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THE SOLID PHASE DEGRADATION OF L-ASCORBIC ACID FORMULATIONS

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ABSTRACT

The basis of the thesis is the hypothesis that the reactivities of chemicals in the solid phase are significantly different from that in solution. The aim of the study was to investigate the chemical kinetics, degradation and means of stabilizing solid formulations of L-ascorbic acid

Initial work investigated non-chromatographic methods for the determination of L-ascorbic acid. Due to their lack of specificity and proneness to interferences by potential degradation products chromatographic methods of analysis were developed.

Samples were quantitatively determined for L-ascorbic acid using suppressed ionization reverse-phase high performance liquid chromatography. An automated precolumn sample preparation system was used for the determination of dehydroascorbic acid. Gradient elution systems were developed and used to chromatograph any degradation products formed in solution or solid formulations. The reagent 2,4-dinitrophenylhydrazine was used to monitor for any carbonyl containing degradation products.

Capillary gas chromatography failed to detect any degradation products in severely discoloured solid samples of L-ascorbic acid degraded by moisture alone although it did show different degradation patterns in solid samples degraded by basic and oxidative reagents. Normal-phase thin layer chromatography resolved coloured degradation products from L-ascorbic acid.

Chemical incompatibility between L-ascorbic acid and tablet excipients was determined using differential scanning calorimetry.

Moisture alone was found to degrade L-ascorbic acid in the solid phase and the degradation was always characterized by discolouration. Tristimulus colorimetry was used to measure the extent of colour change and results compared favourably with a quantitative assay for L-ascorbic acid in a kinetic study. Activation energies of 7.96 and 14.2 kcal/mol were obtained by tristimulus colorimetry and quantitative assay respectively. The reaction was first-order. Differences were shown between degradation in solution and in the solid phase. The coloured degradation products were isolated by gradient elution high performance chromatography. Eight different coloured degradation products were detected. Elemental analysis of the main coloured degradation product showed it to consist of 46.26% carbon, 5.42% hydrogen and 48.32% oxygen. Evidence was provided by pyrolysis of this material that it consisted of polymeric furan compounds.

ACKNOWLEDGEMENTS

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PREFACE

Compound Names, Numbers and Abbreviations used in this Text

Compound Number	Name	Abbreviation
(1)	L-ascorbic acid	-
(2)	dehydroascorbic acid	DHAA
(3)	D-glucose	-
(4)	L-xylosone	-
(5)	cyanohydrin	-
(6)	amide	-
(7)	sorbitol	-
(8)	D-glucitol (L-sorbose)	-
(9)	2,3:4,6-di-*O*-isopropylidene-L-xylo-2-hexulofuranose	-
(10)	acid of (9)	-
(11)	L-xylo-2-hexulosonic acid	-
(12)	methyl ester of (11)	-
(13)	D-glucuronic acid	-
(14)	L-gulonic acid	-
(15)	D-glucofuranurano-6,3-lactone	-
(16)	L-gulono-1,4-lactone	-
(17)	D-xylo-5-hexulosonic acid	-
(18)	calcium D-gluconate	-
(19)	cyclic form of (18)	-
(20)	L-idonic acid	-
(21)	D-gluconic acid	-
(22)	D-threo-2,5-hexodiulosonic acid	-
(23)	1,4-lactone form of (22)	-
(24)	positional isomer of (23)	-
(25)	positional isomer of (23)	-
(26)	tautomer of (23)	-

Compound Number	Name	Abbreviation
(27)	N-bromosuccinimide	NBS
(28)	2,6-dichlorophenolindophenol	-
(29)	leuco form of (28)	-
(30)	diazotized 4-methoxy-2-nitroaniline	-
(31)	1,2-diaminobenzene	-
(32)	fluorophorquinoxaline	-
-	2,4-dinitrophenylhydrazine	2,4-DNP
-	2,3-diketogulonic acid	2,3-DKGA

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CHAPTER 1
INTRODUCTION

1.1 Background to L-Ascorbic Acid

L-Ascorbic acid **(1)** is commonly known as vitamin C. It is a white crystalline compound structurally related to the monosaccharides. The enediol group at carbon atoms 2 and 3 is readily oxidized to a diketo group. The resultant dehydroascorbic acid **(2)** has antiscorbutic properties equal to that of L-ascorbic acid and forms a redox system with L-ascorbic acid.

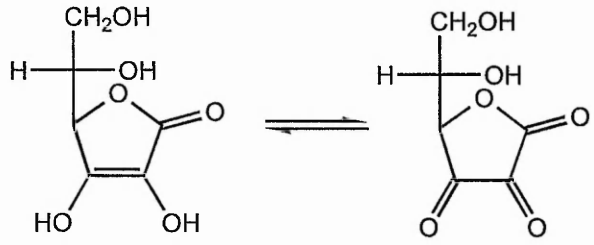

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Figure 1: Redox System Between L-Ascorbic Acid and Dehydroascorbic Acid

It is a water soluble vitamin and structurally one of the simplist of the vitamins. It is found throughout the plant and animal kingdoms and essential to the well being of humans. A few months deprivation produces the unpleasant and ultimately fatal disease, scurvy. Scurvy is a disease which produces haemorrhaging into tissues, bleeding gums, loose teeth, anaemia, weakness and ultimately death.

Scurvy has existed throughout history, however it was not until the very long sea journeys of the early Middle Ages which drew attention to it. The major problem for any ships physician was that the sailors were subject to a large number of different diseases arising from a variety of causes. These were usually poorly described making diagnosis extremely difficult.

Although the consumption of citrus fruits was known to cure scurvy as long ago as the thirteenth century in Spain, this fact was not widely known elsewhere. Lack of communication, the loss of efficacy of fruit and vegetables on storage and the belief that scurvy was a disease of a relatively narrow section of the population all contributed to the failure to recognise the cure for scurvy. Gradually the belief developed that fresh fruit and fruit juices provided an effective treatment for the disease.

James Lind, a surgeon in the Royal Navy demonstrated the effectiveness of fresh fruit and vegetables when he was faced with an outbreak of scurvy onboard HMS Salisbury. He organized a crude clinical trial to test the effectiveness of the various treatments of scurvy in use at that time. He chose twelve men who had contracted scurvy and fed each the same diet throughout the trial which lasted for 6 days. The men were split into 6 groups of 2 and he applied 6 different treatments, one per group, Table 1.

Table 1: Summary of Lind's Clinical Trial of Various Possible Cures for Scurvy.

Group	Treatment
1	One quart of cider
2	25 drops of elixir of vitriol 3 times a day.
3	2 spoonfuls of vinegar before meals.
4	Half a pint of sea water.
5	2 oranges and 1 lemon.
6	A medicinal paste containing a variety of substances such as garlic and mustard seed.

He found that the men in group 5 treated with oranges and lemons quickly recovered. The men in group 1 given a quart of cider recovered to a certain degree but the other treatments had no beneficial effect.

Lind concluded that oranges and lemons were the most effective remedy for scurvy. He made recommendations that ships should carry an extensive store of green vegetables as well as oranges and lemons but even he remained unconvinced that green vegetables and fruit could prevent scurvy and his recommendation was ignored.

It took the influence of Sir Gilbert Blane as physician to the household of the Prince of Wales to get the British Navy in 1795 to issue lemon juice at three quarters of an ounce per day. Scurvy was eliminated from the British Navy as a major problem from then on.

Although the scourge of scurvy had been removed from the British Navy, events on land in the nineteenth century brought outbreaks of scurvy to the British Isles. In Northern Europe, during winter, fresh fruit and vegetables were in short supply and scurvy on land was a seasonal disease appearing in winter and disappearing in spring. For centuries the population of Northern Europe suffered from scurvy remaining undiagnosed due to the many illnesses suffered by the people. In the nineteenth century the focus moved away from the incidence of scurvy at sea to that on land.

A cold wet summer in 1845 across Northern Europe help spread potato blight which resulted in crop failure that year and in the following year. Potatoes, a source of vitamin C, were a staple food and the crop failure resulted in starvation particularly in Ireland. Scurvy quickly began to appear in the weakened people which came as a surprise to the medical establishment. Erroneous beliefs about which foods had antiscorbutic properties were still held but it was gradually accepted that there was a substance in some foods which would prevent scurvy.

Much of the failure to conquer scurvy, even by the early 20th century was due to the reluctance, despite the evidence, to recognise it as a deficiency disease. It was in the first quarter of the 20th century that the antiscorbutic substance present in certain foods was identified, isolated and shown to be an effective cure for scurvy.

1.2 The Discovery of L-Ascorbic Acid

In 1907 Holst and Frölich published a paper concerning the use of guinea pigs in their investigation of the causes of scurvy [1]. Their choice of guinea pig and not the rat, which is the preferred mammal today, was fortunate as the guinea pig is one of the few species of mammal which does not have the ability to biosynthesise L-ascorbic acid. They fed the guinea pigs with a diet they had previously found to produce polyneuritis in pigeons. The symptoms of the disease induced in the guinea pigs were different from polyneuritis and were correctly identified by Frölich as those of scurvy. Frölich and his co-workers tested various diets on the guinea pigs to ascertain the extent of the antiscorbutic activity of different foods. They were able to demonstrate the effectiveness of fresh fruit and vegetables scurvy conclusively. They showed that the fruit and vegetables must be fresh as if dried or cooked the antiscorbutic property disappeared. The only conclusion which could be drawn from this work was that scurvy was produced by a defective diet.

It wasn't until the first World War that Harriette Chick and co-workers working at the Lister Institute in London demonstrated the effectiveness of foods in combating scurvy [1]. Using guinea pigs fed on a balanced diet containing no antiscorbutic foods they demonstrated that lemon juice was powerfully antiscorbutic, fresh potatoes moderately so and fresh milk only slightly. They developed a reliable biological assay for the antiscorbutic factor which provided information and when applied to foods supplied to troops, saved many lives in the war.
The availability of a reliable biological method of assaying the antiscorbutic factor was crucial in the isolation of the vitamin.

1.3 Isolation of L-Ascorbic Acid

L-Ascorbic acid was eventually isolated in 1928 by Szent-Györgyi [2] in crystalline form from the adrenal cortex of the ox and from orange and cabbage juices. The process of extracting the compound was a lengthly one and involved the following steps:

i) extract from chilled and minced adrenal cortex by shaking with methanol and bubbling through with carbon dioxide to prevent oxidation.

ii) filter the extract then mix with lead acetate solution to precipitate as the lead salt.

iii) isolate the precipitate by filtration and suspend in water then add sulphuric acid to precipitate the lead as lead sulphate and dissolve L-ascorbic acid.

iv) evaporate the filtrate to dryness in a vacuum.

v) re-extract the solid with methanol and repeat steps ii), iii), and iv).

vi) dissolve the isolated solid in acetone and precipitate by the addition of petroleum ether.

A visit to the USA at the Mayo Clinic in Rochester, Minnesota enabled Szent-Györgyi to isolate almost 25g of the compound from adrenal glands supplied by the large nearby slaughter houses. Half of this was sent to Professor Norman Haworth in Birmingham for structural determination.

In 1930 Szent-Györgyi returned to Hungary as Professor of Medical Chemistry at Szeged where he was later joined by Svirbely. Svirbely exhausted the supply of isolated crystals as he established that the isolated compound from adrenal glands was "hexuronic acid" [3-6].

Szent-Györgyi discovered that Hungarian paprika was particularly rich in the vitamin and because of the absence of other sugars, its extraction and isolation from this source was much easier than from adrenal glands. Within a week over a kilogram of crystals were isolated and using this material Haworth set about determining the structure. They renamed the compound ascorbic acid because it prevented scurvy.

1.4 Structure Elucidation by Classical Chemical Methods

Hirst joined Haworth and was placed in charge of the structure elucidation of

L-ascorbic acid. They established the molecular formula as $C_6 H_8 O_6$, the presence of an acidic functional group and the facile and reversible nature of its oxidation. This reversible oxidation suggested the possible presence of an ene-diol group. Boiling with hydrochloric acid produced furfural indicating that at least five of the six carbon atoms formed part of an unbranched chain.

In the early 1930s a number of structures [7-14] had been proposed that were considered consistant with the chemical data available. Supporting evidence was provided by it's rapid reaction with diazomethane and with phenylhydrazine, after first oxidizing, to give an osazone, Figure 2.

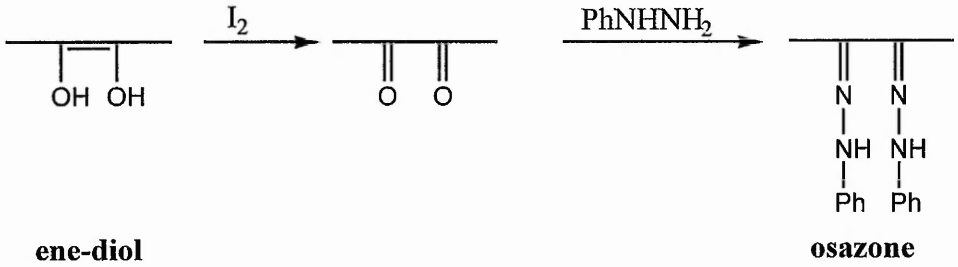
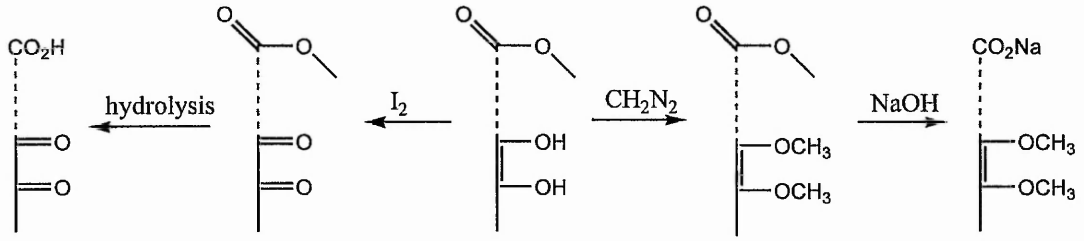


Figure 2: Reaction of the Ene-Diol Group with Phenylhydrazine to Form an Osazone

The absence of a carboxylic acid functional group was shown by oxidizing L-ascorbic acid to dehydroascorbic acid, which was known to be a neutral lactone, followed by hydrolysis which then produced a carboxylic acid. Further proof was given by dimethylascorbic, a neutral compound which reacted with sodium hydroxide to give a sodium salt without loss of a methyl group, indicating ring-opening, Figure 3.



a carboxylic acid | **dehydroascorbic acid (neutral)** | **ene-diol group (acidic)** | **di-*O*-methyl ascorbic acid (neutral)**

Figure 3: Lactone Ring Opening of L-Ascorbic Acid

Reaction of L-ascorbic acid with propanone to give the isopropylidine derivative indicated that there were two alcoholic OH groups present.

The stereochemistry of ascorbic acid was shown to be related to the L-series of sugars as further oxidation of dehydroascorbic acid yielded oxalic and L-threonic acids, Figure 4.

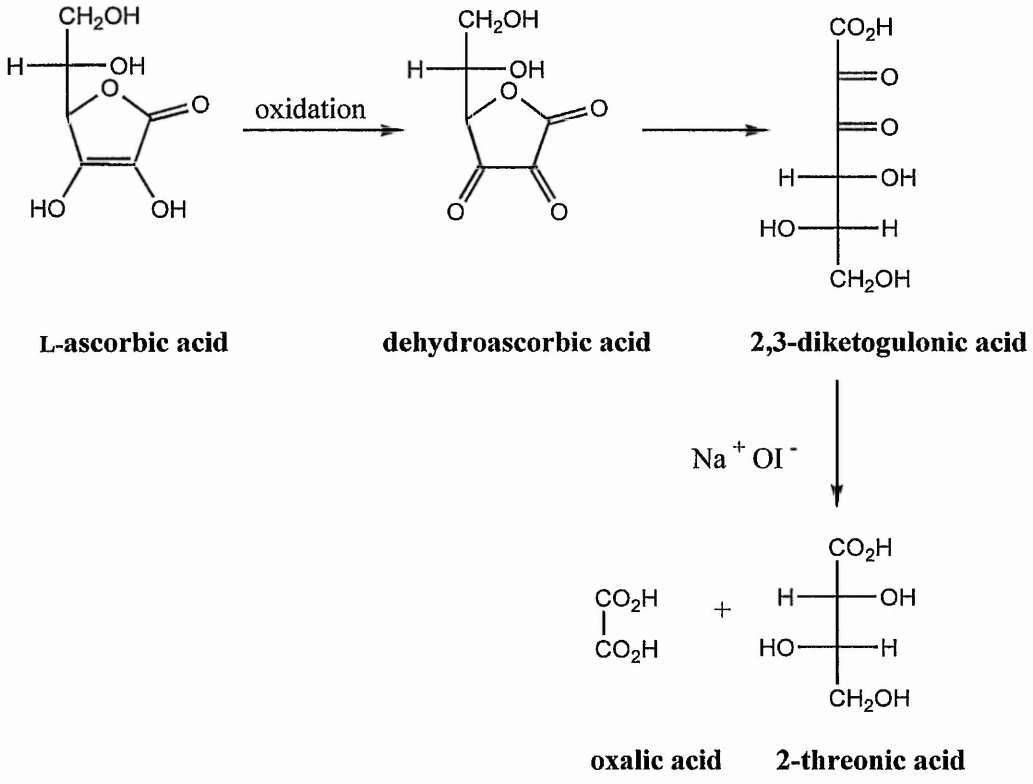
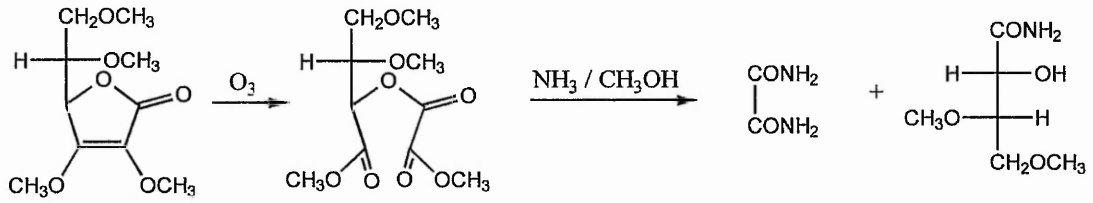


Figure 4: The Oxidative Degradation of L-Ascorbic Acid

Ascorbic acid was shown to be a γ-lactone by the reaction of its tetra-*O*-methylated analog with ozone to give a single, neutral ester. Degradation of this with methanolic ammonia gave oxamide and 3,4-di-*O*-methyl-L-threonamide. This confirmed that the group attached to the lactone ring was at C-4, Figure 5.



tetra-*O*-methylated analog | **neutral ester** | **oxamide** | **3,4-di-*O*- methyl-L-threonamide**

Figure 5: Confirmation of a γ - Lactone

The structure of L-ascorbic acid can be represented in two ways, using Fischer and Haworth projections, Figure 6.

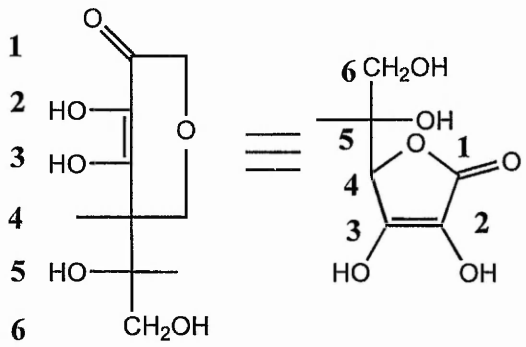


Figure 6: Structure of L-Ascorbic Acid

1.5 Structural Elucidation by Spectroscopic Methods

Spectroscopic data was obtained during the project and specific comments on the features of each technique are presented below.

1.5.1 Ultraviolet Spectroscopy

In 1932, Herbert and Hirst [15] published ultraviolet absorption data which showed an absence of a carboxyl group.

The ultraviolet spectrum of L-ascorbic acid, in water has a λ max of 243 nm at pH 2.0 (ε = 10000 $mol^{-1} dm^3 cm^{-1}$) which undergoes a red shift to 265 nm at pH 7.0 (ε = 16500 $mol^{-1}dm^3 cm^{-1}$), Figure 7, due to the ionization of the C-3 OH proton , Figure 8. These values are consistant with $\pi \rightarrow \pi^*$ excitation of a conjugated carbon-carbon double bond in a five membered lactone ring [1].

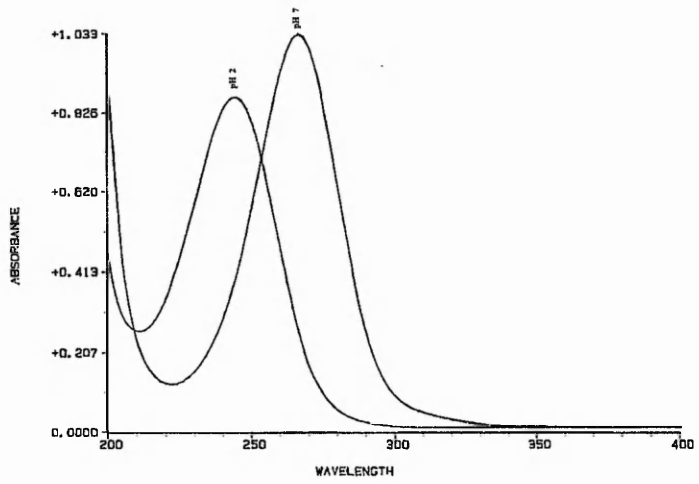


Figure 7: Ultraviolet Spectrum of L-Ascorbic Acid at pH 2.0 and pH 7.0

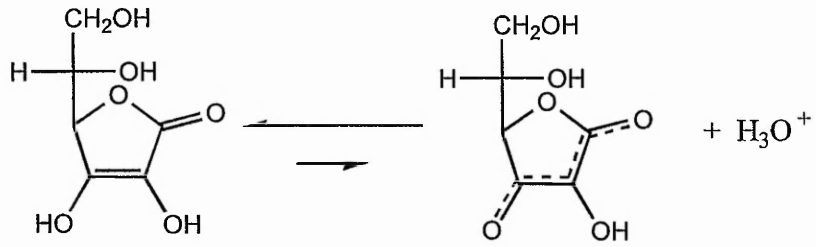


Figure 8: First Ionization of L-Ascorbic Acid

1.5.2 Infrared Spectroscopy

The infrared spectrum of L-ascorbic acid is shown in Figure 9.

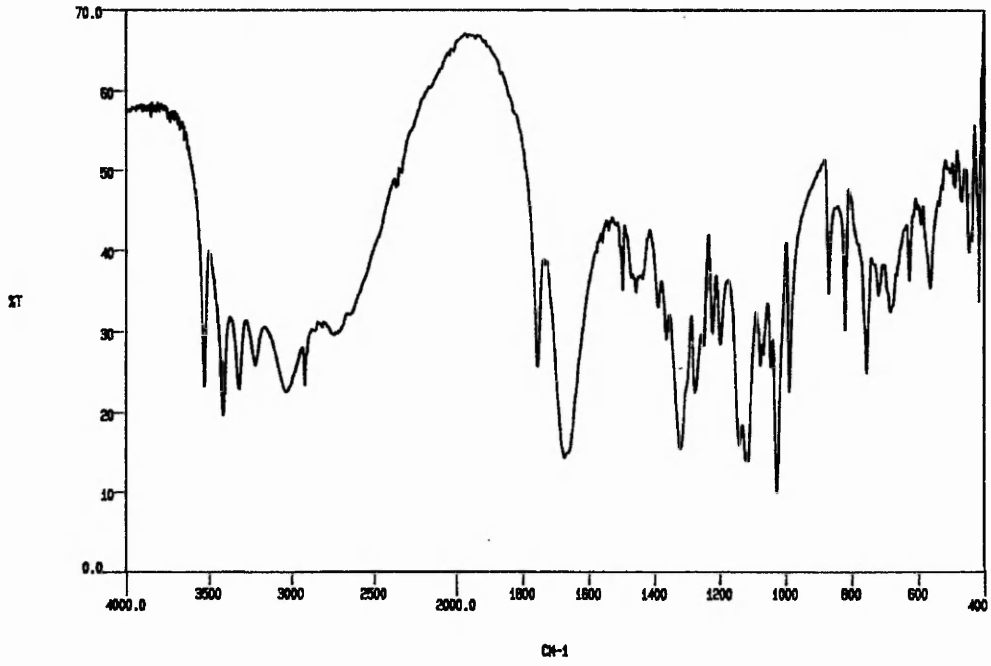


Figure 9: Infrared Spectrum of L-Ascorbic Acid

The four sharp peaks between 3500 - 3200 cm^{-1} are attributed to the side -chain O-H groups stretching vibrations. The enolic O-H groups on C-2 and C-3 have shorter O-O distances and are involved in stronger hydrogen bonding than the C-5 and C-6 O-H groups. This produces a complex series of broader bands in the region 3100 - 2200 cm^{-1}.

The strong absorption at 1754 cm^{-1} has been attributed to C = O stretching of the lactone ring system with the intense doublet at 1675 and 1660 cm^{-1} due to C = C stretching vibration coupled with neighbouring vibrations along the conjugated system [1].

1.5.3. Nuclear Magnetic Resonance Spectroscopy

The proton-decoupled ^{13}C nuclear magnetic resonance (NMR) spectrum shows six signals, Figure 10.

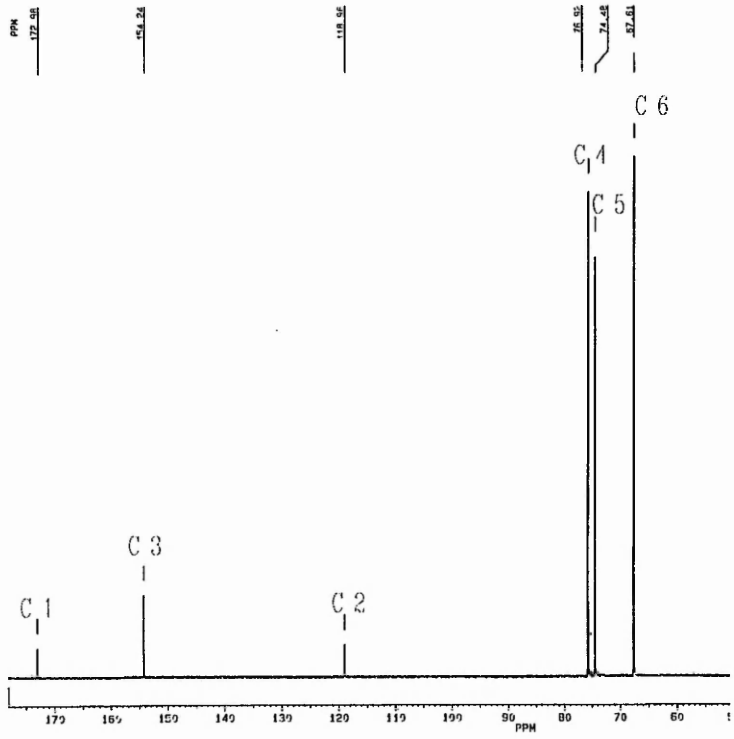


Figure 10: Proton Decoupled ^{13}C NMR Spectrum of L-Ascorbic Acid

The C-4 and C-5 signals were assigned from the proton-decoupled spectrum of the 4-deuterated derivative when the signal at 77δ p.p.m. split into a triplet caused by coupling to deuterium [1].

The chemical shift of C-3 was confirmed by the downfield shift of it's signal when the pH was increased from 2 to 7 causing ionization of the C-3 O-H proton.
The proton NMR spectrum of L-ascorbic acid is simplified when run in D_2O as the four hydroxy protons are exchanged and do not produce signals. The remaining

four protons attached to C-4, C-5 and C-6 comprise an ABMX system as the two protons on C-6 are not equivalent due to the chirality of C-5, Figure 11.

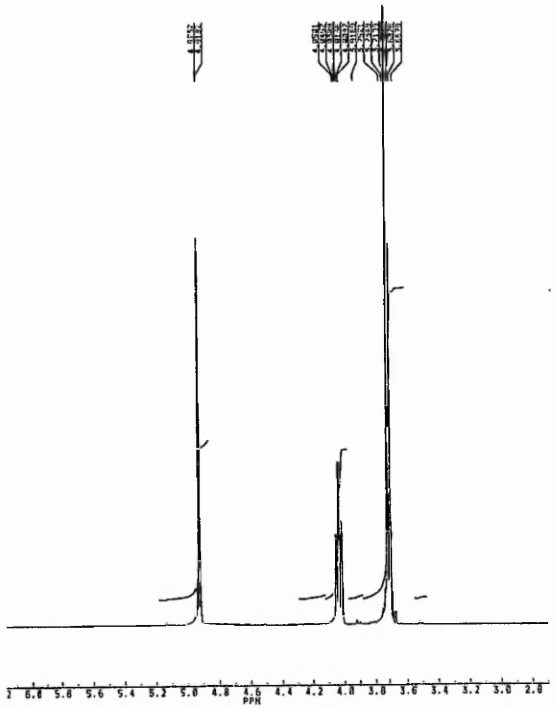


Figure 11: ^{1}H Spectrum of L-Ascorbic Acid in D_2O

1.5.4. X-Ray Diffraction

In 1932, Cox [16] produced the first x-ray data on crystalline L-ascorbic acid revealing that L-ascorbic acid is an almost planar molecule. More recent x-ray and neutron diffraction studies have revealed a monoclinic crystal structure with four molecules per unit cell. The approximate conformation in the crystal is shown by structure 3, see Figure 12. Ogawa and co-workers [17] studied the conformation of L-ascorbic acid-5-d in deuterium oxide by ^{13}C-NMR spectroscopy, and concluded that, in aqueous solution, the most favoured rotamer of L-ascorbic acid is the

structure on the right, Figure 12.

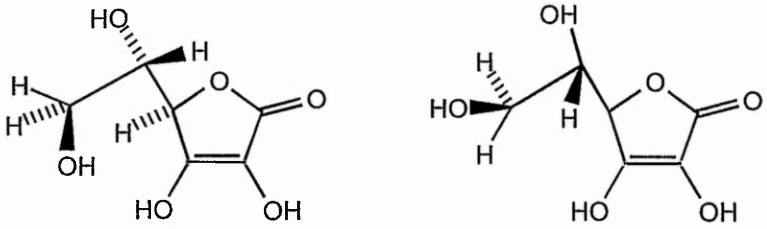


Figure 12: Conformations of L-Ascorbic Acid

1.5.5. Mass Spectrometry

The electron-impact mass spectrum of L-ascorbic acid gives a parent ion at m/e 176 together with a base peak at m/e 116, Figure 13.

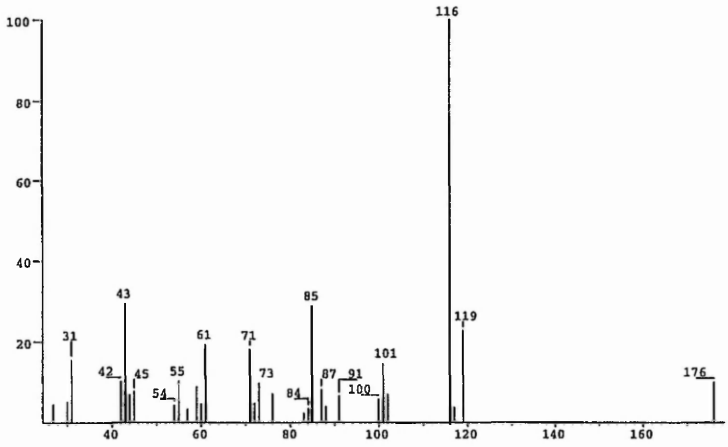


Figure 13: Electron-Impact Mass Spectrum of L-Ascorbic Acid

A possible fragmentation pattern is given in Figure 14.

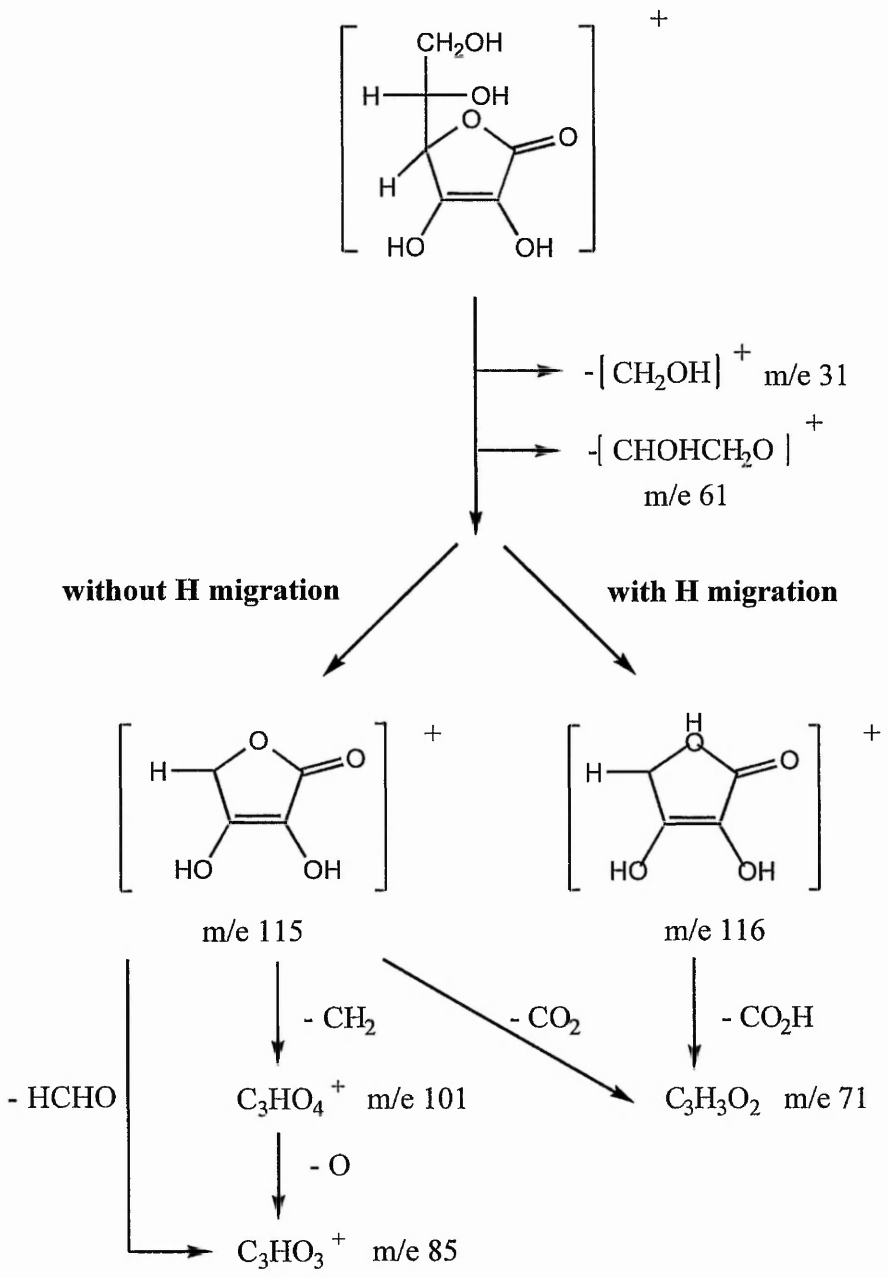


Figure 14: Possible EI MS Fragmentation Pattern of L-Ascorbic Acid

1.5.6. Physical Properties of L-Ascorbic Acid

Nomenclature:	vitamin C, hexuronic acid L-ascorbic acid, L-*xylo*-ascorbic acid L-*threo*,2,3,4,5,6-pentahydroxyhex-2-enoic acid-4-lactone L-*threo*-hexono-1,4-lactono-2-ene
Appearance:	a white crystalline solid
Melting point:	192°C
Relative molar mass:	176.14
Specific rotation:	$[\alpha]_D^{20}$ = +23° in water; $[\alpha]_D^{18}$ = +49° in methanol
pK_a1:	4.17
pK_a2:	11.79
Solubility:	33% $^{w}/_{v}$ in water, 3% $^{w}/_{v}$ in ethanol, 1% $^{w}/_{v}$ in glycerol, insoluble in chloroform, benzene, diethyl ether.
Density:	1.65 g cm^{-3}

1.6 Synthesis of L-Ascorbic Acid

Appendix 1 includes a reference list of compound names and numbers used in this thesis.

Various syntheses routes of L-ascorbic acid (compound 1, Figure 15) have been developed since its structure was determined. The most important starting material is D-glucose (compound 3, Figure 15) which contains the requisite six carbon atoms and is available at low cost for large scale commercial production.

In the biosynthesis of **(1)** D-glucose is converted into **(1)** by two separate pathways [18]. One pathway leads to C-1 of D-glucose becoming C-6 of **(1)** and in the other C-1 of D-glucose becomes C-1 of **(1)**, Figure 15.

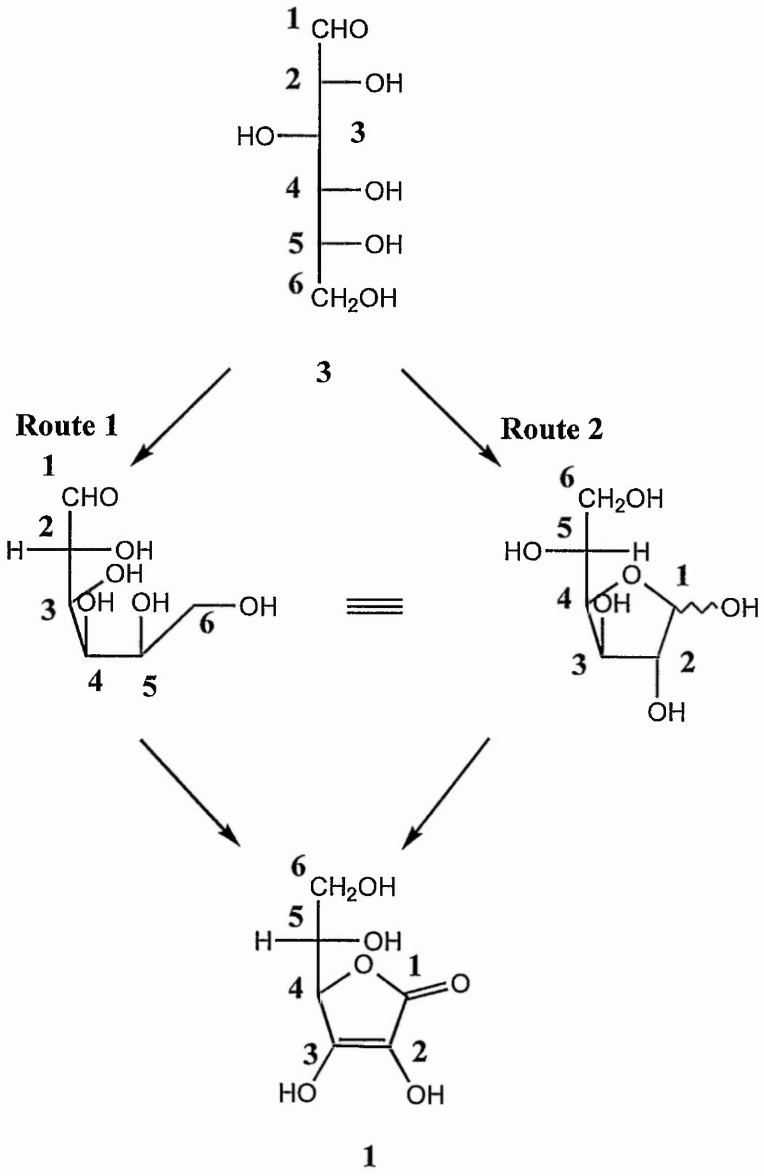


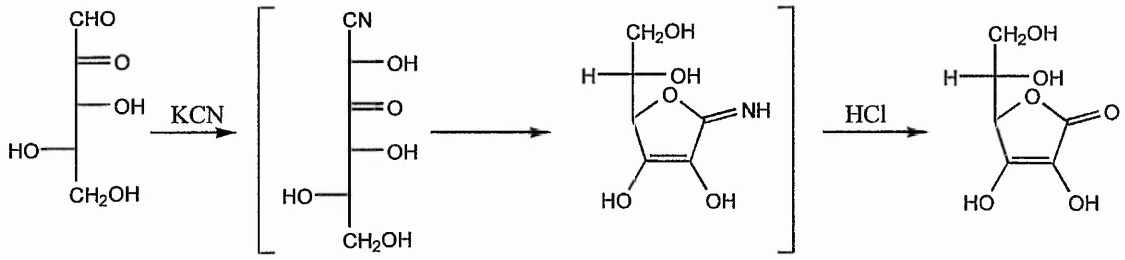
Figure 15: Syntheses Routes of L-Ascorbic Acid

Both of these routes have been used to synthesize L-ascorbic acid. The series of reactions via route 1 (reduction of C-1 oxidation at C-5 and C-6) yields L-xylo-hexulosonic acid which on lactonization affords **(1)**. When route 2 is used

D-glucose must be oxidized at C-1 and C-2 followed by inversion at C-5.

1.6.1 The L-Threo-Pentos-2-Ulose-Cyanide Synthesis

The first synthesis of L-ascorbic acid was reported in 1933 by Reichstein and co-workers [19-23]. Aqueous potassium cyanide solution was added to L-threo-pentos-2-ulose (L-xylosone) **(4)** to afford cyanohydrin **(5)** which under mild conditions, cyclizes to **(6)**. Hydrolysis of **(6)** then gives L-ascorbic acid in 40% yield, Figure 16.



