35	591	0.060	53	1018	0.052	48	684	0.070	31	618	0.051	27	553	0.048
26	32	529	0.060	40	764	0.052	48	692	0.070	27	579	0.047	27	577	0.047
27	32	560	0.057	47	789	0.059	46	669	0.069	27	507	0.054	23	533	0.043
28	31	560	0.056	51	846	0.060	47	713	0.066	34	629	0.054	26	599	0.044
29	33	569	0.058	48	838	0.058	53	722	0.074	32	581	0.055	26	572	0.045
30	44	600	0.074	69	934	0.073	98	848	0.116	60	682	0.088	40	641	0.063
31	46	591	0.078	59	906	0.065	90	796	0.113	49	659	0.074	51	729	0.069
32	54	574	0.094	54	757	0.071	92	766	0.121	42	583	0.073	60	717	0.084
33	51	575	0.089	64	807	0.079	75	686	0.110	46	636	0.072	48	712	0.067
34	45	548	0.082	56	803	0.070	73	745	0.098	60	667	0.090	41	731	0.056
35	46	513	0.090	52	755	0.069	85	804	0.105	50	686	0.072	37	730	0.051
36	39	489	0.080	59	757	0.078	70	674	0.105	57	637	0.090	41	697	0.059
37	52	653	0.080	60	737	0.081	75	779	0.096	41	595	0.070	37	706	0.052
38	58	517	0.113	75	711	0.106	97	743	0.130	45	609	0.074	38	651	0.058
39	74	536	0.138	91	708	0.128	79	720	0.109				46	631	0.072
40	54	503	0.108	60	855	0.071	85	755	0.112				59	676	0.087
41	62	695	0.089	62	851	0.073							38	606	0.063

Week	FX			AK			DL			MS			GH		
	cis	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*	*cis*	*trans*	*cis/trans*
3															
4															
5				27	412	0.066	29	396	0.073				40	505	0.080
6				26	441	0.059	29	431	0.066				36	560	0.064
7				18	303	0.060	30	435	0.069				39	556	0.070
8	39	602	0.064	24	421	0.058	30	449	0.066	25	471	0.053	35	564	0.063
9	36	548	0.066	19	318	0.059	27	431	0.063	34	504	0.068	39	573	0.069
10	32	532	0.060	25	413	0.061	27	462	0.059	35	561	0.061	40	604	0.066
11	39	659	0.059	21	405	0.052	27	479	0.057	25	469	0.053	37	590	0.064
12	35	674	0.051	23	457	0.051	30	499	0.061	30	562	0.054	37	640	0.057
13	37	750	0.049	22	455	0.047	29	523	0.056	31	563	0.055	41	676	0.061
14	33	692	0.047	21	479	0.044	25	560	0.045	29	586	0.050	34	687	0.049
15	29	658	0.044	23	507	0.045	23	564	0.040	31	644	0.048	32	750	0.042
16	28	668	0.042	22	519	0.042	24	602	0.039	37	678	0.054	32	814	0.039
17	29	639	0.045	22	523	0.042	24	561	0.043	32	616	0.052	29	637	0.046
18	32	673	0.048	23	518	0.044	28	578	0.049	36	655	0.056	30	624	0.049
19	31	655	0.047	23	521	0.045	29	599	0.048	37	688	0.054	45	799	0.056
20	38	754	0.050	27	568	0.047	34	669	0.050	49	753	0.065	55	864	0.064
21	32	661	0.049	26	577	0.046	32	682	0.048	45	732	0.061	43	840	0.051
22	32	689	0.047	26	599	0.043	43	693	0.061	46	768	0.059	55	814	0.068
23	32	653	0.049	23	565	0.041	34	660	0.051	41	670	0.061	45	807	0.056
24	30	642	0.046	27	589	0.047	35	686	0.051	47	755	0.062	52	887	0.059
25	32	648	0.049	24	542	0.044	43	690	0.062	39	662	0.059	63	820	0.076
26	27	575	0.046	23	523	0.044	32	681	0.047	35	626	0.057	42	730	0.058
27	28	583	0.048	20	469	0.043	26	651	0.039	34	671	0.051	31	688	0.045
28	32	677	0.047	23	575	0.041	30	733	0.041	39	770	0.051	48	822	0.058
29	30	635	0.047	22	529	0.042	31	701	0.044	35	699	0.050	37	756	0.049
30	55	775	0.071	39	596	0.065	42	744	0.057	51	763	0.067	52	862	0.060
31	53	703	0.076	33	604	0.054	48	725	0.066	46	747	0.061	54	801	0.067
32	50	696	0.072	35	577	0.061	48	729	0.066	58	764	0.076	53	807	0.065
33	50	691	0.072	28	568	0.049	58	773	0.075	42	696	0.060	51	809	0.064
34	42	674	0.062	32	598	0.054	44	700	0.062	44	711	0.062	48	803	0.059
35	44	700	0.062	29	582	0.050	55	867	0.064	50	773	0.065	51	791	0.065
36	47	734	0.064	30	572	0.052	42	778	0.054	56	803	0.070	56	791	0.071
37	46	697	0.066	31	580	0.053	35	688	0.050	54	780	0.070	52	829	0.063
38				37	577	0.064	45	678	0.066	57	700	0.081	50	743	0.068
39				32	481	0.066	58	665	0.087	48	646	0.074	53	657	0.080
40										52	649	0.080	52	714	0.073
41										53	672	0.079	53	721	0.073

Results expressed as ppm acid on sample.

Continued on following page.

Table A3.1 Mixed Juice (continued)

Week	NB			UC			ES			SZ			UK		
	cis	*trans*	*cis/tran*	*cis*	*trans*	*cis/tran*	*cis*	*trans*	*cis/tran*	*cis*	*trans*	*cis/tran*	*cis*	*trans*	*cis/tran*
3	43	592	0.073	26	476	0.054									
4	43	518	0.082	25	480	0.052	30	453	0.066	39	421	0.092			
5	36	540	0.067	25	509	0.049	27	435	0.061	41	459	0.089	25	416	0.060
6	41	611	0.067	24	465	0.051	22	415	0.053	36	485	0.074	39	515	0.076
7	33	578	0.057	22	481	0.047	22	409	0.053	32	426	0.074	34	449	0.076
8	36	619	0.059	23	484	0.047	22	441	0.049	33	453	0.074	33	496	0.066
9	36	622	0.058	28	494	0.056	24	459	0.052	34	448	0.077	36	487	0.074
10	36	603	0.059	26	505	0.052	25	480	0.052	41	495	0.083	36	502	0.072
11	27	498	0.054	20	500	0.040	21	456	0.046	35	472	0.075	32	465	0.068
12	31	588	0.052	23	586	0.039	24	468	0.050	42	558	0.075	33	484	0.069
13	33	599	0.055	24	564	0.042	24	499	0.049	43	566	0.077	34	532	0.063
14	24	543	0.045	22	525	0.041	27	523	0.051	39	548	0.071	37	575	0.065
15	28	661	0.042	28	612	0.045	27	557	0.049	38	573	0.066	33	558	0.059
16	26	676	0.038	27	608	0.045	27	555	0.049	38	566	0.068	32	532	0.060
17	26	687	0.037	27	721	0.038	26	578	0.044	34	535	0.064	32	537	0.059
18	31	715	0.043	34	742	0.046	30	585	0.051	34	548	0.062	32	534	0.061
19	34	720	0.047	30	760	0.040	30	650	0.046	38	589	0.064	33	575	0.057
20	37	724	0.051	37	771	0.048	33	683	0.048	47	650	0.072	37	607	0.061
21	32	694	0.047	33	722	0.046	31	621	0.050	51	667	0.077	41	598	0.068
22	37	738	0.050	31	690	0.044	25	563	0.045	48	672	0.071	39	629	0.062
23	30	632	0.048	26	626	0.042	25	607	0.041	43	616	0.070	44	638	0.068
24	33	629	0.053	30	672	0.044	29	612	0.047	45	634	0.071	41	628	0.065
25	43	647	0.067	34	690	0.049	29	574	0.050	42	648	0.065	36	586	0.061
26	33	588	0.057	30	639	0.047	26	535	0.048	38	604	0.063	33	544	0.060
27	28	559	0.050	27	584	0.046	24	519	0.046	38	587	0.064	32	527	0.061
28	32	654	0.048	28	625	0.044	25	551	0.046	35	583	0.061	29	538	0.054
29	33	597	0.056	25	549	0.046	25	545	0.046	38	547	0.069	34	515	0.065
30	46	680	0.068	38	579	0.066	38	607	0.063	58	654	0.088	52	606	0.085
31	67	679	0.098	36	610	0.060	38	635	0.061	56	622	0.090	45	605	0.074
32	48	598	0.080	38	614	0.061	36	575	0.063	51	601	0.085	58	643	0.090
33	60	608	0.099	33	602	0.055	33	585	0.056	52	620	0.083	46	618	0.075
34	58	671	0.086	41	653	0.063	35	588	0.059	54	624	0.087	54	611	0.089
35	47	663	0.071	40	615	0.065	34	580	0.059	46	574	0.081	57	648	0.088
36	49	589	0.084	36	594	0.061	37	585	0.063	53	672	0.080	47	617	0.076
37	56	641	0.087	39	596	0.066	37	562	0.065	58	691	0.084	51	627	0.081
38	43	603	0.072	46	598	0.076	43	543	0.080	61	642	0.095	60	654	0.092
39	86	596	0.144	38	544	0.069	41	526	0.078	51	572	0.089	53	631	0.084
40	58	616	0.094	50	642	0.078	64	667	0.095	58	619	0.094	57	630	0.090
41	78	561	0.140	39	614	0.064	53	671	0.079	56	611	0.092	53	603	0.088

Results expressed as ppm acid on sample.

Mill		Average *cis/trans*
ML	Malelane	0.070
KM	Komati	0.066
PG	Pongola	0.089
UF	Umfolosi	0.064
EN	Entumeni	0.056
FX	Felixton	0.055
AK	Amatikulu	0.051
DL	Darnal	0.056
MS	Maidstone	0.061
GH	Gledhow	0.061
NB	Noodsburg	0.066
UC	Union Co-op	0.052
ES	Eston	0.056
SZ	Sezela	0.077
UK	Umzimkulu	0.071
AVERAGE :		**0.063**

Table A3.2 Clear Juice

Mill	Month	*cis*	*trans*	*cis/trans*
DL		54	670	0.081
DL		55	730	0.075
ES		28	572	0.049
MS		33	501	0.067
MS		36	554	0.065
MS		22	557	0.040
MS		22	520	0.042
FX		90	945	0.095
MS		128	743	0.172
PG	Aug	59	771	0.071
	Sep	56	685	0.076
	Oct	71	729	0.089
	Nov	65	418	0.135
	Dec	69	650	0.096
FX	May	67	727	0.084
	Jun	56	704	0.074
	Jul	53	694	0.071
	Aug	56	721	0.072
	Sep	64	731	0.081
	Oct	61	698	0.080
	Nov	62	604	0.093
DL	May	53	673	0.073
	Jun	136	669	0.169
	Jul	74	798	0.085
	Aug	56	751	0.069
	Sep	55	672	0.076
	Oct	50	650	0.071
	Nov	50	644	0.072
	Dec	60	704	0.079
NB	Jun	49	719	0.064
	Jul	49	686	0.067
	Aug	51	703	0.068
	Sep	51	673	0.070
	Oct	47	605	0.072
	Nov	52	597	0.080
SZ	May	53	637	0.077
	Jun	102	459	0.182
	Jul	68	756	0.083
	Aug	56	691	0.075
	Sep	45	532	0.078
	Oct	44	530	0.077
UK	Jul	64	736	0.080
	Aug	61	730	0.077
	Sep	60	721	0.077
	Oct	73	667	0.099
	Nov	56	637	0.081
	Dec	69	738	0.086
		Average		**0.083**

Table A3.3 Syrup

Mill	Month	*cis*	*trans*	*cis/trans*
FX		593	3431	0.173
FX		596	3687	0.162
FX		528	3976	0.133
FX		623	3845	0.162
FX		603	4159	0.145
FX		620	3763	0.165
FX		605	3745	0.161
FX		491	3682	0.133
MS		593	3944	0.150
PG	Aug	298	2887	0.094
	Sep	360	3048	0.106
	Oct	354	2947	0.107
	Nov	412	3003	0.121
	Dec	508	2813	0.153
FX	May	474	3217	0.128
	Jun	600	3711	0.139
	Jul	620	3905	0.137
	Aug	490	3879	0.112
	Sep	586	4077	0.126
	Oct	624	4194	0.130
	Nov	652	4107	0.137
DL	May	448	4233	0.096
	Jun	448	2969	0.131
	Jul	570	4968	0.103
	Aug	478	4856	0.090
	Sep	472	4817	0.089
	Oct	468	4882	0.087
	Nov	492	4695	0.095
	Dec	622	5593	0.100
NB	Jun	510	3530	0.126
	Jul	494	3498	0.124
	Aug	452	3799	0.106
	Sep	466	3612	0.114
	Oct	452	3408	0.117
	Nov	464	3247	0.125
SZ	May	564	2816	0.167
	Jun	210	1097	0.161
	Jul	528	3072	0.147
	Aug	488	3066	0.137
	Sep	496	2783	0.151
	Oct	470	2920	0.139
UK	Jul	458	3876	0.106
	Aug	514	4442	0.104
	Sep	626	4912	0.113
	Oct	774	5214	0.129
	Nov	608	5246	0.104
	Dec	650	5359	0.108
		Average		**0.126**

Results expressed as ppm acid on sample.

