Development of a Method of Analysis by High Performance Liquid Chromatography for Products of the Nitric Acid Oxidation of D-Glucose

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Abstract:

This thesis explored the development of a faster and more efficient means of qualitative and quantitative analysis of the products of the nitric acid oxidation of D-glucose and other simple sugars, for the Shafizadeh Rocky Mountain Institute for Wood and Carbohydrate Chemistry.

During the research, analysis was carried out based on previous work completed in a similar area using two Aminex HPX-87H$^+$ cation-exchange columns at different temperatures, and plumbed in series. Standards were filtered and injected onto the columns, then eluted with 5 mM sulfuric acid. A total run time of 33 minutes enabled the elution of all products and by-products of the reaction. Retention times of standards and the use of spiking helped specify and quantify unknowns in samples from a series of oxidation reactions involving D-glucose and other aldoses.

The Prevail™ Organic Acid (OA) column was said to provide "unsurpassed resolution of organic acids". It was therefore investigated, and a method was developed and refined in order to optimize conditions enabling the column's use for the required analyses. The optimized parameters were established as: ambient temperature with an eluent of 10mM KH_2PO_4 adjusted to a pH of 2.1 with phosphoric acid. The sample size was 5 µL with a flow rate of 0.3 mLmin^{-1}, giving a total run time of approximately 13 minutes.

The Aminex HPX-87H$^+$ column method and the Prevail™ OA method were compared to determine the superior method for the analyses intended. While some improvements were made for detection in the Prevail™ OA column method, results were not satisfactory. This was due in part to limits imposed on the Prevail™ OA column method, which prevented the use of gradient elution, the Aminex HPX-87H$^+$ column method outlined herein provides superior resolution for the nitric acid oxidation of D-glucose to D-glucaric acid, and in conclusion it is suggested that the Aminex HPX-87H$^+$ column method be used.

Acknowledgements:

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I hope God blesses you all so hard.

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List of Abbreviations

<	less than
>	greater than
α-	indicates axial position of the anomeric hydroxyl group of a $^4C_1$ D-sugar
β-	indicates equatorial position of the anomeric hydroxyl group of a $^4C_1$ D-sugar
γ-	prefix denoting a 5-membered lactone
δ-	prefix denoting a 6-membered lactone
σ	standard deviation
^{14}C-NMR	carbon nuclear magnetic resonance spectroscopy
^{1}H-NMR	proton nuclear magnetic resonance spectroscopy
4-AcNH-TEMPO	4-acetylamino-2,2,6,6-tetramethyl-1-piperidinyloxy
$^4C_1$	chair conformation of cyclic sugars in which C-4 is situated higher than C-1
C-1, 2, 3 etc	carbon number in a Fischer or cyclic projection
C_{18}	bonded hydrocarbon phase containing 18 carbons
D-	OH group of the highest numbered chiral centre points to the right in Fischer projection in sugars with 6 or less carbons
GC	gas chromatography
HETP	height equivalent to one theoretical plate
HPLC	high performance liquid chromatography
IEC	ion-exchange chromatography
kg/m3	kilograms per cubic metre
L-	OH group of the highest numbered chiral centre points to the left in Fischer projection in sugars with 6 or less carbons
meso-	compound containing chiral centres but is actually achiral due to a symmetry plane
milli-Q	deionised and distilled water
mKg	mono-potassium glucarate

mV	millivolt
N	normality, gram equivalent weight
NBS	*N*-bromosuccinimide
nm	nanometre
NMR	nuclear magnetic resonance
ODS	octadecylsilanized silica
-OH	hydroxyl functional group
PDMS	polydimethylsiloxane
PHPA	polyhydroxypolyamide
PVP	polyvinylpyrrolidone
R^2	coefficient of determination
RI	refractive index
RPC	reversed phase chromatography
SEC	size exclusion chromatography
SPE	solid phase extraction
t_0	void volume
TFA	trifluoroacetic acid
UV	ultraviolet
°C	degrees Celsius
µL	microlitre
µm	micrometre

1.0 Introduction

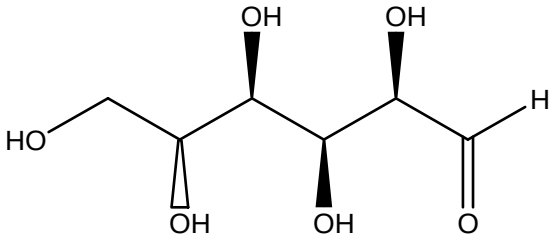
The work for this thesis was carried out in part at the Shafizadeh Rocky Mountain Center for Wood and Carbohydrate Chemistry, The University of Montana, Montana, United States of America and at the University of Waikato in New Zealand. An ongoing research project in the Shafizadeh Center investigates the nitric acid oxidation of monosaccharides to aldaric acids, principally D-glucose to D-glucaric acid, with the ultimate aim of using these bifunctional monomers to form biodegradable polyhydroxypolyamides. Currently no method exists for the accurate quantification of the acidic products of nitric acid oxidation. The work described in this thesis addressed the use of liquid chromatography to achieve quantification.

1.1 D-Glucaric Acid

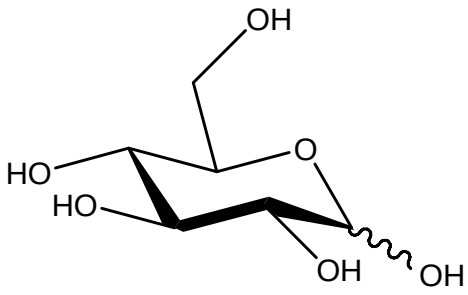
D-Glucose (**Figure 1.1a** and **1.1b**) is the most abundant monosaccharide found in nature (*1*). The ready availability of D-glucose as a precursor to bifunctional D-glucaric acid (**Figure 1.4**) makes D-glucaric acid a viable option for industrial production.

Figure 1.1 Depictions of D-glucose. **a)** Acyclic form **b)** D-Glucopyranose, $^4C_1$ chair conformation.

a)



b)

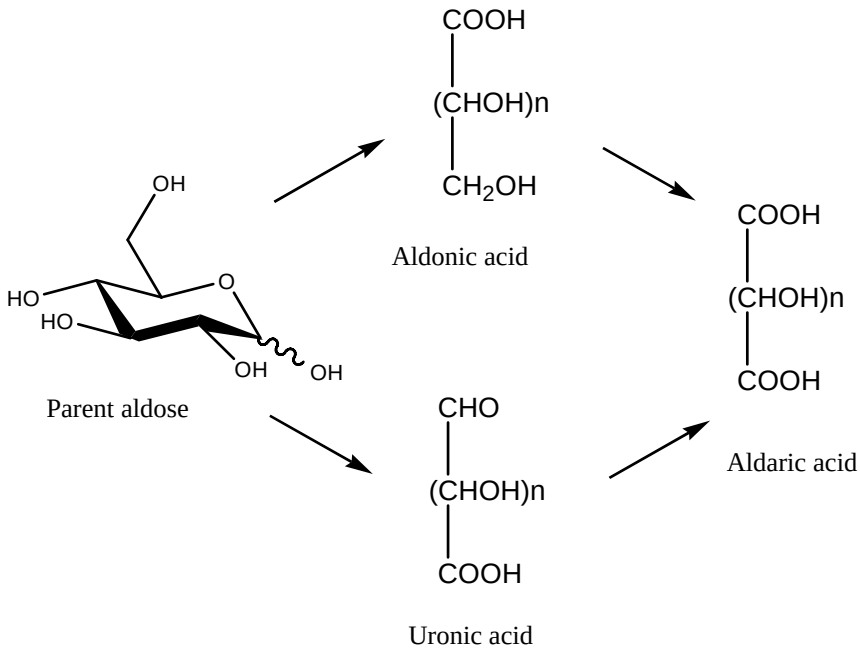


Cellulose and starch are

both naturally occurring polymers of D-glucose and thus potential sources of D-glucaric acid. Starch, a major component of plants and a plant energy reserve, is a mixture of two complex polysaccharides, amylose and amylopectin. Hydrolysis of starch, particularly corn starch, on a large commercial scale produces D-glucose as a commodity chemical (*2*).

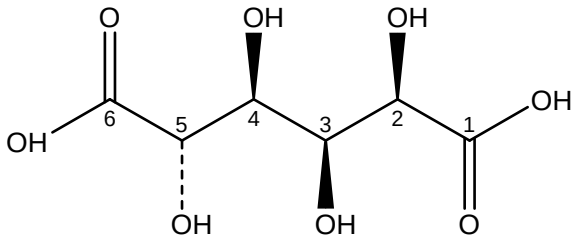
Although cellulose, 40-60% by weight of dry wood, and more than 90% of raw cotton and flax, is the most abundant organic compound produced by biosynthesis (*3*), it is currently not a significantly utilised source of pure D-glucose due to its close association with lignin in plants, which hinders its purification. However, cellulose and these two compounds have the potential to provide an inexhaustible supply of D-glucose for the production of D-glucaric acid (*4, 5*). D-Glucaric acid (or saccharic acid) (**Figure 1.3**) is a naturally occurring aldaric acid or sugar dicarboxylic acid found as a human metabolite and also as a minor constituent of fruits and vegetables (*6, 7*).

Figure 1.2 Oxidation of an aldose to yield aldonic, uronic or aldaric acids.



Oxidation of D-glucose yields D-gluconic, D-glucuronic or D-glucaric acid, an aldonic, uronic or aldaric acid, respectively, or mixtures thereof, depending on the point of oxidation and oxidation conditions (**Figure 1.2**)(*8*).

Figure 1.3 D-Glucaric acid showing its structure and relative stereochemistry.

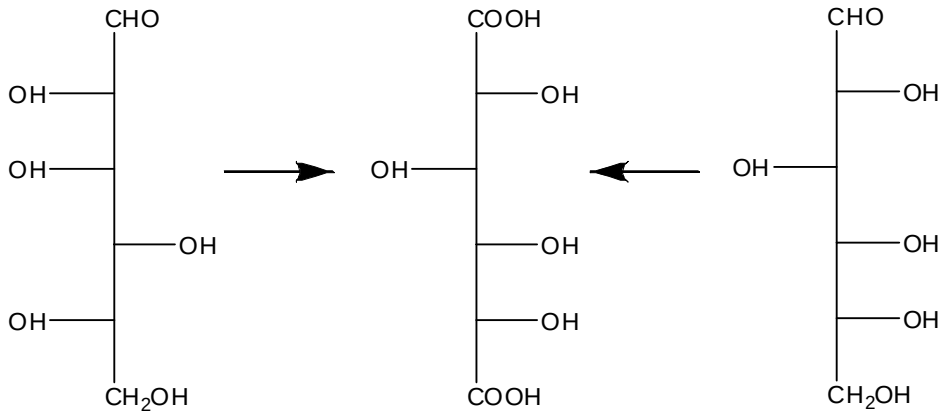


In contrast to D-glucose, D-glucaric acid - and aldaric acids in general- have the same functional group at both ends of their carbon backbone. As a consequence, in some instances, two different aldoses can be oxidised to the same aldaric acid. In the case of D-glucaric acid, it can originate from the oxidation of both D-glucose or L-gulose (**Figure 1.4**), although it is generally produced from D-glucose, it being the more common monosaccharide (L-gulose is an artificial sugar, and not found in nature) (*1*, *8*). Aldaric acids are named from the common aldose from which they are derived.

The characterisation of D-glucose by Fischer in the late 19th century was made via D-glucaric acid, isolated as the mono-potassium salt. In 1912 D-glucaric acid was reported as the magnesium salt produced from the sap of *Ficus elastica* (*9*).

Much work has been carried out in the area of D-glucaric acid production since (*10*, *11*, *12*, *13*, *14*, *15*). D-Glucaric acid has been produced by the oxidation of a number of different D-glucose based carbohydrates, though many methods reported are expensive, frequently give yields of less than 55% (*4*, *5*, *13*, *16*, *17*), and use strong oxidants (*18*). Only starch and D-glucose are viable commercial options for starting material

Figure 1.4 D-Glucaric acid and precursors L-gulose and D-glucose.



(*16*), and D-glucaric acid is now generally made by oxidation of D-glucose, molasses or starch. However, at present there is still no large-scale commercial source of D-glucaric acid.

A number of preparations of D-glucaric acid from oxidation of D-glucose have been described involving oxidants such as nitric acid (*17*) as well as other oxidants. Recently research has been published outlining the preparation of D-glucaric acid by microbial synthesis (*6*).

1.1.1 Uses for D-Glucaric Acid

D-Glucaric acid shows promise as a commodity chemical from the oxidation of D-glucose, based upon the wide natural abundance and commercial availability of this monosaccharide.

D-Glucaric acid can be used as a monomer diacid in the preparation of a group of polyamides, or hydroxylated nylons (PHPAs) (*12*, *19*, *20*,), including polydimethylsiloxane (PDMS) polyamides (*21*). At the Shafizadeh Center PHPAs have been produced from D-glucaric (*12*, *22*, *23*, *24*, *25*), D-mannaric (*22*), galactaric (*22*, *24*) and xylaric acids (*22*). Potential uses include biodegradable polymers (*26*), slow release fertilisers (*26*), and the formation of various films (*27*) and

biodegradable adhesives (*27, 28*). Work is being carried out in an attempt to minimise production costs, and to refine the effectiveness of the methods involved (*29*).

D-Glucaric acid has many other uses besides its use as a monomer. It is produced by the human body (*30*) and is regarded as safe for human use (*16*). As an everyday consumer product chemical, it may be found in metal anti-corrosion and electroplating applications, as a chelator (*31*) and in sugar cane/beet processing. It has also found numerous applications in industrial processes: as calciu- D-glucarate in calcium fortified yoghurt and powdered milk for example, and in medicine (*32*), for treatment of dermatological disorders (*33*), in the cosmetics industry (*33*), and has even been reported to have cholesterol-lowering effects (*7*).

As the calcium salt, D-glucaric acid has been shown to suppress β-glucuronidase, an enzyme produced by colonic microflora. Calcium-D-glucarate is converted in the gut to D-glucaric acid, in equilibrium with the D-glucaro-6,3- and 1,4-lactones. The latter lactone inhibits β-glucuronidase (*34*), a mammalian enzyme that allows the excretion of certain potentially harmful toxins by the body before they can be reabsorbed. β-Glucuronidase activity is associated with an increased risk of various cancers, especially hormone-dependent cancers such as breast, prostate and colon cancers (*34, 35*). The National Cancer Institute has initiated clinical trials in patients at high risk for breast cancer, examining the use of calcium-D-glucarate as an alternative to tamoxifen to block estrogen receptors (*36*).

Studies have shown that in some cases there could be a reduced risk of cancer development by ingesting foods rich in D-glucaric acid or by self-medication with D-glucarates either alone or in combination with other chemopreventive agents (*37*). A 1994 [^{14}C]-D-glucarate study by Webb and co-workers indicated that dietary glucarate, both alone and in combination with retinoids, inhibits the chemical induction and the growth of primary mammary tumors in rats (*38*). Studies have been conducted which indicate its potential as an inexpensive, non-invasive urinary biomarker for reflecting absorption of anticancer drugs and other genotoxins by

health workers (*39*).

These preliminary reports show much promise for D-glucaric acid in many areas of health science. However, mechanisms of effects and the potential use of D-glucaric acid require more thorough investigation before it can be stated with confidence just how useful D-glucaric acid will be.

1.1.2 Properties of D-Glucaric Acid

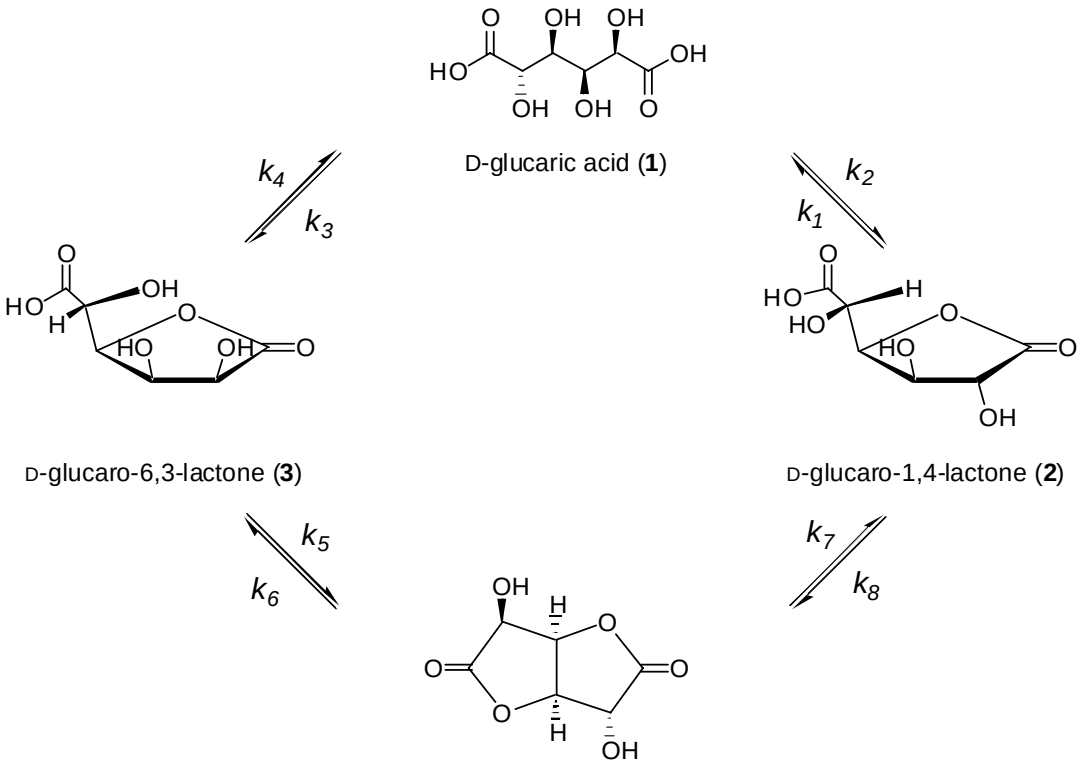
Being a hexaric acid, D-glucaric acid has four chiral centres. It has a melting point of 125.5 °C (*40*), about 20 degrees lower than either α-D- or β-D-glucopyranose, the former anomer having a melting point of 146 °C and the latter of 150 °C. Unlike some other 'extended' acyclic aldaric acids, such as *meso*-galactaric (muccic) acid, crystalline D-glucaric acid has a 'bent' configuration (*2*). The relatively low melting point of D-glucaric acid can be attributed to this configuration, which prevents the extensive hydrogen bonding present in aldaric acids with more extended conformations; for example, the melting point of D-galactaric acid is much higher at 205 °C (*41*).

In a study published in 1982, Horton and Walaszek observed the conformations of D-glucaric acid in solution by ^{1}H- and ^{13}C- NMR. The NMR data showed an equilibrium between one extended zigzag conformation and two sickle conformations (*42*). The sickle, or "bent" conformations are preferred by D-glucaric acid as they prevent an unfavourable 1,3-steric interaction between the C-2 and C-4 hydroxyl groups (*43*).

Unlike unoxidised sugars, aldaric acids cannot form cyclic hemiacetals, but they do frequently form lactones. Bose *et al*. developed a method for the isolation of the 1,4- and 6,3-lactones of D-glucaric acid (**Figure 1.5, 2 & 3**) in the early 1960s (*44*). The conformations of the lactones, including the 1,4:6,3-linked dilactone (**Figure 1.5, 4**), were also observed during the study by Horton and Walaszek (*42*). D-Glucaric acid is easily handled as the crystalline di- or monolactones (*2*).

D-Glucaric acid contains two carboxylic and four alcoholic hydroxyl groups that can potentially coordinate to metal ions. Studies have confirmed that it readily forms coordinates with metals such as vanadium (*45*).

Figure 1.5 D-Glucaric acid and its lactones.



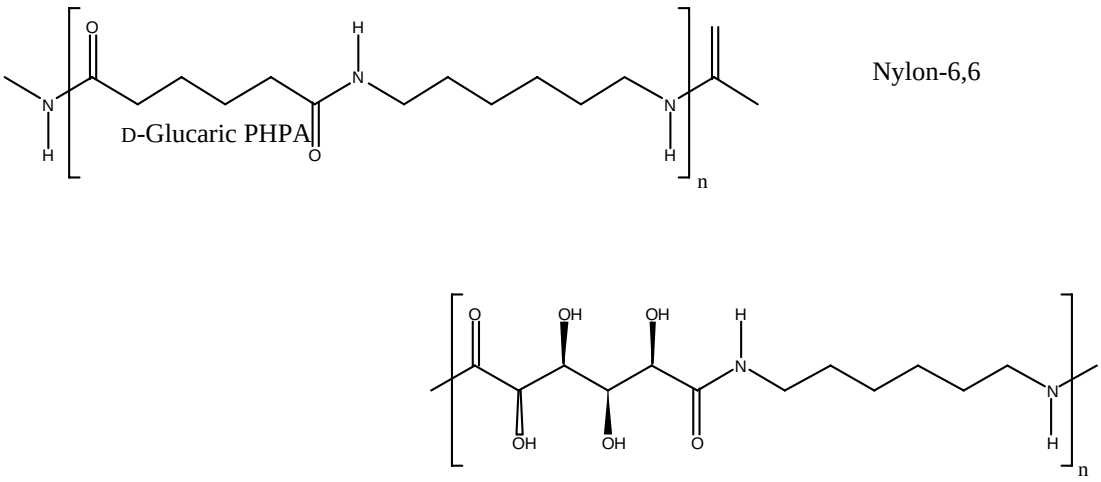
1.1.3 Polymers from D-Glucaric Acid

The large-scale production of carbohydrate-based polymers is foreseen to be based on polymerisation of small activated carbohydrate molecule monomers (*23*). A polyhydroxypolyamide (PHPA) is similar in structure to a nylon, except that the diacid monomer of the nylon is replaced by a suitably modified carbohydrate diacid (*20*). For example, a methanol solution of an activated D-glucaric acid monomer (such as methyl-D-glucarate-1,4-lactone) stirred with triethylamine and 1,6-diaminohexane at room temperature for several hours precipitates a polyamide structurally related to nylon-6,6 (**Figure 1.6**) (*23*).

Due to the "bent" conformation of D-glucaric acid (**Section 1.1.2**), polyamides produced from it tend to be more water soluble and have lower melting points than polyamides produced from aldaric acids such as *meso*-galactaric acid (*2*). In general the differences in water solubility of different PHPAs originates from which combination of aldaric acid and diamine monomers are used to generate the polyamide. These water solubility differences also imply differences in relative biodegradation rates of the polyamides (*22*).

Early reports using carbohydrate molecules in the synthesis of polyamides displayed the need for protection / deprotection steps which complicated matters (*46*). The range of polymers from D-glucaric acid as specified by Kiely *et al.* do not require protection of the carbohydrate hydroxyl groups, and thus provide a convenient method for their synthesis, as well as reducing production costs (*2, 23*).

Figure 1.6 Nylon-6,6 and poly(hexamethylene D-glucaramide), a PHPA.



1.2 Oxidation of Carbohydrates

As detailed previously (**Section 1.1.2**), much work in recent years has been centered

on the role of carbohydrates as precursors in the large-scale production of plastics, both from an environmental focus and due to economical advantages (*15*). Carbohydrate diacids, especially D-glucaric acid, are promising unrefined materials for the formation of many environmentally friendly commodities (*2*), but there remains a shortage of economical processes to develop sugar-based raw materials (*11*).

The oxidation of carbohydrates has been extensively studied for well over two centuries (*4, 5, 47, 48*). Fehling's solution (named for Hermann von Fehling who developed it in the 19th century) causes the precipitation of the brick-red cuprous oxide when the cuprate ion (complexed with tartrate ion) is heated with an aldehyde, caused by the oxidation of the aldehyde to a carboxylic acid (*8, 49*). Bernhard C. G. Tollens (1841 - 1918) is well known for the discovery of Tollens' reagent, ammoniacal silver(I) nitrate, used to oxidise aldehydes to carboxylic acids, with related reduction of silver(I) to silver(0) as a "silver mirror" (*8*). Fischer oxidised arabinose, D-glucose, gulose, mannose and xylose in his elucidation of the structures of the eight aldohexoses and the four aldopentoses, using bromine water, or nitric acid (*8, 50*). Kiliani also carried out extensive work in the area, as outlined in his series of articles entitled "*New (Observations) in the Chemistry of the Sugars*" (*51, 52, 53, 54*) and in his numerous other works (*55*).

1.2.1 Oxidation of Aldoses

Oxidation of carbohydrates at an anomeric hydroxyl group generates aldonic acids or glycosylated aldonic acids derived from disaccharides, oligosaccharides or polysaccharides. These carbohydrates are classified as *reducing sugars*, forming a "silver mirror" when treated with Tollens' reagent, or a brick-red precipitate when heated with Fehling's solution. The corresponding aldonic acid formed is often isolated as a γ-lactone (*56, 57*).

Oxidation of the terminal hydroxyl group, which is accomplished enzymatically but not easily by a chemical oxidation method gives a uronic acid. Using a strong oxidising agent, such as nitric acid, oxidation of both terminal ends yields an aldaric

acid (**Figure 1.2**), which may be isolated as the free diacid, a salt or salts, a monolactone and / or a dilactone (*56*).

1.2.2 Oxidation of Ketoses

While aliphatic aldehydes are readily oxidised to carboxylic acids under mild conditions, ketones are resistant to such oxidation (*1, 58*). However, like aldoses, ketoses give aldonic acids when treated with both Tollens' reagent and Fehling's solution (*1, 8, 49, 57*). Normally ketones are not oxidised by either of these reagents, but in the presence of these basic reagents, ketoses, such as D-fructose, are in equilibrium to some extent with their corresponding aldoses. These are oxidised and give a positive test for a reducing sugar (*59*).

1.2.3 Oxidising Reagents

An extensive range of oxidising agents with varied outcomes have been used in the oxidation of carbohydrates.

The kinetics and mechanism of the *N*-bromosuccinimide (NBS) oxidations of D-arabinose, D-xylose and D-galactose to their corresponding uronic acids in the presence of a Pd(II) catalyst has been researched, and suitable mechanisms proposed (*60*). Interestingly, the NBS oxidation of organic compounds is known to be accompanied by the parallel oxidation of bromine, thus complicating matters by requiring a scavenger for the Br^- ion.

Kabir-ud-Din *et al.* studied the oxidation of the D-sugars fructose, arabinose, xylose and glucose by chromic acid in the presence of $HClO_4$, demonstrating an example in which the modes of oxidation of aldo- and keto-sugars with the same oxidant are completely different (*61*). Aromatic *N*-halosulfonamides are a group of mild oxidising agents which have been widely used in oxidations of organic compounds, including aldehydes. An assortment of pentoses and hexoses have been oxidised to their aldonic acids by chloramine-T in alkaline medium, and kinetics and mechanisms have been suggested (*62*).

In recent years, work has been focused on developing and refining several methods of the oxoammonium oxidation of alcohol groups to aldehydes. Oxoammonium salts can be generated *in situ* from the corresponding nitroxyl radical, or they may be used as stoichiometric oxidants. Using hypohalites in the method as a co-oxidant to yield the nitroxyl radical establishes the selective oxidation of primary alcohols and aldehydes to carboxylates (*15, 63*). The nitroxide-mediated oxidation of D-glucose to its corresponding aldaric acid using hypohalites and 4-acetylamino-2,2,6,6-tetramethyl-1-piperidinyloxy (4-AcNH-TEMPO) has also been reported, and hypohalites have also been implemented in the oxidation of mannose to mannaric acid and galactose to mucic acid (*15*, *64*).

Additional agents for carbohydrate oxidation include nitrogen dioxide (NO_2) (*65, 66*), peroxidases and nitroxy radicals (*67*), sodium hypochlorite and potassium hypochlorite (*13*, *54, 68*). Reports on oxidations using oxygen, combined with a platinum-carbon catalyst have been published (*67*), also using air flow over a vanadium pentoxide catalyst, the latter affording a 63.9% yield at optimum conditions (*70*). Other common methods require the precious metal catalysts Rh and Ru (*15*).

1.2.4 Nitric Acid as an Oxidising Agent

Nitric acid (HNO_3), is commonly used as a laboratory reagent. It is a highly corrosive, toxic liquid with a density of 1.5 gcm^{-3} and is colourless when pure. Nitric acid is subject to increasing light and thermal decomposition with increasing concentration; older samples tend to acquire a yellow cast due to the accumulation of oxides of nitrogen. It is found as a white crystalline sold at -42 ˚C and boils at around 83 ˚C, depending on purity (*71*).

