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Development of an Ion Chromatography Method for the Analysis of Nitric Acid Oxidation Reactions of Common Sugars

A thesis
submitted in partial fulfillment
of the requirements for the degree
of

Master of Science in Chemistry
at
The University of Waikato

by
Cara-Lee Davey

The University of Waikato
2008

Abstract

The large scale nitric acid oxidation of common sugars into their corresponding aldaric acids is being investigated as an important source of potentially useful components for industrial applications such as polymers.

This thesis details the development of an Ion Chromatography (IC) method for the analysis of these oxidation mixtures and related samples from the work-up and purification processes. The method was developed for use with a Dionex ICS2000 IC system equipped with an AS11-HC column and utilising suppressed conductivity detection. IC proved to be a useful, versatile and straightforward method of studying the reactions and their products.

The detected ions include but are not restricted to the anionic salt forms of: D-Glucaric acid, Xylaric acid, Mannaric acid, D-gluconic acid and both keto forms of the same, D-xylonic acid, D-mannonic acid, glycolic acid, oxalic acid, tartaric acid and tartronic acid. Nitrate from the nitric acid used in the oxidation is often observed. The results compare favorably to GC-MS and HPLC analysis of similar samples. An overview of the theory and operation of the instrument along with the method development and results from application to the oxidation mixtures and related samples are presented.

As part of the investigation into the range of utility of IC for studying these reactions, a study was made of the retention behaviour of a large number of simple and low molecular weight (LMW) carboxylic acids eluted by the ion chromatography system in use. The results of this study are included with an explanation of the major factors affecting anion retention on the column.

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APCI	....	Atmospheric Pressure Chemical Ionisation
ASRS	....	Anion Self Regenerating Suppressor
CE	....	Capillary Electrophoresis
CR-TC	....	Continuously Regenerating Trap Column
DVB	....	Divinylbenzene
ECD	....	Electrical Conductivity Detector
EGC	....	Eluent Generator Cartridge
EVB	....	Ethylenedivinylbenzene
GC	....	Gas Chromatography
HPLC	....	High Performance Liquid Chromatography
IC	....	Ion Chromatography
ICP-AES	....	Inductively Coupled Plasma – Atomic Emission Spectroscopy
LMW	....	Low Molecular Weight
LOD	....	Limit of Detection
LOQ	....	Limit of Quantification
MKG	....	mono-potassium glucarate
MS	....	Mass Spectrometry, often attached as a detector to other analysis methods e.g. GC-MS
NMR	....	Nuclear Magnetic Resonance
OA	....	Organic Acid
ppm	....	parts per million. Refers to µmol/mL concentrations unless otherwise stated.
RI	....	Refractive Index
RO	....	Reverse Osmosis (a filtration purification method for water)
RT	....	Retention time.

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IC	…………….	Ion Chromatography
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RI	…………….	Refractive Index
RO	…………….	Reverse Osmosis (a filtration purification method for water)
RT	…………….	Retention time.

1. Introduction

1.1 Background

The Shafizadeh Rocky Mountain Centre for Wood and Carbohydrate Chemistry at the University of Montana, USA has a research program focusing on the use of aldaric acids as commercially exploitable products and as polymer precursors. The director Dr Donald E. Kiely has said[1]:

> "Ultimately, the long range potential economic value of [aldaric acids] can only be realized when there is at least a modest supply of less expensive acid available for further applications testing" (p. 65)

To further this aim, the Shafizadeh Centre has been working on creating and commercializing a large scale oxidation method of producing various aldaric acids[2]. The monitoring and detection of products, unused reactants and byproducts is important in understanding, optimizing and controlling the reaction process. Previously both GC-MS and HPLC have been used for this purpose. However, there are limitations to both of these methods. In this study, Ion Chromatography (IC) was investigated as an analysis method and proved to be a fast and accurate way of following and quantifying these oxidation reactions.

1.2 Aldaric Acids

1.2.1 General description and production by nitric acid

Aldaric acids are the polyhydroxy dicarboxylic acids of sugars (aldoses). They are produced by oxidation of the terminal carbons of the aldoses. This oxidation has been historically performed by nitric acid[3] although several catalysts such as platinum-charcoal and platinum-gold have been used[4].

The production of aldaric acids by the nitric acid oxidation of the corresponding sugars is a very old procedure. A pivotal paper by Bose et al[5] has references to reports

of such oxidations in the late 1880's. A generic nitric acid oxidation can be represented as:

$$CHO(CHOH)_nCH_2OH \xrightarrow{HNO_3} COOH(CHOH)_nCOOH$$

The oxidations of D-glucose along with D-xylose (wood sugar), D-mannose, L/D-arabinose (plants) and galactose (lactose) to their respective acids are considered the best avenues for bulk production[1]. All specific reactions here will be shown in reference to the reaction of D-glucose with nitric acid as this was the primary focus of the method development. D-Glucose can be found as a part of many natural compounds, notably starch, a polysaccharide which can essentially be considered a polymer of D-glucose. Although starch is found in many tuber and cereal type plants, maize (corn) is the most common source for commercially used starch.

The products of the nitric acid oxidation of D-glucose have been identified as D-glucaric acid and its precursor D-gluconic acid along with the byproducts 5-keto- D-gluconic acid, tartaric acid, tartronic acid and oxalic acid[3,6]. (Figure 1) D-Glucaric acid is most commonly isolated as the monopotassium salt, although it can be separated as the disodium salt or the 1,4:6,3-dilactone[2].

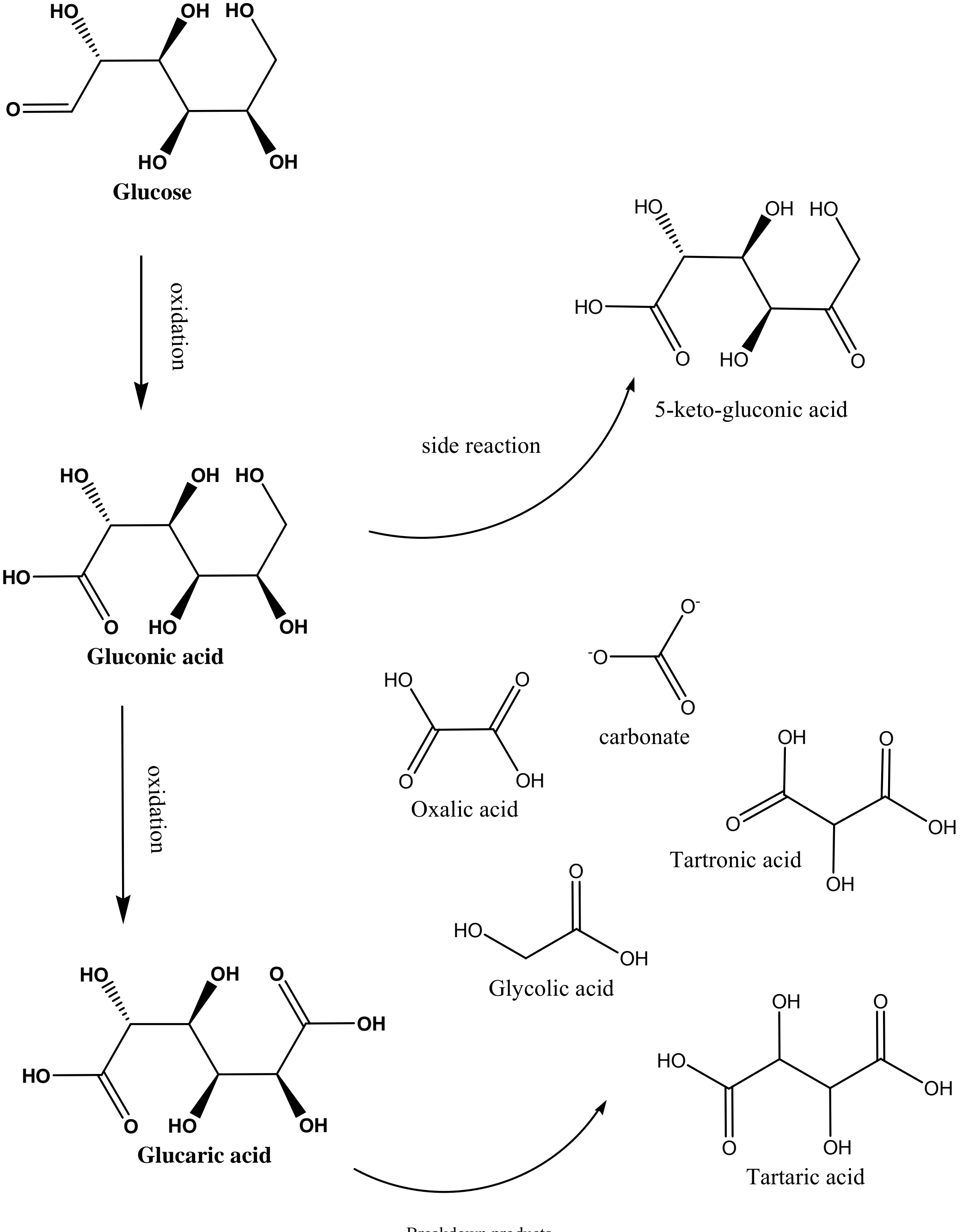

Figure 1: A diagram of the nitric acid oxidation of D-glucose showing the organic byproducts of the reaction including carbonate.

Using nitric acid to oxidize aldoses has one big advantage; as an aqueous solution it can act as the solvent medium and as a reagent. Nitric acid is reasonably selective towards aldose oxidation to aldaric acids. The terminal anomeric hydroxyl and

primary hydroxyl groups are the easiest to oxidize resulting in the diacid. However the α-hydroxyl carbon can also be oxidized resulting in the keto-aldonic acids and carbon-carbon bond breakage is possible ultimately forming oxalic acid or carbonate (Figure 1).

The reaction is very exothermic and evolves large amounts of NO and NO_2 gases which are environmental pollutants. Both of these issues have to be addressed in the development of a commercial process.

In the reaction method used by Kiely et al[2], the nitric acid acts as a catalyst in the oxidation, it is not consumed. This is achieved by pumping oxygen into the system; as the oxygen is consumed in the reaction the nitric acid is regenerated from the dissolved noxious gases before they can be released into the air. The evolution of the visible brown gas, NO_2 is limited during the reaction. The regeneration of nitric acid means that there is often a very high concentration of nitric acid still present in the reaction mixture at the end. Along with analysis issues related to the high content of the inorganic nitrate ion, there is also the opportunity to reclaim this acid for re-use, an attractive proposition for commercial purposes.

The addition of oxygen to the reaction and a controlled introduction of the reactants to the system allow the process to occur at lower temperatures than are often reported in the earlier literature; 25-40°C for the six carbon aldoses and slightly higher for the 5-carbon aldoses (likely due to the lower oxidation potential of the terminal carbons in the latter) [2].

In traditional oxidation methods, a large portion of the final mixture is residual nitric acid which can be time consuming and difficult to remove. There are three techniques commonly used to remove the nitric acid; the first is neutralizing the solution by adding a hydroxide solution and separating the resulting salts. This technique can be very expensive in the use of large amounts of hydroxide. The second option uses an alternating distillation and dilution method which is time and energy consuming as well as not completely effective. Another method uses large volumes of 2-propanol to destroy the residual nitric acid. This technique results in acetone and other components being added to the oxidation product which will also need to be

removed. The latter method requires too many different steps to be commercially successful[2, 6].

The method described by Kiely *et al* includes specifications on nitric acid removal[2]. The first step removes and recovers the bulk of the acid by distillation. The remaining product is made basic with inorganic hydroxide leaving a solution of inorganic nitrate and the salts of the carbohydrate acids. This mixture is separated by nanofiltration resulting in a retentate containing the organic acid salts; this retentate has a sufficiently lowered level of nitrates to make the collection of purified products more successful than other methods.

As a product isolation method; diffusion dialysis has only recently been applied to this separation. It is a low energy process normally used to separate inorganic acids from metal salts. In this application it provides around 90% removal of nitrates with only a small loss of the organic products. Diffusion dialysis does not require any further preparation of the reaction mixture and can be used either before or after the concentration by distillation depending on industrial necessities[2]. After diffusion dialysis, the organic products are obtained by making the mixture basic and collecting the salts of the organic acids.

1.2.2 Commercial uses of aldaric acids

> "Carbohydrate acids, and in particular carbohydrate diacids (aldaric acids) offer significant economic potential as carbon based chemical building blocks for the chemical industry, as safe additives or components of products use in pharmaceutical preparations and food products, and as structural components of biodegradable polymers [2]"

Tartaric acid is one of the most widely used diacids in current commercial products. This is largely due to its ready supply as a byproduct of the wine industry. Potassium bitartrate is the cause of 'wine diamonds', crystals that form on the rim of bottles and

on the sides of maturing casks. Tartaric acid is used in a wide variety of industrial processes:

- As a chelator for dying synthetic hair[7] and absorbing fire retardant chemicals onto wool fabrics[8].
- Derivatives make part of a biodegradable surfactant[9]
- As a fuel additive mixture improving economy and anti-wear properties[10]

As a natural product L-tartaric is used in the food industry (e.g. as a nutrient modifier for cheese[11]) and for medical applications such as a pH balancer in a treatment for urinary system infections[12] as well as for treating Gaucher disease[13].

Since L-tartaric and D-glucaric are both hydroxyl acids and chelators, applications currently limited to the easily available L-tartaric acid could be expanded to include D-glucaric acid.

D-Glucaric has been identified as one of the top ten compounds targeted as potential key chemical building blocks from renewable resources in a report from the US Biomass Program[14]. Xylaric acid and arabinaric acid were also singled out with glucaric acid. Potential uses of glucaric acid include medical and agricultural processes. The amides of glucaric acid and related 5 and 6 carbon aldonic and aldaric acids can be used as fertilizers with improved biodegradation profiles[15]. Since D-glucaric acid is a human metabolite, it is seen as a safe compound for human use[1]; medical applications include complexing with an imaging agent to detect tumor-like growths[16] and as a treatment or preventative for hypercholesterolemia[17]. D-Glucaric acid is also mentioned in a range of industrial processes such as washing the felt layer used in paper-making[18] and as a complexing agent for bleaching and altering keratin fibres (esp. horse hair)[19]. Several of the applications found in patent literature list several of the polyhydroxy acids along with D-glucaric acid as equally effective.

1.2.3 Aldaric acids as Polymer precursors

Many commercial and synthetic polymers in use today are made from monomer units based on petroleum. The low biodegradability of these polymers and the reducing availability of the petroleum feedstocks have made the development of alternative polymers using renewable carbohydrates, a very popular option[20]. Nylon 6,6; the

most well known polymer is an example of synthetic polyamides created through a polymer condensation reaction. Nylon 6,6 is made from hexamethylene diamine and adipic acid. (Figure 2).

Hexamethylene diamine + Adipic acid

Nylon 6,6

Figure 2: Reaction of Hexamethylene diamine and adipic acid to form Nylon 6,6.

Both hexamethylene diamine and adipic acid are commonly produced from benzene sourced from oil refineries[21]. Environmental considerations and the expected decrease in oil supplies have made a search for alternative monomers important. An unstereospecified aldaric acid is shown next to adipic acid below (Figure 3).

A 6 carbon aldaric acid vs. adipic acid

Figure 3: Comparison of a 6 carbon aldaric acid and adipic acid

The correlations in structure between these acids meant that aldaric acids were easily proposed as potential replacements for adipic acid. Aldaric acids can be produced directly from the corresponding simple sugars. These sugars are often easily and cheaply available; D-glucose is a component of starch and as dextrose has long been one of the most accessible.

There are a few conditions for a carbohydrate to be a commercially viable option for the polymerization process; the reaction should take only a few steps, require no functional group protection/deprotection and produce a fairly pure product directly. D-Glucaric acid has been shown to produce this type of reaction with a range of *bis*-primary diamines[20]. The products of these reactions are referred to as polyhydroxypolyamides (PHPA's) or hydroxylated nylons. Mannaric, D-Galactaric and Xylaric acids have all been investigated as monomers for PHPAs[21, 22, 23]. The physical and chemical properties can be adjusted by the choice of diamines and the diacid monomers. The length of the diamine alkyl chain and the configuration of the diacids changed the solubility and melting points of the polymers.
PHPAs have several proposed uses in both industry (paints, safety glass and structural plastics) and synthesis chemistry (precursors and complexing agents)[22]. The ease of preparation, a simple condensation reaction and the suggested biodegradability of the polymers make them potential targets for application in areas such as adhesives, a time release component of fertilisers and in household products such as detergents[1].

1.3 Analysis of Organic Acids

1.3.1 Analysis of General Organic Acid mixtures.

Several analysis methods have been applied to the detection and quantification of organic acids in many different types of mixtures. A very early publication used paper chromatography, relating the concentration of the organic acid to the size of the spot representing that acid[24]. Since then analysis methods have become far more technical and precise. HPLC, IC, capillary electrophoresis (CE) and GC techniques are the most favoured for separation, with varying detection methods used depending on the sample's matrix.

Blake *et al* quantitatively analysed sugar cane process juice for organic acids by HPLC[25]; the acids were first isolated from the mixture on an anion-exchange resin then eluted through two cation exchange columns equilibrated at different temperatures and analysed with RI detection. This paper also includes a table of

HPLC analyses using Aminex® HPX-87H columns, performed on organic acid mixtures between 1981 and 1986. Of the 45 reported methods, 34 detected the analytes by UV absorption between 200nm and 220nm. The others used Refractive Index (RI) detectors or Amperometric detection. These three detectors were sometimes paired together or with conductivity detection. A similar table from a more recent review shows the same type and application of detectors[26]. The main problems found with these methods were poor resolution and identification or quantification of poorly resolved or co-eluting peaks. The authors improved the resolution of the targeted acids by using columns at different temperatures 35°C/85°C, reaching a limit of detection (LOD) of 50μg/mL.

CE, HPLC and GC have all been used to determine the organic acids in coffee which play important roles in the flavour of the beverage[27, 28]. GC and HPLC were directly compared with HPLC being preferred[27]. The GC method was described as tedious several times, a common appellative attached to the system by many investigators. In the GC method, the derivatised organic acids were unstable and had to be injected directly after derivatisation. This restricted the GC to single samples and extended the analysis process considerably. HPLC had higher detection limits than GC although this was not considered a problem since the organic acids were concentrated enough to need dilution before analysis. This comparison tested the detection of eight common organic acids in coffee. The CE method was applied to the successful detection of 17 organic acids[28]. Of the three methods described, CE has the best reported application to the analysis of coffee samples due to its simple sample preparation and greater efficiency. In general application, the greatest disadvantage of CE is the low reproducibility of the migration times[26] which can be greatly affected by the conditions and aging of the capillary[29].

The beverage industry is an area where the analysis of organic acids is very important to test quality, safety and taste. Wine and beer are the two of the most commonly analysed samples. A review of analytical methods that have been applied to the analysis of organic acids in grape juice and wine also covered several of the less common methods[26]. Enzymatic and spectrophotometric analyses were found to be

highly specific and accurate. However both of these methods were only able to analyse for one organic acid at a time, making them very long and tedious. The report did not identify a preferred method although CE and liquid chromatographies such as HPLC received the least criticism.

The literature has consistently identified GC-MS as the method whose high sensitivity and good qualitative properties cannot overcome issues related to the necessary sample preparation steps. Jones and Chalmers[30] addressed a particular example of this in the analysis of the biologically important compound; 3-hydroxy-3-methylglutaric acid. They identified artefacts present in the routine GC-MS analysis of the trimethylsilyl (TMS) derivatised compound. The artefacts are attributed to di- and tri- TMS derivatives and cause a low value for the quantified peak. Procedures to alleviate the artefact issue were proposed and tested, however they all added to the complexity and time required for sample preparation.

One major advantage of mass spectrometry as a detection method is the ability to identify a compound without the need for comparison to standards. The mass fragments and pseudo-molecular ion masses can be used to identify whole compounds and functional groups. This often leads to the identification of a specific compound as the eluting peak. A downside is that when attached to GC separation methods; analytes often require derivatisation for analysis and the derivatisation of small organic acids in aqueous solution is difficult due to the low reactivity of the carboxylate group in water.
A HPLC method was developed for the monitoring of the progress of the Kraft pulping process[31]. The detection method was atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) This system proved a much simpler and faster analysis than the previous GC-MS application, providing nearly real-time monitoring.