Table A3.4 Molasses

Mill	*cis*	*trans*	*cis/trans*
KM	0.14	0.40	0.350
SZ	0.38	1.49	0.255
KM	0.58	1.41	0.411
KM	0.42	1.33	0.316
KM	0.25	1.29	0.194
ML	0.34	1.43	0.238
KM	0.28	1.63	0.172
PG	0.25	1.03	0.243
GH	0.34	1.37	0.248
SZ	0.55	1.10	0.500
UF	0.53	1.52	0.348
MS	0.63	1.56	0.401
GH	0.48	1.36	0.349
NB	0.53	1.37	0.389
UC	0.48	1.66	0.293
ML	0.47	1.66	0.281
DL	0.47	1.64	0.285
SZ	0.43	1.48	0.293
AK	0.36	1.71	0.213
FX	0.41	1.82	0.224
SZ	0.58	1.80	0.324
UK	0.60	2.55	0.234
ES	0.42	1.65	0.255
ES	0.42	1.96	0.212
UC	0.07	0.23	0.279
NB	0.07	0.24	0.268
PG	0.32	1.33	0.242
PG	0.91	2.89	0.317
UF	0.90	3.11	0.290
NB	0.42	0.98	0.426
GH	0.80	3.12	0.257
PG	0.67	2.08	0.324
KM	0.74	2.59	0.284
ML	0.62	2.23	0.277
KM	0.41	1.03	0.397
AK	0.42	2.38	0.175
EN	0.63	2.58	0.242
PG	0.61	2.10	0.292
DL	0.51	2.71	0.190
MS	0.65	2.36	0.274
MS	0.84	4.65	0.182
FX	0.65	2.78	0.235
	Average :		**0.285**

Results expressed as % acid on sample.

Table A3.5 Raw Sugar

Mill	*cis*	*trans*	*cis/trans*
ML	51	310	0.165
ML	55	288	0.191
ML	46	212	0.217
ML	6	30	0.200
ML	68	385	0.177
ML	39	240	0.163
ML	39	210	0.186
ML	22	91	0.242
ML	67	364	0.184
ML	68	350	0.194
SZ	54	289	0.187
SZ	43	236	0.182
KM	53	304	0.174
KM	51	277	0.184
KM	77	291	0.265
SZ	57	206	0.277
SZ	60	310	0.194
KM	48	267	0.180
KM	50	262	0.191
KM	67	230	0.291
KM	5	24	0.208
KM	4	22	0.182
DL	13	61	0.213
DL	23	94	0.245
DL	26	107	0.243
DL	55	315	0.175
SZ	47	254	0.185
SZ	62	264	0.235
SZ	7	36	0.194
SZ	39	229	0.170
SZ	39	223	0.175
SZ	58	199	0.291
DL	9	39	0.231
DL	5	26	0.192
AK	45	265	0.170
AK	41	238	0.172
AK	36	242	0.149
AK	38	244	0.156
AK	41	274	0.150
AK	40	258	0.155
	Average :		**0.198**

Results expressed as ppm acid on sample.

APPENDIX 4

ISOMERISATION METHOD REPEATABILITY

Table A4.1

Concentration data for reproducibility of isomerisation runs at 90°C, pH 5

Time (min)	Replicate 1 (mM)		Replicate 2 (mM)		Replicate 3 (mM)	
	cis	*trans*	*cis*	*trans*	*cis*	*trans*
5	0.002	0.279	0.003	0.282	0.003	0.280
35	0.017	0.261	0.020	0.264	0.016	0.257
65	0.028	0.252	0.033	0.249	0.027	0.246
95	0.037	0.239	0.044	0.237	0.037	0.237
125	0.046	0.231	0.053	0.228	0.045	0.227
155	0.052	0.221	0.060	0.220	0.051	0.222
185	0.058	0.218	0.065	0.214	0.057	0.216
215	0.062	0.213	0.069	0.209	0.061	0.212
245	0.065	0.207	0.073	0.204	0.065	0.208
275	0.066	0.206	0.075	0.201	0.068	0.203
305	0.072	0.203	0.077	0.199	0.070	0.201
335	0.072	0.197	0.079	0.198	0.072	0.198
365	0.074	0.198	0.080	0.196	0.074	0.196
395	0.076	0.196	0.081	0.194	0.075	0.194
425	0.076	0.195	0.082	0.192	0.076	0.194
455	0.077	0.193	0.082	0.192	0.078	0.191
485	0.078	0.191	0.082	0.191	0.078	0.190
515	0.076	0.194	0.083	0.189	0.078	0.189
545	0.076	0.194	0.083	0.189	0.079	0.190
575	0.076	0.194	0.083	0.189	0.079	0.188
605	0.076	0.193	0.083	0.188	0.080	0.187
Statistics						
R	0.9989	0.9988	0.9999	0.9994	0.9999	0.9978
Variance explained %	99.79	99.76	99.98	99.87	99.99	99.57
Equilibrium concentration (mM)	0.082	0.188	0.085	0.187	0.082	0.188
Calculated k_e (x 10^{-5})	10.61		11.23		10.98	

Rate constants calculated as described in Section 2.6.1

APPENDIX 5

ISOMERISATION CONCENTRATION DATA

Table A5.1

Concentration data for pH 4, 97°C at varying phthalate buffer concentrations

10mM			20mM			25mM			50mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
0	0.002	0.280	0	0.002	0.300	0	0.002	0.312	0	0.003	0.341
900	0.017	0.259	900	0.023	0.278	900	0.024	0.289	600	0.022	0.318
1800	0.032	0.251	1800	0.040	0.261	1800	0.042	0.267	1200	0.037	0.300
2700	0.044	0.240	2700	0.053	0.249	2700	0.057	0.252	1800	0.051	0.286
3600	0.055	0.230	3300	0.061	0.239	3600	0.068	0.240	2400	0.062	0.275
4500	0.063	0.222	4500	0.074	0.226	4800	0.080	0.224	3000	0.071	0.264
5400	0.070	0.213	5700	0.083	0.214	6000	0.087	0.212	3600	0.079	0.255
6300	0.075	0.206	6900	0.091	0.204	7200	0.093	0.201	4500	0.088	0.244
7200	0.079	0.197	8100	0.095	0.198	9000	0.099	0.191	5400	0.094	0.234
9000	0.086	0.188	9900	0.099	0.188	10800	0.101	0.180	6300	0.099	0.226
10800	0.090	0.178	11700	0.102	0.180	14400	0.103	0.167	7200	0.104	0.221
14400	0.095	0.166	15300	0.103	0.168	18000	0.099	0.156	9000	0.107	0.211
18000	0.095	0.155	18900	0.101	0.158	21600	0.096	0.148	10800	0.108	0.205
23400	0.092	0.143	22500	0.097	0.150	25200	0.092	0.140	14400	0.108	0.196
27900	0.091	0.141	26100	0.094	0.143	30600	0.084	0.128	18000	0.105	0.188
32400	0.087	0.133	29700	0.090	0.136	87300	0.031	0.047	21600	0.101	0.181
86400	0.040	0.057	32280	0.088	0.131	108900	0.019	0.033	25200	0.098	0.175
			86400	0.039	0.055	116400	0.016	0.030	82800	0.053	0.092
			108000	0.026	0.039						

Table A5.2

Concentration data for pH 5, 70°C at varying acetate buffer concentrations

25 mM			50 mM			75 mM			150 mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.002	0.294	0	0.000	0.312	300	0.001	0.284	300	0.001	0.293
3900	0.008	0.292	1800	0.004	0.312	3900	0.008	0.269	3900	0.015	0.269
7500	0.013	0.290	3600	0.008	0.313	7500	0.016	0.264	7500	0.026	0.258
11100	0.016	0.285	5400	0.011	0.305	11100	0.022	0.256	11100	0.035	0.249
14700	0.020	0.282	7200	0.014	0.301	14700	0.028	0.252	14700	0.043	0.243
18300	0.024	0.276	9000	0.018	0.302	18300	0.033	0.247	18300	0.049	0.236
21900	0.028	0.274	10800	0.020	0.297	21900	0.037	0.242	21900	0.054	0.231
25500	0.032	0.270	12600	0.023	0.293	25500	0.041	0.238	25500	0.058	0.226
29100	0.035	0.266	14400	0.026	0.290	29100	0.045	0.234	29100	0.062	0.223
32700	0.038	0.263	16200	0.029	0.288	32700	0.048	0.232	32700	0.065	0.220
36300	0.041	0.260	18000	0.031	0.285	36300	0.051	0.228	36300	0.067	0.217
39900	0.044	0.257	19800	0.034	0.283	39900	0.054	0.226	39900	0.069	0.215
43500	0.047	0.255	21600	0.036	0.281	43500	0.056	0.223	43500	0.072	0.213
47100	0.050	0.252	23400	0.038	0.278	47100	0.059	0.221	47100	0.073	0.212
50700	0.052	0.249	25200	0.040	0.275	50700	0.061	0.219	50700	0.074	0.210
54300	0.054	0.247	27000	0.043	0.273	54300	0.063	0.217	54300	0.075	0.210
57900	0.057	0.245	28800	0.044	0.271	57900	0.064	0.215	57900	0.076	0.209
61500	0.059	0.243	82800	0.077	0.236	61500	0.066	0.214	61500	0.077	0.208
65100	0.060	0.240	86400	0.080	0.234	65100	0.067	0.213	65100	0.078	0.208
68700	0.062	0.239	90000	0.080	0.234	68700	0.068	0.212	68700	0.078	0.207
72300	0.064	0.238	93600	0.082	0.233	72300	0.069	0.210	72300	0.078	0.206
75900	0.065	0.236	97200	0.082	0.232	75900	0.070	0.209	75900	0.079	0.207
79500	0.067	0.235	100800	0.082	0.232	79500	0.071	0.208	79500	0.079	0.206
83100	0.068	0.233	106200	0.084	0.231	83100	0.072	0.208	83100	0.079	0.206
86700	0.070	0.232	109800	0.084	0.230	86700	0.073	0.207	86700	0.080	0.206
90300	0.071	0.231	112800	0.083	0.228	90300	0.073	0.207	90300	0.080	0.206
93900	0.072	0.229	345600	0.081	0.226	93900	0.074	0.206			
97500	0.074	0.229									
101100	0.074	0.226									
104700	0.075	0.225									
108300	0.076	0.224									
111900	0.077	0.223									
115500	0.078	0.223									
119100	0.079	0.221									
122700	0.080	0.221									
126300	0.080	0.220									
129900	0.081	0.220									
133500	0.082	0.219									
137100	0.082	0.219									
140700	0.083	0.219									
144300	0.083	0.217									
147900	0.083	0.216									
151500	0.084	0.216									
155100	0.084	0.216									