Nitric acid is completely miscible in water in any proportion and ionises almost completely in this medium to the nitrate ion, NO_3^-, and a hydronium ion, H_3O^+:

$$HNO_3 + H_2O \rightarrow H_3O^+ + NO_3^- \quad \text{..... Eqn. 1}$$

Thus, it is considered to be a very strong acid, with a9 pK_a (acid dissociation constant) of -2. It is a rapid and potent oxidizing agent, and can nitrate many organic compounds, thus making it useful for the production of various chemicals.

Some reactions of nitric acid can be dangerous. Reactions with compounds such as carbides and metal powders may be explosive, caused by nitric acid's strongly oxidising attributes. Reactions of nitric acid with a number of organic compounds are combustible and extremely violent.

Acid concentration, temperature and the reducing agent all influence the end products of oxidation with nitric acid. Nitric acid will react with all metals except the precious metal series and various alloys. Generally, oxidising reactions that use concentrated nitric acid are inclined to favour the formation of nitrogen dioxide (NO_2). With the dilute acid, the preferential product tends to be nitrogen oxide (NO). Nitric acid usually oxidises non-metals to their highest oxidation state, with the exception of bromine, chlorine, nitrogen, oxygen and silicon.

While other frequently used carbohydrate oxidation methods involve precious metal catalysts or require methodologies that hinder their ease of use in industrial settings, the nitric acid oxidation of carbohydrates is relatively cheap and sufficiently uncomplicated for it to be a very commonly used method (*13*). However, it is usually not selective and typically gives multiple products (*64*).

Selectivity of oxidation reactions is not always a simple matter in the absence of protecting groups (*64*), but with simple aldoses the anomeric hydroxyl group is by far the easiest group to oxidise, and terminal primary hydroxyls for the most part are then preferentially oxidised. Nitric acid oxidation converts primary alcohols and anomeric hydroxyl groups to carboxylic acids. Kiliani extensively studied the oxidations of many carbohydrates and reported that aldoses may be oxidised to aldonic or aldaric acids, which subsequently form lactones (*51*, *52, 53, 54*).

One drawback in the nitric acid oxidation of compounds is the residual nitric acid

which remains in the oxidation product. For many oxidation reactions, removal of residual nitric acid is necessary to achieve purity of products. This is especially noted in the nitric acid oxidation of alcohol-containing compounds such as carbohydrates, although there are some exceptions, such as those involving crystallization of galactaric acid, a highly insoluble product (*29*).

Several methods exist for the removal of nitric acid residues. Aldaric acids may be isolated from nitric acid by their neutralisation and subsequent separation of the resulting inorganic and organic salts (*72*). Regeneration of the aldaric acid in a neutralisation process requires acidification of the aldaric acid salt with a strong acid (*29*). Other methods require complicated or many steps, making them economically unsuitable (*29*).

1.2.4.1 Mechanism of Oxidation

Although nitric acid has often been used in the oxidation of alcohols to organic acids, only a few studies have been made to try to define the oxidation path (*73*) and a comprehensive mechanism for many of these nitric acid oxidations still has not been entirely elucidated. Oxidations with nitric acid are in general very complex and usually several intermediates are formed. A range of different reacting species have been proposed, such as N_2O_4 by Horvath *et al*. (*74*), and NO_2 by Camera *et al*. (*75*).

Oxidation of galactose using concentrated nitric acid initially exhibits suppression; an inhibition which is overcome by the addition of various nitrites (*10*). Urea is known to remove nitrous acid (HNO_2), and is found to be an effective inhibitor of the reaction. This suggests that nitrous acid rather than nitric acid itself is the actual oxidising agent.

The oxidation of benzyl alcohol to benzaldehyde (which proceeds in dilute HNO_3) and of benzaldehyde to benzoic acid (which proceeds only under strongly acidic conditions) have both been studied. It has been suggested that the radical ion, $HNO^{\bullet}{}_2^+$ is the oxidising agent in the former reaction, and for the latter, a mechanism was proposed involving hydrogen abstraction by protonated nitrogen dioxide

(HNO_2^+), followed by nitrite formation between the resulting organic radical and NO_2. The nitrite ester could then hydrolyse to the aldehyde (*73*).

Levitt stated that $H_2ONO_2^+$, NO_2^+ or NO_2 were the most likely oxidation species in a nitric acid medium, with H_2ONO^+, NO^+ or NO being the most likely oxidation species in a nitrous acid medium (*76*). Strojny *et al.* proposed that the nitrosonium ion (NO^+) is the oxidising agent in the oxidation of 2-methoxyethanol to methoxyacetic acid in a nitric acid solution (*73*). Others have proposed a chain reaction involving ion radicals or radicals (*73*).

The nitric acid oxidation of cellulose has been studied extensively for many years (*77*). It has been shown that during the oxidation of cellulose by nitrogen dioxide in the presence of carbon tetrachloride, the addition of nitric acid causes considerable nitration of the cellulose as a preliminary step in the oxidation process. The nitration in this reaction is essentially limited to the primary hydroxyl, with nitric acid appearing catalytic - although it is suggested that nitric acid could in fact be the reactive agent in the conversion of the nitrate ester to a carboxyl group (*78*).

1.2.5 Nitric Acid Oxidation of D-Glucose

The nitric acid oxidation of D-glucose has generated a lot of interest, and has been studied in depth for some time (*10*). Nitric acid is known to convert aldoses into terminal dicarboxylic acids (*1*). The oxidation of D-glucose to D-glucaric acid is a relatively straightforward procedure. There are certain obstacles though; the reaction generally proceeds with low regiospecificity, and gives only a moderate yield of D-glucaric acid, typically isolated as a salt (*15*). At the present time the large-scale production of D-glucaric acid has been prevented because there is no economically viable process for synthesizing and isolating D-glucaric acid (*11*). Nevertheless, nitric acid remains a cheap oxidant (*13*), making the optimisation of the method an attractive goal over alternative methods of oxidation.

1.3 Products of the Nitric Acid Oxidation of D-Glucose

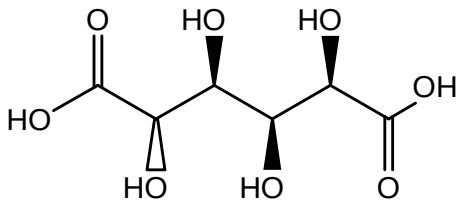
Numerous by-products may be formed in the nitric acid oxidation of D-glucose to D-

glucaric acid reaction (*79*), some of which remain unidentified (**Figure 1.7**).

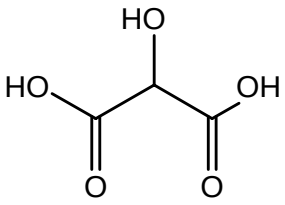
In 1974 Kuridze *et al.* reported the resolution of the products of the HNO_3 oxidation of D-glucose. The final products included D-glucose, D-glucaric acid, D-gluconic acid and 5-keto-D-gluconic acid (*80*).

Prior to this research, workers in the Shafizadeh Center had confirmed the presence of several major products of the reaction with the aid of Gas Chromatography - Mass Spectrometry (GC-MS). These included D-gluconic acid (sodium salt, or sodium gluconate), oxalic, tartronic, *meso*- and L-(+)-tartaric, 5- keto-D-gluconic and D-glucaric acid. Oxalic acid is a common by-product of sugar oxidation, its removal from oxidation products facilitating product crystallization (*29*). Mehltretter and Rist also reported the presence of oxalic acid, L-tartaric acid, and 5-keto-D-gluconic acid (*16*) amongst the oxidation reaction products.

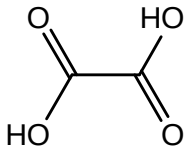
Figure 1.7 Previously identified nitric acid oxidation products of D-glucose.



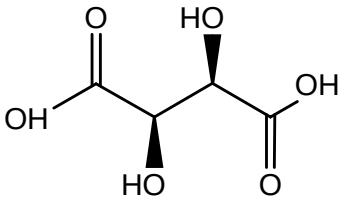
D-Glucaric acid



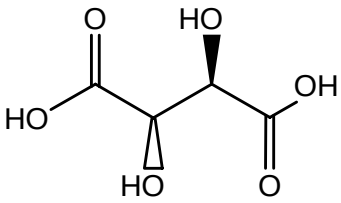
Tartronic acid



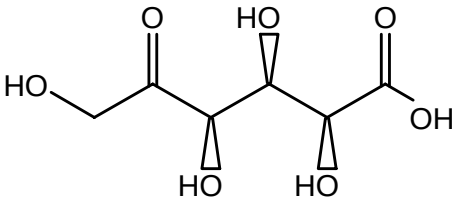
Oxalic acid



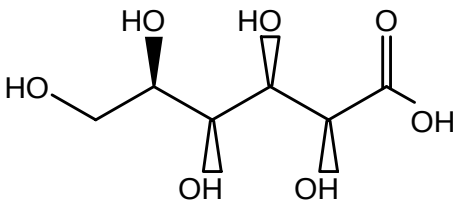
L-(+)-Tartaric acid



Meso-tartaric acid



5-keto-D-gluconic acid



D-Gluconic acid

1.4 Previous Analysis of Organic Acids

A range of chromatographic procedures have been applied to the separation and analysis of organic acids, especially in the areas of soil and environmental chemistry, where low molecular weight organic acids are common (*81, 82, 83*).

Mixtures of low mass organic acids have been successfully separated by capillary zone electrophoresis (*83, 84, 95*), GC-MS (*86*), solid-phase extraction in conjunction with GC-MS (*87, 88*), and GC-FID (*89*). Determination of organic acids has also been carried out using colorimetry (*90*) and by ion chromatography with series bulk acoustic wave detection (*91*).

1.4.1 HPLC Analysis of Organic Acids

A large quantity of work for the analysis of organic acids has been undertaken using High Performance Liquid Chromatography (HPLC), with much of the research carried out also concentrating on soil and environmental chemistries (*81, 82, 92*). In addition, HPLC is a well established analytical technique for sugars and other compounds in sugar industry laboratories (*93*).

Methods tend to involve acidic mobile phases to prevent dissociation of organic acids in aqueous solution, and the use of ion-exchange, ion-exclusion and reversed-phase columns.

1.4.1.1 Ion-Exchange Chromatography

In Ion-Exchange Chromatography (IEC) charged substances are differentiated via column materials that carry an opposite charge. IEC is widely used in separation of carbohydrates and organic acids because, due to their small size and chemical similarities, it can be difficult to apply other methods of separation to them (*94*). Application of simple IEC is primarily to inorganic ions, while organic acids may be separated on the basis of their net charges. Most separation of organic compounds involves (at least partially) other mechanisms as well (*95*).

The ionic groups of IEC columns are covalently bound to the matrix, and are

compensated for by small amounts of counterions, which are provided by the buffer present in the mobile phase. Weakly-bound counterions are displaced when a sample of the opposite charge to the covalently bound functional group (ie one of the same charge as the counterion) is passed over the column, resulting in sample retention on the solid-phase (*96*). It is due to this exchange of the sample with the counterion that the technique is called "ion-exchange". As they are reversibly bound, the molecules of interest can be eluted from the column, a step often accomplished with the aid of a pH or salt gradient. In the absence of other interactions, and along with neutral molecules, molecules having the same charge as the support are eluted in the dead volume of the column (*94*).

Resolution of carbohydrate oxidation reaction products has been successfully carried out on the AV-17 ion-exchange column. The products of the HNO_3 oxidation of D-glucose were separated on the anion-exchange column to give D-glucose, D-glucaric acid, D-gluconic acid and 5-keto-D-gluconic acid, by successive elution with H_2O, 0.01 N HCL, and a gradient of 0.1 to 1.0 N HCL (*80*).

1.4.1.2 Ion-Exclusion Chromatography

Ion-Exclusion Chromatography involves the use of strong anion- or strong cation-exchange resin as an instrument for separation of non-ionic or weakly ionised materials from ionic materials (*97*, *98*). The primary separation mechanism of the sample molecules from the charged stationary phase is Donnan exclusion (*97*), a process which results in the reduction of the concentration of mobile ions within an ion-exchange membrane due to the presence of fixed ions of the same sign as the mobile ions (*99*).

A resin such as the Aminex HPX-87H^+ column resin used in this study has sulfonic groups fixed predominantly on its surface (**Figure 1.8**), thus forming a negatively charged shield on the polymeric surface. The polymer network of the resin entraps some eluent, which is considered to act as the stationary phase. In ion-exclusion chromatography this eluent must remain physically trapped and static within the framework to act as a stationary phase, and is separated from the moving eluent by

the Donnan membrane (*98*).

Once the analyte is introduced to the column, the dissociated fraction is repelled from the Donnan membrane, while the neutral and undissociated organic acid molecules may penetrate the membrane and move into the trapped eluent. Here their movement is retarded, influencing retention times (*98, 100*).

Ion-exclusion chromatography is also known by many other names, including ion-exclusion partition chromatography, Donnan exclusion chromatography and ion-moderated partition chromatography. These labels reflect the principles by which ion-exclusion chromatography works, although there is still some controversy surrounding the exact mechanisms (*98*).

A large number of different parameters have been shown to influence retention of the analyte, including its degree of ionisation, molecular size and structure. The ionic strength of the eluent, eluent concentration and pH, presence of organic solvents in the eluent, and the column temperature and structure also play a part, as do various other parameters (*79, 98*). In principle, for an ion-exclusion column, retention time should be a function of the pK_a value of the sugar being analysed (*100*) (**Section 1.5 (i)**).

Ion-exclusion chromatography has many advantages in organic acid analysis (*101*). Through the use of strongly acidic cation-exchange resins, it has been found useful for separation of low molecular mass carboxylic acids (*102*). It is particularly suited for aqueous matrices and there is no danger of loss of hydrophilic or volatile compounds. There are rarely any complications for thermolabile compounds and generally samples require no pre-treatment such as pre-column derivatisation. Often there are no interferences with inorganic ions, except from very weakly dissociated acids (*82*).

The nitric acid oxidation of biomass residues containing carbohydrates results in several organic acids, including sugar acids. Such combinations as these have been

separated with ion-exclusion chromatography, using the Merck cation-exchange Polyspher OA-HY column at two different parameter sets, both run at 0.5 $mLmin^{-1}$. Parameter set I. had a temperature of 45 °C and an eluent of 0.005 $molL^{-1}$ sulphuric acid. Parameter set II. included a temperature of 10 °C with an 0.05 $molL^{-1}$ sulphuric acid eluent. The second separation parameter set gave higher resolution for D-gluconic, D-glucaric and oxalic acids, as well as giving increased retention times for mono- and dicarboxylic acids (*79, 101*). The investigated reference compounds included oxalic, L-(+)-tartaric, 5- and 2- keto-D-gluconic, D-glucuronic, D-gluconic and D-glucaric acid. It was noted that the retention of sugar acids and lactones were dependent on temperature, with the former having increased retention times at increased temperature, and the latter responding in the opposite manner (*79*).

1.4.1.3 Reversed-Phase Chromatography

In Reversed-Phase Chromatography (RPC) a solute molecule will bind to the nonpolar, hydrophobic stationary phase, with the commencing mobile phase being polar. Decreasing the polarity of the solvent elutes compounds in order of increasing hydrophobicity, though compounds are also retained according to their hydrogen bonding capacity, net charge and degree of ionisation (*94, 96*).

The retention of ionisable compounds in RPC can be modified by mobile phase pH, with the degree of ionisation depending on the pH of the mobile phase. A low pH is generally maintained to suppress the ionisation of the acidic groups in the analyte molecules. Suppression of the ionisation of weak acids enhances RP separation. This is performed by adding a water-miscible organic solvent (organic modifier) to the eluent, thus lowering the polarity of the aqueous mobile phase. In RPC, the eluting strength is greater with lower polarity of the eluent. The elution order can be affected by changing the type of organic modifier.

Ding and coworkers optimised the analysis of low molecular weight organic acids in soil solution by RP-HPLC using an Atlantis™ C_{18} column, enabling the determination of 11 acids. The method was run at 0.8 $mLmin^{-1}$, using 10 mM

KH_2PO_4 - CH_3OH (95:5, pH 2.7 with H_3PO_4) as the eluent. The use of the organic modifier CH_3OH ensuring that all the acids were protonated and thus neutral, successfully separated the acids, including oxalic and tartaric acid (*92*).

The separation of organic acids in wine has been achieved by RP-HPLC, following removal of polyphenols and neutral compounds by polyvinylpyrrolidone (PVP) and solid phase extraction (SPE) respectively. Isocratic elution with 0.02 M potassium dihydrogen phosphate (pH 2.88) and a 2% methanol organic modifier were used on a RP ODS-2 column to separate 8 organic acids, including L-(+)-tartaric acid (*103*).

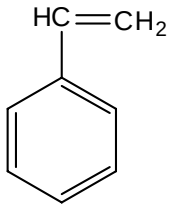
1.4.2 Previous Use of the Aminex HPX-87-H^+ Column

The Aminex HPX-87H^+ is a cation-exchange column. It has an organic support consisting of a sulfonated polystyrene-divinylbenzene co-polymer (**Figure 1.8**) (*95, 104*). It was first assessed by Wood *et al.* and Jupille *et al.* early in the 1980s (*95, 105*), and has since been used for a broad range of applications, including the determination of uronic and aldonic acids, their lactones (*106*), organic acids, sugars and their derivatives (*107, 108, 109*) and other small molecular weight analytes (*104*).

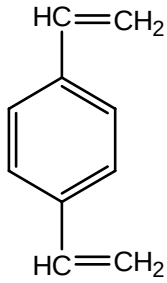
A study of three different columns by Albarran and Collins (the Aminex HPX-87H^+, Aminex A-5 and AG50W x 12 columns) maintained at 37 °C, showed that formic, oxalic, glyoxylic, glycolic and acetic acid were separated successfully, using 5×10^{-4} M H_2SO_4 as the mobile phase. Of these columns, the Aminex HPX-87H^+ eluting at 0.6 $mLmin^{-1}$ gave the best resolution. It was found that lowering the concentration of the sulfuric acid caused the oxalic and glycolic peaks to superimpose, while at higher concentrations glycolic, formic and acetic acids were not well resolved (*102*). It was also noted that several different interactions were involved in the separations on the columns, including normal- and reversed-phase partition and ion-exclusion interactions (*102, 105*).

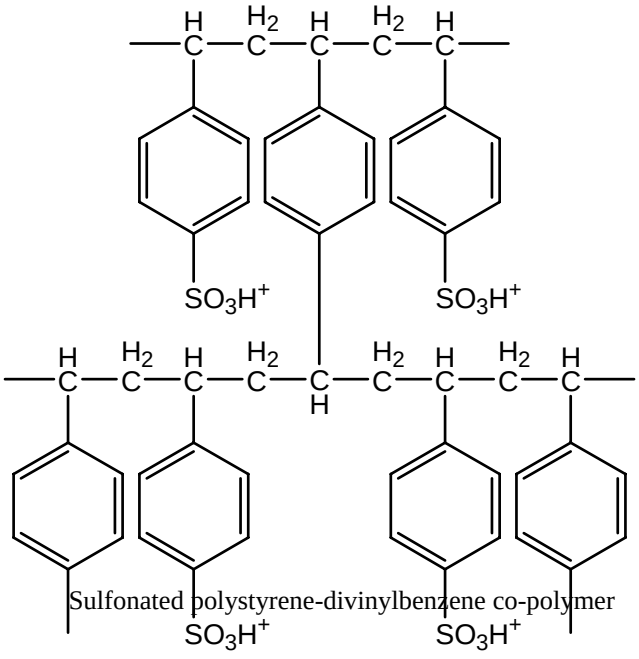
Figure 1.8 Sulfonated polystyrene-divinylbenzene co-polymer (*95*).

Vinylbenzene



Divinylbenzene





Sulfonated polystyrene-divinylbenzene co-polymer

A method using the Aminex HPX-87H$^+$ column was optimised for the determination of organic acids and sugars in fruit juices. Samples were compared with and without sample clean-up using Bond Elute® Sax cartridge. Results favoured the method that included sample clean-up, with 17 of the 18 analytes tested, including D-glucose, showing good precision. Oxalic acid precision was low due to its low pK_a, which meant it was almost totally ionised. This resulted in its elution very near to the column exclusion region, and therefore obstructed the recovery calculations. The method included an eluent of 0.005 *N* phosphoric acid, which was preferred over

sulfuric acid, as it resulted in superior baseline stability and lower background noise. The eluent was run at 0.4 $mLmin^{-1}$. Two different organic modifiers (acetonitrile and methanol) were also tested (*107*).

Hicks and coworkers used the Aminex HPX-87H^+ column in the analysis of 21 compounds including D-glucuronic acid, D-gluconic acid (potassium salt), 2-keto-D-gluconic acid (calcium salt) and 5-keto-D-gluconic acid (potassium salt). The method used 0.009 *N* sulfuric acid as the eluent at 0.6 $mLmin^{-1}$, adjusted to pH 2, and included running the column at 25, 35, 50 and 65 °C. At the highest temperature it was noted that several acids had much longer retention times which were either identical to, or very close to those of their respective aldono-1,4-lactones. It is well known that at low pH and high temperatures aldonic acids are partially converted into their lactones (*101, 106*). This data suggests that many aldonic acids and their 1,4-lactones can be detected individually at low temperatures and collectively at higher temperatures (*106*). Uronic acids and lactones were well separated by this system, as were the 2- and 5- "keto"-D-aldonic acids. Under the conditions it was shown that ion-exclusion and size-exclusion both played important parts in the separation mechanism (*106*).

Blake *et al.* investigated the organic acids in sugar cane process juice by running two Aminex HPX-87H^+ columns in series and at different temperatures. This showed improved resolution over operation of only one column, or even two at the same temperature. The organic acids analysed were first separated on DEAE-Sephadex A-25 anion-exchange resin. They included oxalic, tartronic, 2- and 5-keto-D-gluconic, D-glucuronic, L-(+)-tartaric, D-gluconic, D-gluconic acid-δ-lactone and glycolic acid, and were eluted at 70 °C, 0.5 $mLmin^{-1}$ with 5 mM sulfuric acid (*109*).

1.4.2.1 Use of the Aminex HPX-87H^+ Column for the Analysis of the Nitric Acid Oxidation of D-Aldoses

A rapid and accurate method to help determine the composition of aliquots taken from oxidation reactions involving various D-aldoses, especially D-glucose, is

required. Previous work in the Shafizadeh Center used GC-MS as the instrument of choice for analytical analysis, as it could provide useful information about the structure of compounds involved, as well as reaction rate and reaction pathway information. However, GC-MS affords some drawbacks, and the method of analysis required extensive sample preparation, including silylation and considerable drying time. HPLC circumvents this.

The oxidation reactions to be investigated involved nitric acid, which was often present in the sample to be analysed, and thus had to be taken into consideration when selecting the appropriate column. Ion-exclusion chromatography is determined to be a good choice when organic acids are in the presence of strong inorganic acids (*79*) and the Aminex HPX-87H$^+$ cation-exchange column has been reported to give good results, while remaining very stable even after repeated use (*109*).

The products of the oxidation of D-glucose were determined by Rajakyla using the Aminex HPX-87H$^+$ column at 65 °C eluted with 0.006*N* sulphuric acid. D-Gluconic acid, L-(+)-tartaric acid and other acids formed in the biochemical or catalytic oxidations of D-glucose were separated at 0.5 mLmin^{-1} (*110*). This method gave reasonable resolution of the products, but it experienced problems with co-elution and product identification.

1.5 Parameters for Assessing the Efficacy of HPLC Methods

Determination of efficiency of peak separation in an HPLC method specifies whether a method is efficient or not. For this, specific factors must first be discerned.

(a) Void Volume (t_0)

t_0 is the void volume of the column, or the time required for the unretained material to pass through the column.

Uric acid was used to determine the void volume of the Prevail™ OA column, and was included in all standards run. As uric acid does not interact with a reversed-phase column, the point at which the baseline began to rise significantly due to the

uric acid peak was used as t_0 (*94*). For the Aminex HPX-87H$^+$ column, the point at which the baseline began to fall significantly due to the negative solvent peak was used as t_0.

(b) Peak Resolution (R_s)

Peak Resolution is the separation of two peaks in terms of their average peak width at base ($t_{R2} > t_{R1}$). The R_s of two peaks can be defined as:

$$R_s = \frac{2\left(t_{R2} - t_{R1}\right)}{w_2 - w_1} \quad \text{..... Eqn. 2}$$

where w_1 and w_2 are the peak widths measured at their respective bases. These are expressed in the same time units as the retention times.

(c) Plate Number (N)

This number is indicative of column performance, or efficiency, and is given by either of the following three equations:

$$N = \left(\frac{t_R}{\sigma}\right)^2 \quad \text{..... Eqn. 3a}$$

$$N = 16\left(\frac{t_R}{w_b}\right)^2 \quad \text{..... Eqn. 3b}$$

$$N = 5.545\left(\frac{t_R}{w_h}\right)^2 \quad \text{..... Eqn. 3c}$$

where t_R is the retention time, and σ is the standard deviation of the band in time units, calculated from the peak width at base, w_b, or peak width at half height, w_h. Measurement of the peak width at half its height is most often used to determine plates because it is more accurate than at the baseline where ambiguities caused by

noise and tailing are often present (*94*). However, when a peak is tailed, *N* using half height is considerably larger than when using *N* at baseline, and thus *N* at w_b is more sensitive to column performance change than *N* at w_h (*113*).

A large value of *N* indicates an efficient column, which is potentially capable of producing sharp peaks (*94*).

(d) Effective Plate Number (N_{eff})

The effective plate number, or the *Number of Effective Plates*, is calculated as for the plate number, but uses the adjusted retention time, t_R', instead of the total retention time, t_R. t_R' is calculated by:

$$t_R' = t_R - t_0 \quad \text{..... Eqn. 4}$$

(e) Plate Height (*H*)

This is also known as the *Height Equivalent to One Theoretical Plate*, or HETP. It is obtained by dividing the column length (*L*) by the plate number:

$$H = L/N \quad \text{..... Eqn 5}$$

(f) Effective Plate Height (H_{eff})

The H_{eff}, or *Height Equivalent to One Effective Plate*, is the column length divided by the effective plate number:

$$H_{eff} = L/N_{eff} \quad \text{..... Eqn. 6}$$

(g) Retention Factor *(k)*

The retention factor or capacity factor, *k*, is the number of dead volumes or mobile phase volumes required to elute the molecule of interest. It is a measure of the time the sample spends in the stationary phase relative to the time it spends in the mobile

phase, and indicates how much longer the sample takes to travel through the column than it would take if it was not retarded by the stationary phase (*111*). It may be determined using the formula:

$$k = \frac{t_R - t_0}{t_0} \quad \text{..... Eqn 7}$$

For an analysis method to be commercially viable, the first sample needs to elute at around 5 minutes, and the run time needs to be minimised (*112*). This is to ensure the maximum number of analyses may be carried out within a realistic time frame.

Convenient *k* values fall between 1 and 5, thus giving the best compromise of resolution and retention time (*96*). If the capacity factor is clearly less than 1, compounds are eluting too quickly since their retention times differ little from the void volume. There are marginal benefits from increasing *k* above about 5, rather this only serves to increase analysis time (*94*).

(h) Asymmetry (A_x)

Peak asymmetry is an important factor in determining how much, if any, a peak deviates in shape from a normal distribution:

$$A_x = b/a \quad \text{..... Eqn. 8}$$

where the subscript *x* refers to the percentage of peak height at which the asymmetry is determined, *b* is the distance between the perpendicular connecting the baseline to the peak maximum and the peak tail, and *a* is the distance between the perpendicular connecting the baseline to the peak maximum and the peak front. The two most commonly used values for percentage of peak height are A_{10} and A_5, at 10 and 5% peak height respectively (*94, 113*).

For a tailed peak $A_x > 1$, for a fronted peak $A_x < 1$, and for a symmetric peak, $A_x = 1$.

Fronting peaks (i.e. values of asymmetry (A_x) that are less than one) are sometimes due to overloading, but may also be caused by column packing problems (*94*). Asymmetry may also be due to poorly made tubing connections (*113*).

(i) Dissociation Constant (K_a), and (pK_a)

The Henderson -Hasselbalch equation, **Eqn. 9**, relates pK_a to pH

$$pH = pK_a + \log_{10} \frac{[A^-]}{[HA]} \quad \text{..... Eqn 9}$$

Rearranging the Henderson-Hasselbalch equation, **Eqn. 9**, to give pH = pK_a, that is, when:

$$\frac{[HA]}{[A^-]} = 1 \quad \text{..... Eqn. 10}$$

it can be deduced that at a pK_a value equal to the pH, only 50% of the analyte species are charged. When the pH is ± 1 unit from the pK_a, then the analyte is 90% charged. For 99% conversion to the charged form, the pH must be ± 2 units from the pK_a. This establishes that the range of pH within which there is partial dissociation of the acid is about pK_a ± 2 (*113*).