For the specific use of IC with Organic acids see Section 1.5.

1.3.2 Previous analysis methods for Nitric acid oxidation of aldoses

Prior to the use of IC for these analyses, HPLC was investigated in detail[6, 32]. The Shafizadeh centre routinely used an HPLC system for analysis comprising two Aminex® HPX-87H columns in series, RI detection and a 0.5mM sulfuric acid eluent. The basis of this system was taken from a reported method for analyzing sugar cane process juice[25]. The literature has a similar method reported in 1981 using one column and UV detection for the analysis of D-gluconic acid and other related compounds resulting from the biochemical or catalytic oxidation of glucose[33]. This report stated that column materials comprising amino phases (e.g. Aminex) were particularly applicable to D-gluconic acid analysis.

A comparison of the Aminex columns with a Prevail Organic Acid (OA) column for the analysis of nitric acid oxidations of D-glucose was made recently[6]. This involved a detailed study of both systems. Detection was performed by RI in both cases. The Aminex columns in series were ultimately found to be more effective than the Prevail OA column. The main reasons for this were the better resolution achieved with the Aminex columns and the inability to use gradient elution methods with the Prevail OA.

On the routinely used system of the two Aminex HPX-87H columns, oxalic acid presented an analysis problem due to a characteristically skewed shape which inhibited peak calculations including those for calibration. The presence of oxalic acid in the reaction mixtures could not be confirmed by other methods (GC-MS). The two cyclised lactone forms of D-glucaric acid eluted at separate retention times with HPLC. This meant that quantifying the overall concentration of D-glucaric acid was complicated and made less precise as the potential errors in three peak calibrations could be contributing.

The retention factor is a parameter used to judge the efficiency of an analysis method. It relates the retention time of a compound to the amount of time a non-retained molecule takes to elute (dead volume). The ideal value for this factor is between 1 and 5. While a lower value suggests that the resolution from the non-retained compounds can suffer, a higher value than 5 suggests that the method takes too

long[34]. The *k* values for both the Aminex HPX-87H and Prevail OA methods were below 1, significantly below in the cases of some analytes. This was most likely caused by the relatively large dead volume of the HPLC systems; over 13 minutes in the case of the Aminex HPX-87H columns.
In the end, the main disadvantages of using this HPLC system as an industrial analysis method for nitric acid oxidations was the time and effort involved in starting up the system and equilibrating the columns. It took around 3 hours each time for the columns to reach stability at the programmed temperatures. This is far too long to be effective in a commercial setting.

GC-MS was also routinely used as an analysis method prior to HPLC; however the limitations mentioned earlier were still present. The sample preparation was long and complicated. The sample was made basic and an internal standard added; this mixture was freeze-dried which was often done overnight (12hrs). The freeze-dried sample was per-O-trimethylsilylated by Tri-Sil; a process taking nearly two hours before it could be analysed. Quantification was not possible without the addition of an internal standard (xylitol). The method has a run time of 30 minutes and included a temperature re-equilibration time of 17 minutes necessary for every sample[6]. Due to the lack of a suitable internal standard and problems with the silylation and volatility of oxalic acid, the GC-MS was unable to quantify oxalic acid. This lack of application to a significant analyte is an example of GC-MS's unsuitability for this purpose.
NMR could be used to identify components in samples only if there was a peak that was specific to the component. Quantification has been performed with NMR using internal standards[35] however the need for deuterated solvents and the cost of an NMR instrument make this a very expensive method. GC-MS and NMR are useful as confirmatory methods but would be less than ideal as the primary analysis method.

1.4 Ion Chromatography (IC)

1.4.1 History and development

Ion Chromatography is a phrase that refers to any chromatographic system used to analyse inorganic or organic ions[36]. In modern usage it implies a high performance ion-exchange chromatography system. The term was first used in reference to ion-exchange chromatography systems developed in the early 1970's using continuous detection for ion analysis. That type of system; employing an ion-exchange column coupled with eluent suppression and conductivity detection is still the most easily recognised 'ion chromatography' system.

The Beginning

The original separations using ion-exchange chromatography were done for isolation purposes; for example, rare elements were isolated in gram quantities by F.H. Spedding in the late 1940's (as cited in ref 36). Subsequently a great many methods were created and reported that used ion-exchange media. Most of these were large particle resins using gravity eluent flow combined with fraction collecting and manual detection of the analytes of interest. Inorganic analysis in particular relied on laborious wet chemistry methods for ion detection which were time consuming and error-prone[37]. Some applications, such as the separation and detection of metal cations were an exception. Spectrometric methods were used successfully in conjunction with forced flow ion-exchange chromatography to separate and detect iron down to very low levels[38].

The first exploration into the field of high performance systems was prompted by the demand for a simpler, more accurate and sensitive method of inorganic ion analysis. This was due to the increasing need for detecting and monitoring the levels of common ionic species in aqueous systems such as water supplies, waste streams and fluids of biological importance[39]. These important species were often anionic, e.g. F^-, NO_3^-, SO_4^{2-}, and although many cations already had reasonably good accepted methods for determination, no such general method existed for anions, particularly at low concentrations[40].

The use of ion-exchange columns for the type of simple and efficient analysis required was limited by a lack of suitable continuous detectors. The nature of the eluting solution, necessarily ionic in form, restricted the choices. For those analytes possessing a property that sufficiently differentiated themselves; such as UV absorption, detection could be relatively easy. However, many important species such as the alkali metal ions and halides did not fall into this category. Conductivity detection, which could be universally applied to ions, was stymied by the presence of the eluent. This was stated well and resolved by Small *et al*[39] in the landmark 1975 paper:

> "It would be desirable to employ some form of conductimetric detection as a means of monitoring ionic species in a column effluent since conductivity is a universal property of ionic species in solution and since conductance shows a simple dependence on species concentration. However, the conductivity from the species of interest is generally "swamped out" by that from the much more abundant eluting electrolyte. We have solved this detection problem by using a combination of resins which strips out or neutralizes the ions of the background electrolyte leaving only the species of interest as the major conducting species in the effluent. This has enabled us to successfully apply a conductivity cell and meter as the detector system." (p. 1802)

The most important concept in the evolution of modern Ion Chromatography was the creation of this Suppressor system. Initially a combination of suitable resins, the device has become an essential yet often invisible part of the modern IC system.

The Rise of the Suppressor

Originally called the eluent stripper column or stripper; the suppressor suppresses the background conductivity of the eluent by transforming it via an ion-exchange reaction into a neutral or weakly conducting form. This allows for the detection of other

conductive ions in the eluting solution. Suppressors are normally a separate device placed directly in the flow between the separation column and the detector.
A second ion-exchange column inserted in the system was the first evolution of this suppression method. In anion analysis; a cation-exchange column was placed after the anion-exchange separation column and used to change the nature of the eluting solution. For example, a sodium hydroxide eluent would undergo the following reaction with the suppressor resin:

$$NaOH + RsH^+ \longrightarrow RsNa^+ + H_2O$$

The associated analytes A^- would be turned into the H^+A^- form which is a better conductor than the sodium salt for many anionic species. Cation analysis simply reversed the above procedure. These early versions increased the dead volume of the system and produced some peak broadening. They also required periodic regeneration as the resin became exhausted. To lengthen the useful life of the suppressor, low capacity separator columns had to be used so that the needed concentration of eluent was kept low.
Theoretically, hydroxide was highly favoured for anion elution as it suppressed to water. However due to the high concentrations made necessary by its poor displacement potential for some ions, the use of hydroxide quickly depleted early versions of the suppressor. Therefore the first anion systems studied in detail used carbonate/bicarbonate or phenate eluents.

The next big step for suppressor technology was the development of the membrane suppressor. This version uses an external source of exchange ions to continuously regenerate the suppressor system. The separator columns output enters the suppressor between two cation-exchanging membranes. On the other side of the membranes, an acid is flowing counter current to the eluent solution. The cation-exchange membranes allow the passage of the counter ions of the eluent; Na^+ in the case of NaOH and block the anions. H^+ ions from the acid regenerant exchange with the sodium ions and combine with the OH^- ions to form water. The preferred regenerating solution is sulphuric acid as the doubly charged sulphate ions are more effectively excluded than a singly charged anion such as chloride. Thus with the

addition of a reservoir of concentrated acid, the membrane suppressor can be used for long series of analyses without requiring regeneration.
The use of electrochemical reactions to create the suppressor effect was the next improvement. Dionex Corporation holds the patent for suppressor technology and uses these reactions in their ASRS® Suppressors. The operation of this system for the suppression of NaOH is shown in Figure 4. The only necessary addition by the user is a reservoir of de-ionised water. This is much easier to source and handle than the concentrated acids the membrane suppressors require.

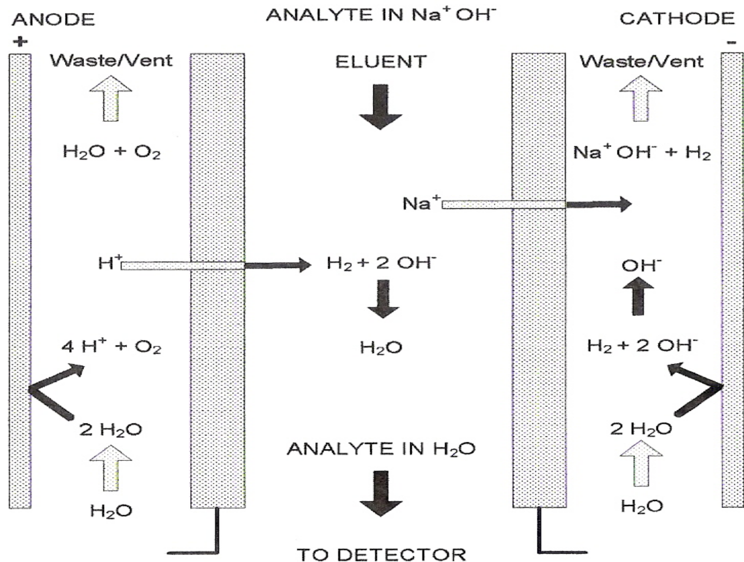

Figure 4: Operational details of the Dionex ASRS Suppressor system (with permission from Dionex Corporation[41])

The most recent versions of the suppressor are self-regenerating systems; requiring no dedicated source of water, some simply use the eluent flow after the detection module to regenerate themselves, requiring little or no attention to continue performing. Thus the suppressor can be a completely non-intrusive yet vital part of the system.

A separate technique referred to as non-suppressed or single column chromatography (SIC) was also developed in order to allow the normal HPLC system to be used for IC simply by the addition of a conductivity detector. Certain disadvantages to the early suppressor columns (see above) also gave impetus to this technique. SIC uses

ion-exchange resins of very low capacity allowing the eluent to be much more dilute. The nature of the eluent is also carefully considered and the goal is to maximise the conductivity difference between the analyte and eluent. In SIC, the elution of the analyte is detected by the difference in background conductivity, either a peak (increase) or a dip (decrease) depending on the relative conductivity of the analyte and eluent. SIC has a lower signal to noise ratio than suppressed chromatography and so the system has lower sensitivity as well. Suppressed chromatography also has a greater dynamic range as the choice of eluent is far more independent of the analytes' nature. SIC's main advantage is the lack of specialised equipment needed; although very weak acid anions e.g. borate are best detected without suppression as their suppressed acid form have such a low dissociation constant.

With these and so many other various modes of operation, ion chromatography has become a very popular rapid analysis method for many types of inorganic and organic ions. This work deals solely with conductivity detection, however the IC system is able to be modified for use with almost every type of HPLC detection.

1.4.2 The principles of operation for the ICS2000

The Dionex Corporation's ICS2000 ion chromatography system was used for all IC work reported here. Figure 5 shows a basic overview of the ICS2000 system.

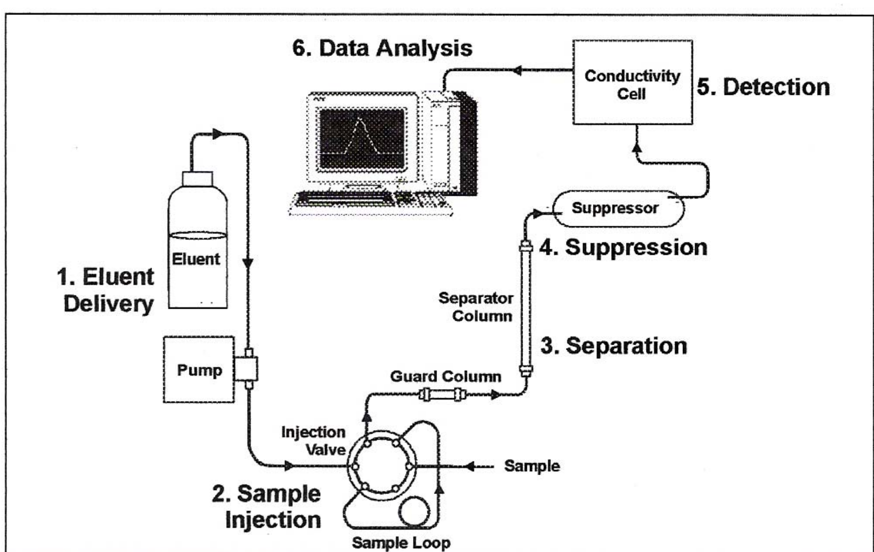

Figure 5: ICS2000 system overview diagram (with permission from Dionex Corporation[42])

1.4.2.1. Eluent Delivery

The system was supplied with a Millipore water reservoir continuously degassed by Nitrogen. All other eluent generation was done by the system itself using a concentrated reservoir of NaOH in the Eluent Generator Cartridge (EGC40) [Figure 6]. The EGC operates using the same electrochemical ideas as previously mentioned for the suppressor.

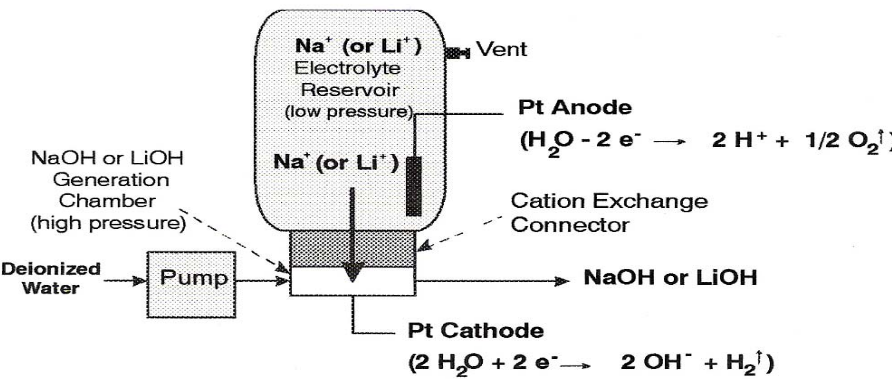

Figure 6: Principle of operation of EGC40 (with permission from Dionex Corporation[43])

The eluent solution output from this system is cleaned of impurities by a trap column, in this case the CR-ATC [Figure 7]. This removes a certain amount of the dissolved carbonate, although it is not a 100% removal. Figure 8 is a chromatogram showing the remaining carbonate appearing on the elution method used for the nitric oxidation samples.

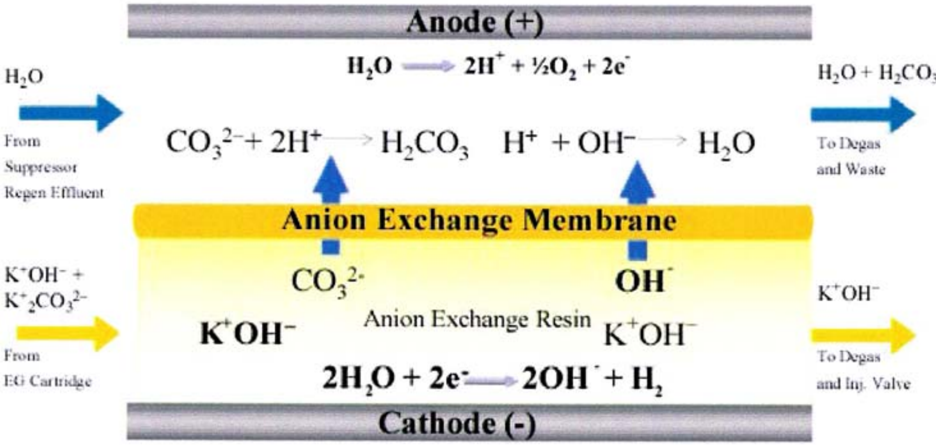

Figure 7: Principle of operation of the CR-TC trap column (with permission from Dionex Corporation[44])

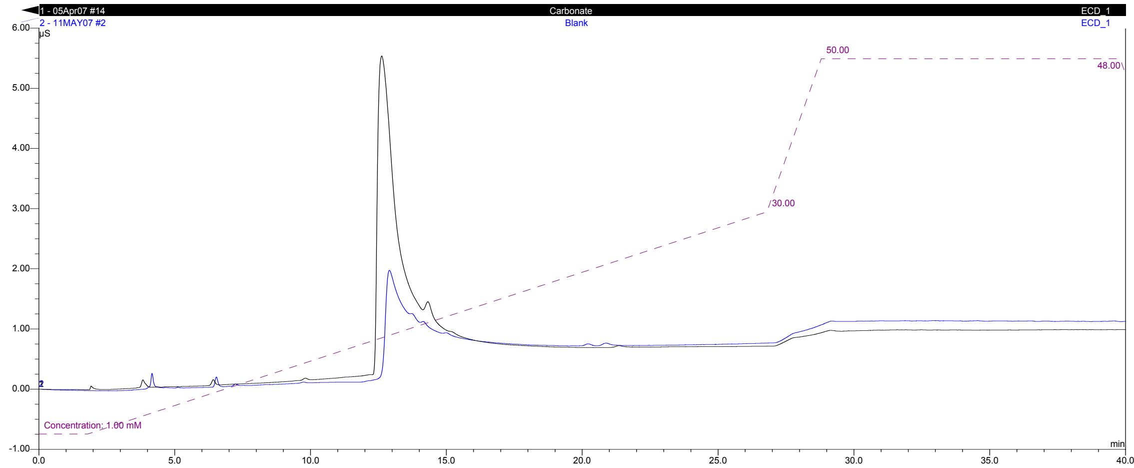

Figure 8: Carbonate peak present at 13min: (blue) average blank water sample run, (black) Blank water sample spiked with sodium carbonate.

1.4.2.2 Sample Delivery

The Injection valve is a simple Rheodyne 6-port, electrically activated valve. Samples can be injected directly into the valve with a syringe or by means of an attached auto-sampler.

1.4.2.3 Separation

Full details on separation are included in Chapter Three. The guard column (AG11-HC 4mm) and the analytical separation column (AS11-HC 4mm) are enclosed in a heat controlled chamber to ensure reproducibility of results. This also allows for manipulation of the retention times with warmer conditions corresponding to slightly shorter elution times. The AS11 column has quaternary ammonium functional groups. An Alkanolamine functionalized resin is based on ethylene-vinylbenzene (EVB) cross-linked to a 55% divinylbenzene (DVB). This type of exchange resin is hydroxide-selective and is particularly suited to use with hydroxide ion eluents[45]. HC stands for High Capacity, the AS11-HC 4mm, has a column capacity of 290µeq/column compared to 45µeq/column for the AS11 4mm[46, 47]. Thus the column is well suited to solutions where one or more components may be present in much higher concentrations than others, allowing analysis without needing detection limiting dilutions.

1.4.2.4 Suppression

This part of the system is the most important with respect to the utility of Ion Chromatography; the component turns the highly charged background of NaOH eluent into water. A de-ionised water background means that the only significant signal should proceed from the eluting analytes. Details of this system have already been covered in Section 1.4.1 and Figure 4.

1.4.2.5 Detection

The analytes are detected with a DS6 Heated Conductivity Cell. The void volume of the system at the typical run rate of 1.5mL/min is 2.7mL from injector to detection.