Table A5.3

Concentration data for pH 5, 80°C at varying acetate buffer concentrations

25 mM			50 mM			75 mM			150 mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.002	0.294	0	0.001	0.289	300	0.002	0.294	300	0.003	0.305
2100	0.006	0.289	1800	0.009	0.281	2100	0.013	0.275	2100	0.021	0.287
3900	0.010	0.283	3600	0.016	0.273	3900	0.023	0.265	3900	0.035	0.276
5700	0.014	0.278	5400	0.022	0.265	5700	0.031	0.255	5700	0.046	0.266
7500	0.018	0.276	7200	0.026	0.260	7500	0.038	0.248	7500	0.055	0.258
9300	0.022	0.271	9000	0.034	0.255	9300	0.044	0.245	9300	0.063	0.250
11100	0.026	0.268	10800	0.039	0.249	11100	0.049	0.239	11100	0.068	0.244
12900	0.029	0.264	12600	0.043	0.243	12900	0.054	0.231	12900	0.073	0.239
14700	0.032	0.260	16200	0.051	0.235	14700	0.058	0.229	14700	0.077	0.236
16500	0.035	0.257	18000	0.054	0.232	16500	0.061	0.225	16500	0.080	0.233
18300	0.038	0.254	19800	0.057	0.227	18300	0.064	0.219	18300	0.082	0.230
20100	0.040	0.251	21600	0.060	0.225	20100	0.067	0.219	20100	0.084	0.229
21900	0.044	0.249	23400	0.062	0.223	21900	0.069	0.216	21900	0.086	0.227
23700	0.046	0.246	25200	0.065	0.221	23700	0.071	0.213	23700	0.088	0.226
25500	0.048	0.244	82800	0.086	0.199	25500	0.073	0.211	25500	0.088	0.224
27300	0.050	0.242	86400	0.085	0.195	27300	0.074	0.210	27300	0.089	0.223
29100	0.052	0.240	90000	0.086	0.198	29100	0.076	0.209	29100	0.090	0.223
30900	0.053	0.237	93600	0.085	0.196	30900	0.077	0.207	30900	0.090	0.223
32700	0.056	0.236	106200	0.085	0.196	32700	0.077	0.206	32700	0.090	0.222
34500	0.057	0.234				34500	0.079	0.205	34500	0.091	0.222
36300	0.059	0.232				36300	0.079	0.204	36300	0.091	0.221
38100	0.060	0.231				38100	0.080	0.203	38100	0.091	0.221
39900	0.061	0.229				39900	0.080	0.202	39900	0.091	0.220
41700	0.063	0.228				41700	0.081	0.202	41700	0.091	0.220
43500	0.064	0.226				43500	0.081	0.200	43500	0.091	0.220
45300	0.066	0.225				45300	0.082	0.201	45300	0.091	0.220
47100	0.066	0.224				47100	0.082	0.200	47100	0.091	0.220
48900	0.068	0.223				48900	0.082	0.200	48900	0.091	0.220
50700	0.068	0.221				50700	0.082	0.199	50700	0.091	0.219
52500	0.070	0.220				52500	0.082	0.200	52500	0.091	0.220
54300	0.069	0.219				54300	0.083	0.200	54300	0.091	0.219
56100	0.069	0.218				56100	0.083	0.199	56100	0.091	0.219
57900	0.072	0.217				57900	0.083	0.199	57900	0.091	0.219
59700	0.072	0.217				59700	0.083	0.198	59700	0.091	0.219
61500	0.073	0.215				61500	0.083	0.199	61500	0.091	0.219

Table A5.4

Concentration data for pH 5, 90°C at varying acetate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.002	0.296	300	0.003	0.280	300	0.003	0.309	300	0.003	0.269
2100	0.012	0.284	2100	0.016	0.257	2100	0.023	0.286	2100	0.027	0.248
3900	0.022	0.274	3900	0.027	0.246	3900	0.039	0.273	3900	0.044	0.229
5700	0.030	0.266	5700	0.037	0.237	5700	0.052	0.261	5700	0.051	0.216
7500	0.038	0.259	7500	0.045	0.227	7500	0.059	0.251	7500	0.059	0.209
9300	0.044	0.251	9300	0.051	0.222	9300	0.067	0.242	9300	0.066	0.204
11100	0.050	0.246	11100	0.057	0.216	11100	0.074	0.237	11100	0.072	0.202
12900	0.055	0.240	12900	0.061	0.212	12900	0.076	0.232	12900	0.076	0.199
14700	0.059	0.235	14700	0.065	0.208	14700	0.080	0.229	14700	0.078	0.200
16500	0.063	0.231	16500	0.068	0.203	16500	0.082	0.224	16500	0.077	0.198
18300	0.066	0.227	18300	0.070	0.201	18300	0.085	0.224	18300	0.075	0.199
20100	0.069	0.224	20100	0.072	0.198	20100	0.085	0.221	20100	0.079	0.198
21900	0.071	0.221	21900	0.074	0.196	21900	0.086	0.220	21900	0.077	0.197
23700	0.074	0.218	23700	0.075	0.194	23700	0.088	0.218	23700	0.078	0.197
25500	0.075	0.217	25500	0.076	0.194	25500	0.088	0.218	25500	0.076	0.196
27300	0.077	0.214	27300	0.078	0.191	27300	0.089	0.216	27300	0.081	0.197
29100	0.078	0.212	29100	0.078	0.190	29100	0.090	0.216	29100	0.081	0.196
30900	0.080	0.211	30900	0.078	0.189	30900	0.089	0.215	30900	0.079	0.196
32700	0.081	0.209	32700	0.079	0.190	32700	0.088	0.215	32700	0.078	0.196
34500	0.082	0.207	34500	0.079	0.188	34500	0.089	0.215	34500	0.079	0.195
36300	0.082	0.206	36300	0.080	0.187						
38100	0.083	0.205	38100	0.080	0.186						
39900	0.084	0.204	39900	0.080	0.187						
41700	0.084	0.203	41700	0.080	0.185						
43500	0.085	0.202	43500	0.080	0.186						

Table A 5.5

Concentration data for pH 5, 97°C at varying acetate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.014	0.289	300	0.004	0.287	300	0.014	0.296	300	0.014	0.269
1320	0.020	0.284	1320	0.018	0.271	1320	0.029	0.276	1320	0.037	0.239
2340	0.028	0.275	2340	0.031	0.259	2340	0.044	0.260	2340	0.054	0.222
3360	0.034	0.264	3360	0.041	0.250	3360	0.055	0.247	3360	0.066	0.211
4380	0.041	0.258	4380	0.049	0.243	4380	0.063	0.239	4380	0.072	0.206
5400	0.047	0.253	5400	0.056	0.236	5400	0.070	0.232	5400	0.077	0.201
6420	0.051	0.247	6420	0.062	0.231	6420	0.075	0.225	6420	0.080	0.197
7440	0.056	0.240	7440	0.067	0.225	7440	0.078	0.221	7440	0.081	0.195
8460	0.061	0.236	8460	0.071	0.221	8460	0.080	0.217	8460	0.082	0.194
9480	0.062	0.232	9480	0.074	0.217	9480	0.082	0.214	9480	0.082	0.193
10500	0.065	0.229	10500	0.077	0.214	10500	0.084	0.211	10500	0.082	0.193
11520	0.067	0.225	11520	0.080	0.210	11520	0.085	0.209	11520	0.083	0.192
12540	0.070	0.221	12540	0.081	0.207	12540	0.086	0.208	12540	0.083	0.191
13560	0.071	0.218	13560	0.082	0.205	13560	0.086	0.206			
14580	0.072	0.215	14580	0.084	0.203	14580	0.087	0.205			
15600	0.074	0.213	15600	0.084	0.201	15600	0.087	0.204			
16620	0.075	0.210	16620	0.085	0.199	16620	0.087	0.202			
17640	0.075	0.208	17640	0.085	0.198	17640	0.086	0.202			
18660	0.076	0.207	18660	0.086	0.197						
19680	0.077	0.205	19680	0.086	0.197						
20700	0.077	0.203	20700	0.087	0.195						
22980	0.078	0.200	21720	0.086	0.194						
			22740	0.086	0.194						
			23760	0.086	0.193						

Table A5.6

Concentration data for pH 5, 110°C at varying acetate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
0	0.001	0.297	0	0.001	0.296	0	0.003	0.300	0	0.011	0.322
600	0.013	0.291	600	0.023	0.286	600	0.022	0.280	600	0.058	0.242
1200	0.023	0.279	1200	0.038	0.251	1200	0.042	0.259	1260	0.080	0.215
1800	0.031	0.268	1800	0.048	0.238	1800	0.058	0.245	1800	0.089	0.202
2400	0.040	0.258	2400	0.058	0.226	2400	0.065	0.231	2400	0.092	0.192
3000	0.046	0.250	3000	0.064	0.216	3000	0.073	0.221	3000	0.091	0.186
3600	0.051	0.242	3600	0.069	0.208	3600	0.078	0.214	3600	0.090	0.180
4500	0.057	0.231	4500	0.073	0.194	4500	0.083	0.205	4500	0.088	0.173
5400	0.063	0.223	5400	0.076	0.188	5400	0.085	0.198	5400	0.085	0.165
6300	0.067	0.215	6300	0.077	0.180	6300	0.086	0.192	6300	0.081	0.157
7200	0.069	0.208	7200	0.077	0.175	7200	0.085	0.188	7200	0.079	0.155
8100	0.072	0.200	8100	0.078	0.173	10800	0.083	0.175	10800	0.067	0.134
11700	0.076	0.182	9000	0.076	0.165	14400	0.080	0.166	14400	0.058	0.115
15300	0.074	0.170	9900	0.075	0.162	18000	0.075	0.159	18000	0.051	0.100
20700	0.072	0.157	10800	0.075	0.160	21600	0.072	0.154	21600	0.045	0.091
26100	0.068	0.147	14400	0.069	0.146	26400	0.069	0.148	86040	0.017	0.036

Table A5.7

Concentration data for pH 6, 70°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
600	0.002	0.299	600	0.002	0.307	600	0.002	0.296	600	0.000	0.299
7800	0.005	0.295	7800	0.008	0.300	7800	0.009	0.287	7800	0.013	0.289
15900	0.008	0.291	15900	0.013	0.295	15900	0.016	0.280	15900	0.023	0.278
22200	0.011	0.287	22200	0.018	0.289	22200	0.021	0.275	22200	0.029	0.271
30300	0.014	0.285	30300	0.023	0.286	30300	0.027	0.270	30300	0.035	0.266
88200	0.029	0.269	88200	0.045	0.262	88200	0.048	0.249	88200	0.050	0.250
117300	0.035	0.264	117300	0.051	0.257	117300	0.052	0.245	117300	0.051	0.250
172500	0.045	0.255	172500	0.058	0.251	172500	0.056	0.242	172500	0.052	0.249
202800	0.049	0.253	202800	0.060	0.250	202800	0.057	0.243	202800	0.052	0.251
258300	0.055	0.249	258300	0.062	0.248	258300	0.058	0.241	258300	0.053	0.251
290100	0.057	0.246	290100	0.063	0.248	290100	0.058	0.242	290100	0.053	0.251
347400	0.060	0.242	347400	0.064	0.247	347400	0.058	0.241	347400	0.053	0.251
376500	0.062	0.241	376500	0.064	0.246	376500	0.058	0.240	376500	0.053	0.250
606600	0.067	0.235	606600	0.064	0.244						
635400	0.068	0.235	635400								
694200	0.068	0.235	694200								