The retention of ionisable compounds in RPC can be modified by mobile phase pH, with the degree of ionisation depending on the pH of the mobile phase. A low pH is generally maintained to suppress the ionisation of the acidic groups in the analyte molecules, as suppression of the ionisation of weak acids enhances RPC separation. For RPC, mobile phase pH should be selected so that it is at least ± 1.5 pH units from the analyte's pK_a. This ensures that the analytes are either 100 % ionised or 100 % non-ionised (*113*).

1.6 Project Objectives

The nitric acid oxidation of various aldoses to their corresponding aldaric acids, with

emphasis on D-glucose to D-glucaric acid, was under exploration at the Shafizadeh Rocky Mountain Institute for Wood and Carbohydrate Chemistry. The final reaction mixtures of the oxidation reactions, and aliquots taken at specific time intervals during the reaction had to be analysed in order to determine a) the compounds produced in the reaction, and b) to monitor reaction progress.

The objective of this project was to develop a convenient analytical method for monitoring these conversions using the Prevail™ OA column, and to compare the method with a slightly modified previously published Aminex HPX-87H$^+$ column method, assessing which available column was better suited to the analysis. The method was required to replace an existing GC-MS method.

Prior to undertaking the the research outlined in this thesis, the main method of analysis was by GC-MS, sometimes used in conjunction with Nuclear Magnetic Resonance Spectroscopy (NMR). These methods proved arduous and time-consuming, and did not always give definitive results. With much further work to be completed in this area of research, a suitably enhanced method was required to replace, or at least complement the existing methods. HPLC was thought to be appropriate for this purpose.

The Prevail™ OA column was purported to give superior results compared to the Aminex HPX-87H$^+$ column for the purpose intended. It was hoped that acceptable parameters could be established with the new column, thus developing a novel and more advanced method to meet the requirements.

The Prevail™ Organic Acid column was said to provide “unsurpassed resolution of organic acids” in considerably more favorable times than other columns of a similar nature (*114*). Because the Prevail™ OA column is a silica-based column, flow rates can be turned up much higher than for a polymer-based column without the need for expensive column heaters. This was a distinct advantage over the Aminex HPX-87H$^+$ column, which frequently took three hours to reach the required temperature and degree of equilibration. Combined with the fact that the Prevail™ Organic Acid

column method only used one column, not two in series, these reasons indicated that the latter column would be an advantageous choice for analyses, both economically and time-wise.

2.0 Experimental

2.1 Method Development

2.1.1 General Procedures

Samples were weighed on a Mettler Toledo 4-figure balance unless otherwise stated. Small solvent quantities were measured with Rainin Pipet lite pipettes.

The HPLC systems were kept in rooms maintained at constant temperature to avoid fluctuations in pH of the solvents and samples and therefore in retention times.

2.1.1.1 Apparatus and Reagents

(a) Chemicals

Analytical grade samples of the following standards were obtained from Aldrich Chem. Co., and used as supplied: formic acid, D-galactono-1,4-lactone, D-galactose, D-gluconic acid (potassium salt, or potassium gluconate), D-gluconic acid (sodium salt, or sodium gluconate), D-glucose, D-glucuronic acid (sodium salt), lactic acid, malic acid, mono-potassium glucarate (mKg), oxalic acid, sodium bicarbonate, sodium nitrate, L-(+)-tartaric acid, tartronic acid, uric acid.

The following standards were purchased from Sigma Chem. Co. and used as supplied: D-gluconic acid-δ-lactone, glycolic acid, 5-keto-D-gluconic acid (hemicalcium salt), 2-keto-D-gluconic acid (hemicalcium salt), $LiNO_3$.

For mobile phase manufacture, sulfuric acid ampules were purchased from Aldrich Chem. Co.. TFA, KH_2PO_4 and NaOH were obtained from Sigma Chem. Co.

(b) Solvent / Liquids

i) Aminex HPX-87H$^+$ Column Method

Aqueous solutions were prepared with ultrapure Milli-Q water (Millipore Simplicity™ 185, Molsheim, France). The 5 mM sulfuric acid eluent was made using volumetric solution ampules to produce stock solutions, with subsequent

dilution to the desired concentration by addition of Milli-Q water. The eluent was stirred on a hot plate at around 70 °C, and was degassed as described (**Section 2.1.1.1 (d)**).

ii) Prevail™ OA Column Method

Eluents were run through an inline degasser (**Section 2.1.1.1 (e)**) .

KH_2PO_4 was weighed out accurately and made up to the desired concentration with Milli-Q water. The eluent was sonicated to ensure complete dissolution, and buffer added until the desired pH was reached. The eluent was sonicated for 5 minutes to degas.

(c) Glassware

Glassware was acid washed and rinsed three times with tap water, followed by rinsing in distilled water three times, then oven-dried.

(d) General Instruments

Sonication was carried out using an Astron 8HTE sonicator. For pH measurement a Series 3 EDT BA 350 pH meter was used; buffers used to calibrate the pH meter were prepared by a laboratory technician

(e) HPLC Instruments and Parameters

i) Aminex HPX-87H$^+$ Column Method

The liquid chromatography system consisted of a PMC 720 series Dataplate® digital hot plate/stirrer, an Alphatech Degasys Populaire DP4010 inline degasser and a Waters 515 HPLC pump. Data was acquired by a Waters 2414 differential refractive index detector with an internal temperature setting of 30 °C for compensation, and a Waters 996 photodiode array detector. Empower Pro software was used to process data. A Rheodyne 7725i injector, loop volume 5 μL, was used to inject samples on to two Aminex HPX-87H$^+$ cation-exchange columns (300 x 7.8 mm I..D.) connected in series and protected by a guard column (Cation H, 40 x 3.6 mm I.D.). Columns were kept at their respective temperatures by Waters column ovens. The eluent was

5 mM sulfuric acid in milli-Q water, flowing at 0.5 $mLmin^{-1}$.

ii) Prevail™ OA Column Method

The HPLC system included a Waters 515 HPLC pump, Alphatech Degasys Populaire DP4010 inline degasser and Rheodyne 7725i injector with 5 µL loop. Detection was by a Waters 2996 photodiode array detector and a Waters 2414 refractive index detector with an internal temperature setting of 30 °C for compensation. The column being investigated was a Prevail™ Organic Acid, 5 µm, 150 x 4.6 mm column. Data was acquired and processed with Empower Pro 2002 Waters Corporation Version 2.0 operating software.

(f) Standards and Sample Preparation

Standards were accurately weighed and subsequently dissolved in a pipetted volume of the mobile phase, unless stipulated otherwise. They were not made up to one consistent concentration due to their variable solubilities in the eluents, but were kept within the range of between 5 and 10 $mgmL^{-1}$ to maintain peak symmetry as much as possible. RP columns have lower loading capacities than do ion-exchange (*91*), thus concentrations were kept below those used on the Aminex HPX-87H^{+} column, which generally showed adequate symmetry.

Standards were sonicated for 5 minutes using an Astron 8HTE sonicator and passed through a 13 mm 45 µm Millex filter membrane into clean vials. If not injected the same day as prepared, standards were stored at 0 °C until required. Other standards were made up to a predetermined concentration in Milli-Q water, or in some cases in 2 M NaOH solution.

Samples taken from the reactor during and after oxidation runs were prepared by laboratory technicians and were treated as follows: 0.2 mL of sample were placed into a clean vial, and 1.0 mL of 2 M NaOH added. Samples were shaken, passed through a 13 mm 45 µm Millex filter membrane and stored at 0 °C until required.

Uric acid was added to all Prevail standards to act as an internal reference for void

volume (t_0) determination.

3.0 Results and Discussion

3.1 Development of Aminex HPX-87H$^+$ Column HPLC Method

Previous work in a research field similar to the one undertaken suggested a method that might give good results for our purposes. Blake *et al.* had investigated separation of organic acids (**Section 1.4.2**) by running two Aminex HPX-87H$^+$ columns in series and at different temperatures (*109*). Due to the superior resolution and ease of use, this method was chosen to be further investigated for the analysis of products from nitric acid oxidation of D-glucose. It should be noted that the original research involved removal of non-acidic components from samples on DEAE-Sephadex A-25 anion-exchange resin. This step was not necessary for our purpose and was subsequently omitted.

3.1.1 Individual Standards

Each standard was analysed individually using the HPLC method described in the experimental (**Section 2.1.1.1** (**e**) *i)*).

3.1.1.1 Retention times

Individual retention times for standards were obtained by injecting each standard (**Appendix 1**) in triplicate and calculating the estimated mean retention time for each substance. An estimate of the standard deviation of each mean retention time was also calculated (**Appendix 2**). A summary of the results obtained during the research of this thesis shows that each triplicate retention time falls within 3 standard deviations of the estimated mean (**Table 1**). It can be estimated that the retention time for 99.73% of injections for each standard will fall within 3 standard deviations of the estimated mean value given.

Inspection of the estimated means and their corresponding standard deviations indicates that the majority of the retention times are separated by more than three standard deviations either side of the estimated mean. However, the third confidence intervals of some retention times do overlap. The confidence intervals of D-galactono-1,4-lactone and of L-(+)-tartaric acid intersect, as do those of D-

glucose and D-gluconic acid. While this would be of importance if each of these compounds were a starting material, product or by-product of the nitric acid oxidation of D-glucose to D-glucaric acid, D-galactono-1,4-lactone is not a component of this reaction (though it may be a product of the nitric acid reaction of D-galactose to muccic acid). D-Gluconic acid is a component, but it co-elutes almost simultaneously with D-glucose, and has been treated as such in chromatograms for the research presented. Thus, it can be said that for the research presented within, the retention times of the compounds involved in the reaction differ from each other enough to be certain of peak origin.

The salts KNO_3 and $NaNO_3$ were included in the analysis (**Table 1**) as prospective nitric acid residues expected to be contained in the final reaction mixture (**Section 1.2.4**). Their retention times were essentially the same, and were treated as being one since in the aqueous acid eluent would be converted to nitric acid. When both were injected simultaneously, the resultant chromatogram showed only one peak. The fact that both retention times were so similar was not of any importance as both compounds are waste products which would need to be removed and, in all probability, would be reclaimed for reuse or discarded.

D-Galactono-1,4-lactone, D-mannose, D-mannaric acid, D-xylose, and xylaric acid were also included in the standards run because the oxidation of various D-sugars were to be investigated using the refined method following this research. The remaining standards analysed were included as potential by-products of the nitric acid oxidation of D-glucose to D-glucaric acid. Glycolic acid has been largely omitted from the Aminex HPX-87H$^+$ column research because at the time of investigation it had not been identified as a possible product of D-glucose oxidation.

The standards analysed with the Aminex HPX-87H$^+$ column method (**Section 2.1.1.1 (e)** *i)*) displayed slightly different retention times from data published by Blake *et al.* (**Table 2**), however, the order of elution of comparable compounds remained the same. These negligible differences in retention times are likely to be caused by previous utilisation, treatment and age of the columns employed - all factors which

may influence degradation of the stationary phase. The retention time differences had no effect on the outcome of the experiment as they are relevant only to the specific column used.

Table 1. Retention times (in minutes) of standards for Aminex HPX-87H$^+$ column HPLC method.

Standard	Retention Time[a]
KNO_3	14.574 σ 0.004
$NaNO_3$	14.594 σ 0.005
Oxalic acid	16.367 σ 0.016
D-Glucaric acid	18.828 σ 0.005
2-keto-D-gluconic acid	18.973 σ 0.004
Tartronic acid	19.213 σ 0.004
Xylaric acid	19.292 σ 0.005
D-Glucuronic acid	19.586 σ 0.006
5-keto-D-gluconic acid	19.649 σ 0.014
D-Glucaro-1,4-lactone	20.045 σ 0.009
L-(+)-Tartaric acid	20.095 σ 0.022
D-Glucaro-6,3-lactone	20.254 σ 0.007
D-Glucose	20.955 σ 0.009
D-Gluconic acid	20.974 σ 0.007
D-Mannaric acid	21.632 σ 0.011
D-Mannose	22.248 σ 0.009
D-Xylose	22.412 σ 0.021
Glycolic acid	28.396[b]

a. Values shown include mean and standard deviations of three injections.
b. Due to it not being considered in the potential reaction components, glycolic acid was not analysed as fully as the other standards, and only one retention time was recorded.

The retention times of the standards all fall within 25 minutes of injection, although there are several additional, very small, unknown peaks that elute at 26, 28 and 32 minutes. This gives a total run time of around 33 minutes for all compounds (known and unknown) to elute. The Aminex HPX-87H$^+$ column HPLC method affords a total run time that is at least 7 minutes shorter, and it does not require the re-equilibration time of the GC-MS instrument between injections (additional time of approximately 17 minutes per sample). From this it may be concluded that HPLC is a more efficient method for the analysis intended than is the equivalent GC-MS method.

Table 2. Retention times (in minutes) of standards for Aminex HPX-87H$^+$ column HPLC method, and their corresponding literature values.

Standard	Retention time[a]	Literature ret. time[b] (*106*)	Difference[c]
KNO_3	14.574	-	-
$NaNO_3$	14.594	-	-
Oxalic acid	16.367	15.94	+0.427
D-Glucaric acid	18.828	-	-
2-keto-D-gluconic acid	18.973	19.11	-0.137
Tartronic acid	19.213	18.72	+0.493
Xylaric acid	19.292	-	-
D-Glucuronic acid	19.586	19.9	-0.314
5-keto-D-gluconic acid	19.649	20.05	-0.401
D-glucaro-1,4-lactone	20.045	-	-
L-(+)-Tartaric acid	20.095	20.41	-0.315
D-Glucaro-6,3-lactone	20.254	-	-
D-Glucose	20.955	-	-
D-Gluconic acid	20.974	21.36	-0.386
D-Mannaric acid	21.633	-	-
D-Mannose	22.248	-	-
D-Xylose	22.412	-	-

a. Retention times shown are mean of three injections, for standard deviation see (**Table 1**) and (**Appendix 1**).
b. Where available.

3.1.1.2 *k* Values

The *k* values of each standard were calculated, and with the exception of glycolic acid, all were found to fall below 1 (**Appendix 3**). In theory this is far from ideal (**Section 1.5** (**g**)), especially since almost every value was below 0.5. However, in practice the standard peaks seem to be reasonably well separated, and the compounds found to be in the reaction mixture (with the exception of D-glucose and D-gluconic acid (**Section 3.1.1.1**)) are sufficiently spaced so as to be discernable. The low *k* values are attributed to the high value of the retention time of the void volume, which is used in *k* value calculations. It was thought that calculations for t_0 may have been incorrect, thus giving low *k* values for each standard. However, Blake *et al.* gave a very similar value for the void volume retention time, and as a result, a time of around 14.58 minutes was accepted as correct.

3.1.1.3 Peak Asymmetry

Peak asymmetry values for each standard were calculated. Results are presented in (**Table 3**). Calculations were based on measurements taken at A_{10}, or 10% of peak height (**Section 1.5** (**h**)).

Table 3. Asymmetry values for Aminex HPX-87H$^+$ column standard retention times at A_{10}.

Standard	a	b	A_x = b/a
D-Glucaro-1,4-lactone	26.50	28.75	1.08
2-keto-D-gluconic acid	34.00	32.00	0.94
5-keto-D-gluconic acid	24.75	33.00	1.33
D-Glucaro-6,3-lactone	30.25	33.00	1.09
D-Glucose	39.50	47.50	1.28
D-Glucaric acid	30.00	30.75	1.03
D-Gluconic acid	21.50	31.50	1.47
D-Gluconic acid-δ-lactone	23.75	27.50	1.16
Glycolic acid	44.50	49.50	1.11
Oxalic acid	75.75	20.25	0.27
L-(+)-Tartaric acid	44.50	41.75	0.94
Tartronic acid	75.75	23.25	0.31

Peaks were found to be relatively symmetrical, with the exception of oxalic and tartronic acid. The peaks of both these standards exhibited fronting, which can often be attributed to column overloading or packing problems (**Section 1.5** (**h**)). In this instance both reasons seem unlikely for either standard. Other standards yielded symmetrical peaks, making a packing problem unlikely. At lower concentrations both oxalic and tartronic acid peaks retained their characteristic skewed shape and other standards injected at higher concentrations resulted in symmetrical peaks, leading to the conclusion that overloading is improbable. It is possible that oxalic acid peak asymmetry may be caused by early elution times (**Section 3.1.2**), but the reason for the fronted tartronic acid peak remains unclear, although there is a

possibility that it could be due to equilibration of the tartronic acid in the mobile phase.

3.1.1.4 Plate Number

The efficiency (plate number and effective plate number) of the Aminex HPX-87H$^+$ column method (**2.1.1.1.** (**e**) *i)*) was calculated for four standards: oxalic acid, glycolic acid, D-glucaric acid, and D-glucose (**Section 1.5** (**c**) and (**d**)). The latter two standards were chosen due to their importance in the reaction (starting material and final product). Oxalic acid was used because it was the earliest eluting of the known standards, and glycolic acid was used because it was the latest eluting of the known standards.

Table 4. Efficiency of Aminex HPX-87H$^+$ column method showing plate number and effective plate number of some compounds.

Standard compound	Plate number (N)	Effective plate number (N_{eff})
Oxalic acid	6367	169
D-Glucaric acid	3503	261
D-Glucose	13672	1671
Glycolic acid	20678	5498

From (**Table 4**) it can be seen that the plate number and effective plate number for oxalic acid are low. This is to be expected due to the fact that oxalic acid is almost totally ionised in the eluent and therefore elutes close to the void volume of the column (**Section 1.5** (**i**)). The calculated value of D-glucaric acid is also low when using the unadjusted retention time. Use of the adjusted retention time, t_R', to give N_{eff} yields a more precise value, though it is still low due to the large void volume value (**Section 3.1.1.2**).

3.1.2 Peak Identification

Peaks in the final reaction mixture were identified by comparison with standards.

This was achieved by injecting reaction mixtures and cross-referencing peak retention times with the standards' retention times to assign peaks. Reaction mixtures were also co-injected (spiked) with individual standards for further peak confirmation. In this way D-glucaric acid, D-glucose, glycolic acid, 5-keto-D-gluconic acid and oxalic acid were confirmed as being present in the final reaction mixture of the nitric acid oxidation of D-glucose (**Appendix 4.1 - 4.5**).

The reaction mixtures displayed two unknown peaks of significant size, as well as several smaller peaks (**Peaks A** and **B,** and **c - f Figure 3.1**). Unknown peak **A (Figure 3.1**) remains unresolved. It was thought that this peak could possibly relate to glycolic acid, which is is known to undergo typical oxidation reactions to give glyoxylic and oxalic acid, and is further oxidised to carbon dioxide and formic acid (*116*). This hypothesis was further investigated, and glycolic acid was injected on to the Aminex HPX-87H$^+$ column set-up. This produced a peak with a retention time of 28.396 minutes; much later than unknown peak **A**, and corresponding instead to one of the smaller previously unidentified peaks instead (unknown peak **f**). It is interesting to note that in the study of three different columns by Albarran and Collins (**Section 1.4.2**), lowering the concentration of the eluent caused superimposition of the oxalic and glycolic acid peaks, which had retention times of 5.5 and 11.5 minutes respectively. In the Aminex HPX-87H$^+$ column method of this research the two compounds elute too far apart for this to occur, although lowering of the eluent concentration was not researched. The discrepancies between the corresponding retention times of Albarran and Collins and those of the presented research can be attributed to the flow rate difference and the fact that only one column was used in their study. In comparison, glyoxylic acid eluted at 8.5 minutes in the study by Albarran and Collins, indicating that it is improbable that unknown peak **A** from this research is glyoxylic acid, as glyoxalic acid eluted *after* the oxalic acid peak in their study.

Comparison of retention times with the original published method (**Table 2**) lead to the proposition that one of the unknown peaks - unknown peak **B** - could be oxalic acid (*109*). Oxalic acid is a commonly produced compound in the oxidation of

sugars (*29*), signifying a high probability of it being present in the final reaction mixture.

For oxalic acid peak assignment, peak shape was one factor used for verification. Reaction mixture chromatograms showed a skewed, fronted peak (**Section 1.5** (**h**)) at the time oxalic acid was believed to elute. The oxalic acid standard peak showed the same characteristic fronted shape (**Figure 3.2**). Although another standard - tartronic acid - also presented this skewed shape, its mean retention time, at 19.213 minutes (compared to 16.367 minutes for oxalic acid) is vastly different, indicating that the earlier peak cannot be tartronic acid.

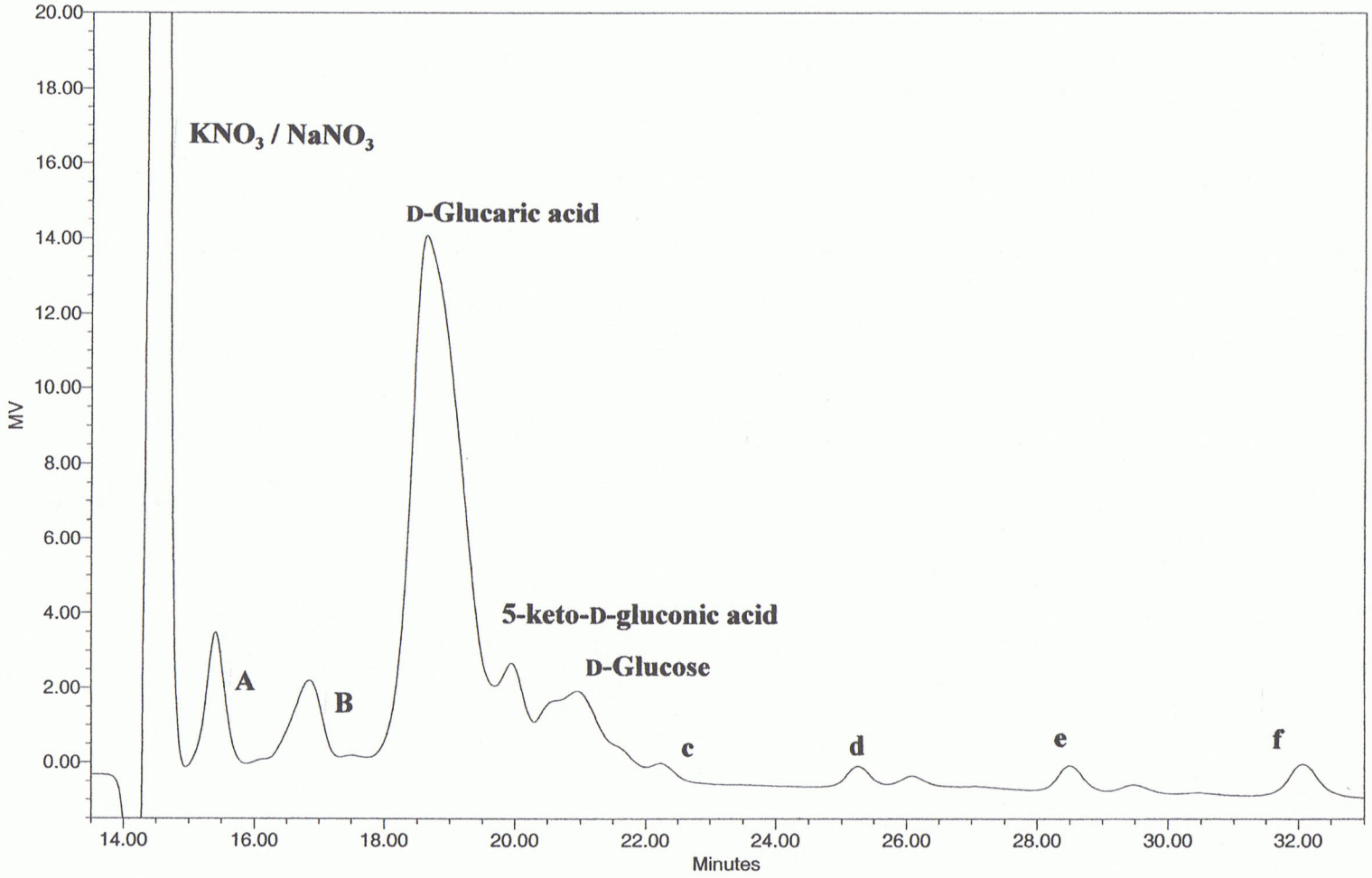
Table 5. Some pK_a values at 25 °C of potential products of the nitric acid oxidation of D-glucose to D-glucaric acid

Standard	**pK_1**
Oxalic acid	1.38±0.54
L-(+)-Tartaric acid	3.07±0.34
5-keto-D-gluconic acid	3.24±0.19
D-Glucuronic acid	3.30±0.35
Glycolic acid	3.74±0.11
Tartronic acid	1.98±0.34
D-Gluconic acid	3.35±0.35
D-Glucaric acid	2.99±0.35

All pK_a values are from SciFinder, and are calculated values, calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (©1994-2007 ACD/Labs) (*40*).

The calculated pH of a 5mM solution of sulfuric acid is around 2.3. As shown (**Table 5**), the low pK_a value of oxalic acid is around one pH unit below the pH of the eluent, ensuring the almost total ionisation of oxalic acid (**Section 1.5** (**i**)). This would result in its elution near to the column exclusion region (*102*), and may have

Figure 3.1 Chromatogram of final reaction mixture.



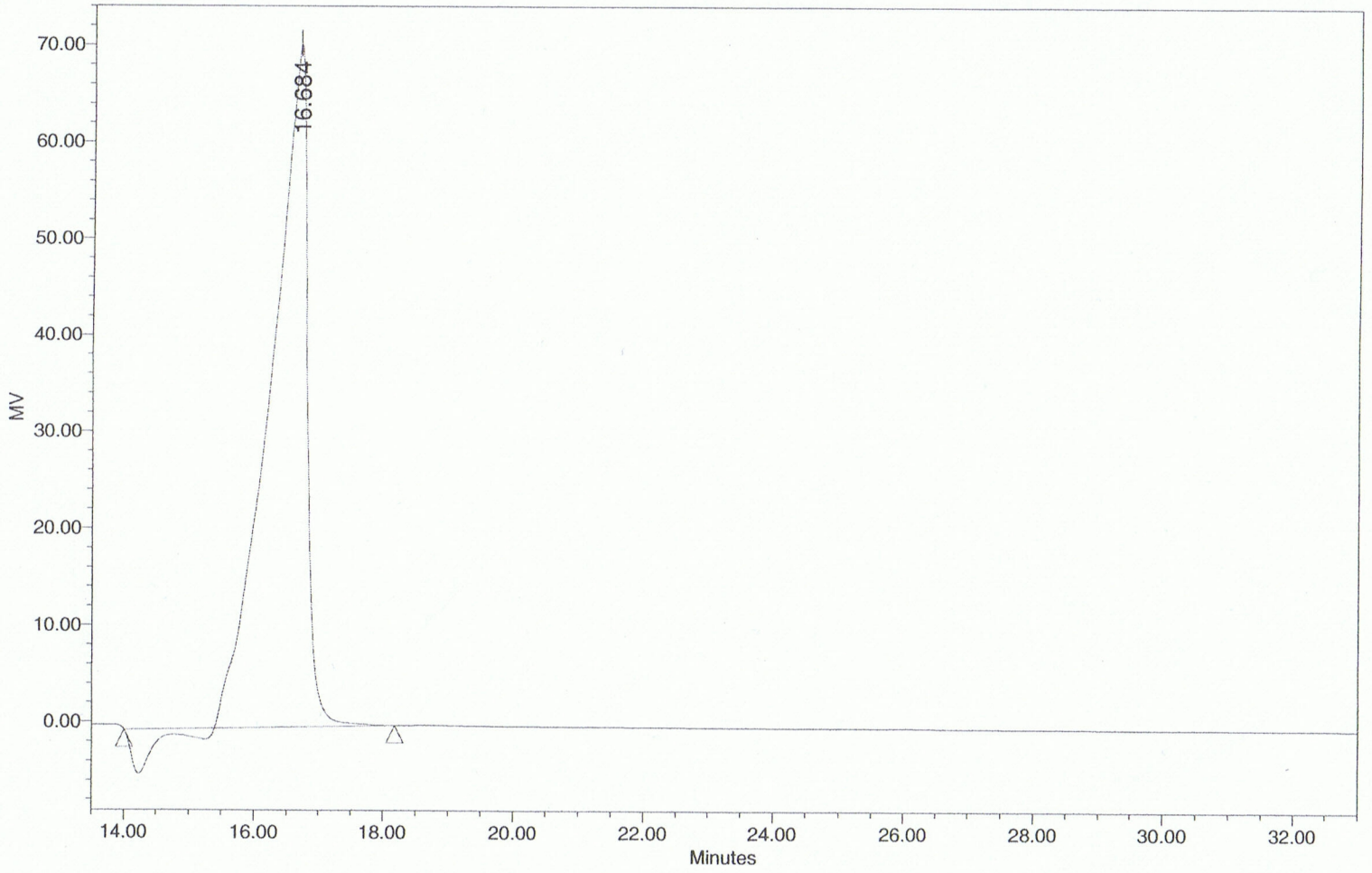
induced the skewed oxalic acid peak shape. As mentioned previously, the skewed peak shape could also have been attributed to column overloading, or even packing problems (**Section 1.5 (h)**), though both are somewhat unlikely as other peaks in the chromatogram show very good symmetry (**Section 3.1.1.3**). The skewed oxalic acid peak obstructed peak calculations, including the asymmetry calculations and peak area values for calibration curve assessment.