1.4.3 Expanding Applications

Due to the versatility and reliability of the basic procedure, the uses of Ion Chromatography systems are continually expanding, with new columns and more efficient and advanced equipment. Some other recently reported applications include:

- Measuring trace levels of atmospheric molecules in Antarctic ice cores[48]
- Separating Single-walled Carbon Nanotubes according to their specific electrical properties[49]
- Isolating the cellular fuel molecule adenosine triphosphate, using a stationary phase with a similar structure to ice cream[50]

1.5 Previous IC work with Organic acids

Much has been written about the use of IC in both organic and inorganic ion analysis. Separations have been performed on various classes of the less complex organic acid anions, usually in conjunction with study of one specific type of analyte solution. There are two rough ‘types’ of anion specific ion chromatography, pure ion-exchange as in the system described in this study and ion-exclusion which can be considered a direct opposite to ion-exchange. In ion-exclusion, the elution order is mostly reversed compared to from ion-exchange with the smaller, more negatively charged anions eluting first. Neutral molecules which elute with the void volume in ion exchange normally elute last in ion exclusion. This method applies acidic eluents and utilizes negatively charged stationary phases, commonly sulphonated resins. Strong acids generally elute before weak acids and the effect of eluent concentration is opposite to that in ion-exchange[51]. An increase in concentration of the acidic eluent causes the weak acids to be less dissociated, decreasing their exclusion from the stationary phase and increasing their retention times.

In some applications both of these methods are used simultaneously to fully characterize a mixture containing several co-interfering ions. One such application was the analysis of fountain solutions used in the printing industry[52]. The target analytes were mono-, di-, and hydroxycarboxylates, alkylbenzenesulfonates and polyphosphates. The system included ICP-AES analysis for the determination of total sulfur and total phosphorus. Ion exchange quantified the inorganic anions and the alkylbenzenesulfonates as well as some of the organic carboxylates. Comparison of peak areas and identification between the ion-exchange and ion-exclusion results allowed the quantification of all other analytes. This system is very effective for the type of mixed component solutions presented to it but it would be overkill on the relatively simpler organic acids and few inorganic ions in the nitric acid oxidation mixtures.

Ion exclusion has been used for oxidation mixtures as well[53]. The Fenton oxidation process is used for the oxidation and breakdown of organic pollutants. The analysis of

this process is important as some oxidation compounds may be more toxic than their precursors. Ion exclusion chromatography was considered for the analysis because of the potential organic/inorganic analyte interference possible with ion-exchange chromatography. Oxalic and maleic acids presented continuous analysis challenges with all of the eluent systems tested. In addition, high fronting of the oxalic acid peak caused it to merge even more with the peak from the solvent front at the void volume. An anion-exchange method had to be appended to the analysis for quantitative determination of oxalic and maleic acids.

Ion chromatography has been used to monitor and control fermentation processes where accurate information about the composition of the fermentation broths is important[54, 55]. The water from a drinking water plant has been analysed simultaneously for organic and inorganic anions using a classic ion chromatography system comprising little sample preparation besides filtration, direct injection and fast analysis with a high pH eluent and conductivity detection. Ion chromatography has been called the "method of choice"[56] for environmental water samples.

Other detection methods besides conductivity have been applied. The Dionex AS11 column, the same column used in this method development, was paired with electrospray ionisation mass spectrometry (ESI-MS) for the analysis of 18 aliphatic (poly)hydroxy (poly)carboxylic acids including D-glucaric and D-gluconic acids[57]. These acids were considered environmentally important and the method provided a reasonable separation of most of them. Some poorly resolved peaks could be improved by adding modifiers to the eluent. The method proved to allow for sensitive detection, identification and quantification of all analytes tested.

Sarzanini and Bruzonitti have published several studies on the retention behaviour of carboxylic acids in ion chromatography and created retention models for a carbonate eluent system[29, 58, 59]. There are some differences between a hydroxide eluent's retention order on the same column as used in ref 59 however the basic theory is similar enough that these papers were used as a guideline for the analysis detailed in Section 2.1.1.

2. Results and Discussion

2.1 General Organic Acid Behaviour

The retention behaviour of several simple carboxylic acids was studied in order to provide context for the analysis of the nitric acid oxidation. This study was made to provide a guide to the expected behaviour of such anions in the investigated system, with the intention of being able to predict and understand retention orders and give direction to characterising unknowns.

The largest anion studied was the mellitate ion; the smallest anion was formate. Structures of all of the acids eluted by the column are included in Appendix One. (The structures of the relevant inorganic anions are included, e.g. nitrate, nitrite, carbonate)

2.1.1 Results and basic empirical rules

Initially, it was felt that the acidity of a compound measured as its pKa would be directly relatable to the compound's retention time (RT). A partial list of pKa values is included in Table 1 mainly to show that there is no direct correlation between retention time and pKa. This is principally due to the nature of the eluent; strongly alkaline NaOH ensures that all compounds would be completely in their conjugate base form making the compounds ability to sustain that form (i.e. pKa) irrelevant. However, factors that influence the pKa of a compound, such as induction or resonance, could have an effect on the retention times for different reasons. A factor which affects pKa usually does so through a movement of electron density towards or away from the acid centre (carboxylate group). This electron movement could have effects on that carboxylate's interaction with the stationary phase of the column.

The column interacts with the eluting molecules in several ways; the two interactions with the most direct effect on retention time are electrostatic interactions of charged entities and hydrophobic/hydrophilic interactions with the stationary phase and mobile phase.

Table 1 is a list of eluted compounds in their order of elution. The method of elution used to obtain these times is a constant 1mM/min increase of NaOH concentration starting at 2mM. The entries are identified by font as to the broader group of organic acids into which they fall; **Bold** is di-acid, *Italics* is a mono-acid and Normal font is the acids attached to rings. ***Citric acid*** is in bold italics since it is the only straight chain tri-acid on the list.

The reported times in Table 1 are all for single component sample runs. The reported variance in retention time of 0.2min is due to varying conditions; particularly that of backpressure differences between days. Peak identification in a mixture is not usually affected as all peaks change by the same amount.

Table 1 : Elution Order List of eluted compounds

Acid	Elution Time (RT)	NaOH	pKa where available
	(min)±0.2	(mM)	±0.2
xylonic acid	3.79	2.99	
D-gluconic acid	3.91	3.11	3.86
4-hydroxybutyric acid	3.93	3.13	4.72
quinic acid	3.94	3.14	
3-hydroxy butyric acid	3.96	3.16	4.70
mannonic acid	4.00	3.20	
3-hydroxy propionic acid	4.02	3.22	
lactic acid (2-hydroxy prop. acid)	4.07	3.27	3.86
glycolic acid	4.35	3.55	3.83
acetic acid	4.37	3.57	4.76
levulinic acid (4-oxopentanoic acid)	4.42	3.62	4.59
formic acid	4.75	3.95	3.75
propionic acid	4.79	3.99	4.87
2-keto- D-gluconic acid	4.89	4.09	
isobutyric acid	5.00	4.20	4.9
oxalacetic acid	5.21	4.41	2.22, 3.89
pyruvic acid	5.23	4.43	2.39

Acid	Elution Time (RT)	NaOH	pKa where available
	(min)±0.2	(mM)	±0.2
butyric acid	5.29	4.49	4.83
crotonic acid	5.97	5.17	4.74
3-chloropropionic acid	6.27	5.47	
glyoxalic acid	6.36	5.56	3.30
monochloroacetic acid	6.47	5.67	2.85
D-glucuronic acid	6.59	5.79	3.30
2-furoic acid	8.08	7.28	3.17
5-chlorovaleric acid	8.53	7.73	
hexanoic acid	8.97	8.17	
5-keto- D-gluconic acid	9.57	8.77	3.24
3-bromopyruvic acid	9.85	9.05	
o-chlorobenzoic acid	12.32	11.52	2.94
glutaric acid	12.82	12.02	4.34, 5.27
adipic acid	12.97	12.17	4.41, 5.28
D-glucaric acid	12.99	12.19	2.99
malic acid	13.10	12.30	3.40, 5.05
xylaric acid	13.11	12.31	
succinic acid	13.13	12.33	4.21, 5.64
benzoic acid	13.13	12.33	4.18
mannaric acid	13.46	12.66	
tartaric acid	13.76	12.96	2.98, 4.34
malonic acid	13.79	12.99	2.86, 5.70
maleic acid	14.64	13.84	2.00, 6.50
tartronic acid	14.81	14.01	2.37, 4.74
p-anisic acid	15.48	14.68	4.47
mesoxalic acid	16.04	15.24	
oxalic acid	16.06	15.26	1.27, 4.27
m-toluic acid	17.99	17.19	4.27
p-toluic acid	18.13	17.33	4.37

Acid	Elution Time (RT)	NaOH	pKa where available
	(min)±0.2	(mM)	±0.2
syringic acid	21.64	20.84	
trichloroacetic acid	21.91	21.11	0.77
phthalic acid	21.92	21.12	2.98, 5.28
vanillic acid	22.24	21.44	4.52
p-hydroxybenzoic acid	22.96	22.16	4.58
3-nitrophthalic acid	25.18	24.38	
citric acid	26.22	25.42	3.13, 4.76, 6.40
p-chlorobenzoic acid	29.29	28.49	3.98
salicylic acid	31.53	30.73	2.97
1,2,3-benzenetricarboxylic acid	36.70	35.90	
1,2,4-benzenetricarboxylic acid	37.60	36.80	
1,2,4,5-benzenetetracarboxylic acid	53.06	52.26	
mellitic acid (hexacarboxylic benzene)	60.59	59.79	
3, 5-dinitrosalicylic acid	above 66mM		

3, 5-dinitrosalicylic acid has not been given a reported elution time as the yellow colouration of the compound only appears in the exit stream after the method has finished; during the high concentration rinse. A response peak was not able to be identified for certain. Further refinements to find an identifiable retention time were not undertaken since the main focus of the investigation; the compounds in the nitric acid oxidation mixes, all eluted before 60mM.

The behaviour of 3, 5-dinitrosalicylic acid appears very different to all other compounds covered. The nitro groups make the compound a very strong acid and also present extra concentrations of negative charge comparable to the benzenetricarboxylate ions. However there must be other factors involved as 3, 5-dinitrosalicylic acid appears to elute far later than both these acids and the tetra- and hexa-benzenecarboxylate ions.

Combination samples were also made up and used to study the interaction and separation of closely eluting compounds. The multi-components samples were chosen deliberately to allow for the maximum possible separations of peaks; e.g. about every third compound from the list below. Also those compounds showing strong tailing or aberrant behaviour were avoided. The largest combination contained 21 compounds. On the standard run, 17 compounds could be identifiably separated within 40 minutes with 11 of the analytes having baseline resolution [Figure 9].

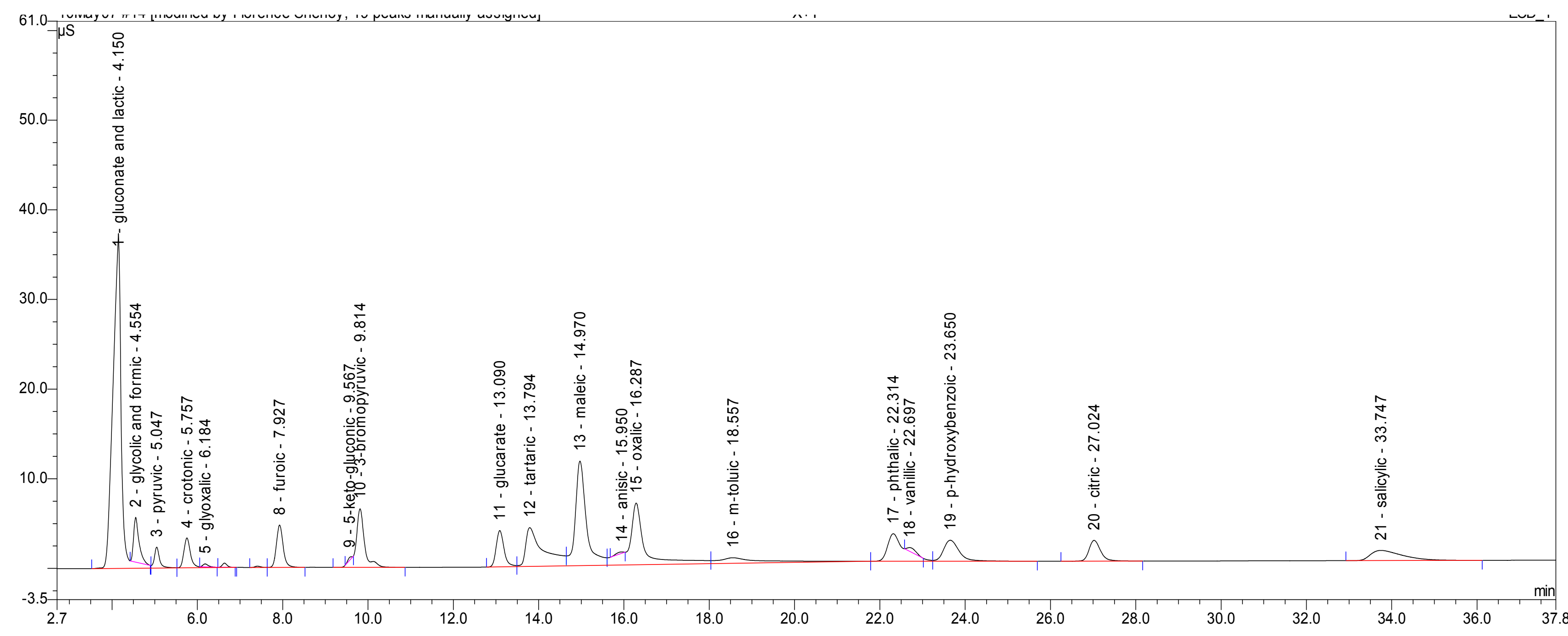

Figure 9: Combination sample run of Organic acids

The behaviour of isomers varied as to the type of isomers studied. Two of the three structural isomers of toluic acid were indistinguishable; *o*-toluic was not available to be compared [Figure 10]. The similarity in these isomers' RT can be explained by the minimal difference between their pKa's (4.27 and 4.37). The effect of the methyl group changes very little between *p*- and *m*- position. Therefore any other factors affecting RT would change little as well.

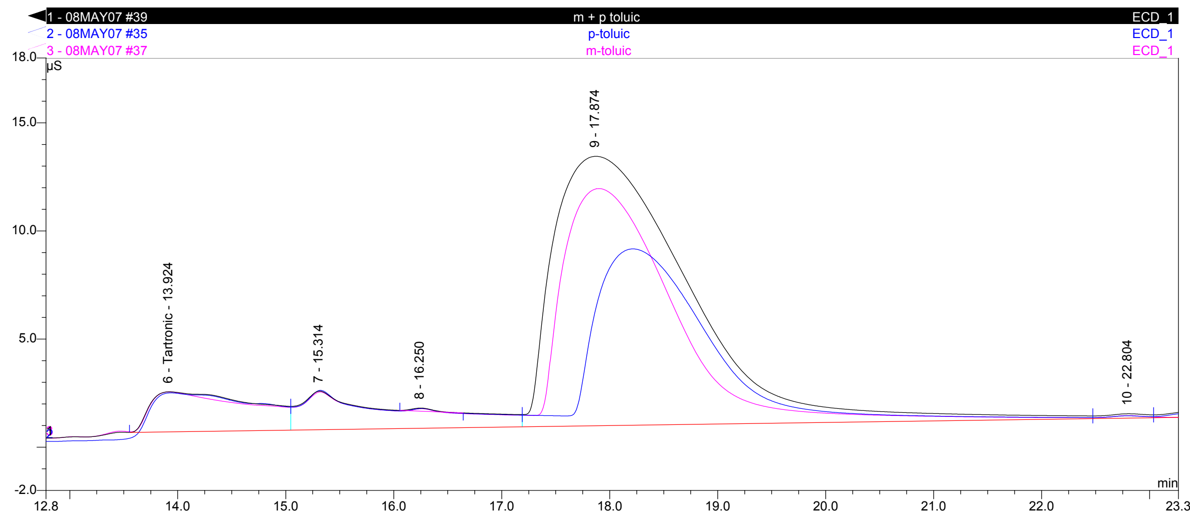

Figure 10: The isomers of toluic acid. Black line is a mixture, with *p*-toluic (pink) and *m*-toluic (blue) also run separately.

However, the diastereoisomers D-glucaric and mannaric acid could be separated at most concentrations [Figure 11]. The only difference between these compounds is the orientation of one hydroxyl group which changes the conformation of the molecule. D-Glucaric acid has a sickle conformation but mannaric acid prefers to be in double or even triple sickle conformation[60]. Xylaric and D-glucaric acids have a similar conformation overall even though xylaric acid has one less CH_2OH group. These two molecules have less differentiability than may have been expected if simply considering the size difference. [Figure 12]

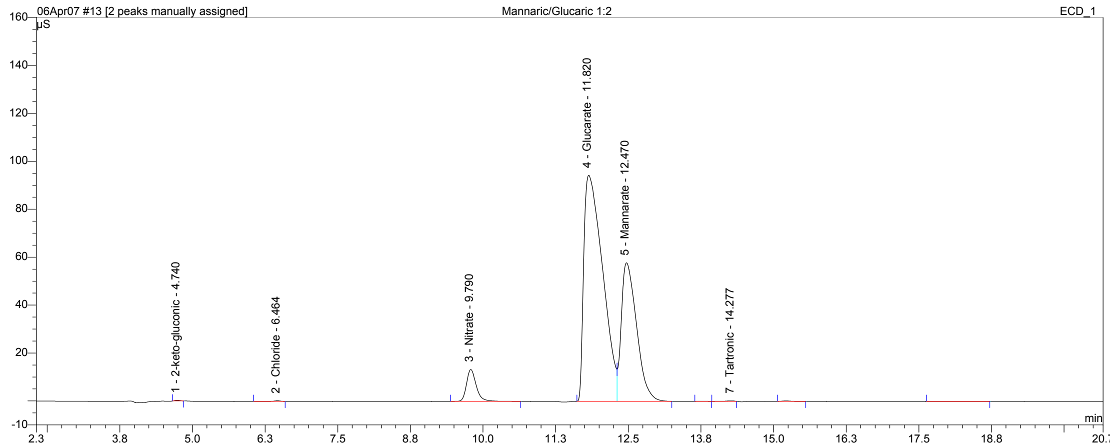

Figure 11: Mannaric acid and D-Glucaric acid mixture, showing the differentiability of the isomers.

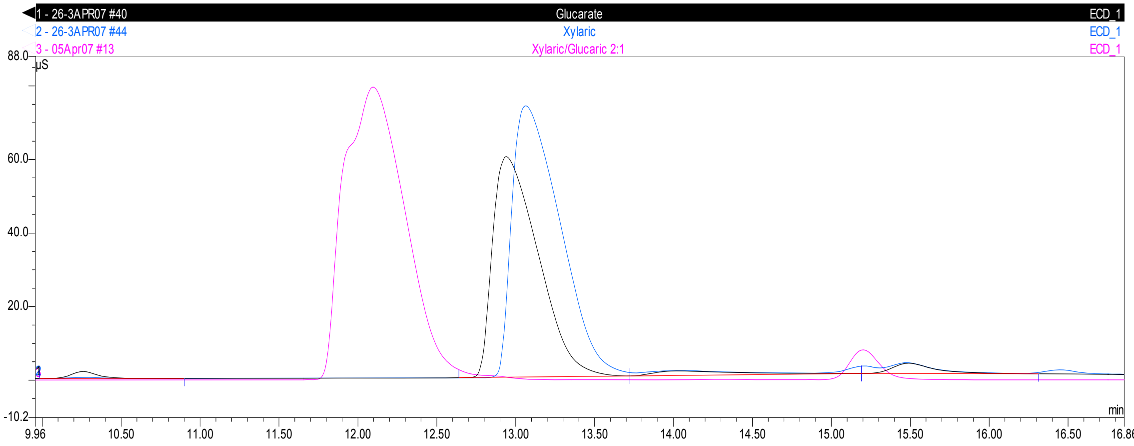

Figure 12: Xylaric and D-glucaric acid comparison, (black) glucarate, (blue) xylarate, (pink) mixture run on a slightly earlier gradient.

The two carbon compounds eluted in the order:

acetate 4.3min, glycolate 4.3min, glyoxalate 6.4min, monochloroacetate 6.5min, oxalate 16.1min, trichloroacetate 21.9min

These six compounds show most of the retention difference effects caused by modifying the same base structure. The acetate and the glycolate ions very nearly co-elute, the hydroxyl group has almost no effect on the retention of the glycolate ion.