4 **5** **6** **1**

Figure 16: The L-Threo-Pentos-2-Ulose-Cyanide Synthesis

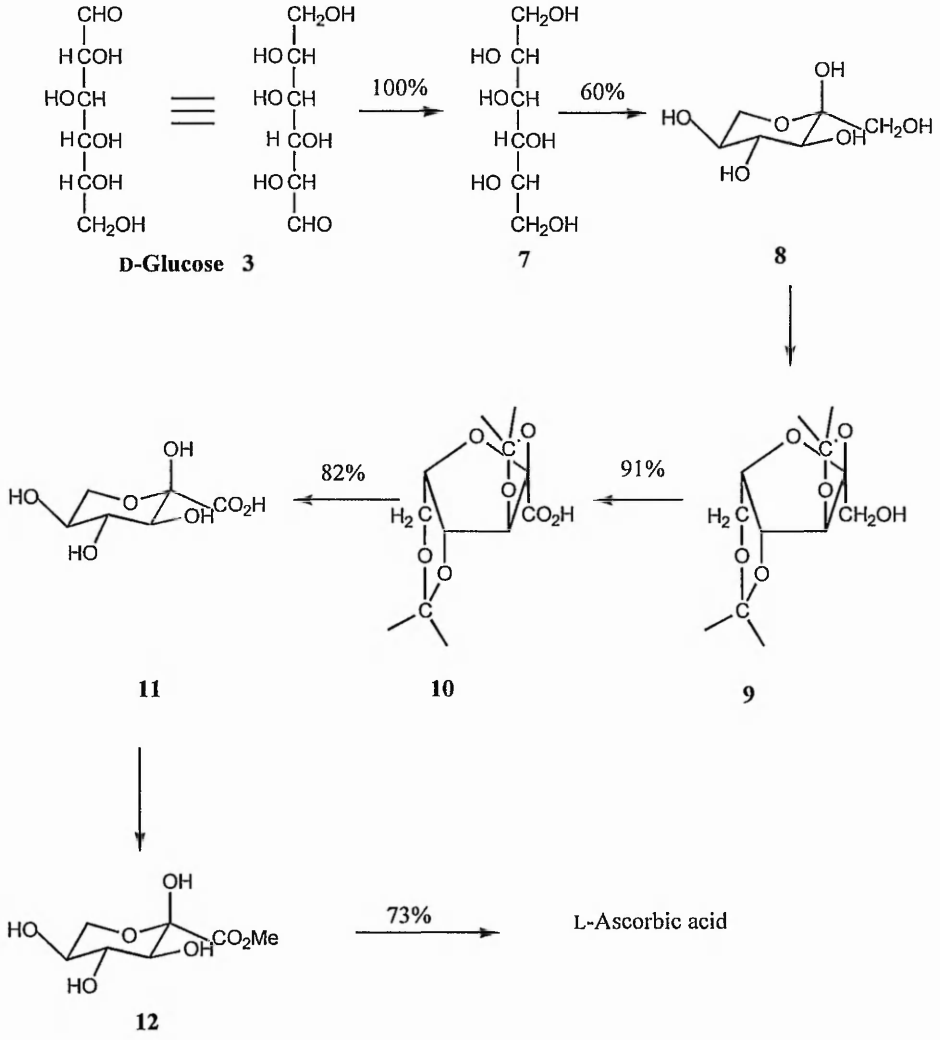
This general synthesis - the addition of cyanide to an aldos-2-ulose, followed by cyclization and hydrolysis - proved of great value in the preparation of analogs of ascorbic acid [24], but is not financially viable with later synthesis of L-ascorbic acid because a cheap source of **4** has not yet been developed.

1.6.2 The Reichstein-Grüssner Synthesis

First reported in 1934 [25] this reaction involves the inversion of D-glucose **(7)**. D-glucose **(7)** was hydrogenated to D-glucitol **(8)** which was microbiologically oxidized to L-xylo-2-hexulopyranose **(9)** with *acetobacter xylinum*. On treatment of **9** with acetone in the presence of sulphuric acid, 2,3:4,6-di-*O*-isopropylidene-L-xylo-2-hexulofuranose **(10)** was isolated. Compound **10** was oxidized to the

corresponding acid **(11)** with potassium permanganate. When **11** was heated in water, it afforded L-xylo-2-hexulosonic acid **(12)** and L-ascorbic acid **(1)** was obtained from **12** by heating in water at 100°C (13-20% yield). The overall yield of **(1)** from D-glucitol **(8)** was 15-18% thus this synthesis provided an efficient method for the preparation of L-ascorbic acid. It also became the industrial method. for the preparation of L-ascorbic acid.

During the succeeding years, a number of modifications and improvements in yield were made. As a result, this synthesis remains the current industrial method for the preparation of L-ascorbic acid (Scheme 1).



Scheme 1: Industrial Preparation of L-Ascorbic Acid

The following is a summary of the relevant chemistry of each step in the synthesis; Scheme 1.

a) D-Glucose to Sorbitol

The hydrogenation of D-glucose **(3)** to sorbitol **(7)** currently uses a Raney nickel catalyst and the yield is essentially quantitative [26-28].

b) Sorbitol to L-Sorbose

The microbiological oxidation of sorbitol **(7)** to L-sorbose **(8)** was initally reported by Bertrand [29] in 1904. A practical procedure was described in 1933 by Schlubach and Vowerk [30] using *acetobacter xylinum* and this afforded **(8)** in 50 to 75% yield. This transformation has also been achieved with *acetobacter* **suboxydans** [31-37] which provides L-sorbose in >95% yield.

c) 2,3:4,6-Di-*O*-isoproylidene-L-xylo-hexulofuranose **(9)**.

It was found in the original work reported by Reichstein and Grüssner [25] that, when L-sorbose **(8)** is treated with acetone and sulphuric acid, its 2,3:4,6-diisopropylidene acetal **(9)** is formed, accompanied by the 2,3-isopropylidene acetal.

A mixture of the 2 acetals was relatively easily separated and **(9)**could be recycled. When the reaction was carried out at - 8 to + 4°C, **(9)** is then formed in 85% yield [38]. Other aldehydes and ketones have been used for preparing protected derivatives of L-sorbose that are structurally analogous to **(9)**. These include formaldehyde and benzaldehyde [25, 39]. The resulting acetals have been converted into L-ascorbic acid.

d) 2,3:4,6 - di-*O*-isopropylidene-L-xylo-hexulofuranosonic acid **(10)**.

The oxidation of **(9)** as originally performed with potassium permanganate in water, afforded **(10)** in 90% yield [25, 39]. Several different, electrochemical oxidations of **(9)** to **(10)** have been reported. Using a variety of electrodes **(9)** was oxidized in aqueous potassium hydroxide solution containing potassium chromate or permanganate, to afford **(10)** in 70 to 85% yield [40, 41].

Following the initial report by Reichstein and Grüssner [25] on the oxidation of **(9)** to **(10)**, a number of other methods using potassium permanganate or related manganese salts as the oxidant, under a variety of conditions, appeared [42-44]. Weijlard [45, 46] found that **(9)** could be converted to **(10)** in 90% yield with a catalytic amount of nickel chloride and one molar equivalent of sodium hypochlorite, an oxident much cheaper than potassium permanganate.
The oxidation of **(9)** to **(10)** has also been achieved in good yield using air, or oxygen, and a metal catalyst such as palladium on carbon [47-49], palladium or platinum [50] and platinum on carbon [51].

e) Conversion of 2,3:4,6-di-*O*-isopropylidene-L-xylo-hexulofuranosonic acid **(10)** into L-ascorbic acid **(1)**.
Since Reichstein and Grüssner [25] first reported the conversion of **(10)** into **(1)**, much work has been carried out to improve this final stage of the synthesis. The work can be divided into two main areas:

i) hydrolysis of **(10)** to L-xylo-2-hexulosonic acid **(11)** followed by esterification and based-catalyzed cyclization to **(1)**, or
ii) hydrolysis of **(10)** to **(11)**, followed by acid-catalyzed cyclization to **(1)**.

The most successful base catalyzed, cyclization procedure appears to be that using the salt of a weak acid, such as sodium hydrogen carbonate or sodium acetate. Concentrated hydrochloric acid is the favoured reagent for the acid-catalyzed cyclization of **(11)** to **(1)**.
The catalyzed cyclizations of **(10)**, **(11)** or **(12)** by aqueous acid leads to the decomposition of **(1)** as it is formed [52].
Reichstein [53] found that, in an essentially non-aqueous system compounds **(10)**, **(11)** and **(12)** are converted into **(1)** in good yield. Bassford and co-workers [54] discovered that high yields of relatively pure L-ascorbic acid was readily obtained by heating **(10)**, **(11)** or **(12)** in a non-aqueous solvent such as benzene, tetrachloroethane or toluene containing concentrated hydrochloric acid without an alcholic co-solvent. Presumably, these conditions are successful because the

L-ascorbic acid precipitates from the mixture as fast as it is formed and therefore avoids decomposion.

In summary, the synthesis of L-ascorbic acid as described in Scheme 1 by way of D-glucose **(3)**→D-glucitol **(7)**→L-sorbose **(8)**→2,3:4,6-di-*O*-isopropylidene-L-xylo-2-hexulofuranose **(9)**→2,3:4,6-di-*O*-isoproylidene-L-xylo-2-hexulofuranosonic acid **(10)**→**(1)** maybe achieved in excellent yield. Commercially this synthetic route affords **(1)** in greater than 50% overall yield [55].

Clearly, an improved synthesis of L-ascorbic acid would result from the direct oxidation of L-sorbose to L-xylo-2-hexulosonic acid thus eliminating the protecting-deprotecting steps required in the Reichstein-Grüssner synthesis (Scheme 1). Efforts to perform this oxidation can be divided into two categories, namely, chemical and fermentative.

1.6.3 Chemical Oxidation of L-Sorbose to L-Xylo-2-Hexulosonic Acid (11)

Haworth and co-workers [56,] reported the first direct oxidation of L-sorbose **(8)** to L-xylo-2-hexulosonic acid **(11)** by use of hot, aqueous nitric acid. The oxidation has been achieved with a number of oxidants such as oxygenated halogen compounds, the halogens, hydrogen peroxide, chromium dioxide-potassium permanganate.

The most promising of the direct, chemical-oxidation procedures for the conversion appears to be air oxidation catalyzed by platinum or related metals. Heyns and co-workers [57-60] found that using 5% platinum on carbon in aqueous sodium hydrogen carbonate gave a yield of **(12)** up to 62%. Unfortunately, under the conditions of the reaction **(11)** can be further oxidized to oxalic and L-threonic acids.

1.6.4 Fermentative Oxidation of L-Sorbose

The first fermentative oxidation of L-sorbose **(8)** to L-xylo-2-hexulosonic acid **(11)** was reported using micro-organisms of the genus **pseudomonas**. When the

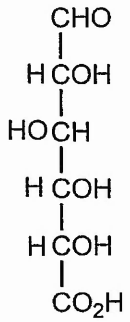
fermentation was started at pH 7-8 with a 2% w/v of **(8)**, compound **(11)** was present at 0.3% w/v after 80 hours. Other micro-organisms have been reported to produce the oxidation [61]. Different mechanisms have been proposed for the conversion depending on the organism used for the fermentation.
At the present time the direct chemical or fermentative oxidation of L-sorbose to L-xylo-2-hexulosonic acid is not efficient enough to compete with the Reichstein-Grüssner method.

1.6.5 Alternative Synthesis Routes To L-Ascorbic Acid

Other synthesis routes to L-ascorbic acid include the following:

a) Conversion of D-glucitol (sorbitol) to L-xylo-2-hexulosonic acid.
If an efficient fermentative oxidation of **(7)** into **(11)** could be developed it would be more economical than the Reichstein-Grüssner route. Motizuki and co-workers [62] found that micro-organisms from the genera *acerbacter, bacterium* and *pseudomonas* can achieve this conversion. Low concentrations (2-5%) of D-glucitol coupled with poor yields (12% or less) and a mixture of products suggest this approach is not efficient enough to become a viable proposition.

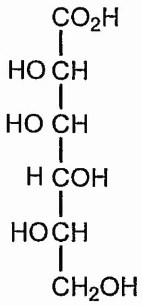
b) Synthesis from D-glucuronic acid **(13)**



13

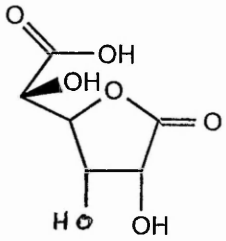
Conversion of D-glucose into **(13)** followed by reduction at C-1 then oxidation at C-5 should lead to the formation of **(11)**.
This approach had not been adopted on an industrial scale as after oxidation of **(13)** at C-5 various intermediates are formed on the way to **(11)**.

c) Synthesis From L-Gulonic Acid (L-Gulono-1,4-Lactone) **(14)**.



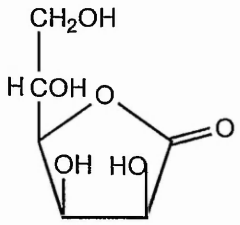
14

L-Ascorbic acid may be prepared by converting L-glucose **(3)** into D-glucuronic acid **(13)** followed by reduction of **(13)** to L-gulonic acid **(14)** which is then converted into L-xylo-2-hexulosonic acid **(11)** and this into **(1)**.
Probably the most efficient method for the preparation of **(14)** is the hydrogenation of D-glucofuranurano-6,3-lactone **(15)**.



15

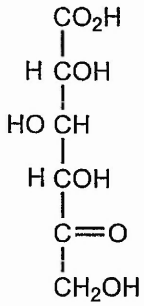
Reduction of **(15)** with hydrogen and Raney nickel catalyst, followed by removal of water yields L-gulono-1,4-lactone **(16)**.



16

The enzymatic oxidation of **(16)** produces a 40% yield [63].
The direct oxidation of **(16)** to **(1)** in 10% yield has been reported using Fentons reagent. Later it was found [64] that treatment of **(16)** with ionizing radiation affords **(1)** in low yield.

d) Synthesis From D-Xylo-5-Hexulosonic Acid **(17)**

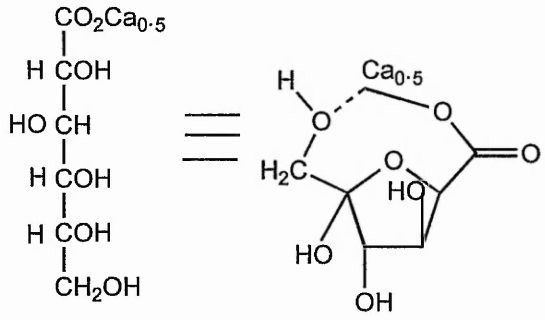


17

All previous synthetic routes have at some stage involved reduction of D-glucose at C-1 and oxidation at C-5 and C-6, resulting in inversion of the carbon chain. This and the next synthetic route does not involve the inversion of the D-glucose carbon chain and C-1 of D-glucose becomes C-1 in L-ascorbic acid.

D-xylo-5-hexulosonic acid **(17)** can be prepared in low yield by the direct oxidation of D-glucose with nitric acid [65] or bromine [66].

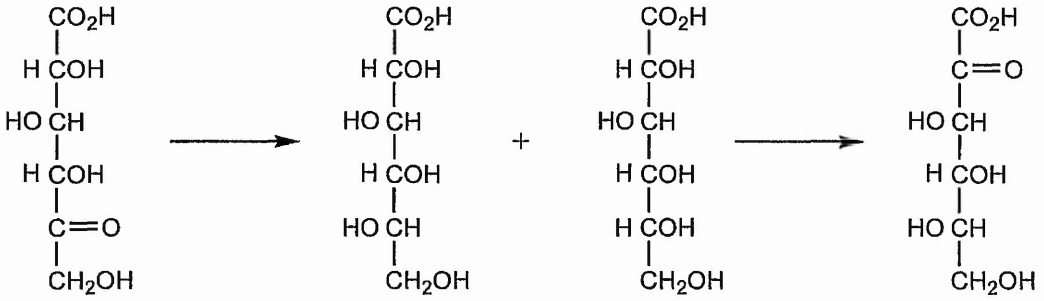
The fermentative oxidation of D-glucose to the calcium salt of **(17)** in 90% yield using *acetobacter* species has been reported [67, 68]. The conditions for the fermentation have been studied by Yamazaki [69] who found that the formation of calcium D-gluconate **(18)** is not affected by the proportion of carbonate added to the fermentation mixture or by the pH of the medium.



18 **19**

The conversion of **(18)** into **(17)** is greatly dependent on the pH of the medium.

The next stage of the synthesis involves the reduction of **(17)** to L-idonic acid **(20)** followed by the oxidation of **(20)** to L-xylo-hexulosonic acid **(11)**.



17 **20** **21** **11**

Reduction of **(18)** invariably leads to a mixture of **(20)** and calcium D-gluconate **(21)**. Using Raney nickel catalyst the calcium salt of **(18)** was converted by Sato and co-workers [70] in good yield into an almost 1:1 mixture of **(20)** and **(21)**. Attempts by fermentative reduction of **(18)** to selectively produce L-idonic acid **(20)** have been reported [71, 72]. An enzyme system isolated from *fusarium oxysporum* has been reported [71] to reduce **(18)** to **(20)** in approximately 80% yield. As yet no practical method for achieving this enzyme reduction has been developed.

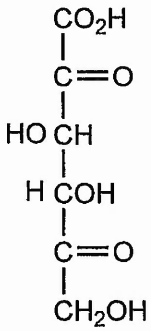
Mixtures of **(20)** and **(21)** can be converted into L-xylo-2-hexulosonic acid **(11)** by the simultaneous, fermentative oxidation of **(20)** to **(11)** and decomposition of **(21)**. Gray [73] was the first to report the conversion of **(20)** into **(11)** by fermentation. Using *pseudomonas mildenbergii* a yield of >80% was achieved.

Leibster and co-workers [74] and Faerber *et al* [75] found that, when the mixture of **(20)** and **(21)** is fermentated with an activated strain of *pseudomonas chromospirans* **(20)** is converted into **(11)** and **(21)** is entirely decomposed.

Thus D-glucose may be efficiently converted into calcium D-xylo-5-hexulosonate which, on hydrogenation, affords an approximate 7:3 mixture of L-iodic acid and D-gluconic acid that may be efficiently converted into L-xylo-2-hexulosonic acid with an overall yield of about 50%.

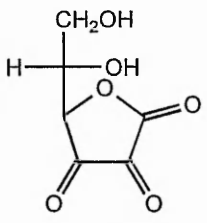
If a more selective reduction of the calcium salt of **(17)** can be found then this approach would be a competitor to the Reichstein-Grüssner synthesis.

e) Synthesis from D-Threo-2,5-Hexodiulosonic Acid (2,5-Diketo-D-Gluconic Acid)
The structure of D-threo-2, 5-hexodiulosonic acid **(22)** was initally determined by Katznelson and co-workers [76].



22

The isolation of salts of **(22)** in pure form is difficult owing to their instability. It has been reported [77] that the calcium salt of **(22)** is stable for several months at 6°C. In solution, **(22)** decomposes at high or low pH. Katznelson and co-workers [76] noted that **(22)** is a positional isomer of dehydro-L-ascorbic acid **(2)** as can be seen if **(22)** is drawn in the 1,4-lactone form **(23)** which could also exist as **(24)** and **(25)**.



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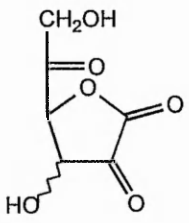

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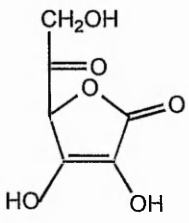

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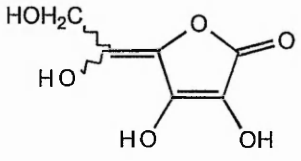
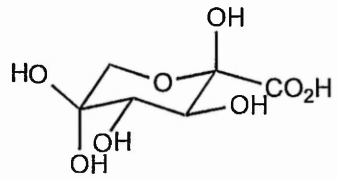


25

In solution ^{13}C-nmr data suggests structure **(26)** is the major tautomeric form.

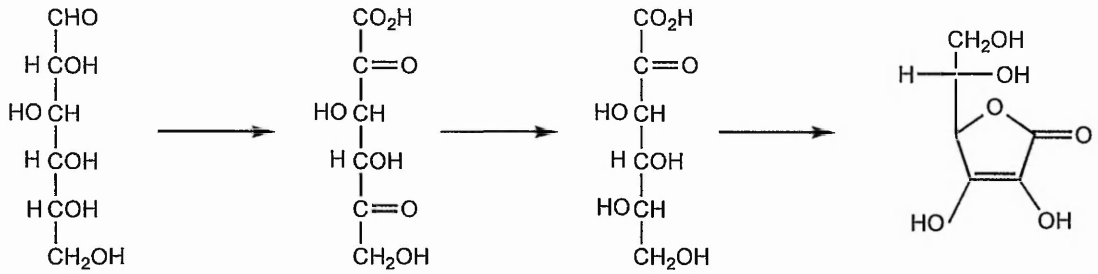


26

The preparation of **(22)** was first prepared in 1953 by Katznelson and co-workers [76] as a product of fermentative oxidation of D-glucose. A variety of micro-organisms have been used to convert D-glucose into **(22)** including *acetobacter melanogenum, rubininosus and suboxydans.*

Oga and co-workers [78, 79] found that *acetobacter fragum* readily produces calcium D-threo-2,5-hexodiulosonate.

If D-threo-2,5-hexodiulosonic acid could be regio and stereo - selectively reduced to L-xylo-2-hexulosonic acid a three step synthesis of L-ascorbic acid from D-glucose would be possible (Scheme 2).



3 **22** **11** **1**

Scheme 2: Three Step Synthesis of L-Ascorbic Acid From D-Glucose

Both chemical and fermentative reductions have been reported. Ikawa and co-workers [80] obtained the greatest yield of about 50% for fermentative reduction. Both Raney nickel [81] and sodium borohydride [80, 82] have been investigated for the chemical reduction of **(22)** but **(11)** was always the minor product.

Until a successful means of reducing **(22)** to **(11)** can be found the Reichstein-Grüssner synthesis will remain the industrial process for the manufacture of L-ascorbic acid.

1.7 Solid Phase Degradation

1.7.1 Theory of Solid Phase Degradation

Any reaction occuring in the solid phase will exhibit different characteristics i.e. reaction rate and order and reaction products, from the same reaction proceeding in either a liquid or a gaseous state.

The solid phase reaction is mainly initiated on the external surface and leads to the formation of an array of product nuclei which in general have a unit cell differing in geometric form and in dimensions from the original substance. These set up strains in the crystal surface which are relieved by the formation of cracks. At the mouth of these cracks, decomposition will be favoured because of lattice imperfections; reaction therefore spreads down the crevices into the crystal. Covering of the surface of these cracks produces lateral strains which ultimately lead to further cracking from the inner surface. A series of "branchings" will occur and planes of strain will form. Along these planes the deformation effect of the product on the electronic structure of an adjacent molecule of unreacted substance will be a maximum and decomposition will be favoured. A chain reaction develops which becomes subject to interference since, when a plane of product molecules in encountered, the chain is broken.

The number of product nuclei which form often depends on the history of the solid and the way they develop depends on the directional properties of the crystal. The growth may take place in 1 dimension (to give a needle), in 2 dimensions (to give a disc) or in 3 dimensions (to give a sphere or cube), Figures 17 and 18.

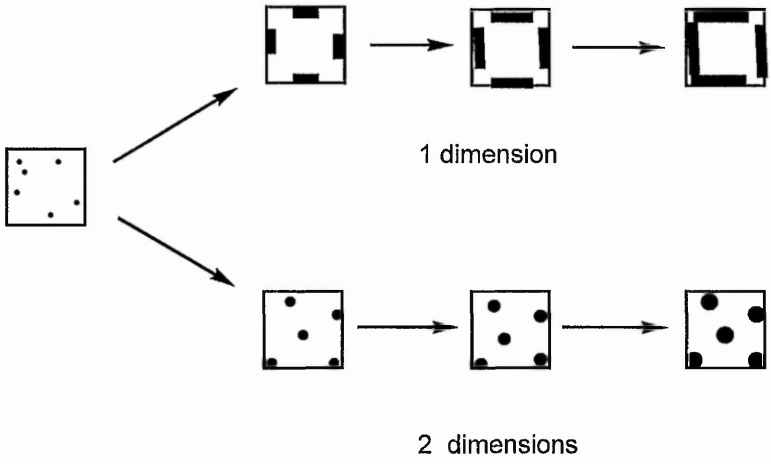


Figure 17: Dimensions of Growth

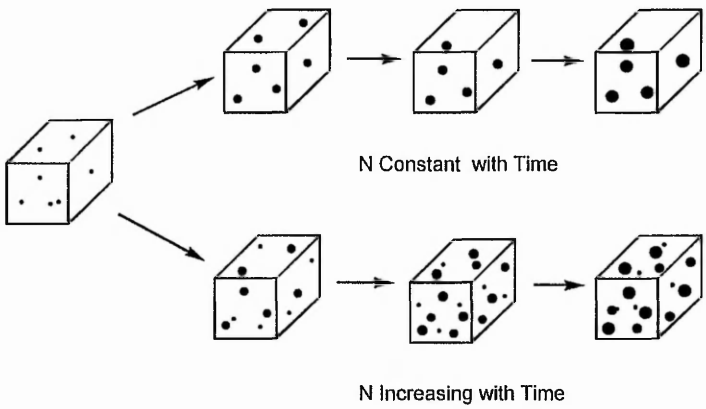


Figure 18: Time Dependence of Nucleation

According to Garner [83, 84] there are three main types of reactions, Figure 19.

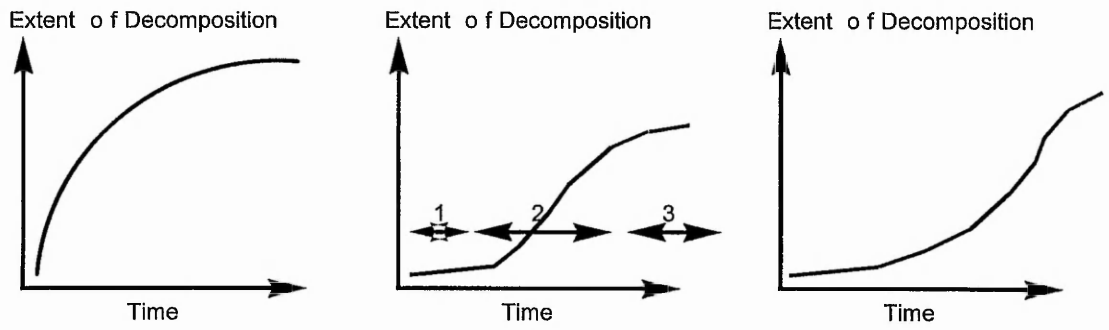


Figure 19: Three Main Types of Solid Phase Reactions

Solid reactions involving nucleation have been classified according to the shape of the thermal decomposition - time curves.

Curve a) the most rapid rate of reaction occurs at the start where the nucleation process of the new phase is very efficient and there is a rapid coverage of the external surface with a file of small nuclei of the solid product.
Curve b) there is an induction period (1) caused by the slow rate of production of nuclei which is followed by the rapid acceleration period (2) and ending with a decay period (3). If the decomposition product is removed by vacuum or exposure to a humid atmosphere the acceleration period is maintained without the onset of the decay period.
Curve c) slow evolution of gas is followed by a rapid acceleration period.
Where melting occurs during the decomposition the curve may not correspond with any of the decomposition curves. Ng [85] has argued that most thermal decompositions can be defined by Equation 1.

$$\frac{d\alpha}{dt} = \bar{k}\ \alpha^{1-\rho}(1-\alpha)^{1-q} \qquad \text{Equation 1}$$

where α = fractional decomposition

k = composite rate constant

ρ = parameter related to the reaction mechanism

q = parameter related to the reaction mechanism

The reaction defined by Equation 1 assumes that all decomposition occurs initially at nuclei on the crystal surface (nuclei are stress points, imperfections or dislocation points in or on the crystal).
Degradation causes an increase in the stress or dislocations in the crystal thereby increasing the number or nuclei. After a certain time the number of dislocations will decrease as a deceleratory phase is entered which depends on the value of q. If both p and q = 1 then Equation 1 simplifies to Equation 2 and zero - order kinetics are observed.

$$\frac{d\alpha}{dt} = \bar{k} \qquad \text{Equation 2}$$

If ρ = o and q = 1 then Equation 3 results:

$$\frac{d\alpha}{dt} = \bar{k}\alpha \qquad \text{Equation 3}$$

and the degradation follows exponential kinetics.

If p and q both equal 0 then Equation 4 results:

$$\frac{d\alpha}{dt} = \bar{k}\alpha(1-\alpha) \qquad \text{Equation 4}$$

and the degradation will follow a sigmoidal curve.

1.7.2 Factors Influencinging Solid Dosage Stability

There are eight factors influencing the stability of solid dosage forms:

a) Moisture
b) pH
c) Oxidation
d) Light
e) Polymorphic structure
f) Particle size and shape
g) Mechanical stress
h) Solid - solid interactions
i) Temperature

Moisture

Moisture is perhaps the single most important factor affecting stability of compounds in the solid phase as absorption of moisture onto the surface may induce hydrolytic degradation. Generally, degradation arises as a function of the free and not the bound moisture. Free moisture can be described as that being loosely associated with a solid e.g. arising from crystallization from an aqueous solution. Bound moisture is that present due to the compound existing as a hydrate.

Solid phase hydrolysis involves the solubilization of surface molecules in an adsorbed layer of water. For a poorly water insoluble compound undergoing solid phase hydrolysis the solid will dissolve to a limited concentration in the surface moisture. The amount dissolved will depend on the volume of water in the surface film and the rate of hydrolysis will follow zero-order kinetics.

A more soluble compound will behave similarly provided the amount of free water present is insufficient to completely dissolve the compound. If the compound completely dissolves in the water then first-order kinetics are followed.

Leeson and Mattocks [86] demonstrated that in the presence of moisture (in the vapour phase) the degradation of acetylsalicylic acid crystals took place in the sorbed moisture layer. The sorbed moisture is believed to become saturated with the compound and this saturated solution then becomes the media for degradation, Figure 20.

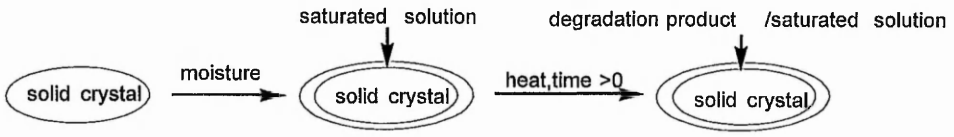


Figure 20: Absorption of Moisture onto a Water Soluble Crystal

pH

The presence of moisture can alter the microscopic pH environment and hence influence solid state degradation. The acid strength of a solid surface has been defined by Walling [87] as the ability of the surface to convert an absorbed neutral base to its conjugate acid. In the solid state the ionization of excipients, drug or degradation products in the presence of moisture can influence surface pH. Kornblum and Zoglio [88] have shown how the presence of an alkali stearate forms an aqueous soluble salt of aspirin. This salt formation acts as the driving force and accelerates the degradation of aspirin. Castello and Mattocks [89] showed that alkaline lubricants cause discoloration of tablets containing amphetamine sulphate in a spray dried lactose base. The alkalinity of the lubricant converts some of the amphetamine salt to the free base, which then reacts with the lactose causing discoloration.

In solid dose drug formulations known to be susceptible to moisture and pH effects a judicious choice of additives can stabilize the pH.

Oxidation

Many organic compounds are oxidized on exposure to dry heat in the presence of oxygen. Oxidation can be prevented by flushing the headspace of the container with nitrogen. Drug formulations can include antioxidants to scavenge oxygen and chelating agents may also be added to remove catalytic metal ions.

The presence of moisture can accelerate oxidation with the degradation taking place in the adsorbed moisture on the surface.

Solvates or hydrates are usually more chemically stable than the anhydrous form due to the solvent or water stabilizing the crystal lattice resulting in a higher melting

point.

Light

There are many pharmaceutical compounds, including vitamins that degrade via photolytic mechanisms. The degradation is frequently auto-oxidative and catalyzed by metal ions. Elevated levels of moisture can accelerate the degradation which can also be influenced by pH.

A simple mechanism for photolysis in the solid phase was demonstrated by Eble and Garrett [90] and is shown in Equation 5.

$$A + h\nu \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A^* + A \xrightarrow[k_3]{} \rho \qquad \text{Equation 5}$$

A molecule A absorbs a quantum of light to form an excited species A* with a rate constant k_1. This excited species can either return to the ground state with a rate constant k_2 or react with a second molecule to degrade into ρ with rate constant k_3. Under steady state conditions, dA*/dt = 0 and Equation 6 results.

$$k_1 * I_{abs} = (k_2 + k_3 A) A^* \qquad \text{Equation 6}$$

Where:

I_{abs} = intensity of light absorbed.

The rate-determining step may be the step where the activated molecule, A*, reacts with, or transfers its energy to, another molecule in the crystalline lattice. The probability of deactivation occuring increases as the number of molecules adjacent to the activated molecule decreases.

First order kinetics result when $k_3 >> k_2$.

Both the intensity and wavelength of the light affect the rate of photolysis with the shorter wavelengths inflicting the most damage.

Photolytic degradation of materials in solid dosage froms is essentially a surface phenomena and is limited to only a small fraction of the depth from the surface.

Light sensitive formulations can be protected from photolysis by packaging in amber glass bottles. If in tablet form the incorporation of a light blocking coating will prevent photolysis. Formulations in capsules can be protected if they are filled into opaque capsule shells.

Polymorphic Structure

A polymorph is a solid crystalline phase of a compound resulting from the possibility of at least two different arrangements of the molecules of that compound. The temperature, solvent used for crystallization and rate of cooling are important factors in determining the type of polymorph obtained. Polymorphs of a drug dissolve to give identical solutions, and are therefore indistinguishable in solution. In the solid state polymorphs are distinct entities, clearly distinguishable by x-ray diffraction fingerprints and differential scanning calorimetry. Each possesses its own physical properties e.g. solubility, melting point, density, hardness, crystal shape and vapour pressure.

Many crystalline organic compounds can exist as more than one polymorphic structure. If the molecules of such a compound assume a different relative geometry by breaking old or forming new bonds during transformation from one polymorph to another, the transformation can be considered to be a solid state chemical reaction. The reactant and product polymorphs might exhibit different chemical and physical properties. A high melting point polymorph will exhibit greater chemical stability than its lower melting point counterpart. Crystalline compounds are generally more chemically stable than their amorphous counterparts. Crystalline potassium penicillin can withstand elevated temperatures for several hours without significant degradation, while its amorphous form under the same conditions loses considerable activity [91]. Absorbed water generally increases the rate of reaction of an organic compound. The anhydrous form of a compound will be more stable than its hydrate unless it is amorphous or has a lower melting point. A hydrate can be more stable providing the water of hydration is tightly bound and the crystal lattice does not become ruptured.

Particle Size and Shape

As a rule the greater the surface area of a compound the faster the rate of degradation. Therefore smaller crystals are less stable than large ones.
Smaller sized samples dehydrate faster than larger sized samples.
In certain instances larger particle sized samples are more reactive than smaller ones. One example is for aspirin anhydride [92] where the larger particles contained trapped solvents which dissolved the solid compound and hastened its decomposition. This explanation was later challenged [93] as when the larger crystals were heated under vacuum to remove any entrapped solvent the decomposition profile did not change. It was suggested [93] that the larger crystals contained more reaction nuclei than the smaller ones.
Crystal shape can also affect the reactivity of solids. When acetyl-5-nitrosalicylic acid [94] was recrystallized from solution both columnar and ramified crystal shapes were obtained. The crystals were sorted, under a microscope, into their different types and exposed to elevated levels of humidity. The ramified crystals were about 5 times more reactive than the columnar ones. When viewed under polarized light the ramified crystals exhibited many more cracks and defects on their surface than the columar ones. These cracks and defects provided regions for growing product nuclei and were the cause of the greater instability of the ramified crystals.

Mechanical Stress

Mechanical handling such as grinding and compression can cause compounds to become more chemically unstable than their unstressed counterparts. Solid-solid interactions, polymorphic transformations and desolvation can occur during mechanical handling.
Moisture can be driven out of hydrated crystals during compression and if the water molecules preferentially exit from the ends of crystals, then mechanical handling which breaks the crystals longitudinally will accelerate dehydration by reducing the length of the tunnels through which the molecules have to negotiate to escape [95]. The bonding force between the water of crystallization and the drug is weakened by

grinding and the liberated water is then free to hydrolyze the drug.

Solid-Solid Interactions

The types of bonding involved in these interactions can be as follows:

1) Van der Waals forces
2) hydrogen bonding
3) covalent bonding

Inclusion compounds can also be present in the solid phase where the following reactions can occur:

1) free radical
2) polar
3) charge transfer

There are two limiting types of absorption also to consider i.e. physical and chemical. Adsorption is the tendency of a material to accumulate at a surface. It occurs because the atoms on a surface are subjected to balanced forces of attraction perpendicular to the surface plane and therefore the surface posseses a degree of unsaturation. Physical adsorption occurs when the valency requirements of each atom on the surface are satisfied by bonding to adjacent atoms. The forces involved are of the van der Waals type and are weak with adsorption and desorption accuring rapidly. Chemisorption occurs when the valency requirements of the surface atoms are not fully satisfied by nearby atoms. Therefore bonding between adsorbate and adsorbent may result in the formation of a new species. Chemisorption involves electron transfer and requires an appreciable activation energy and therefore occurs at higher temperatures than physical adsorption. Chemisorption can be promoted by heat, pressure and mechanical stress.

Frequently these interactions promote degradation of a drug in a solid dose formulation. Potential drug-excipient interactions can be determined by differential

scanning calorimetry (DSC). The evaluation of thermograms of mixtures, even binary mixtures, is difficult. In the evaluation of the thermograms the following points have to be considered [96]:

i) DSC is concerned with the melting ranges of compounds and reactions or transformations occuring at elevated temperatures may not happen at room temperatures.

ii) Compatibility is assumed if the curve of the mixture is a superposition of those of the single compounds.

The selection of the most stable excipient for cephradine was made using DSC studies of the drug and a selection of excipients [97]. Only the sodium carbonate/cephradine mixture exhibited similar thermograms to the original individual components.

Reactions can also occur between metallic adjuvants and organic compounds. Interactions of this type that have been reported include those of oxytetracycline, anthracene, phenothiazine and salicylic acid with magnesium salts [98]. An inert excipient may contain surface impurities, such as metal ions that may act as a catalyst or react with and degrade the drug in a formulation. Traces of heavy metals can be found in talc, a common tablet excipient therefore batch to batch variation in drug/excipient stability may be found.

If a drug is found to interact with all possible excipients then a higher melting point polymorph or salt form should be tried.

Drug/excipient complexation can occur which may either increase or decrease the stability of a formulation. Cyclodextrin, hydroxypropylmethyl cellulose and polyvinylpyrrolidone have all been used to improve via complexation, the stability of labile drugs.

Thermolysis

Utilizing the Arrhenius equation:

$$k = Ae^{-E_{a}/RT} \qquad \text{Equation 7}$$

Where:

k = reaction rate constant

A= frequency factor constant

E_a = activation energy

R = gas constant

T = absolute temperature

It can be seen that an increase in temperature will increase the rate of reaction. Rates of solid-state thermal reactions are between 2 to 100 times slower than corresponding reactions in solution [99].

Heating solid dose formulations if not carried out in a gas tight vessel, can lead to loss of free moisture which may be the cause of degradation. Instead of seeing an increase in the rate of reaction a decrease may be observed due to the loss of this free moisture.

Accelerated stability studies carried out at elevated temperatures in closed vessels, to minimize loss of moisture, will limit the availability of oxygen. Hence care must be taken when extrapolating data from stability studies of solid phase formulations carried out at elevated temperatures.

A solid-state thermal reaction involves four stages [100];

1) molecular loosening (disorder of reacting crystal at reaction site);
2) molecular change;
3) solid solution formation;
4) separation of product.

The first stage allows the tightly packed reactant molecules sufficient space in

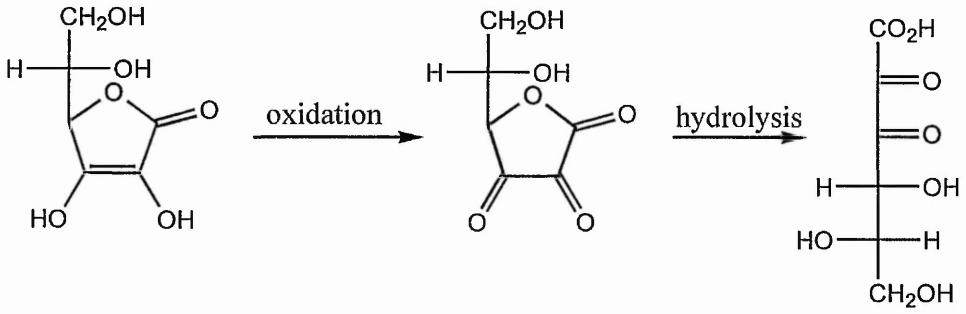
which to react. The second stage involves conversion of the reactants to products. As the first product molecules are formed a solid solution of product molecules in the reactant crystals is formed giving rise to stage 3.

After the solubility of the product in the reactant crystal is exceeded crystallization of the product from the solid solution occurs (stage 4). The crystalline product is usually highly disordered as crystallization occurs at many sites.

1.8 Degradation of L-Ascorbic Acid

1.8.1 Degradation in Solution

The degradation of L-ascorbic acid in solution can occur via an aerobic or anaerobic pathway giving rise to different degradation products. In aqueous solution it is readily oxidized (reversibily) to give dehydroascorbic acid which can undergo hydrolysis to produce 2,3-diketogulonic acid [101], Figure 21. Degradation is pH dependent with the maximum degradation rate occuring at pH 4 and maximum stability at pH 3 and pH 6. The rate of aerobic reaction is also dependent on the oxygen concentration and is catalyzed by certain metal ions, especially Cu^{2+} and Fe^{3+}.



L-ascorbic acid **dehydroascorbic acid** **2,3-diketogulonic aci**

Figure 21: Aerobic Degradation of L-Ascorbic Acid in Aqueous Solution

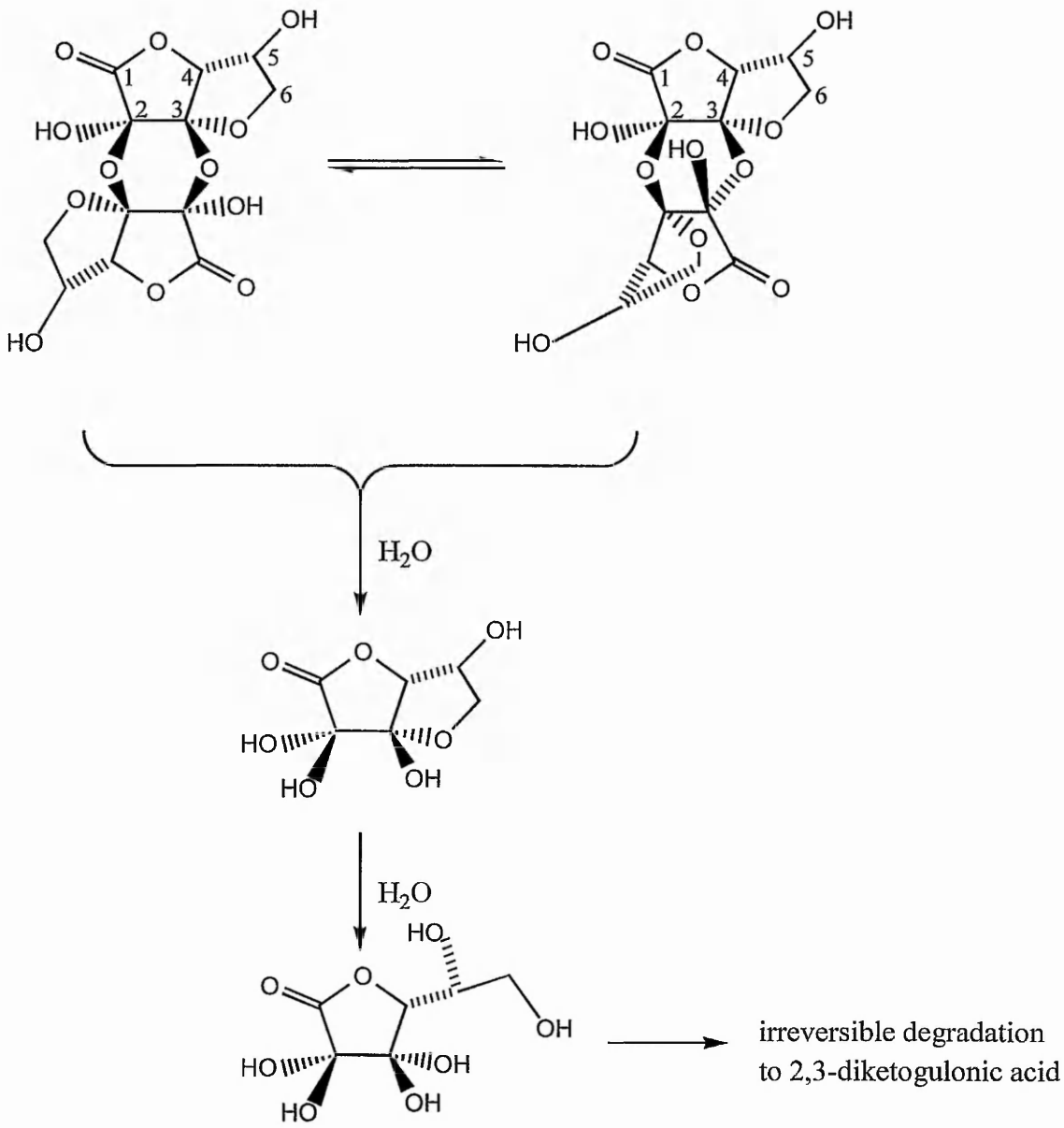


Figure 22: Irreversible Degradation to 2,3-Diketogulonic Acid

Dehydroascorbic acid exists as a dimer in solution which can react with water to form a bicyclic hydrated monomer [102] which then undergoes irreversible degradation to 2,3-diketogulonic acid, Figure 22.