There was some doubt surrounding the assignment of the oxalic acid peak, as GC-MS analysis of the final reaction mixture by other researchers did not support its presence
as a product. However, GC-MS is not always representative of quantity since it depends on ease of ionisation, and must use an internal standard to be a quantitative method. Thus, GC-MS spectra showing very little oxalic acid is not definitive proof of the absence of significant amounts in the actual sample. When the oxidation mixture was spiked with oxalic acid, the proposed peak did enlarge (**Appendix 4.5**). It is still possible that the original unspiked peak could have been caused by the co-elution of another unknown product with the same retention time as the oxalic acid spike, though it is thought to be somewhat unlikely.

It should be noted that although oxalic acid has the lowest pK_a value and it has the lowest retention time, pK_a is not the only deciding factor in the elution order of the standards analysed. Other considerations must be taken into account, as a comparison of the pK_a values in (**Table 5**) and the retention times in (**Table 1**) show.

None of the unknown peaks correspond directly to any of the other standards analysed. There remains the possibility of an indirect correlation though, such as degradation products of the lactones of D-glucaric acid, or similar.

Figure 3.2 Oxalic acid fronted peak.



3.1.3 Quantitation

Calibration curves were created for D-glucaric acid, D-gluconic acid, D-glucose, $NaNO_3$, and oxalic acid in order to measure the ratios of starting materials to products during reactions. At least four separate samples of varying concentration were made up for each standard to be analysed. Samples were prepared as outlined previously (**Section 2.1.1.1 (f)**). Calibration curves were generated by plotting peak area (in mV x mins) against concentration (in $mgmL^{-1}$). The results were linear over the expected range, with no calculated R^2 value falling below 0.995 (**Appendix 5.1 - 5.5**).

As a consequence of the fronting nature of the oxalic acid peaks, the calibration curve generated for oxalic acid may not be as valid as the other calibration curves produced.

3.1.3.1 Reaction Mixture Analysis

Peak retention times and calibration curves were used in conjunction by colleagues to gain valuable information about the progress of the nitric acid oxidation reactions and to ascertain whether or not the reactions were proceeding to completion, i.e. if all the D-glucose was being consumed.

Chromatograms of samples taken at various intervals during reaction runs clearly show depletion of the D-glucose peak and corresponding enlargement of the D-glucaric acid peak (**Figure 3.3**). The 5-keto-D-gluconic acid and the unknown peaks **A**, **B** (now identified as oxalic acid), **c, d** and **e** (now identified as glycolic acid), also enlarge as the reaction proceeds. The aliquots were taken at time intervals as follows:

#1 = 5 minutes after glucose added
#2 = 180 minutes
#3 = 270 minutes
#4 = 389 minutes (final reaction mixture)

(**Figure 3.4**) shows the increasing and decreasing concentrations of products and

Figure 3.3 Reaction mixture at 4 time intervals.

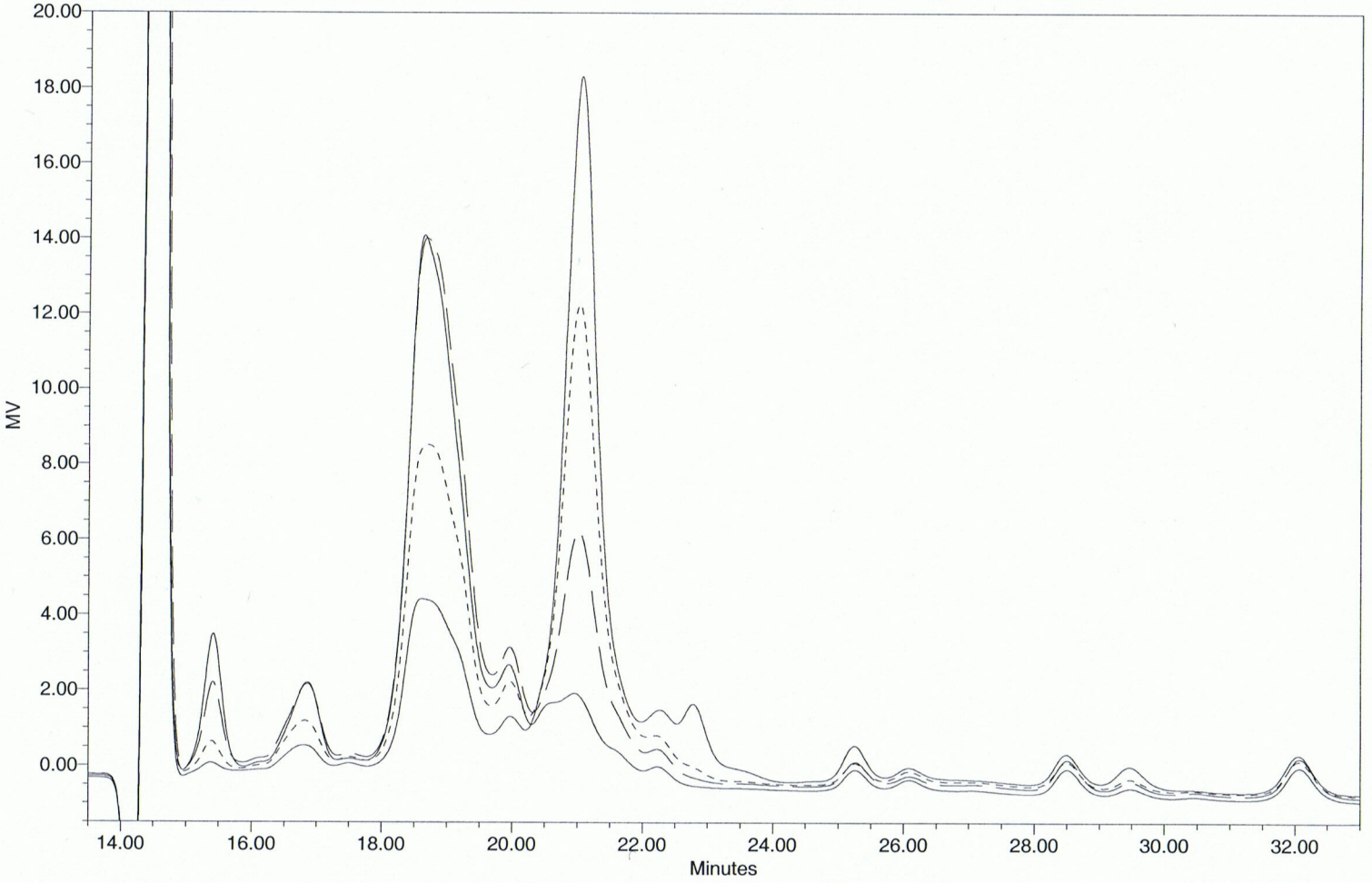
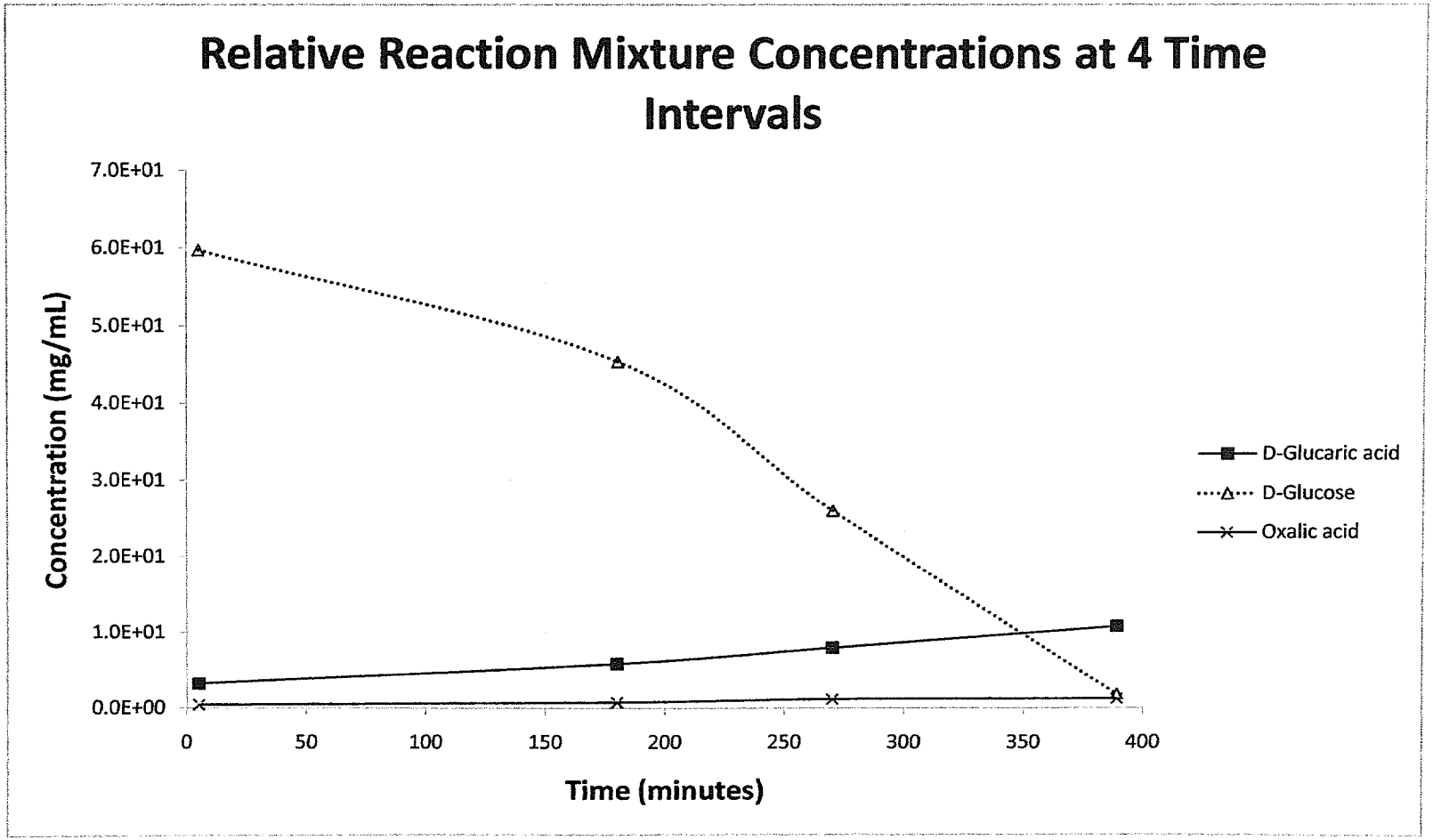


Figure 3.4 Relative concentrations of 5-keto-D-gluconic acid, D-glucaric acid, D-glucose and oxalic acid at 4 time intervals.



3.2 Development of Prevail™ Organic Acid Column HPLC Method

The starting point for experimental design for the development of the Prevail™ OA column method was based on parameters indicated by literature that accompanied the column when purchased (*114*). These guidelines included:

1. 25mM KH_2PO_4 (potassium phosphate - monobasic) eluent, adjusted to pH 2.5 with phosphoric acid
2. Flow rate 1.0 $mLmin^{-1}$
3. UV detection at 210 nm

Other published template methods suggested using an organic modifier to adjust the eluent (*92*). A decision was made to omit this step from trial runs because the final method needed to be as simple - and therefore as easy to set up - as possible. The use of gradient elution was also considered, but was not put in place for the same reason.

Initially each standard was analysed using HPLC and an isocratic elution method, to elucidate their retention times (**Section 2.1.1.1 (e)** *ii)*). Different parameters were tested in attempts to gain better resolution of the standards (**Table 6**). Parameter 1 involved use of 26.5 mM KH_2PO_4, adjusted to pH 2.5 with phosphoric acid, with a flow rate of 0.3 $mLmin^{-1}$. Parameter 2 adjusted the eluent concentration to 10 mM. Parameter 3 raised the pH of the eluent to 3.0. Parameter 4 lowered the pH of the eluent to 2.1. Parameter 5 increased the flow rate to 0.4 $mLmin^{-1}$. Parameter 6 decreased the flowrate to 0.2 $mLmin^{-1}$.

All values are taken from chromatograms generated using the 'max plot' function of Empower™, except in the case of wavelength detection investigation. A 'max plot' is a special chromatogram which is produced by plotting the maximum spectral absorbance measured at each time point in the data file, and thus gives the best possible chromatogram for any given run.

Table 6. Experimental design for the Prevail™ OA column method showing the different parameters standards were subjected to.

Factors	Concentration of KH_2PO_4 (mM)	pH of mobile phase, adjusted with H_3PO_4	Flow rate (mL min^{-1})	Detection Wavelength (nm)	Temperature	Sample size (µL)
Effect of mobile phase concentration on *k*	10, 26.5	2.5	0.3	Max plot	Ambient	5
Effect of pH on *k*	10	2.1, 2.5, 3.0	0.3	Max plot	Ambient	5
Effect of flow rate on separation	10	2.1	0.2, 0.3, 0.4	Max plot	Ambient	5
Best wavelength for detection	10	2.1	0.3	210, 220, 240	Ambient	5

Void volume determination was calculated for each parameter set by calculating the mean retention time of 12 injections (**Table 7**). Unlike conventional retention times, in which the retention time is taken as the highest point of a peak, the void volume, t_0, is calculated from the point where the baseline begins to alter due to the unretained material peak (**Section 1.5 (a)**).

Table 7. Uric acid injections to determine t_0 of the Prevail™ OA column at 0.3 mLmin^{-1}.

	Parameter Set			
	1	**2**	**3**	**4**
t_0*	4.461	4.586	4.549	4.483

* Values are the mean of 12 injections (see Appendix 6).

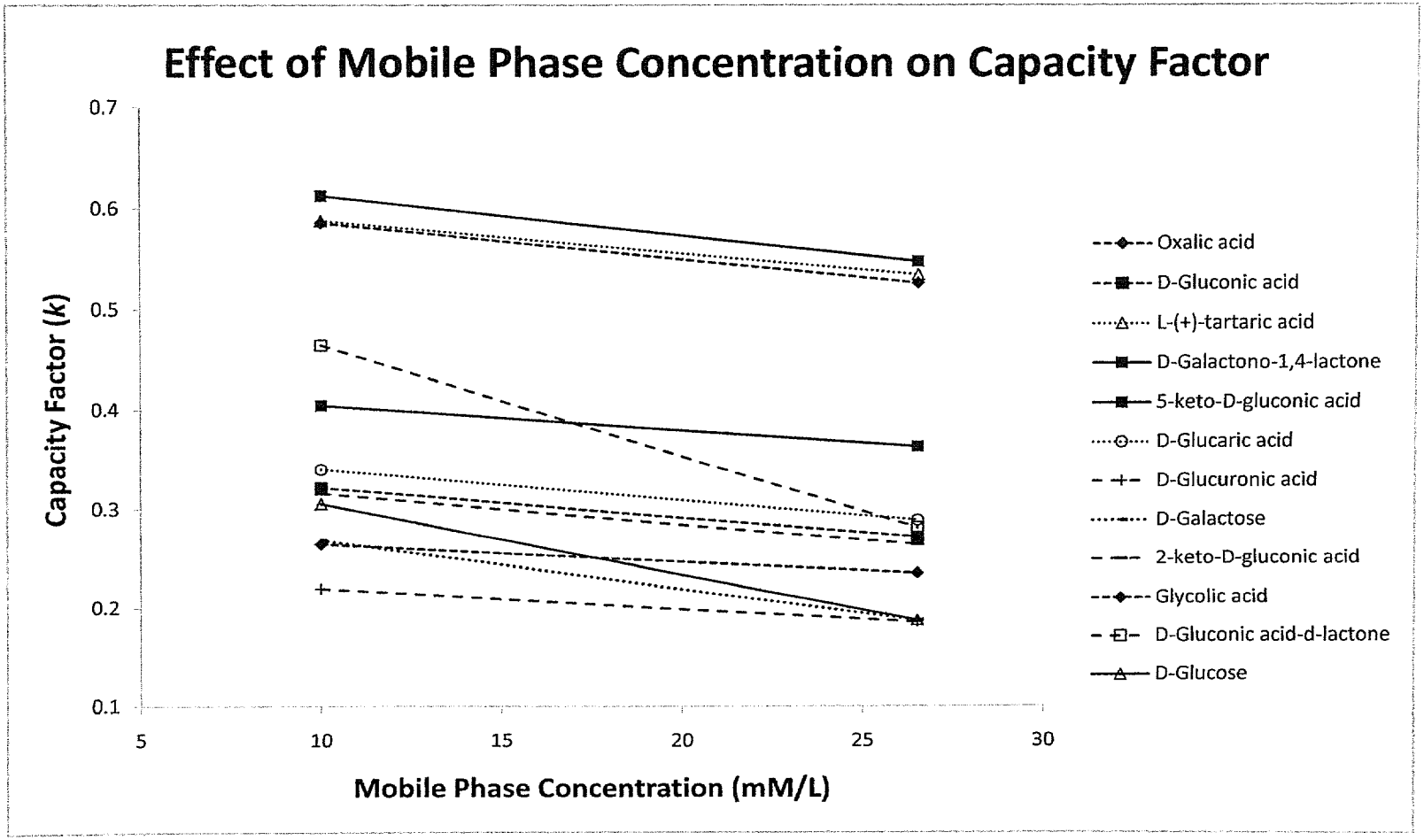
Of the 12 retention time values collected for each t_0, (i.e. 48 total) all except two values fell within 2 standard deviations of the calculated mean. Both of these values fell within 3 standard deviations of the mean (**Appendix 6**), giving a good estimate of the t_0 values.

3.2.1 Effect of Mobile Phase Concentration on *k*

The literature accompanying the Prevail™ OA column stated that using KH_2PO_4 as the eluent enabled accurate analysis of organic acids, therefore it was employed for this purpose in this research.

An acidic mobile phase is essential in the case of HPLC organic acid analysis where water is the main constituent of the mobile phase due to the fact that acids undergo dissociation in aqueous solution (**Section 1.4.1.3**). Partial or full dissociation prevents adequate interaction of the analyte with the solid phase, and may result in analyte elution at the void volume, inaccurate elution times or broadened peaks. Peaks produced by the acids in their molecular state are more likely to be sharp and well defined. Using an acidic phase moderates this dissociation, allowing retention of molecules on the silica-based RP solid phase, and means they can be elucidated accordingly (*92*).

Figure 3.5 Effect of mobile phase concentration on k for the Prevail™ OA column standards.



KH_2PO_4 has a UV cutoff of <200nm at 10mM, and showed no absorbance at any of the wavelengths investigated.

Idealistic *k* values should fall between 1 and 5 (**Section 1.5 (g)**). (**Figure 3.5**) shows that the best results for *k* in the context of this study (i.e. they fall closer to the optimum range) are found when the mobile phase is at a concentration of 10 mM. Oxalic acid, D-gluconic acid, L-(+)-tartaric acid, D-galactono-1,4-lactone, 5-keto-D-gluconic acid, D-glucaric acid, D-glucuronic acid, D-galactose, 2-keto-D-gluconic acid and glycolic acid all showed a slight improvement in *k* when subjected to 10 mM KH_2PO_4, as opposed to when they were subjected to 26.5 mM KH_2PO_4. D-Gluconic acid-δ-lactone and D-glucose showed much higher increases in *k*. As a result, the mobile phase concentration chosen was 10 mM KH_2PO_4.

While the *k* was improved for every standard under these conditions on the Prevail™ OA column, for all of them it still falls below 1, though values are somewhat higher for L-(+)-tartaric acid, D-galactono-1,4-lactone, glycolic acid and for D-gluconic acid-δ-lactone. Since the *k* for D-glucuronic acid was significantly less than the others in 10 mM KH_2PO_4, it was hoped that adjusting other factors would improve its value.

3.2.2 Effect of Mobile Phase pH on *k*

Mobile phase pH is an important consideration in method development (**Section 1.5 (i)**). Buffering mobile phase pH in the range between 2-5 can inhibit the ionisation of acid groups, thus allowing the desired molecules to be retained in their neutral form. Increasing the pH will permit gradual dissociation of the organic acids (*92*).

While phosphoric acid was specified as the buffer in the manufacture method (**Section 3.2**) of the Prevail™ OA column, the use of trifluroacetic acid (TFA) as a buffer was also investigated during this research to see if peak resolution could be improved upon with an alternative buffer. Instead, it lead to a decrease in resolution of the standards. This is thought to arise from the ability of TFA to form strong ion-pairs with positively charged species due to its very low pK_a of 0.3. This is

commonly found in the case of TFA/organic acid combinations (*113*). The formation of ion-pairs at values much less than 100% would complicate the chromatogram by increasing the number of peaks detected.

Using phosphoric acid as the buffer showed superior peak shape compared with a TFA buffer (**Appendix 7**). Moreover, TFA has a smaller buffer range than phosphoric acid, and phosphoric acid was deemed to be the more effective buffer for the analysis. While the baseline was slightly more irregular using phosphoric acid over TFA, the former yielded better peak detection. This enabled the detection of much lower concentrations. (**Appendix 7**) shows the same injection of D-glucose, with a much larger peak generated using phosphoric acid as the buffer.

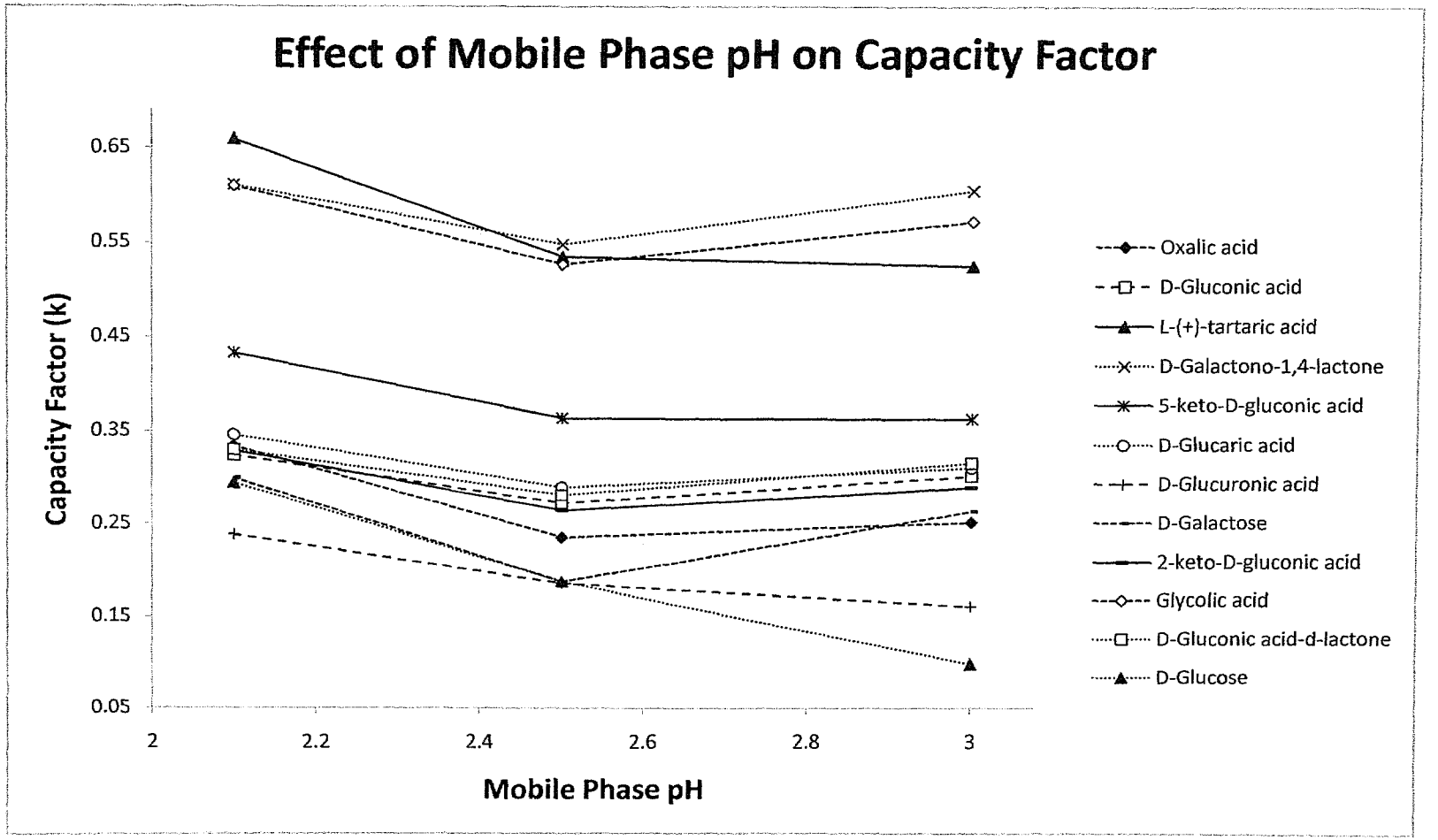
From a starting value of 2.5, the pH was raised to 3.0 and then lowered to 2.1 to ascertain which pH gave the best results for the Prevail™ OA column.

(**Figure 3.6**) shows that lowering the pH of the mobile phase to 2.1 with phosphoric acid improves *k* for all standards run. While the increase in *k* was relatively small for most standards, it was especially pronounced for D-glucose. L-(+)-Tartaric acid also showed a marked positive increase at pH 2.1, as did D-galactose, which also retained the increase in *k* when the pH was subsequently increased to 3.0, although they are all still outside the optimum range.

Increasing the pH to 3.0 from the starting point of 2.5 also showed some positive scaling of *k*, but not for L-(+)-tartaric acid, 5-keto-D-gluconic acid, D-galacturonic acid, or D-glucose, and it was very minimal for oxalic acid. As D-glucose is the starting material, and 5-keto-D-gluconic acid and oxalic acid have been identified as products in the nitric acid oxidation of glucose, a pH of 2.1 was the preferred choice. Overall, the lower pH of 2.1 gave better results than either the starting pH of 2.5 and the higher pH of 3.0.

It was also noted that upon lowering the pH from 2.5 to 2.1, the three lowest *k* values

Figure 3.6 Effect of mobile phase pH on *k* for the Prevail™ OA column standards.



(D-glucuronic acid, D-galactose and D-glucose), were raised by 28%, 60% and 57%, to 0.24, 0.30 and 0.30 respectively. These figures show improvements for k, but remain very low. These results correspond to the poor resolution shown by the column under these conditions.

Lower pHs were not investigated because at pHs of below 2 the column could be subject to degradation over a period of time (*94*).

3.2.3 Effect of Flow rate on Separation

Flow rate can alter the width of a peak in a chromatogram as it can directly affect the ability of the analyte to interact with the stationary phase. In the case of some flow sensitive detectors such as a UV detector, the flow rate may also affect peak area (*94*). Under isocratic conditions, however, flow rate generally has a limited effect on the plate height of small molecules such as alcohols.

As expected, the retention times of the standards were decreased with increasing flow rate, and increased with decreasing flow rate on the Prevail™ OA column. The different flow rates investigated were 0.2, 0.3 and 0.4 $mLmin^{-1}$.

Chromatograms of the final oxidation reaction mixture at all three times were basically the same, with the same number of peaks appearing in each run. However, peak resolution was slightly better with decreasing flow rate. This can be attributed to the individual molecules having more time to interact with the stationary phase, and thus allowing them to be separated more completely. While the fundamental resolution was not significantly better at the lower flow rate, it was not compromised at the higher flow rate either.

Running the solvent at 0.2 $mLmin^{-1}$ on the Prevail™ OA column resulted in an entire run time of 17.8 minutes to elute all peaks, while at 0.4 $mLmin^{-1}$ it only took 7.7 minutes. Eluent flow rate of 0.3 $mLmin^{-1}$ gave a complete run time of 12.2 minutes; an improvement of greater than 20 minutes when compared with the Aminex HPX-

87H$^+$ columns - not taking into consideration the time needed for equilibration or heating of the Aminex HPX-87H$^+$ column, both of which add a substantial amount of time to the Aminex HPX-87H$^+$ column method.

k values (**Table 8**) were best at 0.4 mLmin^{-1}, but were very low, with all values appearing well below 1. L-(+)-Tartaric acid had the best *k* value, of 0.67, while D-glucuronic acid had the lowest, at 0.24.