This is most likely due to two effects competing to change the retention time in opposite directions. The hydroxyl group can interact by hydrogen bonding with both the aqueous mobile phase and with the alkanol groups on the column. In this molecule's case the two effects essentially cancel out.

The inductively withdrawing chloride group on the monochloroacetate is a large contributor to the 2mM difference from acetate of the strength of NaOH necessary for elution. The induction draws the electron density towards the chlorine. This could be expected to weaken the attraction of the carboxylate group to the column by decreasing its negative charge. However, because the retention time increases, an increase in column interaction must occur. Since some of the electron density has moved onto the chlorine, one could consider the chlorine as another centre of negative charge, which could then interact with the column on its own. This would create a sort of bidentate analyte similar to the di-acid oxalic acid. The inductively charged chlorine is obviously a much weaker negative charge than the second carboxylate on oxalic acid and this can be seen in its earlier retention time. This Inductive Effect can be seen even more exaggerated in the much longer retention of trichloroacetate, a mono-acid compound which elutes far later than all the di-acids. In this case, the electron density would be almost solely concentrated on the three chlorines, suggesting that they and not the carboxylate group would be the primary interactors with the column.

Inductive effects fall off with distance from the acid centre and this could be taken as the main reason that 3-chloropropanoate needs only an increase of 1.5mM NaOH strength to elute compared to propanoate. A halide substituent has the same effect of lengthening retention times on all straight chain variants tried. Another contributor to the lessened effect of the halide substituent could be conformational. The added carbons between the two charged centres make it harder for them to bind simultaneously to the column. By the time the chain length increased enough to allow a curved bidentate interaction, the halide would be too far from the carboxylate group to be effectively inductive.

In comparison, the effect of this substituent varies depending on position, *ortho*- (*o*-), *meta* (*m*-) or *para* (*p*-) when placed on an aromatic ring. This adds to the theory that it

is an inductive effect as the impact of an inductive group changes nature and strength based on its relative position on an aromatic ring. The rationale of why this induction has an effect on RT is because a highly electronegative chloride group could be attracted to the stationary phase adding to the retention time on its own. Induction plays a part in this explanation by causing the delocalization of negative charge onto the chlorine which would enhance its negative character. Distance would have the effect of lessening the delocalisation and lessening the change in retention time.

An interesting aromatic example of several effects occurring at once is the retention difference between benzoic acid and two of its halogenated derivatives. *o*-Chlorobenzoic acid elutes at *ca*. 12mM NaOH whereas *p*-chlorobenzoic acid elutes at *ca*. 28.5mM; a difference of more than 15mM. (Figure 13) Benzoic acid elutes at 12.3mM.

Cl O $^{-}$O vs. $^{-}$O O Cl

Figure 13: o-chlorobenzoic acid vs. p-chlorobenzoic acid

A phenyl group has a resonance donation effect when considered as a substituent. It can donate electron density to any suitable group which is co-planar with it[61]. This should increase the negative character of the carboxylate moiety in benzoic acid and increase the RT. Compared to acetic acid (3.57mM) benzoic acid does show a marked increase in RT.

In the chlorobenzoic acids' case, chlorine has two effects acting *on* the phenyl group; it is inductively withdrawing but also has an electron-donating resonance effect. Then there is the consideration of chlorine as another concentration of negative charge. The inductive effect dominates for the *ortho*- version because the chlorine is close to the centre of charge. The *Ortho* Effect is based on steric hindrance; resonance requires a co-planar molecule and the bulky chlorine can push the carboxylate group out of alignment[61]. The inductive effect alone in this case results in very little change from benzoic acid, the retention times are within the variation range of each other. The *p*-chlorobenzoic acid experiences such a large change in retention characteristics

because all three theorized effects could be acting. The two distance effects (induction and resonance) combine to move the electron densities out towards the two substituents on the benzene ring. Induction pulls electron density towards the chlorine at the same time that it donates electron density to the carboxylate by resonance. Therefore the largest retention effect must be the presentation to the column of two strongly negative charge centers.
p-Hydroxybenzoic acid (22.2mM) on the other hand is mainly an example of hydrophilically enhanced interaction with the stationary phase; both the aromatic centre and the hydroxyl group increase the retardation effect by the stationary phase. The previous application of induction and resonance effects cannot be used to explain the increased RT of this molecule because the hydroxyl's inductively withdrawing effect is overpowered by its electron donation with resonance. This interplay of effects only produces some movement of electron density onto the carboxylate group which may play a small part in the increased RT.

A general rule for diacid vs. monoacid retention can also be seen in the two carbon series; due to the larger negative charge concentration; the oxalic anion (16min) has a predictably longer retention time than the acetate (4.4min). The increase in retention time is predominantly due to the greater strength of the interaction of oxalate with the positively charged stationary phase. The aromatic acids (those including rings) elute on average later than both the mono- and di-acids. This is caused by the interaction of the aromatic rings with the hydrophobic styrene backbone of the stationary phase (see Section 1.4.2.3).

In the aliphatic di-acid series; $HOOC(CH_2)_nCOOH$, n = 0 to 4, the retention time decreases as n increases. This is attributed to the decreasing polarity of the molecules as the distance between the acid centres increases. The time difference between n = 0, oxalic acid and n = 1, malonic acid is the greatest of the series. The intervals decrease as the chain lengthens, showing that the effect becomes less pronounced.

The mono-acid series CH3(CH2)nCOOH, n = 0 – 2, 4 shows the opposite response; as the length of the aliphatic chain increases, so does the interaction with the

hydrophobic backbone of the stationary phase; increasing the retention times. The retention time for n = 3, pentanoic acid can be inferred from its chloro derivative, 5-chlorovaleric acid. The chloro- group is isolated enough to have very little effect on the retention time. If this holds then pentanoic acid fits into the trend identified above. Also as n increases the interval between it and n-1 is greater.
The effect of hydroxyl groups on these two series is also opposite. For mono-acids; D-gluconic acid is the fully hydroxylated version of hexanoic acid and the addition of so many hydrophilic groups removes the interaction with the column substrate and creates an increased affinity with the aqueous mobile phase, meaning that D-gluconic acid's retention time is one of the earliest, a decrease of about 5 minutes or 5mM NaOH. For di-acids; the change to a fully hydroxylated aliphatic chain increases the retention time as it increases the polarity of the molecule and increases the hydrogen-bonding interactions between the molecule and alkanol groups on the column; partially counteracting the effect attributing to the faster elution mentioned earlier.

A carbonyl group generally increases the retention time; the most appropriate example of this is the difference between D-gluconic acid (3.9min) and its two carbonyl derivatives; 2-keto- D-gluconic acid (4.9min) and 5-keto- D-gluconic acid (9.6min). The carbonyl group interacts with the alkanol moiety on the stationary phase through H-bonding. One exception to this is the decrease in retention time shown when adding a single carbonyl group to succinic acid. This could be caused by the delocalization of the negative charge on the carboxylate ion to be shared with the carbonyl group. This would present a more diffuse charge concentration to the substrate, decreasing its attraction.

All of the described elution orders in this section were based on a pure and continuous gradient system. Some improved separation of specific molecules can be achieved with isocratic analysis, e.g. using a 45mM isocratic run, *m*-toluic and *p*-toluic could be eluted *ca*. 2 minutes apart. In the isocratic case, the affinity for the eluent has a greater effect; whereas, starting from a low value as with gradients, puts stationary phase interactions into dominance.

2.2 Nitric Acid Oxidation of Sugars

The object of this method development was to characterise the reaction products during the course of the reaction and the work-up steps. A main focus was on refining the identification and quantification of unused reactant and by-products. Calibrations and results are quoted in ppm, representing in this study µmol/mL concentration.

2.2.1 Method development

The starting method was a simple 15 minute isocratic run at 30mM NaOH tested on monopotassium glucarate, 5-keto D-gluconic acid, tartaric acid and oxalic acid. This elution method did separate the four components that were used for the initial study, however it was found to be inadequate to sufficiently separate the full complex mixture; the glycolate and D-gluconate ions co-eluted at the dead volume. A gradient system was judged to be the better avenue for study. A series of runs were made to determine the highest concentration at the start of the elution that would still allow adequate separation between the glycolic acid and D-gluconic acid peaks. These peaks could be separated at the 5mM NaOH concentration.

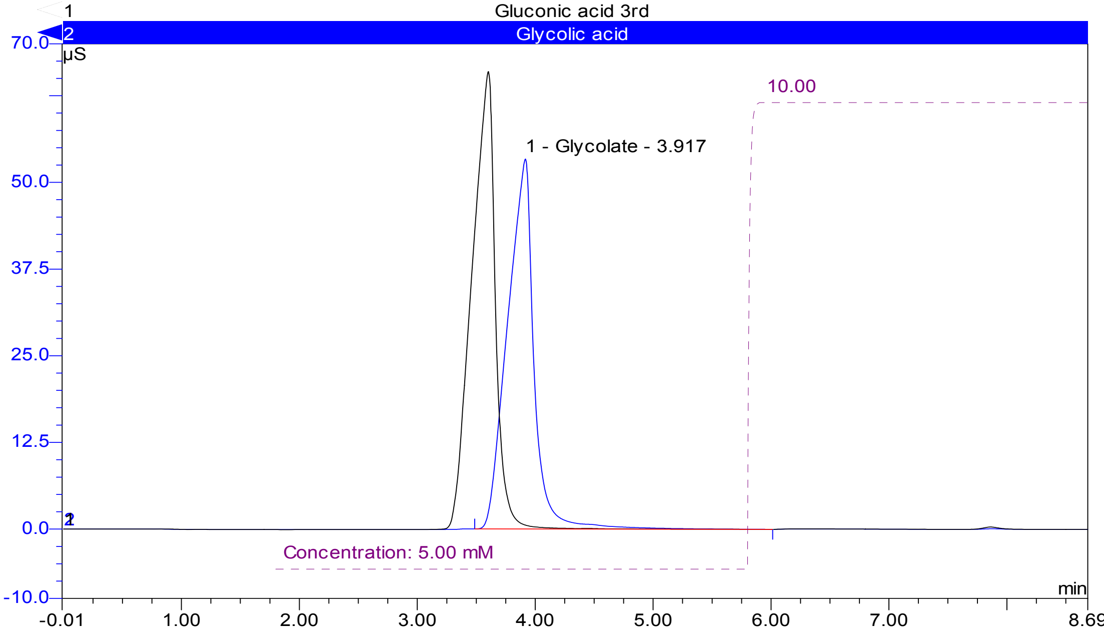

Figure 14: Overlaid chromatographs of glycolic acid (labelled) and D- gluconic acid (unlabelled peak) showing gradient conditions

From Table 1 in Section 2.1.1, it can be seen that the longest retained anion of interest in this reaction is oxalic which elutes at ~15mM NaOH concentration. To provide certainty that all analytes are eluted, this number was doubled to provide the upper concentration value for the elution gradient; 30mM NaOH. A high concentration 'rinse' was added to the elution method so ensure that all potential contaminants were flushed off the column before the next sample. The final elution method is shown [Figure 15] as a graph of eluent concentration against time. The graph shows equilibration at 1mM NaOH for 4 minutes with sample injection occurring at t = 0; followed by a ramp up to 30mM over 25 minutes, then a sharp rise over 2 minutes to the rinse concentration of 50mM which is held for 10 minutes before returning to 30mM over 2 minutes. The complete method takes 45 minutes including autosampler injection. Figure 16 shows a typical sample of the end products of the oxidation analysed by the elution method. Six of the seven expected major components are present and clearly identified.

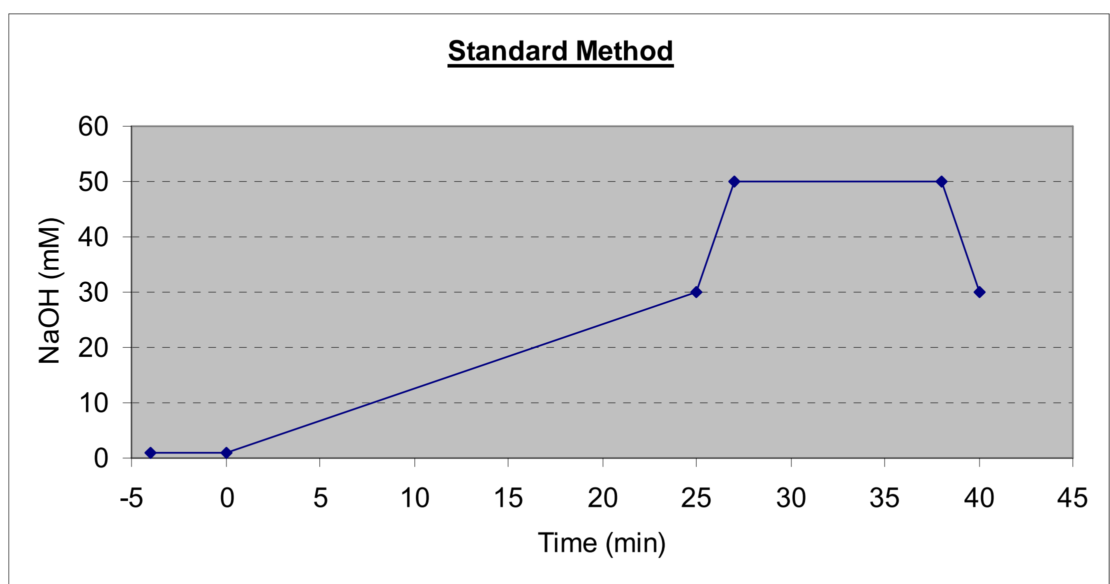

Figure 15: The Standard method used to elute the majority of reported oxidation mixtures, shown as a function of NaOH concentration (mM) against run time (min).

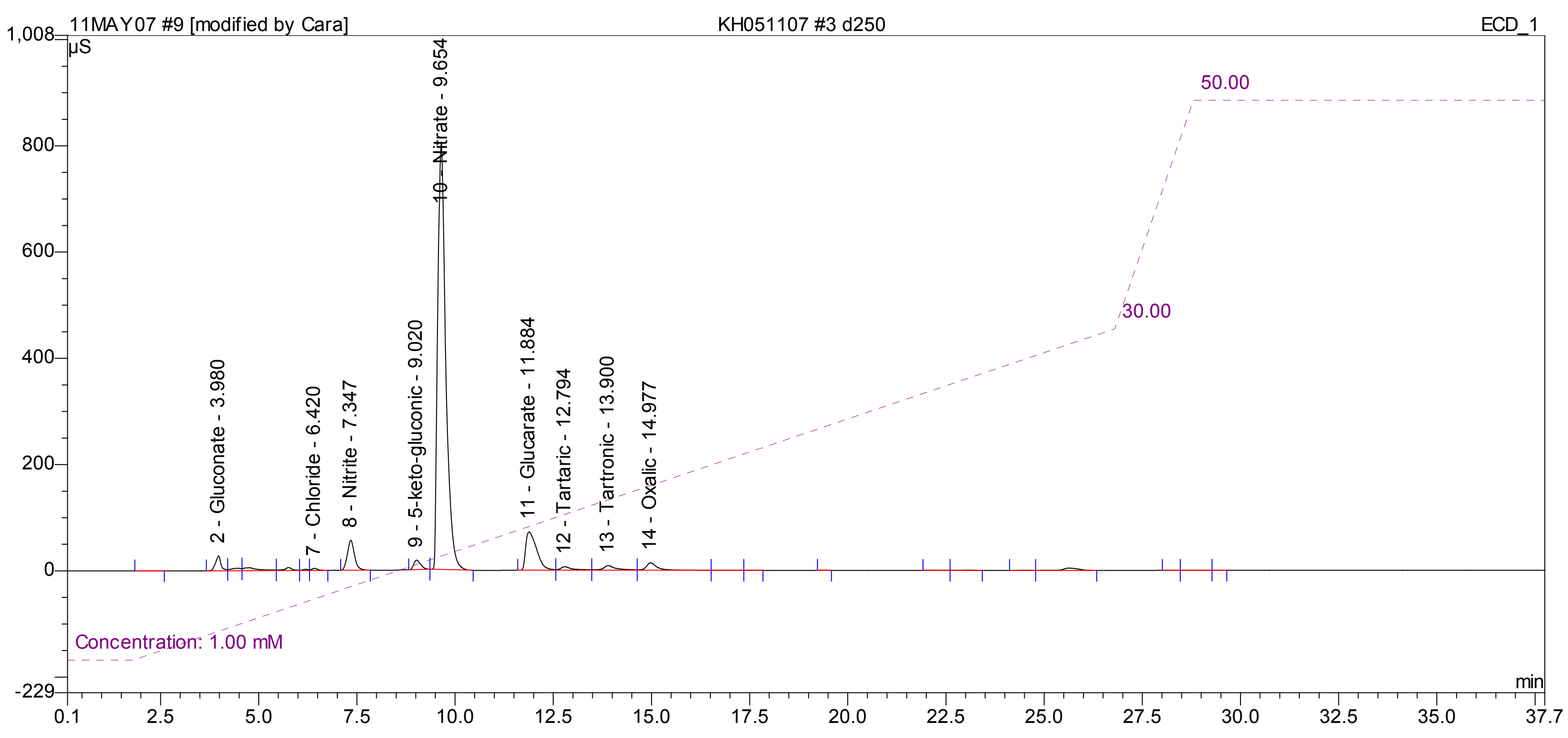

Figure 16: An example of a typical D-glucose oxidation sample showing the gradient overlaid. KH051107 #3 d250 run on 11 May 07 #9

Early test runs established the elution order and behaviour of the important analytes. 2-keto- D-gluconic had been proposed as a by product of the reaction, however no identifiable amount was found in the initial samples and so it was not included in the calibration. It was never identified as a component in any D-glucose oxidation. product mixture.
5-keto- D-gluconic was identified as being present in the D-glucose oxidation mixture. It presented as three peaks, proposed to be the straight chain and the two furanose ring forms. The first eluting of these peaks was the largest and baseline resolved from all surrounding peaks. It was taken to be the straight chain version, as the ring forms would be at lesser concentrations and based on section 2.1; have a greater retention time. Over several runs and tests, the ratio of the three peak areas remained constant allowing the resolved peak to be used on its own for calibration. The low level of the 5-keto- D-gluconic acid present meant that the two peaks which interfered with other analyte positions were very small and were not included in the quantification method. The inter-conversion between D-glucose and its lactone forms might have been thought to produce multiple peaks as with 5-keto- D-gluconic acid. However, samples of D-glucaro-6,3-lactone were tested on the system early in the process and consistently appeared in the same place as the monopotassium glucarate standard. The theory was that the highly basic solution promoted the hydrolysis of the lactone and prevented the straight chain from lactonising. This was recently proven by NMR determination of the D-glucaro-lactones in dilute alkaline solutions [62]. The lactones opened out to the straight chain extremely quickly in reasonably alkaline conditions (pH >13). This single compound elution is an advantage as it allows for the complete quantification of the main analyte, D-glucaric acid from a single peak rather than two or even three as with HPLC.

An interesting reversal of retention order occurred upon the change to a gradient system. In the isocratic and stepped gradient runs; nitrate eluted after the aldaric acids, D-glucaric and xylaric acid, whereas in the ramp gradient elution the order was reversed. When nitrate eluted before the aldaric acids, the interval between the two peaks increased by more than a minute; improving the resolution; nitrate also eluted

in a gap in the organic acid peaks in this case. When nitrate eluted after the aldaric acids, there was a large overlap with the tartaric acid peak. (Figure 17 to Figure 19)

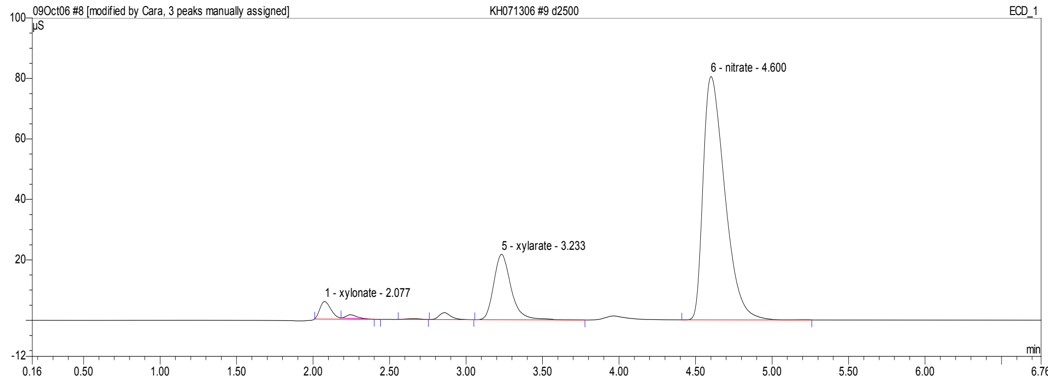

Figure 17: A xylose oxidation reaction mixture eluted with isocratic 30mM. Nitrate elutes after xylarate.