Pfeilsticker and co-workers [103] consider that in aqueous solution dehydroascorbic acid exists as 3,6-anhydro-L-xylo-hexulono-1,4-lactone hydrate, Figure 23. This compound could be formed by the elimination of water between C-3 and C-6 with ring closure.

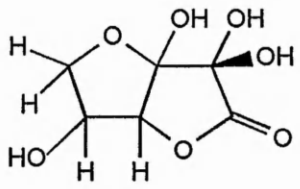
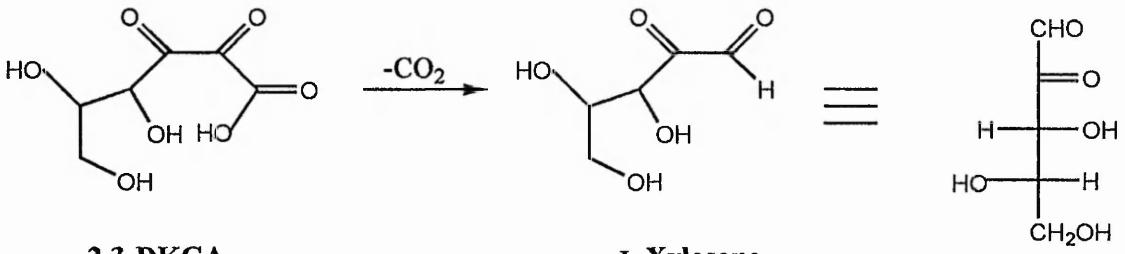


Figure 23: Hydrate of Dehydroascorbic Acid

The 2,3-diketogulonic acid (2,3-DKGA) can lose a mole of CO_2 to form L-xylosone which can then degrade further to a host of degradation products as described by Niemelä [104], Figure 24.



2,3-DKGA **L-Xylosone**

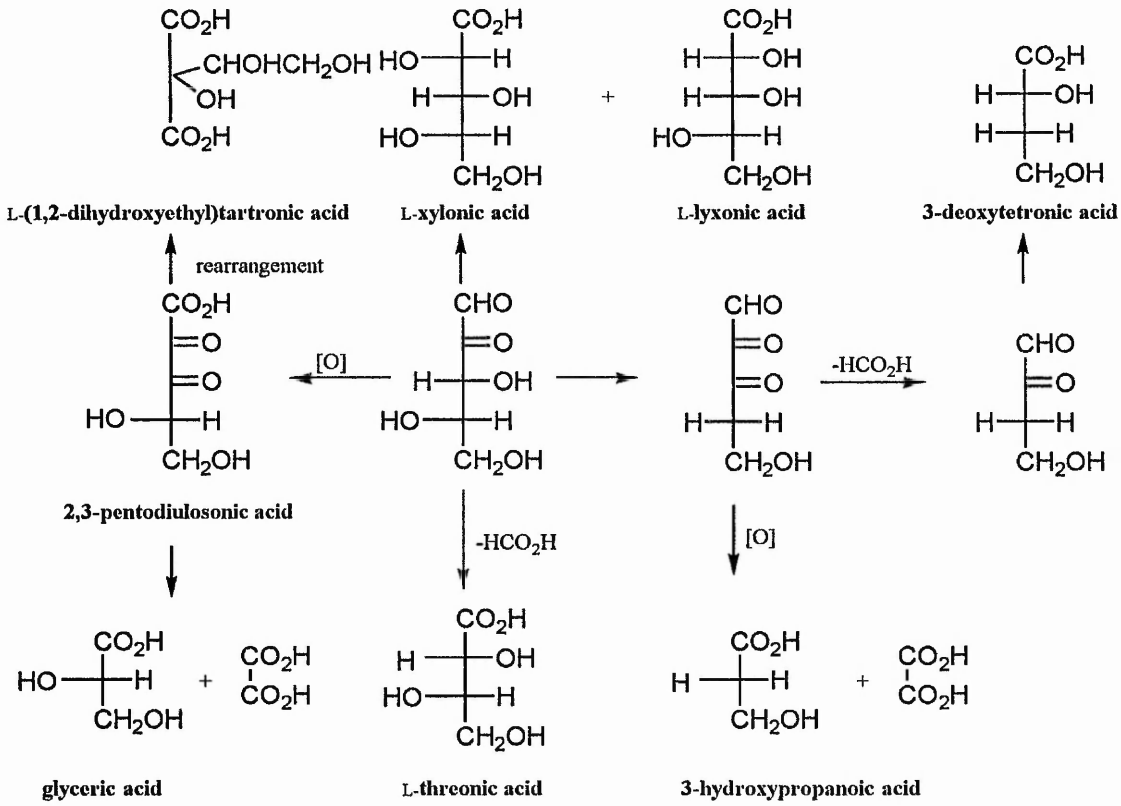


Figure 24: Aqueous Degradation of L-Xylosone

The 2,3-DKGA can also degrade under high pH conditions to form a different set of reaction products, Figure 25.

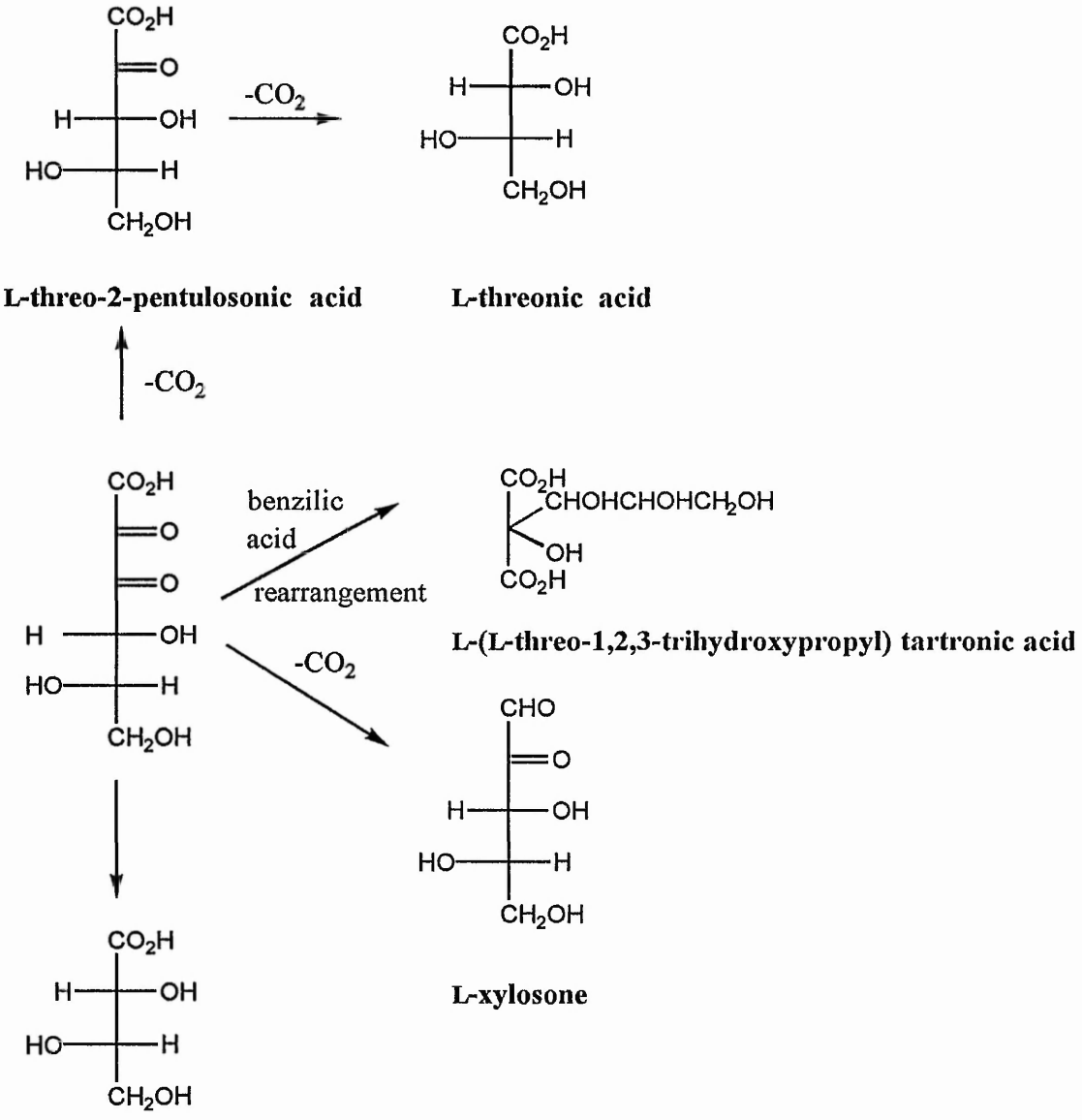


Figure 25: Aqueous Degradation of 2,3-DKGA Under High pH Conditions

L-Ascorbic acid can degrade under anaerobic conditions leading to the formation of furfural and carbon dioxide [105], Figure 26.

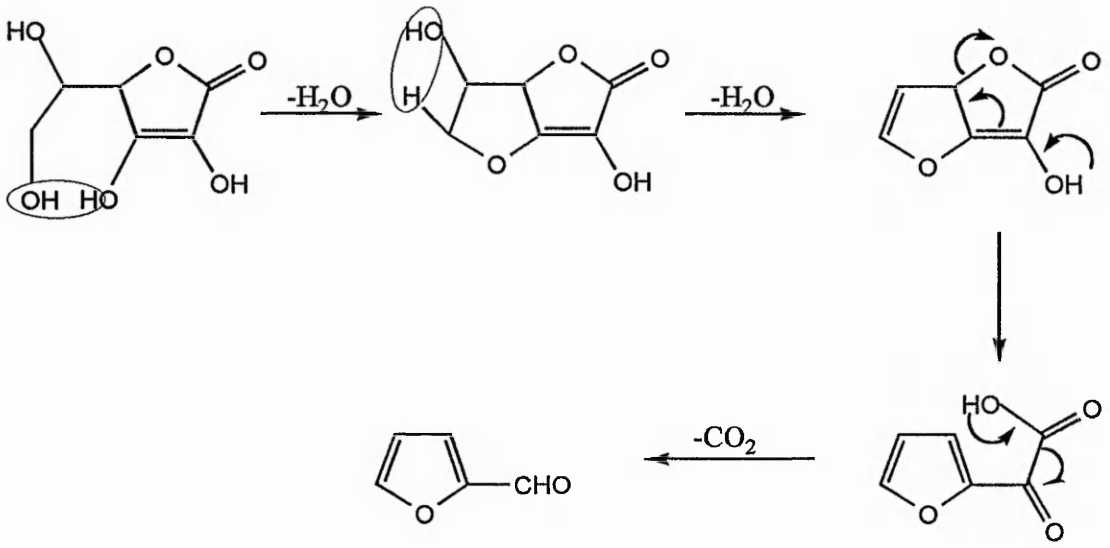


Figure 26: Anaerobic Degradation of L-Ascorbic Acid

Tatum, Shaw and Berry [106] isolated ten furan-type compounds, two lactones, three acids and 3-hydroxy-2-pyrone when L-ascorbic acid was heated in aqueous solution. The degradation of L-ascorbic acid is characterized by a discolouration of the solution. This discolouration is believed to be caused by furan-type compounds producing unknown brown pigments.

1.8.2 Degradation In The Solid Phase

L-Ascorbic acid tablets discolour during storage under ambient conditions of temperature and humidity. Tablets progressively turn from white to yellow to brown. Wortz [107] used a tristimulus colorimeter to measure this discolouration. Measurements were made using blue, amber and green light. He studied the effect of eight commonly used lubricants on the colour stability of L-ascorbic acid tablets. Lubricants low in metal ion content conferred maximum colour stability to the tablets. Alkaline lubricants such as magnesium and calcium stearate and minerals such as talc

were shown to cause excessive discolouration. Colour stability, as measured by tristimulus colorimetry, was found to be closely related to chemical stability, as measured by assay of L-ascorbic acid content.

This browning of products, including food and fruit, containing L-ascorbic acid has been the subject of many studies over recent years. The browning reactions are classified as;

a) enzymic;
b) non-enzymic.

Enzymic browning reactions are concerned with food products.

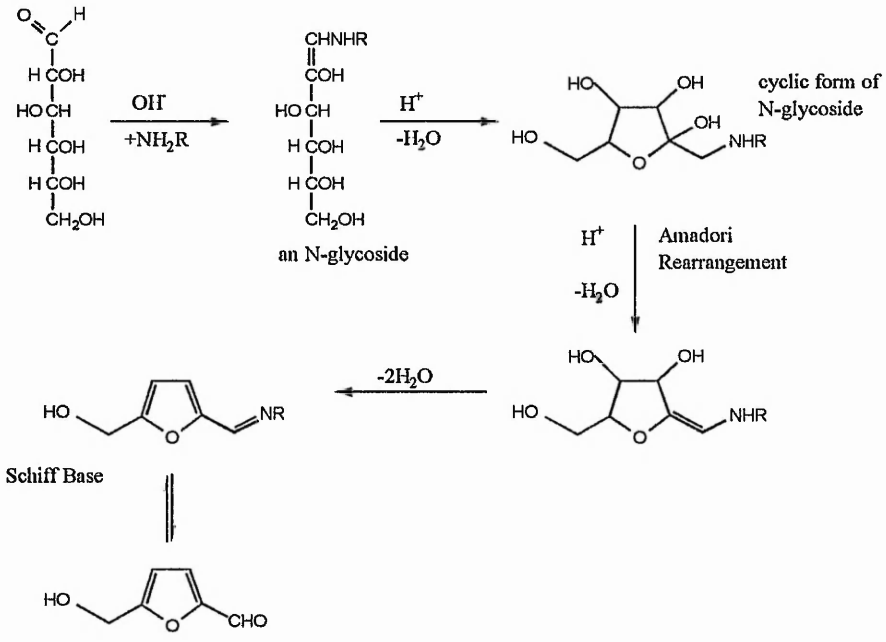
As this thesis is predominantly concerned with the pure compound, L-ascorbic acid in the presence or absence of pharmaceutical excipients only, this introduction will describe only the non-enzymic browning reactions.

Various theories have been advanced to explain the mechanism of non-enzymic browning reactions;

a) Maillard;
b) caramelization;
c) ascorbic acid.

a) Maillard

The Maillard, or melanoidin condensation theory involves the condensation of amino acids and reducing sugars to give rise to the formation of dark coloured substances. Maillard [108] was the first to record that solutions of amino acids and sugars turned brown on heating. The following reaction scheme (Scheme 3) is suggested [109].



Scheme 3: Melanoidin Condensation Reaction

Addition of an amino acid to a reducing sugar yields a N-glycoside. The cyclic form of this can undergo an Amadori rearrangement which then loses two moles of water to produce a Schiff base and 5-hydroxymethylfurfural. This then further degrades/polymerizes to give rise to brown coloured components - melanoidins. The Maillard reaction does not require elevated temperatures and can occur at sub-ambient temperatures. The reaction rate, however, increases markedly with increase in temperature and with decrease in moisture level. It was demonstrated by Hodge and Rist [110] that un-substituted N-glycosides readily degrade into a dark brown tar. If the C-2 hydroxyl was substituted then no discolouration occured.

The Amadori rearrangement of N-substituted glycosylamines involvement in the browning reactions was demonstrated by Gottschalk [111] and Hodge and Rist [110] in 1952. Both ketoses and aldoses condense with aliphatic amines with ketoses doing so more readily than aldoses [112]. Conversely, aldoses condense with aromatic amines more readily than do ketoses according to Barry and Honeyman [113].

After condensation of the sugar and the amine and rearrangement to the deoxyaminoketose dehydration occurs to form the Schiff base of the furfural. The amine is subsequently liberated from the Schiff base by hydrolysis however not all the Schiff base undergoes this reaction by hydrolysis. The unchanged Schiffs base can polymerize to form brown compounds associated with the browning reaction melanoidins. The sugar in sugar - amine browning reactions can be dehydrated in two ways. In neutral or acidic aqueous systems, furfurals are formed. In anhydrous conditions reductions occur. L-Ascorbic acid is a reductone and it is the dehydro form of a reductone i.e. dehydroascorbic acid that is the prime source of browning. Aldol condensations, aldehyde-amine polymerization and the formation of heterocyclic nitrogen compounds are the ultimate reactions in the final stage of browning in Maillard reactions.

In contrast to the relatively good understanding of the early stages of Maillard browning the later stages which lead to the discolouration are still ildefined. The products melanoidins contain a number of different functional groups and the composition of the products depends on the reaction conditions. The molar ration of starting materials, pH and temperature can all influence the final composition of the melanoidins.

The melanodins comprise a mixture of discrete products which have been separated from glucose and ammonia into six components [114]. Elemental analysis of melanoidins may be used to calculate the degree of hydration and loss of carbon dioxide as reactants, sugars and amines, are converted into melanoidins. If a and b moles of aldose and amine react and liberate x and y moles of carbon dioxide and water then the empirical formula for the melanoidin is given by Equation 8.

$$C_{la+pb-x}\ H_{ma+qb-2y}\ O_{na+rb-2x-y}\ N_b \qquad \text{Equation 8}$$

where: l, m, n = respectively, the numbers of carbon, hydrogen and oxygen atoms in the aldose and p, q, r corresponding numbers in the amine

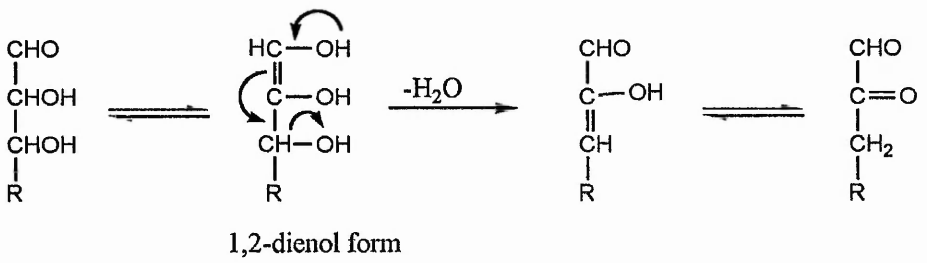
Infrared and ultraviolet spectroscopy does not provide unambiguous evidence concerning the structure. Infra-red measurements indicate that melanoidins probably contain hydroxy, C = O and C = C functional groups [115]. Hydrolysis of the melanoidins formed from the glucose-ammonia reaction liberate substituted hydroxypyridines [116] and pyrazines [117] but these compounds were believed to have only a weak association with the polymer. Oxidation with permanganate of the melanoidin yielded oxamic acid as the main product. Pyrolysis of the polymeric melanoidin followed by gas chromatography of the volatiles liberated has been used to study their composition [118] but only one or two products have been identified. Hydroxyl groups may be determined by acetylation and, if prior to acetylation the melanoidin is reduced with sodium borohydride, the extent of acetylation yields the sum of hydroxyl and carbonyl groups and thus the number of carbonyl groups may be found by difference.

Kato and Tsuchida [118] have used this method to determine the number of hydroxy and carbonyl groups in some sugar-amine reaction products. They found between 0.6 and 1.0 hydroxyl groups per sugar molecule and only one carbonyl per 3 - 9 sugar molecules. Based on this information some possible structures were suggested for the polymers. Porretta [119] has reviewed the chromatographic analysis of Maillard reaction products. After extraction of the pigments reversed-phase high performance liquid chromatography is used to resolve the components from the mixture.

b) Caramelization

Caramelization reactions also lead to discolouration of sugar based systems in the absence of amines. When sugars are heated at elevated temperatures they turn brown then black. The reaction involves 1,2 - enolization, dehydration of the sugar to furfurals and sugar fusion. Much more vigorous reacation conditions are required for this type of reaction than for Maillard reactions. The sugar, or carbohydrate, is usually boiled in dilute acid when the formation of acyclic enediols in the initial

reaction followed by dehydration, Figure 27.



1,2-dienol form

Figure 27: Caramelization Reaction

The 1,2 - enolization of reducing sugars can occur at high, neutral or low pH and is catalyzed by organic acids and their sodium salts. Furfural formation predominates at low pH (below pH 3) but can occur to a degree at elevated temperatures up to pH 6.7 [120] and sugar fusion predominates at higher pH. Obviously with caramelization the coloured pigments, unlike with the Maillard reaction, are nitrogen free.

c) Ascorbic Acid

The degradation of L-ascorbic acid, as discussed previously, (section 1.8) often leads to the formation of brown coloured pigments. The mechanism for the formation of coloured products is still uncertain but decarboxylation occurs in the presence, or absence of amino compounds which are known to catalyze the colour development. If the L-ascorbic acid degrades under aerobic conditions then it must degrade via dehydroascorbic acid. Dehydroascorbic acid is extremely unstable in aqueous solution and rapidly degrades to 2,3 - diketogulonic acid and then to unknown brown pigments. Presumably high molecular weight products are produced from condensation reactions in the absence, or presence of amines. Koppanyi et al [121] made the observation that dehydroascorbic acid reacts rapidly with α - amino acids to produce strongly coloured (reddish to brown) complexes. The reaction is specific for dehydroascorbic acid. Kurata and co-workers [122] suggest that a Strecker reaction between dehydroascorbic acid and the amino acid followed by oxidation of the product to an imine. Transalkylidenation of the imine with the unoxidized amine

produces a red pigment. The mechanism of formation of the red compound from dehydroascorbic acid is not fully understood but would appear not to involve ring opening.

Anaerobic degradation of solutions of L-ascorbic acid, as described in section 1.8.1, gives rise to furfural and carbon dioxide and is accompanied with colour formation, the rate of which is accelerated by the presence of amino compounds. The furfural can then condense with the amino compound and/or polymerize to form brown resinous materials.

Aims and Objectives

The basis of this thesis is the hypothesis "the reactivity of L-ascorbic acid in the solid state is significantly different from that in solution". As a consequence of the importance of L-ascorbic acid in solid state formulations, such as vitamin tablets, and mega dose regimes of the vitamin in tablet form the study of its reactivity in the solid state is of considerable interest.

Initial work will focus on identifying chemical interactions between L-ascorbic acid and tablet excipients. Once chemical interactions or conditions conducive to the degradation of the vitamin have been identified these particular reactions will be studied in detail. Means of stabilizing L-ascorbic acid to the identified degradation conditions will be sought.

The objectives of the study will be to:

a) chemically characterize major degradation and interaction products
b) establish chemical degradation pathways
c) obtain kinetic data for the reactions

A literature search (1975 to present) has highlighted a serious lack of knowledge on L-ascorbic acid solid phase stability and excipient interaction. Most work has been carried out in solution and there is evidence that there is considerable difference in

the chemical reactivity between the two phases. Some degradation products formed in solution have been identified by other workers but all are colourless compounds. Solid phase degradation of L-ascorbic acid is characterized by severe discoloration, the cause of which has not yet been identified, and this work aims to characterize this degradation product or products.

The study will utilize the following techniques in order to chemically characterize reaction products, obtain kinetic data and study the mechanism of the solid state reactions:

a) chromatography, to obtain orders of reaction and rate constants. If a literature search does not produce suitable chromatographic methods then new chromatographic systems will be developed. Chromatography will be used to resolve and isolate any degradation products. Comparison of any newly developed methods against established ones will be made.

b) tristimulus colorimetry, to monitor the discoloration of samples of L-ascorbic acid that is characteristic of its degradation. Kinetic studies will be performed using tristimulus colorimetry and results compared to those obtained using established techniques such as high performance liquid chromatography (HPLC). Use of tristimulus colorimetry is still a novel means of measuring chemical kinetics.

c) thermal analysis, to measure the changes in a samples physical or chemical properties as a function of temperature. The use of differential thermal analysis to screen for the compatibility of the vitamin with excipients will be explored. Predictions made by this technique will be compared to actual values obtained by established methods such as HPLC assay.

d) semi-preparative HPLC will be used to resolve and isolate any degradation/interaction products in high purity on a milligram scale.

e) structures will be determined primarily by using hyphonated mass spectroscopy (HPLC-MS or GC-MS).

Due to the increasing use of L-ascorbic acid, especially in mega-dose regimes to prevent cancer, results of this study will be of particular importance to the pharmaceutical industry.

To date, the structure of the solid phase degradation product of L-ascorbic acid has not been determined or a means of monitoring its formation determined. This novel study aims to isolate the degradation product or products, characterize it, elucidate the degradation pathway and identify means of preventing its formation.

Chapter 2

The Non-Chromatographic Determination Of L-Ascorbic Acid

2.1 Non-Chromatographic Methods

With the exception of outdated bioassays, most of the non-chromatographic methods for the determination of L-ascorbic acid fall into two categories

a) determination of the reduced form
b) determination of the oxidized form

The dienolic configuration of L-ascorbic acid involving atoms C-2 and C-3, confers properties of special interest for analytical purposes. The hydrogen atoms of the two enol groups are readily oxidized thus confering strong reducing properties on the compound. Measurement of the reducing properties of L-ascorbic acid forms the basis of a considerable number of methods for its determination [122 - 128]. The methods are not specific as any compounds present that have similar reducing properties will also be included in the determination. Oxidation of the enol group exposes two ketonic groups which may be derivatized with suitable reagents [129 - 131].

Spectroscopic measurements were amongst the earliest methods developed for the determination of L-ascorbic acid and infrared spectroscopy [132] has been used to monitor for its degradation.

In the following sections various non-chromatographic methods used for the determination of L-ascorbic acid will be described. Three of the methods were studied in detail in order to gain a greater understanding of the determination of L-ascorbic acid and the problems associated with its instability.

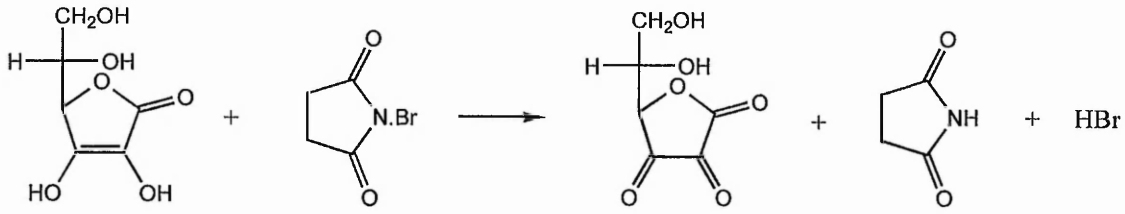
2.2 Spectroscopic Methods

The UV absorbance spectrum of L-ascorbic acid in neutral aqueous solutions has a maximum at 265 nm which shifts to 245 nm in acidic solutions. Dehydroasorbic has a maxima around 200 nm with a weak absorbance at 300 nm. Clearly at these wavelengths many substances absorb and interferences will occur. Unless pure solutions of L-ascorbic acid and dehydroascorbic acid are used for absorbance readings then errors will occur.

2.3 Titrimetric Methods

2.3.1 Oxidimetric Titration With N-Bromosuccinimide

This reagent was first described by Barakat et al [126]. It utilizes the oxidative property of N-bromosuccinimide **(27)** to oxidize L-ascorbic acid **(1)** to dehydroascorbic acid **(2)**. The reaction proceeds quantitatively in equimolar concentrations according to the reaction:



1 **27** **2**

After preferentially oxidizing L-ascorbic acid excess **(27)** liberates iodine from potassium iodide in an aqueous acetic acid medium. Starch indicator is used to detect the end-point which is observed by the appearance of a blue colour.

Experimental

The procedure as outlined was followed and is briefly described below:

An aqueous 0.09% w/v N-bromosuccinimide solution was standardized against 10 ml aliquots of a solution of L-ascorbic acid (0.2% in 10% w/v aqueous acetic acid). Potassium iodide solution and starch indicator solution were added to obtian an end-point. The solution was titrated with the N-bromosuccinimide solution with continuous shaking until the first permanent blue colour was observed. A blank titre was performed substituting water for the aliquot of ascorbic acid solution.

Results

The precision of the technique was measured by performing 10 replicate assays of stock solution of L-ascorbic acid. Results were similar to those obtained by Barakat et al [126], Table 2.

Table 2: Precision for the N-Bromosuccinimide Assay of L-Ascorbic Acid

Range (mg)	Mean (mg)	Standard Deviation	Coefficient of Variation	Actual Mass (mg)	% Actual
499.22-502.13	500.68	1.0477	0.21%	500.4	100.06

2.3.2 Oxidimetric Titration Using 2,6-Dichlorophenolindophenol

The classical L-ascorbic acid assay is that of Tillmans [133] in which the vitamin is titrated with 2,6-dichlorophenolindophenol **(28)**. This dye is blue in the solid and in neutral of alkaline solution. In acidic solutions it is pink and is reduced to the colourless leuco form **(29)**. L-ascorbic acid **(1)** is oxidized by the dye to dehydroascorbic acid **(2)**, Figure 29. The dye therefore acts as the indicator for the titration procedure.

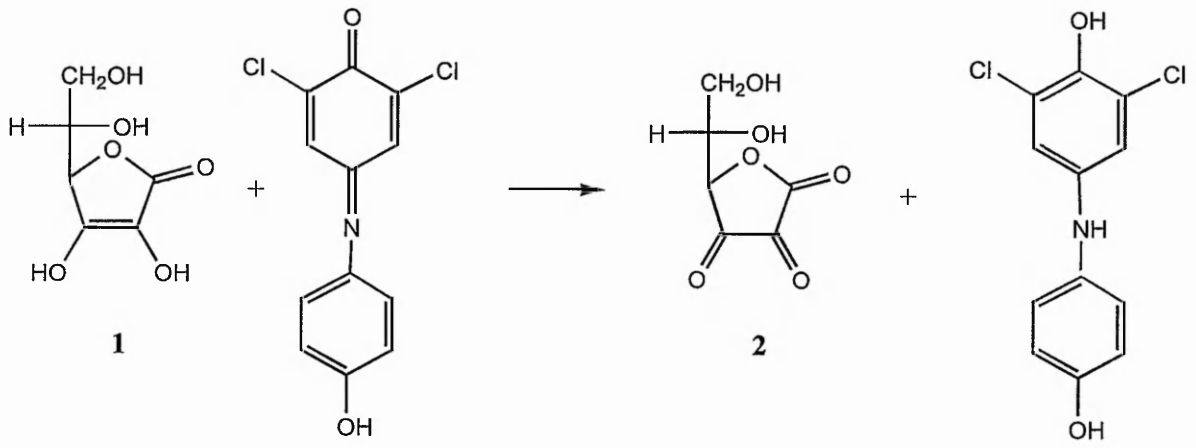


Figure 29: Reaction of L-Ascorbic Acid and 2,6-Dichlorophenolindophenol

A photometric procedure also exists [122] where the absorbance of a solution of the dye is measured at 520 nm before and after adding L-ascorbic acid solution. The change in absorbance, ΔA, due to reduction of the dye by L-ascorbic acid is calculated from Equation 9.

$$\Delta A = (RB - RB_b) - (S - S_b) \qquad \text{Equation 9}$$

where :

RB	=	absorbance of the reagent blank
RB_b	=	absorbance of the reagent blank after addition of L-ascorbic acid
S	=	absorbance of the sample solution
S_b	=	absorbance of the sample solution after addition of L-ascorbic acid

ΔA is linearly related to L-ascorbic acid concentrations and the concentration in a sample solution is obtained by comparison of ΔA with a standard curve.

Limitations encountered with 2,6-dichlorophenolindophenol methods, whether titrimetric of photometric include lack of specificity and dye instability. Since the methods are dependent upon the reduction of the dye by L-ascorbic acid, it is evident that any substance having a reduction potential lower than that of the dye

(0.210V) is a potential source of interference. Such compounds include cysteine, pyrogallol and sulphite, ferrous and cuprous ions.

Experimental

A 0.025% w/v solution of 2,6-dichlorophenolindophenol was standardized against aliquots of the same solution of L-ascorbic acid as used for the N-bromosuccinimide determination.

Results

The precision of the technique was measured by performing 10 replicate assays of the solution of L-ascorbic acid as used for the N-bromosuccinimide determination to enable a direct comparison of the two titrants to be made.
Results (Table 3) showed the 2,6-dichlorophenolindophenol titrant not to be as accurate or precise as the N-bromosuccinimide titrant.

Table 3: Results for the Assay of L-Ascorbic Acid Using 2,6-Dichlorophenolindophenol and N-Bromosuccinimide

Titrant	Range (mg)	Mean (mg)	Standard Deviation	Coefficient of Variation	% Actual Mass
2,6-DCP	498.51-504.78	501.18	2.22	0.44	100.19
N-BS	499.22-502.13	500.68	1.05	0.21	100.06

2.4 Colorimetric Methods

Several methods of analyses are based on the reactions of L-ascorbic acid and dehydroascorbic acid to produce osazones or furfural. For high sensitivity and greater specificity the 2,4-dinitrophenylhydrazine method to form an osazone was often used. Interference from certain metal ions, reductones and some sugars may

produce high results [134].

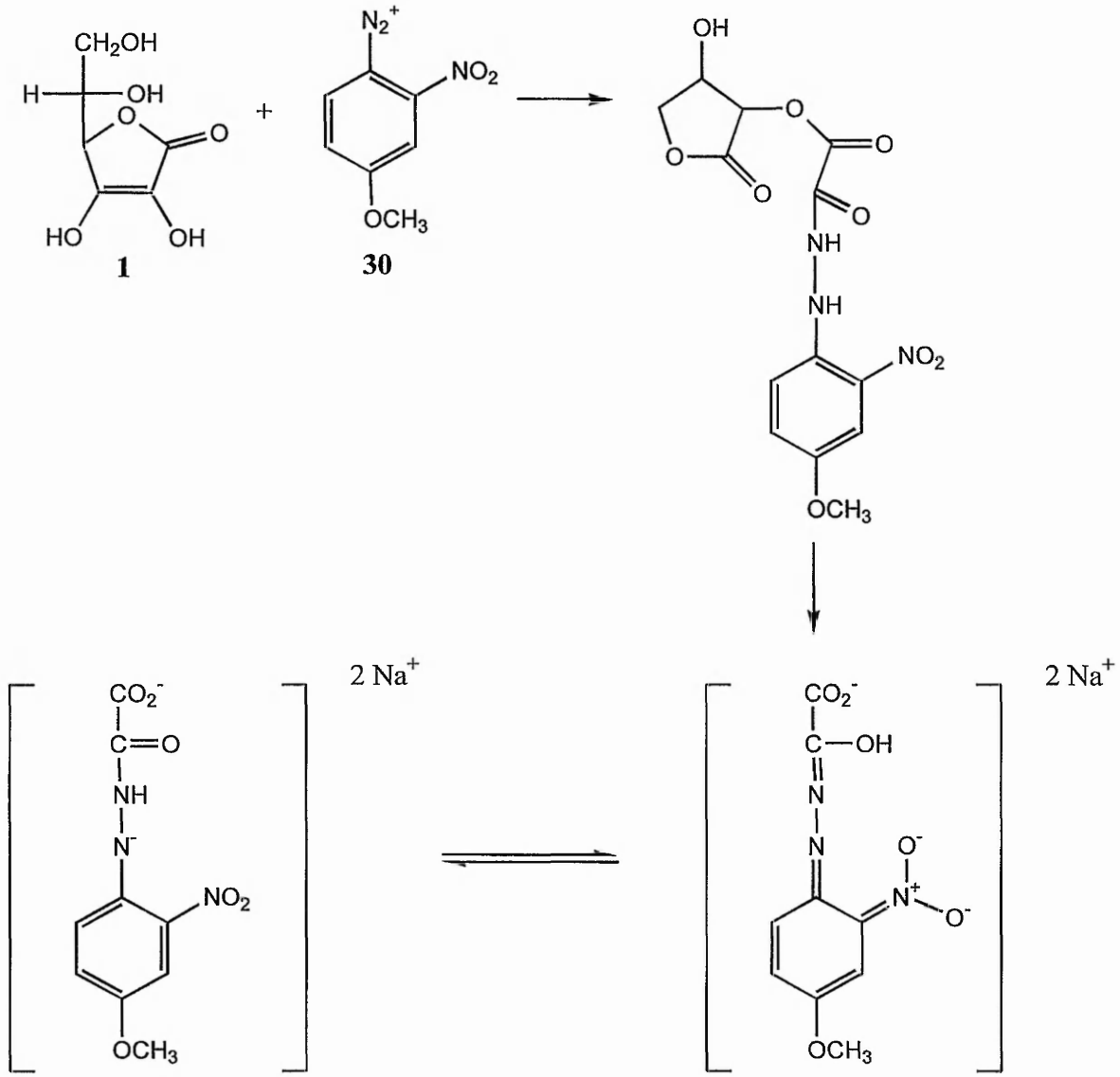
The reduction of 2,6-dichlorophenolindophenol at pH 1 to 4 produces the colourless leuco form which forms the basis of another colormetric method [134]. This only measures the reduced ascorbate whereas the 2,4-dinitrophenylhydrazine method measures dehydroascorbic acid.

The reduction of ferric ion to ferrous ion by L-ascorbic acid followed by the determination of the ferrous ion as the red orange $\propto$ -$\propto^1$ - dipyridyl complex. In the presence of orthophosphoric acid at pH 1 - 2 interfering compounds are inhibited [122].

2.4.1 Colorimetric Determination Using 4-Methoxy-2-Nitroaniline

Colorimetry offers another approach to the determination of L-ascorbic acid. Most colorimetric methods for the determination of L-ascorbic acid are based on the reduction of 2,6-dichlorophenolindophenol or coupling with 2,4-dinitrophenyl hydrazine after first oxidizing L-ascorbic acid to dehydroascorbic acid [122, 128, 135]. Both of these methods are subject to interferences from other reductones.

A more specific method involves the reaction of L-ascorbic acid with diazotized 4-methoxy-2-nitroaniline **(30)**. This reaction was first studied by Schmall and co-workers [130]. They investigated various diazonium salts to find an intensely coloured reaction product with L-ascorbic acid. When diazotized 4-methoxy-2-nitroaniline was reacted with the vitamin a vivid blue colour was obtained in alkaline medium. The blue colour is reversible upon acidification and has an absorption maximum at 570 nm. The reaction mechanism is a hybrid redox - derivatization and is illustrated in Scheme 4.



Reaction Scheme 4

The Reaction Between L-Ascorbic Acid and Diazotized 4-Methoxy-2-Aniline

Derivatization is rapid and the absorbance of the alkaline blue solution is proportional to the L-ascorbic acid concentration. As the absorbance is not linear at low concentrations a calibration graph must be constructed when greater accuracy is required.

2.4.2 Experimental

Calibration Graph

A calibration graph was constructed from absorbance measurements of standard solutions of L-ascorbic acid prepared in the following way:

To a series of 200 ml volumetric flasks the following reagents were added in the order given as described by Schmall *et al* [130].

To 2 ml amino reagent (500 mg 4-methoxy-2-nitroaniline in 125 ml glacial acetic acid diluted to 250 ml with 10% w/v sulphuric acid) add 2 ml nitrite reagent (0.2% w/v aqueous sodium nitrite solution) and 75 ml ethanol, swirl to mix. Standard solutions of L-ascorbic acid (2, 4, 6, 8 and 10 ml of a 0.200 mg/ml solution in 0.5% w/v oxalic acid solution) were pipetted into each volumetric flask and the solutions swirled to mix. After 5 minutes the solutions were made alkaline by the addition of 25 ml 10% w/v sodium hydroxide solution. The solutions were then diluted to volume with purified water and thoroughly mixed. The absorbance of the solutions was recorded at 570 nm after 1 minute against a purified water reference. The calibration graph was found to be non-linear throughout the concentration range (Figure 30).

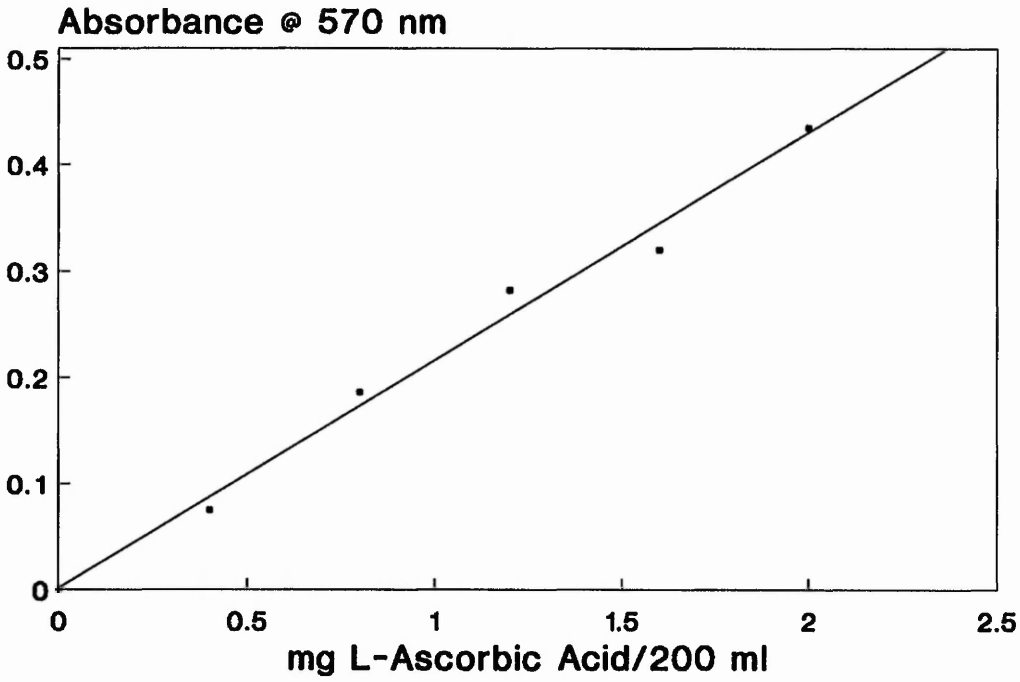


Figure 30: Linearity of L-Ascorbic Acid Diazotized with 4-Methoxy-2-Nitroaniline

Precision

Using the procedure as outlined for the calibration graph ten replicates of 3.0 ml aliquots of the standard solution were reacted and their absorbances recorded at 570 nm. A mean absorbance of 0.233 and a coefficient of variation of 7.71% were recorded.