Among the standard components, D-glucuronic acid eluted first at 4.12 minutes for a flow rate of 0.4 mLmin^{-1} and this increased to 8.29 minutes for the flow rate of 0.2 mLmin^{-1}. L-(+)-Tartaric acid eluted last of the standards for all runs, with retention times of 11.16 minutes for 0.2 mLmin^{-1} and 5.55 minutes for 0.4 mLmin^{-1}. The t_0 values for 0.2 mLmin^{-1} and 0.4 mLmin^{-1} were 6.82 minutes and 3.32 minutes, respectively, based on uric acid as eluting at the void volume.

Table 8. Prevail™ OA column standards' *k* values at different flow rates.

	k		
Standard	0.2 mLmin^{-1}	0.3 mLmin^{-1}	0.4 mLmin^{-1}
2-keto-D-gluconic acid	0.31	0.33	0.33
5-keto-D-gluconic acid	0.41	0.43	0.46
D-Galactono-1,4-lactone	0.59	0.60	0.62
D-Galactose	0.28	0.30	0.32
D-Glucaric acid	0.32	0.34	0.37
D-Gluconic acid	0.30	0.32	0.34
D-Gluconic acid-δ-lactone	0.31	0.33	0.34
D-Glucose	0.27	0.29	0.32
D-Glucuronic acid	0.22	0.24	0.24
Glycolic acid	0.58	0.61	0.62
L-(+)-Tartaric acid	0.64	0.65	0.67
Oxalic acid	0.31	0.33	0.35

0.3 $mLmin^{-1}$ was chosen as the flow rate as a compromise between the slightly better resolution and k values of 0.2 $mLmin^{-1}$ and the slightly shorter run time of 0.4 $mLmin^{-1}$. A higher flow rate of the mobile phase would also cause a reduction in the number of theoretical plates (**Section 1.5** (c)- (f)). The elected flow rate of 0.3 $mLmin^{-1}$ could easily be replaced by a faster or slower rate, depending on circumstances such as time factors or in the case of molecules co-eluting.

3.2.4 Optimum Detection Mode

It was proposed that UV be the mode of detection for the determination of organic acids. Results were obtained using a diode array detector, but three wavelengths were investigated for cases where a fixed wavelength detector is necessary. The three wavelengths were 210 nm, 220 nm and 240 nm.

Table 9. Optimum wavelength for detection in the UV on the Prevail™ OA column.

	Peak Height (μV)[a]		
Standard	210.0 nm	220.0 nm	240.0 nm
2-keto-D-gluconic acid	4.68E+05	4.50E+05	9.44E+04
5-keto-D-gluconic acid	1.01E+05	4.99E+04	4.83E+03
D-Galactono-1,4-lactone	2.99E+05	3.46E+05	6.38E+04
D-Galactose	4.00E+03	1.89E+03	7.52E+02
D-Glucaric acid	7.09E+05	5.34E+05	6.06E+04
D-Gluconic acid	4.52E+05	3.19E+05	2.99E+04
D-Gluconic acid-δ-lactone	4.79E+05	3.39E+05	3.24E+04
D-Glucose	2.60E+03	1.50E+03	5.98E+02
D-Glucuronic acid	3.39E+05	2.45E+05	3.42E+04
Glycolic acid	5.32E+05	2.93E+05	1.67E+04
L-(+)-Tartaric acid	1.26E+06	1.01E+06	7.34E+04
$NaNO_3$	1.82E+03	9.73E+02	4.12E+02
Oxalic acid	2.96E+06	2.54E+06	4.42E+05
Sodium bicarbonate	6.77E+03	5.54E+03	4.64E+03

[a] It should be noted that the peak heights of different standards are not directly comparable to each other as concentrations differ, while still falling withing the range of 5-10 $mgmL^{-1}$; however, the concentration of each standard is the same for the three different wavelengths analysed for each standard.

(**Table 9**) shows that peak height (in μV) increased with decreasing wavelength. Detection at 210 nm generated the tallest peaks for every standard, while peak height fell sharply when switching from 220 nm to 240 nm. At 210 nm, compared with 220 nm, peaks shapes were more likely to be slightly more irregularly shaped (**Section 1.5 (h)**), with minimally better resolution (**Appendix 8**). Therefore 220 nm was determined to be the best wavelength for detection with a single wavelength.

Peaks were also analyzed with a refractive index (RI) detector to draw a comparison between the different detectors. RI gave a much smoother baseline and peaks were more inclined to be symmetrical than when detection was with the UV detector. Unfortunately, with RI, negative solvent peaks were often present, interfering with peak analysis. In addition, UV analysis is a more sensitive technique than RI and is often more convenient, lacking a reference cell and therefore eliminating the need to flush and keep it clean. These factors helped influence the choice of detector.

3.2.5 Testing of Parameters

Once the parameters were established, a solution containing each of the 12 standard compounds was injected on to the Prevail™ OA column, and eluted at 0.3 $mLmin^{-1}$ in 10 mM KH_2PO_4, adjusted to pH 2.1 with phosphoric acid. The resulting chromatogram yielded only 6 distinct peaks (**Appendix 9**), one of which is uric acid, implying that the method parameters do not give adequate resolution for the analyses intended.

3.3 Quality Assurance for Methods

i) Aminex HPX-87H⁺ Column Method

For quality assurance of the Aminex HPX-87H⁺ column method, a reference standard was used as supplied by the column manufacturer, and was stored according to manufacturer specifications. The reference standard was injected regularly between

analyses to monitor column degradation (if any).

(**Table 10**) shows that there were no significant differences between retention times of peaks in the reference standard throughout the Aminex HPX-87H$^+$ column analyses, as all retention times fell within three standard deviations of the estimated mean for each standard. Peak asymmetry was also monitored, and found to be maintained throughout the time of analysis (**Appendix 10**).

Table 10. Retention times in minutes, of organic acid peaks in standard test mixture for Aminex HPX-87H$^+$column method.

	Standard Compounds					
Date Injected	Oxalate	Citrate	Malate	Succinate	Formate	Acetate
14/06/2005	14.356	16.530	18.768	22.319	27.142	34.698
25/07/2005	14.344	16.357	18.665	22.222	27.062	34.688
01/08/2005	14.401	16.476	18.715	22.272	27.099	34.738
18/08/2005	14.389	16.550	18.747	22.317	27.179	34.837
19/09/2005	14.293	16.700	18.675	22.258	27.124	34.779
25/10/2005	14.219	16.313	18.773	22.414	27.285	34.846
22/11/2005	14.189	16.483	18.905	22.591	27.496	34.990
Mean	14.313	16.487	18.750	22.342	27.198	34.797
STDev (σ)	0.076	0.119	0.075	0.116	0.138	0.098

ii) Prevail™ OA Column Method

A reference standard specific to the Prevail™ OA column (*114*) was made up and injected regularly to ascertain whether the column was being degraded due to the parameters and / or the samples being run.

This standard was kept shielded from light and at room temperature. It was injected at the beginning, halfway through, and at the end of each set of parameters tested.

Peaks in the standard injection mixture were checked for symmetry, and each set of

three injections were compared for signs of column degradation.

(**Table 11**) shows that the retention times of the acids in the specific standard did not alter significantly during analyses, thus column integrity was maintained. (**Appendix 10**) shows peak asymmetry values for each peak. Asymmetry data supports the retention time data, by indicating there was very little or no change in the test chromatograms. All retention times fall within 2 standard deviations of the mean.

Table 11. Retention times in minutes, for organic acid peaks in standard test mixture for Prevail™ OA column method at 0.3 $mLmin^{-1}$.

		Standard compounds			
Parameters	Place in run	$LiNO_3$	Formic	Malic	Lactic
26.5 mM/pH 2.5	Beginning	5.753	8.398	10.635	12.381
	Middle	5.753	8.401	10.634	12.371
	End	5.682	8.320	10.586	12.310
	Mean	5.729	8.373	10.618	12.354
	STDev (σ)	0.033	0.037	0.023	0.031
10mM/pH 2.5	Beginning	5.654	7.443	9.360	11.096
	Middle	5.663	7.422	9.341	11.123
	End	5.641	7.463	9.375	11.085
	Mean	5.653	7.443	9.359	11.101
	STDev (σ)	0.009	0.017	0.014	0.016
10mM/pH 2.1	Beginning	5.792	7.560	9.776	11.319
	Middle	5.783	7.547	9.749	11.294
	End	5.795	7.553	9.740	11.286
	Mean	5.790	7.553	9.755	11.300
	STDev (σ)	0.005	0.005	0.015	0.014
10mM/pH 3.0	Beginning	5.577	7.278	8.822	10.697
	Middle	5.586	7.300	8.908	10.754
	End	5.596	7.308	8.929	10.760
	Mean	5.586	7.295	8.886	10.737
	STDev (σ)	0.008	0.013	0.046	0.028

4. Final Discussion and Conclusion

4.1 Evaluation and Summary of Findings

The goal during this research project was to utilise an existing HPLC method for qualitative and quantitative analysis of the products of the nitric acid oxidation of D-glucose to D-glucaric acid, to develop an alternative, faster HPLC method for the same analysis, and to compare the two methods.

i) Aminex HPX-87H$^+$ Column Method

The research conducted on the Aminex HPX-87H$^+$ column gave the desired results for separation of the products and by-products of the nitric acid oxidation reaction investigated. The method proved to be appropriate for the analysis of the products in the reaction mixtures, as well as providing a suitable tool for determining the ratios of reactants to products in order to monitor the reactions.

While the method does provide acceptable results, potential barriers to it becoming a routine method of analysis remain. Setting up of the Aminex HPX-87H$^+$ column method and running samples on it are both time consuming procedures. Equilibration of the columns in the mobile phase at the correct temperatures proved to take up to 3 hours each time the set-up was run. This is unacceptable if the process must be undertaken regularly. If the columns are able to be equilibrated and left to run at the desired temperatures for long periods of time, it becomes less of a problem. However, the method still demands a good deal of time for each individual sample analysed; at least 33 minutes per sample is needed to ensure all products and by-products of the reaction have eluted from the column.

The production of rather low k values also persists for the Aminex HPX-87H$^+$ column method.

ii) Prevail™ OA Column Method

The desire for a fast, quantitative and yet uncomplicated analysis meant that certain useful modifications had to be waived from the Prevail™ OA column method.

Despite this, detection limits and *k* for several organic acid standards were improved upon under the new method. The new set of parameters include a mobile phase of 10mM KH_2PO_4, adjusted to a pH of 2.1 with phosphoric acid, run at a flow rate of 0.3 $mLmin^{-1}$ at ambient temperature and with a sample size of 5μL. If detection at a fixed wavelength is deemeddeems to be necessary, 220nm was determined to be the best wavelength at which to undertake the analysis.

Results are not conclusive. The Prevail™ OA column is not particularly practical for the intended purpose under the restricted conditions of isocratic elution, and as a result there was no point in attempting to utilise the column for quantitative analysis.

When run individually, the standards afforded retention times that were extremely close to one another and, as a result, when they were run as a mixture they were not adequately resolved by the column. A solution containing 12 different compounds run on the Prevail™ OA column set-up produced a chromatogram yielding only 6 distinct peaks. When the column is used either as intended (for the reaction mixture), or for the solution containing different standards, co-elution is a major problem.

Another difficulty with the Prevail™ OA column method is the preparation of samples taken from the reactor. These samples are made basic prior to injection in order to open any ring forms present, as under the acidic conditions of the nitric acid reaction the diacids tend to be in the lactone forms. This was considered to some degree in the testing of the parameters, with specific standards run after being made basic with 2M NaOH to simulate conditions. Resulting chromatograms showed many more peaks than there were compounds present in the injected sample, the baseline was significantly disrupted and the peaks tended to be much more asymmetrical than when samples were not treated with NaOH. The column specifications indicate that the Prevail™ OA column can be run at a pH of up to 7.5 (*115*), but at higher pHs there would be considerable dissociation of acids present, once again causing undesirable peaks.

If the Prevail™ OA column is to be employed for the analysis of the reactions of the nitric acid oxidation of D-glucose to D-glucaric acid, the method would require further modification, because the parameters outlined previously (**2.1.1.1** (e) *ii)*) do not give sufficient resolution of the products and by-products.

The retention times of the standards on the Prevail™ OA column are much shorter than those on the Aminex HPX-87H$^+$ column, which resulted in a suitably shorter analysis time. However, the standards run on the Prevail™ OA column produced peaks that overlapped significantly between standards, resulting in extremely poor and in some cases nonexistent, resolution. Consequently it was decided that the Aminex HPX-87H$^+$ column is the superior option for the analyses specified.

Sample preparation for the HPLC methods is more time-efficient than sample preparation for the corresponding GC-MS method, which involves extensive sample handling and requires derivatisation of non-volatile compounds by silylation.

While there would be some further work left to do if either of these two HPLC methods were to be applied to the analysis of the nitric acid oxidation of D-glucose to D-glucaric acid, in general they are more efficient than the GC-MS method previously employed, and with the added advantage of the Aminex HPX-87H$^+$ column also giving quantitative results, whereas GC-MS does not, unless an internal standard is used.

4.2 Recommendations for Further Work

i) *Aminex HPX-87H$^+$ Column Method*

Glycolic acid was omitted from the Aminex HPX-87H$^+$ column analyses because it was not yet recognised as a by-product of the reaction. It therefore needs to be injected under the conditions specified (**Section 2.1.1.1** (e) *i)*) to determine its retention time in the case of future analyses.

It remains to be determined whether or not column degradation occurs if the columns

are run continuously for extended periods of time at the specified temperatures. During the course of the research contained within this thesis, the columns were regularly stored at room temperature without eluent flow when they were not in use. This meant they were not under high pressures and remained at ambient temperature intermittently over the time period. If the columns can remain stable at the required running temperatures and pressure over long periods of time, this would limit equilibration time, making the method much more favourable.

If the D-glucose to D-glucaric acid process were to be made industrially viable, it might be appropriate to determine the limits of detection of the Aminex HPX-87H$^+$ method in order to give more precise by-product / impurity levels in the final reaction mixtures.

The reason behind the skewed, fronted shape of the tartronic acid peak could be further investigated, as it has been identified as a by-product of the oxidation reaction. This is arguably an unnecessary step as the peak shape may actually have a negligible effect on the outcome of analyses.

The k values of individual standard peaks are much lower than ideal, a problem which could potentially be rectified with the use of gradient elution. Increasing the sulfuric acid concentration of the eluent could be a possible change to the method, as research indicates that doing so would increase retention times of some molecules present (**Section 3.1.2**). This would make the time interval between the void volume and peaks of interest larger, which in turn could have an effect on the oxalic acid peak shape if it is caused by its elution too close to the void volume time.

ii) *Prevail™ OA Column Method*

Tartronic acid was omitted from the Prevail™ OA column analyses, due to its unavailability at the time the research was carried out. For future research with the Prevail™ OA column on this project, it will be necessary to subject a sample of it to the Prevail™ OA column method in order to ascertain its behavior and retention

time.

There are other adjustments which could be made to the method to make the column more suitable for the analysis required. While the original method outlined in the literature accompanying the column (*114*) has been developed to give better results for the analysis required, the limitation of isocratic elution results in inadequate separation of the mixture of standards. This issue needs to be addressed.

A gradient separation yields a higher peak capacity than an isocratic separation, and refocuses a peak, generating sharper peaks and allowing for better detection (*114*). Using a gradient in the place of isocratic elution would complicate the method, which was required to be kept as simple as possible. The re-equilibration of the column in the original solvent of the gradient, however, would be time-consuming and would be necessary after every injection, thus affecting turnaround time. If the analysis were refined to give adequate baseline resolution with gradient elution, the flow rate could be accelerated to compensate for any extra equilibration time taken. This requires consideration and needs to be explored further before the Prevail™ OA column is dismissed as being unsuitable for the required analyses.

The effects of adding an organic modifier to the mobile phase could be explored to ascertain improved resolution. Improvements for peak resolution have been demonstrated using methanol as an organic modifier in a KH_2PO_4 mobile phase on a RP column. Significant reduction of retention times was also noted with use of the organic modifier (*92*).

One of the main goals for the Prevail™ OA column is its use in an industrial setting to give real-time estimates of how reactions are proceeding. Running actual samples from the reactions on the Prevail™ OA column would be a significant step in determining how effective it is for this purpose. The Prevail™ OA column method was developed in a laboratory based at the University of Waikato, in New Zealand, whereas the nitric acid oxidations of D-glucose reactions were carried out at the

Shafizadeh Center at the University of Montana in America. Samples were therefore not available for analyses on the Prevail™ OA column, and as a consequence, this was out of the scope of the research presented.

The Prevail™ OA column stationary phase is also available with dimensions 250 x 4.6 mm, 100 mm longer than the column used in this research. A longer stationary phase results in more theoretical plates, and thus better resolution. Literature has shown better separation by 0.11 minutes between oxalic and tartaric acid at flow rates of 1.0 $mLmin^{-1}$ using the longer column, and involved using water adjusted to pH 2.5 with formic acid as the eluent (*114*). Trial of this longer column could afford better resolution and could be further investigated.

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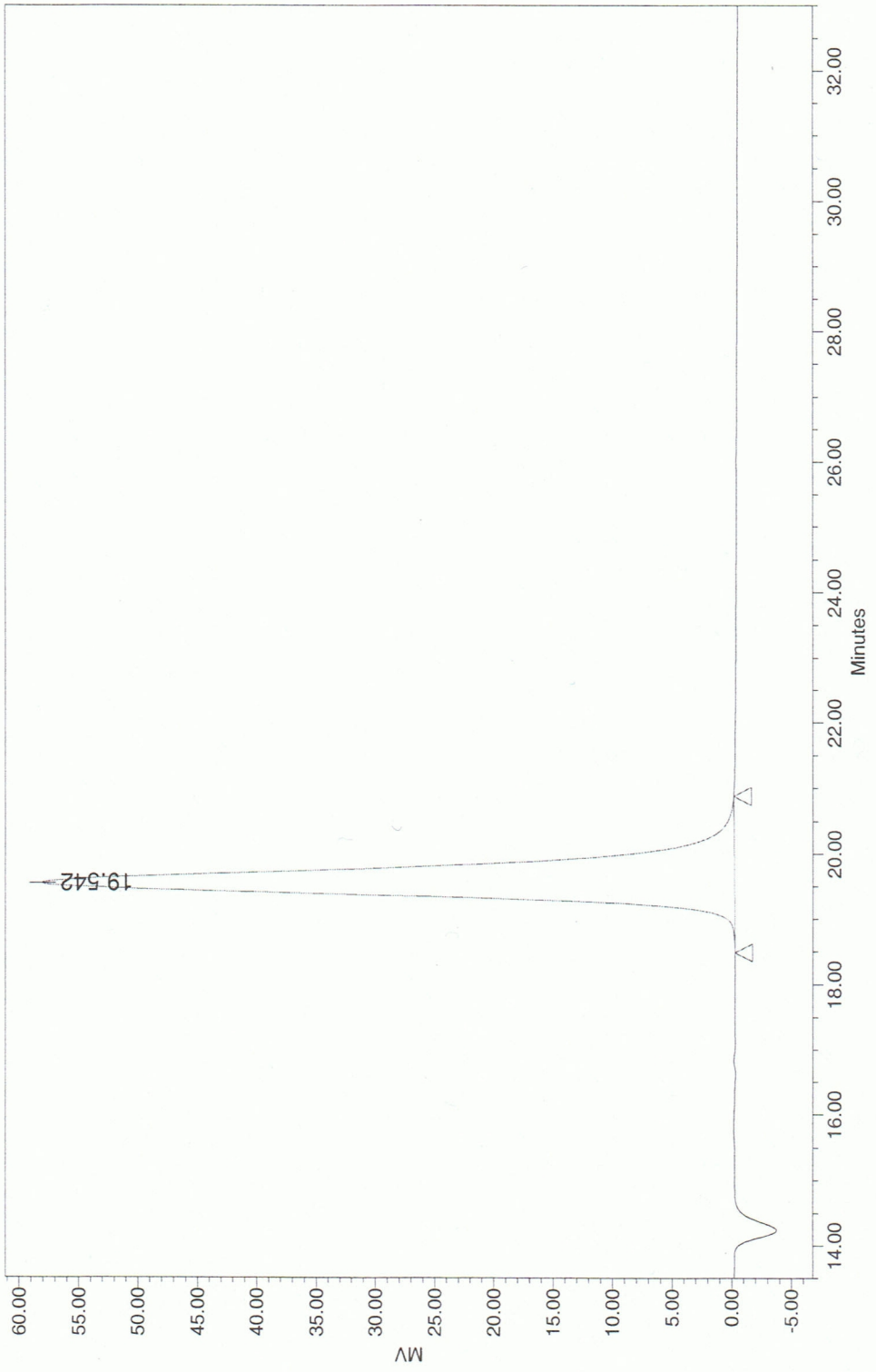
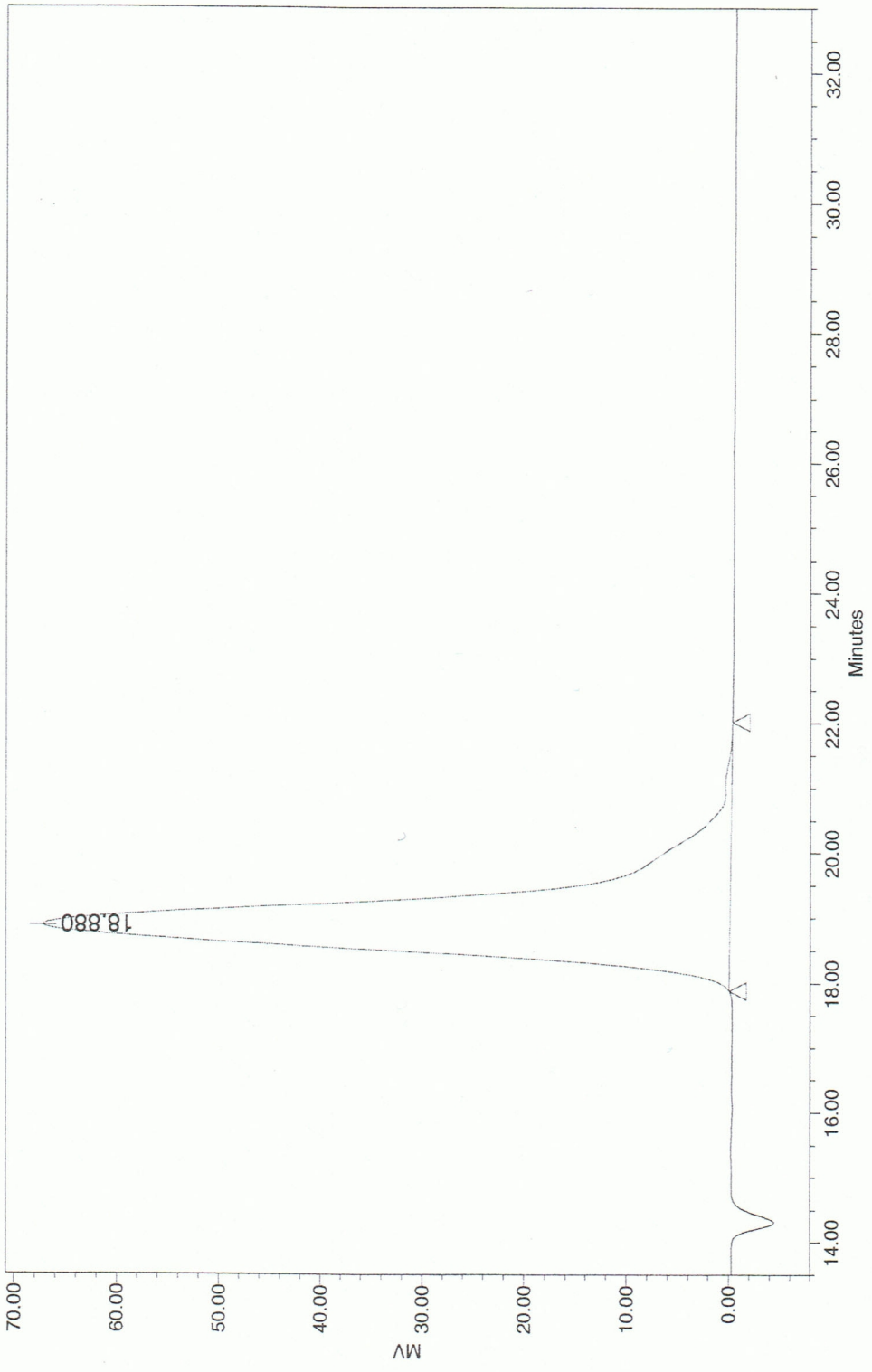
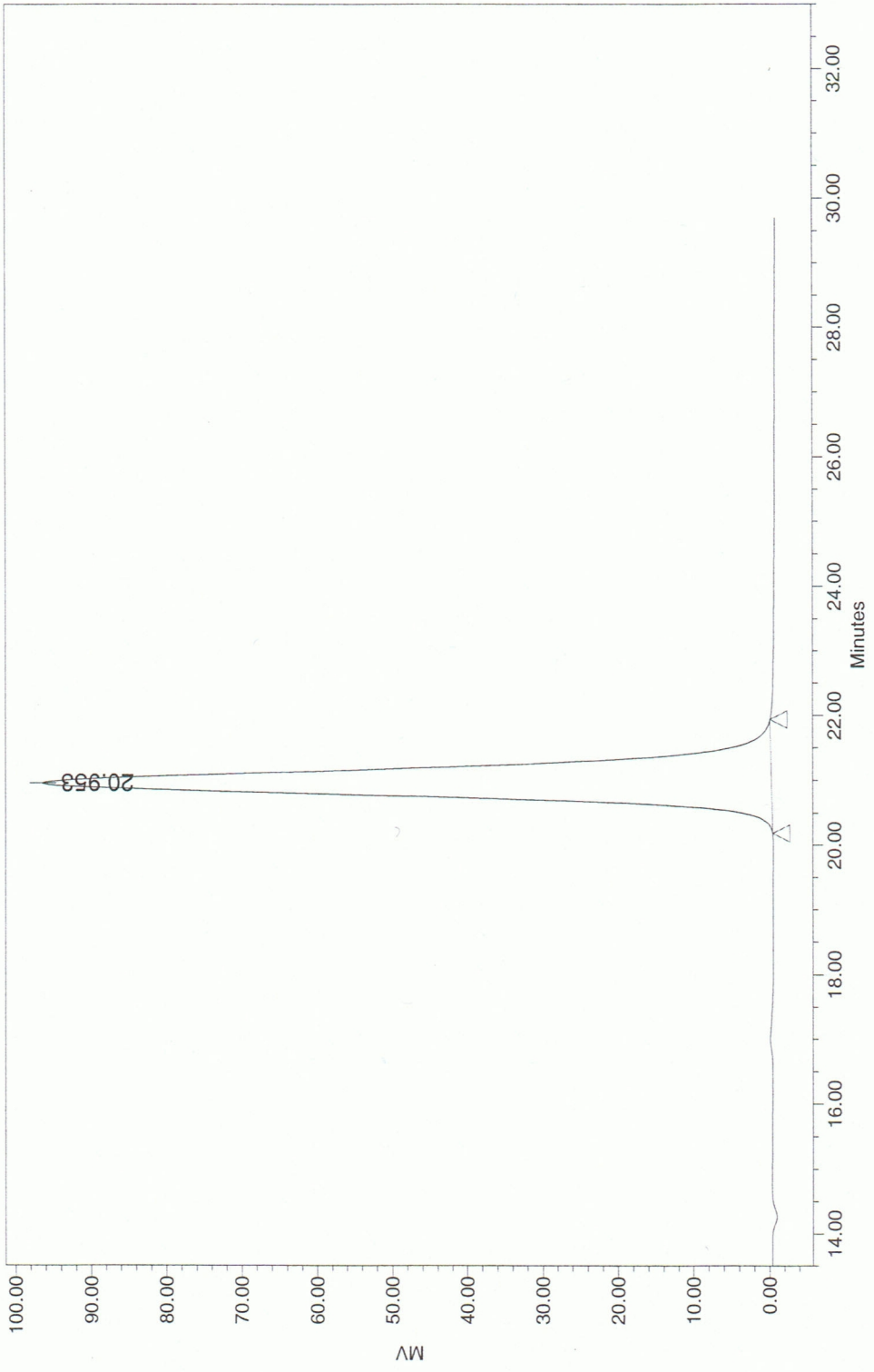
Appendix 1.1 5-keto-D-gluconic acid standard for Aminex HPX-87H$^+$ column.