Table A5.8

Concentration data for pH 6, 80°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.003	0.313	300	0.003	0.295	300	0.003	0.318	300	0.001	0.291
7500	0.010	0.307	7500	0.014	0.285	7500	0.019	0.303	7500	0.026	0.266
14700	0.016	0.302	14700	0.023	0.276	14700	0.031	0.292	14700	0.039	0.254
21900	0.021	0.299	21900	0.030	0.270	21900	0.039	0.285	21900	0.045	0.248
29100	0.026	0.292	29100	0.035	0.264	29100	0.045	0.277	29100	0.049	0.244
85200	0.050	0.267	85200	0.055	0.242	85200	0.062	0.261	85200	0.054	0.239
101100	0.054	0.265	101100	0.057	0.241	101100	0.062	0.259	101100	0.053	0.235
115500	0.057	0.262	115500	0.058	0.239	115500	0.062	0.261	115500	0.053	0.239
173400	0.064	0.254	173400	0.059	0.235	173400	0.063	0.260	173400	0.053	0.239
186900	0.065	0.254	186900	0.059	0.234	186900	0.063	0.260	186900	0.054	0.239
201000	0.066	0.253	201000	0.059	0.235	201000	0.063	0.261	201000	0.054	0.239
257400	0.068	0.250	257400	0.058	0.231	257400	0.063	0.260	257400	0.053	0.239
273900	0.068	0.251	273900	0.058	0.232	273900	0.063	0.259	273900	0.053	0.239
344400	0.069	0.249	344400	0.057	0.229	344400	0.062	0.259	344400	0.054	0.239
376800	0.069	0.251	376800	0.057	0.227	376800	0.062	0.260	376800	0.054	0.238
615900	0.068	0.247	615900	0.055	0.217	615900	0.063	0.258	615900	0.054	0.240

Table A5.9

Concentration data for pH 6, 90°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.004	0.285	300	0.002	0.243	300	0.006	0.274	300	0.003	0.285
10800	0.021	0.267	10800	0.027	0.219	10800	0.037	0.245	10800	0.046	0.237
25200	0.035	0.244	25200	0.041	0.203	25200	0.053	0.234	25200	0.053	0.230
79500	0.059	0.220	79500	0.052	0.193	79500	0.059	0.224	79500	0.054	0.227
97500	0.060	0.217	97500	0.053	0.191	97500	0.059	0.223	97500	0.054	0.226
111600	0.061	0.214	111600	0.054	0.187	111600	0.059	0.224	111600	0.054	0.227
147900	0.062	0.207	147900	0.054	0.191	147900	0.059	0.223	147900	0.054	0.226
165600	0.061	0.205	165600	0.054	0.191	165600	0.059	0.222	165600	0.054	0.226
180300	0.062	0.204	180300	0.054	0.191	180300	0.059	0.223	180300	0.053	0.222
236700	0.060	0.199	236700	0.056	0.186	236700	0.059	0.222	236700	0.054	0.224
252900	0.061	0.197	252900	0.052	0.193	252900	0.058	0.224	252900	0.051	0.222
266400	0.060	0.197	266400	0.052	0.193	266400	0.058	0.224	266400	0.053	0.216
320700	0.059	0.193	320700	0.052	0.191	320700	0.058	0.223	320700	0.053	0.215
339300	0.059	0.192	339300	0.052	0.192	339300	0.058	0.223	339300	0.052	0.215

Table A5.10

Concentration data for pH 6, 97°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.002	0.307	300	0.002	0.283	300	0.001	0.291	300	0.002	0.287
2100	0.008	0.301	2100	0.012	0.272	2100	0.014	0.279	2100	0.023	0.265
3900	0.014	0.294	3900	0.022	0.263	3900	0.025	0.267	3900	0.036	0.251
5700	0.019	0.290	5700	0.030	0.255	5700	0.035	0.259	5700	0.044	0.244
7500	0.024	0.285	7500	0.036	0.249	7500	0.042	0.252	7500	0.049	0.239
9300	0.029	0.281	9300	0.042	0.244	9300	0.047	0.248	9300	0.052	0.237
11100	0.033	0.278	11100	0.046	0.240	11100	0.052	0.242	11100	0.053	0.236
12900	0.037	0.274	12900	0.050	0.236	12900	0.055	0.239	12900	0.054	0.235
14700	0.040	0.270	14700	0.053	0.233	14700	0.056	0.237	14700	0.055	0.235
16500	0.043	0.266	16500	0.055	0.231	16500	0.058	0.235	16500	0.055	0.235
18300	0.046	0.264	18300	0.057	0.229	18300	0.060	0.234	18300	0.055	0.234
20100	0.049	0.261	20100	0.059	0.228	20100	0.060	0.232	20100	0.055	0.234
21900	0.051	0.258	21900	0.060	0.226	21900	0.061	0.232	21900	0.055	0.234
23700	0.054	0.255	23700	0.061	0.225	23700	0.061	0.230	23700	0.055	0.233
25500	0.056	0.253	25500	0.062	0.224	25500	0.062	0.229	25500	0.055	0.234
27300	0.057	0.251	27300	0.063	0.223	27300	0.062	0.229	27300	0.055	0.234
29100	0.059	0.249	29100	0.064	0.221	29100	0.062	0.228	29100	0.055	0.233
30900	0.061	0.247	30900	0.064	0.221	30900	0.062	0.229	30900	0.055	0.233
32700	0.062	0.246	32700	0.064	0.220	32700	0.062	0.227	32700	0.055	0.233
34500	0.063	0.244	34500	0.065	0.220	34500	0.062	0.227			
36300	0.065	0.243	36300	0.065	0.220	36300	0.062	0.227			
38100	0.066	0.242	38100	0.065	0.219	38100	0.062	0.226			
39900	0.067	0.241	39900	0.065	0.220						
41700	0.068	0.240	41700	0.065	0.219						
43500	0.068	0.238	43500	0.065	0.219						
45300	0.069	0.238	45300	0.065	0.218						
47100	0.070	0.236									
48900	0.070	0.235									

Table A5.11

Concentration data for pH 6, 110°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
0	0.001	0.312	0	0.001	0.292	0	0.002	0.306	0	0.004	0.321
1200	0.013	0.300	1560	0.024	0.265	1200	0.030	0.278	1200	0.047	0.281
2400	0.024	0.290	1800	0.025	0.247	2400	0.047	0.260	2400	0.062	0.265
3600	0.032	0.280	2700	0.035	0.252	3600	0.057	0.249	3600	0.067	0.256
4800	0.040	0.271	3600	0.040	0.231	4800	0.062	0.242	4800	0.069	0.253
6000	0.045	0.265	4500	0.048	0.241	6000	0.066	0.238	6000	0.069	0.253
7200	0.050	0.260	5400	0.048	0.217	7200	0.067	0.234	7200	0.069	0.250
10800	0.060	0.246	6300	0.053	0.220	10800	0.069	0.230	10800	0.069	0.247
14400	0.066	0.236	7200	0.056	0.220	14400	0.069	0.227	14400	0.068	0.245
18000	0.070	0.229	8400	0.056	0.210	18000	0.068	0.223	18000	0.067	0.241
21600	0.071	0.223	9600	0.060	0.218	21600	0.067	0.222	23400	0.065	0.235
25200	0.072	0.219	10800	0.061	0.216	25200	0.066	0.220	28200	0.062	0.224
28200	0.072	0.215	12000	0.061	0.211						
			13200	0.060	0.205						
			14400	0.063	0.212						
			18000	0.061	0.203						

Table A5.12

Concentration data for pH 7, 70°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
600	0.001	0.284	600	0.000	0.290	600	0.000	0.305	600	0.000	0.281
7800	0.001	0.282	7800	0.001	0.288	7800	0.001	0.304	7800	0.001	0.279
15900	0.001	0.281	15900	0.002	0.287	15900	0.002	0.302	15900	0.003	0.276
22200	0.002	0.281	22200	0.002	0.287	22200	0.003	0.302	22200	0.004	0.274
30300	0.002	0.280	30300	0.003	0.285	30300	0.004	0.300	30300	0.006	0.273
88200	0.005	0.277	88200	0.008	0.281	88200	0.011	0.295	88200	0.016	0.264
117300	0.006	0.277	117300	0.010	0.279	117300	0.013	0.292	117300	0.019	0.261
172500	0.009	0.274	172500	0.013	0.275	172500	0.018	0.288	172500	0.024	0.257
202800	0.010	0.276	202800	0.015	0.275	202800	0.020	0.288	202800	0.026	0.257
258300	0.012	0.274	258300	0.017	0.272	258300	0.023	0.285	258300	0.029	0.254
290100	0.013	0.272	290100	0.019	0.270	290100	0.025	0.283	290100	0.030	0.254
347400	0.015	0.271	347400	0.021	0.267	347400	0.027	0.281	347400	0.031	0.253
376500	0.016	0.270	376500	0.022	0.267	376500	0.029	0.280	376500	0.032	0.253
606600	0.023	0.265	606600	0.028	0.257	606600	0.034	0.276	606600	0.033	0.251
635400	0.023	0.265	635400	0.028	0.257	635400	0.034	0.276	635400	0.034	0.251
694200	0.024	0.264	694200	0.029	0.255	694200	0.035	0.275	694200	0.034	0.251

Table A5.13

Concentration data for pH 7, 80°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.001	0.300	300	0.001	0.284	300	0.000	0.286	300	0.000	0.309
7500	0.002	0.300	7500	0.002	0.283	7500	0.002	0.280	7500	0.000	0.305
14700	0.003	0.300	14700	0.003	0.283	14700	0.004	0.279	14700	0.006	0.300
21900	0.003	0.297	21900	0.005	0.282	21900	0.006	0.278	21900	0.010	0.298
29100	0.004	0.297	29100	0.006	0.280	29100	0.008	0.276	29100	0.013	0.294
85200	0.010	0.291	85200	0.014	0.273	85200	0.017	0.266	85200	0.028	0.281
101100	0.012	0.291	101100	0.016	0.270	101100	0.019	0.265	101100	0.030	0.277
115500	0.013	0.289	115500	0.017	0.269	115500	0.021	0.263	115500	0.032	0.278
173400	0.018	0.284	173400	0.022	0.265	173400	0.026	0.258	173400	0.036	0.273
186900	0.019	0.283	186900	0.023	0.264	186900	0.027	0.257	186900	0.036	0.274
201000	0.020	0.283	201000	0.024	0.263	201000	0.028	0.257	201000	0.037	0.273
257400	0.023	0.280	257400	0.028	0.260	257400	0.031	0.255	257400	0.038	0.272
273900	0.024	0.279	273900	0.028	0.260	273900	0.031	0.255	273900	0.038	0.272
344400	0.027	0.281	344400	0.030	0.257	344400	0.033	0.253	344400	0.038	0.273
376800	0.028	0.276	376800	0.031	0.259	376800	0.033	0.256	376800	0.038	0.275
615900	0.034	0.275	615900	0.035	0.258	615900	0.036	0.257	615900	0.039	0.277

Table A5.14

Concentration data for pH 7, 90°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.001	0.269	300	0.000	0.267	300	0.000	0.280	300	0.000	0.281
10800	0.004	0.268	10800	0.005	0.261	10800	0.007	0.275	10800	0.011	0.273
25200	0.007	0.267	25200	0.009	0.257	25200	0.013	0.275	25200	0.020	0.263
79500	0.019	0.254	79500	0.022	0.243	79500	0.027	0.254	79500	0.033	0.247
97500	0.022	0.251	97500	0.025	0.241	97500	0.030	0.251	97500	0.034	0.245
111600	0.024	0.250	111600	0.026	0.240	111600	0.031	0.251	111600	0.034	0.243
147900	0.030	0.245	147900	0.031	0.235	147900	0.035	0.247	147900	0.035	0.243
165600	0.031	0.245	165600	0.032	0.235	165600	0.035	0.246	165600	0.035	0.242
180300	0.032	0.244	180300	0.032	0.233	180300	0.035	0.246	180300	0.035	0.243
236700	0.035	0.241	236700	0.034	0.232	236700	0.036	0.247	236700	0.035	0.242
252900	0.032	0.237	252900	0.034	0.236	252900	0.036	0.249	252900	0.034	0.234
266400	0.033	0.237	266400	0.034	0.235	266400	0.037	0.249	266400	0.034	0.234
320700	0.034	0.235	320700	0.035	0.234	320700	0.037	0.249	320700	0.034	0.233
339300	0.035	0.235	339300	0.035	0.235	339300	0.037	0.249	339300	0.034	0.235