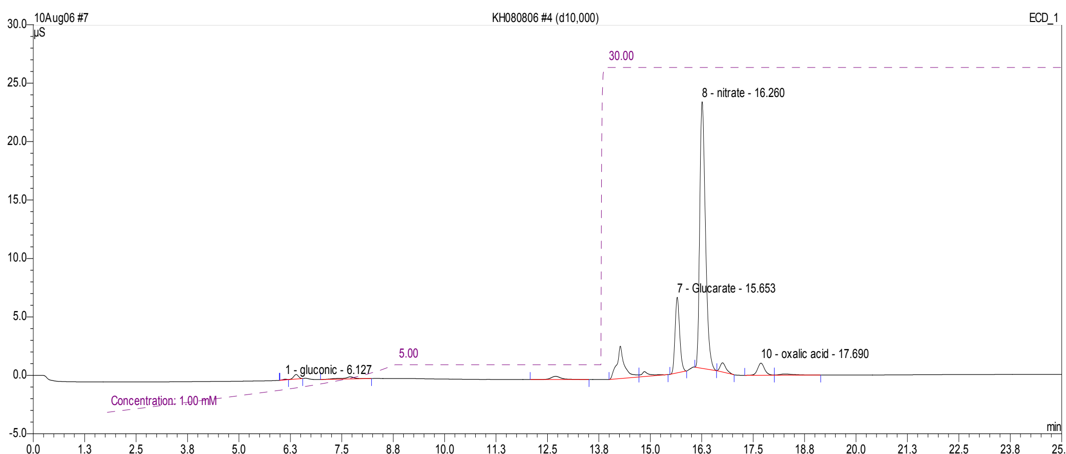

Figure 18: A D-glucose oxidation run at high dilution with a stepped gradient, nitrate elutes after glucarate and co-elutes with an unlabelled tartaric peak.

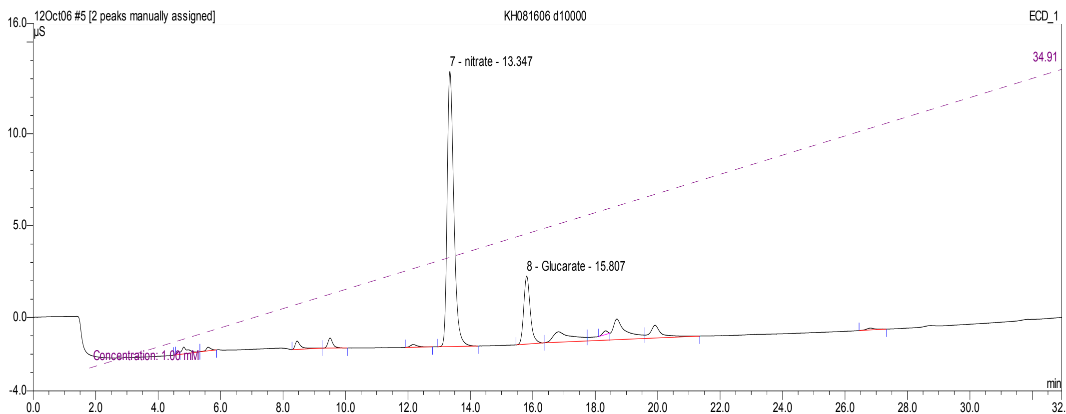

Figure 19: A D-glucose oxidationrun at high dilution with a ramp gradient, nitrate elutes before glucarate and apart from any significant analyte peaks.

The presence of carbonate in the eluent appeared as an uneven rise in the baseline (Figure 20). The presence of this carbonate peak rather than a constant contribution to the baseline is due to the gradient profile. The start of the method is a low enough concentration that the carbonate ions can accumulate on the column to be finally flushed out once [OH^-] becomes high enough to flush them out[57]. The sudden step up in the baseline after the peak, unrelated to an equal raise in eluent concentration, can be explained as the carbonate contribution as the suppressor is optimised for neutralisation of the hydroxide concentration.

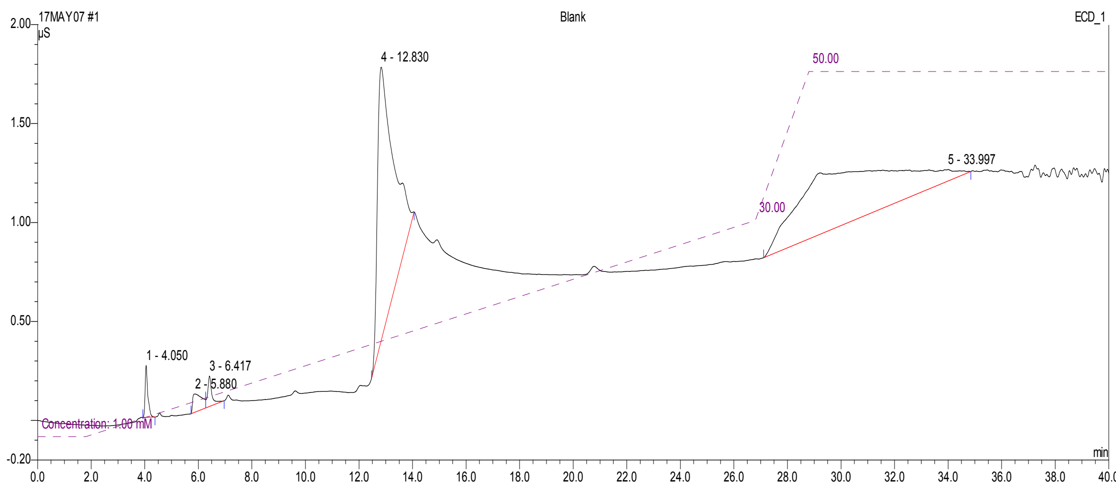

Figure 20: Blank matrix sample run showing the carbonate 'peak' at 12.8min. (maroon) gradient shown as overlay.

The water reservoir was placed under nitrogen to slow the absorption of carbon dioxide; this reduced the peak size by roughly 50%. Other sources such as small amounts in the eluent generation set-up and more significant amounts in the samples are more difficult to address. A trap column is included in the eluent generation set-up and is designed to remove most of the carbonate from that source (section 1.4.2.1). The carbonate in the samples is harder to manage without introducing excessive complication to sample preparation. Reasonable attempts were made to limit the time a sample remained exposed to the air. However, some carbonate was actually expected within the reaction mixture as the final oxidation result.
Quantification was not unduly affected by this carbonate artefact as a cancellation effect was included by an automatic subtraction of blank 'matrix' samples.

2.2.2 Quantification/ Calibration

Calibration was performed entirely by external standards and managed through the Chromeleon software. The use of combination standards was dictated by the software constraints and the need to include possible interactions and interferences from other analytes. As a direct consequence of this need, each standard was made of analytes in varying concentration rather than creating standards that had all components at the same concentration. For example making nitrate be the same concentration within the standards as the organic acids would be unwise as the majority of samples would have nitrate at a level at least twice the concentration of the organic acids. An estimate of the ratio of expected components was created. This was partly based on early unquantified samples of the reactions.

After the first set of standards was applied, these calculations were refined with subsequent standards to create standards that mirrored even more closely the analytes. The only exception to this matching was acidity; no attempt was made to control the pH of the standards although the oxidation reaction samples were all measurably acidic. This was due to the decision not to introduce another anion such as chloride or sulphate in with the acid. Nitric acid was considered but the need for very precise concentrations of nitrate, and the easier attainment of that precision with sodium nitrate meant that nitric acid was not fully studied.

Values of pH higher than 2 appeared to have little effect on the eluting analytes although very acidic (pH ≤ 1) samples showed decreases in retention times. Oxidation samples had a pH >1 once diluted at least 100-fold, so the pH level was not considered to be a factor. In addition, the majority of other samples presented for analysis were neutral or only weakly acidic.

Table 2 shows the Chromeleon retention table used for identification and quantification. Chloride, nitrite and 2-keto- D-gluconic acid were included simply for identification of peaks that may occur in the mixture occasionally.

Table 2: Glucose Oxidation Peak identification table

Peak Name	Ret. Time	Window	Standard	Int. type	Cal. Type[a]	Coeff det. R^2 (%)
Gluconate	3.850 min	0.150 AG	External	Area	0QOff	99.93
Glycolic	4.100 min	0.200 AG	External	Area	0QOff	99.77
2-keto-gluconic	5.000 min	0.200 AG	External	Area		
Chloride	6.700 min	0.200 AG	External	Area		
Nitrite	7.750 min	0.200 AG	External	Area		
5-keto-gluconic	8.900 min	0.200 AG	External	Area	0QOff	99.83
Nitrate	9.700 min	0.300 AG	External	Area	0QOff	99.85
Glucarate	12.000 min	0.300 AG	External	Area	0QOff	99.90
Tartaric	12.700 min	0.200 AG	External	Area	0QOff	99.70
Tartronic	13.800 min	0.200 AG	External	Area	0QOff	99.70
Oxalic	14.900 min	0.200 AG	External	Area	0QOff	99.85

[a]: 0QOff = quadratic analysis including a (0,0) point.

Calibration was performed using the peak areas of external standards. The calibration points were interpreted by a quadratic analysis including a (0,0) point for curve fitting. Quadratic analysis has been shown to greatly lower the analysis error in IC[63]. The calibration curves all have an R^2 value of above 99%. The values range from 99.7% to 99.9%. The calibration curves of all eight analytes are graphed below with the 99% confidence limits shown in red (Figure 21 to Figure 28). The graphs show the concentration of the analyte in μmol/mL versus the area of the analyte peak. The curves are listed in the order of elution.