2.4.3 Discussion of Results

Schmall *et al* quoted a co-efficient of variation of only 0.8%. Possible sources of error that would account for the much higher co-efficient of variation obtained in this work were;

a) insufficient mixing of reagents;

b) incomplete colour development (reaction time);

c) unstable colour.

Insufficient Mixing of Reagents

Using the same procedure as outlined for the calibration graph ten replicates of 5.0 ml aliquots of a standard solution of L-ascorbic acid solution were determined after throughly mixing the solution after each addition of reagent.
A mean absorbance of 0.431 and a coefficient of variation of 3.01% were recorded.

A thorough mixing after addition of each reagent resulted in a halving of variance but did not reduce it to the precision obtained by Schmall *et al.* Other, factors must have caused the higher variance.

Reaction Time

Using the procedure outlined for the calibration graph seven 5.0 ml aliquots of the L-ascorbic acid solution were reacted but the time before the reaction mixture was rendered alkaline was varied. A reaction time of 10 minutes was found necessary to fully develop the colour, the time quoted by Schmall, Figure 31.

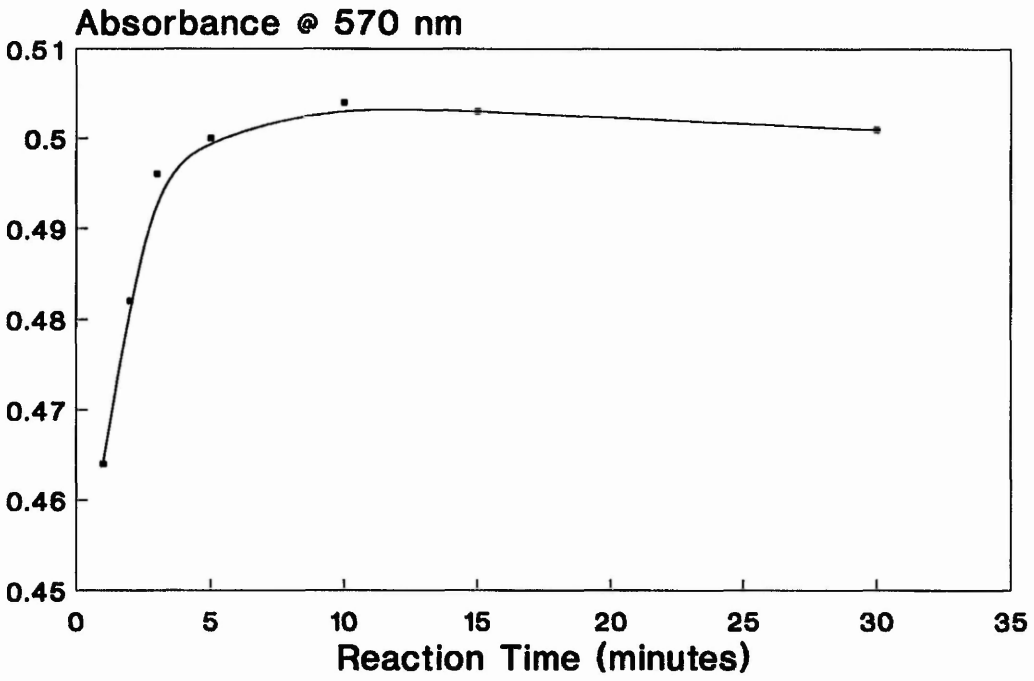


Figure 31 : Reaction Time for L - Ascorbic Acid Diazotized with 4-Methoxy-2-Nitroaniline

Precision After a Ten Minute Reaction Time

a) Reproducibility

Ten replicate aliquots of L-ascorbic acid (equivalent to 1 mg) were reacted using the modified reaction conditions and their absorbances measured, a mean absorbance of 0.235 and a coefficient of variation of 0.58% were recorded.

Precision was now acceptible and linearity was repeated using the modified reaction conditions.

b) Linearity

Aliquots of L-ascorbic acid solutions equivalent to 0 to 4 mg were reacted using the modified reaction conditions. The resulting calibration graph, Figure 32, was shown to be linear throughout the concentration range with a correlation co-efficient of 0.9989 and the equation of the plot

$$y = 0.2451\ x - 0.0067$$

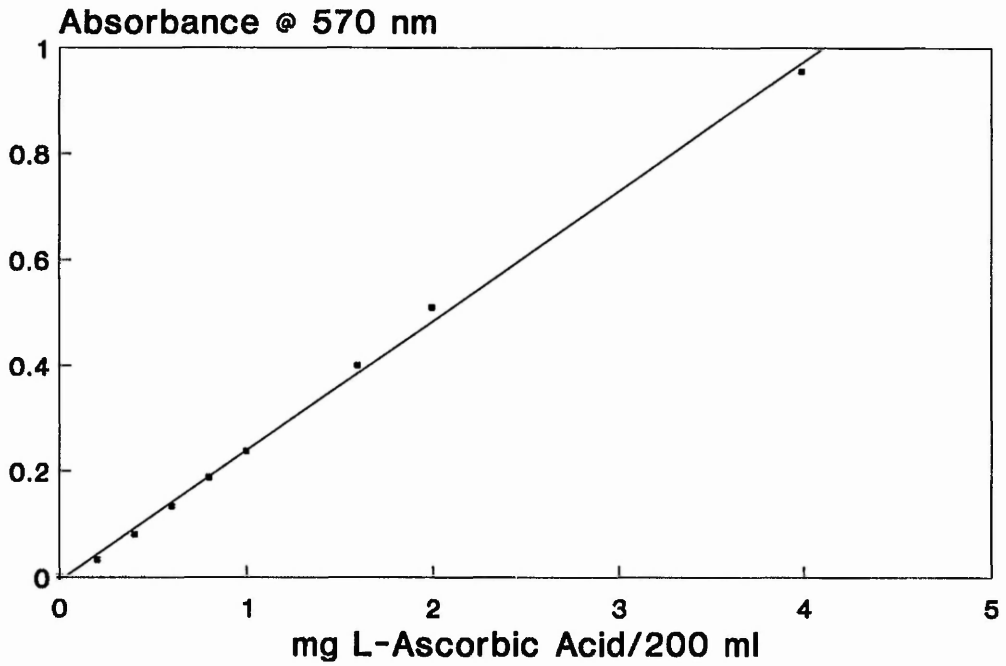


Figure 32: Linearity of L-Ascorbic Acid Diazotized with 4-Methoxy-2-Nitroaniline

2.4.4 Comparison of 4-Methoxy-2-Nitroaniline Method with the N-Bromosuccinimide Method

The stability indicating properties of the oxidimetric titrant, N-bromosuccinimide, the titrant giving superior results to 2,6-dichlorophenolindophenol, was compared to the colorimetric reagent 4-methoxy-2-nitroaniline. Both reagents were used to assay an aqueous solution of L-ascorbic acid before and after storing at 20°C for 7 days. Results expressed as a percentage of L-ascorbic acid remaining are given in Table 4.

Table 4: Assay of L-Ascorbic Acid Solution Stored for 7 Days by Colorimetric and Titrimetric Methods

Method	% L-Ascorbic Acid Remaining
4-methoxy-2-nitroaniline	98.64
N-bromosuccinimide	98.30

Results obtained by both methods were found to be in close agreement with each other.

2.4.5 Fluorometric Methods

One of the most specific methods for the determination of L-ascorbic acid and dehydroascorbic acid is the fluorometric method introduced by Deutsch and Weeks [136] which is an official method of analysis of the Association of Official Analytical Chemists. It is based on the oxidation of L-ascorbic acid to dehydroascorbic acid **(2)** and its condensation with 1,2-diaminobenzene **(31)** to form the fluorophorquinoxaline **(32)**, Figure 33.

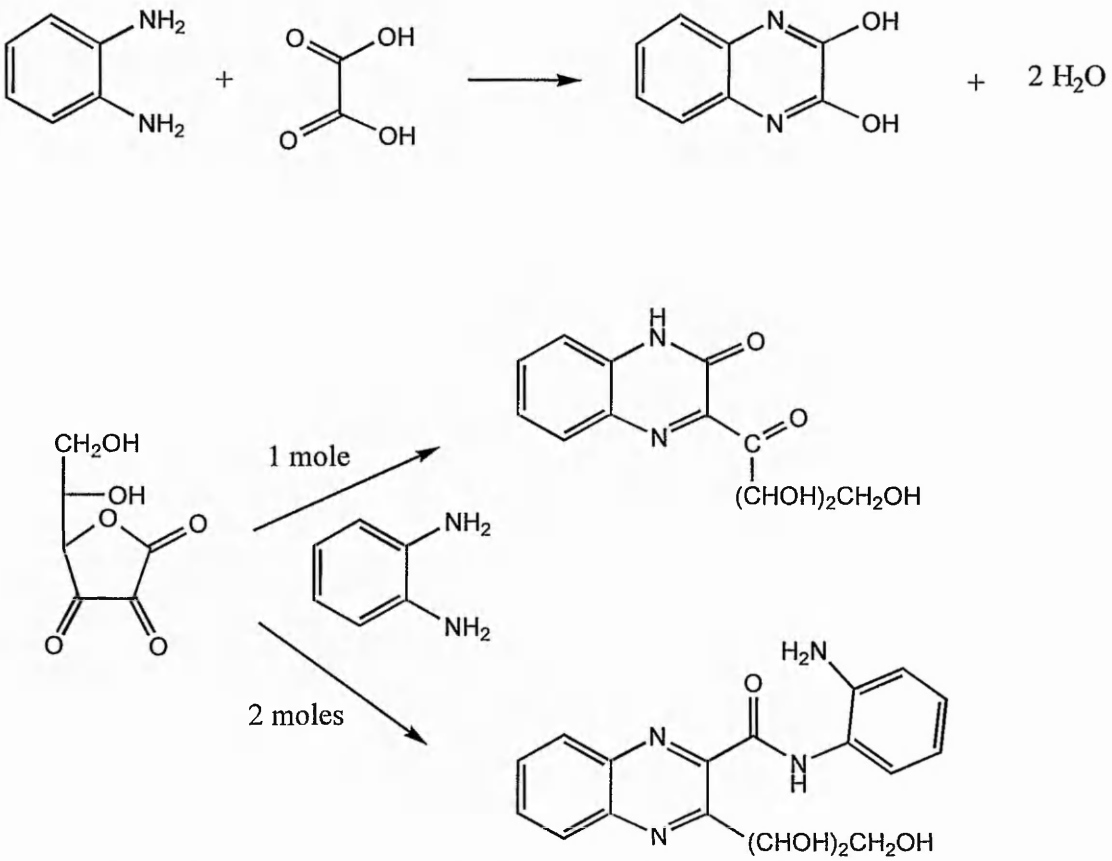


Figure 33: Reaction of the Carbonyl Group with 1,2-DAB

For the oxidation of L-ascorbic acid to dehydroascorbic acid, various chemical oxidants such as iodine, N-bromosuccinimide and charcoal (Norit) have been used. The presence of reducing substances or highly coloured compounds do not interfere with the reaction of dehydroascorbic acid and 1,2-diaminophenol and so the procedure can be used in the food industry to measure the level of L-ascorbic acid in foods.

In general the spectrofluorometric assay procedures involve fluorescent measurements on accidified samples at 435 - 450 nm with an excitation wavelength of 348 - 365 nm.

Although non-chromatographic methods were found to have satisfactory accuracy and reproducibility their lack of specificity and proneness to interferences is problematic. Chromatographic methods of analysis were therefore developed. These would also be compatible with the degradation studies discussed in subsequent chapters.

CHAPTER 3

The Chromatographic Determination of L-Ascorbic Acid

3.1 Introduction to Chromatographic Methods

Although many diverse methods exist for the determination of L-ascorbic acid only chromatographic methods offer the selectivity required when monitoring levels of L-ascorbic acid in the presence of possible interferring substances such as degradation products and tablet excipients. A simple, fast, automated, reproducible and selective method for the assay of L-ascorbic acid is required for the repetative multiple analysis of formulations that will be generated in the study.

The chromatographic determination of L-ascorbic acid has developed along with the advancement of different chromatographic techniques. The different chromatographic techniques used for its determination can be described under the following headings:

i) paper chromatography
ii) thin layer chromatography
iii) gas liquid chromatography
iv) ion chromatography
v) high performance liquid chromatography

The selection of a specific method to be used depends upon the information desired and the nature of the sample.

i) Paper Chromatography

Initial paper chromatographic methods were concerned with identifying suitable visualizing reagents and optimizing the developing solvents. Partridge reported the first qualitative separation of L-ascorbic acid and dehydroascorbic acids [137] using

cold ammoniacal silver nitrate to visuallize the spots. Mobile phases have included phenol-acetic acid [138], butanol [139] and butanol - acetic acid [140]. The location of L-ascorbic acid on the chromatograms can be revealed by several development agents including 2,6-dichlorophenolindophenol, iodine vapour, ammonium molybdate and molybdophosphoric acid.

ii) Thin Layer Chromatography (TLC)

Thin layer chromatography offers greater resolutions over paper chromatography. Brenner et al [141] described a TLC system giving excellent separation of various isomers of L-ascorbic acid. A silica gel stationary phase was used with a mobile phase of acetonitrile/butyronitrile/water in a ratio of 66:32:2 v/v. Saari *et al* [142] studied the oxidation of L-ascorbic acid using a ^{14}C-labeled compound. The chromatographic conditions resolved L-ascorbic acid from dehydroascorbic acid, oxalic acid and 2,3-diketogulonic acid. Advances in high performance TLC has lead to a resurgence in the technique due to increased sensitivity and resolution, ability to run several chromatograms in paralled and the capability of using densitometric and fluorimetric detection.

Klaus *et al* [143] used high performance TLC to resolve L-ascorbic acid from acids found in fruit juices. Using an amino-modified silica gel stationary phase reagent-free detection of the acids was accomplished. The acids in the fruit juices produced fluorescent derivatives on contact with the active surface of the porous sorbent under the influence of heat. The derivatives fluoresce during irradiation with short- or long-wave ultraviolet radiation.

iii) Gas-Liquid Chromatography (GC)

Sweeley and co-workers [144] reported the first gas-liquid chromatographic analysis of L-ascorbic acid. Polar compounds such as L-ascorbic acid are non-volatile and are not directly amenable to gas-liquid chromatography. In order to impart volatility to polar compounds silyl ethers are formed by the reaction of the

hydroxy groups of the polar compound with silylating reagents such as trimethylchlorosilane. L-Ascorbic acid may be derivatized at any one of its four hydroxyl groups.

Vecchi and Kaiser [145] characterized the silyl derivative of L-ascorbic acid with GC-mass spectrometry and concluded that the tetra-trimethylsilylether derivative was formed. Various reagents including n-trimethylsilylacetamide and N,*O*-bis(trimethylsilyl)acetamide have been used for derivatization. After derivatization chromatography is carried out on a non-polar stationary phase such as methyl polysiloxane with flame ionization detection. Degradation products of L-ascorbic acid such as dehydroascorbic acid and 2,3-diketogulonic acid can be chromatographed and resolved from each other using GC.

Löwendahl and Petersson [146] studied the conversion of dehydroascorbic acid to a branched hexaric acid in neutral and alkaline aqueous solution using GC-mass spectrometry. Analysis of the trimethylsilyl derivatives on a QF-1 stationary phase at 160°C permitted the separation of the major reaction product from interfering compounds.

iv) Ion Exchange Chromatography

Ion exchange chromatography has successfully been applied to the resolution of many organic acids [147 - 149]. When coupled to a conductivity detector, problems of detecting these UV inactive species are overcome.
Two modes of ion chromatography can be used for the separation of ionic species:

i) High Performance Ion Chromatography Exclusion (HPICE)
ii) High Performance Ion Chromatography (HPIC)

HPICE is the mode of choice for organic species and HPIC for inorganic species. When the 2 techniques are combined, refered to as HPICE-HPIC maximum information about the type of ionic species is obtained, for example strong acids

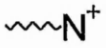
are separated as a class from weak acids.

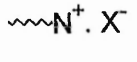
Detection of the ions is accomplished by a suppressed conductivity detector which provides much higher sensitivity for these non-chromophoric compounds than UV detection even at short wavelengths.

Typical ion-exchange stationary phases contain either acid groups, such as sulphonic acid or carboxylic acid for the separation of cations, or basic groups such as amine or quaternary amine for the separation of anions. The ion-exchange process may be represented, Figure 34, which describes the process for anion exchange:

Figure 34 : Anion Exchange Process

 represents an anion exchange site,

 an ionic site on the stationary phase

The associated counter ion, NO^-_3 can be displaced by a solute ion, X^-, to give the ion pair  .

Ion exchange resins can be bonded to a styrene-divinyl-benzene copolymer or with the ion-exchange groups chemically bonded to microparticulate porous silica gel. Ion-exchange chromatography has been used to resolve L-ascorbic acid from other water soluble vitamins [150], in foodstuffs, pharmaceuticals and body fluids [151 - 152]. Both weak anion exchange, with $-NH_2$ bonded phases and strong ion exchange with quaternary ammonium bonded phases have been used to chromatograph L-ascorbic acid [153 - 155]. Both these types of stationary phases

were found to suffer from loss of performance when degraded L-ascorbic acid samples were chromatographed.

v) High Performance Liquid Chromatography (HPLC)

Procedures vary in the type of stationary phase, mobile phase, detection system and means of stabilizing the extracts. The use of a solution medium suitable for chromatographing solutions of L-ascorbic acid and also stabilizing the vitamin is of vital importance to the development of automated HPLC analysis. The use of metaphosphoric acid (HPO_3) solutions for the extraction of L-ascorbic acid from plant and animal tissues was first proposed in 1935 by Fujita and Iwatake [156]. They compared the stability of L-ascorbic acid solutions in metaphosphoric acid, trichloroacetic acid and water. Oxalic acid has also been used to stabilize L-ascorbic acid solutions [157]. Metaphosphoric acid inhibits the catalytic oxidation of L-ascorbic acid by metal ions such as copper and iron. It also inactivates enzymes that oxidize the vitamin and also reduces the tendency for hydrolysis of the lactone ring. Solutions of metaphosphoric acid should be used fresh as the acid undergoes hydrolysis to orthophosphoric acid (H_3PO_4) which has reduced stabilization properties compared to metaphosphoric acid. Metaphosphoric acid (0.1% w/v aqueous) can serve as both the solvent and mobile phase for reverse phase HPLC [158].

Methods of retaining L-ascorbic acid by HPLC are varied e.g. anion exchange, ion-pair reagents, reverse-phase and polymer stationary phases. Ion-pair reagents have been used to retain L-ascorbic acid on reverse-phase stationary phases [159-162]. Reagents used include tridecylammonium formate, hexadecyltrimethylammonium bromide, tetrabutylammonium phosphate, dodecyltrimethylammonium chloride and octylamine salicylate. Sood *et al* [159] described the first ion-pair HPLC method for determining L-ascorbic acid in foods and multivitamin products. Several ion-pair reagents were evaluated, tridecylammonium formate giving the best separation. Modifications of the method have been used to determine L-ascorbic acid levels in potatoes [160]. Some known

degradation products of L-ascorbic acid solutions have also been resolved by ion-pair HPLC [161, 162].

Kennedy and co-workers have resolved L-ascorbic acid from many of its known aqueous solution degradation products using polymer reverse-phase columns [163, 164]. The method utilizes a high surface area, small pore size, polymeric reverse-phase adsorbent, PLRP-S 100 Å with a low pH aqueous mobile phase.
Using an acidic mobile phase to suppress the ionization of L-ascorbic acid and hence increase its retention on a reverse-phase C18 silica based stationary phase, Ziegler *et al* quantified levels in rose hips at a detection limit of 1.4 ng. The same authors used the method to also determine dehydroascorbic acid using a post column reduction system [165].

Derivatization procedures have been used to increase the sensitivity of HPLC methods. Garcia-Castineiras and co-workers [166] first oxidized L-ascorbic acid to dehydroascorbic acid which was then derivatized with 2,4-dinitrophenylhydrazine to give the osazone derivative. This was better retained on a reverse-phase column than the underivatized acid. A fluorescent assay was used to determine total L-ascorbic acid in whole blood [167]. The assay consisted of derivatization of L-ascorbic acid with 1,2-diaminobenzene to the quinoxaline after oxidation with ascorbic acid oxidase.

Due to the highly polar character of L-ascorbic acid it is virtually unretained by straight reverse-phase HPLC. Ziegler er al [165] found, on optimizing chromatography conditions, that ion-pair reagents were not required in order to retain the acid sufficiently and resolve it from interfering substances. By suppressing the dissociation of L-ascorbic acid using a strongly acidic aqueous mobile phase the maximum retention that could be obtained on a C18 stationary phase was 3.8 minutes with a flow rate of 1 ml/minute. No degradation of performance was observed after hundreds of determinations. For the study of L-ascorbic acid formulations two HPLC systems will be required;

a) a stability indicating assay method;
b) a screening method capable of resolving and detecting degradation products.

The assay method must resolve any close eluting impurities that may form during the degradation of L-ascorbic acid or formulation excipients from it, i.e. it must be specific for L-ascorbic acid.

The degradation screen must be capable or resolving and detecting any degradation products.

3.2 Methods for the Assay of L-Ascorbic Acid

The study is concerned with the resolution and detection of degradation products of L-ascorbic acid samples. Previous studies have found that weak and strong anion columns had a very limited life when degradated samples of L-ascorbic acid were chromatographed. Initial work carried out using these stationary phases with degraded aqueous solutions of L-ascorbic acid confirmed that chromatographic performance was irreversibly impaired rendering them of little use for the analysis of degraded samples. The following HPLC techniques were investigated;

a) reverse-phase ion-pair partition chromatography;
b) suppressed ionization partition chromatography.

3.2.1 Reversed Phase Ion-Pair Partition Chromatography

Introduction

Anion ion-pair reagents are frequently used to retain a polar anion on a reverse phase stationary phase. The pH of the mobile phase must be such that the polar

analyte is ionized and the ion-pair reagent protonated in order for ion-pairing to occur. The partition equilibrium can be represented by Equation 10.

$$B^{+}aq + P^{-}aq = (B^{+}P^{-})\ org \qquad \text{Equation 10}$$

where B^{+} is the protonated base

P^{-} is the anion of an acid

$(B^{+}\ P^{-})$ is the ion pair

The reduced polarity of the ion-pair has an increased affinity for the organic reverse-phase stationary phase resulting in an increase in retention time. This approach was successfully applied to L-ascorbic acid by Sood *et al* [159] who investigated six different anion ion-pair reagents using a μBondapak C18 reverse-phase column. Using this stationary phase and the anion ion-pair reagent tetrabutylammonium hydroxide the chromatography of aqueous solutions of L-ascorbic acid were investigated.

Experimental

A 0.2 mg/ml aqueous solution of L-ascorbic acid was chromatographed using the following conditions:

stationary-phase	:	μBondapak C18
column dimensions	:	30 cm x 3.9 mm
mobile phase	:	tetrabutylammonium hydroxide, 2×10^{-3}mol/l pH 5.0 in acetonitrile
flow rate	:	1.5 ml/min
wavelength	:	245 nm
injection volume	:	10 μl

Results

The solution of L-ascorbic acid was chromatographed isocratically varying the percentage of acetonitrile in the mobile phase between each injection. The retention time of the acid was recorded.

The L-ascorbic acid eluted as sharp symmetrical peak under all conditions. Predictably reducing the acetonitrile concentration lead to an increase in its retention time. At 0% v/v acetonitrile concentration a retention time of 6.3 minutes was obtained, Figure 35.

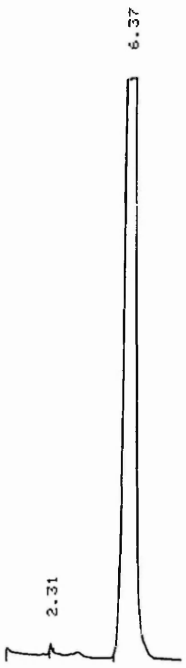


Figure 35: Chromatogram of L-Ascorbic Acid Using Reverse-Phase Ion-Pair Chromatography

To demonstrate whether the chromatography conditions could indicate if the analyte solutions were stable, indicating the solution of L-ascorbic acid was

degraded by heating at 95°C for 1 hour then chromatographed using the mobile phase with 0% acetonitrile concentration. No additional peaks were observed but the L-ascorbic acid peak was much reduced in area (typically by 10%) indicating degradation had occurred. Attempts at developing this system into a degradation screen using a gradient elution failed due to the presence of many "ghost" peaks caused by the ion-pair reagent.

Conclusion

A potential stability indicating HPLC assay method had been developed for the assay of L-ascorbic acid based upon reverse-phase ion-pair partition chromatography. Due to the presence of many "ghost" peaks during gradient elution the chromatography conditions could not be developed into a degradation screen.

An HPLC system capable of both assaying L-ascorbic acid and resolving and detecting its degradation products is required therefore other chromatography conditions were investigated.

3.2.2 Suppressed Ionization Reverse-Phase Chromatography

Introduction

Due to its highly polar character L-ascorbic acid is only slightly retained by reverse-phase stationary phases especially when dissociated. The retention time of L-ascorbic acid strongly depends on the pH of the mobile phase and on the column temperature.

By suppressing unfavourable dissolution with strongly acidic aqueous mobile phases (hydrophobic chromatography) L-ascorbic acid elutes as a sharp peak with a satisfactory retention time for analysis.

Ziegler *et al* [165] used this approach to retain L-ascorbic acid for 3.8 minutes using a reverse-phase C18 column and a mobile phase of 1% w/v aqueous metaphosphoric acid. Using a polymer reverse-phase column and the same mobile phase, Kennedy et al [163], also successfully retained L-ascorbic acid long enough to resolve it from certain known degradation products.

As the pKa, of L-ascorbic acid is 4.19 and a pH < 1.5 units below the pKa of an acid is required to suppress all ionization then any acidic mobile phase with a pH < 2.7 will be acceptable.

Metaphosphoric acid, used to both stabilize the sample solutions and act as the mobile phase [165] is not compatible with MS and as HPLC-MS may be carried out on samples in the study an alternative acid is required to acidify the mobile phase.

Trifluoroacetic acid is an organic acid compatible with HPLC-MS and is a sufficiently strong acid to produce a pH of 2.7 at a concentration of 0.1% v/v aqueous therefore this was adopted as the mobile phase replacing metaphosphoric acid used by other workers.

Ziegler *et al* [165] investigated a number of reverse-phase stationary phases using L-ascorbic acid as a probe compound to determine the phase giving the longest retention time. Lichrosorb RP-18 produced distinctly longer retention times than others investigated.

Study of the Characteristics of the Reverse-Phase Stationary Phases

A selection of reverse-phase stationary phases including those investigated by Ziegler and Kennedy were investigated. The majority were C18 produced by different manufacturers, others were of intermediate polarity or of a polar nature. Two of the phases, YMC ODS-AQ and a porous graphitic carbon are of recent development and not previously investigated by other workers. ODS-AQ from

YMC, of Japan, is a reverse-phase stationary phase specifically designed for the analysis of polar compounds such as L-ascorbic acid.

Novel silane bonding chemistry produces a stationary phase with a high carbon loading and relatively hydrophilic endcapping. Hydrophilic endcapping imparts a stationary phase with a surface that can be wetted with aqueous mobile phases. In the presence of an aqueous mobile the C18 ligands on the ODS-AQ stationary phase are lifted off the silica surface by the action of water penetrating down to the hydrophilic endcap. More ligand is available for interactions with a polar analyte than with conventionally endcapped ODS stationary phases where the C18 ligands mat down due to repulsions with the polar water.

The Hypercarb Porous Graphitic Carbon Stationary Phase is one of the more interesting novel reverse-phases to emerge recently. It is produced by the graphitization of a phenol-formaldehyde resin impregnated silica gel. Dissolution of the silica gel prior to graphitization imparts a porous structure. Retention properties are reported to resemble that of conventional reverse-phase stationary phases. Applications are still being found for this stationary-phase therefore it was considered worthwhile including it in the study.

The specifications required for a stationary phase to be used in this work were;

a) a satisfactory separation of the components of interest in a minimum time;

b) stable surface characteristics for reproducible chromatography and an analytically robust system;

c) isocratic operating conditions are prefered, especially for the assay determination, in order to reduce the analysis time of repetative assays and to simplfy the mobile phase preparation.

The approach to developing suitable separation methods for this work involved a review of the literature for stationary phases capable of separating potential degradation products. This was followed by a study into improving the separation achieved by these stationary phases. The optimization of the separation required a minumum analysis time with satisfactory resolution and peak symmetry. There are a number of mathmatical definitions of peak resolution (Appendix 1). Resolution may be considered to be a function of relative retention and the number of theoretical plates present on a column. Alternatively, the factors which may be controlled to improve peak resolution are selectivity and efficiency. In order to improve chromatography these factors were studied separately.

The efficiency of a column may be considered in terms of the number of theoretical plates i.e. equilibrium steps present. A theoretical plate is an imaginary length of the chromatographic column wherein complete equilibriation of the solute between the mobile and stationary phases is attained. The number of theoretical plates is determined by dynamics and the physical characteristics of the column. The van Deemter rate theory permits the efficiency of a column to be quantified in terms of the physical dimensions of the column, column packing and the thermo-dynamic retention process. This involves plotting a van Deemter graph which may be expressed by Equation 11.

$$h = A + \frac{B}{u} + c.u \qquad \text{Equation 11}$$

where A, B and c are constants

u = the average linear mobile phase velocity

h = height equivalent to a theoretical plate

Alternatively column efficiency can be expressed using Equation 12.

$$N = a\,(\frac{t_r}{W})^2 \qquad \text{Equation 12}$$

where N = number of theoretical plates

a = 5.54 when peak width measured at ½ height

t_r = retention time of peak

W = peak width at a given peak height, commonly ½ height

Experimental

The suitability of a variety of stationary phases, Table 5, for the isocratic separation of components in a 1% w/v solution of dehydroascorbic acid was studied. The stationary phases were purchased pre-packed into stainless steel columns. The following chromatography conditions were used throughout the study:

Instrument	:	Hewlett Packard HP1090M with diode array detection
Mobile phase	:	0.1% v/v trifluoroacetic acid, aqueous
Flow rate	:	1.0 ml/minute
Column temperature	:	40°C
Wavelength	:	245 nm
Injection volume	:	10 µl
Sample solutions	:	1% w/v aqueous metaphosphoric acid 0.01% w/v L-ascorbic acid 1% w/v dehydroascorbic acid solution

The following stationary phases were assessed, Table 5.

Table 5: Stationary Phases Assessed for Assay of L-Ascorbic Acid

Manufacturer/Column Name	Type	Particle Size (μ)	Column Length/ID (cm)
1) Merck Lichrospher	C18	5	25/0.46
2) Merck RP Select B	C8	5	25/0.46
3) Waters μBondapak	C18	5	30/0.39
4) Waters Nova-Pak	C6	4	15/0.4
5) Spherisorb Hexyl	C18	5	25/0.46
6) Hichrom ODS	C18	5	25/0.46
7) Hypersil ODS	C18	5	25/0.46
8) YMC-Pak ODS-AQ	C18	5	25/0.46
9) Polymer Labs PLRP-S	PSDVB	5	15/0.46
10) Spherisorb CN	CN	5	25/0.46
11) Merck Lichrospher	DIOL	5	25/0.46
12) Spherisorb Methyl	C1	5	25/0.46
13) Shandon Hypercarb	PGC	7	10/0.46

Results and Discussion

The 1% w/v solution gave rise to three close eluting impurity peaks, all eluting prior to L-ascorbic acid. Resolution, R_s, between the two major peaks in the mixture and the tailing factor, T, of L-ascorbic acid were measured. Comparison of the retention properties of different stationary phases requires the determination of the column dead-time, t_m. Injection of a 1% w/v solution of metephosphoric acid, an unretained solute, enabled measurement of t_m to be made hence the partition ratio, k^1, was calculated for DHAA and L-ascorbic acid using Equation 13.

$$k^1 = \frac{t_r^1}{t_m} \quad given \quad t_r^1 = t_r - t_m \qquad \text{Equation 13}$$

where t_r^1 = sample reduced retention time in seconds

t_m = retention time of unretained solute

Selectivity, α, between L-ascorbic acid and DHAA was calculated for each column, Equation 14.

$$\alpha = \frac{k^1}{k^{11}} \qquad \text{Equation 14}$$

where k^1 = partition ratio L-ascorbic acid

k^{11} = partition ratio DHAA

If the retention time of L-ascorbic acid was 3 minutes or less not all the parameters were measured as a retention time greater than 3 minutes was required to ensure it would be adequately resolved from any polar degradation products. To prove that suppressing the ionization increases retention time the L-ascorbic acid solution was chromatographed using the same chromatography conditions as used with the Lichrospher 100-RP C18e stationary phase equilibriated to a mobile phase of 0.1% v/v trifluoroacetic acid, pH 5.7 (pK_a + 1.5). Retention time was reduced from 3.58 minutes to 2.02 minutes for the dissociated ascorbic anion. It can be seen, Table 6, that k^1 for DHAA would be greatest for the Hypercarb column. Unfortunately the L-ascorbic acid was irreversibly bound to this stationary phase. The column was washed with a variety of mobile phases with and without organic modifiers and at pH's above and below 7 but the acid was not detected in the column eluent. This stationary phase is therefore unsuitable for the chromatography of L-ascorbic acid.

Table 6: Results for the Chromatography of L-Ascorbic Acid

Manufacture	t_m (mins)	t_r (mins) AA	t_r (mins) DHAA	T_{AA}	k^1AA	k^1DHAA	α	Rs
1	1.74	3.58	1.84	1.15	1.0575	0.0575	18.3913	3.52
2	2.40	3.73	2.54	1.17	0.5545	0.0583	9.5060	2.00
3	2.14	3.35	2.26	1.35	0.5654	0.0561	10.0784	1.66
4	0.88	1.21	0.88	1.57	0.3750	0	0	0.59
5	1.87	3.13	1.95	1.63	0.6738	0.0428	15.7430	2.82
6	2.22	3.87	2.29	1.55	0.7432	0.0315	23.5937	coeluted
7	2.52	3.97	2.92	1.45	0.5754	0.1587	3.6257	3.15
8	2.54	4.87	3.89	-	0.9173	0.5315	1.7259	-
9	1.44	2.28	2.01	-	0.5833	0.3958	1.4736	-
10	2.13	2.48	-	-	0.1643	-	-	-
11	2.15	3.05	-	-	0.4186	-	-	-
12	1.91	3.04	-	-	0.5916	-	-	-
13	1.25	did not elute	3.43	-	-	1.7400	-	-

The greater retentive powers for polar compounds of the YMC ODS-AQ stationary phase was demonstrated and initially this column was adopted for the chromatography of L-ascorbic acid. Unfortunately despite claims from YMC that the stationary phase is exceptionally stable towards hydrolysis when operated under highly aqueous conditions, retention times for L-ascorbic acid decreased to a value typical to that obtained on other conventional C18 reverse-phase stationary phases. Therefore as the Merck Lichrospher 100-RP C18e column exhibited good retentive properties, high k and α values for L-ascorbic acid this column was adopted for the quantification of L-ascorbic acid.

3.2.3 Precision of the Suppressed Ionization Reverse Phase Chromatography Isocratic Assay

The precision of the isocratic HPLC method was determined by measuring the following parameters;

a) linearity;
b) limit of detection;
c) reproducibility;
d) specificity;
e) comparison with titrimetric assay.

a) Linearity

Aliquots of L-ascorbic acid solutions equivalent to 0 to 1.0 mg/ml were chromatographed using the conditions described in 3.2.2. Linearity was investigated using a conventional UV detector and a diode array detector (DAD). The resulting calibration graphs were linear up to 0.10 mg/ml for the conventional detector and 0.5 mg/ml for the DAD, Figure 36.

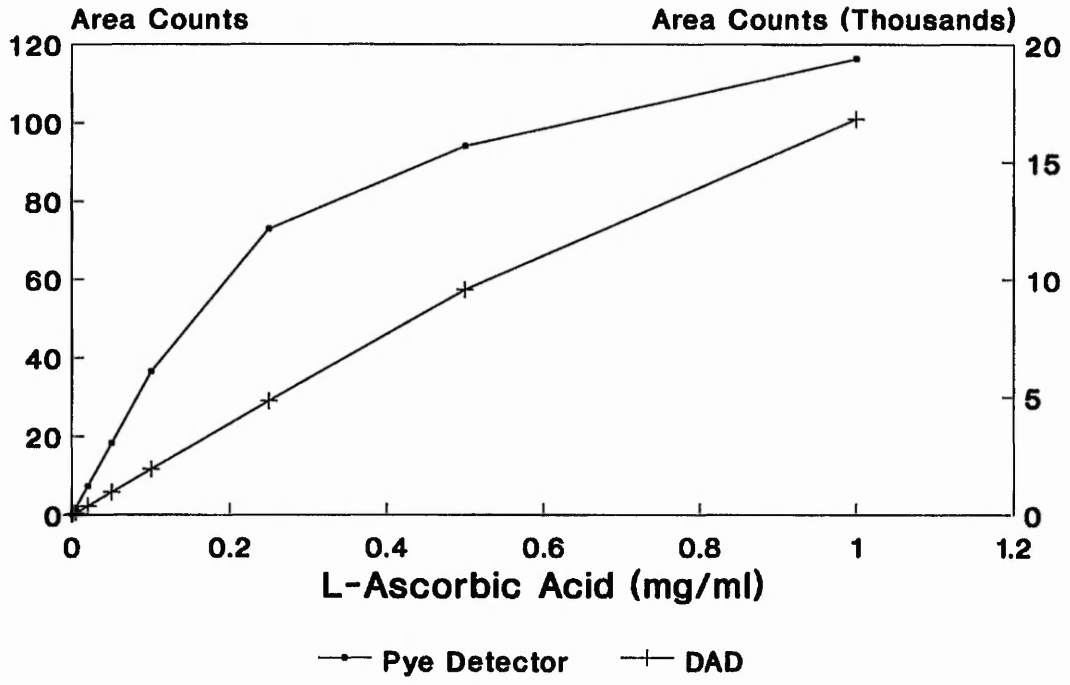


Figure 36: Linearity of L-Ascorbic Acid

b) Limit of Detection

A standard solution of L-ascorbic acid was sequentially diluted to produce solutions of reducing concentration to 100 ng/ml. These solutions were chromatographed using the conditions described in 3.2.2 using both the conventional UV detector and a DAD. A concentration of 500 ng/ml and 100 ng/ml could be detected three standard deviations above the noise signal for the DAD and conventional detectors respectively.

c) Reproducibility

Ten replicate weights of a commercially available L-ascorbic acid tablet (500mg/tablet) were assayed for L-ascorbic acid content against an external standard . Results were as follows:

Range	:	536.11 to 578.14 mg/tablet
Mean	:	544.97 mg/tablet
CoV	:	2.31%

The covariance was higher than when the tablets were assayed by titrimetric methods e.g. 0.44% and 0.21% for 2,6-dichlorophenolindophenol and N-bromosuccinimide resepectively. Reasons for the higher covariance obtained by HPLC could include injector problems on the chromatograph.

d) Specificity

In order to demonstrate the stability indicating properties of the assay method various concentrations of L-ascorbic acid solutions were chromatographed fresh then again after storage at ambient temperature in the light for 5 and 12 hours. Peak areas were recorded and compared and any reduction in area after storage expressed as a percentage of the initial value solutions were made in the following solvents;

i) deionized water;
ii) 1% w/v metaphosphoric acid, aqueous, pH 2;
iii) 0.1% w/v sodium metabisulfite solution, aqueous;

Various workers have investigated the stability of L-ascorbic acid in different solvents. Ponting [168] found oxalic acid solution stabilized L-ascorbic acid for a period of several hours. Trichloroacetic acid and metaphosphoric acid have also been investigated and found to be superior to oxalic acid [169, 170] with metaphosphoric acid being adopted by most workers. The degradation of L-ascorbic acid in solution is known to be catalyzed by transition metal ions and microbial contamination. Metaphosphoric acid stabilizes the L-ascorbic acid by complexing with any metal ions present and also acts as a deproteinator thus destroying any microbial contamination.

L-ascorbic acid is a vigorous scavenger of oxygen. Sodium metabisulphite is an antioxident frequently used in solution to stabilize easily oxidized compounds. Solutions of L-ascorbic acid were made in 0.1% w/v aqueous sodium metabisulphite to determine whether the L-ascorbic acid would be more stable in this solution than in 1% w/v aqueous metaphosphoric acid. Unprotected L-ascorbic acid in deionized water was included to demonstrate the stability indicating properties of the method, Table 7.

Results

Table 7: Assay Results by HPLC for L-Ascorbic Acid Dissolved in Various Solvents

Solvent	Concentration (mg/ml)	% Remaining After 5 Hours	% Remaining After 12 Hours
Deionized water	0.02	75.26	59.12
"	0.05	43.95	20.25
"	0.10	88.58	80.37
"	0.20	74.69	62.17
1% HPO_3	0.02	96.88	96.15
"	0.05	99.18	99.00
"	0.10	99.47	99.73
"	0.20	99.81	99.95
0.1% metabisulphite	0.02	44.38	0.0
"	0.05	72.91	55.12
"	0.10	60.51	35.57
"	0.20	90.57	84.66

L-Ascorbic acid was stable for at least 12 hours in 1% w/v metaphosphoric acid. After only 5 hours the L-ascorbic acid had degraded noticibly in both deionized water and 0.1% w/v sodium metabisulphite solution. Dilute solutions (0.02 mg/ml) were the least stable. The method was found to be stability indicating with no degradation products co-eluting with the L-ascorbic acid peak.

e) Comparison With Titrimetric Assay

A stock 1.0 mg/ml ascorbic acid solution in water was heated at 95°C for up to 24 hours in order to promote degradation. Aliquots were taken at 1, 2, 3, 5 and 24 hours and assayed for levels of ascorbic acid by the HPLC method and by a titrimetric method previously found to be accurate and precise, namely using N-Bromosuccinimide (N-BS). Three commercially available ascorbic acid tablet formulations were also assayed by both methods. Results were as follows, Table 8.