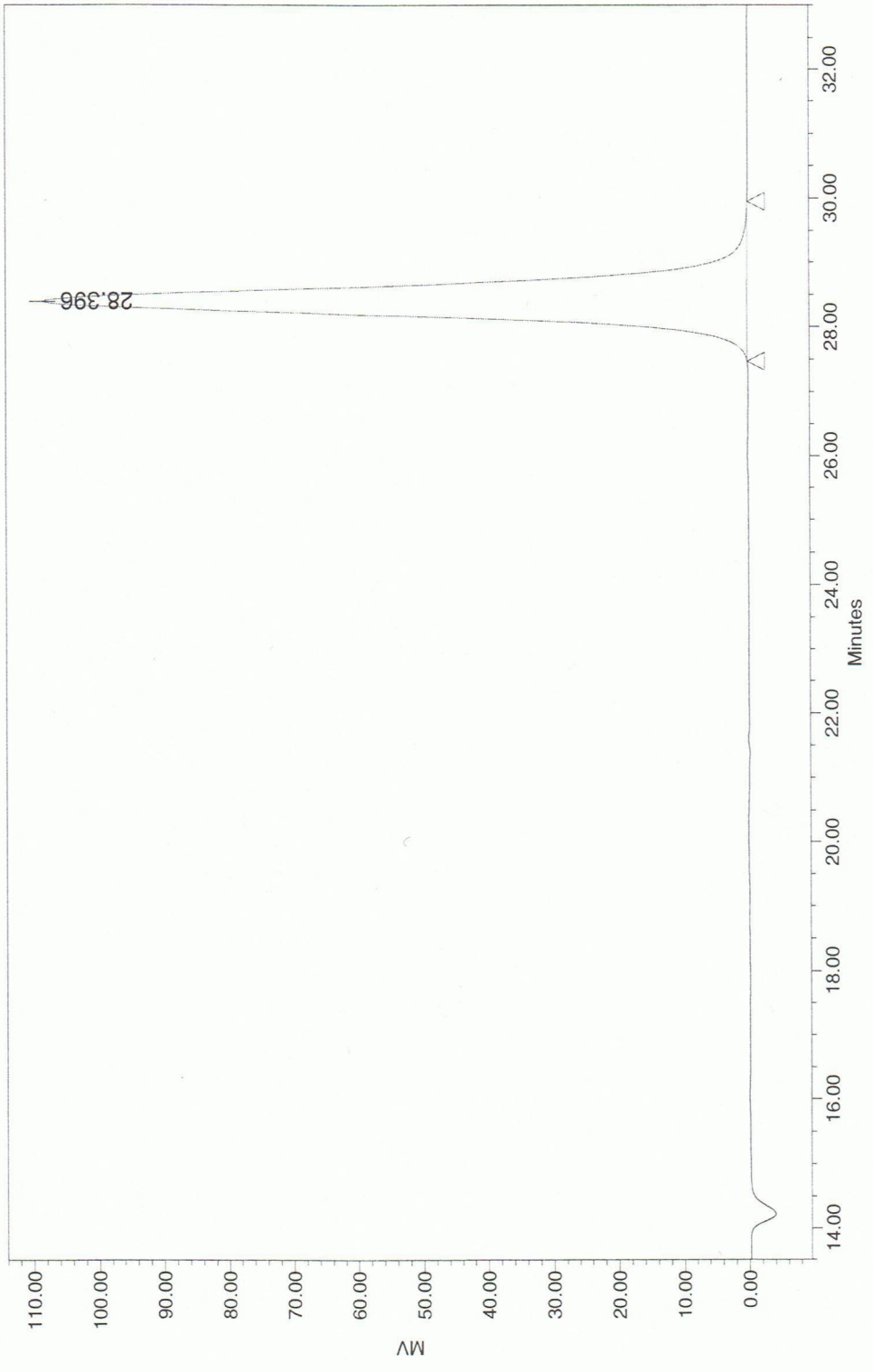
Appendix 1.2 D-Glucaric acid standard for Aminex HPX-87H$^-$ column.



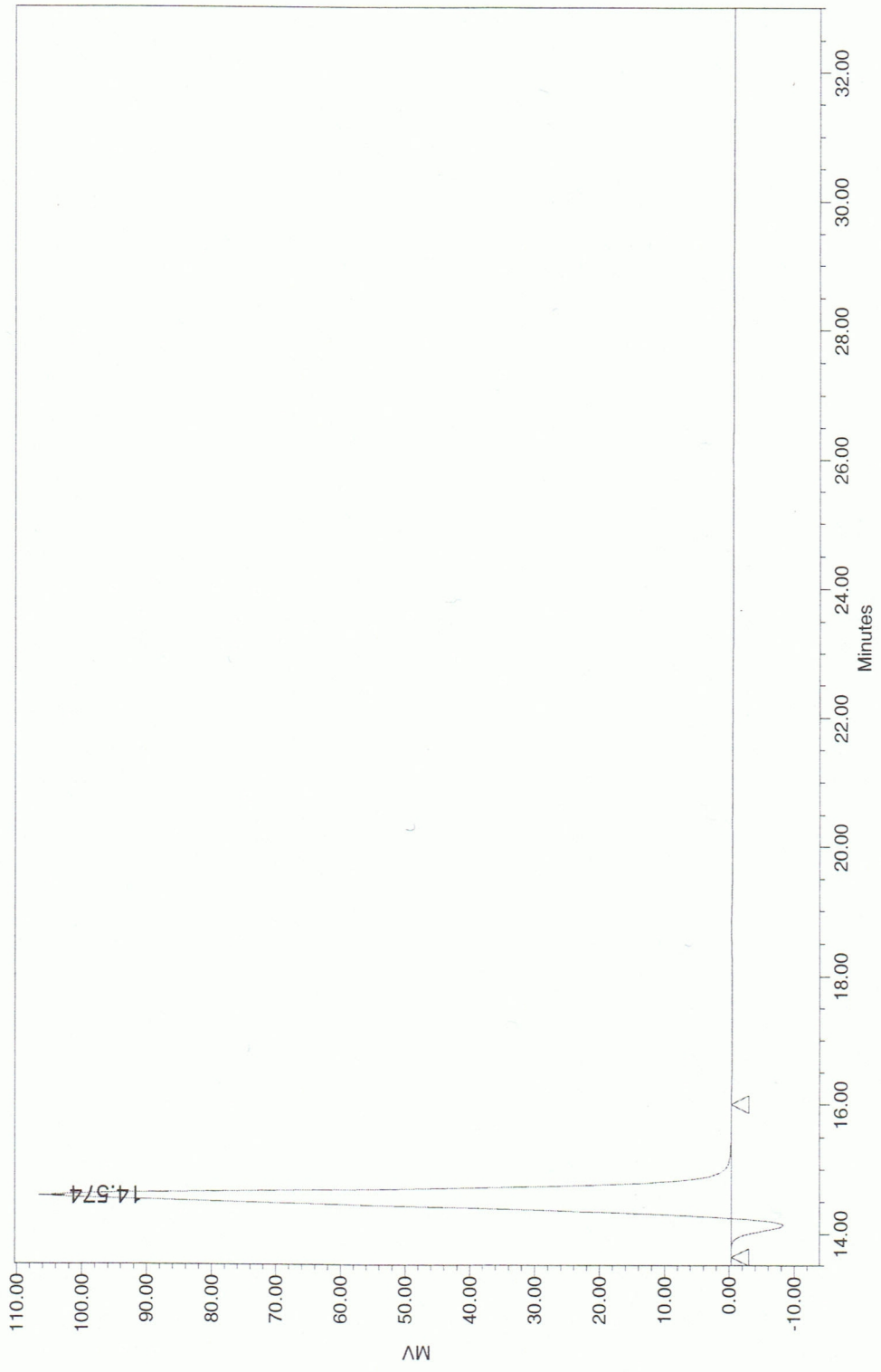
Appendix 1.3 D-Glucose standard for Aminex HPX-87H$^+$ column.



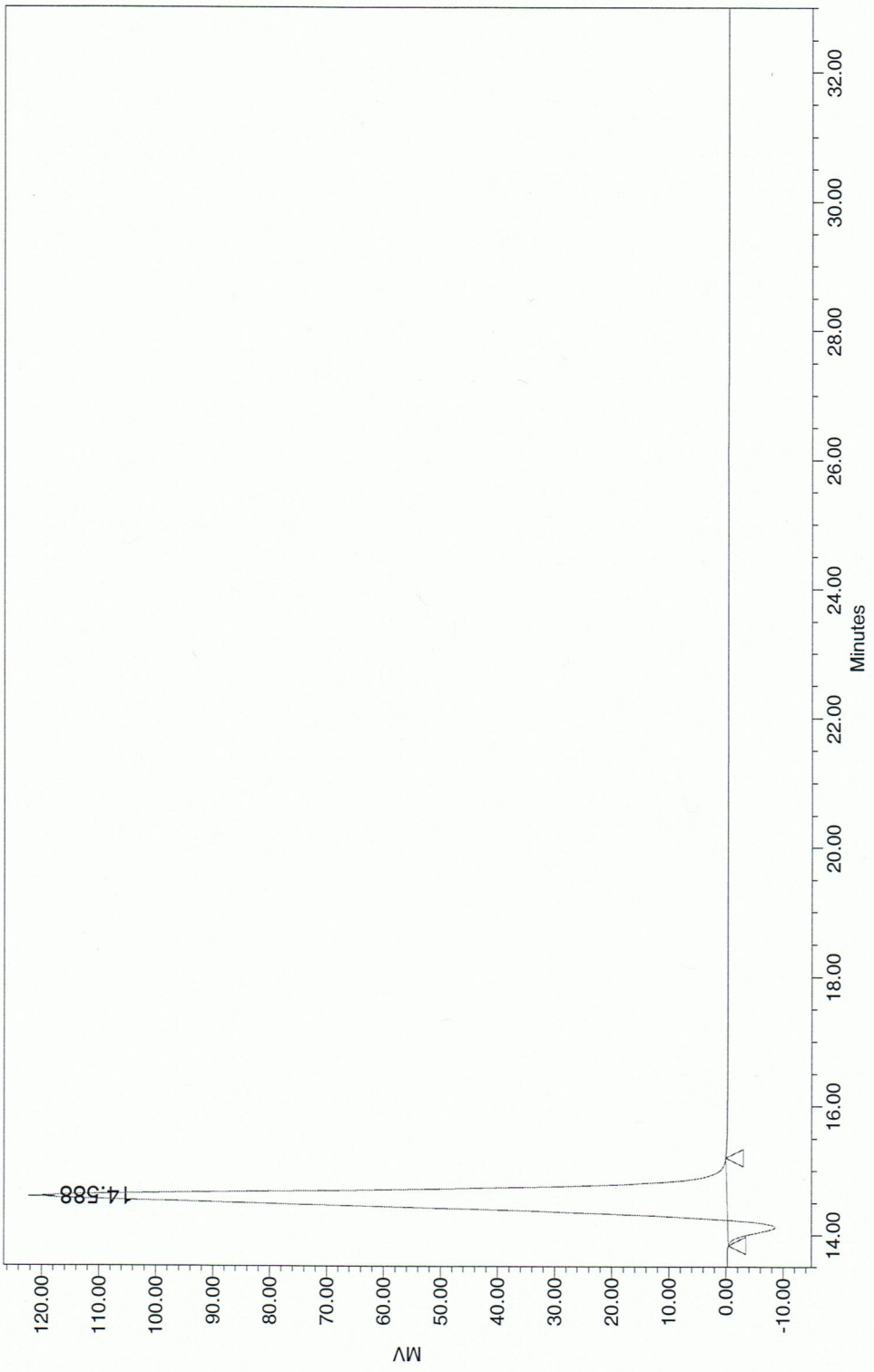
Appendix 1.4 Glycolic acid standard for Aminex HPX-87H$^+$ column.



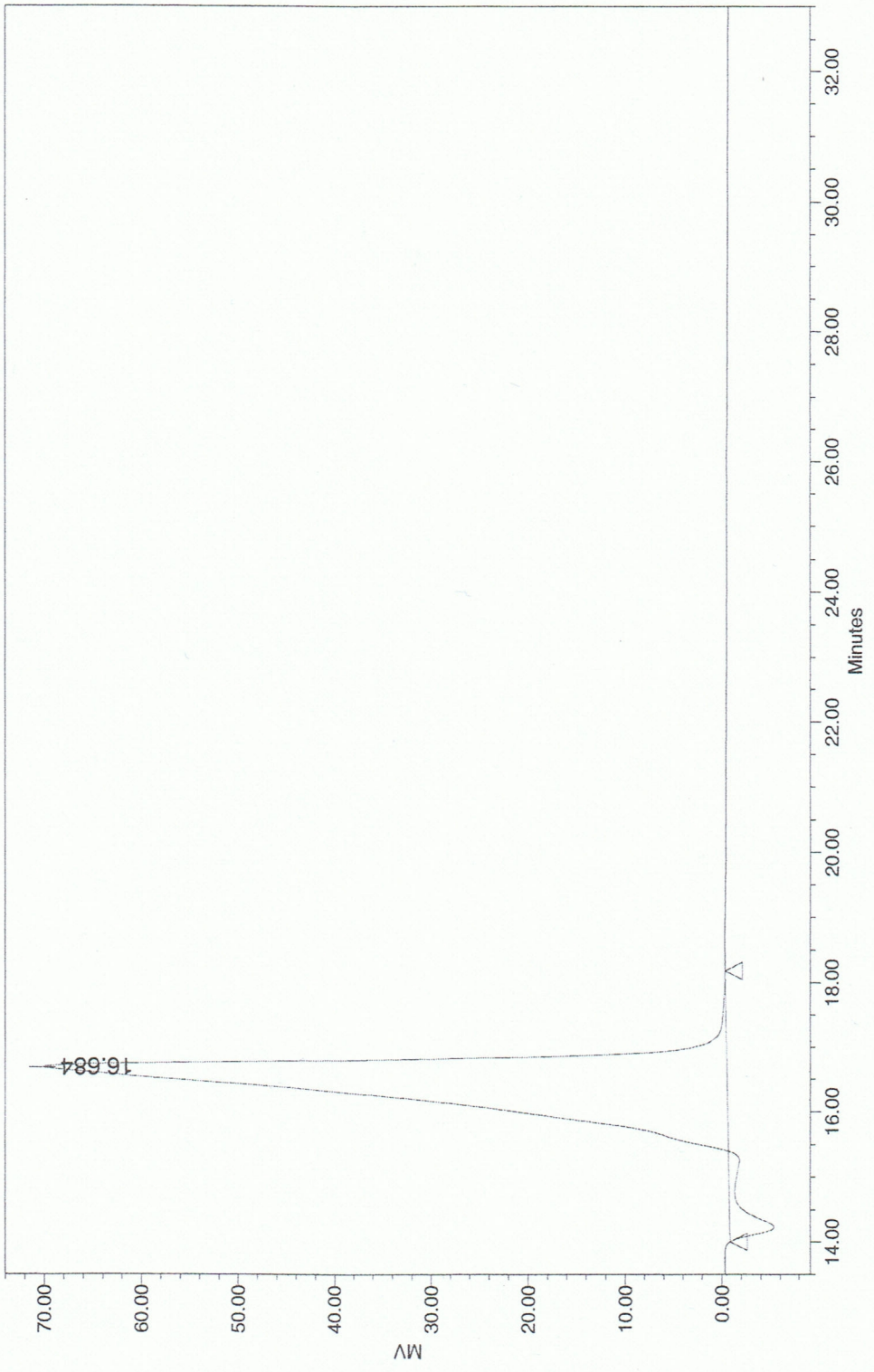
Appendix 1.5 KNO_3 standard for Aminex HPX-87H$^+$ column.



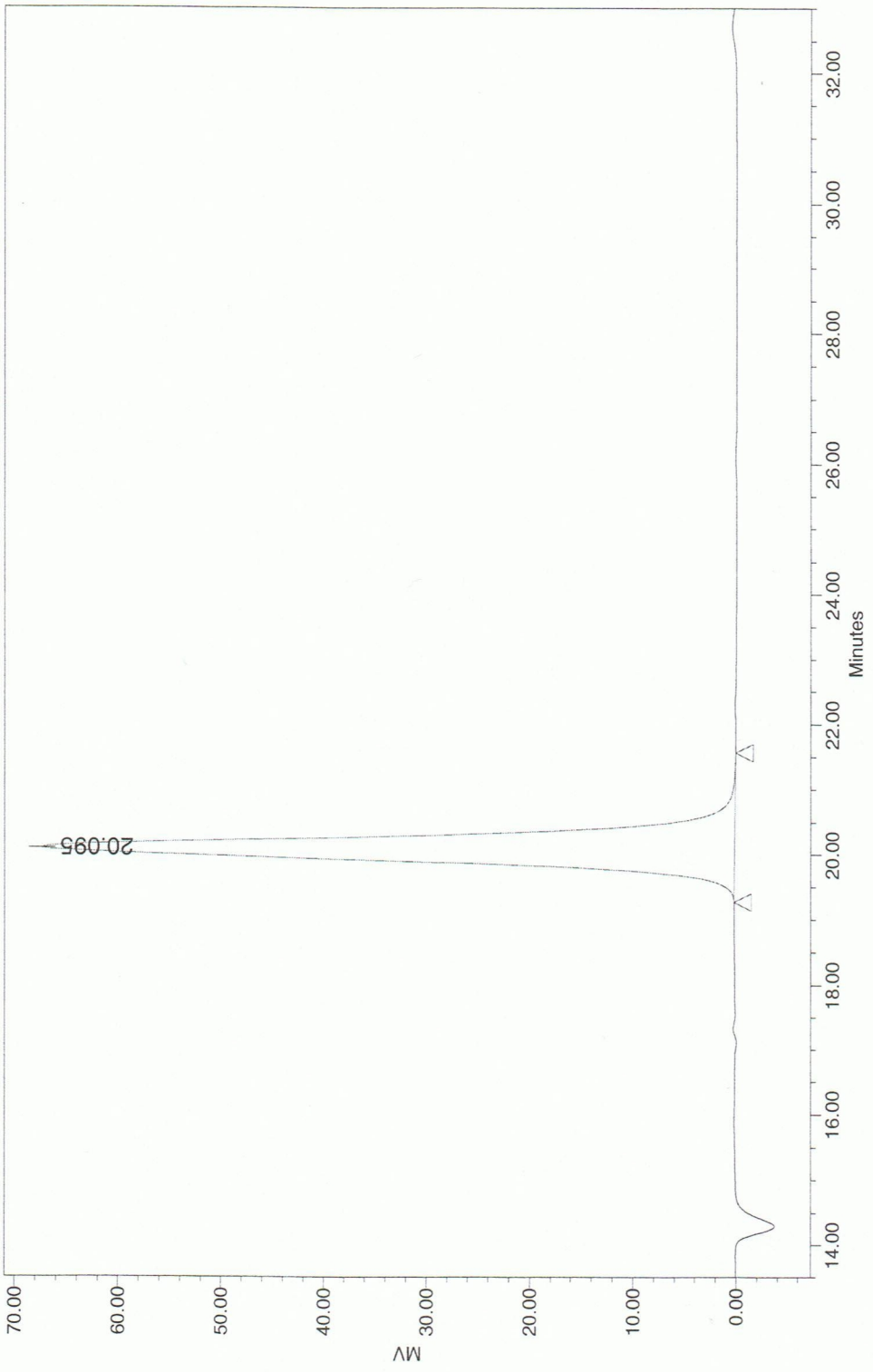
Appendix 1.6 $NaNO_3$ standard for Aminex HPX-87H$^+$ column.



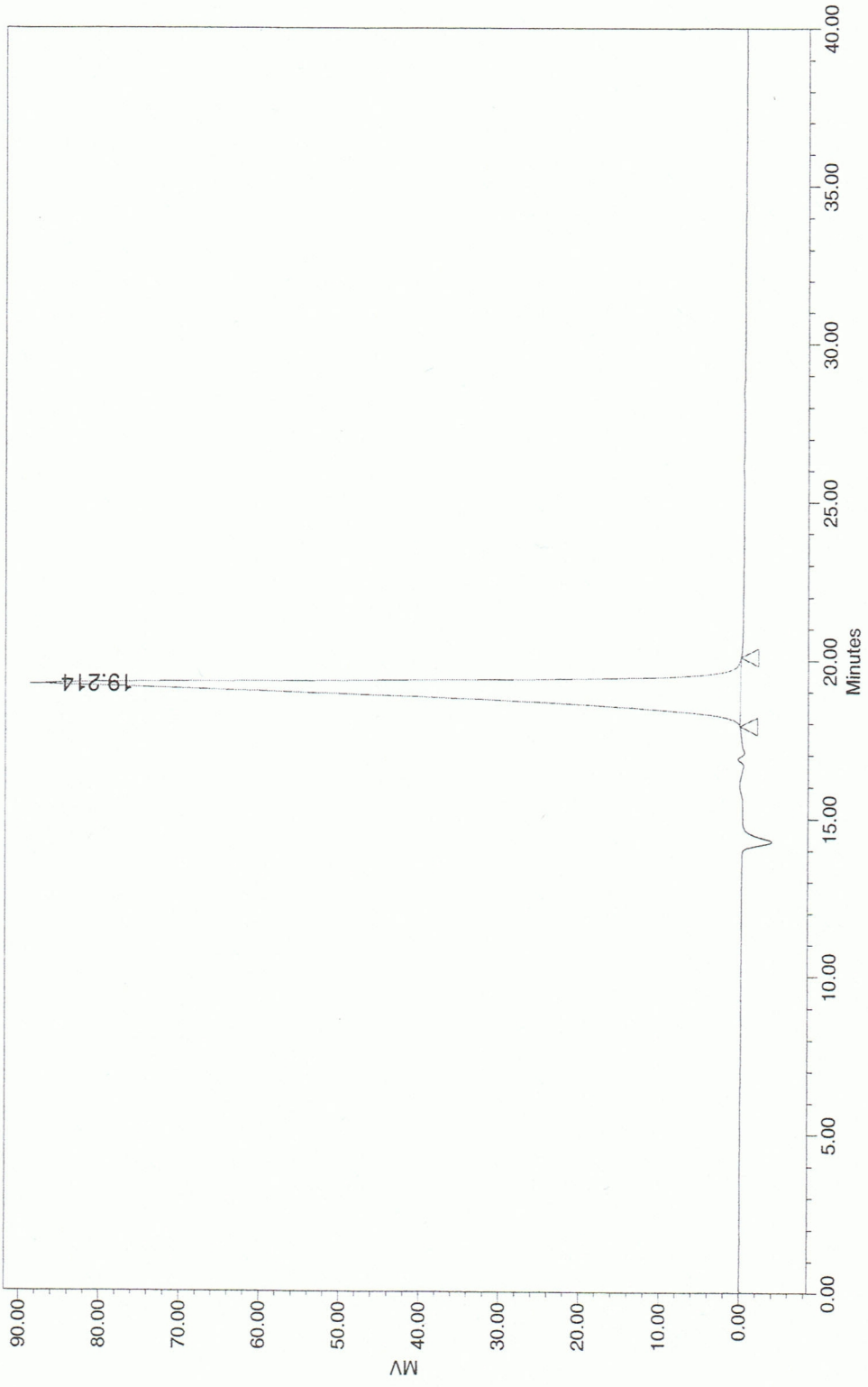
Appendix 1.7 Oxalic acid standard for Aminex HPX-87H+ column.



Appendix 1.8 L-(+)-Tartaric acid standard for Aminex HPX-87H$^+$ column.



Appendix 1.9 Tartronic acid standard for Aminex HPX-87H+ column.



Appendix 2.1 Triplicate standard retention times of Aminex HPX-87H$^+$ column standards and their estimated standard deviations.

Standard	Retention Times (min)	Mean and STDev	Mean ± 2σ	Do RTs fall within range?
KNO_3	14.571 14.572 14.579	14.574 0.004	14.567 - 14.581	Y
$NaNO_3$	14.588 14.601 14.593	14.594 0.005	14.583 - 14.605	Y
Oxalic	16.364 16.349 16.389	16.367 0.016	16.334 - 16.400	Y
Glucaric	18.825 18.824 18.836	18.828 0.005	18.817 - 18.839	Y
2-Keto-D-gluconic	18.977 18.967 18.975	18.973 0.004	18.964 - 18.982	Y
Tartronic	19.207 19.217 19.214	19.213 0.004	19.204 - 19.221	Y
Xylaric	19.288 19.288 19.299	19.292 0.005	19.281 - 19.302	Y
Glucuronic	19.578 19.591 19.589	19.586 0.006	19.575 - 19.597	Y
5-Keto-D-gluconic	19.662 19.654 19.630	19.649 0.014	19.621 - 19.676	Y

table continued overleaf

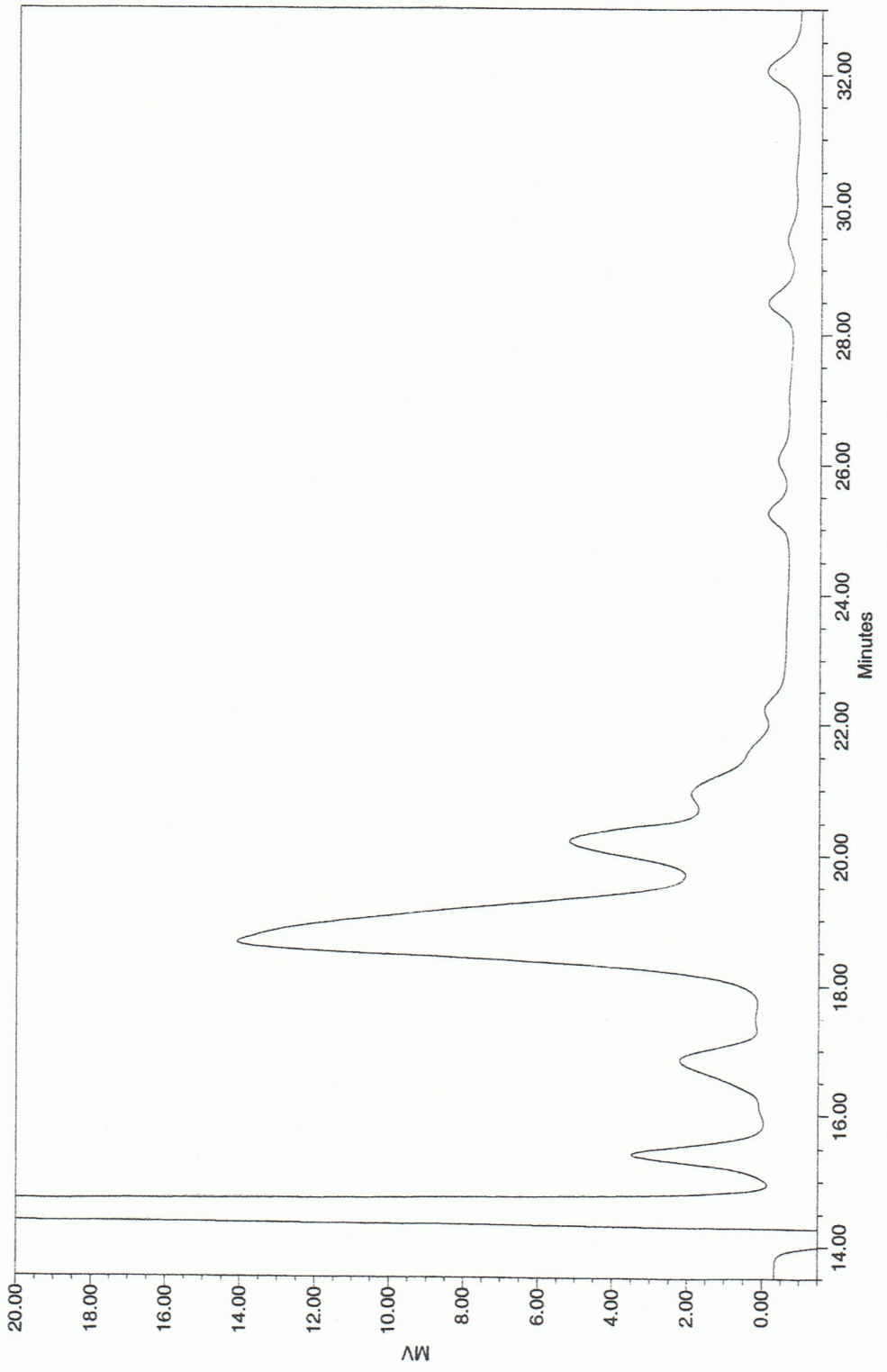
Appendix 2.1 *continued...*

Standard	Retention Times (min)	Mean and STDev	Mean ± 2σ	Do RTs fall within range?
1,4-lactone	20.032 20.051 20.052	20.045 0.009	20.027 - 20.063	Y
Tartaric acid	20.086 20.073 20.125	20.095 0.022	20.050 - 20.139	Y
6,3-lactone	20.256 20.244 20.261	20.254 0.007	20.239 - 20.268	Y
Glucose	20.967 20.953 20.945	20.955 0.009	20.937 - 20.973	Y
Gluconic	20.964 20.980 20.977	20.974 0.007	20.960 - 20.988	Y
Mannaric	21.632 21.645 21.619	21.632 0.011	21.611 - 21.653	Y
Mannose	22.241 22.261 22.242	22.248 0.009	22.230 - 22.266	Y
Xylose	22.401 22.394 22.441	22.412 0.021	22.371 - 22.453	Y