Table A5.15

Concentration data for pH 7, 97°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.001	0.292	300	0.000	0.286	300	0.000	0.299	300	0.000	0.309
20400	0.010	0.282	3900	0.003	0.282	3000	0.002	0.297	3000	0.000	0.306
77700	0.029	0.263	7500	0.006	0.278	5700	0.005	0.294	5700	0.007	0.299
105900	0.033	0.260	11100	0.008	0.276	8400	0.008	0.290	8400	0.012	0.295
164100	0.038	0.256	14700	0.011	0.273	11100	0.011	0.288	11100	0.016	0.291
192000	0.039	0.255	18300	0.013	0.272	13800	0.014	0.286	13800	0.019	0.288
253800	0.041	0.253	21900	0.015	0.270	16500	0.016	0.284	16500	0.022	0.286
279600	0.041	0.252	25500	0.017	0.267	19200	0.018	0.280	19200	0.024	0.283
351900	0.041	0.252	29100	0.019	0.266	21900	0.020	0.281	21900	0.026	0.281
438300	0.042	0.250	32700	0.021	0.265	24600	0.022	0.278	24600	0.028	0.280
525900	0.042	0.251	36300	0.022	0.263	27300	0.023	0.277	27300	0.029	0.278
615300	0.042	0.250	39900	0.024	0.263	30000	0.025	0.276	30000	0.030	0.275
703200	0.042	0.250	43500	0.025	0.261	32700	0.026	0.274	32700	0.031	0.276
			47100	0.026	0.260	35400	0.028	0.272	35400	0.032	0.275
			50700	0.027	0.260	38100	0.029	0.271	38100	0.033	0.275
			54300	0.028	0.259	40800	0.030	0.272	40800	0.033	0.274
			57900	0.029	0.258	43500	0.031	0.272	43500	0.034	0.274
			61500	0.030	0.257	46200	0.032	0.270	46200	0.034	0.273
			65100	0.031	0.258	48900	0.033	0.271	48900	0.035	0.275
			68700	0.032	0.256	51600	0.033	0.268	51600	0.035	0.274
			72300	0.032	0.256	54300	0.034	0.269	54300	0.035	0.274
			75900	0.033	0.256	57000	0.034	0.268	57000	0.035	0.275
			79500	0.034	0.256	59700	0.035	0.269	59700	0.035	0.274
			83100	0.034	0.257	62400	0.035	0.268	62400	0.035	0.274
			86700	0.034	0.256	65100	0.036	0.267			
			90300	0.035	0.256	67800	0.036	0.267			
			93900	0.035	0.256	70500	0.037	0.267			
			97500	0.036	0.256	73200	0.037	0.268			
			101100	0.036	0.255	75900	0.037	0.268			
			104700	0.036	0.255	78600	0.038	0.266			

Table A5.16

Concentration data for pH 7, 110°C at varying phosphate buffer concentrations

50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*
0	0.000	0.310	0	0.001	0.310	0	0.000	0.308
3600	0.010	0.298	3600	0.015	0.295	3600	0.024	0.283
7200	0.017	0.290	7200	0.026	0.289	7200	0.034	0.266
10800	0.023	0.284	10800	0.032	0.278	10800	0.039	0.259
14400	0.027	0.277	14400	0.036	0.272	14400	0.041	0.253
18000	0.030	0.273	18000	0.040	0.269	18000	0.041	0.250
21600	0.033	0.269	21600	0.042	0.267	21600	0.041	0.248
27000	0.036	0.265	25200	0.043	0.263	25200	0.041	0.245
84600	0.037	0.234	28800	0.044	0.263	27900	0.041	0.242
171000	0.036	0.228	86400	0.045	0.254	84600	0.034	0.208
			108000	0.044	0.252	100800	0.033	0.202
			171000	0.042	0.245			

Table A5.17

Concentration data for pH 8, 97°C at varying phosphate buffer concentrations

25mM			50mM			75mM			150mM		
Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)		Time (s)	Conc (mM)	
	cis	*trans*		*cis*	*trans*		*cis*	*trans*		*cis*	*trans*
300	0.003	0.283	300	0.003	0.302	300	0.002	0.297	300	0.000	0.285
20400	0.005	0.279	20400	0.005	0.298	20400	0.006	0.294	5100	0.001	0.280
77700	0.012	0.272	77700	0.014	0.290	77700	0.015	0.286	15900	0.004	0.278
105900	0.014	0.270	105900	0.017	0.288	105900	0.019	0.284	26700	0.006	0.277
164100	0.018	0.268	164100	0.022	0.284	164100	0.023	0.280	37500	0.008	0.276
192000	0.020	0.267	192000	0.024	0.283	192000	0.025	0.278	48300	0.011	0.275
253800	0.023	0.264	253800	0.027	0.279	253800	0.028	0.275	59100	0.013	0.275
279600	0.024	0.262	279600	0.028	0.280	279600	0.029	0.274	87900	0.014	0.270
351900	0.025	0.263	351900	0.031	0.278	351900	0.032	0.274	99300	0.018	0.267
438300	0.023	0.259	438300	0.033	0.277	438300	0.034	0.273	159600	0.024	0.261
525900	0.031	0.259	525900	0.034	0.276	525900	0.035	0.272	187200	0.026	0.259
615300	0.032	0.257	615300	0.036	0.276	615300	0.036	0.272	245100	0.028	0.257
703200	0.033	0.257	703200	0.036	0.276	703200	0.036	0.272	264900	0.028	0.257

APPENDIX 6

Chromatographic raw data

Table A6.1 Phthalate buffer, 97°C

10 mM				20 mM				25 mM				50 mM			
	Peak Area				Peak Area				Peak Area				Peak Area		
Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	Itaconic	Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	itaconic
0	254215	39651552		0	184950	41768800		0	228408	43254816	35770	0	331411	42045920	
15	2053721	36728864		15	2631910	38682656		15	3165301	39961760	48430	10	2571722	39187296	
30	3820210	35622400	38099	30	4622288	36262176		30	5501616	36968256	65625	20	4359645	37060256	
45	5164010	34007680	62249	45	6156989	34653152	77429	45	7478605	34918240	102271	30	5941411	35343296	
60	6430989	32575776	113934	55	7114743	33307399	113934	60	8933459	33197408	145929	40	7276877	33911040	71004
75	7436435	31421072	162770	75	8643456	31423200	181047	80	10491088	31055552	223370	50	8320621	32640928	87765
90	8213773	30269424	208594	95	9697510	29802176	271439	100	11503216	29315952	295872	60	9218182	31429536	157507
105	8797331	29219872	261586	115	10572824	28434880	349916	120	12297176	27886992	372148	75	10266464	30120368	231793
120	9347827	27969072	308770	135	11104048	27586400	443581	150	13047760	26384560	506615	90	10968352	28924912	287652
150	10165872	26639984	445105	165	11610728	26145024	563052	180	13301792	24922640	644368	105	11509712	27931440	356989
180	10571288	25262912	546719	195	11943856	25088208	719973	240	13503928	23110944	919270	120	12079440	27294016	445219
240	11228024	23605712	844498	255	12042152	23339312	1002908	300	13082048	21616544	1171931	150	12440944	26047168	575456
300	11233744	21947232	1068342	315	11832624	21996928	1284904	360	12585544	20469120	1419523	180	12620736	25346160	719941
390	10820456	20288784	1449890	375	11348120	20891824	1551258	420	12074808	19388608	1660085	240	12560712	24197600	994942
465	10701712	19940512	1600075	435	11025272	19890112	1839900	510	11121704	17692368	1994807	300	12206640	23200272	1232025
540	10218864	18918816	1871149	495	10554544	18861120	2103202	1455	4028934	6464333	4171536	360	11798008	22373200	1486271
1440	4708122	8057171	4288349	538	10240104	18246864	2289013	1815	2497374	4605581	4507965	420	11430440	21628944	1753395
				1440	4528157	7670650	4556800	1940	2133642	4184120	4587731	1380	6160259	11332488	4668563
				1800	3036770	5362186	5020714								
Standards															
Conc (mM)	0.2483	0.2782	0.1537		0.2483	0.2782	0.1537		0.2483	0.2782	0.1537		0.2483	0.2782	0.1537
Area	29295040	39448096	7073002		29003920	38709600	6947101		32691984	38525472	6842739		28958704	34333728	7210547

Table A6.2 Acetate buffer pH 5, 70°C

25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	313433	41161504	0	0	846072	5	199231	40619968	5	202795	42165376
65	1072131	40823008	30	9246	846342	65	1245530	38402112	65	2130128	38659648
125	1809829	40531008	60	17647	849394	125	2280150	37702560	125	3775966	37032192
185	2269008	39878336	90	24960	827452	185	3212464	36572960	185	5125712	35790880
245	2890962	39464256	120	32547	817197	245	4042434	36094176	245	6227389	34882784
305	3480270	38649536	150	41345	819564	305	4788330	35292992	305	7109331	33926304
365	4027382	38325184	180	47064	804276	365	5421325	34568800	365	7856195	33198512
425	4524464	37677152	210	53682	795276	425	6042208	34087040	425	8505715	32533552
485	5047808	37134240	240	61408	786547	485	6582019	33509952	485	9030458	32060752
545	5454346	36789312	270	67777	779550	545	7060765	33167120	545	9498803	31562768
605	5891898	36321664	300	72376	772623	605	7526595	32660592	605	9859258	31197136
665	6296061	35867232	330	79709	767530	665	7916880	32265504	665	10156440	30932736
725	6695738	35673792	360	83493	760634	725	8250829	31861872	725	10453800	30641184
785	7051069	35174784	390	89356	754403	785	8648454	31580512	785	10641504	30531792
845	7402269	34800480	420	94070	744505	845	8983059	31358112	845	10829144	30246192
905	7735578	34487200	450	98984	739925	905	9205978	31053680	905	11036616	30114288
965	8051651	34300736	480	103035	734051	965	9410451	30688208	965	11111352	29978000
1025	8343075	33916064	1380	179997	638798	1025	9663059	30588832	1025	11237128	29946944
1085	8599763	33527248	1440	184897	633751	1085	9823891	30441472	1085	11331032	29833696
1145	8858029	33407312	1500	186862	634431	1145	10028464	30262992	1145	11413216	29763344
1205	9057850	33267696	1560	190129	632267	1205	10140032	30069392	1205	11468096	29656928
1265	9289683	32984256	1620	191218	629989	1265	10284800	29921088	1265	11500504	29723968
1325	9504954	32898848	1680	191000	627834	1325	10479768	29797936	1325	11516424	29583216
1385	9694810	32544848	1770	194467	625676	1385	10558152	29775574	1385	11554080	29623840
1445	9927936	32439344	1830	194701	623116	1445	10759840	29642096	1445	11625272	29582944
1505	10075568	32285456	1880	193552	619374	1505	10779648	29523744	1505	11638792	29681200
1565	10255688	32006944	5760	188951	613290	1565	10930320	29421024			
1625	10460992	31998144									
1685	10579600	31635072									
1745	10718144	31481936									
1805	10863120	31358336									
1865	10958120	31227520									
1925	11115936	31120816									
1985	11210040	30939456									
2045	11348760	30892960									
2105	11438400	30778288									
2165	11483616	30716528									
2225	11611624	30638944									
2285	11693672	30554000									
2345	11765728	30604208									
2405	11839760	30402352									
2465	11876720	30264304									
2525	11952288	30238320									
2585	12018560	30138544									
2645	12048088	30132576									
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29787904	39929728		486417	774206		30760496	40835008		30600720	41053824