Table 8: Assay Results by HPLC and a Titrimetric Assay Method

Solutions @ 95°C (hours)	HPLC method (mg/ml)	N-BS method (mg/ml)
1	0.9156	0.9260
2	0.8757	0.8796
3	0.8544	0.8543
5	0.8356	0.8332
24	0.7153	0.7362
Solid formulation (mg/tab)		
1	42.48	38.39
2	41.40	39.04
3	37.02	36.89

3.2.4 Determination of Dehydroascorbic Acid

The first stage in the oxidative degradation of L-ascorbic acid is to dehydroascorbic acid (DHAA). This reaction is reversible and reduction of DHAA will yield L-ascorbic acid. As this oxidation reaction occurs in solution it may or may not occur in the solid phase degradation of L-ascorbic acid formulations. Therefore DHAA must be assayed during the study of the solid phase degradation of L-ascorbic acid and a method is required for this analysis.

Dehydroascorbic acid is difficult to quantify due to its instability. Due to the highly polar nature of dehydroascorbic acid it is poorly retained by reverse-phase high performance liquid chromatography (HPLC). Further, due to its low UV absorptivity a short wavelength must be used. At short wavelengths interferences from other compounds can be a serious problem with the quantification of dehydroascorbic acid. Due to these problems HPLC assays have been developed which measure dehydroascorbic acid indirectly after reduction. The L-ascorbic acid content of the sample is first measured directly, the sample is then reduced using a mild reducing agent and the sample re-assayed for L-ascorbic acid. Any increase in the L-ascorbic acid content is due to dehydroascorbic acid [171 - 176]. Various reagents have been used for the reduction of dehydroascorbic acid to L-ascorbic acid. Nagy and Degrell [171], Ziegler *et al* [165], Kim [173] and Sapers *et al* [175] used DL-dithiothreitol. Graham and Annette [176] and Nyyssönen *et al* [172] used DL-homocysteine and Dhariwal *et al* [174] used 2,3-dimercapto-1-propanol. The sample reduction procedures either involved a lengthy manual step prior to chromatography or a post-column reaction [165]. This work was supported by an automated pre-column reduction reaction using an automated precolumn sample preparation system.

Chromatography of both the L-ascorbic acid and reduced DHAA (reduced back to L-ascorbic acid) will be identical assuming the reducing agent is resolved from the L-ascorbic acid

The assay of L-ascorbic acid and DHAA combined is often referred to as the total L-ascorbic acid content. Using standard solutions of L-ascorbic acid a method was validated for reaction conditions and recovery of DHAA.

Experimental

i) *Assay for total L-ascorbic acid and dehydroascorbic acid*

Approximately 100 mg of each sample was accurately weighed into a series of 100 ml volumetric flasks and dissolved in 1% w/v aqueous metaphosphoric acid solution to produce 1 mg/ml sample solutions. These sample solutions (1 mg/ml) were diluted five fold with 1% w/v aqueous metaphosphoric acid solution to produce 0.2 mg/ml L-ascorbic acid solutions for quantitative analysis. A 10 µl aliquot of each of the 0.2 mg/ml solutions was chromatographed isocratically in duplicate using reverse-phase HPLC with spectroscopic detection at 245 nm. The sample solutions were assayed initially for total L-ascorbic acid (dehydroascorbic acid and L-ascorbic acid) using the automated precolumn sample preparation system and then for L-ascorbic acid only against an external standard solution of 0.2 mg/ml L-ascorbic acid.

Reducing Reagent

The precolumn reducing reagent was prepared by dissolving 1.135 g of trisodium orthophosphate dodecahydrate, 0.665 g of sodium dihydrogen orthophosphate dihydrate and 15 mg dithiothreitol in 50 ml deionized water to produce a solution buffered at pH 7.6 [165].

An aliquot of reagent solution was drawn into a heated capillary tube followed by an aliquot of sample solution and finally another aliquot of reagent solution. Once in the heated capillary tube the solutions were mixed together by cycling backwards and forwards ten times. After heating for one minute the whole volume was injected onto the chromatographic column.

Chromatography Conditions

i) Total L-ascorbic acid

Automated precolumn sample preparation system

Injector program

Micro-oven temperature	:	50°C
Slowdown Draw and Eject	:	2
Mix	:	2
Hold after Draw and Eject	:	0

Line #	Function					
1	Draw	:	2.5 µl from	Vial #	:	100
2	Draw	:	5.0 µl from	Sample		
3	Draw	:	2.5 µl from	Vial #	:	100
4	Mix	:	10.0 µl	Cycles	:	10
5	Wait	:	1.0 minutes			
6	Inject					

Column	:	Lichrospher RP 18e, 25 x 0.46 cm, 5 µm
Mobile Phase	:	0.1% v/v trifluoroacetic acid, aqueous
Flow Rate	:	1.5 ml/minute
Column Temperature:		40°C
Wavelength	:	245 nm
Injection Volume	:	10 µl
Run Time	:	4 minutes

ii) L-ascorbic acid only

Chromatography conditions were identical to those used for total L-ascorbic acid except the automated precolumn sample preparation system was bypassed and 5 µl aliquots of the same sample and standard solutions were chromatographed.

Using a mobile phase of 0.1% v/v trifluoroacetic acid, aqueous, the dissociation of L-ascorbic acid was completely suppressed and maximum column retention obtained without the use of ion-pair reagents. A retention time of approximately 1.0 and 2.2 minutes were obtained for DL-dithiothreitol and L-ascorbic acid respectively, further L-ascorbic acid eluted as a sharp symmetrical peak, Figure 37.

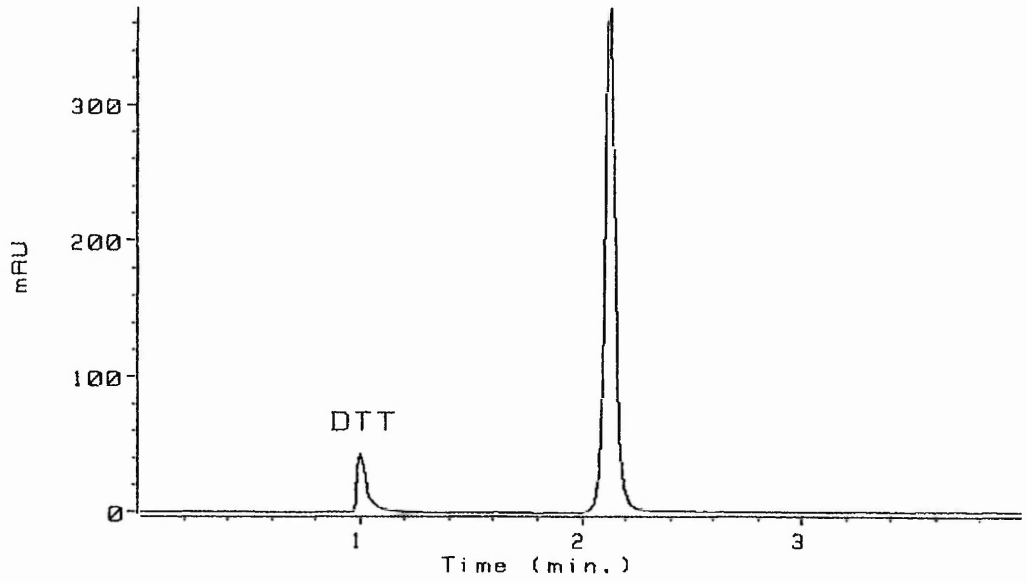


Figure 37: Typical Sample Chromatogram for Total L-Ascorbic Acid

Use was made of the automated precolumn sample preparation system fitted to a Hewlett Packard HP 1090 Series M HPLC. This unit enables precolumn derivatizations to be carried out at between ambient temperatures and 99°C, Figure 38.

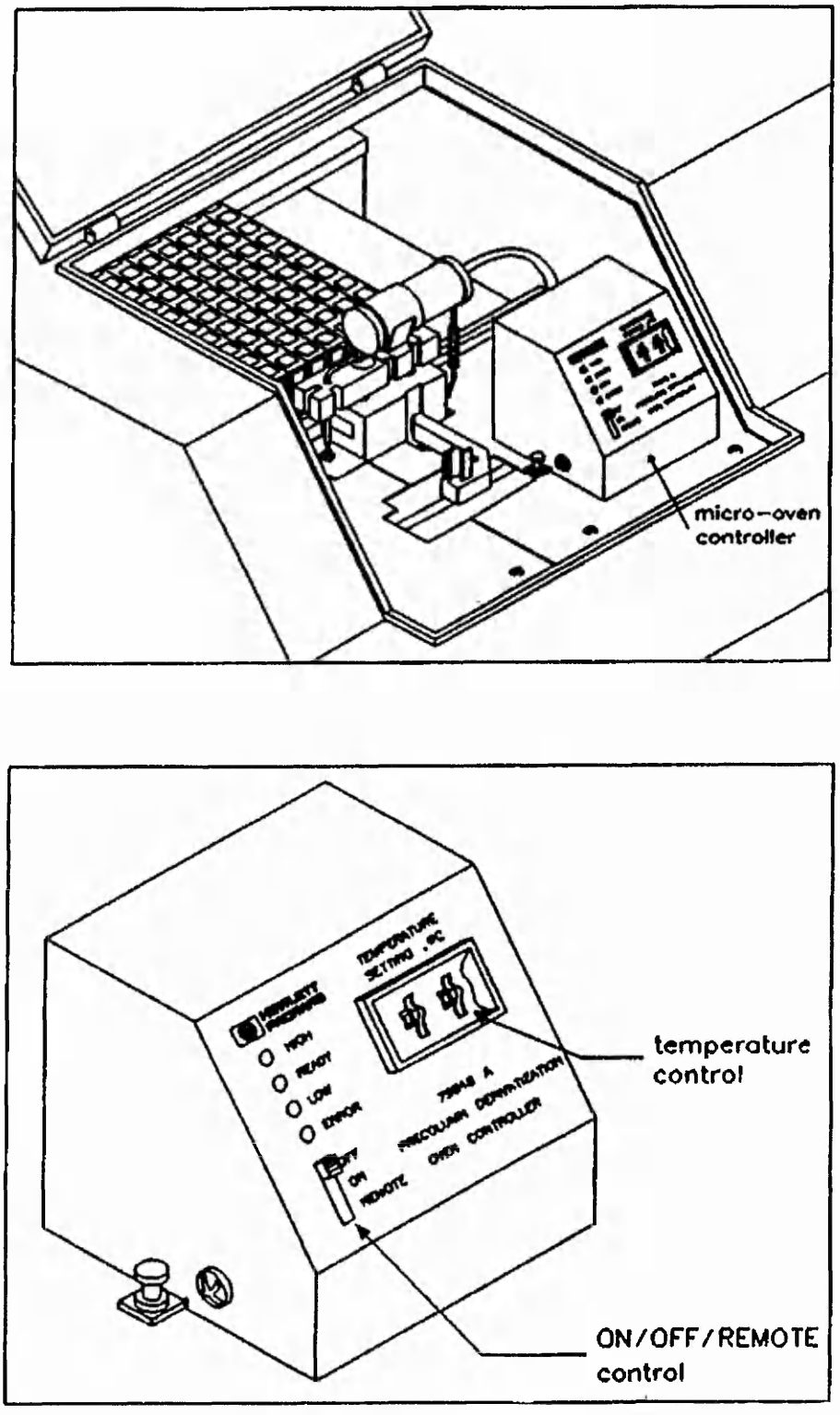


Figure 38: The Automated Precolumn Sample Preparation System

Utilizing the injector program, user defined volumes of liquid from different vials can be drawn into the reaction capillary where they are mixed and heated at a specified temperature and duration before being injected onto the analytical column. The reaction capillary is heated in a micro-oven , Figure 39.

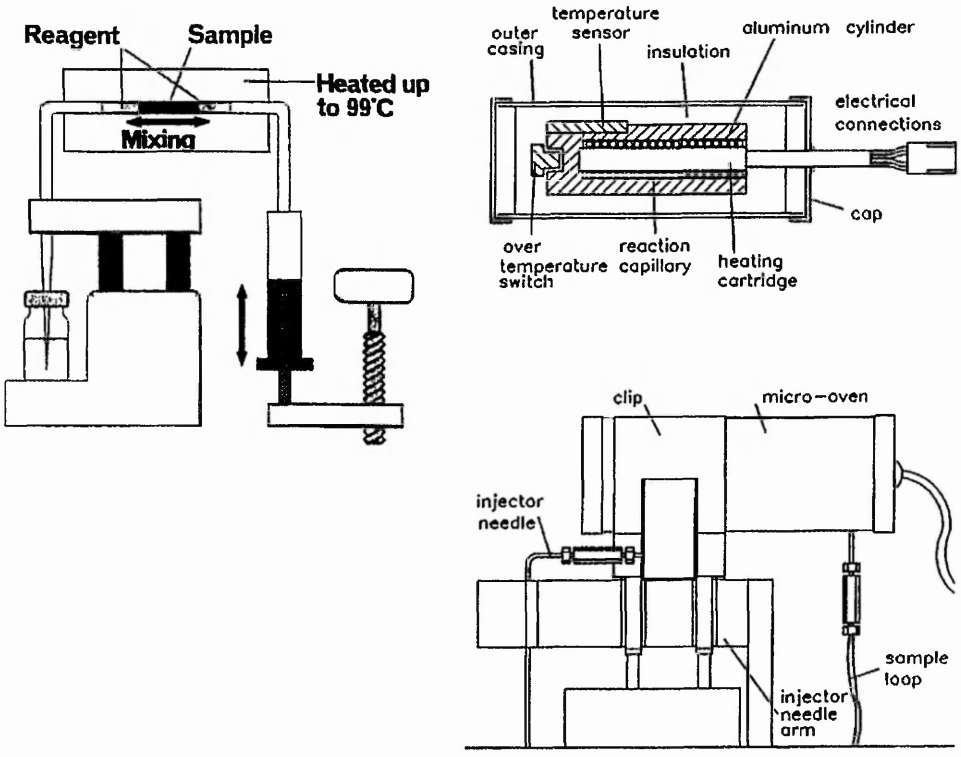


Figure 39: Schematic Diagram of the Micro-Oven

Results and Discussion

i) Assay for L-ascorbic acid and dehydroascorbic acid

The reduction rate of dehydroascorbic acid by DL-dithiothreitol reaches a maximum at pH 6.5 - 8.0 [177]. Buffering the reagent solution to pH 7.6 neutralized the metaphosphoric acid in the sample and standard solutions. In order

to determine the recovery of the reaction a 50 ml aliquot of a 0.2 mg/ml standard L-ascorbic acid solution in 1% w/v metaphosphoric acid was mixed with 0.5 ml of 3% v/v aqueous bromine solution. The addition of the bromine solution caused partial oxidation of the L-ascorbic acid solution to dehydroascorbic acid.

An unoxidized aliquot of the standard solution was retained as a control solution. The solutions were assayed for both L-ascorbic acid only and for total L-ascorbic acid using the DL-dithiothreitol reagent to reduce the dehydroascorbic acid formed on oxidation back to L-ascorbic acid. Fixing the reaction temperature at 50°C, a temperature above 45°C is required for complete conversion within one minute [165], the reaction time and number of mixes were optimized. After mixing for 10 cycles a reaction time of 60 seconds was found to give a recovery of 98.7%, Figure 40.

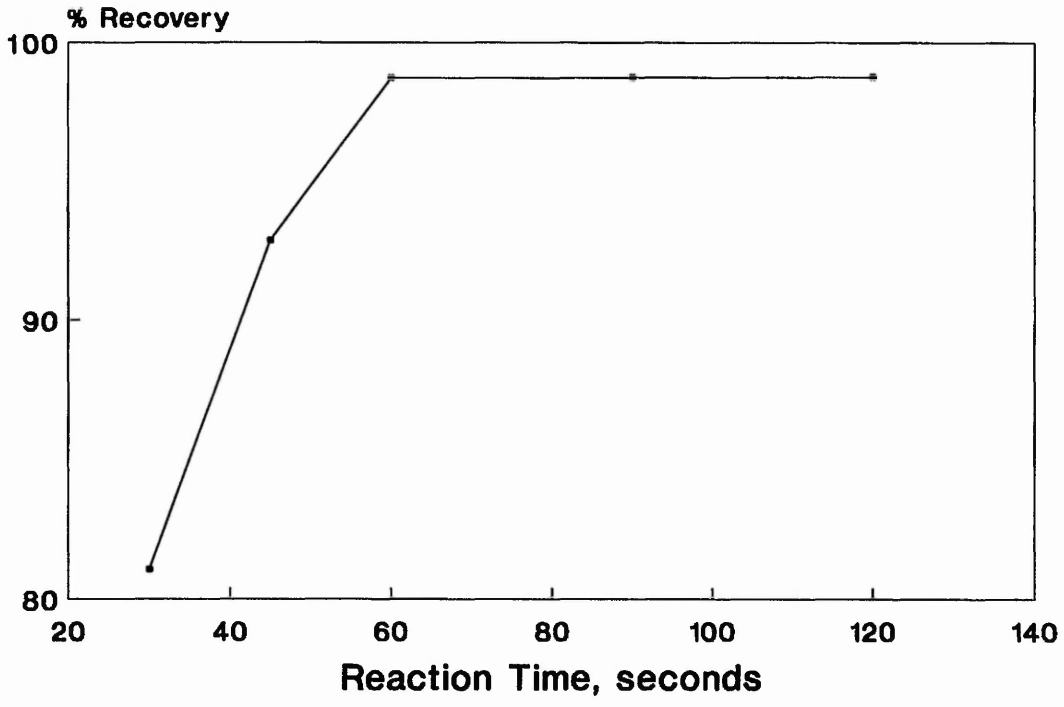


Figure 40: Recovery of Dehydroascorbic Acid at 50°C and 10 Mixing Cycles

The repeatability of injection was found to be 0.10% for six injections when a

standard unoxidized solution was chromatographed via the automated precolumn sample preparation system and 0.57% (n=6) when a partially oxidized standard solution was chromatographed

Conclusion

An automated and rapid precolumn reduction reaction was developed for the determination of total L-ascorbic acid (L-ascorbic acid and dehydroascorbic acid). A reaction time of only 60 seconds at 50°C was required for the reduction of dehydroascorbic acid to L-ascorbic acid.

3.2.5 Summary of Conclusions

Reverse-phase ion-pair partition chromatography was investigated and L-ascorbic acid was retained for up to 6.3 minutes. The method was demonstrated to be stability indicating but was not pursued further as a system for both the assay and degradation screen of L-ascorbic acid was required.

Various reverse-phase stationary phases were investigated using suppressed ionization reverse-phase chromatography. Also, studying various chromatography parameters a column, Lichrospher RP 18e, was adopted for the quantification of L-ascorbic acid. The precision of the method was demonstrated. The method was found to be linear up to 0.10 mg/ml and 0.5 mg/ml for variable wavelength and diode array detection respectively. The limit of detection was 100 and 500 ng/ml for variable wavelength and diode array detection respectively.

Reproducibility was carried out on commercially available L-ascorbic acid tablets. A coefficient of variation of 2.31% was achieved. This value was higher than those obtained by titrimetric methods. The reason for this was believed to be due to a faulty injector as the instrument typically gave a coefficient of variation of <1%.

The method was demonstrated to be specific for L-ascorbic acid using degraded solutions of L-ascorbic acid. Metaphosphoric acid was found to stabilize L-ascorbic acid for at least 12 hours.

The HPLC method is less time consuming than the non-chromatographic methods studied and is not subject to operator error caused by, for example, the incorrect determination of end-points. The chromatography stage of the HPLC assay is readily automated which will enable large numbers of samples generated in the study to be quantified.

An automated sample preparation system was validated for the reduction of DHAA to L-ascorbic acid using DL-dithiothreitol. A recovery of 98.7% was obtained at 50°C with a 60 second reaction time.

3.3 Methods For the Detection of the Degradation Products of L-Ascorbic Acid

A chromatographic analytical procedure is required in order to isolate degradation products that may be formed by the solid phase degradation of L-ascorbic acid. HPLC is the preferred technique as the method can be scaled up to the semi-preparative scale to enable milligram quantities of degradation products to be obtained in high purity for characterization.

3.3.1 Reverse-Phase High Performance Liquid Chromatography

An investigation of the degradation products of L-ascorbic acid required a chromatographic method to both resolve and detect the products. Four reverse-phase stationary phases and two aqueous buffer based mobile phases were compared using various gradients with acetonitrile as the organic eluent. Degraded samples of L-ascorbic acid, both solutions and solids, were chromatographed alongside known solution degradation products [106] for identification purposes.

Experimental

Chromatograph	:	Hewlett Packard 1090M with ChemStation
Detector	:	diode array
Columns	:	1) Waters μBondapak, C18, 5μ, 30 x 0.39 cm
		2) Shandon Hypersil, C18, 5μ, 25 x 0.46 cm
		3) Merck Lichrospher 100-RP C18e, 5μ, 25 x 0.46 cm
		4) Spherisorb Hexyl, 5μ, 25 x 0.46 cm
Chromatography conditions:		
Mobile Phase A	:	1) 0.1% v/v trifluoroacetic acid (TFA)
		2) 0.1% w/v metaphosphoric acid (HPO_3)
Mobile phase B	:	'A' in 80% v/v acetonitrile
Flow rate	:	1.0 ml/min
Wavelengths	:	220, 245 and 280 nm

Injection volume	:	10 µl	
Temperature	:	40°C	
Run time	:	30 minutes	
Gradient	:	Time (minutes)	% 'B'
		0	0
		5	0
		25	20
		30	20

Authentic possible degradation compounds chromatographed alongside samples;

DHAA;
oxalic acid;
gulonic lactone;
5,6-diketogulonic acid;
2-ketogulonic acid.

The UV spectra of 0.001% w/v solutions in 0.1% v/v aqueous TFA were measured in 1cm cuvettes on a scanning spectrophotometer. From the spectra the choice of two wavelengths, 220 and 245 nm, was made as all compounds exhibited maximum absorbance, λ_{max}, at one of these wavelengths, Table 9.

Table 9: Wavelength of Maximum Absorbance of Possible Degradation Compounds

Compound	λ_{max}
DHAA	220
oxalic acid	220
gulonic lactone	220
5,6-diketogulonic acid	245
2-ketogulonic acid	220

A wavelegth of 280 nm was included to detect any furan type compounds which are also known solution degradation products.

Using the Lichrospher column resolution between L-ascorbic acid and degradation products eluting either side of it, Rs_1 and Rs_2, was measured for both buffers along with the tailing factor, T of a major late eluting degradation peak, Table 10.

Results

Table 10: Resolution and Tailing Factors Using Two Buffers and a Lichrospher 100-RP C18 Stationary Phase

Buffer	Rs_1	Rs_2	T
0.1% HPO_3	2.58	2.67	3.50
0.1% TFA	3.55	3.05	1.60

Based on these data the buffer giving greatest resolution, i.e. TFA, was chosen for the mobile phase with which the four stationary phases were assessed. Resolution between L-ascorbic acid and two impurities found in the heat degraded solution eluting either side of it, Rs_1 and Rs_2, were measured for each stationary phase, Table 11.

Table 11: Resolution for Peaks Using Four Stationary Phases

Stationary Phase	Rs_1	Rs_2
Waters µBondapak C18	2.49	1.66
Shandon Hypersil C18	2.13	1.54
Merck Licrospher 100-RP C18e	3.55	3.05
Spherisorb Hexyl C6	1.50	1.28

Discussion

Greater resolution and sharper peak shapes were obtained using the TFA based mobile phase therefore the four reverse-phase stationary phases were compared using TFA based mobile phases. The Merck Lichrospher 100-RP C18e stationary phase produced greater resolution between the impurities eluting either side of the L-ascorbic acid peak. Similar retention times for degradation products and an identical number of peaks was obtained for all four columns, Figure 41.

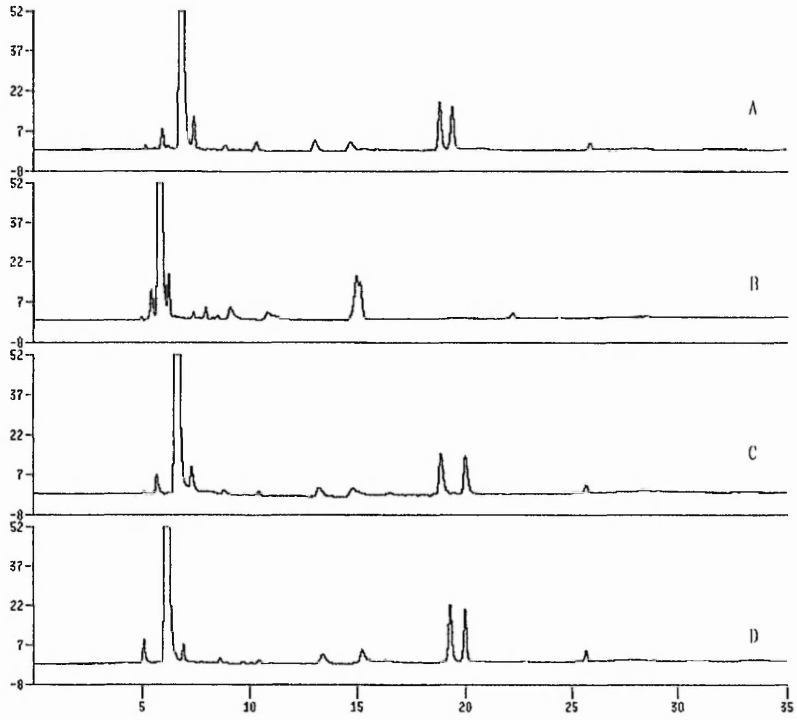


Figure 41: Chromatography Using Four Different Stationary Phases,
A = Waters µBondapak C18 B = Spherisorb Hexyl
C = Hypersil C18 D = Lichrospher 100-RP C18e

Up to 30 degradation peaks were detected in the heat degraded solution, Figure 42.

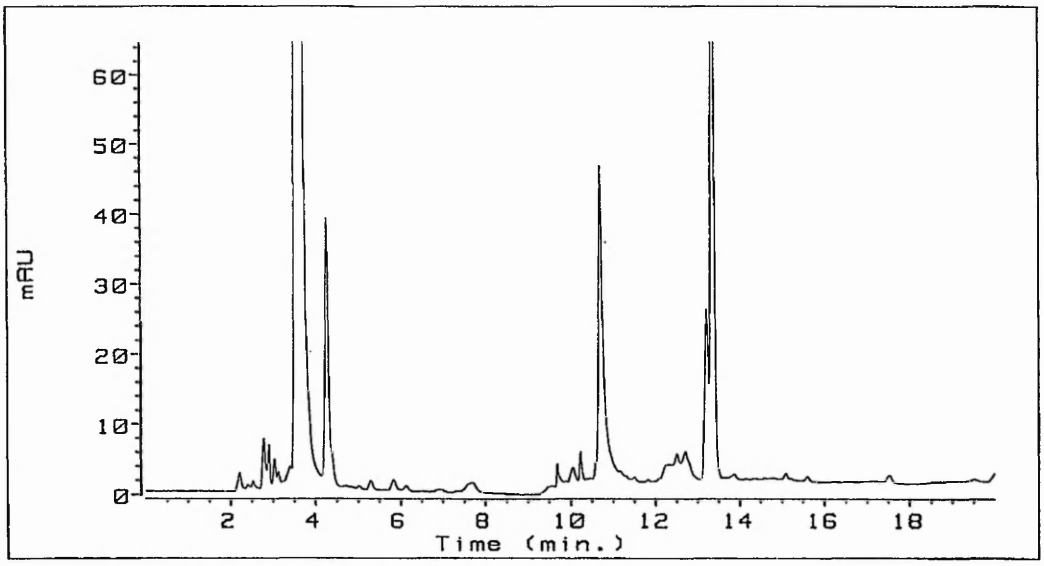


Figure 42: Reverse-Phase HPLC of a Heat Degraded Solution of L-Ascorbic Acid

Many fewer degradation peaks were detected in the alkaline degraded solution, Figure 43.

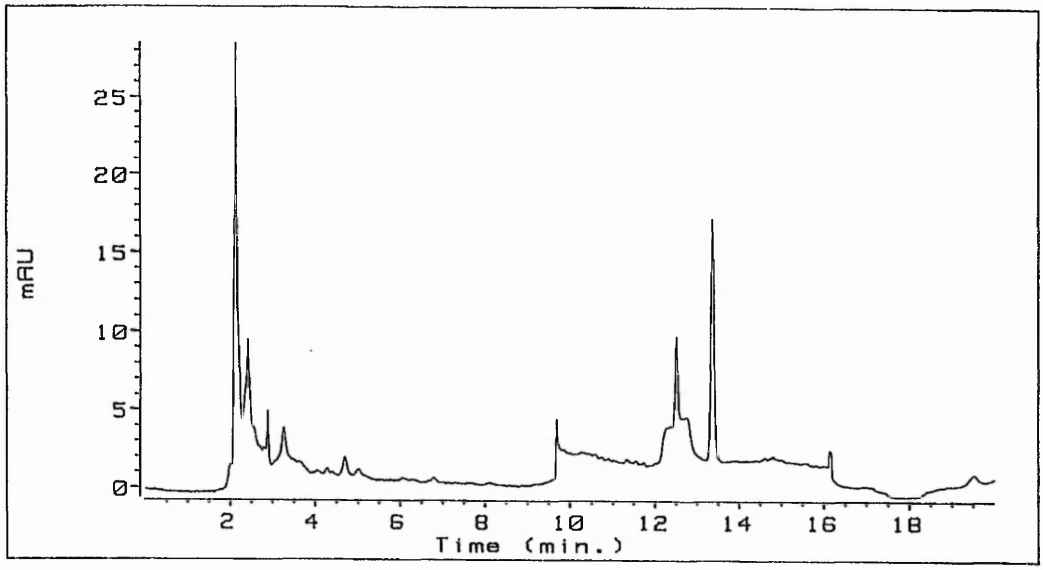


Figure 43: Reverse-Phase HPLC of an Alkaline Degraded Solution of L-Ascorbic Acid

Polar degradation products were poorly retained and resolved by all columns. Two of the compounds could only be detected at 220 nm. At this low wavelength baseline problems due to solvent UV cut-off were encountered making peak assignment difficult.

Conclusion

The effect of two different mobile phase buffers on peak resolution and shape was studied. A TFA based mobile phase gave superior chromatographic performance to that of HPO_3 buffer. Four reverse-phase stationary phases were studied using a TFA based mobile phase and degraded L-ascorbic acid solutions as test samples. Merck Lichrospher 100-RP C18e was chosen as the stationary phase with which to continue the degradation studies based on better resolution of impurities.

Work continued with the Merck Lichrospher 100-RP C18e stationary phase and TFA buffer based mobile phases to give greater retention and resolution of the more polar compounds found in the degraded test solutions.

Investigation Into Increasing Retention Time and Resolution of Polar Degradation Products

As the early part of the gradient was purely aqueous (0% B) and buffered at pH 2.5 i.e. any ionization should be suppressed the mobile phase cannot be made to be any more retaining on the reverse-phase stationary phase. Options available for investigation to increase the retention time and resolution of the polar degradation products are;

1) a more retentive stationary phase;
2) reduce the column temperature;
3) reduce mobile phase flow rate.

1) A More Retentive Mobile Phase

Ziegler et al [165] studied a range of reverse-phase stationary phases and determined that the Merck Lichrospher RP-100 C18e 5μ, 25 x 0.46 cm column retained and resolved L-ascorbic acid and dehydroascorbic acid the longest. This column was found (Table 5, Section 3.2.2) to give superior chromatographic performance out of 13 different columns investigated. There would be no advantage in changing from the Merck Lichrospher RP-100 C18e column.

2) Reduce The Column Temperature

The effect of reducing the column temperature from 40°C to 0°C was studied. This study was combined with the investigation into reducing the eluent flow rate.

3) Reduced Mobile Phase Flow Rate

The effect of reducing the mobile phase flow rate on retention time and resolution of polar degradation products was studied, flow rates of 1.0 and 0.6 ml/minute were investigated.

Experimental

The column temperature was reduced to 0°C by immersing the column in melting ice.

Chromatograph	: Hewlett Packard HP1082
Column	: Lichrospher 100-RP C18e, 5μ, 25 x 0.46 cm
Mobile Phase	: 0.1% TFA in deionized water
Flow Rate	: 0.6 and 1.0 ml/minute
Column Temperature	: 0 and 40°C
Wavelength	: 245 nm
Injection Volume	: 10 μl

Samples	:	oxalic acid
		2-ketogulonic acid (2-KGA)
		gulonic lactone
		dehydroascorbic acid (DHAA)
		5, 6-diketogulonic acid (5,6-DKGA)
		L-ascorbic acid
		HPO_3

Sample solutions (0.2 mg/ml) in deionized water, 1% w/v HPO_3 was used to measure t_m.

Results

In order to quantify results k and α were calculated for each compound in order of elution for each set of conditions. Results of the study are given in Table 12.

Table 12: Effect of Reducing Temperature and Mobile Phase Flow Rate

Compound	1 ml/min @ 40°C			1 ml/min @ 0°C			0.6 ml/min @ 40°C			0.6 ml/min @ 0°C		
	t_r	k^1	α	t_r	k^1	α	t_r	k^1	α	t_r	k^1	α
Oxalic Acid	2.237	0.3964	-	2.425	0.4223	-	4.144	0.2890	-	4.625	0.4377	-
2-KGA	2.340	0.4607	1.1622	2.585	0.5161	1.2221	4.400	0.3686	1.2754	4.819	0.4980	1.1378
Gulonic Lactone	2.459	0.5350	1.1613	2.792	0.6375	1.2352	4.565	0.4199	1.1392	5.229	0.6254	1.2558
DHAA	2.874	0.7940	1.4841	3.559	1.0874	1.7057	5.168	0.6075	1.4468	6.737	1.0942	1.7496
5,6-DKGA	3.578	1.233	1.5529	6.094	2.5742	2.3673	6.100	0.8974	1.4772	11.500	2.5748	2.3531
Ascorbic Acid	3.607	1.2516	1.0151	6.825	3.0029	1.1665	6.135	0.9082	1.0120	12.877	3.0028	1.1662
HPO_3 (t_m)	1.602	-	-	1.705	-	-	3.215	-	-	3.217	-	-

Discussion

Resolution increased for both flow rates when the column temperature was reduced to 0°C. Reducing the flow rate at both 40°C and 0°C had a deleterious effect on separations.

Conclusion

Reducing the column temperature from 40 to 0°C marginally improved selectivity but due to the impracticalities of maintaining the column at 0°C a temperature of 40°C was retained. No improvement in selectivity was obtained by reducing flow rate.

Polar, far UV absorbing degradation products could be better retained, resolved and detected at low concentration levels. Other techniques for this purpose must be investigated. Reverse-phase HPLC with conductivity detection was also studied to determine whether improved detection of more polar degradation products of L-ascorbic acid could be achieved.

Reverse-Phase HPLC With Conductivity Detection

Diode array detection enabled the simultaneous monitoring of three wavelengths, i.e. 220 nm for compounds having no chromophore, 245 nm for maximum sensitivity for L-ascorbic acid and 280 nm for furfural related compounds.
The reverse-phase degradation screen developed in 3.3.1 involved gradient elution and the lowest wavelength that could be used without giving rise to 'ghost' peaks, artefacts of the system, was 220 nm. A lower wavelength, e.g. <200 nm, would be preferred for weakly absorbing species potentially present but an irregular and inconsistant baseline at these wavelengths made sub 200 nm work impracticable. A conductivity detector is capable of greater sensitivity than UV detection for non-chromophoric species.

Conductivity detection is based upon the electrical conductivity of an ionic solution when placed between two oppositely charged ecectrodes. The presence of ions in the solution allows electrical current to flow between the electrodes, completing the circuit. At low ionic concentrations, conductivity is directly proportional to the concentration of conductive species in the solution. The total ionic concentration of the solution in the cell and the temperature of that solution affect the linearity of this relationship. Use of a temperature compensation factor helps eliminate baseline variations caused by changes in the temperature of the solution in the cell. This factor normalizes the measured solution conductivity to 25°C

The Effect of Concentration on Conductivity Detection

The electrical conductance of a solution depends upon the type and concentration of all ions present. Electrical current is carried by both cations and anions in solution. Ideally, conductance increases linearly as the total ionic concentration increases. In reality the linearity is limited by the degree of dissociation, the mobility of the ions in solution and the formation of ion-pairs in solution.
For weak electrolytes e.g. weak acids and bases, the primary factor limiting detection linearity is the degree of dissociation or ionization. The degree of dissociation represents the fraction of total solute which is available to carry electrical current and depends on both the solute concentration and the solvent. Weak electrolytes are not completely ionized in solution and some molecules will be present in their un-ionized forms. As concentration increases the ratio of ionized to un-ionized species decreases. The un-ionized molecules cannot carry current and, therefore, the concentration of detected ions is less than the total concentration of the species in solution. For weakly dissociated species, the linearity range varies and is dependent on the pK. For most ions, linearity will be observed at low (ppm) concentrations where dissociation is essentially complete.

For strong electrolytes e.g. strong acids and bases and their salts, which are completely dissociated in solution, the primary factor limiting detection linearity is ionic mobility. Factors affecting the mobility of an ion are the retardation of ion

movement by the solvation sphere surrounding each ion and the total ionic concentration of the solution. In dilute solutions each ion in the mobile phase carries a large fraction of the current at any one time. As the total ionic concentration increases the fraction of the available current carried by each ion is reduced. The result is an apparant lower concentration of each ion, which in turn, results in a lower detector response, Figure 44.

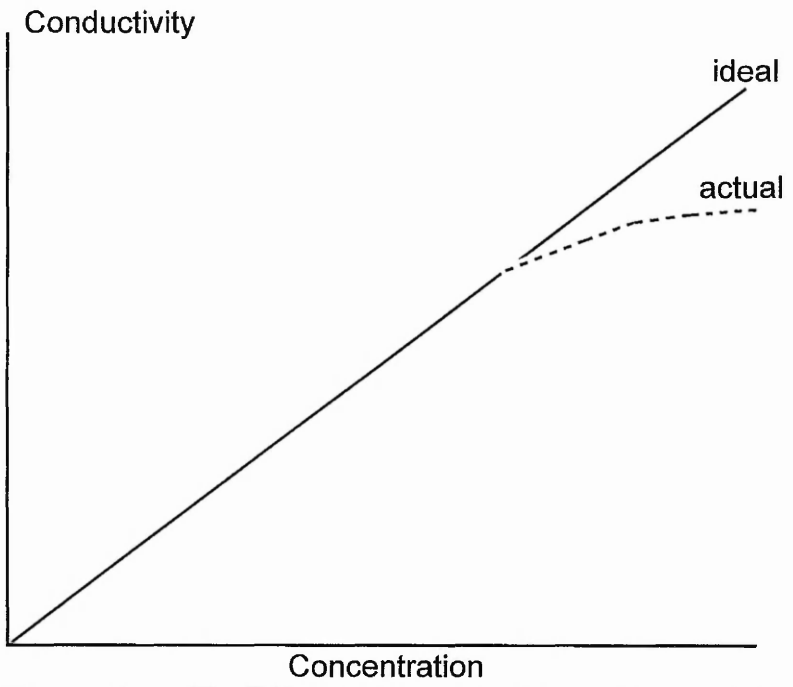


Figure 44: The Effect of Concentration on Conductivity

For both weak and strong electrolytes, detection linearity is influenced by the formation of non-conducting ion-pairs in solution. The ion pairs form as a result of electrostatic attraction between oppositely charged ions. Although each ion-pair has a finite life-time, at any instant, the total number of free ions capable of carrying current is lowered by the continual interchange of ions in solution. Under these conditions, a species may be completely ionized but will not be completely dissociated. As a result, the conductivity of the solution will decrease and the

concentration of the detected species will appear lower than it is.

In ion-chromatography, use of a suppressor device replaces the background concentration of highly conductive eluent ions with species which are significantly less conductive. This reduces the effect of the eluent on the mobility of the ions to be detected and expands the range of concentrations over which detection is linear. In addition, ions of interest are converted to their corresponding acids or hydroxides as they pass through the suppressor. The exceptionally high mobilities of H^+ and OH^- in water increase the conductivity of most species, resulting in increased detector sensitivity. Reduction of the background conductivity also increases the detector sensitivity and reduces the influence of temperature on measured conductivity.

The Effect of Temperature on Conductivity Detection

The conductivity of a solution is directly affected by its temperature, i.e. a change in temperature, causes a change in the solution conductivity. As the solution conductivity increases the effect of temperature changes becomes more pronounced. This change is observed as a regular oscillation in the baseline that is directly related to the room temperature i.e. the baseline will exhibit a positive shift with increasing temperature. The change may effect the reproducability and linearity of a determination.

Anion Analysis Using Eluent Suppression

The ion chromatograph used in this study, Dionex 2010i, utilizes an anion self-regenerating suppressor to provide high capacity suppression of the eluent to expand the detection limits of anion exchange chromatography. The suppressor comprises two regenerant compartments and one eluent compartment separated by ion exchange membranes. The eluent flow is in a countercurrent direction to the regenerant flow, Figure 45.

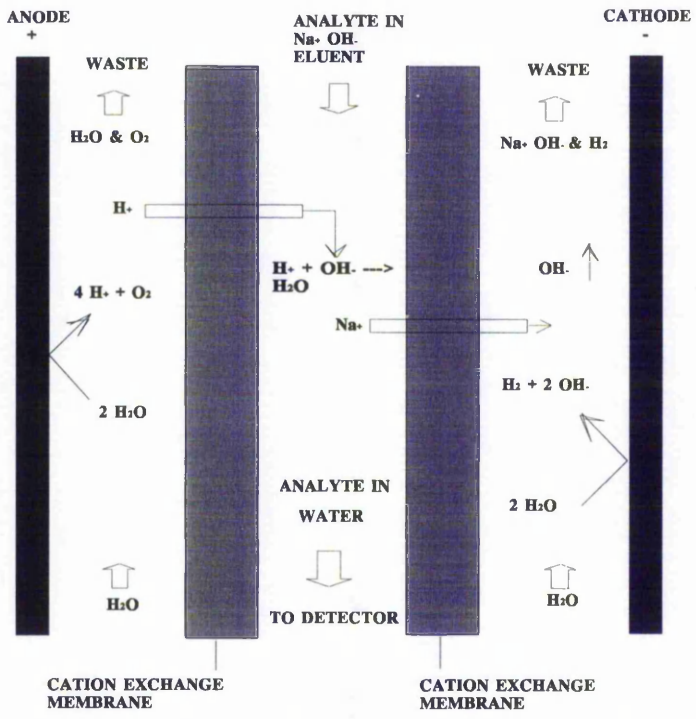


Figure 45: Schematic illustration of the Ion Exchange Suppressor

Electrodes are placed along the length of the regenerant channels. When an electrical potential is applied across the electrodes, water from the regenerant channels is electrolyzed, supplying hydronium ions (H_3O^+) for the neutralization reaction. The hydronium ions pass through the membrane into the eluent chamber thus weakly ionizing the eluent electrolyte. Eluent cations are simultaneously passed into the regenerant chamber to maintain charge balance.