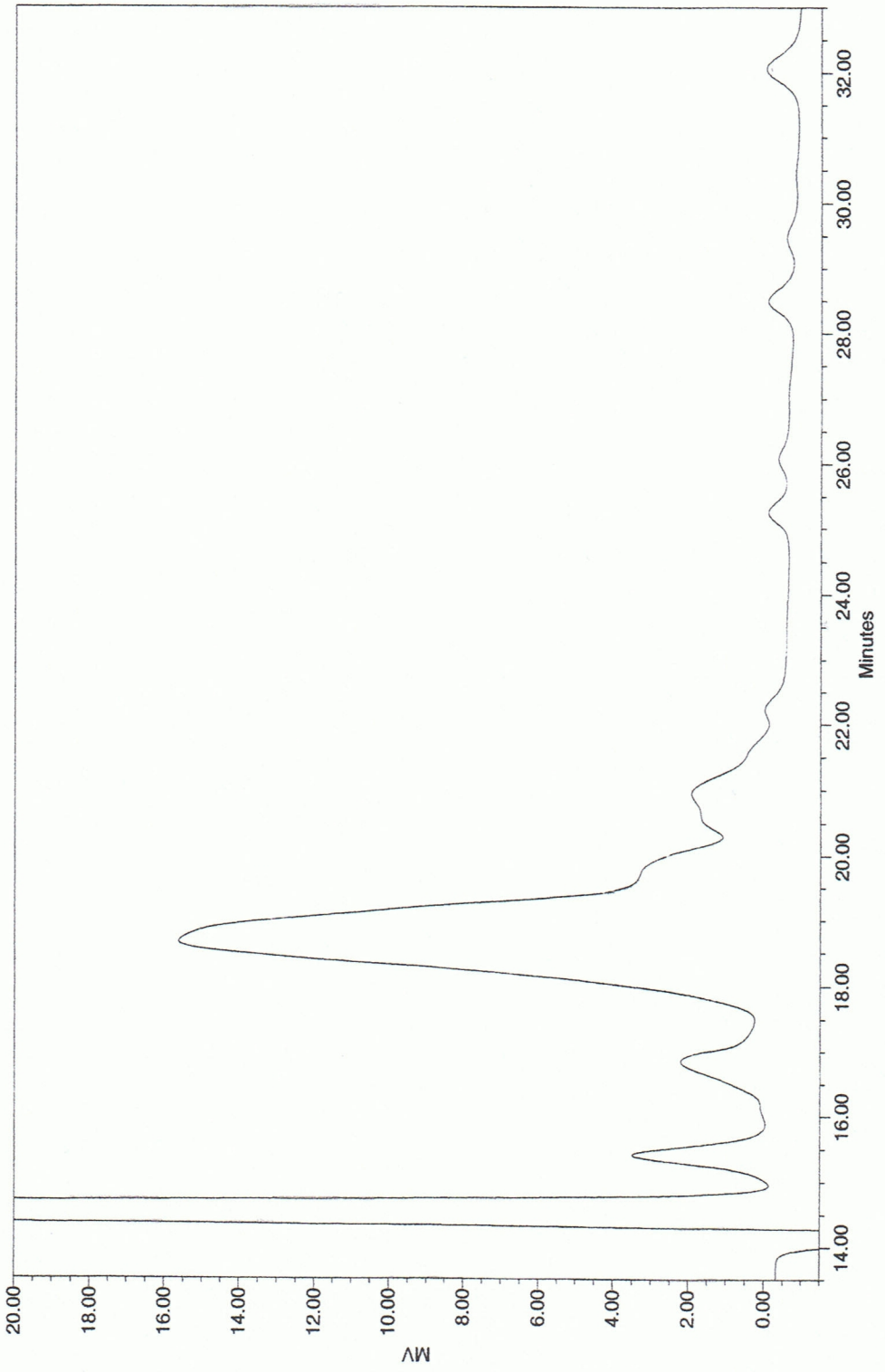
Appendix 3.1 *k* Values of Aminex HPX-87H$^+$ column standards.

Standard	t_0	t_R	$k = t_R - t_0/t_0$
Oxalic	13.698	16.367	0.195
Glucaric	13.685	18.828	0.376
2-Ketogluconic	13.656	18.973	0.389
Tartronic	13.724	19.213	0.400
Xylaric	13.788	19.292	0.399
Glucuronic	13.752	19.586	0.424
5-Ketogluconic	13.652	19.649	0.439
1,4-lactone	13.602	20.045	0.474
Tartaric	13.645	20.095	0.473
6,3-lactone	13.722	20.254	0.476
D-Glucose	13.628	20.955	0.538
Gluconic	13.617	20.974	0.540
Glycolic	13.753	28.396	1.065

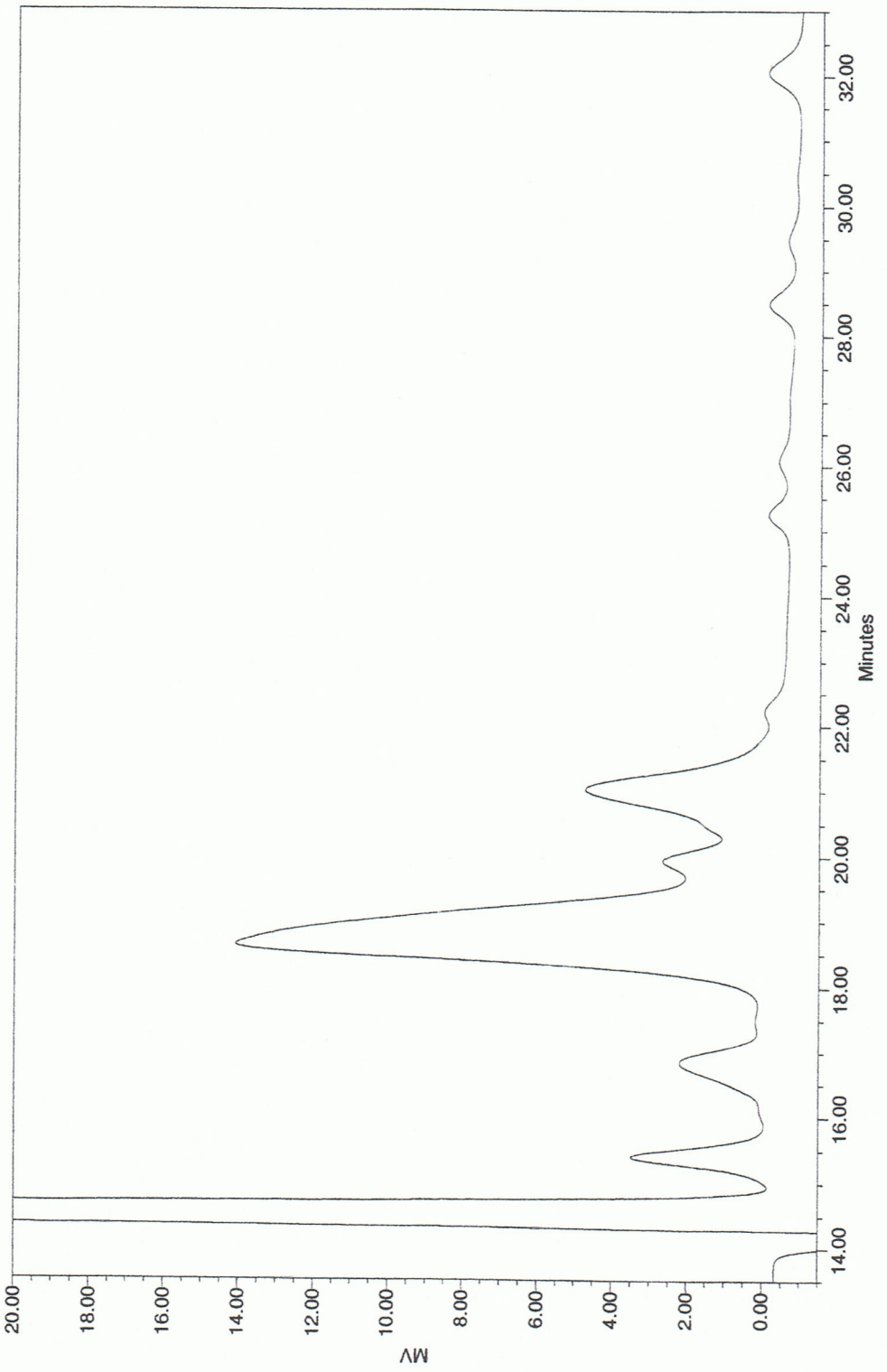
Appendix 4.1 Final reaction mixture spiked with 5-keto-D-gluconic acid.



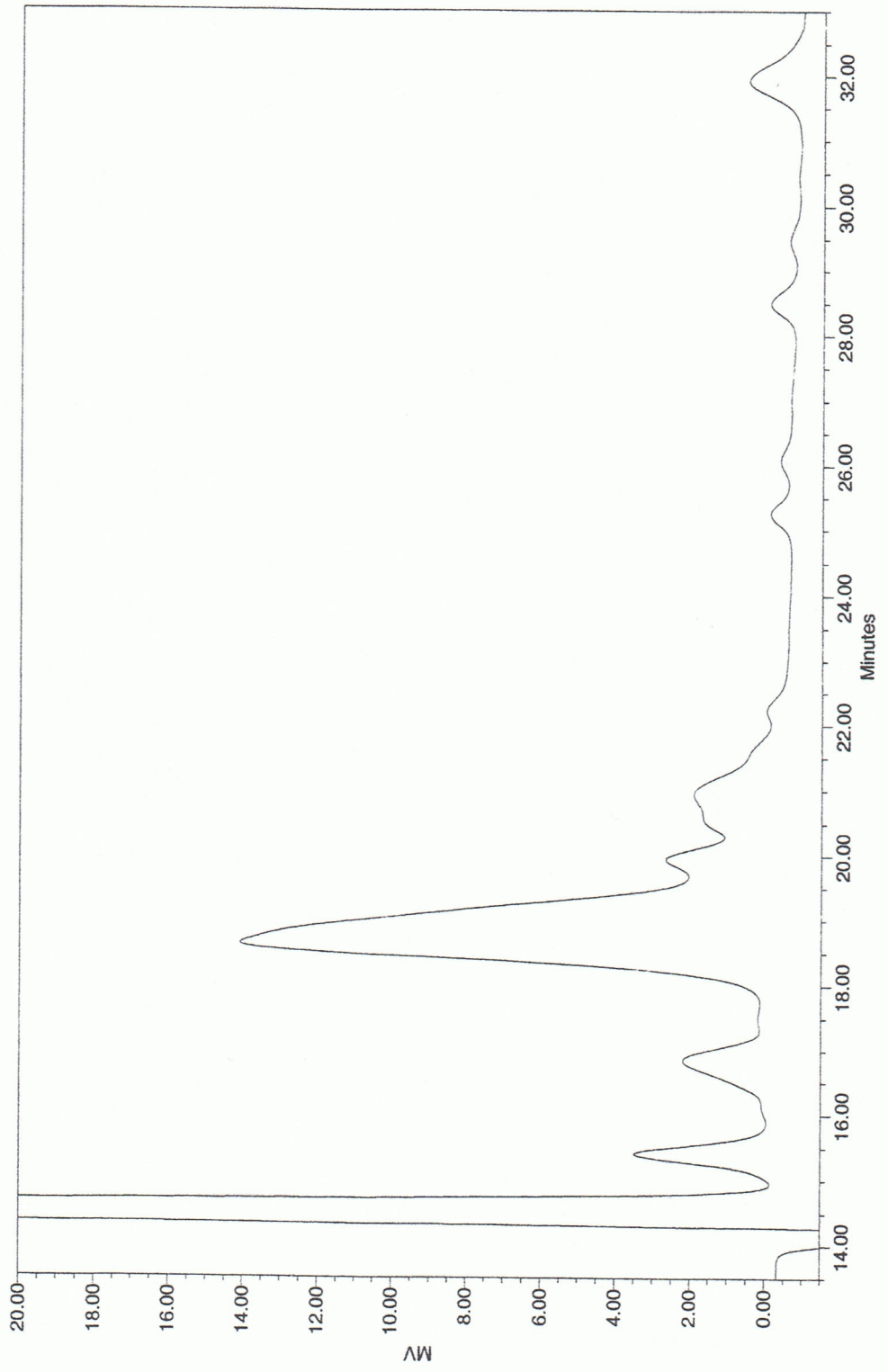
Appendix 4.2 Final reaction mixture spiked with D-glucaric acid.



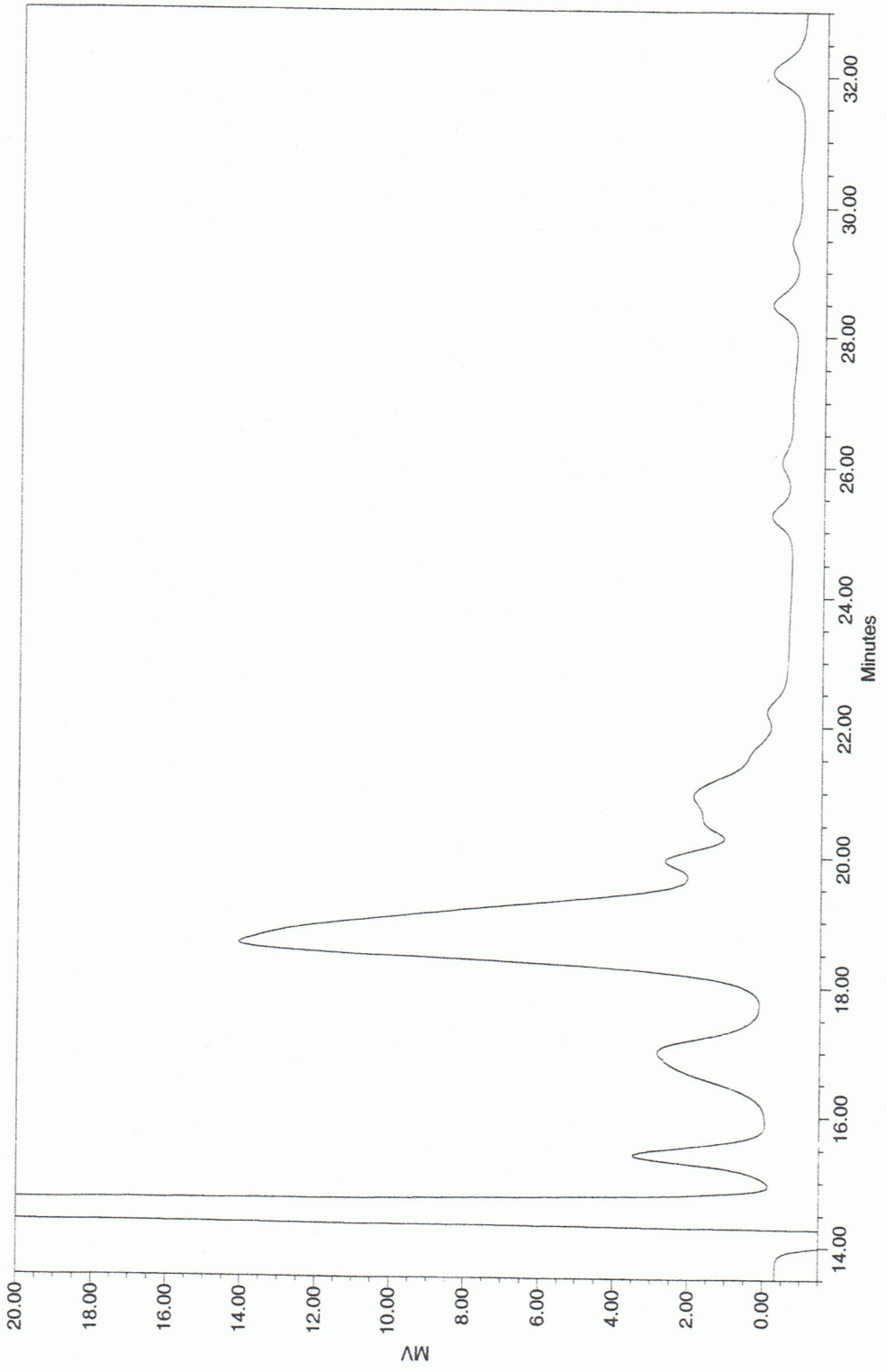
Appendix 4.3 Final reaction mixture spiked with D-glucose.



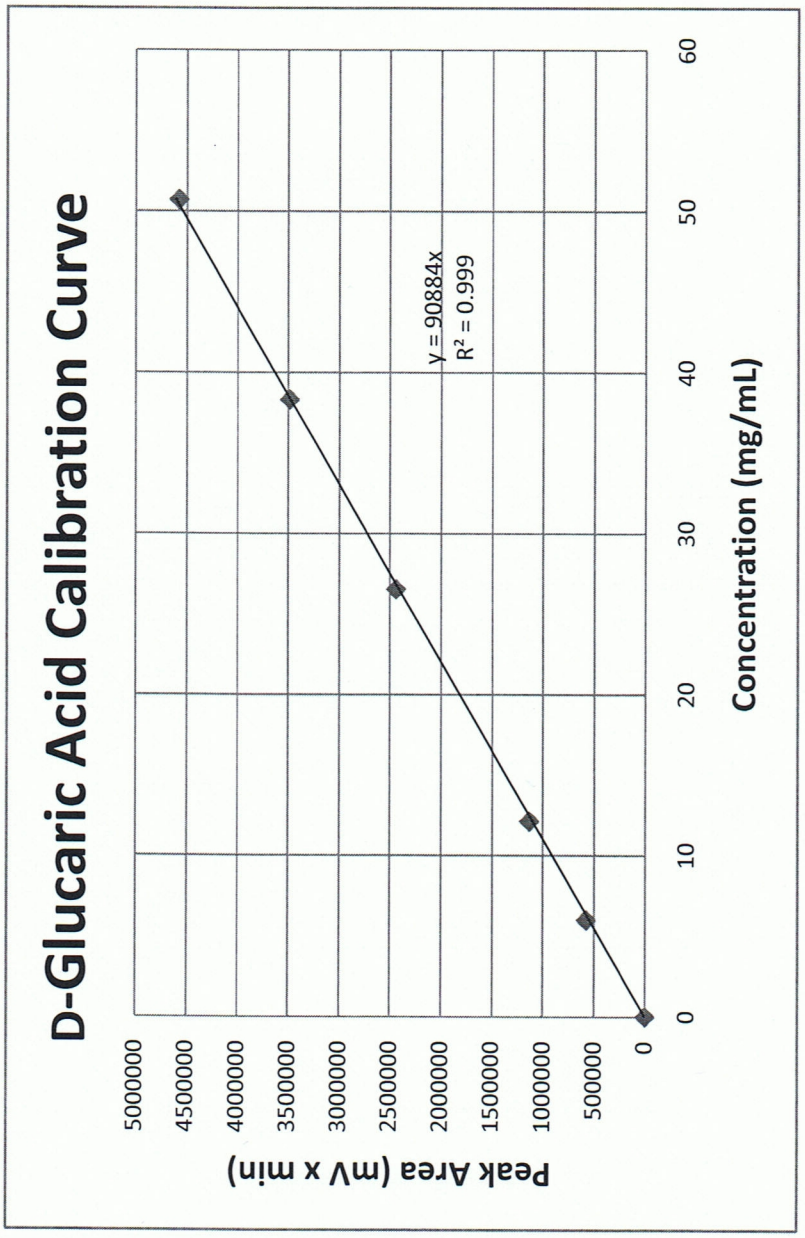
Appendix 4.4 Final reaction mixture spiked with glycolic acid.



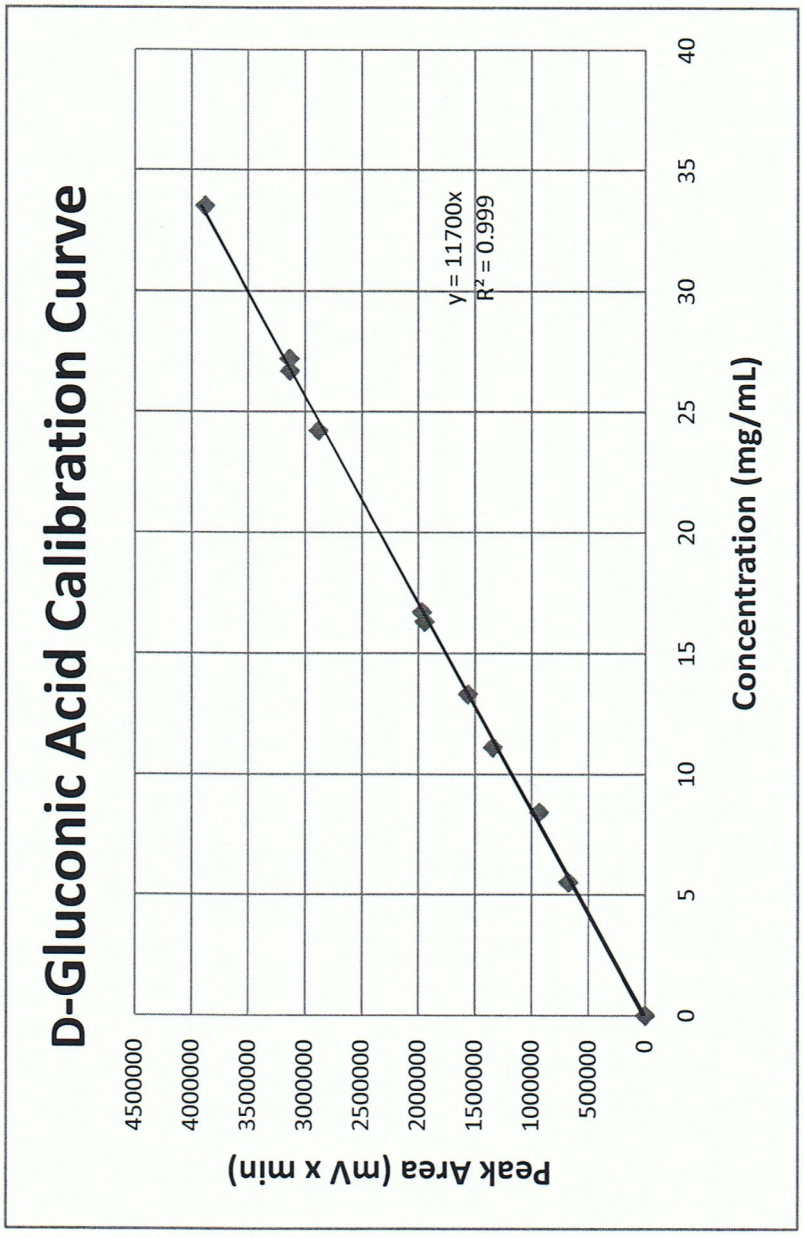
Appendix 4.5 Final reaction mixture spiked with oxalic acid.



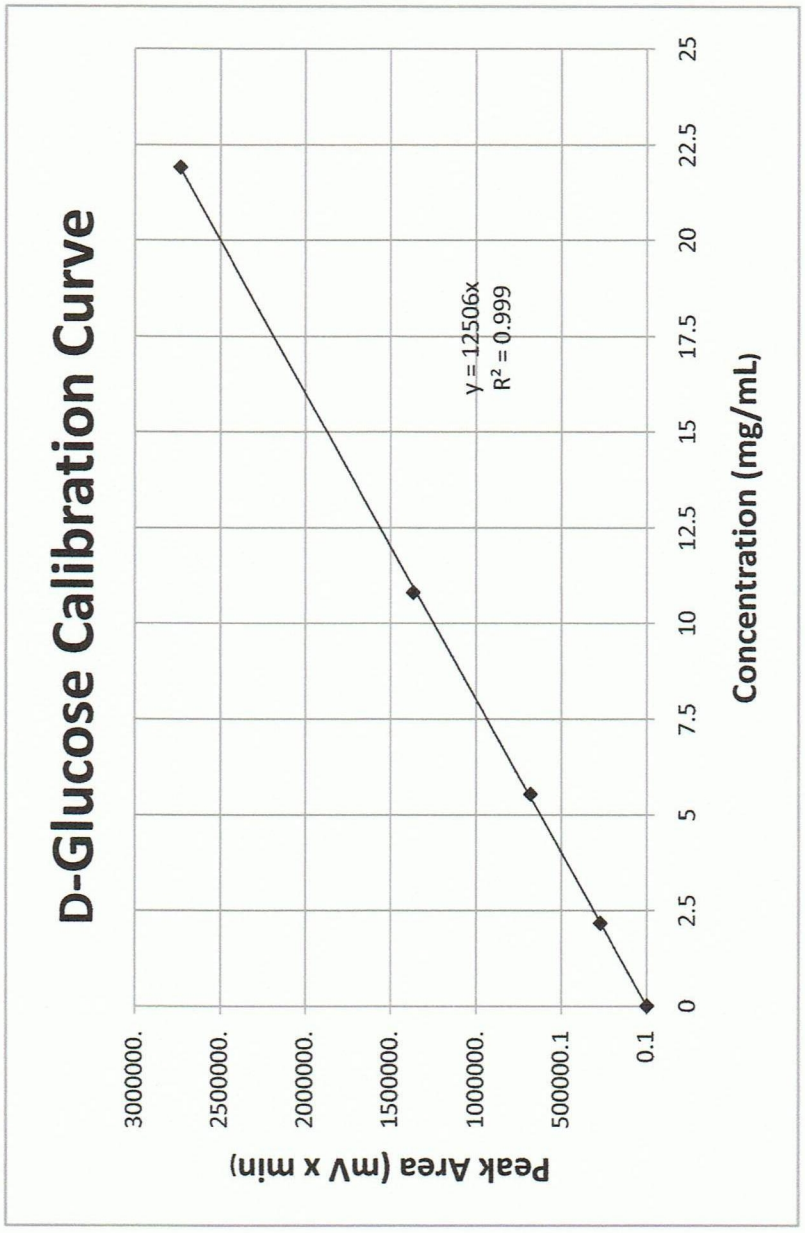
Appendix 5.1 D-Glucaric acid calibration curve.



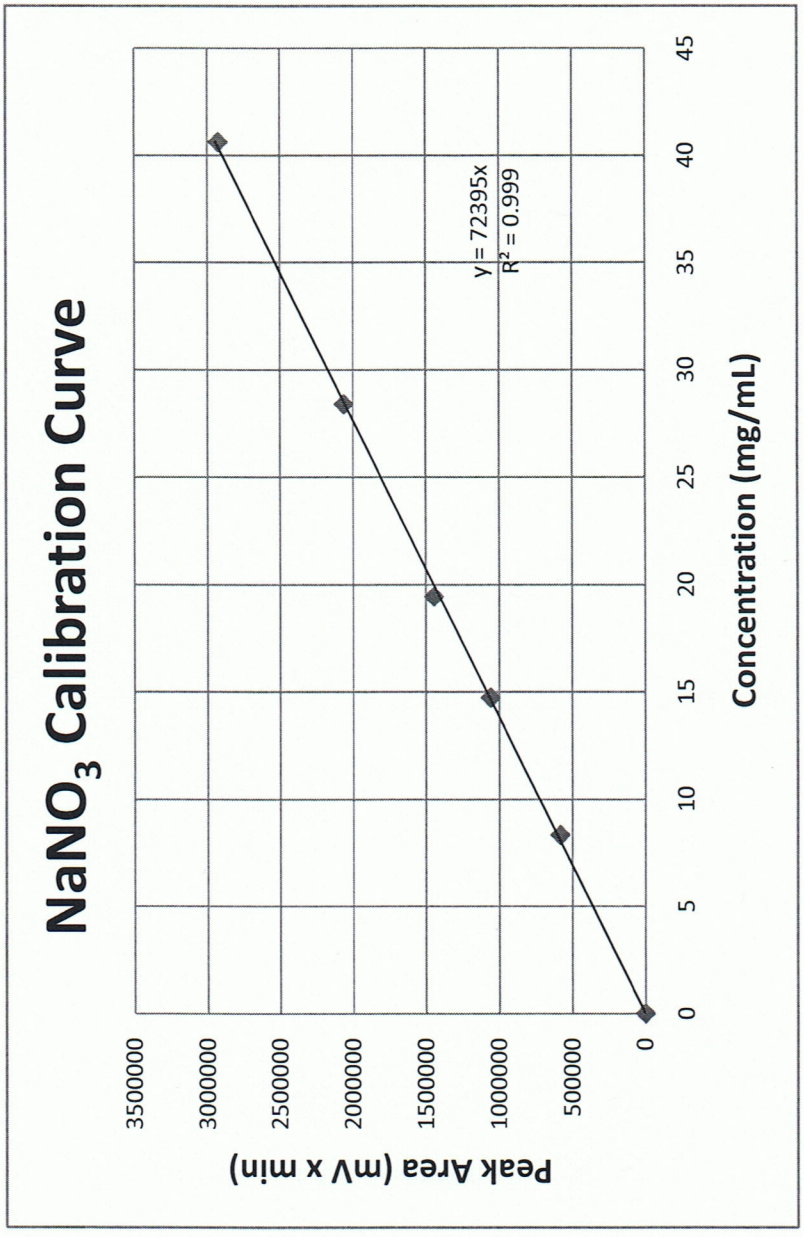
Appendix 5.2 D-Gluconic acid calibration curve.