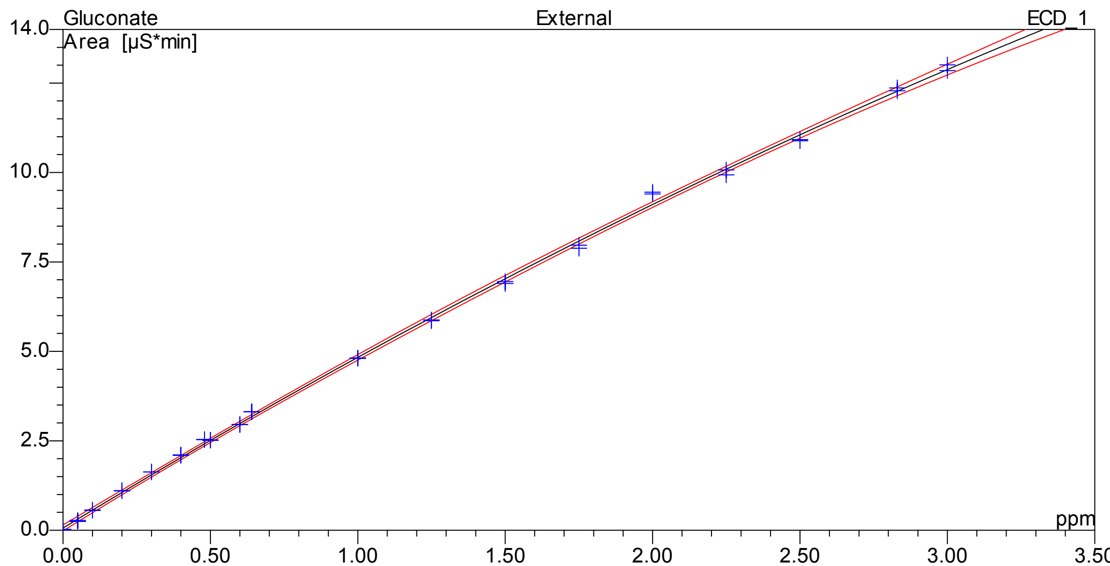

Figure 21: D-Gluconate calibration curve

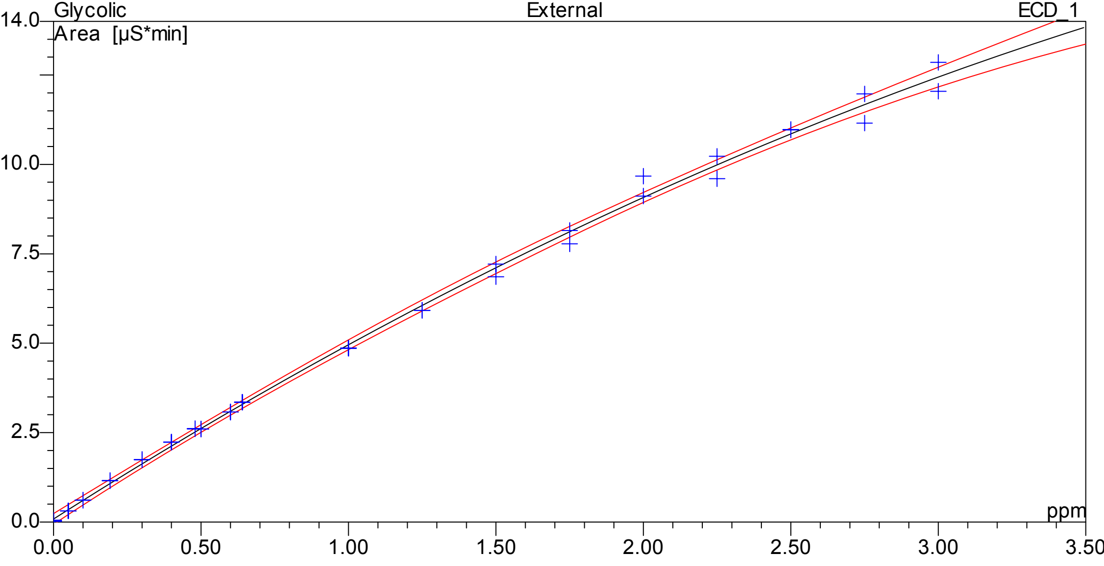

Figure 22: Glycolate calibration curve

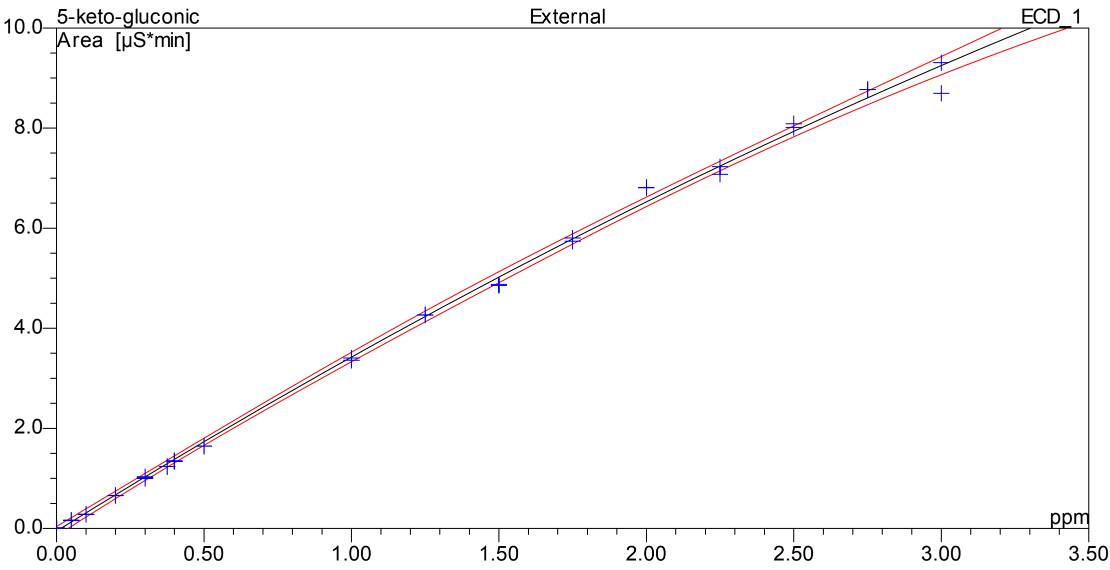

Figure 23: 5-keto- D-gluconate calibration curve

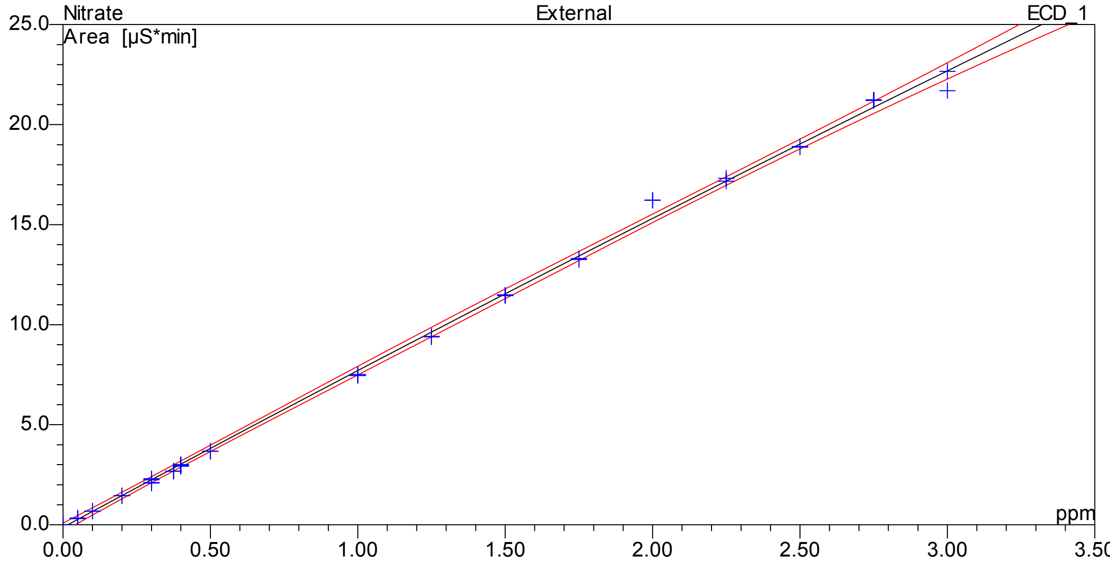

Figure 24: Nitrate calibration curve

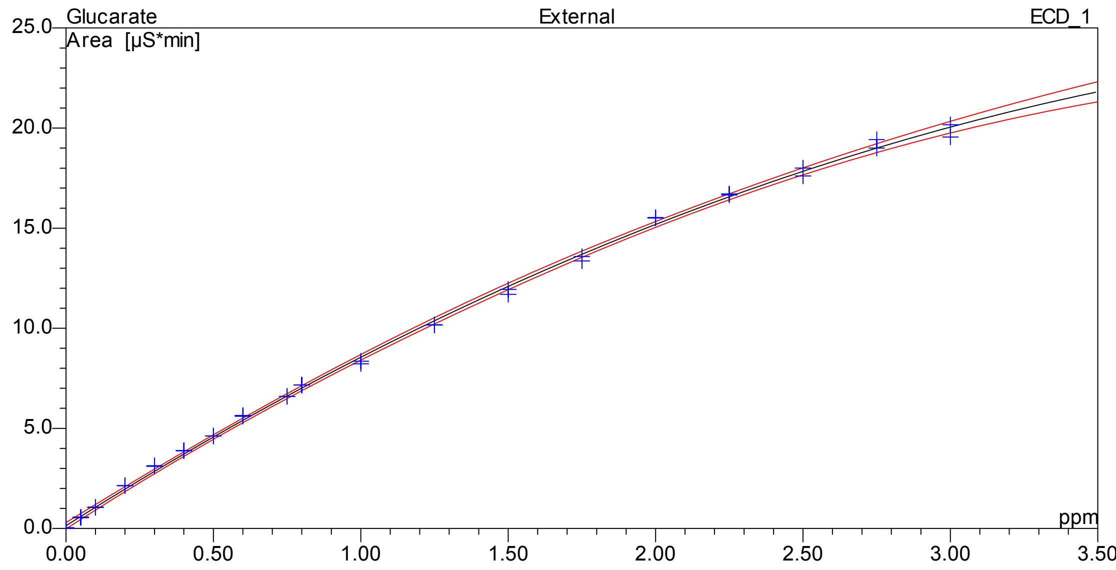

Figure 25: D-Glucarate calibration curve

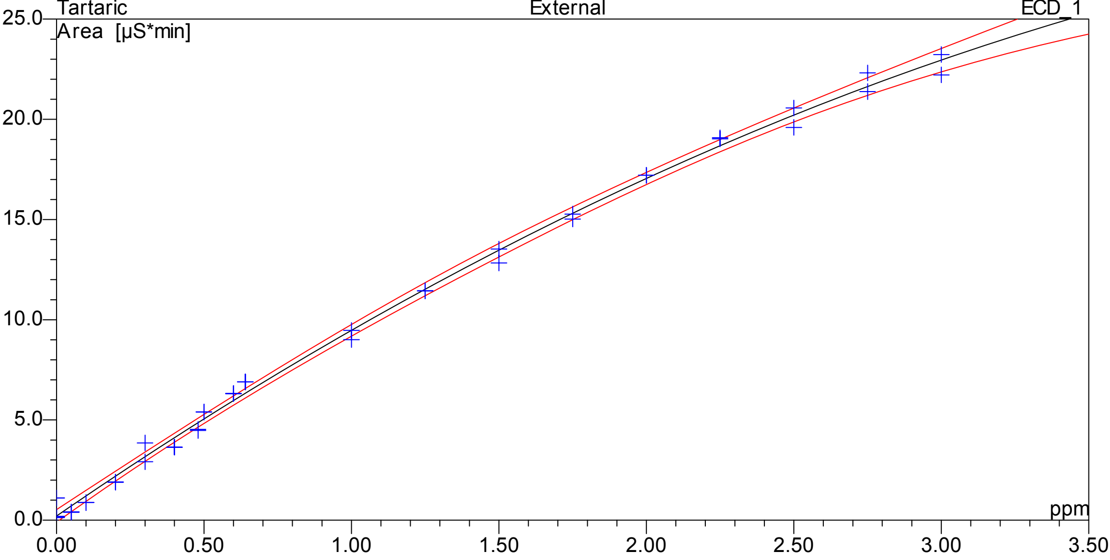

Figure 26:Tartrate calibration curve

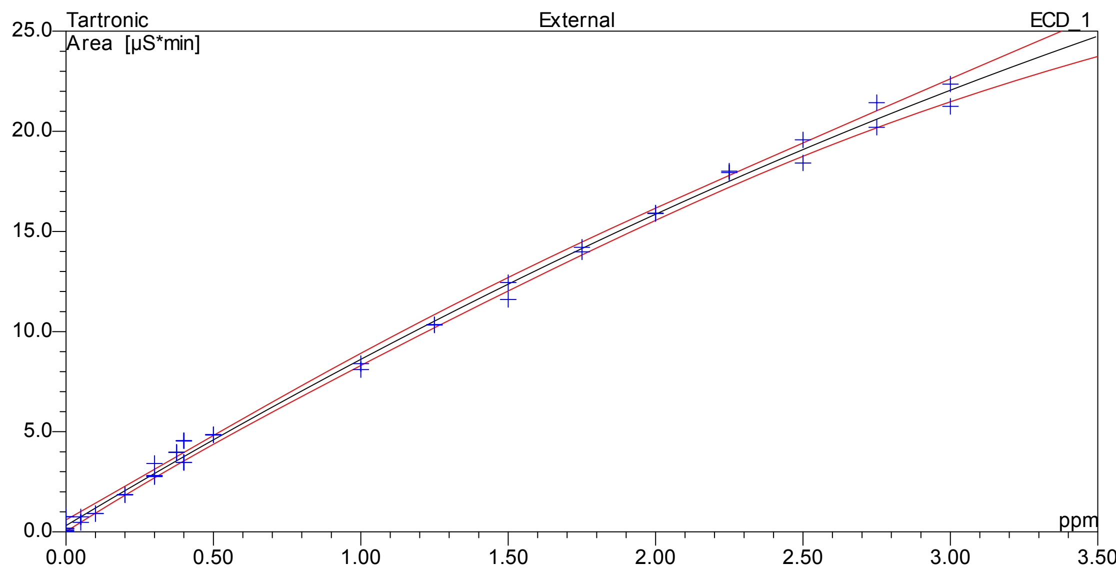

Figure 27: Tartronate calibration curve

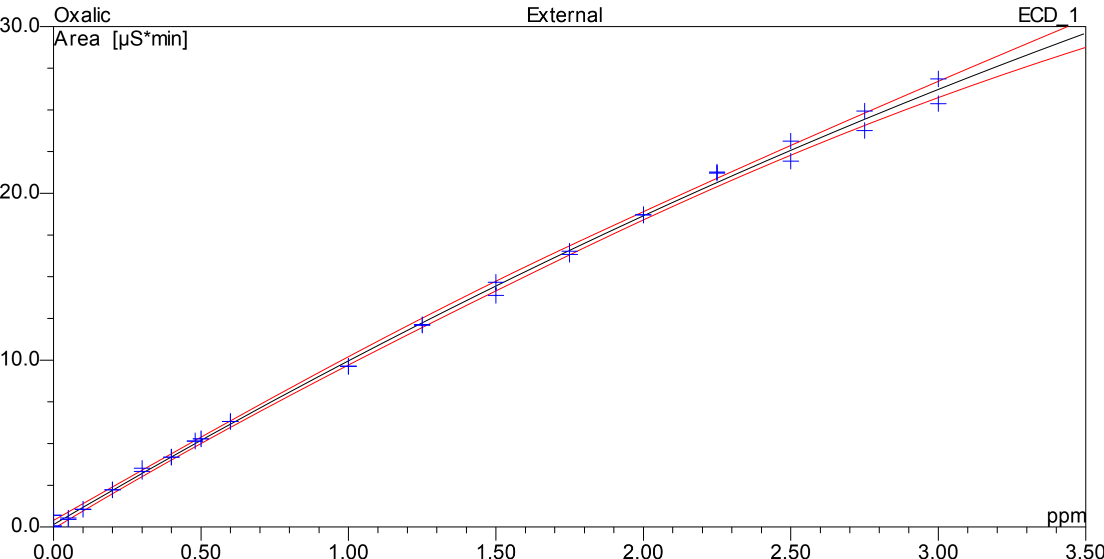

Figure 28: Oxalic Calibration Curve

The non-linear nature of some of the calibration curves results from the increasing formation of the neutral acid in the suppressed elution mixture. Nitrate has a purely linear calibration plot because it is a very strong acid. This effect is most exaggerated for the weaker acids such as D-glucaric acid. The likelihood of the acid form occurring increases as the concentration of the anion increases. The early part of the calibration is linear before the concentration increases to the point of the acid forming. Once the neutral acid form occurs, it decreases the apparent concentration of the analyte anion causing the values to read low. Most concentrations of acids in the diluted reaction mixtures fell within the linear part of the calibrations. Nitrate was occasionally above the calibrated level but tests showed that the calibration continued linear beyond the 5ppm level. Tartronic and Tartaric show some larger errors in the lowest concentrations as they elute on top of the carbonate artefact. Slight variations in this carbonate 'peak' elude the blank subtraction correction and contribute to acid peak areas at very low levels.

The calibrations for the D-mannose and D-xylose oxidations were carried out in exactly the same manner as for the D-glucose oxidations. The peak identification tables and the calibration curves for analytes specific to these oxidations are shown in Table 3 and Table 4, and Figure 29 to Figure 32.

Table 3: D-Xylose Oxidation Peak identification table

Peak Name	Ret. Time	Window	Standard	Int. type	Cal. Type	Coeff det. R^2 (%)
Xylonate	3.800 min	0.200 AG	External	Area	0QOff[a]	99.96
Chloride	7.000 min	0.300 AG	External	Area	LOff[b]	
Nitrate	9.700 min	0.300 AG	External	Area	LOff	99.99
Xylaric	12.100 min	0.300 AG	External	Area	0QOff	99.91
Tartronic	13.800 min	0.200 AG	External	Area	LOff	99.97
Oxalic	14.900 min	0.200 AG	External	Area	LOff	98.62

[a]: 0QOff = quadratic analysis including a (0,0) point.

[b]: LOff = linear analysis

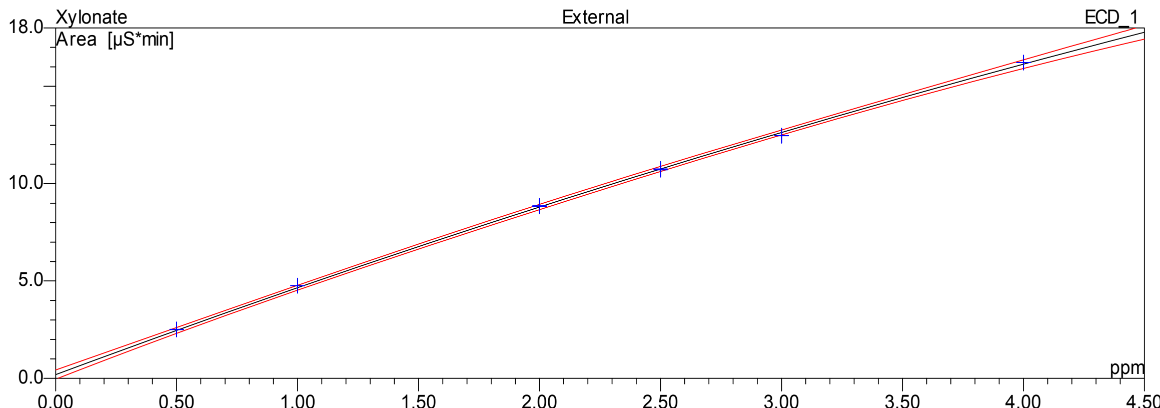

Figure 29: D-Xylonic acid Calibration curve

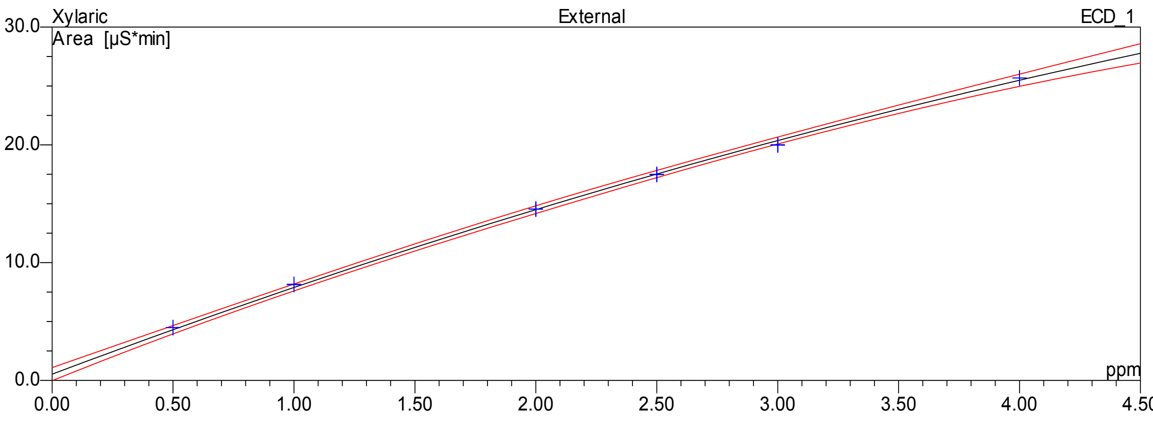

Figure 30: Xylaric acid Calibration curve

Table 4: D-Mannose Oxidation Peak identification table

Peak Name	Ret. Time	Window	Standard	Int. type	Cal. Type	Coeff det. R^2 (%)
Mannonate	4.100 min	0.100 AG	External	Area	0QOff[a]	99.96
Chloride	6.700 min	0.200 AG	External	Area	LOff[b]	
Nitrite	7.850 min	0.200 AG	External	Area	LOff	
Nitrate	9.700 min	0.300 AG	External	Area	LOff	99.72
Mannaric	12.500 min	0.300 AG	External	Area	0QOff	99.76
Tartronic	13.800 min	0.200 AG	External	Area	0QOff	99.85

[a]: 0QOff = quadratic analysis including a (0,0) point.

[b]: LOff = linear analysis

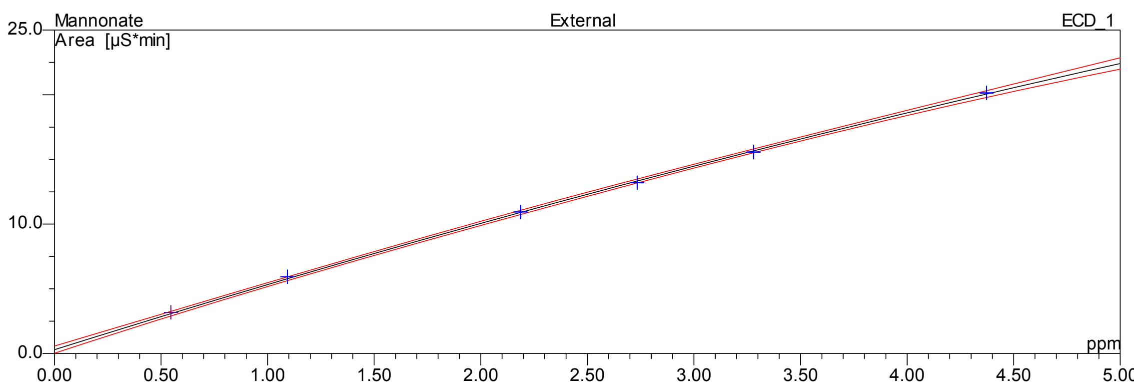

Figure 31: D-Mannonic acid Calibration curve.

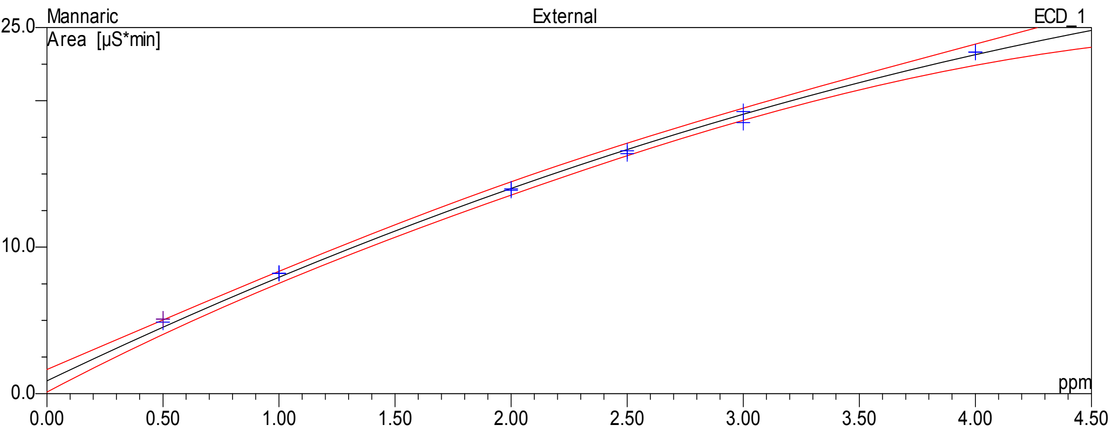

Figure 32: Mannaric acid calibration curve.

In both the D-mannose and D-xylose oxidation calibrations, the oxalic was more difficult to calibrate than for the D-glucose oxidations. This is possibly due to an error in the standard preparation process although all other peaks were calibrated to an R^2 value of at least 98%. The oxalic calibration curve resulting from the mannaric oxidation mixture is shown in Figure 33. Another source of the calibration error could be from residual oxalic acid left in the aldaric and aldonic acid samples. The sources of these compounds were extracted mixtures of previous nitric acid oxidations. Thus they were not at commercial grade although IC analysis with NMR confirmation showed a ~90% purity for most samples. The possible contribution to the oxalic standard level from the residual component in the aldonic and aldaric acid standards was not included in the calibration calculations. The D-mannonic and mannaric acid standards had a greater level of residual oxalic acid than the D-xylonic and xylaric acid standards and this was shown in the greater error present in the D-mannose oxidation calibrations.

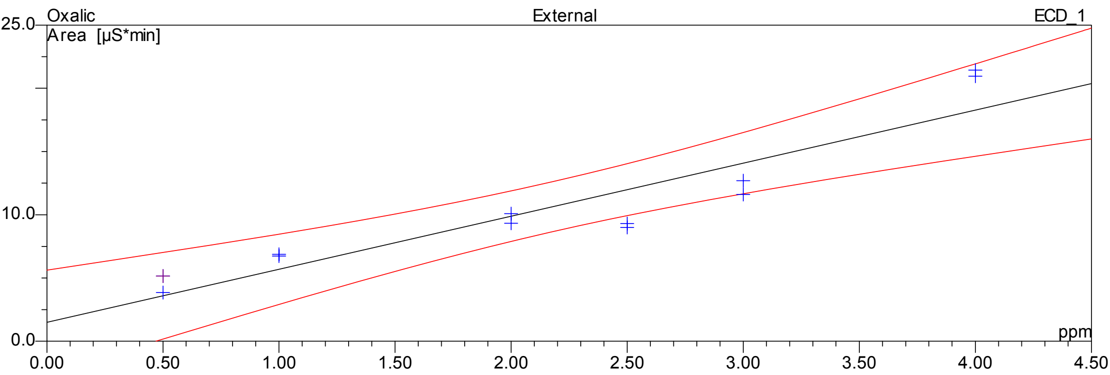

Figure 33: Oxalic Calibration Curve from Mannose oxidation standards showing the large error within the 99% confidence interval.

The calibrations for the mannose and xylose oxidations were not optimised for oxalic acid as it was not considered a major factor in their analysis at that time.

2.2.3 Results

The IC proved an invaluable analytical tool requiring no sample derivatisation and in most cases providing easily interpretable results within an hour, including sample prep and set-up. The IC system was kept operating 5 days a week with at least 30 samples a day for over 3 months. There was no obvious decrease in performance over this time, showing that the system has a very robust nature, requiring only the daily refilling of the water reservoir.

Previously oxalic acid had been a difficult component to identify and quantify but the IC system makes it easily separable and an analyte to which the conductivity detector is very sensitive due to the double charge on the small (2-carbon) molecule. The identification of the oxalic peak in the HPLC chromatographs had been presenting some difficulties. Using fractions collected at appropriate times from the HPLC, the peak was confirmed as oxalic acid using the IC. A sample of an oxalic acid standard was run on the HPLC; the peak was collected. A real oxidation mixture was analysed on the same system and the peak tentatively identified as oxalic was collected as it eluted. These samples were at low concentrations in a strongly acidic (5mM H_2SO_4) matrix. The low concentration of the analyte meant that dilution of the acidic mixture was counterproductive when there was a standard in the same matrix available for comparison. Figure 34 and Figure 35 show two separate elution methods used to confirm the identity of the ambiguous peak in HPLC as oxalic acid.