A constant supply of water through the regenerant channels is required to maintain suppression. The water regenerant undergoes electrolysis to form hydrogen gas and hydroxide ions in the cathode chamber while oxygen gas and hydronium ions are formed in the anode chamber. Cation exchange membranes allow hydronium ions to move from the anode chamber into the eluent chamber to neutralize hydroxide ions. Sodium ions in the eluent are attracted to the cathode chamber and more across the membrane to the cathode chamber to maintain electronic neutrality with the hydroxide ions at the electrode. The analyte then passes to the detector in water and not the eluent used for the chromatographic separation and hence lower

levels of detection can be obtained using the suppressor.

Experimental

The ion chromatograph had no gradient facility therefore only isocratic elution was possible. Most of the known degradation products of L-ascorbic acid, are more polar e.g. oxalic acid, and thus elute before L-ascorbic acid when chromatographed on a reverse-phase stationary phase. The critical region of the separation therefore is the early stage and hence lack of a gradient system is not detrimental.

Compounds such as oxalic acid have no chromophore present and cannot be detected at low concentration using ultra-violet wavelengths. These compounds are ideally suited to detection by a conductivity detector. This study was carried out to determine whether conductivity detection coupled to reverse-phase chromatography could detect any degradation products in L-ascorbic acid.

Samples;
- oxalic acid
- hydrochloric acid (HCl)
- heat degraded L-ascorbic acid solution
- L-ascorbic acid, fresh solution
- HPO_3

Sample solutions were at a concentration of 0.2 mg/ml in deionized water. The hydrochloric acid was chromatographed to obtain t_m.

Chromatography conditions ;

Column	:	Lichrospher 100-RP 50 DS, 250 x 4.6 mm
Mobile phase	:	0.1% v/v TFA, aqueous
Flow rate	:	1 ml/min
Regenerant	:	5 mM tetrabutyl ammonium hydroxide
Regenerant flow rate	:	2 ml/min
Injection volume	:	50 µl

Results

The mobile phase of 0.1% v/v TFA produced a background conductivity of >1500µS. This resulted in an unusable system. The mobile phase was changed to 1 mM TFA (pH = 2.85, therefore ionization of L-ascorbic acid would still be suppressed). The background conductivity with the suppressor was reduced to a usable 90 µS.

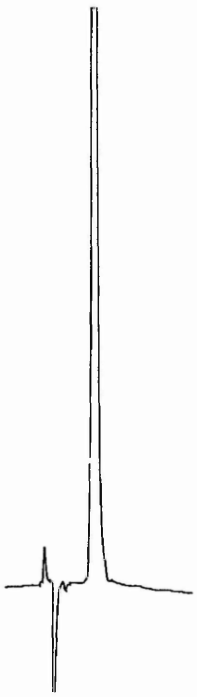


Figure 46: Chromatogram of L-Ascorbic Acid Using Conductivity Detection

The L-ascorbic acid eluted as a sharp symmetrical peak, Figure 46, and the heat degraded solution eluted as a complex mixture with only 12.7% of the original amount remaining.

Retention times of the compounds chromatographed are given, Table 13.

Table 13: Retention Times Obtained Using Reverse - Phase HPLC and Conductivity Detection.

Retention Time (minutes)	L-Ascorbic Acid	Oxalic Acid	HPO_3
	4.7	2.1	1.9

Discussion

Many more polar degradation peaks were detected in the heat degraded solution by conductivity detection than by UV detection. Most of the polar components were poorly retained but this was due to the stationary phase/chromatography conditions rather than the method of detection.

Summary of Conclusions

The use of reverse-phase HPLC with gradient elution and UV detection has been investigated. Using diode array detection three wavelengths were simultaneously monitored for the detection of any degradation products. Four different reverse-phase stationary phases and two different mobile phase buffers were studied. Greatest resolution was obtained using a TFA buffer and Merck Lichrospher 100-RP C18e stationary phase. Polar degradation products were poorly retained and two known degradation products could only be detected at 220 nm. Methods of retaining polar compounds longer were studied. Reduced flow rate and temperature produced no improvement on chromatography.

A conductivity detector was investigated to enhance detection of UV inactive polar degradation products using a reverse-phase stationary phase and eluent suppression. Many polar degradation products were detected but they were poorly retained and resolved.

3.3.2 High Performance Ion Chromatography Exclusion

Introduction

A further high performance chromatographic technique, high performance ion chromatography exclusion (HPICE) has been studied to assess its suitability for analyzing known degradation products of L-ascorbic acid. Such compounds are carboxylic acids which are extremely polar and contain no chromophore rendering them difficult to retain and detect using reverse-phase HPLC and UV detection.

Ion chromatography has successfully been applied to the resolution of many organic acids [179-181]. When coupled to a conductivity detector problems of detecting these non-chromophoric compounds are overcome.

Two types of ion chromatography can be used for the separation of anion species:

a) high performance ion chromatography exclusion (HPICE)
b) high performance ion chromatography (HPIC)

Both types of ion chromatography use resins as column packing. The five major properties of ion chromatography resins are;

i) type of polymeric material;
ii) crosslinking;
iii) particle size;
iv) functional group identity;
v) capacity factor.

i) The resin is based upon a polystyrene coplymer made by copolymerizing styrene and divinylbenzene. The crosslinking comes about by the functional groups reacting to link two polystyrene chains together making them rigid and able to withstand the pressures and viscous forces generated during chromatography.

ii) The degree of crosslinking affects the property of the resin. The higher the crosslink, the less a resin will swell in a liquid environment and the lower the total pore volume of the resin. Ions having large radii can pass more easily through low crosslinked resins than through highly crosslinked resins. Highly crosslinked resins are the most rigid and produce a lower pressure drop across the column than low crosslinked resins.

iii) The smaller the particle size of the resin the greater the efficiency of separation due to shorter diffusion paths. Small particle size resins produce greater back pressures than larger size resins.

iv) Resins having functional groups throughout the resin bead are termed 'porous' (HPICE resins). Resins with groups only on the bead surface are pellicular (HPIC resins). Sulphonic and quaternary amine functional groups are the most common.

v) The capacity of an ion exchange resin is defined as the number of functional groups per unit volume and is quoted as milliequivalents per gram. The higher the capacity of a resin the greater the retention time of an analyte.

HPICE stationary phases employ a narrow range particle size sulphonated cation exchange resins. Separation is dependent upon three different mechanisms;

i) Donnan exclusion;
ii) steric exclusion;
iii) adsorption/partition.

Donnan exclusion causes strong acids to elute in the void volume of the column.

Weak acids which are partially ionized in the eluant are not subject to Donnan exclusion and can penetrate into the pores of the packing material. Separation is accomplished by differences in acid strength, size and hydrophobicity. The Donnan exclusion limits the ability of a charged species to move across a pseudo semi-permeable membrane into the pore volume of the resins. The functional groups of the resin tend to form a "semi-permeable membrane" between the eluent around the resin particles and the occluded liquid inside the resin pores. A highly ionized species such as sulfate cannot penetrate this membrane and is not retained and elutes in the void volume of the system. Non-ionized species are not subject to Donnan exclusion and can permeate the ionic membrane to reach the volume within the resin pores. Retention then becomes a function of total pore volume and surface area of the resin. As in steric exclusion chromatography the solute enters the pores of the packing material then migrates back into the eluent. In ion exclusion chromatography retention can be controlled by controlling the degree of ionization of the solute.

The ionization of a weak organic acid can be controlled by the pH of the eluent. The higher the pH, the greater the ionization, and the less the retention by ion exclusion as the anion is excluded by the Donnan membrane. Conversely, the lower the pH the less the ionization, and therefore the greater the retention in the ion exclusion mode. Depending on the complexity of the sample, the pH of the eluent can be used to control the retention of ionic species and thus resolution and analysis time.

Chromatographic separation is principally dependent upon the distribution of the species between the occluded resin pore liquid (stationary phase) and the interstitial liquid (mobile phase). The ultimate HPICE separation in dependent upon factors which can affect this distribution. These factors are;

i) solute pK_a - the larger the pK_a, the longer the retention;
ii) temperature - elevated temperatures will increase ionizsation and decrease retention;

iii) concentration of solute - important for weak electrolytes since ionization is a function of concentration;

iv) eluent pH - the lower the pH, the longer the retention of a weak electrolyte since ionization is reduced;

v) crosslinking of resin - the degree of crosslinking affects the total pore volume of the resin. The higher the crosslink, the lower the volume and reduced separation is obtained;

vi) nature of the resin - results are dependent upon polymer type, their relative hydrophobicities and functional group;

vii) flow rate - since separation is diffuse dependent, slow flow rates are desirable, typically 0.8 ml/minute.

In summary, the expected elution sequence can be partially predicted from the pK_a, of the solute i.e. the lower the pK_a the shorter the retention time. However, as solutes become more aliphatic or aromatic, Van der Waals forces become dominent and elution sequences are more difficult to predict.

HPICE enables the rapid separation of organic acids and group and separation of inorganics from organics. When HPICE combined with HPIC maximum information can be obtained about anionic species in a mixture e.g;

i) strong acids are separated as a class from weak acids;

ii) weak acids can be analyzed free of interference;

iii) strong acids can be concentrated on a guard column and analyzed using ion chromatography.

HPIC involves the use of low capacity pellicular ion exchange materials in a separation mode dominated by ion exchange. The functional groups are sulfonic acid groups covalently bonded to a copolymer. As the functional groups are on the surface diffusion paths are short, efficiencies high but stationary phase capacities are low. The ion exchange mechanism is based upon the exchange of ions between the mobile phase and ionic sites on the resin. At any point, during the ion

exchange process, the neutrality of the fixed ionic charge on the resin is preserved by the exchangeable eluting ion exchanging with sample ions. When it is paired with the fixed charge on the resin the sample ion does not move with the flow and because different ions have different affinities for the resin then mixtures of different ions can be separated. The ion exchange process may be carried out in aqueous or non-aqueous solvents. The mobile phase contains counter ions of opposite charge to the resin ionic group. The counterion is in equililbrium with the resin as an ion pair. When a solute ion of the same ionic charge as the counter ion is present an ion exchange equilibrium is set up. The higher the value of an ions partition coefficient, k, the stronger the ion- resin interaction and the longer the ion is retained. The partition coefficient is a function of;

i) ionic charge;
ii) ionic size;
iii) ionic strength of the eluent;
iv) pH of eluent;
v) resin type.

In general, the greater the valence of the sample ion, the greater the affinity for the ion exchange sites e.g. trivalent ions have a greater affinity than divalent ions and divalent ions a greater affinity than monovalent ions. The typical elution order therefore is monovalent ions followed by divalent then trivalent ions.

For different ions of the same valence, the larger the ionic radius the more strongly the attraction to an ion exchange site and hence the longer the retention.

The stronger the ionic strength of the eluent the shorter the analyte retention time due to ions in the eluent competing for the ion exchange sites.

Eluent pH affects the equilibrium distribution of multivalent ions. The phosphate ion can be mono, di or trivalent by increasing the pH of the eluent. Therefore the retention time of the phosphate ion is dependent upon the pH of the mobile phase.

The selectivity of an ion exchange resin plays a major role in HPIC separations due to it directly affecting the equilibrium distribution of sample and eluent ions. Changes in the selectivity of an HPIC anion resin can be accomplished by varying the crosslink, size or the functional group of the resin.

HPIC mobile phases for the separation of carboxylic acids and other anions are generally bicarbonate/carbonate solutions of typically 3 mM $NaHCO_3$/2.4 mM Na_2CO_3 concentration. Using these mobile phases both mono and divalent anions can be resolved in a single analysis. Sodium hydroxide can also be used as a weak eluent and sodium iodide as a strong eluent. Organic modifiers such as methanol and acetonitrile can also be added to the mobile phase to reduce analysis time.

Detection of the anions is accomplished by a suppressed conductivity detector which provides much greater sensitivity for these non-chromophoric compounds than UV detection even at low wavelengths.

These two techniques (HPICE and HPIC) were investigated for their potential to resolve and detect the polar and UV inactive species believed to be present in the degraded solid formulations of L-ascorbic acid.

Experimental

A total of four different HPICE chromatographic systems were assessed (HPICE 1 to 4) and one HPIC system, Table 14. Samples chromatographed were known degradants and L-ascorbic acid solutions and solid mixtures stressed with sodium hydrogen carbonate and Cu^{2+} ions which were found to degrade L-ascorbic acid, Chapter 4.

Samples:

1) oxalic acid solution, 1 mg/ml
2) 2-ketogulonic acid solution, 1 mg/ml
3) DHAA solution, 1 mg/ml
4) L-ascorbic acid solution, 1 mg/ml
5) L-ascorbic acid + 1000 ppm Cu^{2+} solution
6) solid L-ascorbic acid + 1000 ppm Cu^{2+}, 2 weeks at 55°C
7) 1:1 molar ratio L-ascorbic acid/$NaHCO_3$ solution, 2 weeks at 55°C
8) 1:1 molar ratio L-ascorbic acid/$NaHCO_3$ solid, 2 weeks at 55°C

Table 14: Chromatographic Systems Assessed for HPICE and HPIC

Parameter	HPICE, 1	HPICE, 2	HPICE, 3	HPICE, 4	HPIC
Column	ICE AS1	ICE AS1	ICE AS1	ICE AS5	AS4A
Mobile Phase	1mM TDFHA	1.5 mM TDFHA	1mM OSA	1mM TDFHA	3mM $NaHCO_3$ 2.4 mM Na_2CO_3
Flow Rate (ml/minute)	0.8	0.8	0.8	0.5	1.5
Regenerant	5 mM TBAOH	5 mM TBAOH	5 mM TBAOH	5 mM TBAOH	25 mM H_2SO_4
Regenerant Flow Rate (ml/minute)	2	2	2	2	2
Detector Range (μS)	30	30	30	30	30
Injection Volume (μl)	50	50	50	50	50

Note: TDFHA = tridecafluoroheptanoic acid
OSA = octanesulfonic acid
TBAOH = tetrabutylammonium hydroxide

Results

HPICE system 1 was initially assessed as this represented standard HPICE chromatographic conditions. The retention time and capacity factor, k, were measured for each probe compound, Table 15.

Table 15: Chromatographic Data for HPICE and HPIC Systems

Probe Compound	HPICE, 1		HPICE, 2		HPICE, 3		HPICE, 4		HPIC	
	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	k
L-Ascorbic Acid	8.67	0.618	8.54	0.611	8.61	0.649	6.66	1.622	1.86	2.207
Oxalic Acid	6.30	0.175	6.37	0.202	5.77	0.105	2.59	0.020	10.54	17.172
2-KGA	6.29	0.174	6.30	0.189	6.42	0.230	3.21	0.264	1.49	1.569
DHAA	5.90	0.101	5.63	0.062	5.70	0.092	2.95	0.161	1.57	1.707
t_m	5.36	-	5.30	-	5.22	-	2.54	-	0.58	-

The retention time of an unretained solute, t_m, was measured using water. When the samples were dissolved in water a large negative peak resulted. Therefore all solid samples were dissolved in the mobile phase which produced no negative void peak. The solution samples had the mobile phase salt added to produce a concentration equivalent to the mobile phase concentration.

HPICE system 1 failed to resolve oxalic acid and 2-KGA, two possible degradants. Noticeable differences were seen in the peak profile of solid and solution samples of the same formulation, Figure 47, for formulation with sodium hydrogen carbonate, Figure 48, for formulation with 1000 ppm Cu^{2+} ions. Chromatography could be improved upon as many early eluters almost coeluted. From knowledge of the mechanism by which HPICE operated the early eluting components were believed to be strong acids and the late eluting compounds weak acids.

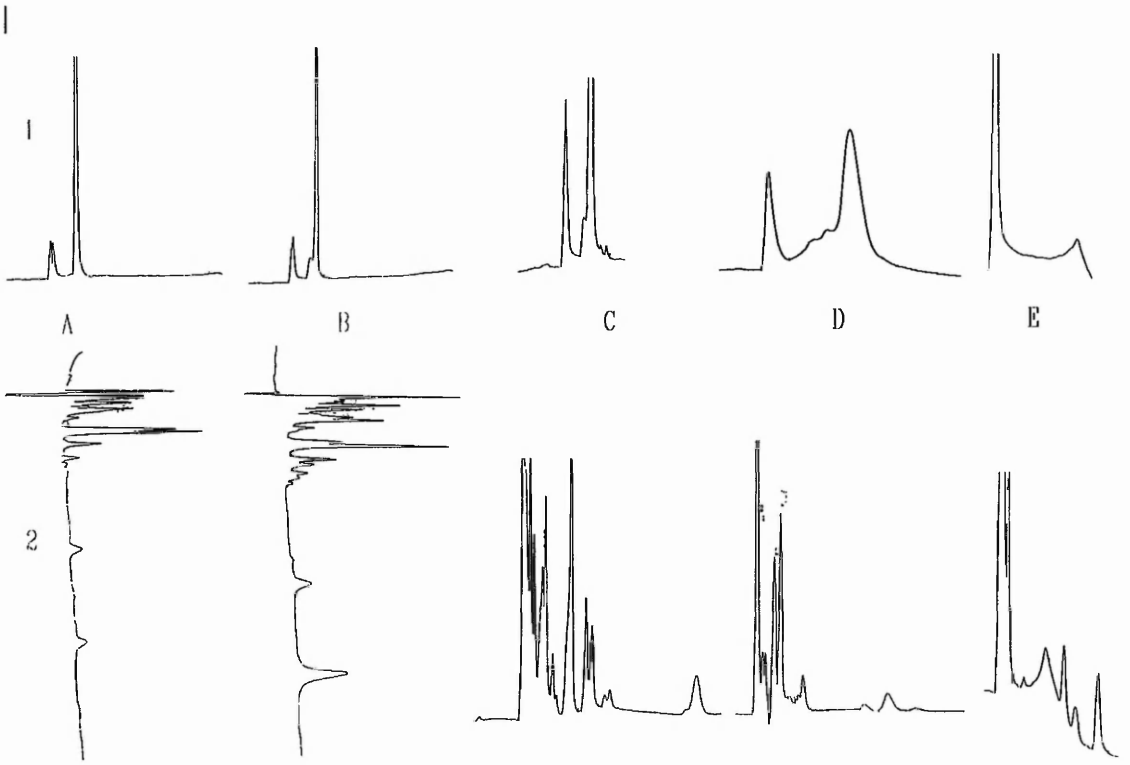


Figure 47: Ion Chromatography of Formulation with $NaHCO_3$

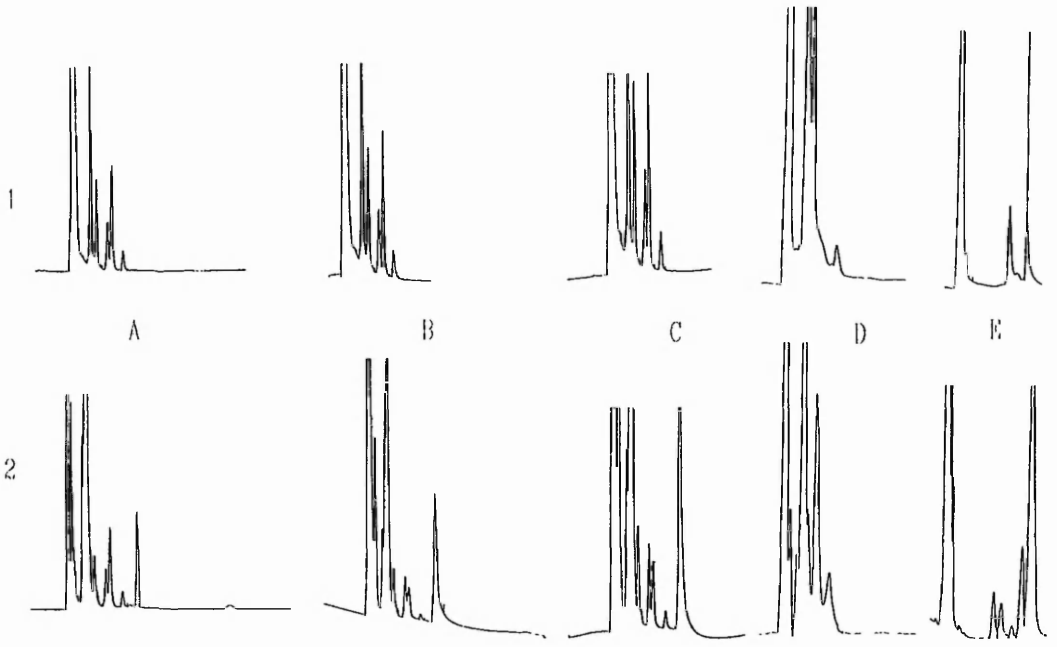


Figure 48: Ion Chromatography of Formulation with 1000 ppm Cu^{2+} Ions (Solid = Series 1, Solution = Series 2)

Key for Figures 47 and 48 is as follows:
A = HPICE 1, B = HPICE 2, C = HPICE 3, D = HPICE 4, E = HPIC

Lowering the pH of the mobile phase should reduce ionization of electrolytes and increase retention time. HPICE system 2 was an attempt at this by increasing the concentration of TDFHA from 1 to 1.5 mM (an increase to 5 mM resulted in an unacceptibly high background current). Retention times of probe compounds and sample formulations were virtually unchanged. Changing the mobile phase from TDFHA to an OSA based system should have retained and resolved early eluters better, HPICE 3. Chromatography was identical to that obtained using TDFHA based mobile phases and no improvement in resolution between earlier eluting impurities was achieved, Figures 47 and 48, Series 1 and 2A, B and C.

Mobile phase changes had no effect on the chromatography therefore the stationary phase was changed from ICE AS1 to ICE AS5, HPICE system 4. L-Ascorbic acid now eluted as a poorly shaped peak prone to fronting. Degraded samples were even less well resolved than when chromatographed on the AS1 column. Retention times were much reduced than with the AS1 column, approximately half, Figures 47 and 48, Series 1D and 2D.

As HPICE failed to retain and resolve degradation products in sample formulations use of HPIC was made to determine whether chromatography could be improved over HPICE. Although oxalic acid was now very well resolved form 2-KGA, L-ascorbic acid now eluted very close to DHAA and 2-KGA. The solid and solution formulations exhibited different chromatographic profiles demonstrating chemical degradation was occurring by different pathways, Figures 47 and 48, Series 1E and 2E.

Conclusion

HPICE and HPIC with suppressed conductivity detection resolved and detected many degradation products in stressed solution and solid formulations of L-ascorbic

acid. HPIC was found to retain oxalic acid strongly and resolve it from L-ascorbic acid and certain degradation products, although 2-KGA and DHAA virtually coeluted. Four HPICE chromatography systems were assessed but none was satisfactory due to poor retention of many degradation products. Both HPIC and HPICE suffered from poor chromatography due to the broadness of peaks and tailing. Ion chromatography was considered inferior to reverse-phase HPLC due to the broadness of peaks and tailing.

Conductivity detection could readily detect non-chromophoric compounds such as oxalic acid and 2-KGA. These compounds could only be detected at high concentration by UV detection.

When reverse-phase HPLC was coupled to a conductivity detector no improvement was obtained in chromatography as polar degradation products, such as oxalic acid, were poorly retained and resolved although they were easily detected. A chromatographic system is required that has the high efficency of reverse-phase HPLC, be able to retain and resolve polar components and be capable of detecting non-chromophoric compounds. With these objectives defined the use of derivatizing reagents was investigated.

3.3.3. Use of Derivatizing Agents For Reverse-Phase HPLC

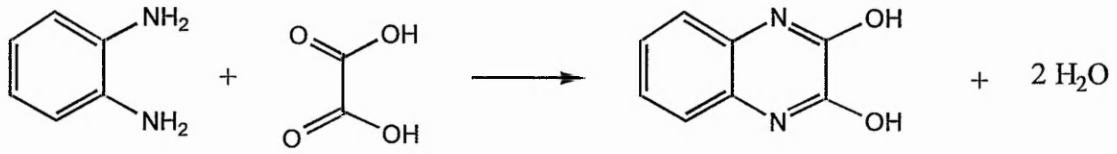
Attempts at retaining and resolving known polar degradation products of L-ascorbic acid such as oxalic acid, 2-KGA, 2, 3-DKGA and DHAA using reverse-phase HPLC and ion-chromatography (HPIC and HPICE) have failed. The use of suitable derivatizing agents was studied to both retain and resolve derivatized degradants by reverse-phase HPLC and to enhance their detection by a UV detector. Most degradants of L-ascorbic acid contain the carbonyl group either as an aldehyde, ketone or carboxylic acid. Reagents known to form stable derivatives with these classes of compunds are;

a) 1,2-diaminobenzene (1,2-DAB)

b) 2,4-dinitrophenylhydrazine (2,4-DNP)

a) 1,2-DAB

This reagent was suggested by Hughes [182] for the determination of urinary oxalate. The method involves the reaction of oxalic acid with 1,2-DAB in 1M hydrochloric acid at 130°C for 15 minutes. After cooling the pH of the solution is adjusted to pH 4.0 and the level of the 2,3-dihydroquinoxaline formed determined at 312 nm after chromatographing on a reverse-phase HPLC system. Although the method was developed specifically for the determination of oxalic acid other ∝ dicarbonyl compounds will undergo analogous reactions, Figure 49.



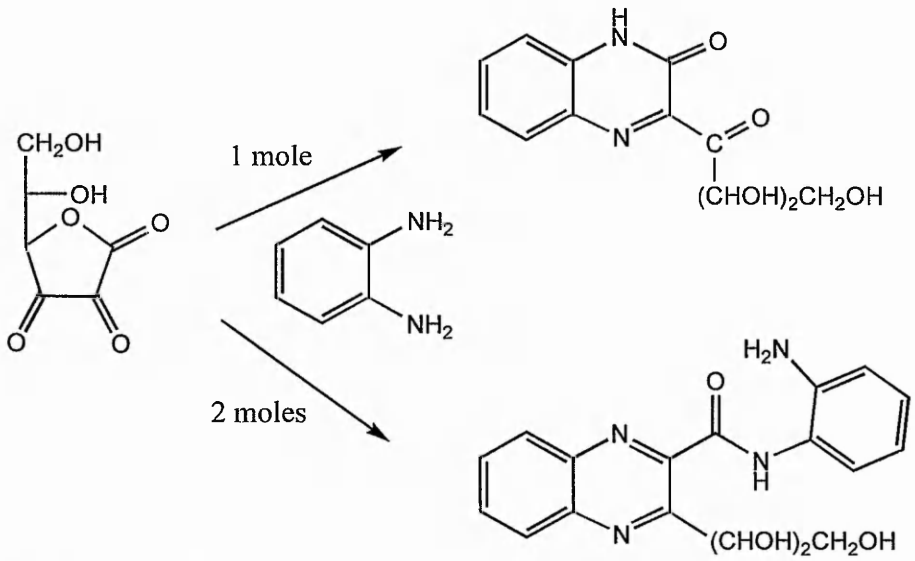
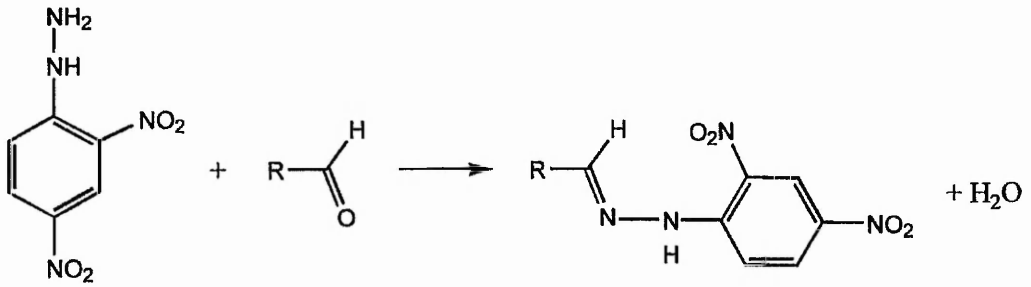


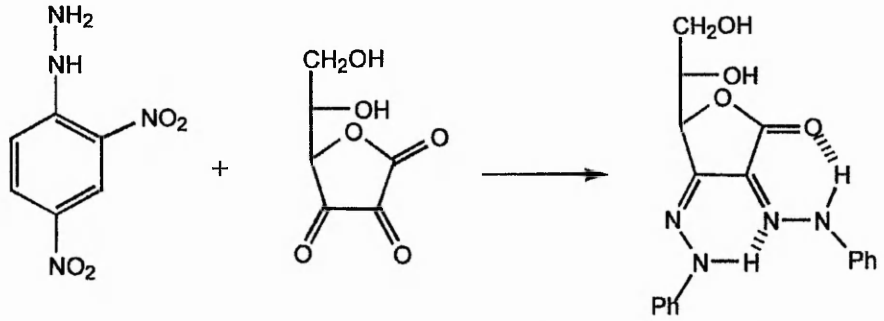
Figure 49: Reaction of the Carbonyl Group with 1,2-DAB

b) 2,4-DNP

This reagent is well known to organic chemists as a spot test reagent for derivatizing carbonyl compounds to form the 2,4-dinitrophenylhydrazone or an osazone, Figure 50. The melting point of these derivatives can then be determined and referenced for identification.



a 2,4-dinitrophenylhydrazone



osazone of DHAA stabilized by intramolecular hydrogen bonding

Figure 50: Reaction of the Carbonyl Group with 2,4-DNP

Experimental

a) 1,2-DAB

i) Reaction Time

The reaction time was studied using oxalic acid. A 3 ml aliquot of an aqueous

stock solution of oxalic acid equivalent to 1.5 mg anhydrous oxalic acid was pipetted into a 25 ml volumetric flask containing 3 ml concentrated hydrochloric acid, diluted to volume with purified water, and mixed well. A 5.0 ml aliquot was transfered into a series of Pierce Reactivials and 2.0 ml of a 6% aqueous solution of 1,2-DAB, dihydrochloride, added. The vials were stoppered and heated at 130°C for pre-determined times. After reacting each vial was cooled and the pH of the solution adjusted to 4.0 by dropwise addition of 50% aqueous sodium hydroxide solution. The contents of the vial were quantitatively transfered to a 25 ml volumetric flask and diluted with water. Aliquots from each flask were chromatographed in duplicate using the following conditions.

Column	:	Lichrospher 100-RP C18, 5µ, 25 x 0.46 cm
Mobile Phase 'A'	:	0.1% v/v TFA, aqueous
Mobile Phase 'B'	:	'A' in 80% v/v acetonitrile, aqueous
Flow Rate	:	1.5 ml/minute
Oven Temperature	:	40°C
Injection Volume	:	20 µl
Wavelength	:	312 nm
Gradient	:	Time 0 % B = 10
		5 % B = 10
		25 % B = 80
		30 STOP
Retention Time Reagent:		2.51 minutes
Retention Time Product:		5.23 minutes

The level of 2,3-dihydroxyquinoxaline was determined by assaying against an external standard solution of authentic material equivalent to a theoretical 100% conversion. The percentage conversion was plotted against time, Figure 51.

ii) Linearity

A calibration graph was constructed by reacting, at 130°C for 50 minutes, a series of anhydrous oxalic acid solutions with the reagent using the reaction conditions described above. Duplicate injections were chromatographed of each solution and mean areas plotted against concentration.

Degraded solid formulations of L-ascorbic acid with sodium hydrogen carbonate and Cu^{2+} ions were reacted along with known degradation products such as DHAA and 2-KGA. A sample of fresh undegraded solid of L-ascorbic acid was also reacted under the reaction conditions described in above. In each case 100 mg sample was reacted for 50 minutes at 130°C.

Results

The reaction reached completion after 50 minutes, Figure 51, considerably longer than the 15 minutes quoted by Hughes [182].

A calibration graph was constructed and found to be linear up to 0.08 mg/ml, the highest concentration tested, Figure 52.

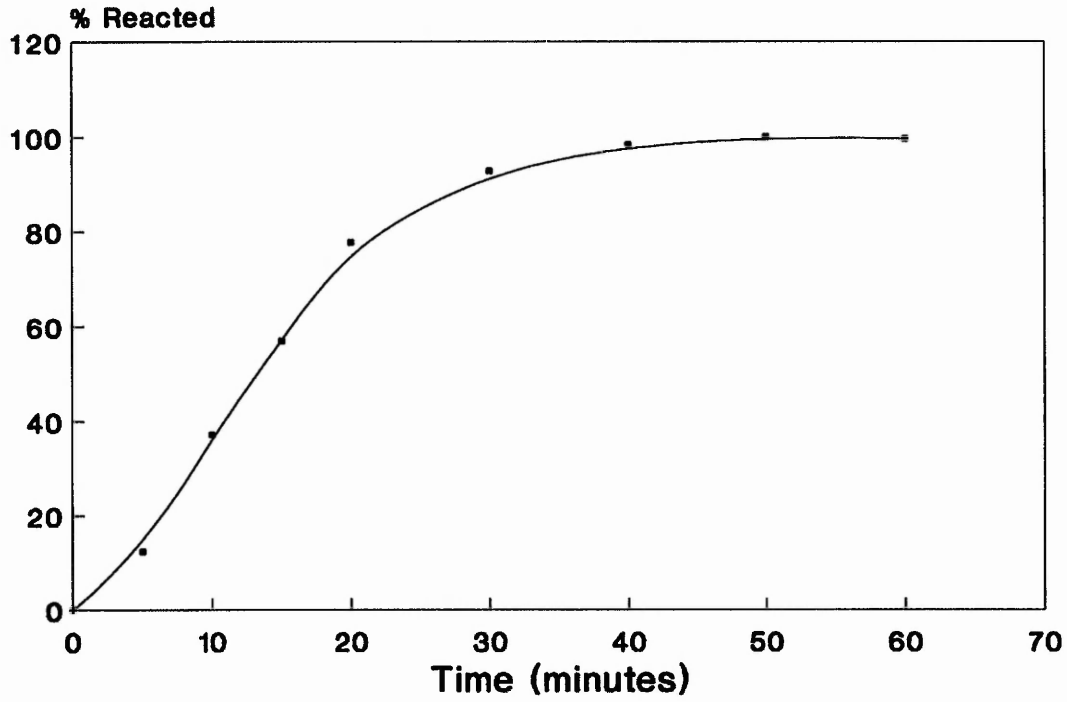


Figure 51: Reaction Time for Formation of 2,3- Dihydroxyquinoxaline

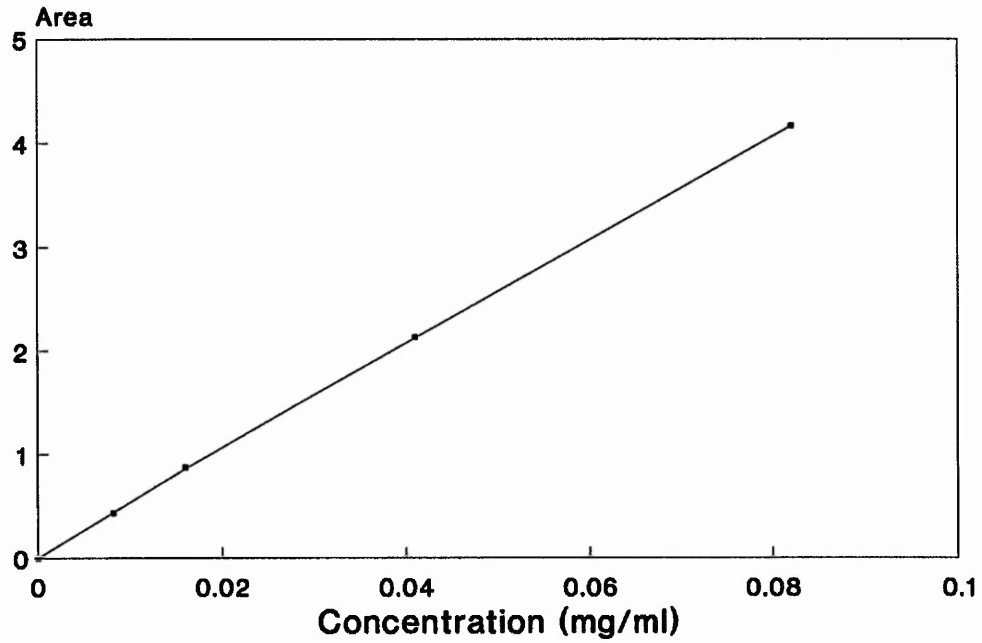


Figure 52: Linearity of Oxalic Acid when Reacted with 1,2-Diaminobenzene

The degraded formulations, known degradation products and undegraded L-ascorbic acid all formed a dark brown/black solution after reacting. This was in contrast to oxalic acid standard solutions which were a yellow colour, only slightly darker than a reagent blank. On cooling and adjusting to pH 4.0 much insoluble material was observed in the solutions that had turned dark brown/black. A mixture of acetonitrile and water was required to dissolve the precipitate. The formulation with degraded sodium hydrogen carbonate was found to contain 15.80% oxalic acid with none present in the formulation with Cu^{2+} ions. The pure undegraded L-ascorbic acid exhibited seven major peaks and many minor, Figure 53.

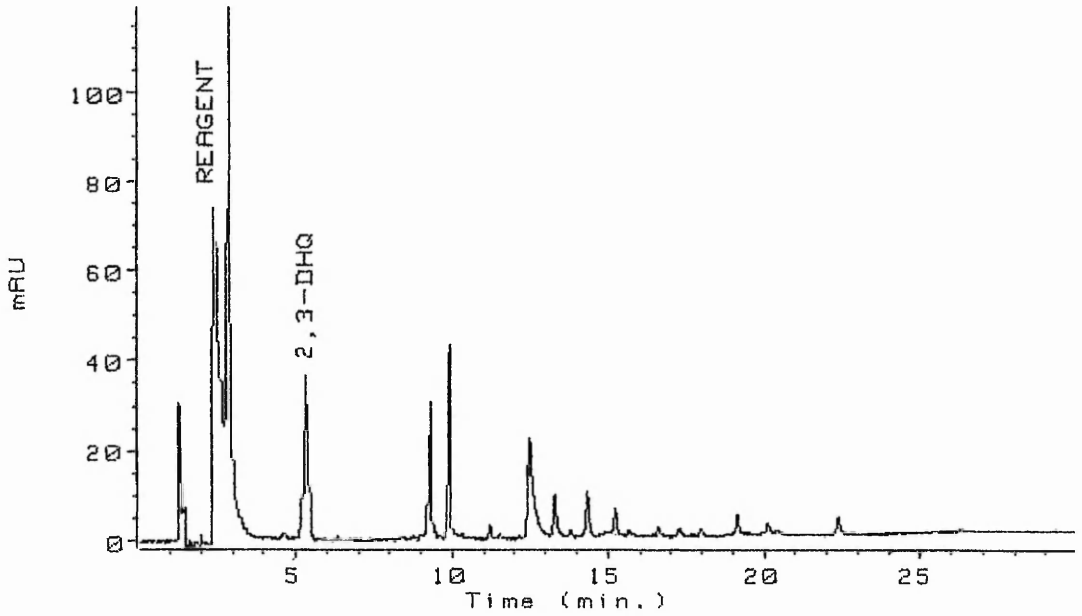


Figure 53: Undegraded L-Ascorbic Acid Reacted with 1,2-Diaminobenzene at 130°C for 50 Minutes

Using standard solutions of L-ascorbic acid and quantitatively assaying for remaining L-ascorbic acid by HPLC, and/or determining the peak profile of the reaction mixtures, studies involved detailed investigations under the following headings:

i) Reaction Medium

Using mixtures of hydrochloric and metaphosphoric acids, L-ascorbic acid was reacted with reagent to determine the minimum amount of hydrochloric acid required to maintain the reagent in solution and also the concentration of metaphosphoric acid to prevent L-ascorbic acid degradation at 130°C for 50 minutes.

ii) pH Adjustment

The pH adjustment step to increase the pH to 4.0 involves addition of 1M sodium hydroxide solution. The addition of such a strong base could be responsible for the degradation of L-ascorbic acid. This study determined the extent of L-ascorbic acid degradation at 130°C for 10, 20, 30, 40 and 50 minutes in the presence of no reagent and reagent followed by pH adjustment to pH 4.0, reagent and no pH adjustment i.e. after reaction the mixture was cooled and diluted to volume with 1% w/v metaphosphoric acid solution.

iii) Reaction Temperature and Time

This study investigated the effect of lowering the reaction temperature and reducing reaction time. Temperatures studied were 60, 80 and 100°C and reaction times of 10, 20, 30, 40, 50 and 60 minutes. Standard amounts of oxalic acid were also reacted and the percentage yield conversion to 2,3-DHQ quantified.

Results

i) Reaction Medium

The mixture with no hydrochloric acid present gave the cleanest reaction by HPLC, Table 16. No differences were observed between mixtures with 1 or 3 ml hydrochloric acid. These two mixtures turned a very dark brown colour.

ii) pH Adjustment

Table 16: Percentage of L-Ascorbic Acid Remaining

Reaction Time (minutes)	No Reagent, pH Adjustment	Reagent, No pH Adjustment	Reagent, pH Adjustment
10	97.73	97.42	93.70
20	92.51	96.98	94.92
30	89.14	86.97	84.99
40	81.80	81.00	83.31
50	84.41	78.56	76.64

Assay results, Table16, show the pH adjustment step gave rise to the greatest initial loss of L-ascorbic acid. After a 20 minutes reaction time results were similar for all conditions.

iii) Reaction Temperature and Time

Assay results, Table 17, show greatest loss of L-ascorbic acid at higher reaction temperatures and with increasing reaction time. There was very little reaction between oxalic acid and reagent at 60°C with similar reaction yields at 80°C and 100°C.

Table 17: Effect of Reaction Temperature and Time on Percentage L-Ascorbic Acid Remaining and Yield of 2,3-DHQ

Time (minutes)	60°C	80°C	100°C
10	100.89	101.10	99.56
20	98.42	99.02	95.14
30	98.02	96.31	93.73
40	96.86	98.20	92.55
50	100.27	95.26	89.56
60	98.29	94.09	86.88
2,3-DHQ (60 minutes)	5.70	21.16	22.99

Loss of L-ascorbic acid was obviously accompanied by degradation which was detected by the HPLC system. Differences in degradation were seen between solid formulations with sodium hydrogen carbonate and with Cu^{2+} ions stressed at 30°C/75% RH for 21 days, Figure 54.