Table A6.3 Acetate buffer pH 5, 80°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	230000	39900000	0	2832	809335	5	367423	43179188	5	466968	43724000
35	839333	39261312	30	20240	786840	35	1981930	40377984	35	3015481	41207552
65	1412325	38494560	60	36223	763691	65	3459792	38849632	65	5030010	39536672
95	2009104	37810560	90	50987	742275	95	4735974	37465344	95	6683904	38071392
125	2569413	37446176	120	60025	728463	125	5803546	36421600	125	7947757	36972192
155	3115205	36792608	150	77908	714823	155	6712221	35915104	155	9029440	35902400
185	3581178	36364800	180	89347	698068	185	7432451	35064896	185	9825146	34975232
215	4066370	35814432	210	99105	679946	215	8093392	33855584	215	10528904	34284672
245	4484304	35375584	270	117546	657114	245	8712698	33617728	245	11075632	33895392
275	4870544	34950208	300	125127	649278	275	9223514	33068080	275	11519760	33423920
305	5283293	34557056	330	131401	636917	305	9672000	32128304	305	11833552	33008880
335	5616400	34167168	360	137645	630427	335	10107424	32175024	335	12133344	32767984
365	6143210	33794976	390	143236	624321	365	10431880	31766464	365	12426304	32489024
395	6395261	33461856	420	148827	619699	395	10697776	31278064	395	12640472	32386176
425	6670797	33112416	1380	197218	556980	425	10990848	31019136	425	12700024	32120688
455	6996912	32831632	1440	195183	545179	455	11238704	30808512	455	12804568	32026272
485	7232368	32555696	1500	197948	553484	485	11478120	30624784	485	12926984	31910480
515	7435283	32274320	1560	196049	547670	515	11594448	30419488	515	13001312	31928272
545	7797720	32073648	1770	196369	550195	545	11714152	30232032	545	13042936	31754416
575	7984768	31759760				575	11889760	30118336	575	13115960	31765168
605	8277987	31517616				605	11990448	29984688	605	13094592	31727856
635	8414106	31332880				635	12045456	29829488	635	13114616	31625920
665	8617312	31133040				665	12134296	29720672	665	13101744	31541488
695	8805562	30968592				695	12215360	29673088	695	13139592	31563936
725	8974906	30656272				725	12254232	29428384	725	13171856	31514128
755	9199424	30545056				755	12323976	29434096	755	13149864	31500608
785	9296982	30433968				785	12398552	29346560	785	13133712	31488352
815	9468806	30240624				815	12436872	29308800	815	13183960	31478280
845	9548390	30090768				845	12426408	29239984	845	13146984	31463232
875	9784902	29954944				875	12472424	29287056	875	13213448	31501568
905	9735763	29799536				905	12559864	29299152	905	13153744	31415136
935	9722528	29648176				935	12518800	29279424	935	13180656	31392832
965	10073608	29544192				965	12523792	29269616	965	13181824	31422352
995	10129192	29474001				995	12555424	29101376	995	13164176	31457184
1025	10229520	29275584				1025	12508504	29143808	1025	13147568	31414208
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29358864	38815456		482140	799974		31645792	41929312		30233088	40946624

Table A6.4 Acetate buffer pH 5, 90°C

	25 mM			50 mM			50mM			50mM			75mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	279337	40468992	5	340534	44144320	0	91921	14855896	5	436603	38705280	5	448299	4.1E+007
35	1790595	38944864	35	2513936	41294688	30	636485	13901568	35	2351976	35487008	35	3286696	3.8E+007
65	3172837	37571424	65	4310323	39052800	60	1064567	13400304	65	4071237	33957920	65	5565312	3.6E+007
95	4367424	36420576	95	5720330	37181184	90	1423070	12899800	95	5483875	32684112	95	7444992	3.5E+007
125	5453296	35418016	125	6823306	35780640	120	1761643	12275064	125	6658611	31369744	125	8506822	3.3E+007
155	6380266	34400640	155	7674992	34408288	150	2009168	11750616	155	7642445	30659968	155	9633184	3.2E+007
185	7173843	33647296	185	8412358	33510784	180	2225426	11594616	185	8420467	29822432	185	10578872	3.2E+007
215	7895533	32922960	215	8900282	32759376	210	2393702	11315344	215	9109786	29310000	215	10862440	3.1E+007
245	8524518	32110400	245	9358419	32019216	240	2479422	11035320	245	9628934	28701840	245	11424688	3.1E+007
275	9050208	31679792	275	9666528	31553920	270	2519654	10964160	275	10076640	28107936	275	11796088	3E+007
305	9516000	31133520	305	9951418	31174368	300	2747646	10819328	305	10418464	27794672	305	12142432	3E+007
335	9956826	30702832	335	10153496	30947328	330	2747672	10490024	335	10663480	27291456	335	12128608	2.9E+007
365	10322416	30248416	365	10305448	30632560	360	2855397	10537136	365	10936048	27025872	365	12324616	2.9E+007
395	10643632	29895264	395	10402352	30362704	390	2898970	10446664	395	11177856	26818160	395	12643584	2.9E+007
425	10865680	29646848	425	10517720	30122768	420	2923160	10361304	425	11276184	26742848	425	12594304	2.9E+007
455	11101504	29347296	455	10606272	30119120	450	2946134	10250584	455	11523912	26393664	455	12700608	2.9E+007
485	11307856	29080384	485	10616048	29981024	480	2993181	10182656	485	11569672	26291056	485	12879544	2.9E+007
515	11499368	28920880	515	10698104	29636368	1440	2995010	9569568	515	11613360	26160400	515	12788120	2.9E+007
545	11649352	28574080	545	10728872	29680832				545	11803816	26208960	545	12684632	2.9E+007
575	11794120	28370096	575	10723080	29574240				575	11759136	25920640	575	12722856	2.9E+007
605	11894064	28223696	605	10694288	29415632				605	11806496	25860288			
635	12043504	28066128	635	10683360	29315584				635	11835544	25714976			
665	12111488	27954848	665	10723560	29242640				665	11883840	25803824			
695	12166832	27805664	695	10744562	29364800				695	11934816	25620208			
725	12210552	27608992	725	10688808	29198016				725	11873408	25632464			
			755	10723672	29196656									
			785	10716608	29110480									
			815	10684984	28973600									
			845	10653808	29004784									
			875	10675840	28941472									
			905	10671496	28862224									
			935	10578536	28821776									
Standards														
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	30222000	39101824		26956448	44744320		8032928	15196896		31083152	39459936		30019328	3.8E+007

	100mM			150mM			250mM			500mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	574175	45445312	5	440887	36451136	5	902067	42635404	5	1561554	43231328
35	3939018	41178080	35	3798352	33564128	35	5587408	36903104	35	7865808	35751840
65	6033869	37745792	65	6320576	31015504	65	7729267	33539696	65	9332582	32762448
95	7554947	35853152	95	7344112	29228736	95	8907027	31803600	95	10193984	32865392
125	8842150	34696384	125	8377229	28357264	125	9548058	31254144	125	10291096	32713760
155	9314752	33323248	155	9461664	27656768	155	9838074	30741808	155	10295920	32777600
185	9857267	32542304	185	10250448	27307216	185	10053304	30429920	185	10418936	32654224
215	10133608	31952496	215	10849432	26991104	215	10069456	30229840	215	10339936	32562256
245	10435160	31444992	245	11217720	27121104	245	10087704	30171680	245	10291856	32582528
275	10563960	31369300	275	11032384	26809104	275	10041280	30074320	275	10214480	32375904
305	10731920	31037952	305	10766936	26916048	305	10127552	30203264	305	10275320	32591360
335	10757600	30632576	335	11317664	26776608	335	10096248	30108944	335	10286080	32500768
365	10792104	30616080	365	11062096	26743728	365	10452136	30624688	365	10285752	32488368
395	10833392	30432320	395	11174216	26676640	395	10357256	30759936	395	10316472	32446336
425	10792264	30198740	425	10918680	26615520	425	10036944	30050400	425	10226784	32448224
455	10813048	30199952	455	11539952	26637424	455	10087224	30128848	455	9920115	31609424
485	10747320	30115744	485	11557912	26597520	485	10097424	29995152	485	10318192	32346480
515	10761752	29926896	515	11281552	26554336	515	10062008	30065456	515	9859251	31145696
545	10776848	30027328	545	11152616	26541744	545	10049536	30224768	545	10261448	32125984
575	10749264	29815200	575	11269552	26468416	575	10095240	30218752	575	10218032	32063264
605	10756000	29848352				605	10011544	30092896	605	9843840	31051520
635	10793432	29752368				635	10008344	30139584	635	10208736	32084704
665	10745784	29593008				665	10026976	29923056	665	10119784	31901824
695	10737112	29943984				695	9974323	30019504	695	10055832	31836016
725	10728440	29952144							725	11873408	25632464
755	10711608	29821616									
785	10627264	29552752									
815	10643472	29800320									
845	10666552	29695168									
875	10657352	29693552									
905	10658768	29566448									
935	10591912	29528896									
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0 2856		0.2093	0.2856		0.2093	0.2856
Area	24397264	42177888		29956304	38707040		24719248	42635104		24870336	42051136

Table A6.5 Acetate buffer pH 5, 70°C

	25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	itaconic
5	1857795	33870144	5	489234	32783472	5	1792798	34510624	5	1744269	30560016	
22	2614720	33269776	22	2227315	30934048	22	3791414	32148816	22	4660189	27223760	
39	3725982	32217504	39	3701234	29508144	39	5708464	30252032	39	6779466	25249536	
56	4452829	30982608	56	4886074	28545264	56	7095552	28729056	56	8218432	23997952	20322
73	5340416	30285184	73	5947645	27685952	73	8140739	27825264	73	9024531	23415424	27067
90	6191402	29641920	90	6748736	26885008	90	9040058	26995712	90	9644192	22888496	49363
107	6670397	28994880	107	7456813	26335344	107	9684966	26209280	107	9989786	22465440	50713
124	7428714	28184880	124	8033450	25698768	124	10121368	25689536	124	10162496	22195040	51286
141	8052266	27709712	141	8504038	25260816	141	10419784	25291872	141	10240984	22055664	69908
158	8137104	27166880	158	8926694	24729088	158	10658880	24911408	158	10276528	21965536	73415
175	8562707	26816368	175	9257050	24385136	175	10903432	24631280	175	10291872	21921760	86976
192	8882547	26396336	192	9579366	23993520	192	11042384	24394832	192	10411904	21870352	102108
209	9162195	25927536	209	9766176	23591312	209	11134624	24174816	209	10417616	21747904	110507
226	9391098	25577936	226	9927520	23358256	226	11220408	24000496				
243	9543936	25255328	243	10077496	23118880	243	11254384	23908752				
260	9728550	24958224	260	10163968	22966496	260	11235320	23761984				
277	9841920	24669120	277	10235672	22751792	277	11242976	23574976				
294	9941862	24407936	294	10299024	22609760	294	11188312	23569968				
311	10040824	24248128	311	10374568	22487712							
328	10141200	24033584	328	10393680	22435728							
345	10119944	23824656	345	10448544	22260080							
383	10313432	23482896	362	10380104	22086160							
			379	10419160	22110672							
			396	10413816	22060864							
Standards												
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856	0.1537
Area	27586048	33507152		25219216	32580672		27182592	33266096		26202736	32490832	6842739

Table A6.6 Acetate buffer pH 5, 110°C

	25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	Itaconic	Time (mins)	*cis*	*trans*	itaconic
0	146144	41623104		0	181072	42356000		0	378655	42249600		0	1374980	45665344	
10	1647001	40810944	77071	10	3068520	40929984	313910	10	2704312	39490496	118847	10	7191341	34393120	77071
20	2899680	39172544	262387	20	4962314	35922656	597172	20	5199645	36450305	312110	21	9987494	30429040	262387
30	3912034	37575360	391872	30	6355280	33954848	862211	30	7261770	34584640	491484	30	11040200	28622320	391872
40	4924592	36167552	552677	40	7643427	32308032	1178262	40	8136666	32487008	584179	40	11405648	27214384	552677
50	5693642	35084512	683717	50	8463085	30808912	1450970	50	9034272	31135760	752342	50	11321488	26355136	683717
60	6386400	33983520	847202	60	9091251	29749696	1714901	60	9693978	30162368	932378	60	11170640	25472144	847202
75	7140035	32464544	1160346	75	9555354	27701072	2088807	75	10293864	28935232	1227917	75	10975888	24493440	1160346
90	7812483	31270080	1399712	90	10002256	26803952	2469838	90	10554344	27888784	1481887	90	10526376	23409056	1399712
105	8282531	30122192	1546393	105	10109056	25707472	2809022	105	10684288	27095680	1716266	105	10020043	22202587	1546393
120	8642573	29199920	1710201	120	10178224	24982880	3140778	120	10806712	26502704	2004328	120	9759590	21980368	1710201
135	8997747	28042400	2076937	135	10202472	24758368	3465525	180	10339336	24624288	2793544	180	8350256	18968400	2076937
195	9521056	25616928	2944944	150	9966842	23612400	3748562	240	9908902	23347520	3167069	240	7184490	16344136	2944944
255	9201920	23880640	3374808	165	9890214	23139360	4049226	300	9396538	22409248	3426974	300	6316051	14223856	3374808
345	8933030	21980336	3801445	180	9853440	22830736	4331674	360	8994426	21673184	3613624	360	5578179	12848832	3801445
435	8466906	20598128	4136413	240	9044211	20886704	5223728	440	8577594	20897584	3774794	1434	2083443	5115984	4136413
Standards															
Conc (mM)	0.2483	0.2782	0.1537		0.24830	0.27820	0.1537		0.2483	0.2782	0.1537		0.2483	0.2782	0.1537
Area	30915344	39050912	6335021		32673088	39744704	6873290		30915344	39195264	6375837		30831360	39464160	6873290