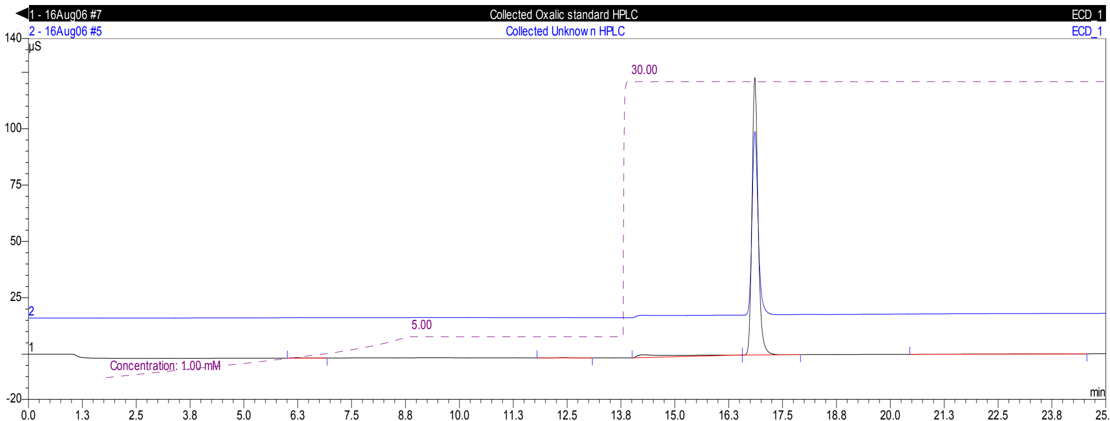

Figure 34: Comparison of Oxalic standard (black) and 'unknown' HPLC peak (blue). The two chromatographs are offset vertically for effect. Eluted with the gradient shown (maroon)

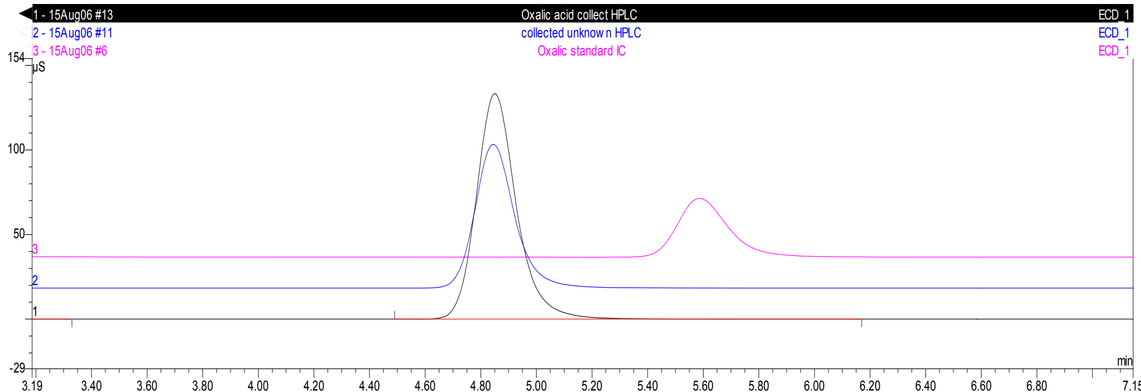

Figure 35: Comparison of Oxalic standard (black) and 'unknown' HPLC peak (blue). The pink line shows the retention of oxalic in a non-acidic matrix. Eluted with 30mM isocratic method.

The precision of the method was tested by comparing ‘known’ concentration standards to the results found with IC. A 10% error was indicated as the upper acceptable value. This test was performed by making up known concentrations of a standard sample mix from pre-prepared solutions and drying them back to solids before following the full sample preparation method. Initial results were within 3 to 5% with D-glucarate and glycolate having the largest errors. The change from a linear calibration to a quadratic one improved the comparison across all compounds. The large error in the glycolate concentration was attributed to the hygroscopic nature of the acid causing the ppm value calculations to be skewed. A procedure for creating sodium glycolate (non-hygroscopic) from the acid was applied (section 3.3) and the calibrations using this compound improved the agreement with the test solutions. Accuracy in the quantifications was improved by analysing duplicates of every sample.

The sensitivity of the method was tested empirically for nitrate and D-glucaric acid by running very low concentration samples, created by dilutions of standards. The limit of detection for D-glucaric acid was 0.002ppm and for nitrate, a peak could be identified manually at 1×10^{-4}ppm although the software had a LOD of 0.001ppm. The other analytes had a software limit of detection between 0.003ppm and 0.005ppm. The limit of quantification (LOQ) was taken to be five times the limit of detection i.e. between 0.02ppm and 0.03ppm for all analytes. This concentration was

far below the concentrations of the analytes of most interest which could also be manipulated by the chosen dilution factor to maintain the concentrations above the limits of the method.

The retention factor (*k*) (section 1.3.2) for all analytes is shown in Table 5. Most fall in the preferred region of 1 to 5. The first four analytes also have a value very close to 1. The value for oxalic acid of 6.1 suggests that improvements can be made in the method for its retention time.

Table 5: *k* values for nitric acid oxidation analytes

Peak	Retention time (min)	k value
Xylonate	3.80	0.81
D-Gluconate	3.85	0.83
Glycolic	4.10	0.95
Mannonate	4.10	0.95
2-keto- D-gluconic	5.00	1.38
5-keto- D-gluconic	8.90	3.24
Nitrate	9.70	3.62
D-Glucarate	12.00	4.71
Xylaric	12.10	4.76
Mannaric	12.50	4.95
Tartaric	12.70	5.05
Tartronic	13.80	5.57
Oxalic	14.90	6.10

The analysis of oxidation mixtures was very successful. An example of typical results for D-glucose is given below for KH052407.

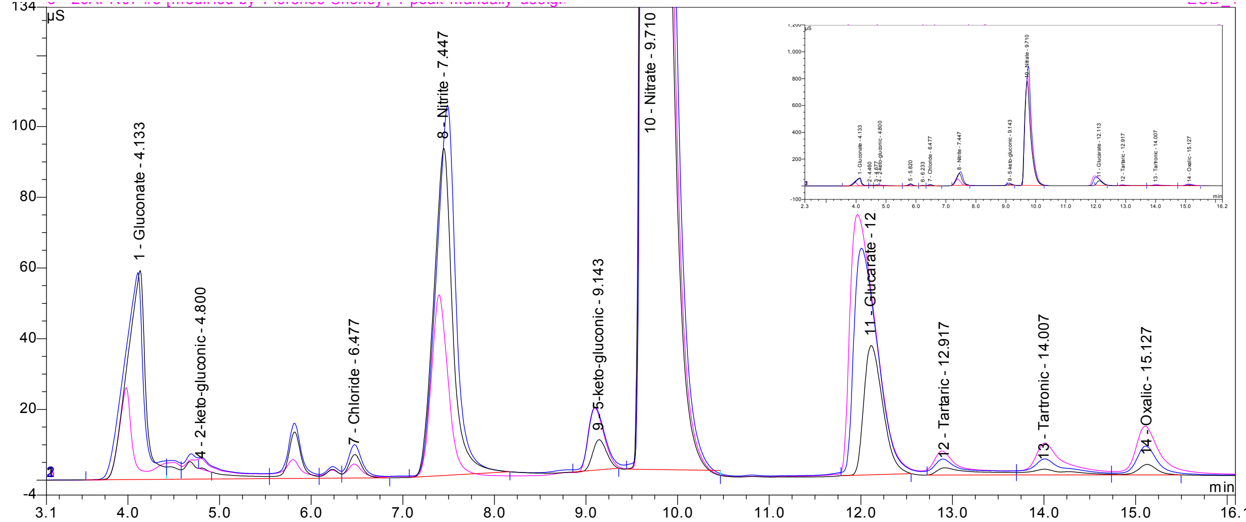

Figure 36: A D-glucose oxidation mixture analysis: samples taken at three points. After D-glucose dosing (Black), after reaching final reaction conditions (Blue) and after 90mins at the reaction conditions (Pink). With inset of full scale chromatograph.

Table 6: Calculated values of components in D-glucose oxidation mixture with columns related to Figure 36. Tartaric was unquantifiable in the first sample. Amounts reported as concentrations in the undiluted samples.

Peak	Amount (ppm, µmol/mL)		
	Black	Blue	Pink
Gluconate	812	825	213
5-keto-gluconic	67	256	273
Nitrate	6250	8000	7000
Glucarate	197	629	998
Tartaric	n.d.	14	26
Tartronic	3	14	54
Oxalic	13	42	88

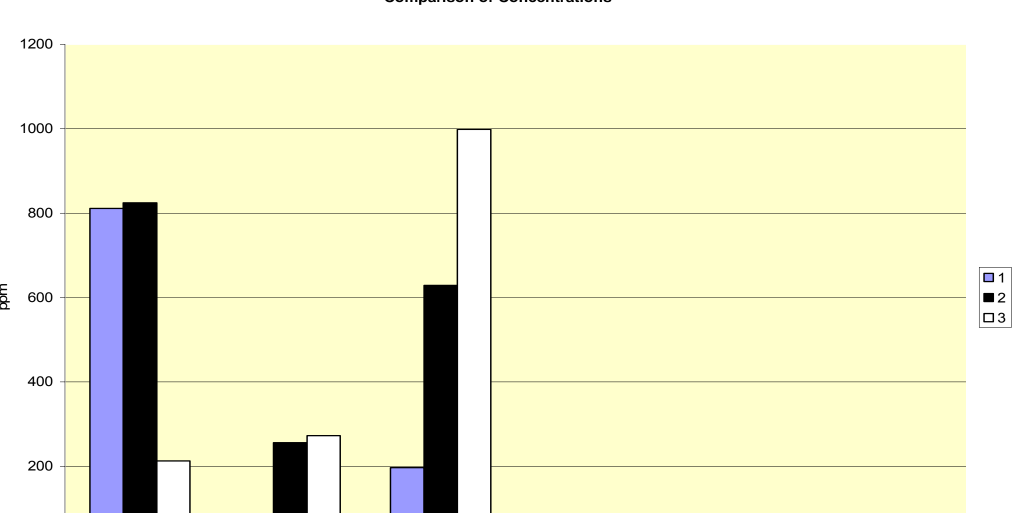

Figure 37: Graph of the values from Table 6 showing changing levels of the analytes. Bar number relates to sample times, increasing reaction time from left to right. Nitrate is excluded due to its high concentration.

Table 6 and Figure 37 show the progression of the reaction clearly. The nitric acid (nitrate) stays relatively constant as suits its role as a catalyst. The variation in the concentrations is largely due to the variable amounts of NO_2 gas released during sample preparation. The concentrations of D-gluconic acid and D-glucaric acid show the D-gluconic acid's role as an intermediate oxidation stage, the D-glucose is initially transformed into D-gluconic acid and then to D-glucaric acid. 5-keto- D-gluconic acid can be identified as the main byproduct of the reaction with the breakdown products of oxalic, tartaric and tartronic acids present in lower concentrations. Glycolic acid could not be identified in this reaction; this was a common result for many glucose oxidations. It was not clear whether this was due to the absence of this compound or its low concentration. Previous analyses[6] had not confirmed the presence of this compound although early samples from the IC method development seemed to indicate a very low level presence.

A series of overlaid chromatographs can be used to visually follow the course of a reaction without quantification. Eleven samples were taken from a D-mannose oxidation mixture and run through the IC over the course of 9 hours. The overlaid chromatographs are shown in Figure 38 and Figure 39. Figure 38 shows the nitrate peaks from the samples overlaid; the variance in peak area is largely a result of sampling and preparative losses of nitrate as NO_2 gas. Figure 39 shows a comparison of the peaks of D-mannonic and mannaric acid. As the sample number and reaction time increases; the concentration of mannaric increases and the concentration of D-mannonic decreases before reaching a plateau at low concentration. The overlaid peaks also show the variances in retention time over a day, all peaks in a chromatograph would shift by the same amount, causing no issue with resolution or identification.

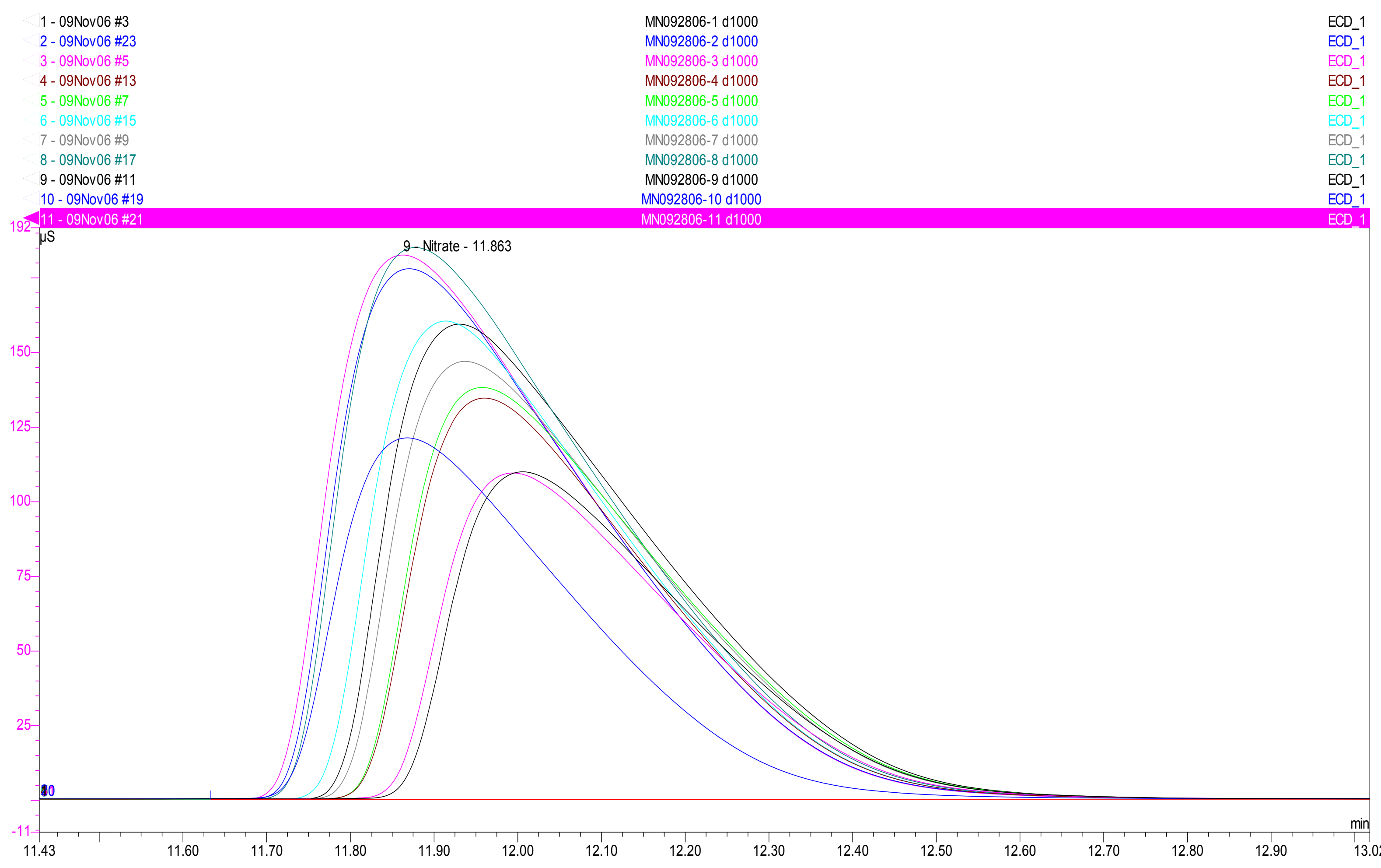

Figure 38: The nitrate peak from 11 D-mannose oxidation samples, as a catalyst it's level does not vary predictably. The fronting of the peak is due to the high concentration of nitrate.

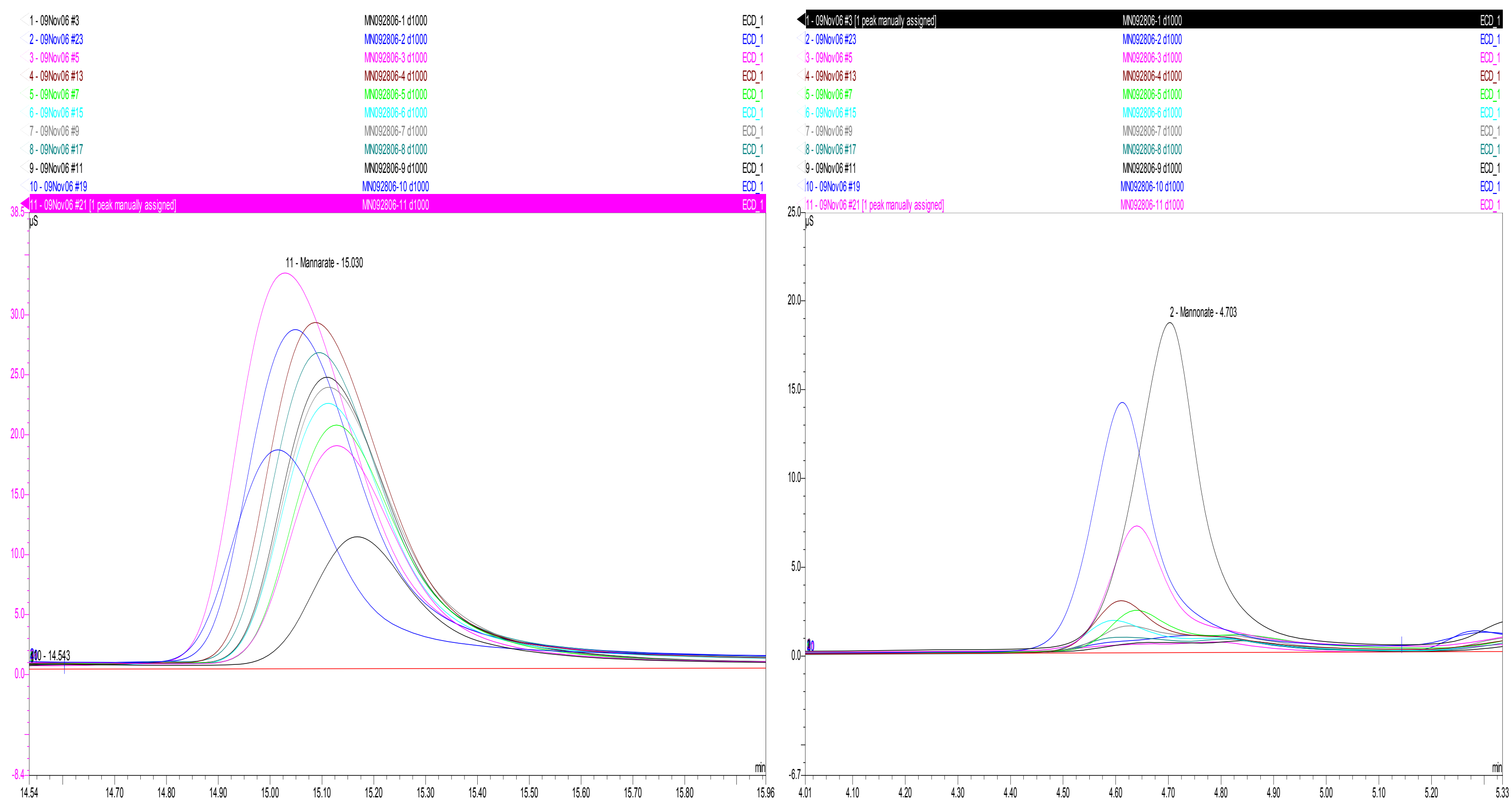

Figure 39: Comparison of D-mannonate and mannarate peaks, showing the opposite behaviour over time.

The effectiveness of the work-up steps (section 3.2.2) was also studied by IC:

Nanofiltration works by restricting the passage of large molecules through the filtration membrane. IC analysis showed that this removed around 75% of the nitrate without affecting the concentrations of the other analytes by much. Figure 40 shows the nitrate and glucarate peaks for the three samples taken:

- Feedstock: original unfiltered mixture (usually post-rotary evaporator).
- Permeate: solution which passes through the membrane
- Retentate: solution which does not pass through the membrane, retained solution.