The reagent showed a different degradation pattern was occuring with a formulation of L-ascorbic acid and sodium hydrogen carbonate (hydrolysis) than with Cu^{2+} ions (oxidation). Due to the instability of L-ascorbic acid during the reaction use of the reagent was not persued.

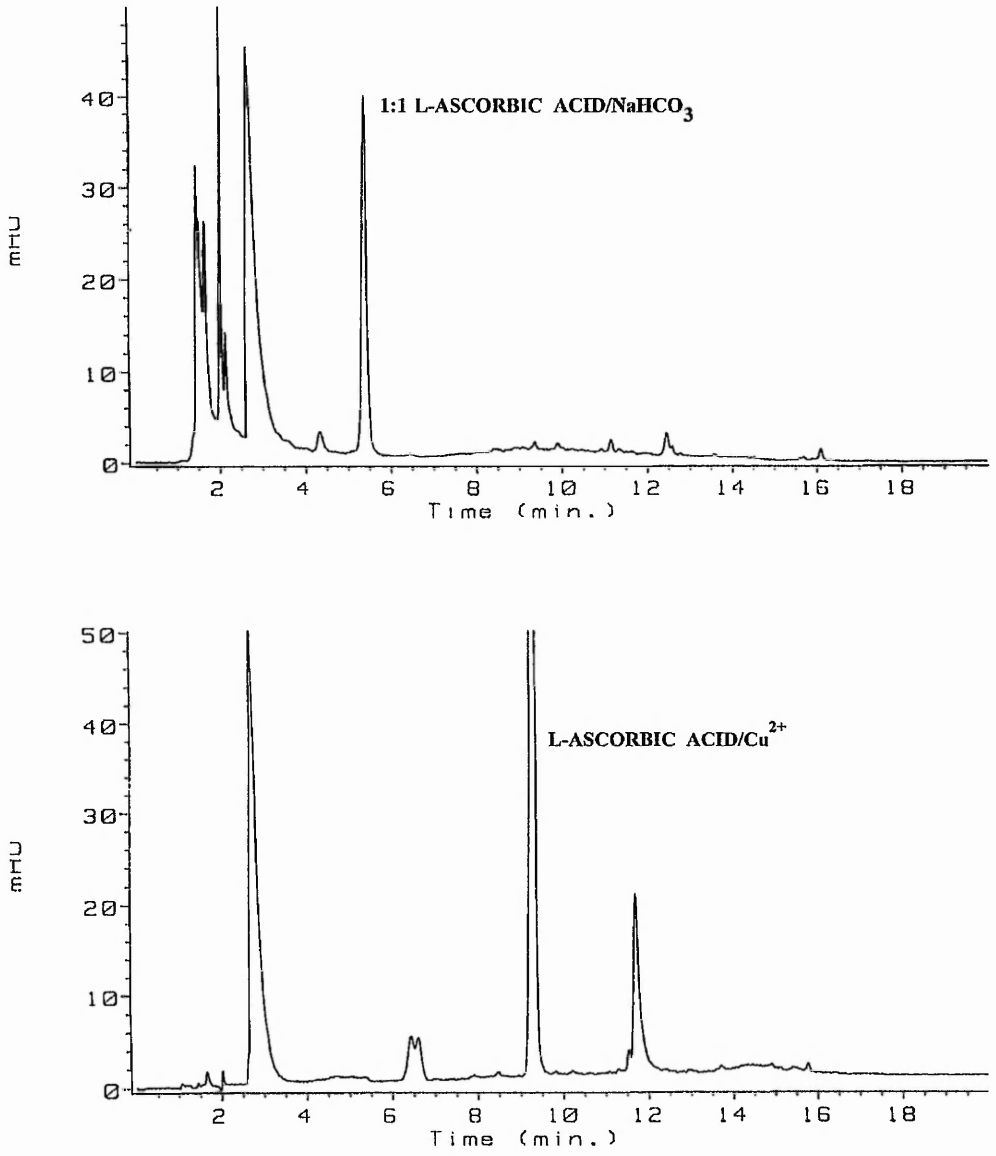


Figure 54: Different Formulations Derivatized with 1,2-Diaminobenzene

Conclusion of Use of 1,2-Diaminobenzene Reagent

A 50 minute reaction time at 130°C was required for complete reaction of oxalic acid, a likely degradant. These conditions degraded L-ascorbic acid to an unacceptable level.

Lower reaction temperatures and shorter reaction times still caused the degradation of L-ascorbic acid and these degradation products formed derivatives with the reagent. Only partial reaction of oxalic acid occurred under these conditions.

This reagent was unsuitable for the study of solid fromulations of L-ascorbic acid.

b) 2,4-DNP

Unknown polyketone compounds are suspected to be responsible for the brown discolouration found in degraded L-ascorbic acid. In addition aldehydes such as furfural and ketones such as 2,3-diketogulonic are known degradation products. The use of an acidic solution of 2,4-dinitrophenylhydrazine (2,4-DNP) as the derivatizing agent for carbonyl groups is well known [183]. The resulting 2,4-dinitrophenylhydrazones can be separated by reverse-phase HPLC using either methanol/water [184, 185] or acetonitrile/water gradients [186, 189]. Using this reagent successful separations of aliphatic C_1 - C_{12} aldehydes [184, 185, 187 - 193, 195], C_3 - C_{11} ketones [184, 185, 190 - 195] and ketoacids [186, 190, 196, 197] containing one or two oxo groups have been described.

Derivatizing Reagent

The reagent was prepared as a solution of 5 g 2,4-DNP in 60 ml orthophosphoric acid, 85%, and 40 ml ethanol [198].

Chromatography Conditions

Column	:	Lichrospher RP 100 RP 18e, 25 x 0.46 cm, 5µm
Mobile Phase 'A'	:	0.1% v/v trifluoroacetic acid, aqueous
Mobile Phase 'B'	:	80% v/v acetonitrile in 0.1% v/v TFA
Flow rate	:	1.5 ml/min
Column temperature	:	40°C
Detector wavelengths	:	245 nm and 350 nm

Injection volume	:	5 µl		
Gradient	:	Time	0 minutes	% 'B' = 30
	:		20 minutes	% 'B' = 80
Run time	:	20 minutes		
Re-equilibriation time:		5 minutes		

Derivatization Conditions

The reaction time and conditions initially investigated were those used for the determination of L-ascorbic acid by reaction with 2,4-DNP [198]. Oxidation of L-ascorbic acid to dehydroascorbic acid followed by reaction with the reagent for 3 hours at 37°C produces the osazone. The stability of L-ascorbic acid under these reaction conditions and at a higher temterature, 50°C, in order to shorten the reaction time, was studied by reacting 50 mg samples of pure L-ascorbic acid for 1, 2 and 3 hours. Samples were weighed into a series of 50 ml volumetric flasks and dissolved in 5 ml 1% w/v metaphosphoric acid. The reagent, 2 ml, was added and the solutions mixed. The flasks were then heated to the prescibed temperatures for 3 hours after which they were removed and sufficient acetonitrile added to dissolve any precipitate. The volume was then adjusted to 50 ml with the addition of 1% w/v metaphosphoric acid and mixed well. Reaction solutions were quantified for remaining L-ascorbic acid against an external standard using conditions outlined in Section 5.1and are expressed as a percentage of a fresh unheated solution, Table 18.

Table 18: Stability of L-Ascorbic Acid, %, with 2,4-DNP Reagent

Reaction Time (hours)	37°C	50°C
1	99.8	98.7
2	99.6	98.2
3	98.0	97.7

The L-ascorbic acid was found to be stable for up to 2 hours at 37°C. The stabilizing effect of metaphosphoric acid was then investigated to determine whether the reaction could be stabilized better at 50°C. Metaphosphoric acid is known to stabilize L-ascorbic acid in solution [199]. The stability of L-ascorbic acid in 2,4-DNP reagent solutions made with 5% w/v 2,4-DNP in metaphosphoric and orthophosphoric acid/solvent mixtures was investigated, Table 19. All reactions were conducted at 50°C for 3 hours.

Table 19: Stability of L-Ascorbic Acid After Reaction for 3 Hours at 50°C

Reagent	L-Ascorbic Acid Remaining (%)
6:4 orthophosphoric acid (85%)/ethanol	97.0
6:4 orthophosphoric acid (85%)/acetonitrile	95.9
6:4 metaphosphoric acid (50%)/ethanol	99.1
6:4 metaphosphoric acid (50%)/acetonitrile	98.1

Greatest stability was achieved using metaphosphoric acid mixtures and the reagent imparting greatest stability, the metaphosphoric acid (50%)/ethanol mixture, was adopted.

Chromatography

In preliminary experiments the system was optimized by using a standard mixture of potential carbonyl degradation products of L-ascorbic acid derivatized with 2,4-DNP. The elution order and their retention times are listed, Table 20.

Table 20: Elution Order and Retention Times of 2,4-DNP Derivatives of Some Degradation Products of L-Ascorbic Acid

Peak	Compound	Retention Time (minutes)
1	Threonic acid	2.7
2	Oxalic acid	2.9
3, 4	Tartaric acid	3.0, 4.4
5	Glyceric acid	3.3
6	2-ketogulonic acid	3.5
7	Glycolic acid	3.7
8	Formic acid	4.3
9	Acetic acid	4.7
10	2,4-DNP	6.3
11 - 14	Glyoxylic acid	6.8, 11.3, 13.3, 17.9
15 - 18	Glyoxal	7.2, 10.3, 14.0, 18.7
19	Glyceraldehyde	11.3
20	2(5H)-furanone	16.1
21	2-furfural	16.6
22	2,3-diketogulonic acid	17.9

A typical mixed standard chromatogram is shown in Figure 55.

Instead of only one peak some compounds gave 2 or 4 peaks due to the formation of isomers of the hydrazone which were resolved under the chromatographic conditions used.

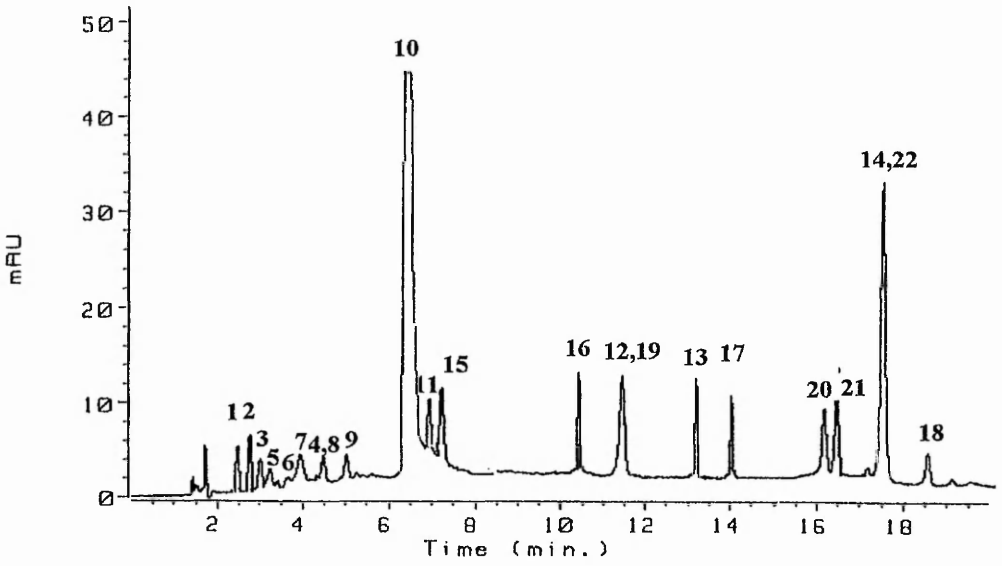


Figure 55: Carbonyl Standards Mixture Derivatized with 2,4 - DNP

Conclusion of Use of 2,4-Dinitrophenylhydrazine Reagent

This reagent was used to study the degradation of solid L-ascorbic acid formulations as L-ascorbic acid was found to be stable with the reagent and the reagent could differentiate between hydrolysis and oxidative degradation.

3.4 Capillary Gas-Liquid Chromatography

Capillary GC has advantages over HPLC due to the greater column efficiency available to resolve complex mixtures and when coupled to MS it is a powerful tool for the elucidation of structures and confirmation of identity. There is no need to isolate components from a mixture as they are resolved by the column before entering the MS directly. Structures can be elucidated from the molecular weight of the parent ion and the fragmentation pattern and comprehensive library searches made of MS fragmentation patterns.

Degradation products that may be present in the samples include carboxylic acids,

both volatile and non volatile, hydroxy acid lactones and furans. To eliminate multiple peaks of hydroxy lactones silylation of the ammonium salt is carried out [200].

Experimental

Sample preparation initially followed the procedure of Alén *et al.* The sample (3mg) was dissolved in water (0.5ml) and passed through a column (100 x 10mm I.D.) filled with weakly acidic cationic exchange resin (Amberlite IRC - 50 NH_4^+). The column was washed with water to obtain an effluent volume of 30 to 40 ml, collecting the eluent in a round bottom flask, this was evaporated to dryness using a rotary evaporator at 35°C. Pyridine (0.5ml) and trifluorobis (trimethylsilane) acetamide (BSTFA) (0.25ml) containing 5% chlorotrimethylsilane (TMCS) were added to the residue. The mixture was shaken for 30 minutes at ambient temperatures.

Due to the lengthy procedure and weak detector response the sample preparation as described by Kim *et al* [201] was followed.

This sample preparation involved dissolving the sample (3mg) in pyridine (0.5ml). Triethylamine (0.25ml) was added to form the ammonium salt followed by the addition of BSTFA containing 5% TMCS (0.25ml). The mixture was heated at 105°C for 5 minutes before chromatographing an aliquot using the following chromatography conditions,

Instrument	:	Hewlett Packard 5890
Column	:	DB-1 (15m x 0.32mm, film thickness 3μ)
Detector Temperature	:	280°C
Injector Temperature	:	280°C
Split Ratio	:	4:1
Injection Volume	:	2 μl
Carrier Gas	:	Helium

Carrier Gas Flow	:	1.8 ml/minutes
Gradient	:	80°C for 2 minutes then ramp to 280°C for 8 minutes

Using authentic samples of potential degradation products of L-ascorbic acid the following retention times were obtained, Table 21.

Table 21: Retention Times of BSTFA Derivatized Potential Degradation Products

Peak Number	Component	t_R (mins)
1	Furfuryl Alcohol	3.0
2	Formic Acid	5.0
3	Lactic Acid	9.0
4	Glycolic Acid	10.2
5	Oxalic Acid	11.5
6	Glyoxylic Acid	15.7
7	Glyceric Acid	18.0
8	Threonic Acid	24.5
9	Tartaric Acid	26.4
10	2, 3-DKGA	27.5
11	Glyceraldehyde	29.2
12	L-Ascorbic Acid	33.5

Many peaks were detected when degraded solid formulations of L-ascorbic acid (1:1 molar ratio with sodium hydrogen carbonate, Figure 56, top chromatogram, and with 1000 ppm Cu^{2+}, Figure 56, bottom chromatogram) stressed for 3 weeks at 37°C/75% RH were treated similarly. Some of these peaks were confirmed by GC-MS, Figure 56.

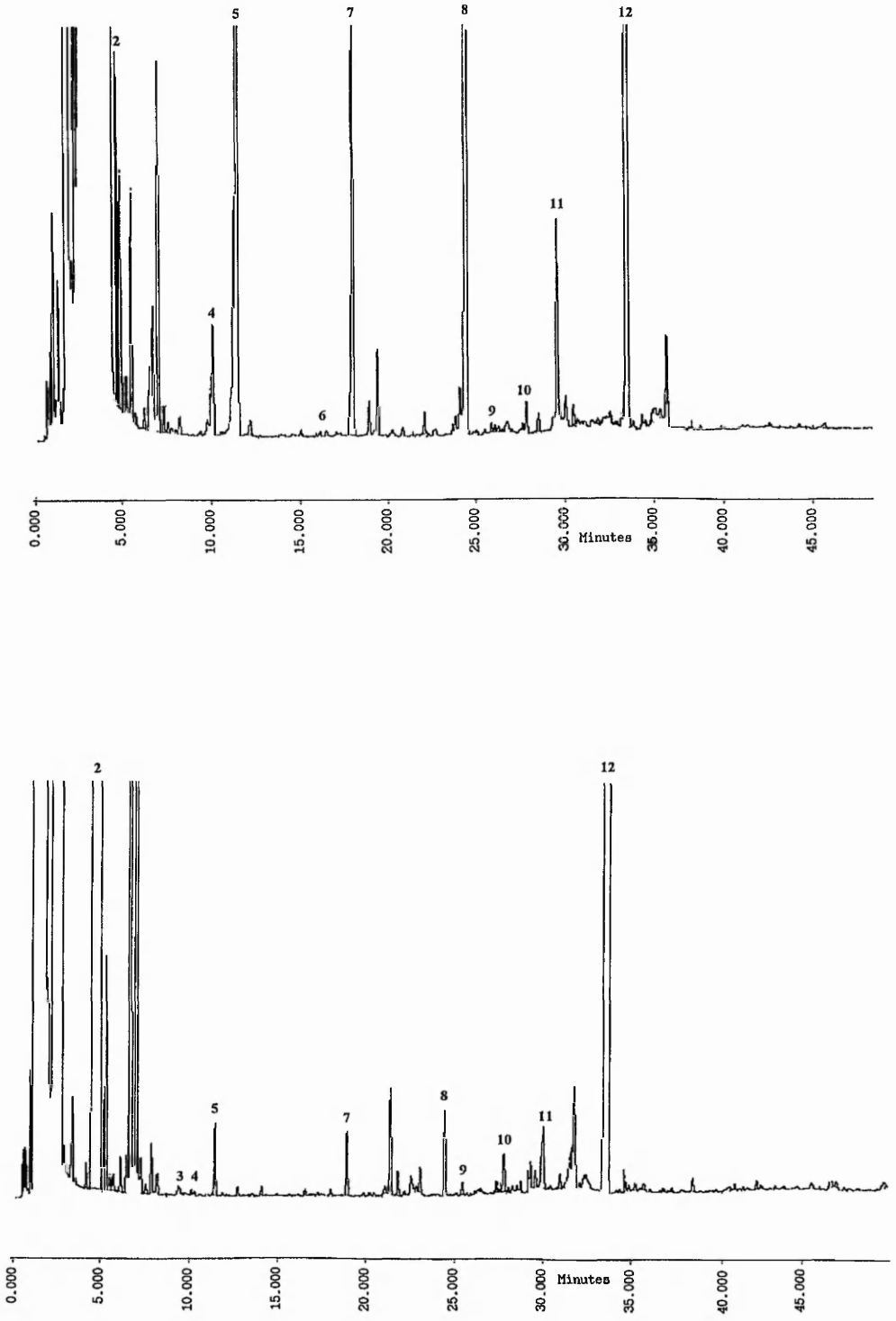


Figure 56: Comparison of Peak Profile of Two Degraded L-Ascorbic Acid Formulations

Two other fused silica open tubular capillary columns were assessed:

i) OV-101, 25 m x 0.2 mm, film thickness 0.33 μ

ii) HP Ultra 1, 12 m x 0.2 mm, film thickness 0.33 μ

Shorter retention times were obtained using the same gradient as used for the DB-1 column, probably due to the thinner film thickness, but the chromatography was inferior to that produced by the thick film DB-1 column. A lower initial temperature, 40°C, enabled furan type compounds to be retained better and with improved resolution, Table 22.

Table 22: Retention of More Volatile Components on a Slower Gradient

Component	t_R (minutes)
Formic Acid	4.3
Furil	6.7
2-Furfural	7.2
Glyoxal	7.7
2-Furoic Acid	8.6
Furfuryl Alcohol	9.1
2-Acetylfuran	9.5

A sample of L-ascorbic acid containing 5% w/w moisture stored at 50°C for 42 days and grossly degraded, indicated by its severe discolouration, an extremely dark brown colour was tested. When this sample was derivatized with BSTFA and chromatographed very little degradation was observed in comparison with the other two degraded formulations, Figure 57.

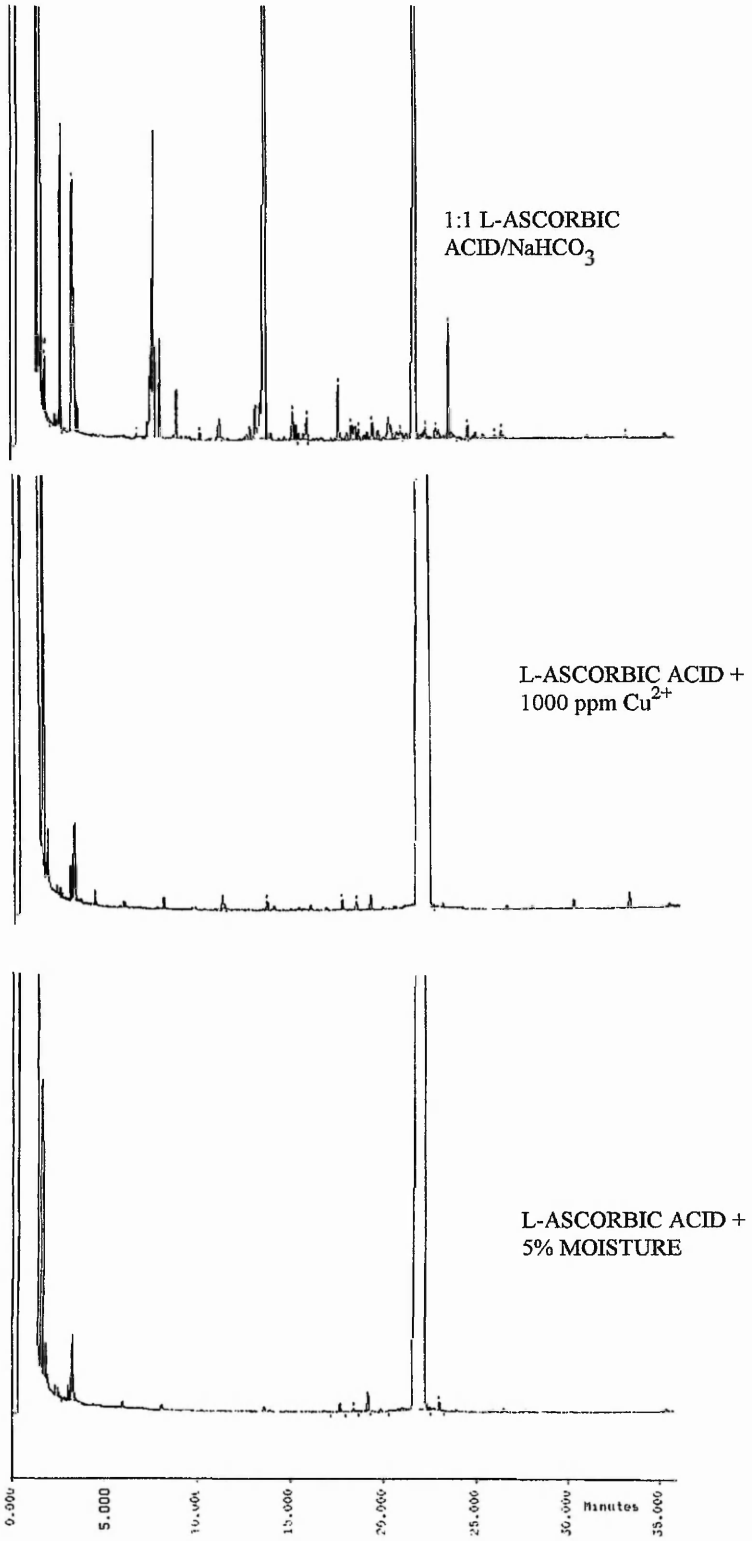


Figure 57: Comparison of GC Chromatograms for Degraded Formulations of L-Ascorbic Acid

Attempts at using higher injection port temperatures (up to 350°C) failed to produce any additional peaks.

Conclusion

GC - MS analysis of BSTFA derivatized formulations of L-ascorbic acid degraded with sodium hydrogen carbonate or Cu^{2+} confirmed the presence of expected degradation products. A visibly grossly degraded sample of L-ascorbic acid exposed to 5% w/v moisture showed no additional peaks when compared to a control sample of undegraded L-ascorbic acid. The degradation products responsible for the discolouration of L-ascorbic acid are either too polar and involatile to be chromatographed by GC or were not silylated to volatile compounds.

3.5 Thin Layer Chromatography

The suitability of thin layer chromatography (TLC) for the analysis of solid-phase degraded L-ascorbic acid was investigated with the intention of scaling up to preparative scale to isolate any bands due to degradation products. Compounds in these isolated bands were extracted from the substrate and assessed for purity by HPLC.

Experimental

In order to assess an established chromatographic system against new systems heat degraded solutions of L-ascorbic acid (1 and 2mg/ml) were loaded onto HPTLC silica gel plates and developed using different mobile phases. After development the plates were dried in a current of cool air and visuallized first by short wave UV light then with a spray reagent (880 ammonia/0.1m silver nitrate/25% sodium hydroxide, in the ratio 1:3:1).

Polar and non-polar mobile phases were prepared and assessed against one quoted

in the literature [202].

Twelve further mobile phases were assessed to resolve the brown discolouration from degraded solid formulations of L-ascorbic acid (1 : 1 molar ratio with sodium hydrogen carbonate and with 1000 ppm Cu^{2+}, both stressed at 37°C/75% RH for 3 weeks) and authentic samples of possible degradants. The most successful mobile phase was used to resolve the bands associated with the discolouration using preparative TLC plates. Preparative TLC was performed using 120 x 20 cm glass backed preparative silica gel plates each loaded with one of two degraded solid formulations. Each sample was dissolved in water (10 ml) and loaded onto a plate by repeatedly streaking the solution using a syringe and allowing the plate to dry in a current of cool air between each loading until all the solution was on the plate. The plates were developed in the solvent system to a height of 15cm. After development the plates were dried in a current of cool air and any coloured bands were scraped off the plate and any compounds extracted from the silica gel with water (10 ml) using sonication. The silica gel was separated from the aqueous extract by centrifugation. The aqueous extract was then assessed for purity using reverse-phase HPLC using the chromatograph conditions described in Section 3.3.1.

The isolated coloured extracts were used to investigate further TLC systems to determine whether the coloured extracts consisted of more than one component. A total of 12 different solvent system combinations were investigated. The possibility of degradation on the plate was investigated by use of two dimensional TLC chromatography. The solvent system found to resolve the greatest number of components was used to chromatograph the two severely degraded solid formulations alongside the system previously found to give the best separation of degradation products.

Comparison of the two systems enabled a choice to be made between the systems. The one producing the best chromatography, as defined by resolving the greatest number of components with minimum of streaking, was used for the preparative TLC of the two severely degraded solid formulations. Individual bands were

scraped off the plates and extracted as previously described. The aqueous extracts were evaporated to dryness using a rotary evaporator at 40°C. The material isolated was weighed then chromatographed by HPLC and TLC using systems previously developed.

Results

The solvent systems, used to resolve heat degraded solutions of L-ascorbic acid, and their performance are given, Table 23.

Table 23: TLC Solvent Investigated to Resolve Heat Degraded L-Ascorbic Acid Solutions

System	Mobile Phase	Solvent Ratio	Result
a	butan-1-ol/ethanol/acetic acid/water	4:1:1:4	Control system
b	Butan-1-ol/acetiic acid/water	4:1:2	4 spots resolved
c	Butan-1-ol/acetic acid/water	6:2:2	4 poorly resolved spots
d	Chloroform/diethyl ether/formic acid	4:1:2	Remained on baseline
e	Propan-1-ol/ethyl acetate/acetic acid/water	5:5:1:1	5 well resolved spots

Systems b, c and e were found to out perform the control system and these were used along with others, Table 24, to chromatograph authentic potential degradants of solid-phase L-ascorbic acid degradation and the two severely degraded solid formulations.

Table 24: TLC Solvent Systems Investigated to Resolve Solid Phase Degraded L-Ascorbic Acid

System	Mobile Phase	Solvent Ratio	Result
b	Butan-1-ol/acetic acid/water	4:1:2	As Table... (above)
c	Butan-1-ol/acetic acid/water	6:2:2	As Table...(above)
e	Propan-1-ol/ethyl acetate/acetic acid/water	5:5:1:1	5 spots from degraded solids
f	Propan-1-ol/ethyl acetate/acetic acid/water	4:4:1:1	5 spots, not as well resolved as e
g	Formic acid	100%	Brown spot, Rf 0.38
h	Dimethyl/sulphoxide	100%	2 spots Rf 0.00 and 1.00
i	Formic acid/dimethyl sulphoxide	1:1	Diffuse spots
j	Formic acid/water	1:1	Diffuse tailing spots
k	1,4-dioxan	100%	1 spot Rf 0.00
l	Tetrahydrofuran	100%	1 spot Rf 0.00
m	1,4-dioxan/water/formic acid	2:1:1	1 spot Rf 0.64
n	1,4-dioxan/formic acid	2:1	All spots Rf 0.51

Of the solvent systems investigated five resolved the brown dissolved material from L-ascorbic acid and from the base line, these systems were g, h, i, j and m. Systems h and m caused severe diffusion of the spots rendering chromatography unusable. The solvent system e was used to preparative TLC the degraded solid formulations.

Approximately 10 mg of a dark brown gum was obtained after preparative TLC from the degraded formulation with copper and 20 mg of a light brown solid from

the formulation with sodium hydrogen carbonate. HPLC analysis of the isolated materials found the copper formulation to consist of 7 polar components and the other degraded formulation contained 9 polar components. Not all components were common to both formulations.

These two isolated materials were used to develop TLC systems capable of resolving them into separate components, Table 25.

Table 25: TLC Solvent Systems Investigated For The Preparative TLC of Isolated Discoloured Material

System	Mobile Phase	Solvent Ratio	Result
o	Butan-1-ol/acetic acid/water	1:1:1	Streak from baseline
p	Methanol/formic acid/water	1:1:1	Diffuse spots
q	Butan-1-ol/methanol/acetic acid/water	1:1:1:1	Streak from baseline
r	methanol/acetic acid/water	1:1:1	Light spots, partial resolution
s	1,4-dioxan/formic acid/water	2:1:1	Streaking
t	1,4-dioxan/formic acid/water	1:1:1	Streaking
u	1,4-dioxan/formic acid/water	5:2:1	streaking
v	ethyl acetate/propan-1-ol/acetic acid/water	1:1:1:1	3 spots, R_f 0.00, 0.48, 0.72
w	propan-1-ol/acetic acid/water	3:1:1	streak from baseline
x	methanol/water	2:1	2 spots, R_f 0.00, 0.73
y	acetonitrile/water	1:1	3 spots, R_f 0.00, 0.54, 0.69
z	acetonitrile/water	1:9	2 bands, R_f 0.75, 0.89

Solvent system "y" was found to give the best chromatography with three distinct brown spots.

In order to demonstrate that system "y" was not causing degradation on the plate

two dimensional TLC was performed in the solvent system using the severely degraded formulation with sodium hydrogen carbonate. The first run produced five spots, R_f of 0.00, 0.60, 0.66, 0.70 and 0.90. The plate was dried, turned through 90° and re-developed in the same solvent system. Again only five spots were detected indicating no degradation had occurred. Using this solvent system and preparative silica gel plates the two degraded solid formulations were chromatographed (200 mg loading). Three different coloured bands developed and a colourless one due to L-ascorbic acid, Figure 58.

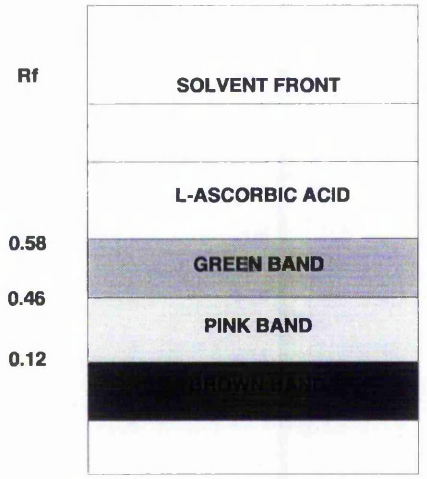


Figure 58: Preparative TLC of Solid Formulation

After extracting the bands off the silica support HPLC analysis of the solutions showed the bands to be impure with up to fourteen components, most of which were virtually unretained and poorly resolved. UV spectra of the peaks showed most to have a λ_{max} at 200 nm with a shoulder around 250 nm. TLC analysis (in system "y") showed up differences between the bands obtained for each formulation. The same coloured bands from the different formulations were different, Figure 59.

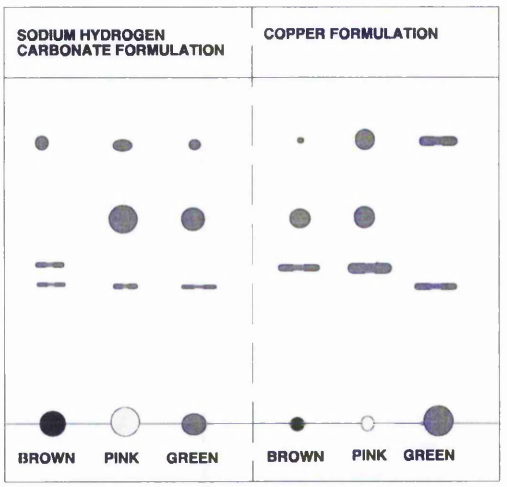


Figure 59: TLC Analysis of Isolated Bands

Conclusion

Two normal phase TLC systems were developed for the analysis of solid phase degraded formulations of L-ascorbic acid. TLC was found to be a means of detecting the brown material in degraded solid samples. Quantification of the brown material was impossible without reference material to quantify against. The brown material was found to consist of more than one component and preparative TLC resolved it into three different coloured bands. HPLC analysis of the coloured bands found them to consist mainly of polar species.

3.6 Summary of Conclusions

A number of different chromatographic techniques were investigated for the chromatographic assay and degradation screen of L-ascorbic acid solid formulations.

Assay methods investigated were reverse-phase ion-pair partition chromatography and suppressed ionization reverse-phase chromatography. Of these techniques the

second one was the preferred choice and was fully validated. This chromatographic technique formed the basis of an automated sample preparation system for the determination of DHAA.

Chromatographic methods for the determination of degradation products were studied. Direct techniques investigated included reverse-phase HPLC with UV and conducting detection, HPIC and HPICE with conductivity detection. These methods were relatively unsuccessful due to the highly polar nature of the degradants.

Two derivatizing agents were investigated to attempt to retain and detect better any degradants. Samples were proved to be stable in the presence of 2,4-DNP and this reagent formed different derivatives with samples having undergone alkaline hydrolysis and oxidation in the solid phase.

Capillary GC was studied and found to show different degradation patterns in samples degraded by different routes in the solid phase. It could not detect any degradation in severely discoloured samples degraded with moisture alone.

Normal phase TLC was investigated and a successful system developed. This system was scaled up to preparative scale and a brown degradation product resolved into three different coloured bands. These bands were found to be impure by HPLC and TLC and consist mainly of very polar compounds.

The use of both the suppressed ionization reverse-phase HPLC systems, isocratic assay and gradient elution system, and the derivatizing reagent 2,4-DNP produced excellent analytical systems for the samples to be studied in this work.

CHAPTER 4

Investigation Into The Solid Phase Degradation of L-Ascorbic Acid

4.1 Introduction

An investigation into the solid phase degradation of L-ascorbic acid required the development of controlled conditions to which the acid was susceptible to degradation. This thesis is concerned with the degradation of L-ascorbic acid formulated with typical tablet excipients and minerals found in commercially available vitamin/mineral supliments. When formulated in a complex matrix such as a tablet or capsule, the various excipients (lubricants, fillers and binders) may act as reactants or catalysts and complicate further any interpretation of results. The purpose of this study was to develop controlled conditions to which L-ascorbic acid would degrade in the solid phase and use the degraded samples to develop chromatographic systems for the detection and resolution of any degradation products. A literature search has highlighted the susceptibility to oxidation of L-ascorbic acid in solution catalyzed by metal ions such as Cu^{2+} and Fe^{3+} [203] it is also susceptible to alkaline degradation [104] and hydrolysis [101].

Metal ions such as Cu^{2+} and Fe^{3+} and basic tablet excipients such as sodium hydrogen carbonate are frequently used in vitamin-mineral suppliment tablets. If formulated as a solution then rapid degradation of L-ascorbic acid occurs. When formulated as a solid formulation degradation is observed as a brown discolouration but which excipient or mineral is responsible for the degradation and what the degradation products are is still unknown. There is obviously an incompatability between L-ascorbic acid and one or more of the excipients. To simplify this study it was necessary to make 1:1 w/w ratio mixtures of L-ascorbic acid with excipients typically found in tablet formulations and to study the mixtures in accelerated stability tests. Minerals were mixed at a concentration of 1000 ppm metal ion with respect to the vitamin. The effect of moisture on the stability of L-ascorbic acid was also studied at levels between 0 and 10% w/w. As

a control aqueous solutions of L-ascorbic acid were made with selected excipients and metal ions.

Differential scanning calorimetry (DSC) was used to predict the stability of the solid mixtures before stressing them at elevated temperatures and humidities. According to the literature DSC is an initial screen to study drug-excipient interactions [95, 204 - 206]. The evaluation of the thermograms is often difficult and should be backed up with a quantitative assay for the drug. Advantages of DSC is that it is quick and requires only a small amount of sample.

4.2 Principle of DSC

Two ovens are linearly heated with one oven containing the sample (typically < 10 mg) in a closed pan and the other containing an inert reference pan. Two methods of measurement can be used, namely "heat flux DSC" and "DSC". With heat flux DSC the sample and reference pans are heated linearly from the same starting temperature. When the sample undergoes a change, such as melting or phase transition, then energy (heat) is absorbed by the sample. This results in an imbalance between the sample and reference pans. This temperature difference is measured and plotted as heat flow against time.

In DSC the sample and reference pans are heated by independent heaters and the individual heating rates are monitored. The pans are heated in such a way that both are kept at the same temperature throughout the heating gradient. When the sample undergoes a change, either endothermic or exothermic, the heating rate is altered to match that of the reference pan. The change in energy input or output is plotted against time.

DSC can be used to determine the purity of a substance based on the shape of the melting point curve. The sharper the curve, the purer the compound as impurities present in a compound will depress the melting point and broaden the curve. The amount of impurities can be calculated from the melting point depression, ΔT, and

by use of the van't Hoff equation, Equation 15.

$$x = \frac{-\Delta T \times \Delta H_f}{RT_o^2} \qquad \text{Equation 15}$$

where x = mole fraction of impurities

ΔT = depression of melting point

ΔH_f = molar enthalpy of fusion of pure compound

R = gas constant, 8.314 J/mol/K

T_o = melting point of pure compound

Polymorphism, that is different crystal forms of the same compound, can be studied using DSC. The different crystalline forms have different thermodynamic properties e.g. melting and sublimation temperatures, kinetics, stability, solubility and dissolution rates. Upon heating, a polymorph can undergo a transition to another polymorph before melting. This transition can be reversible (enantiotropy) or irreversible (monotropy). The energies of these transitions are generally small but are easily detected by DSC.

A polymorph can melt and recrystallize as a different polymorph with a higher melting point. This phenomenum can lead to confusion in interpreting the results. The most easily distinguishable polymorphs are those that melt with no transition from one form to another. The DSC traces from these polymorphic mixtures show distinct peaks for each form.

The DSC instrument used in this study was the Perkin Elmer DSC7, Figure 60.

Figure 60: Perkin Elmer DSC7 Instrument

The instrument is computer controlled and capable of temperature programming linearly over one to fifteen temperature segments. Energy is absorbed or evolved by the sample and this is compensated for by adding or subtracting an equivalent amount of electrical energy to a heater located in the sample holder. Platinum resistance heaters and thermometers are used to accomplish the temperature and energy measurements. The change in power required to maintain the sample holder at the same temperature as the reference pan during a transition is recorded as a positive or negative peak. The peak area indicates the total energy transfer to or from the sample.

4.3 Experimental

The compounds listed in Table 26, found in multivitamin-mineral tablets that discolour badly on storage were studied. The pH of water soluble excipients/mineral salts was measured at a concentration of 5% w/v at a temperature of 25°C. Insoluble excipients, e.g. TiO_2, were measured as a slurry in water as this reflects the true pH of the substance [207].

Table 26: Tablet Excipients and Minerals Studied

Compound	pH	Class of Compound
Stearic Acid	6.40	Lubricant
Sucrose	5.75	Coating Acid, Sweetener
Saccharin Sodium	6.45	Sweetener
Kollidon	4.48	Diluent
Mannitol	5.80	Diluent
Thiamine Mononitrate	7.33	Vitamin
$CaHPO_4 .2H_2O$	7.30	Mineral
$MgCl_2 .6H_2O$	5.50	Mineral
NaCl	6.90	Mineral
TiO_2	6.70	Diluent
$CuCl_2 .2H_2O$	3.67	Mineral
$NaHCO_3$	8.12	Acidity Modifier

A batch of L-ascorbic acid was formulated as 1:1 w/w binary mixtures with the following excipients:

stearic acid
sucrose
saccharin sodium
sodium hydrogen carbonate
kollidon
thiamine mononitrate

A 5 g portion of L-ascorbic acid was weighed into an 8 oz amber jar and 5 g of the excipient added. The jar was capped and blended for 2 minutes using a Turbula T2C blender.

The same batch of L-ascorbic acid was formulated with the minerals at a cation

concentration of 1000 ppm. A mass of the salt was triturated with a calculated mass of L-ascorbic acid in a mortar in order to give a concentration of 1000 ppm of the metal ion. The mixture was transferred to an 8 oz amber glass jar, capped and blended for 2 minutes using a Turbula T2C blender. The binary mixtures were then assayed for L-ascorbic acid by quantitative HPLC and thermograms recorded using a Perkin Elmer DSC7 calorimeter. The individual organic excipients were monitored by heat-flux DSC. As soon as the mixtures were blended the analyses, discussed below, were performed.

Analytical Methods

1. Heat-Flux DSC

Approximately 2 - 3 mg of the mixture or individual excipient were weighed into an aluminium DSC pan and the sample pan closed. The sample was then subjected to heat-flux DSC (Perkin Elmer DSC7) heating from an initial temperatue of 300 K to a final temperature of 500 K (700 K for kollidon) at a rate of 20 K/min. The samples were heated in a constant stream of nitrogen gas. Data was stored on disk and onset and maximum temperatures for each peak on the thermogram recorded.