Table A6.7 Phosphate buffer pH 6, 70°C

	25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	
10	333417	42145056	10	249510	43266592	10	213305	41742304	10	32745	42218624	
130	728219	41573536	130	1058732	42399520	130	1279623	40584128	130	1816387	40733120	
265	1094325	41126176	265	1830116	41667904	265	2247597	39517504	265	3110101	39185952	
370	1451512	40446592	370	2470434	40820288	370	2931698	38763168	370	3981574	38185696	
505	1923877	40176512	505	3148970	40346240	505	3682726	38056288	505	4790240	37557248	
1470	4003706	37995776	1470	6218218	36960512	1470	6598208	35099072	1470	6887594	35297024	
1955	4849578	37239872	1955	7092691	36271680	1955	7218538	34622688	1955	7100285	35264704	
2875	6167040	36010592	2875	8022749	35355360	2875	7718496	34141536	2875	7221994	35094816	
3380	6718717	35727648	3380	8283706	35296448	3380	7878922	34227328	3380	7240208	35365152	
4305	7540803	35103424	4305	8628186	35037632	4305	7993738	34065504	4305	7267242	35350400	
4835	7870973	34756928	4835	8729741	35026816	4835	8011325	34114752	4835	7273194	35462720	
5790	8297709	34109056	5790	8809286	34817184	5790	8049594	34047328	5790	7309549	35434240	
6275	8527718	34055168	6275	8852813	34753248	6275	8042416	33928928	6275	7268858	35344704	
10110	9258003	33103888	10110	8848774	34388352							
10590	9365267	33137568										
11570	9365267	33137568										
Standards												
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856	
Area	28907536	40302240		28907536	40302240		28907536	40302240		28907536	40302240	

Table A6.8 Phosphate buffer pH 6, 80°C

	25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	
5	496140	44654880	5	453821	42054304	5	464500	45301984	5	118351	41450592	
125	1482810	43799936	125	1969802	40670176	125	2587354	43216224	125	3654742	37916704	
245	2342494	43026080	245	3220826	39334592	245	4367146	41594944	245	5511709	36229248	
365	3037518	42611072	365	4229398	38538336	365	5571021	40561056	365	6449821	35375328	
485	3688752	41607424	485	5010170	37695264	485	6422026	39489792	485	7020944	34784128	
1420	7159904	38091808	1420	7852061	34553896	1420	8756698	37275072	1420	7614378	34102336	
1685	7739427	37727424	1685	8121181	34358176	1685	8819174	36903424	1685	7510851	33473744	
1925	8100141	37320352	1925	8207376	34078112	1925	8864749	37155232	1925	7590016	34134368	
2890	9125824	36187744	2890	8364026	33464864	2890	8936486	37068000	2890	7607517	34031872	
3115	9240384	36184512	3115	8377683	33402432	3115	8936352	37006720	3115	7629843	34065696	
3350	9388288	36063616	3350	8377040	33430736	3350	8974682	37224352	3350	7616237	34087840	
4290	9639827	35708960	4290	8296160	32971968	4290	8958528	37115040	4290	7610026	34036832	
4565	9693542	35831200	4565	8297699	33012912	4565	8932352	36982016	4565	7589475	34068096	
5740	9762630	35553952	5740	8178282	32668048	5740	8859738	36940128	5740	7650704	34057952	
6280	9834298	35814176	6280	8114371	32333552	6280	8883763	37021664	6280	7618074	33946656	
10265	9735482	35199968	10265	7818477	30960528	10265	8890822	36811904	10265	7639312	34157216	
Standards												
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856	
Area	29776128	40715648		29776128	40715648		29776128	40715648		29776128	40715648	

Table A6.9 Phosphate buffer pH 6, 90°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	508037	41008224	5	347675	34962752	5	804574	39422400	5	493515	40930464
180	3012117	38431104	180	3801806	31530816	180	5308352	35203648	180	6579504	34031168
420	4991248	35065536	420	5860083	29212096	420	7463011	33593984	420	7497744	33028144
1325	8420000	31600000	1325	7426266	27732496	1325	8350752	32164528	1325	7628333	32692496
1625	8583725	31143232	1625	7477360	27408592	1625	8387053	32035504	1625	7636176	32502288
1860	8728410	30785808	1860	7635491	26802256	1860	8440723	32248256	1860	7620352	32596656
2465	8764346	29725616	2465	7629296	27489968	2465	8399776	32017472	2465	7613587	32436992
2760	8682618	29523664	2760	7677584	27455680	2760	8428781	31904208	2760	7620131	32545520
3005	8741382	29309860	3005	7657088	27508368	3005	8442726	32077792	3005	7565235	31973536
3945	8518406	28626976	3945	7580912	27166240	3945	8393914	31883056	3945	7603274	32239024
4215	8273242	28865936	4215	7130912	28196080	4215	7937597	32777648	4215	7252416	31904272
4440	8238800	28783152	4440	7128947	28178864	4440	7909760	32797904	4440	7214938	31661904
5345	8113018	28234480	5345	7077725	27987456	5345	7860858	32636960	5345	7220339	31526128
5655	8034445	28061760	5655	7085363	28064704	5655	7849040	32651616	5655	7144842	31478720
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29718048	41044992		29718048	41044992		29718048	41044992		29718048	41044992

Table A6.10 Phosphate buffer pH 6, 97°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	306170	42556640	5	237461	39587552	5	163892	40731424	5	299748	40853280
35	1227470	41741568	35	1814359	38077440	35	2105918	39124448	35	3476870	37629984
65	2154110	40817058	65	3313320	36783360	65	3826514	37416288	65	5491408	35755168
95	2870034	40217408	95	4528845	35766336	95	5275056	36257152	95	6683581	34712480
125	3623414	39541024	125	5543171	34888064	125	6408963	35362304	125	7376627	33958144
155	4306538	38966400	155	6366349	34130944	155	7192400	34726944	155	7794144	33739552
185	4948688	38476064	185	7057642	33595552	185	7846480	33901408	185	8029965	33566976
215	5498858	37998656	215	7589139	33046288	215	8276762	33535904	215	8185290	33460624
245	6011920	37403456	245	8042026	32684592	245	8558874	33197792	245	8261552	33384320
275	6520576	36878528	275	8448531	32316832	275	8855226	32907456	275	8228883	33404016
305	6950125	36598688	305	8707507	32105120	305	9034042	32740752	305	8286563	33252880
335	7313421	36146016	335	8960512	31853296	335	9157818	32522576	335	8332419	33276176
365	7684029	35757376	365	9198899	31699136	365	9213792	32501904	365	8343530	33226784
395	8041299	35320608	395	9360384	31469392	395	9312480	32201728	395	8351226	33191568
425	8351936	35128224	425	9511571	31377456	425	9379834	32130864	425	8353146	33273904
455	8632275	34793504	455	9583846	31287920	455	9412422	32101760	455	8354432	33255184
485	8899725	34517056	485	9682451	30938336	485	9439987	32004720	485	8355181	33110784
515	9108256	34269760	515	9738970	30913984	515	9432864	32104928	515	8354051	33138240
545	9349971	34060608	545	9797427	30850368	545	9467731	31743712	545	8354051	33138240
575	9507770	33822496	575	9861760	30776064	575	9466272	31761664			
605	9706886	33654848	605	9865414	30742208	605	9417339	31870240			
635	9839987	33501296	635	9950752	30662512	635	9441184	31894736			
665	10037464	33362960	665	9964179	30762944						
695	10193488	33277680	695	9976243	30704400						
725	10275064	32988144	725	9958502	30646960						
755	10355920	33026320	755	9970867	30564912						
785	10475472	32663200									
815	10573560	32558752									
Standards											
Conc (mM)	0.20932	0.28562		0.20932	0.28562		0.20932	0.28562		0.20932	0.28562
Area	31434960	39587712		31890448	39990272		31764272	40014752		31534090	40610080

Table A6.11 Phosphate buffer pH 6, 110°C

	25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	itaconic	Time (mins)	*cis*	*trans*	itaconic
0	176746	44674240		0	184354	41300256		0	246956	43973696		0	473595	46117568	
20	1659115	42929600	110104	26	3141870	37591328	61045	20	3761742	39968608	37856	20	5883789	40345760	58686
40	2982061	41498048	229398	30	3288638	35033856	80086	40	5863296	37442208	151236	40	7729328	37974752	133530
60	3930157	40142944	143018	45	4625322	35645632	121096	60	7143696	35814880	174764	60	8411418	36744352	167524
80	4976029	38795840	183170	60	5183552	32662368	147592	80	7843251	34769216	161052	80	8649882	36347136	135423
100	5524365	37981184	254862	75	6234003	34094912	226183	100	8334445	34294592	221820	100	8653427	36258912	232065
120	6160781	37176384	264883	90	6269562	30721104	266904	120	8475552	33654816	264371	120	8655744	35832448	239308
180	7389664	35204896	395582	105	6865053	31103088	297871	180	8666330	33024848	421950	180	8573197	35379648	309815
240	8190813	33815648	541818	120	7280829	31207728	368506	240	8653997	32601072	452764	240	8450643	35103648	386899
300	8574899	32850816	653891	140	7347507	29767248	343652	300	8499014	32121328	461174	300	8351264	34652992	264452
360	8718707	31962736	817017	160	7843331	30824560	470906	360	8422438	31954000	525635	390	8063018	33715040	593649
420	8832422	31316864	899641	180	8004525	30573136	515986	420	8356160	31600512	608985	470	7761341	32161296	631463
470	8832422	30725660	960000	200	7963987	29861744	536541								
				220	7838400	29017696	582925								
				240	8195853	29960320	652449								
				300	7990397	28788832	733213								
Standards															
Conc (mM)	0.2483	0.2782	0.1537		0.2483	0.2782	0.1537		0.2483	0.2782	0.1537		0.2483	0.2782	0.1537
Area	30633408	39848224	6757219		32406560	39397408	6873290		31228064	40022592	6860864		30942048	39925216	6875546

Table A6.12 Phosphate buffer pH 7, 70°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
10	98678	40075872	10	61694	40853984	10	14696	43025888	10		39719520
130	131943	39840480	130	145302	40637312	130	128584	42930688	130	168584	39366048
265	200322	39657088	265	251767	40503200	265	331370	42630336	265	384088	38988576
370	239392	39644704	370	328262	40464224	370	447191	42566704	370	577187	38899424
505	316072	39530624	505	426009	40248128	505	596274	42265312	505	860190	38483072
1470	685959	39112384	1470	1048859	39624896	1470	1474890	41581504	1470	2160627	37292000
1955	868816	39141984	1955	1318434	39394112	1955	1845793	41216896	1955	2588678	36873536
2875	1195216	38701376	2875	1790789	38855776	2875	2476026	40694912	2875	3288744	36321024
3380	1388797	38932800	3380	2041204	38776160	3380	2784598	40699264	3380	3558837	36239840
4305	1686816	38729568	4305	2403741	38373728	4305	3232822	40274304	4305	3938470	35899776
4835	1841821	38361248	4835	2598302	38132416	4835	3453584	39984320	4835	4105230	35784480
5790	2108053	38287776	5790	2904608	37665120	5790	3776877	39703392	5790	4308922	35697088
6275	2242526	38085056	6275	3040221	37604960	6275	3945837	39532288	6275	4370346	35677216
10110	3112960	37360928	10110	3809488	36258656	10110	4684416	38978720	10110	4614221	35377120
10590	3206912	37334528	10590	3861872	36282784	10590	4735802	39001632	10590	4630707	35437728
11570	3346237	37223616	11570	3973240	36037472	11570	4794890	38868544	11570	4635226	35403264
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	28907536	40302240		28907536	40302240		28907536	40302240		28907536	40302240