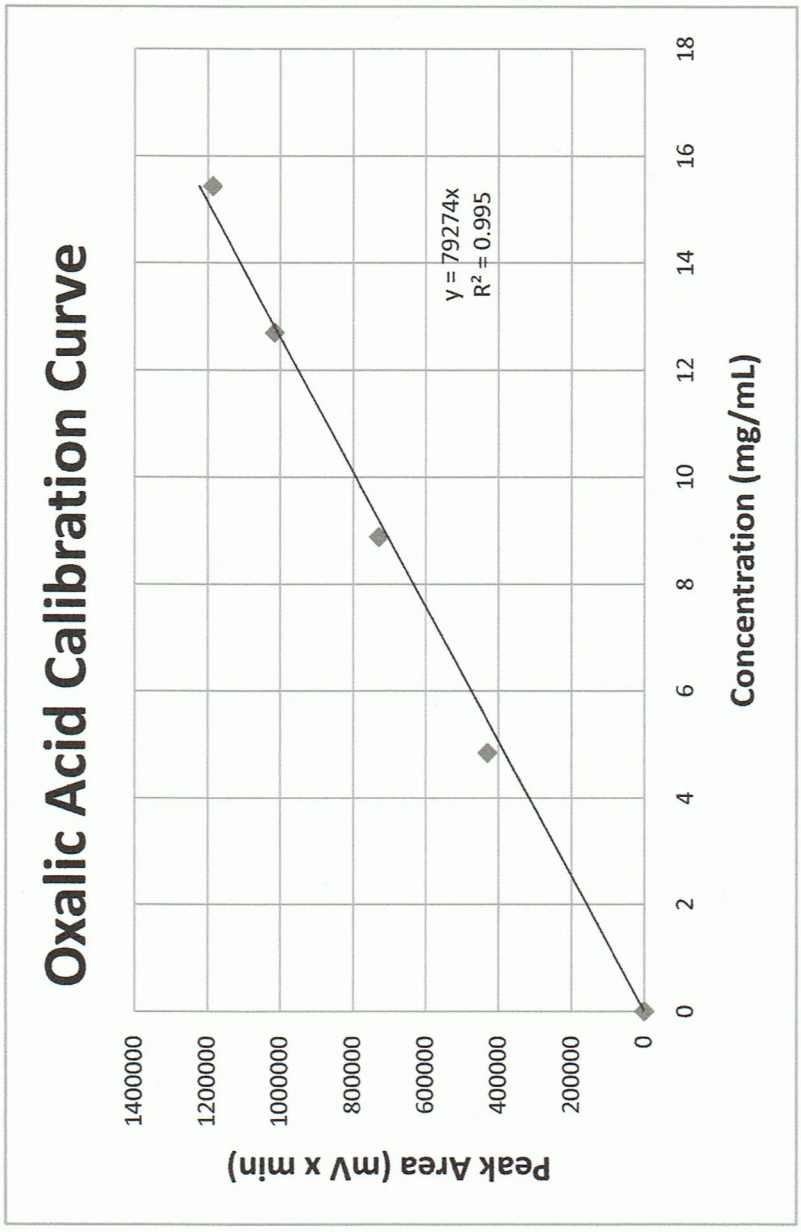
Appendix 5.3 D-Glucose acid calibration curve.



Appendix 5.4 $NaNO_3$ calibration curve.



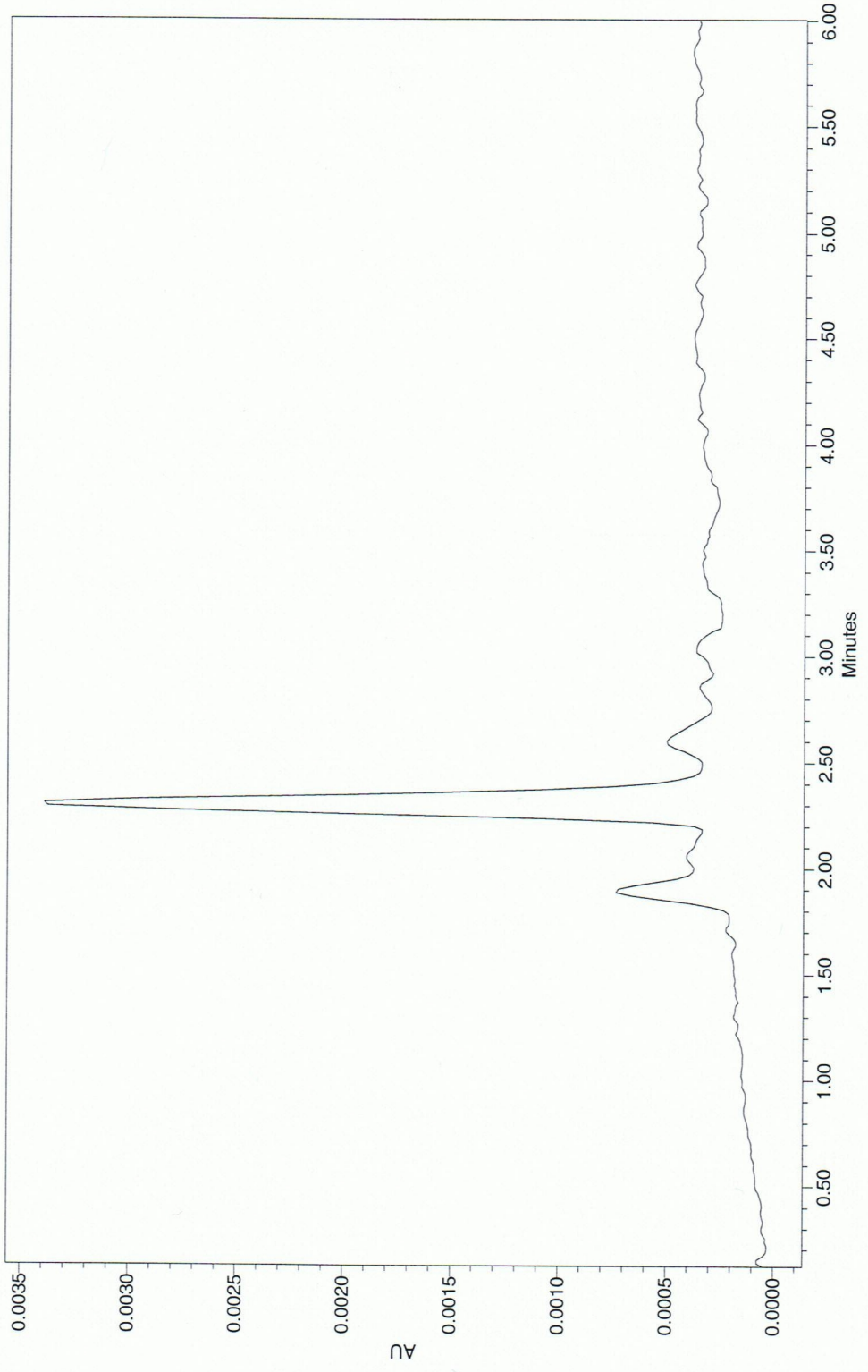
Appendix 5.5 Oxalic acid calibration curve.



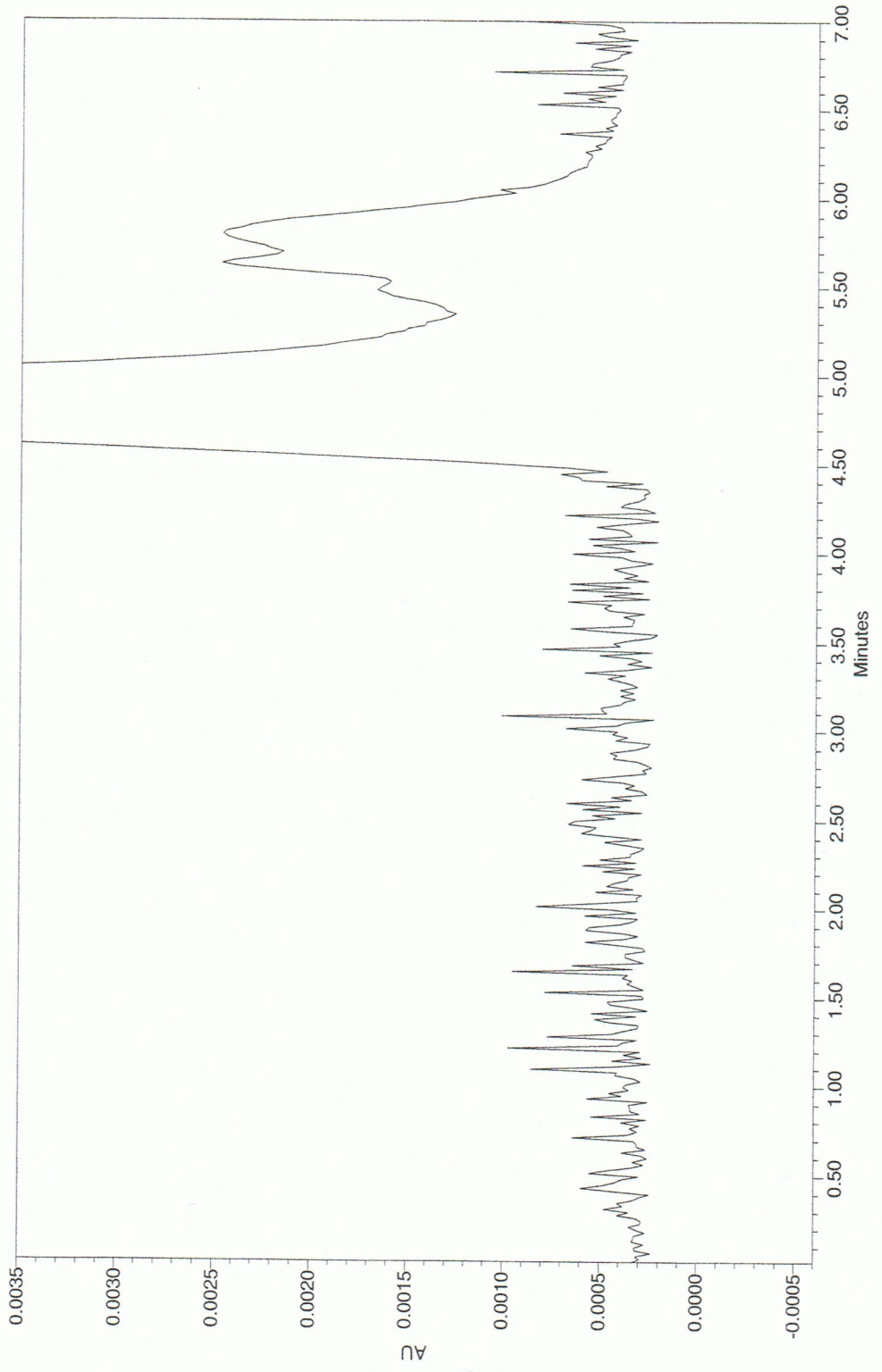
Appendix 6.1 Uric acid injections for t_0 determination on the Prevail™ OA column.

	Parameter Set			
Injection #	**1**	**2**	**3**	**4**
1	4.437	4.601	4.772	4.551
2	4.540	4.585	4.900	4.471
3	4.451	4.578	4.493	4.468
4	4.446	4.615	4.497	4.473
5	4.437	4.548	4.556	4.505
6	4.468	4.603	4.542	4.486
7	4.441	4.609	4.464	4.468
8	4.453	4.592	4.462	4.422
9	4.515	4.594	4.453	4.558
10	4.448	4.560	4.506	4.475
11	4.451	4.572	4.430	4.464
12	4.450	4.576	4.515	4.457
Mean	**4.461**	**4.586**	**4.549**	**4.483**

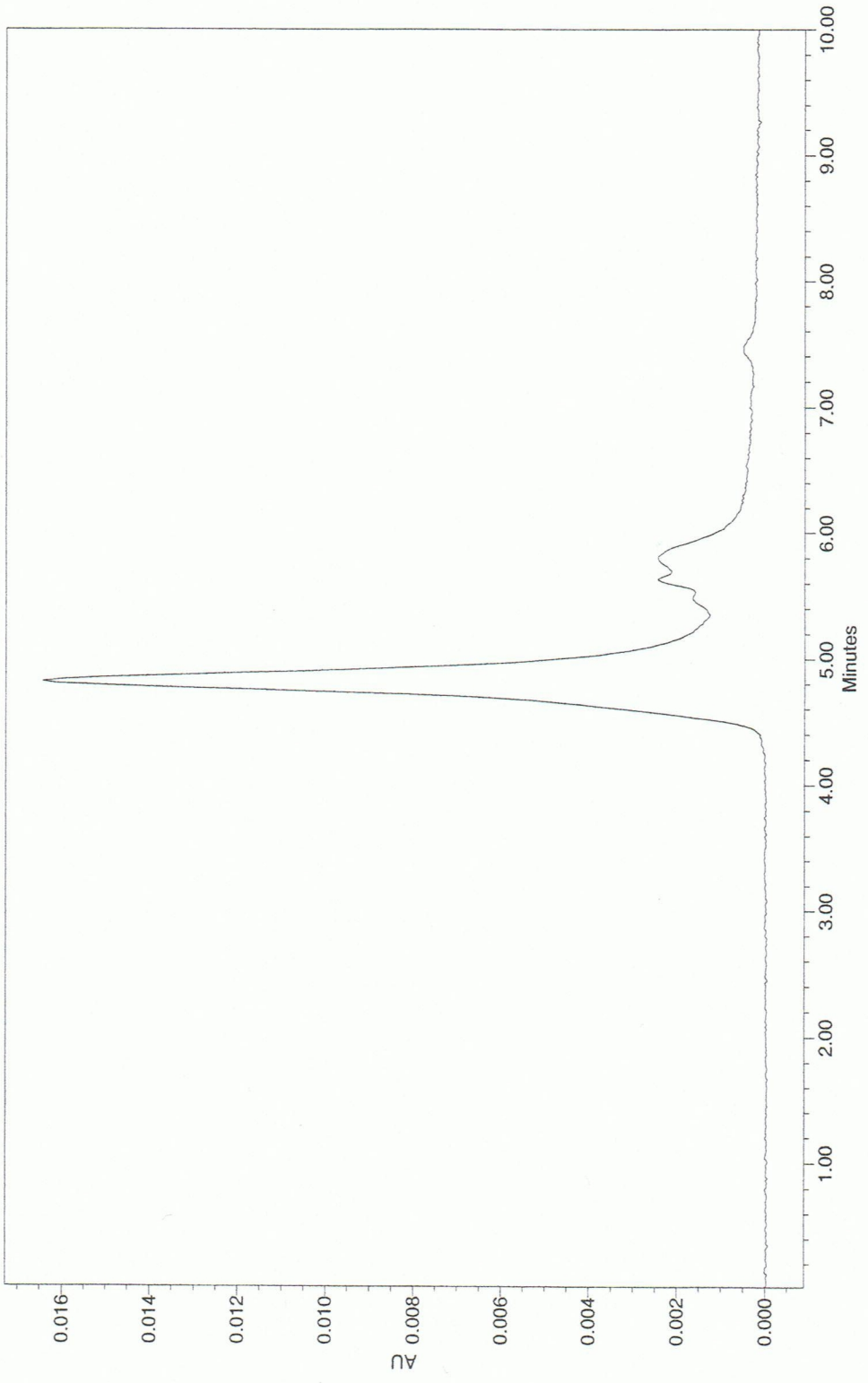
Appendix 7.1 9.8 $mgmL^{-1}$ D-Glucose in KH_2PO_4 with TFA buffer.



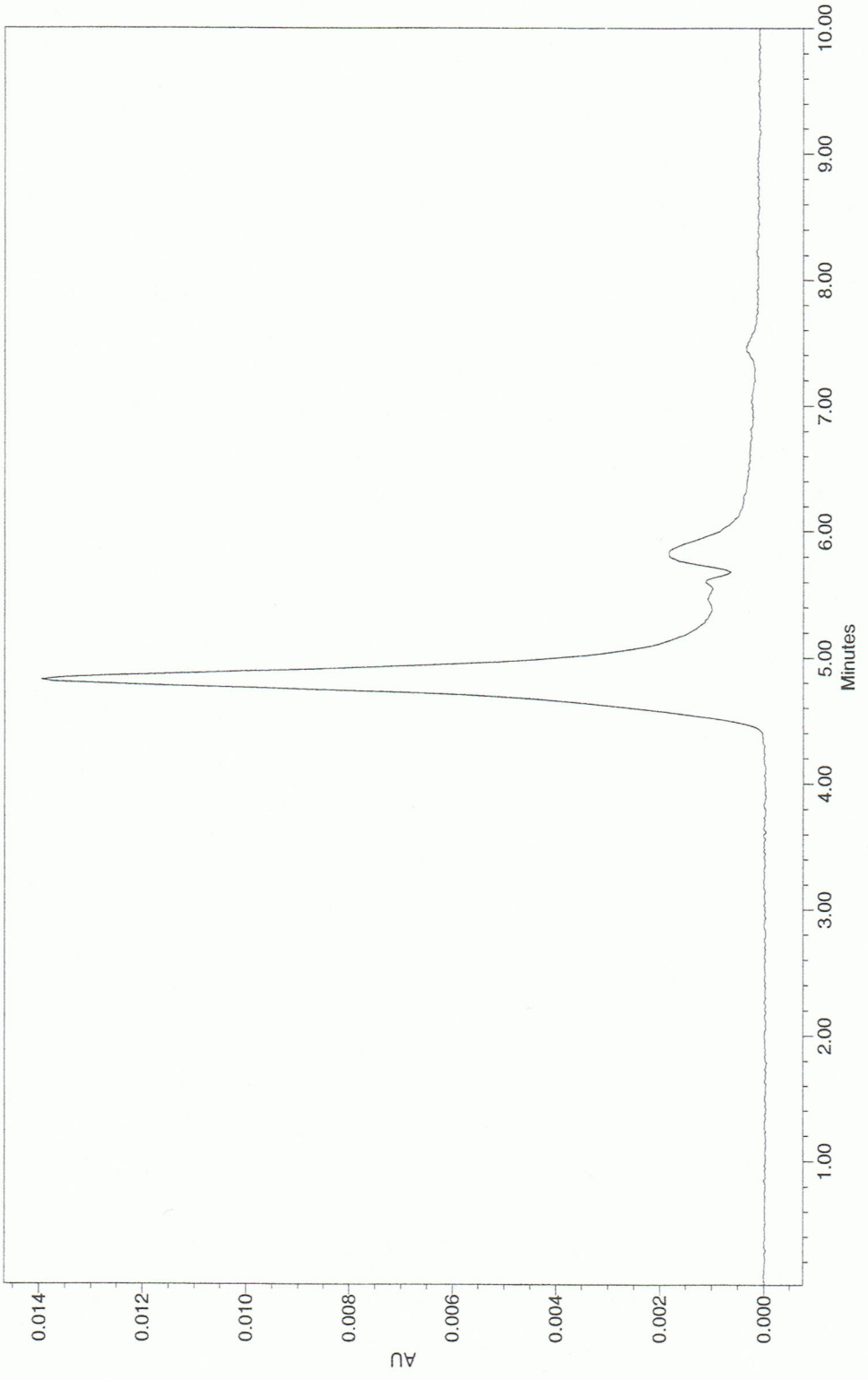
Appendix 7.1 9.8 $mgmL^{-1}$ D-Glucose in KH_2PO_4 with phosphoric acid buffer.



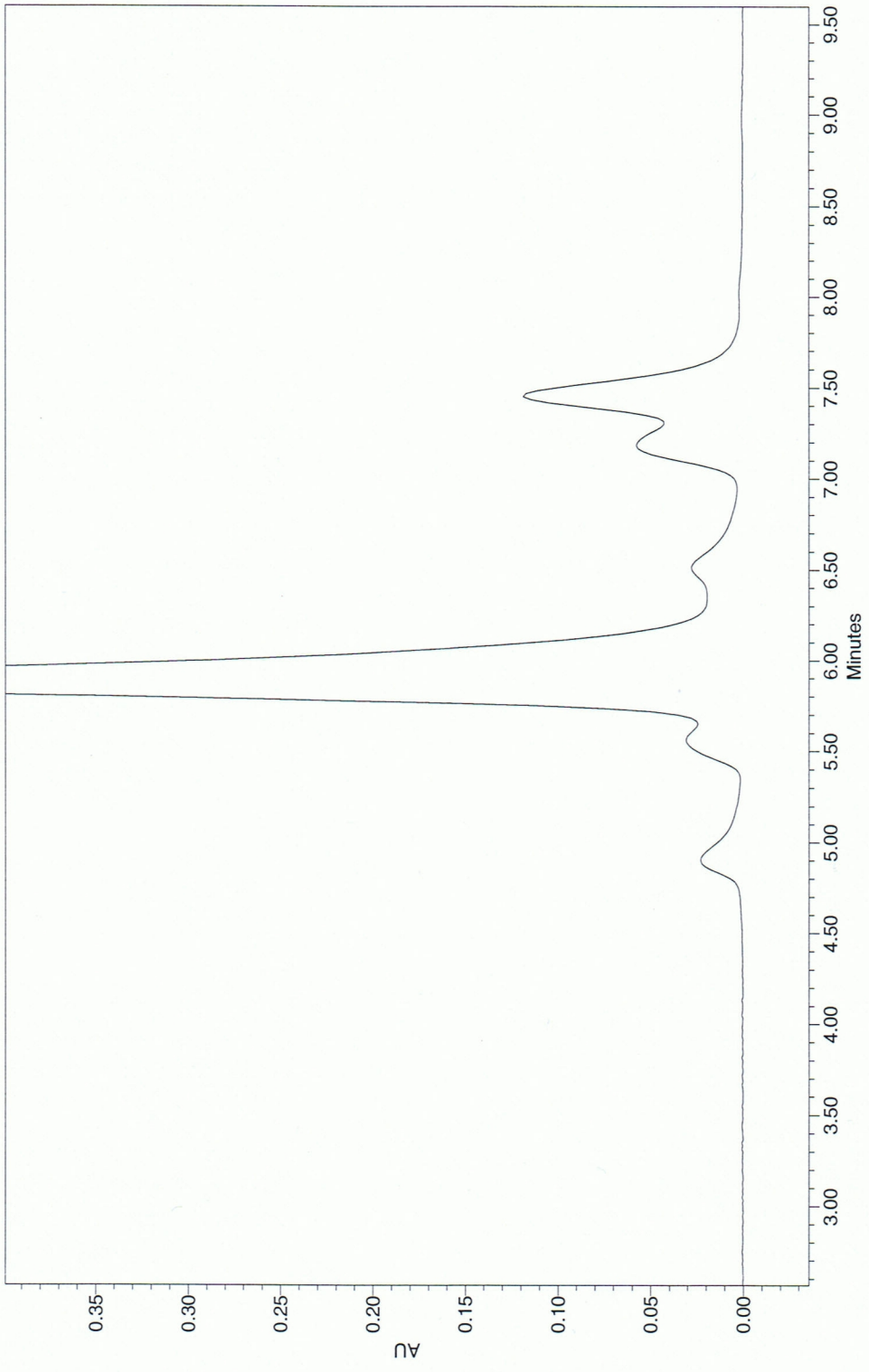
Appendix 8.1 Prevail™ OA column D-Glucose detection at 210 nm.



Appendix 8.2 Prevail™ OA column D-Glucose detection at 220 nm.



Appendix 9.1 Chromatogram of test solution containing 12 standards for Prevail™ OA column testing of parameters.



Appendix 10.1 Peak asymmetry for Aminex HPX-87H$^+$ column standards.

Date Injected	Standard Compounds					
	Oxalate A_x	Citrate A_x	Malate A_x	Succinate A_x	Formate A_x	Acetate A_x
14/06/2005	1.06	1.15	1.01	1.02	0.98	1.09
25/07/2005	1.05	1.14	1.05	1.03	1.00	1.09
01/08/2005	1.09	1.12	1.06	1.05	0.99	1.10
18/08/2005	1.08	1.13	1.08	1.04	0.98	1.09
19/09/2005	1.05	1.13	1.01	1.04	0.97	1.11
25/10/2005	1.07	1.15	1.05	1.02	0.96	1.12
22/11/2005	1.07	1.16	1.03	1.05	0.98	1.11

Appendix 10.2 Peak asymmetry for Prevail™ OA column standards.

P = Parameter set

		Before			Middle			End		
P	**Standard**	**a**	**b**	**A_x = b/a**	**a**	**b**	**A_x = b/a**	**a**	**b**	**A_x = b/a**
P1	Tartaric	4.75	10.50	2.21	18.25	40.75	2.23	9.50	21.25	2.24
	Malic	5.00	5.25	1.05	17.75	19.00	1.07	9.25	9.80	1.06
	Lactic	4.50	6.25	1.39	12.50	17.50	1.40	10.50	15.25	1.45
	Acetic	6.25	7.00	1.12	10.25	11.50	1.12	15.25	17.25	1.13
	Succinic	7.00	9.50	1.36	14.50	19.50	1.34	10.50	14.50	1.38
P2	Tartaric	Not available[a]			16.00	36.25	2.27	8.75	20.00	2.29
	Malic				25.25	27.75	1.10	6.25	7.00	1.12
	Lactic				20.25	28.75	1.42	16.75	24.25	1.45
	Acetic				26.50	29.00	1.09	14.25	14.50	1.02
	Succinic				19.50	26.50	1.36	12.50	16.75	1.34
P3	Tartaric	6.75	15.25	2.26	6.25	14.25	2.28	9.75	22.00	2.26
	Malic	25.25	28.00	1.11	15.50	18.25	1.18	19.00	22.25	1.17
	Lactic	22.50	31.75	1.41	10.25	14.75	1.44	9.75	13.75	1.41
	Acetic	26.75	31.00	1.16	10.50	12.25	1.17	23.25	27.50	1.18
	Succinic	15.25	21.00	1.38	12.75	17.75	1.39	9.25	13.00	1.41
P4	Tartaric	8.5	18.50	2.18	12.50	27.25	2.18	18.75	42.25	2.25
	Malic	27.25	31.25	1.15	13.25	15.75	1.19	16.50	19.00	1.15
	Lactic	21.25	29.25	1.38	14.75	20.06	1.36	6.50	8.50	1.31
	Acetic	32.50	36.00	1.11	15.75	17.25	1.10	12.50	13.75	1.10
	Succinic	29.25	40.75	1.39	16.25	22.50	1.38	16.25	22.00	1.35
P5	Tartaric	N/A[b]			13.50	29.75	2.20	8.75	19.50	2.23
	Malic				26.75	29.25	1.09	14.75	16.25	1.10
	Lactic				22.00	30.75	1.40	8.25	11.25	1.36
	Acetic				23.50	25.75	1.10	12.75	14.00	1.10
	Succinic				27.50	39.25	1.43	9.50	13.25	1.39

Table continued on next page...

Appendix 10.2 continued...

P6	Tartaric	N/A[b]	8.75	19.50	2.23	15.25	34.00	2.23
	Malic		25.25	26.75	1.06	12.50	13.75	1.10
	Lactic		24.25	35.50	1.46	16.50	24.00	1.45
	Acetic		24.50	28.50	1.16	18.50	21.25	1.15
	Succinic		26.75	38.00	1.42	9.75	14.00	1.44

a. These values were unable to be located.
b. These values were not needed as only the flow rate was changed after the last parameter set.