The nature of the procedure is to run the system with RO water which dilutes the filtered solutions by an unmeasured amount. As such the best comparison method is the relative concentrations of the analytes. (Table 7)

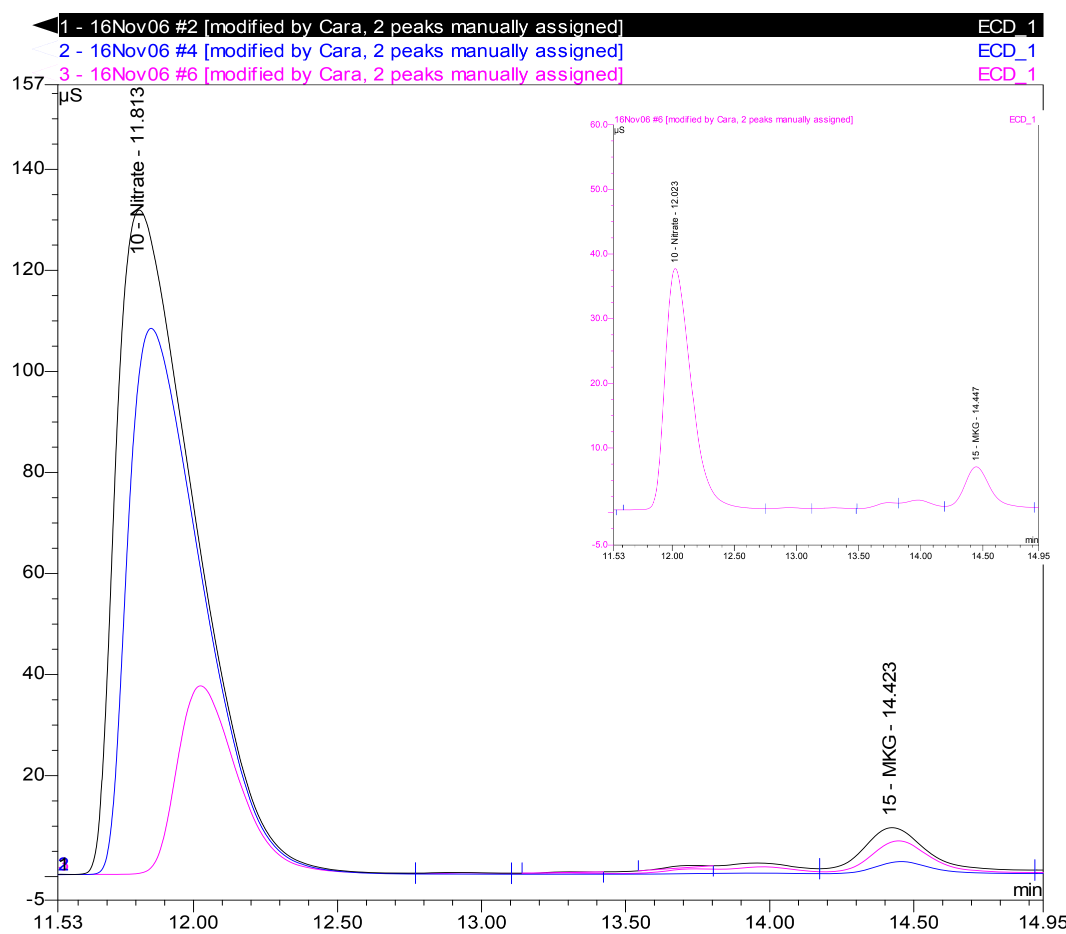

Figure 40: The effect of nano-filtration on a glucose oxidation mixture. (Black) Feedstock, (Blue) Permeate, (pink) Retentate. With inset of retentate.

Table 7: Relative percentage of glucarate peak compared to nitrate peak.

Sample	Area (µS/min)		Relative %
	Nitrate	Glucarate	
Feedstock	41.60	1.5136	3.64%
Permeate	32.40	0.5432	1.68%
Retentate	9.15	1.5136	16.54%

The above table compares areas as the elution method as used at the time of analysis was not calibrated.

Figure 41 shows the effect of repeating the filtrations by running the out put from the filtration column back through the column. As the driving force for the filtration mechanism is the higher concentration of ions on one side of the membrane, the effectiveness of the filtration decreases as the nitrate concentration decreases. The levels eventually reach a plateau and remain roughly constant.

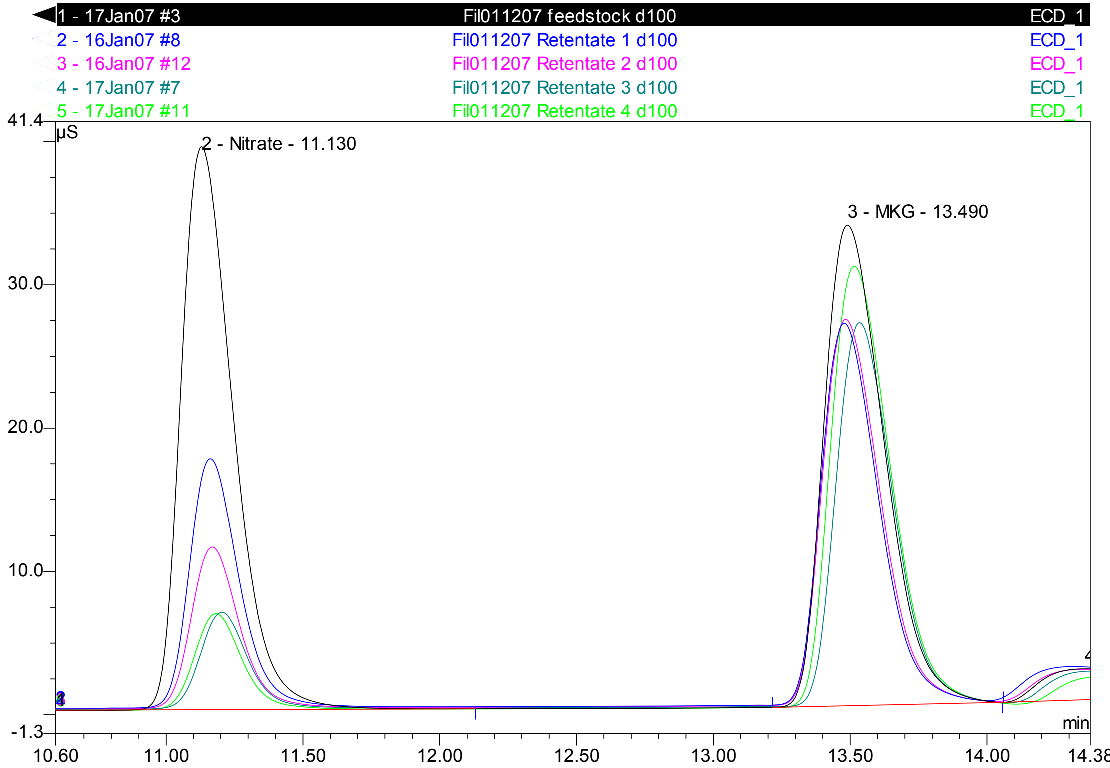

Figure 41: The effect of repeated filtrations, overlaid chromatographs of the feedstock and retentates showing the decreasing level of nitrate.

Diffusion Dialysis was tested as a work-up step during the last stages of this study. Five D-glucose oxidation mixtures were collected and mixed together to provide the volume for a significant test of the system. The end products of the dialysis were a Product mixture containing most of the organic acids and a Reclaimed Acid mixture containing the nitric acid. Table 8 shows the results tabulated for nitrate and D-glucaric acid. This table shows that the majority of the nitric acid has been removed to the reclaimed acid stream. The table also illustrates the reliability of the nitric acid oxidation method in the constant values for the D-glucaric acid present in the reaction mixtures.

Table 8: Diffusion Dialysis results

Oxidation Samples	Nitrate (µmol/mL)	D-Glucarate (µmol/mL)	Ratio of D-glucarate to nitrate
KH042507	24.81	3.99	
KH050807	24.97	3.94	
KH050807	25.05	3.68	
KH050907	24.40	3.94	
KH050907	24.50	3.95	
KH051007	23.97	3.98	
KH051007	24.01	3.98	
KH051107	24.03	3.97	
KH051107	24.08	3.96	
Average	24.42	3.93	16%
After Diffusion Dialysis			
Product	6.02	12.82	213%
Reclaimed Acid	103.70	5.20	5%

3. Experimental

3.1 Ion Chromatograph ICS2000

3.1.1 Apparatus and settings

All of the Ion Chromatographs displayed in this thesis are from the Dionex ICS2000 system used by the Rocky Mountain Shafizadeh Centre for Wood and Carbohydrate Chemistry. The background theory and a detailed description of the IC components are given in Section 1.4 of the Introduction.

The system was run using a 25µL sampling loop. Separation occurred with an AS11-HC 4mm anion separation column fitted with an AG11-HC 4mm guard column at 38°C. The suppressor was an ASRS ULTRA II auto-suppressor system. The samples were supplied to the system by means of an AS40 auto-sampler acting in Constant sampling mode. The eluent was a water/NaOH mixture varying between 1mM and 60mM NaOH, created and controlled with the use of an EG40 eluent generator. The water component of the eluent was provided by a Millipore Simplicity 185 water purifier and kept under Nitrogen.

The elution method used for the nitric oxidation samples was:

Equilibration at 1mM NaOH for 4 minutes with sample injection occurring at time = zero; followed by a ramp up to 30mM over 25 minutes, then a sharp rise over 2 minutes to the rinse concentration of 50mM which is held for 10 minutes before returning to 30mM over 2 minutes. (Figure 42)

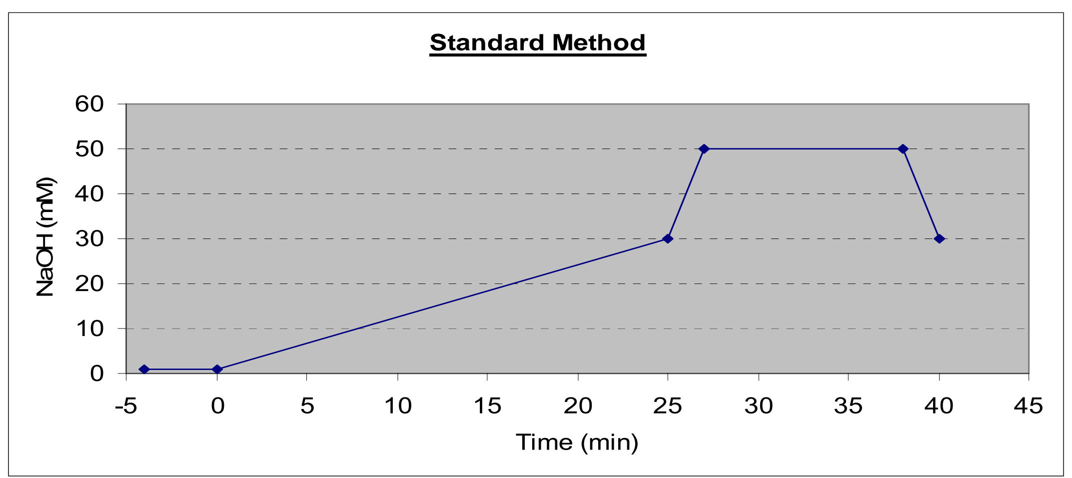

Figure 42: The Standard method used to elute the majority of reported oxidation mixtures, shown as a function of NaOH concentration (mM) against run time (min).

The detector was a DS6 Heated Conductivity Cell at 37°C. The entire system was controlled by Chromeleon 6.8 software. Data acquisition occurred at 5Hz.

3.1.2 Standard and non-oxidation samples compounds

Table 9 shows the providers of all commercially sourced compounds.

Table 9: List of Chemical Sources

Compound	Provider
1,2,3-benzenetricarboxylic acid	Aldrich
1,2,4,5-benzenetetracarboxylic acid	Aldrich
1,2,4-benzenetricarboxylic acid	Aldrich
2-chlorobenzoic acid	Aldrich
2-furoic acid	Aldrich
2-keto- D-gluconic acid (hemiCalcium, monohydrate)	Sigma
3, 5-dinitrosalicylic acid	Sigma
3-bromopyruvic acid	Fluka
3-chloropropionic acid	Aldrich
3-hydroxy butyric acid	Aldrich
3-hydroxy propionic acid	Aldrich

Compound	Provider
glyoxalic acid	J.T.Baker
hexanoic acid	Sigma
isobutyric acid	Aldrich
lactic acid	J.T.Baker
levulinic acid	Acros organics
maleic acid	Aldrich
malic acid	Aldrich
malonic acid	Aldrich
mellitic acid	Aldrich
mesoxalic acid (ketomalonic acid)	Fluka
monochloroacetic acid	Sigma-Aldrich

Compound	Provider
3-nitrophthalic acid	Kodak, Aldrich
4-hydroxy-3-methoxybenzoic acid (Vanillic)	Aldrich
4-hydroxybutyric acid	Aldrich
5-chlorovaleric acid	Aldrich
5-keto- D-gluconic acid (potassium salt)	Sigma
acetic acid (sodium salt)	Fisher Scientific
adipic acid	Aldrich
anisic acid	Aldrich
benzoic acid	Sigma
butyric acid	J.T.Baker
citric acid	Fisher Scientific
crotonic acid	Aldrich
formic acid	Aldrich
D-glucaric acid	Sigma
D-gluconic acid (sodium salt)	Aldrich
D-glucuronic acid	Sigma
glutaric acid	J.T.Baker
glycolic acid	Sigma-Aldrich

Compound	Provider
m-toluic acid	Aldrich
oxalacetic acid	Aldrich
oxalic acid	Sigma-Aldrich
p-chlorobenzoic acid	Kodak
phthalic acid	J.T.Baker
p-hydroxybenzoic acid	Sigma
propionic acid	Aldrich
p-toluic acid	Aldrich
pyruvic acid	Sigma
quinic acid	Aldrich
salicylic acid	Sigma
sodium nitrate and nitrite	EMD Chemicals
succinic acid	Sigma
syringic acid	Aldrich
tartaric acid	Sigma
tartronic acid	Aldrich
trichloroacetic acid	J.T.Baker
xylaric acid	Sigma

Compounds which are not listed in this table were sourced from the purified products of previous reactions, e.g. mannaric and mannonic acids were provided by Chrissie Carpenter (Shafizadeh Centre) from her own work with the identity confirmed by NMR. Xylose reaction products were similarly supplied by Michael Hinton. Standards were made up from stock solutions of the components. The stock solutions were made by weighing out the required mass into volumetric flasks. All concentrations were calculated to 2 decimal places by Microsoft Xcel spreadsheets. As a convention any analysis sample was prefaced by the initials of the person who presented or created it. All samples retained by the investigator were stored at 4°C in capped glass vials or flasks. All glass containers were rinsed three times with Millipore water and oven-dried between uses.

Solid samples were run at 0.5μg/mL for sufficient signal with the least sample amount. Liquid samples were diluted with Millipore water by between 100- and 500-fold according to their nature with highly coloured or acidic (i.e. high counter ion, NO_3^{2-}) samples being diluted more. No attempt was made to control the pH of any sample solution.

Samples were supplied to the Autosampler AS40 in 5mL PolyVial™ plastic sample vials. These vials were washed three times with distilled water, air dried and rinsed with the sample before use.

3.1.3 Oxidation Reaction Samples

The oxidation samples required very little preparation, a benefit of this equipment and method. Oxidation samples were kept at 0°C between sampling and preparation of the sample for analysis. The oxidation samples were diluted with Millipore water 250-fold using an auto-pipette and 10mL volumetric flasks.

Samples from the reaction product work-up e.g. filtration were diluted with Millipore water 100-fold due to their less concentrated nature.

3.2 Nitric Acid Oxidations

3.2.1 LabMax Reactor

All nitric acid oxidations were performed in a Mettler Toledo RC-1 Labmax Reactor. The software used to operate the reactor was CamileTG v1.2. The system had a maximum capacity of 1L of which only 350mL was routinely used, equaling a reactant amount of 135.12g for D-glucose; with similar amounts used for the other sugars.

The reaction methods used vary slightly based on the sugar being oxidized. The reaction stages for glucose can be outlined as:

- Set up: adding nitric acid and setting starting conditions
- Add D-glucose solution in two steps with stabilization breaks between.
- Gradual change to reaction conditions (duration up to 60 minutes)
- Hold at reaction conditions for up to 180 minutes

The technician in charge of the reactor (Kirk Hash) took samples of about 200µL by glass pipette into 8mL sample vials at three points;

- after the final addition of D-glucose (#1),
- at the point when the final reaction conditions are reached (#2),
- after holding those conditions for a pre-determined amount of time (#3)

Samples are identified by the date (USA convention) on which the reaction was performed and the sampling point, #1, 2 or 3. E.g. KH051107 #3 is the last sample from the reaction performed by **K**irk **H**ash on the 11th of May 2007.

3.2.2 Post Reaction Work-up Steps

Two procedures were assessed by IC for the work-up of the reaction mixture.

A. The reaction product was reduced in volume using a rotary evaporator specially modified for removing nitric acid by adding a cooling system using a 60/40 mixture of ethylene glycol and water to cool the condensor. The first fraction was NO_x gases at *ca*. 23-34°C and 50-120millibar. A liquid distillate resulted at around 26-39°C and 26-43millibar and had properties compatible with nitric acid. The concentrated reaction product was made basic and subjected to nanofiltration to remove residual inorganic nitrate. The filtration system used Reverse Osmosis (RO) water as the second flushing solution.

B. Diffusion Dialysis was performed using a Mech-Chem Diffusion Dialysis Acid Purification System, lab scale Model AP-L05. Reverse Osmosis (RO) filtered water was used as the removal phase. The organic stream was collected and made basic.

4. Conclusions and Further Work

The method developed herein for ion chromatography has proven to be a very valuable tool in the analysis of nitric acid oxidation of sugars. The method outperforms the previous GC-MS and HPLC analysis methods in speed, simplicity and sensitivity.

The survey of retention times for various organic carboxylic acids has identified several trends and behaviours that will be valuable in identification and rationalisation of unknown peaks.

Further avenues to explore for possible improvement in the analysis method for nitric acid oxidations include:

- Better separation of glycolic acid and D-gluconic acid should be possible with further adjustments to the early gradient system. This would allow the more precise analysis of D-gluconic acid and a better test for the presence or absence of glycolic acid in the oxidation mixture.
    - Addition of organic solvents to the elution system was not considered previously as the aim of the method was for uncomplicated continuous testing in an industrial setting. However, glycerine 0.2mM addition has been shown to especially increase the retention time of glycolic acid, with half the effect on the other polyhydroxy carboxylic acids[58]. (Figure 43)

OH
HO
OH
glycerine

Figure 43: Structure of glycerine

-
    - This molecule is neutral so there would not be an extra interfering peak or background conductivity increase.

- The separation of the carbonate peak/sharp rise in baseline from the analyte peaks of D-glucaric acid, tartaric acid and tartronic acid would be a worthwhile modification to the elution method. This is likely to be difficult without increasing the run time of the method too much.

- Continuation of reducing contaminant levels (esp. carbonate). The current method of keeping the reservoir under nitrogen is inefficient and could be improve with a more sophisticated set up.
    - If carbonate contamination levels could be kept sufficiently low and steady, an investigation into the quantification of the carbonate present from the oxidation reaction would be possible.

Appendix One: Structures of Eluted Carboxylic acids

Acids, in alphabetical order	Structures shown as conjugate base anions
1,2,3-benzenetricarboxylic acid	
1,2,4-benzenetricarboxylic acid	
1,2,4,5-benzenetetracarboxylic acid	
2-chlorobenzoic acid	
2-furoic acid	
2-keto-gluconic acid	
3, 5-dinitrosalicylic acid	

Acids, in alphabetical order	**Structures shown as conjugate base anions**
3-bromopyruvic acid	
3-chloropropionic acid	
3-hydroxybutyric acid	
3-hydroxypropionic aid	
3-nitrophthalic acid	
4-hydroxybutyric acid	
5-chlorovaleric acid	
5-keto-gluconic acid	
acetic acid	
adipic acid	
anisic acid	

Acids, in alphabetical order	Structures shown as conjugate base anions
benzoic acid	
butyric acid	
carbonate	
citric acid	
crotonic acid	
divinylbenzene (DVB)	
ethylene vinylbenzene (EVB)	
formic acid	
glucaric acid	
gluconic acid	

Acids, in alphabetical order	Structures shown as conjugate base anions
glucuronic acid	
glutaric acid	
glycolic acid	
glyoxalic acid	
hexanoic acid	
isobutyric acid	
lactic acid (2-hydroxy prop. acid)	
levulinic acid (4-oxopentanoic acid)	
maleic acid	
malic acid	
malonic acid	

Acids, in alphabetical order	Structures shown as conjugate base anions
mannaric acid	HO OH O ⁻O O HO OH
mannonic acid	HO OH HO ⁻O O HO OH
mellitic acid	O⁻ O O O⁻ ⁻O O O O⁻ ⁻O O O ⁻O
mesoxalic acid	O ⁻O O O O⁻
monochloroacetic acid	O Cl O⁻
m-toluic acid	CH_3 ⁻O O
nitrate	O⁻ ⁻O N^+ O
nitrite	N O O⁻
oxalacetic acid	O O⁻ ⁻O O O

Acids, in alphabetical order	**Structures shown as conjugate base anions**
oxalic acid	
p-chlorobenzoic acid	
phthalic acid	
p-hydroxybenzoic acid	
propionic acid (propanoic acid)	
p-toluic acid	
pyruvic acid	
quinic acid	
salicylic acid	
succinic acid	

Acids, in alphabetical order	Structures shown as conjugate base anions
syringic acid	
tartaric acid	
tartronic acid	
trichloroacetic acid	
vanillic acid	
xylaric acid	
xylonic acid	

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