Table A6.13 Phosphate buffer pH 7, 80°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	155752	42725888	5	80191	40511040	5	51896	40800480	5		44014816
125	286271	42730336	125	326999	40379424	125	333010	39977312	125	58124	43410720
245	374171	42740224	245	486637	40389376	245	598291	39798464	245	915397	42751488
365	490273	42352096	365	651900	40143520	365	851820	39671776	365	1433114	42510976
485	620572	42285664	485	819657	39849696	485	1070102	39343808	485	1892763	41978848
1420	1456213	41517792	1420	1978747	38903616	1420	2468060	37947808	1420	3933742	40016800
1685	1665727	41474688	1685	2236938	38471648	1685	2772510	37815808	1685	4257098	39508064
1925	1855891	41215200	1925	2445990	38405024	1925	2998470	37545664	1925	4529328	39597312
2890	2534493	40512800	2890	3197504	37760992	2890	3749016	36803584	2890	5074336	38904256
3115	2681112	40390368	3115	3331736	37637120	3115	3876994	36598368	3115	5185200	39052224
3350	2801910	40348128	3350	3479928	37468192	3350	4002590	36677152	3350	5205309	38910272
4290	3302800	39873248	4290	3912173	37010080	4290	4351770	36378560	4290	5351882	38817376
4565	3420222	39767232	4565	4015770	36997600	4565	4456714	36305760	4565	5356851	38823808
5740	3905709	40077600	5740	4314954	36629664	5740	4665408	36134368	5740	5426509	38892579
6280	3974754	39411872	6280	4444045	36911552	6280	4763437	36437312	6280	5455418	39138560
10265	4874442	39228800	10265	4941866	36802912	10265	5050771	36673536	10265	5553027	39553312
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29776128	40715648		29776128	40715648		29776128	40715648		29776128	40715648

Table A6.14 Phosphate buffer pH 7, 90°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	75000	38610304	5	58845	38320480	5	39881	40167048	5		40421888
180	513481	38453824	180	674287	37550400	180	944035	39555904	180	1565167	39239264
420	982894	38318336	420	1323911	36963840	420	1872372	39462720	420	2888798	37738720
1325	2687197	36534560	1325	3105554	34950592	1325	3834176	36477120	1325	4647520	35495904
1625	3103742	36108224	1625	3509853	34640896	1625	4227168	36094400	1625	4815507	35217632
1860	3398746	36179296	1860	3743112	34544448	1860	4431027	36037600	1860	4878666	34964000
2465	4203923	35202304	2465	4360243	33787136	2465	4909840	35555136	2465	4952253	34948128
2760	4391718	35211392	2760	4480064	33736992	2760	4960099	35371040	2760	4950653	34777568
3005	4541469	35024896	3005	4581459	33532256	3005	5020429	35345536	3005	4974099	34856672
3945	4952861	34615008	3945	4771290	33357312	3945	5143034	35434464	3945	4990653	34839264
4215	4423014	34640416	4215	4636595	34478080	4215	4943533	36403456	4215	4597741	34289664
4440	4481475	34704512	4440	4649816	34427360	4440	4981693	36446176	4440	4688906	34187296
5345	4682624	34446688	5345	4721933	34242840	5345	4991578	36453440	5345	4633997	34144224
5655	4727626	34371616	5655	4716688	34312576	5655	5005037	36399168	5655	4644880	34359616
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	28555536	41784928		28555536	41784928		28555536	41784928		28555536	41784928

Table A6.15 Phosphate buffer pH 7, 97°C

	25 mM			50 mM			75mM			150mM	
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	84132	40946304	5	36925	39702816	5		41253536	5		43027136
340	1476997	39432384	65	411827	39097184	50	370549	41033088	50	63597	42606048
1295	4039264	36811872	125	834721	38534496	95	815005	40577888	95	1020736	41668440
1765	4670547	36439104	185	1238635	38293568	140	1254942	40015296	140	1763791	41097408
2735	5344909	35866432	245	1611178	37860160	185	1652224	39818304	185	2375941	40560512
3200	5533299	35729920	305	1961355	37766016	230	2021289	39457920	230	2902856	40144576
4230	5738989	35366656	365	2283078	37469120	275	2355501	39210464	275	3310693	39846400
4660	5787130	35216608	425	2570038	37135552	320	2702032	38636448	320	3688526	39431360
5865	5819872	35305024	485	2858256	36938656	365	2974936	38785216	365	3955114	39188096
7305	5857507	35045504	545	3106214	36804128	410	3243678	38443936	410	4197360	39051712
8765	5926733	35112416	605	3337066	36526128	455	3471277	38282624	455	4433184	38761856
10255	5965277	35066432	665	3553814	36506576	500	3731368	38076992	500	4588698	38322912
11720	5965277	35066432	725	3761181	36268224	545	3936206	37835072	545	4718224	38429888
			785	3941568	36079808	590	4135450	37619200	590	4861792	38396640
			845	4102810	36085824	635	4290765	37472704	635	4944755	38368032
			905	4263194	35905216	680	4444355	37545952	680	4995658	38198336
			965	4392666	35821728	725	4578288	37584736	725	5082570	38194112
			1025	4529072	35727104	770	4720080	37278464	770	5151315	38042528
			1085	4653149	35757792	815	4855587	37392608	815	5221856	38337504
			1145	4739850	35612288	860	4929242	37040064	860	5269626	38176320
			1205	4854352	35614720	905	5026755	37092800	905	5301117	38231616
			1265	4951933	35504128	950	5122637	37026496	950	5351824	38295168
			1325	5029338	35552224	995	5212656	37103808	995	5308989	38233376
			1385	5116781	35666432	1040	5278787	37020480	1040	5331440	38222112
			1445	5150723	35598816	1085	5365021	36884832			
			1505	5238992	35520480	1130	5402272	36820544			
			1565	5295581	35613312	1175	5453488	36932256			
			1625	5352045	35590976	1220	5522797	37049216			
			1685	5409568	35440768	1265	5545498	36953856			
			1745	5449818	35417824	1310	5601485	36805888			
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29462800	39990272		31311440	39662016		31133872	39456288		31653872	39826432

Table A6.16 Phosphate buffer pH 7, 110°C

25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
			0	22493	44115232	0	113781	44922240	0	19489	43449600
			60	1254274	42364352	60	1821283	42817408	60	2915238	39865152
			120	2202488	41145088	120	3162382	41911168	120	4202544	37563744
			180	2935842	40341696	180	3883096	40373088	180	4743600	36516960
	No experiment		240	3490861	39436896	240	4459670	39522592	240	5008432	35652128
			300	3915526	38786496	300	4838640	38999360	300	5073411	35190080
			360	4246218	38196992	360	5078675	38756384	360	5050064	34931872
			450	4596077	37599616	420	5244003	38164096	420	5045738	34528960
			1410	4768659	33238800	480	5344906	38092064	465	5044784	34161664
			2850	4663139	32383632	1440	5449965	36856576	1410	4168394	29343088
						1800	5372506	36506848	1680	4064680	28482544
						2850	5194858	35584096			
Standards											
Conc (mM)				0.2483	0.2782		0.2483	0.2782		0.2483	0.2782
Area				31974768	39537760		30369408	40361024		30385024	39232224

Table A6.17 Phosphate buffer pH 8, 97°C

25 mM			50 mM			75mM			150mM		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	471457	39587136	5	397234	42270112	5	335442	41587776	5	0	39290848
340	770407	39089888	340	765256	41729920	340	871187	41126400	85	173750	38587840
1295	1649439	38092640	1295	2010630	40563232	1295	2168397	40024800	265	514596	38335872
1765	2028895	37835136	1765	2426738	40264080	1765	2633238	39694528	445	865434	38156896
2735	2578656	37493440	2735	3110072	39831840	2735	3282557	39199968	625	1212165	38035072
3200	2807045	37364128	3200	3339042	39588032	3200	3526944	38976000	805	1563363	37850816
4230	3225226	36905984	4230	3794029	39124352	4230	3969072	38441728	985	1869452	37910080
4660	3410382	36713408	4660	3970781	39223744	4660	4122986	38336512	1465	2099615	37294241
5865	3584758	36776032	5865	4336115	38922432	5865	4458403	38385568	1655	2607290	36850087
7305	3219322	36297536	7305	4633578	38745056	7305	4749661	38257248	2660	3450737	36010916
8765	4332064	36238176	8765	4844218	38654528	8765	4866597	38061488	3120	3747899	35707418
10255	4530429	36012288	10255	5011818	38635712	10255	5059162	38071936	4085	4153061	35415089
11720	4644938	35999936	11720	5094554	38593984	11720	5108125	38091104	4415	4055726	35384389
Standards											
Conc (mM)	0.2093	0.2856		0.2093	0.2856		0.2093	0.2856		0.2093	0.2856
Area	29462800	39990272		29462800	39990272		29462800	39990272		30531616	39380192

Table A6.18 Cations and Acetate buffer pH 5, 90°C

9 mM Calcium			9 mM Magnesium			4.5 mM Ca + 4.5 mM Mg			50 mM Potassium		
Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*	Time (mins)	*cis*	*trans*
5	251907	43546752	5	394547	43150592	5	312887	42092896	5	442167	38699328
35	2275261	41724928	35	2185741	41257472	35	2078709	40409408	35	2620826	37038336
65	4063574	39718592	65	3979630	39312288	65	3770130	38594688	65	4427750	35427040
95	5568717	37969344	95	5422707	37582272	95	5538010	36986216	95	5897546	33987328
125	6755715	36646464	125	6558938	36376320	125	6300835	35707648	125	7139389	32394896
155	7702669	35452992	155	7579379	35287328	155	7279741	34779744	155	8055411	31687200
185	8540147	34662784	185	8460288	34408032	185	8051082	33665824	185	8979206	30941344
215	9114784	33813440	215	9109222	33580672	215	8708397	33017824	215	9601786	30537840
245	9659955	33180944	245	9700691	32961632	245	9387424	33047040	245	10180152	29728208
275	10115872	32678320	275	10132608	32446912	275	9686842	32048560	275	10643536	29249008
305	10475688	32175440	305			305	10057952	31552976	305	10899968	28669168
335	10778624	31901664	335	10782456	31655824	335			335	11097872	28463696
365	11025760	31603168	365	11035648	31360432	365	10548496	31033744	365	11374072	28110032
395	11097440	31346480	395	11265424	31151936	395	10881152	31167360	395	11656480	27808176
425	11283864	31027664	425	11453896	31001840	425	11098032	31028192	425	11822672	27646016
455	11384272	30895104	455	11601784	30774464	455	11125488	30466064	455	11984272	27496368
485	11527168	30931456	485	11679808	30648000	485	11219216	30255616	485	12093512	27435520
515	11572952	30696560	515	12050152	29420496	515	11323488	30239040	515	12293184	27119408
545	11603352	29319936	545			545	11709280	29490752	545	12284808	27212624
									575	12267040	27008512
									605	12432480	26925120
									635	12431248	26946592
									665	12367536	26655856
									695	12497176	26738064
									725	12427648	26475424
Standards											
Conc (mM)	0.2483	0.2782		0.2483	0.2782		0.2483	0.2782		0.20932	0.28562
Area	34899360	40469568		34512640	41546304		34281920	40765696		30459440	39975136