2. Quantitative Assay by HPLC for L-Ascorbic Acid

Chromatography conditions were those outlined in Section 3.2.2.

a) Sample Preparation for HPLC Assay

i) 1:1 w/w Binary Mixtures

Approximately 200 mg of the mixture was accurately weighed into a 100 ml volumetric flask, dissolved in and made to volume with 1% w/v aqueous HPO_3. A 20 ml aliquot was pipetted into a 100 ml volumetric flask and diluted to volume

with 1% w/v aqueous HPO_3 to produce a working sample solution. A 20 µl aliquot of the working sample solution was chromatographed in duplicate and quantified against an external standard of L-ascorbic acid using chromatography conditions outlined in Section 3.2.2.

ii) L-Ascorbic Acid and 1000 ppm Cations

These samples were treated in the same way as the 1:1 w/w binary mixtures as in *i)* but taking a 100 mg sample weight.

b) Standard Preparation

Approximately 100 mg L-ascorbic acid, analytical grade, ex Fisons Scientific Equipment, of known purity and moisture content was weighed into a 100 ml volumetric flask. It was dissolved in and diluted to volume with 1% w/w aqueous HPO_3. A 20 ml aliquot was pipetted into a 100 ml volumetric flask and diluted to volume with 1% w/v aqueous HPO_3 to produce a working standard solution. Duplicate 20 µl aliquots of the solution were chromatographed after every sixth sample vial for quantification.
The repeatability of injection was confirmed by injecting six replicate standard solutions.

A concomitant standard solution was prepared in the same way as the working standard solution and chromatographed as a sample. Limits for the concordance of standards was set at 99 - 101% of the working standard solution based on the calculation, Equation 16.

$$\frac{A_{std1}}{A_{std2}} \times \frac{Wt_{std2}}{Wt_{std1}} \times 100 = \%\ concordance \qquad \text{Equation 16}$$

where $A_{std\ 1}$ = mean area standard 1 peak

$A_{std\ 2}$ = mean area standard 2 peak

$Wt_{std\ 1}$ = weight standard 1

$Wt_{std\ 2}$ = weight standard 2

The ability of the chromatographic system to resolve any possible degradation products was confirmed by chromatographing a 1 mg/ml aqueous solution of L-ascorbic acid previously heated to 95°C for 3 hours. Resolution, Rs, was calculated between the main peak and the impurity peak eluting immediately after. The analysis was continues if Rs was > 1.5.

3. HPLC Degradation Screen

Chromatography conditions used were those described in Section 3.3.1. The undiluted (1 mg/ml) solutions prepared for the assay determination were used as sample solutions. A 25 ml aliquot of each solution was chromatographed alongside a 1% v/v dilution of each sample solution in order to quantify any degradation peaks in the strong solution, Equation 17.

$$\%\ impurity = \frac{area\ impurity\ peak}{area\ 1\%\ impurity} \qquad \text{Equation 17}$$

A heat degraded (95°C for 7 days) aqueous solution of L-ascorbic acid (1 mg/ml) was also chromatographed along with known degradation products i.e. oxalic acid, 2-KGA and DHAA (0.1 mg/ml solutions) in order to identify any impurities detected.

After the initial analysis each mixture and organic excipient was divided into three parts and the following carried out:

a) The bulk of the sample was spread into a petri dish and placed on storage at 37°C/75% relative humidity (RH) for 3 weeks in order to stress the samples to high temperature and humidity and an oxidizing atmosphere - conditions condusive to degradation.

b) A smaller portion was placed in a small glass jar and stored at 55°C/ambient RH for 3 weeks to reproduce the maximum storage temperature likely to be encountered.

c) A second small portion was placed in a smalll glass jar and stored at 5°C for 3 weeks to act as a control.

The samples were visually evaluated before, during and after storage and assayed by HPLC for remaining L-ascorbic acid. The control samples were only analyzed if thermograms of samples stored at 37°C/75% RH and 55°C differed. Moisture levels of all mixtures were determined by loss on drying overnight at 105°C.

4.4 Results and Discussion

4.4.1 Heat-Flux DSC

All three batches of L-ascorbic acid were found to be of the same polymorphic form, Figure 71 page 177, therefore only one batch was used throughout for the study.

Evaluation of DSC Thermograms

The evaluation of thermograms of mixtures, even binary mixtures, is difficult. Results from the DSC should be used more as a guide than an absolute certainty that a mixture is unstable at room temperature. In the evaluation of the thermograms the following points have to be considered, Van Doreen, [96];

i) DSC is concerned with the melting ranges of compounds and reactions or transformations occuring at elevated temperatures may not happen at room temperatures.

ii) Compatibility between drug and excipient is assumed if the curve of the mixture was a super-imposition of those of the single components.

iii) Extra peaks in a curve before the peak of the lower melting component in a mixture may indicate incompatibility. This also applies when one of the component peaks disappears completely.

iv) If one of the components degrades before the melting point of the higher melting point substance then the latter substance may react with the degradation products. Prediction of incompatibility is then impossible.

v) If a eutectic or other mixed melting point entity if formed then the DSC curve of the mixture is of that entity. It is uncertain whether the stability of the entity would affect the stability of the mixture.

vi) Predictions should be confirmed by assaying for the drug of interest before and after storage.

The DSC thermograms for individual components and before and after storage are shown in Figures 61 to 71 and discussed below.

i) Mixtures With 1000 ppm M^{n+}

Only the formulation with Cu^{2+} after storage at 37°C/75% RH for 3 weeks, Figure 61, exhibited slight incompatability as indicated by the L-ascorbic acid peak being broader and tailing on the upslope. All other formulations with metal cations had unchanged thermograms indicating stable formulations.

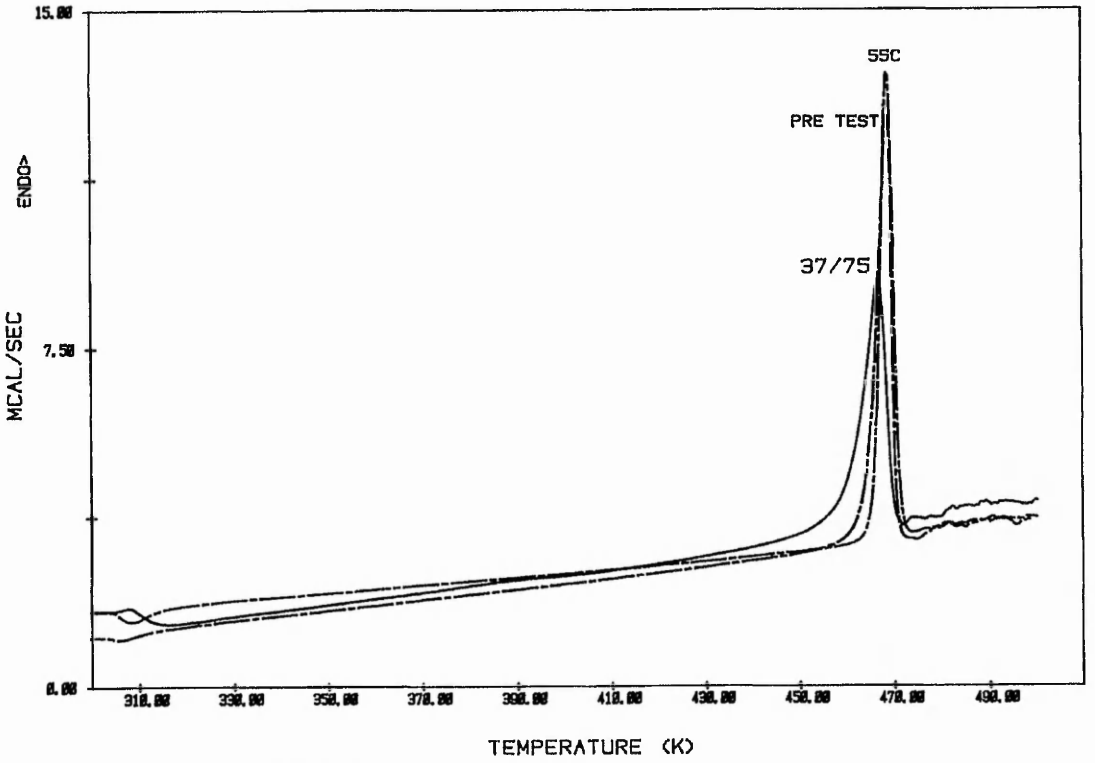


Figure 61: DSC Thermogram for a 1000 ppm Formulation of Cu^{2+} with L-Ascorbic Acid

ii) Stearic Acid Mixture

This mixture was a perfect example of the mixture curve being a super-imposition of those of the single components. The DSC curves, Figure 62, were unchanged after storage at both conditions hence the mixture was assumed to be stable.

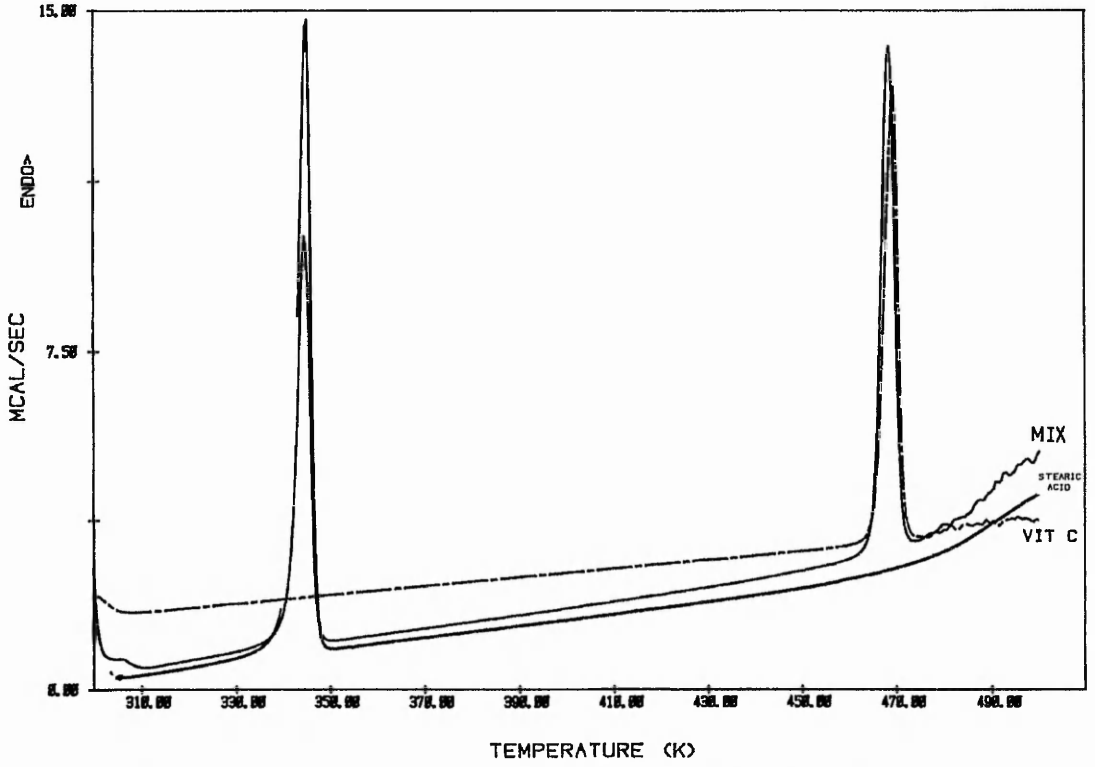


Figure 62: DSC Thermogram of Stearic Acid, L-Ascorbic Acid and a 1:1 w/w Mix

iii) Sucrose Mixture

Due to the melting points of L-ascorbic acid and sucrose being very similar, 469.2K and 465.6K respectively, the mixture thermogram was not a superposition of the two individual components. A broadening and merger of the two peaks was observed. The fresh mixture exhibited an extra peak to that of the individual components at a much lower temperature than the melting points of the individual components. After storage at 55°C this peak was still present but the larger broad main peak had all but disappeared to be replaced by a much smaller split peak. At 37°C/75% RH the small peak was absent but the main broad peak was now reduced in size with the addition of more than one impurity peak at higher temperatures, Figure 63. It was concluded that this mixture was unstable.

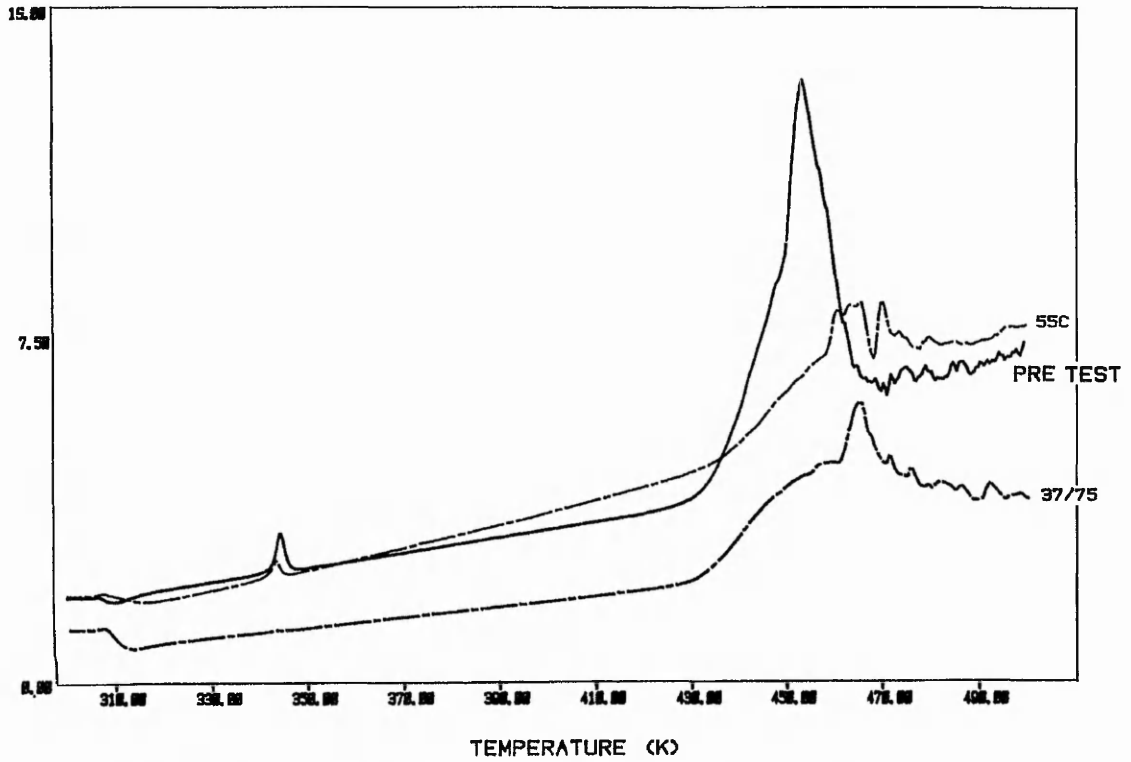


Figure 63: DSC Thermogram of Sucrose/L-Ascorbic Acid Mixtures Before and After Storage

iv) Saccharin Sodium Mixture

The saccharin sodium was found to be polymorphic as five peaks were seen in the individual component thermogram. When stored alone at 37°C/75% RH and 55°C the compound transformed into one polymorph as shown by only one peak remaining on the thermograms, Figure 64. The mixture when stored at 37°C/75% RH and 55°C showed much less complicated thermograms as the polymorphs disappeared, Figure 65. This mixture was assumed to be compatible.

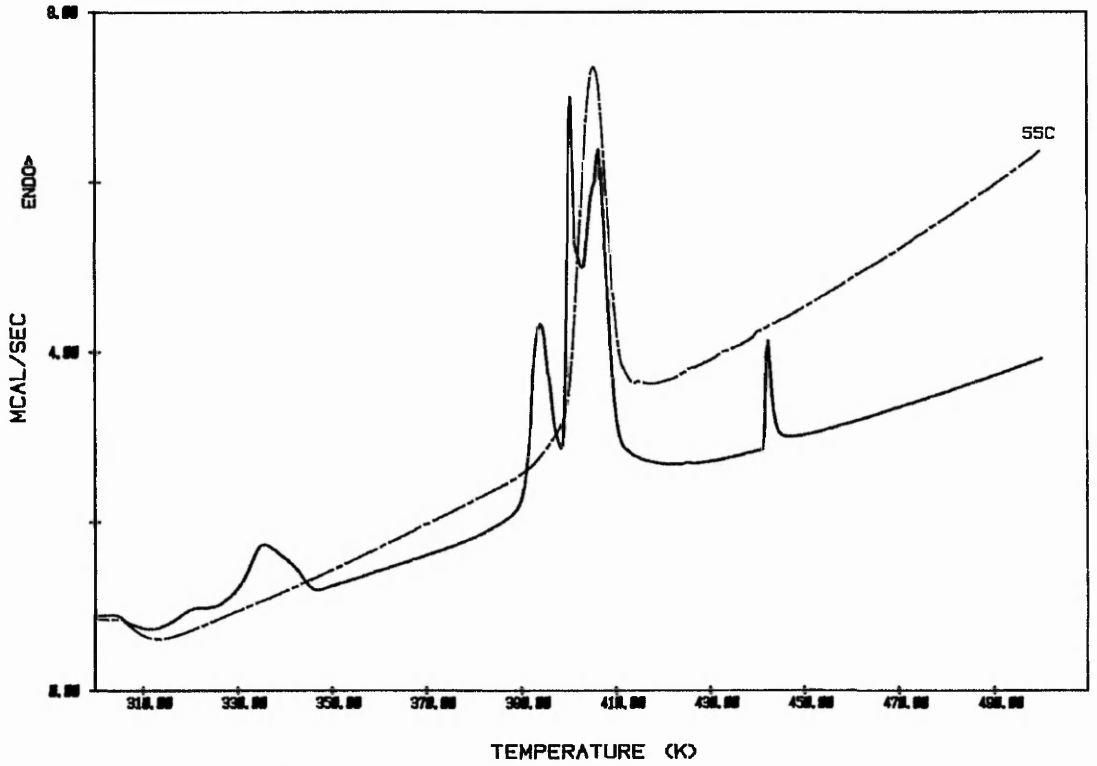


Figure 64: Polymorphs of Saccharin Sodium Before and After Storage at 55°C

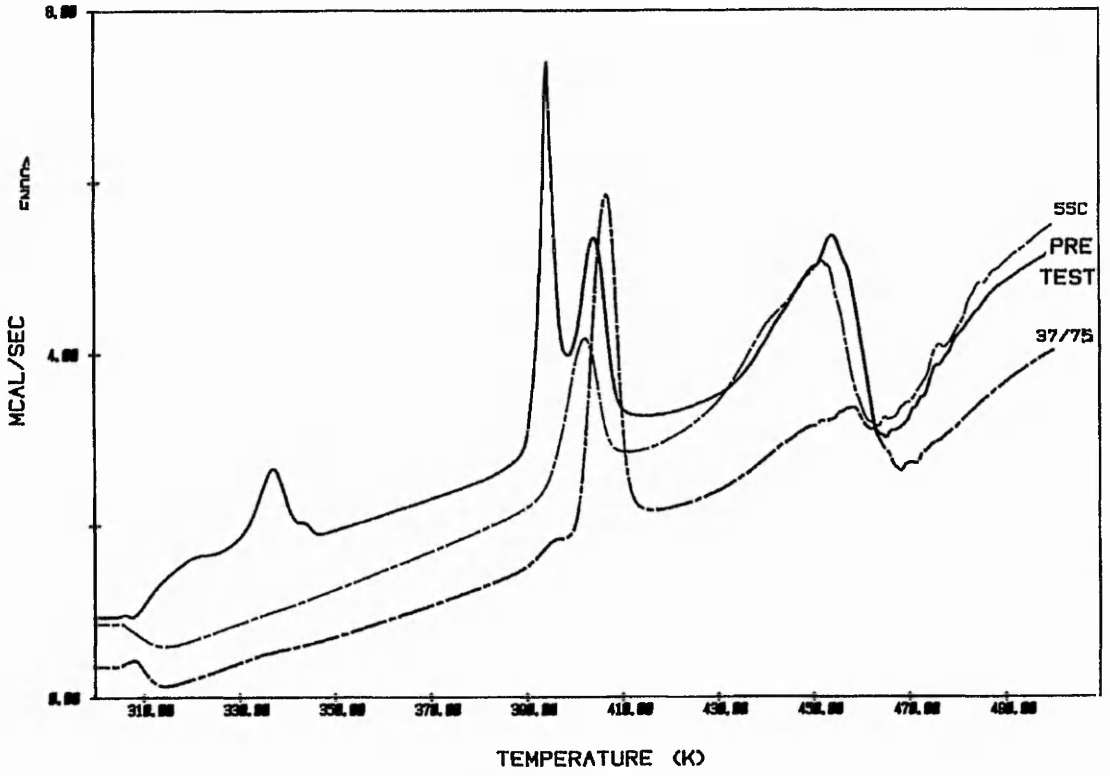


Figure 65: Saccharin Sodium Mixture with L-Ascorbic Acid

v) Kollidon Mixture

When the mixture was stored at 37°C/75% RH a smaller peak due to L-ascorbic acid was seen but this could have been due to a dilution effect caused by the uptake of moisture. The mixture, when stored at 55°C, was unchanged from the initial thermogram, Figure 66. It was concluded this was a compatible mixture.

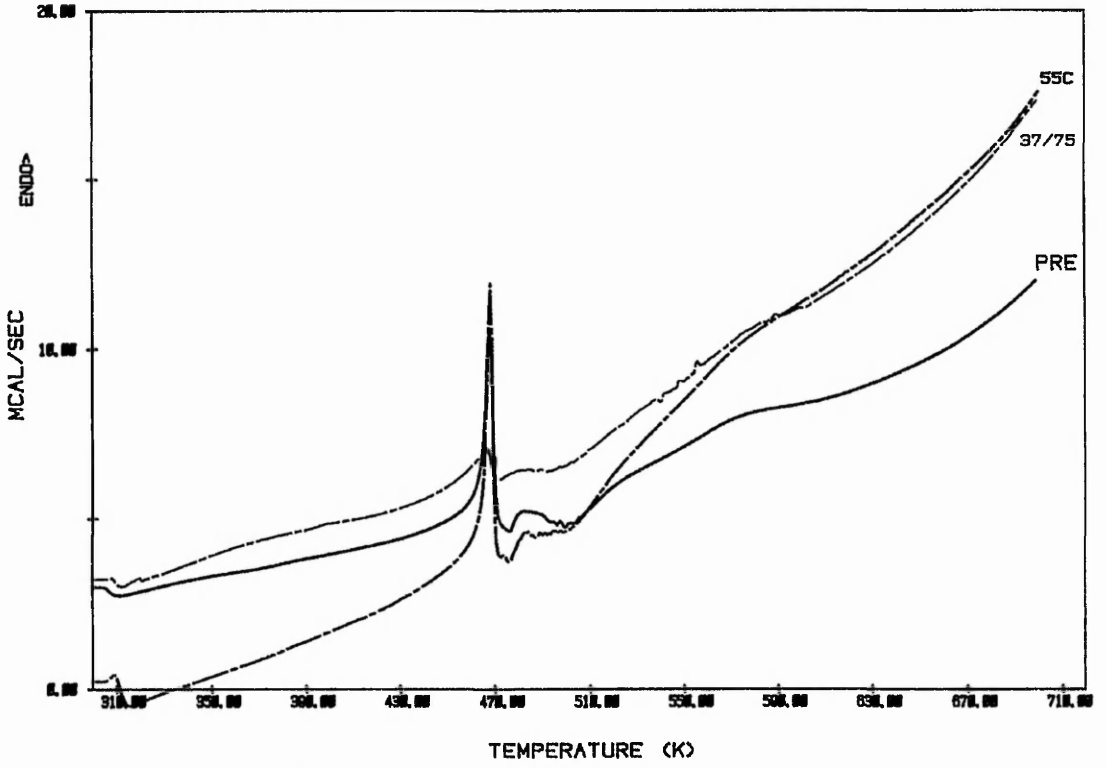


Figure 66: DSC Thermogram of Kollidon-L-Ascorbic Acid Mixture

vi) Thiamine Mononitrate Mixture

This was difficult to interpret as the mononitrate salt oxidized on heating giving rise to a negative peak. As the basic shape of the first peak remained similar on storage it was concluded that this mixture was stable, Figure 67.

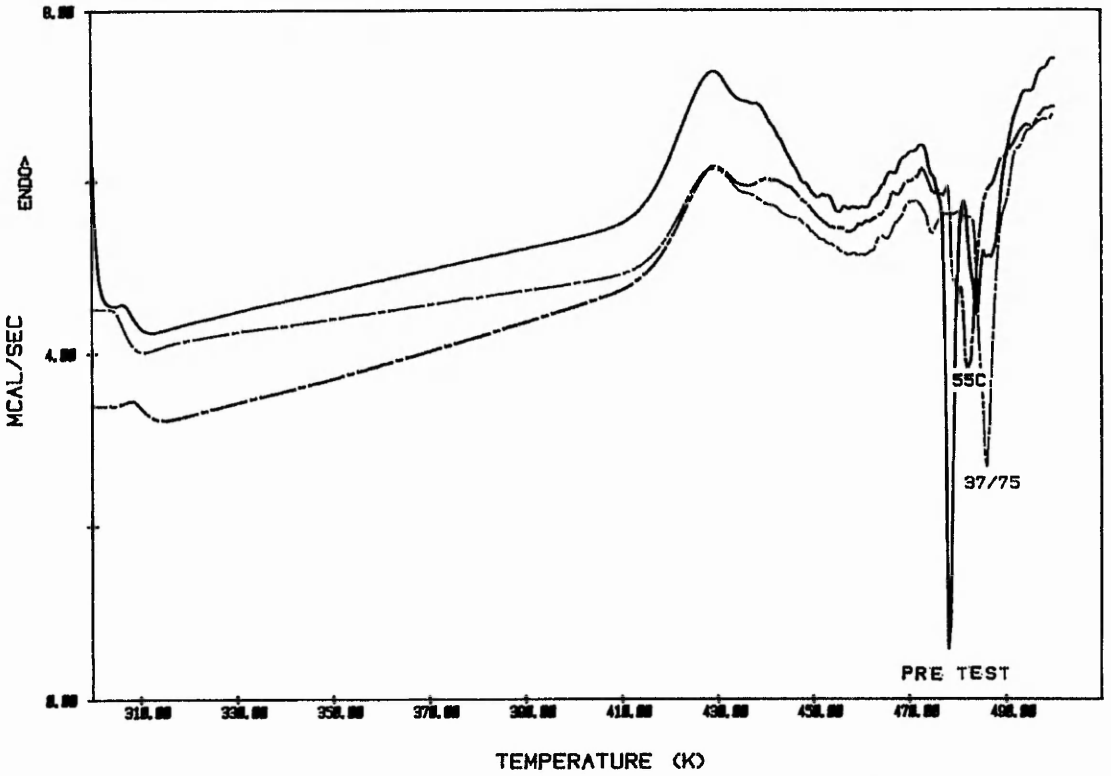


Figure 67: DSC Thermograms of Thiamine Mononitrate Mixtures

vii) Mannitol Mixture

An example of a possible eutectic or mixed melting point entity formed as the initial mixture gave a broad peak with a minor one on the downslope, Figure 68. This minor peak increased in size three or four fold on storage under both sets of storage conditions, Figure 69. It was concluded that this mixture was probably incompatible.

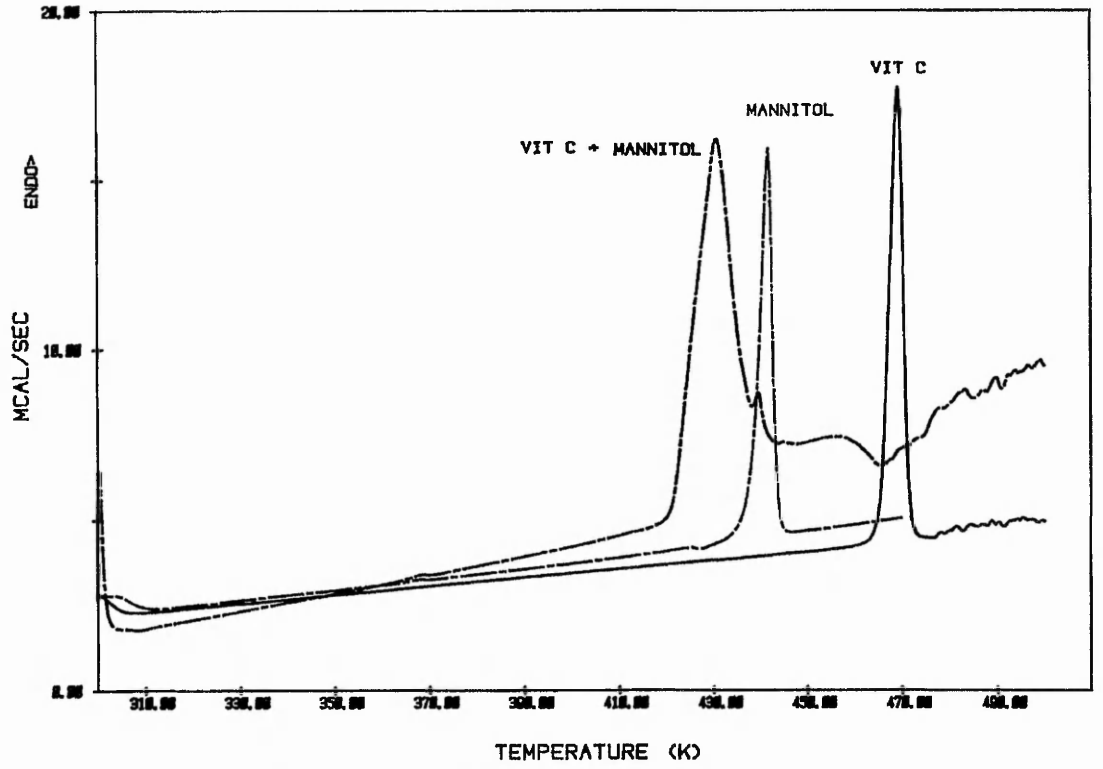


Figure 68: DSC Thermogram of Pre-Test Mannitol Mixture Showing Possible Eutectic

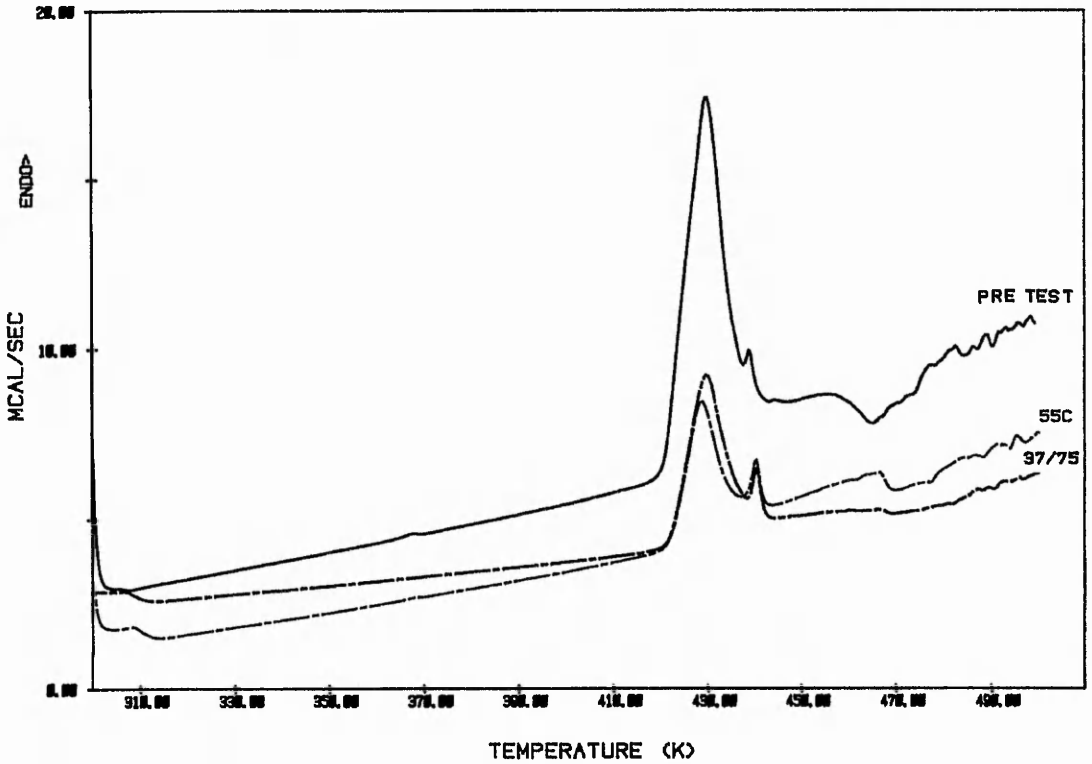


Figure 69: DSC Thermogram of Post-Test Mannitol Mixture

viii) Sodium Hydrogen Carbonate Mixture

As the pre-test and mixture stored at 55°C both gave the same two peaks it was concluded the mixture was compatible at 55°C. At 37°C/75% RH these two peaks were absent and a new broad shallow peak was now present at 60 - 80K below the temperature of the pre-test mixture peaks, Figure 70. It was concluded that at 37°C/75% RH the mixture was unstable. This was almost certainly due to the effect of moisture as when stored dry at 55°C this mixture was shown to be stable by DSC.

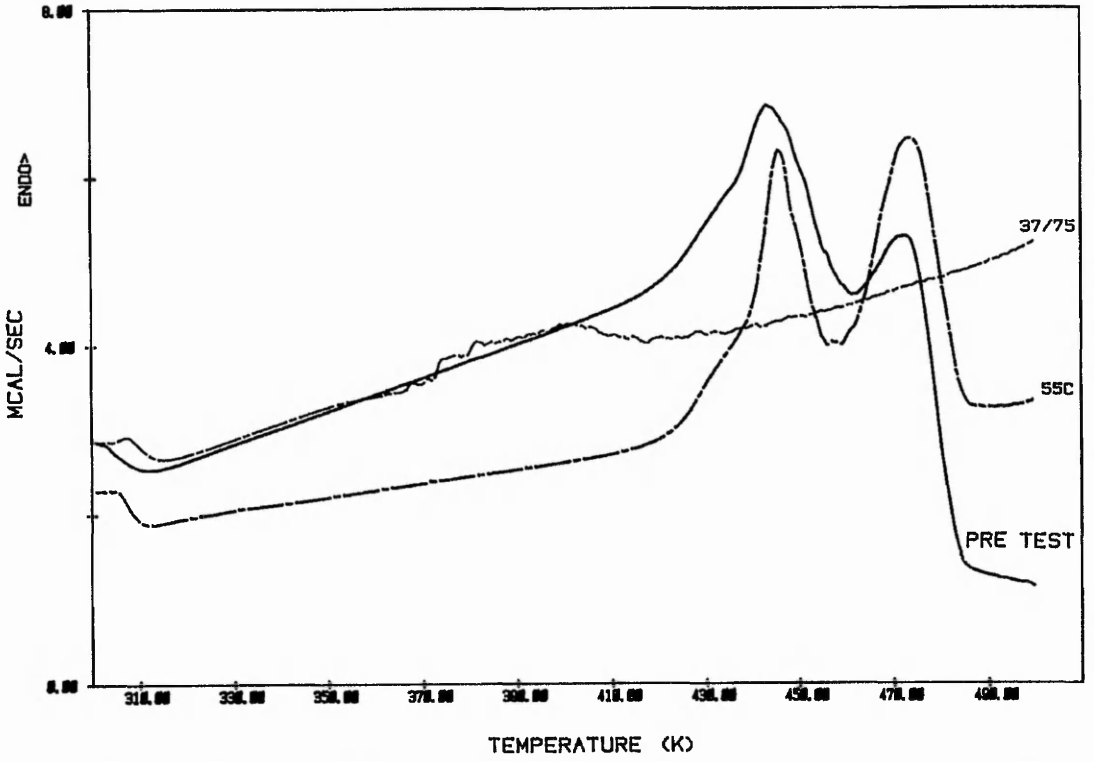


Figure 70: DSC Thermograms of Sodium Hydrogen Carbonate Mixtures

ix) L-Ascorbic Acid

Various batches of L-ascorbic acid were found to be of the same crystal form by DSC and therefore only one batch was used throughout the study. The sample of pure L-ascorbic acid was found to be stable by DSC, Figure 71.

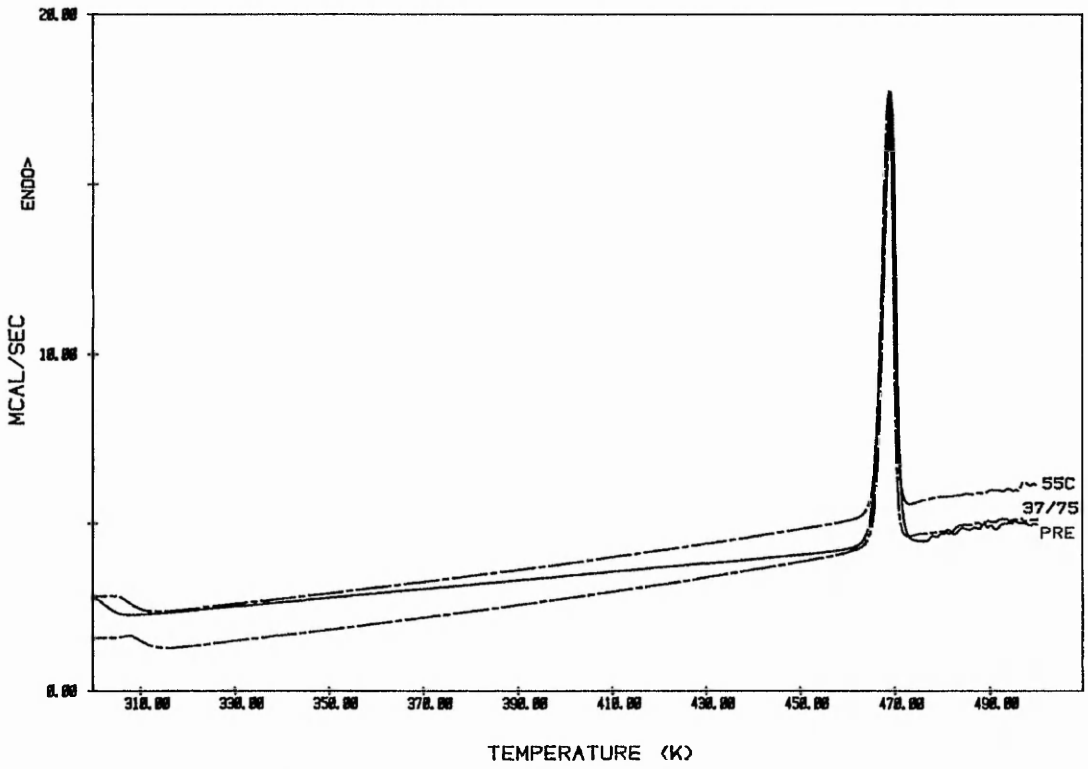


Figure 71: DSC Thermogram of L-Ascorbic Acid

4.4.2 Moisture

It was important to monitor for the uptake of moisture as the presence of moisture is critical in vitamin tablet formulations [208]. Results are shown in Table 27.

Table 27: Moisture in L-Ascorbic Acid and Its Mixtures

Sample	Initial (% w/w)	3 weeks at 37°C/75% RH (% w/w)	3 weeks at 55°C (% w/w)
L-Ascorbic Acid	0.09	0.39	0.09
+1000 ppm Na^{+}	0.20	0.45	0.20
+1000 ppm Mg^{2+}	0.22	1.27	0.39
+1000 ppm Ca^{2+}	0.21	0.44	0.30
+1000 ppm Cu^{2+}	0.25	2.03	0.35
+1000 ppm Ti^{4+}	0.18	0.55	0.18
Stearic Acid Mixture	0.25	0.27	0.20
Sucrose Mixture	0.20	0.45	0.21
Saccharin Sodium Mixture	7.27	3.18	5.03
Kollidon Mixture	2.75	7.38	1.57
Thiamine Mononitrate Mixture	0.24	0.82	0.67
Mannitol Mixture	0.18	0.37	0.29
Sodium Hydrogen Carbonate Mixture	0.42	24.78*	0.21

* This result is high probably due to loss of CO_2

Initial and 55°C sample levels were almost identical for the majority of samples. Only the saccharin sodium and kollidon mixtures had levels which were much

higher than the other mixtures. The saccharin sodium moisture content dropped on storage under both sets of conditions, unexpectedly more at 37°C/75% RH than at 55°C, no reason could be given for this anomaly. The moisture content of the kollidon mixture was high after storage at 37°C/75% RH. The extremely high value of almost 25% for the sodium hydrogen carbonate mixture was almost certainly due to loss of CO_2 caused by the acid reacting with the bicarbonate and was not a true reflection of the moisture content in the mixture.

4.4.3 HPLC Assay for L-Ascorbic Acid

The mean assay results of duplicate sample weight are shown in Table 28.

Table 28: Assay For L-Ascorbic Acid In Mixtures Before And After Storage

Sample	Initial (% w/w)	3 weeks at 37°C/75% RH (% w/w)	3 weeks at 55°C (% w/w)
+1000 ppm Na^{+}	100.26	100.16	101.59
+1000 ppm Mg^{2+}	100.48	100.29	100.67
+1000 ppm Ca^{2+}	100.50	100.90	101.34
+1000 ppm Cu^{2+}	99.17	89.22	100.63
+1000 ppm Ti^{4+}	100.50	100.88	100.46
Stearic Acid Mixture	48.59	51.58	49.46
Sucrose Mixture	47.92	45.22	48.03
Saccharin Sodium Mixture	51.15	51.63	52.70
Kollidon Mixture	51.88	52.70	60.58
Thiamine Mononitrate Mixture	52.18	50.30	53.55
Mannitol Mixture	50.67	49.17	53.12
Sodium Hydrogen Carbonate Mixture	48.76	5.69	49.84
L-Ascorbic Acid	101.21	100.89	101.31

All of the formulations were found to be stable at 55°C as the initial and 3 week assays were almost identical. The kollidon mixture assayed much higher than the initial result, no reason could be found for this as duplicate weights gave similar results.

At 37°C/75% RH the formulations with Cu^{2+} and sodium hydrogen carbonate were noticibly lower in L-ascorbic acid than the initial assay results with the latter formulation approximately one tenth of the initial strength.

4.4.4 Appearance of Samples on Storage

Initally all samples were white crystalline mixtures. The control samples stored at 5°C were unchanged from their initial appearance. Some samples stored at elevated temperatures and humidities did discolour, Table 29.

Table 29: Appearance of Samples Stored at 37°C/75% RH

Sample	1 week	2 weeks	3 weeks
+1000 ppm Na^{+}	no change	no change	surface more yellow than substrate
+1000 ppm Mg^{2+}	no change	no change	no change
+1000 ppm Ca^{2+}	no change	no change	no change
+1000 ppm Cu^{2+}	pale brown	brown	dark brown with lighter patches, odour of burnt sugar
+1000 ppm Ti^{4+}	no change	very pale straw colour	very pale straw colour, small dark brown flecks
Stearic Acid Mixture	no change	no change	no change
Sucrose Mixture	extremely pale straw colour	very pale straw colour	pale straw colour
Saccharin Sodium Mixture	pale brown	mottled brown	mottled brown
Kollidon Mixture	very pale brown plastic	pale brown plastic	pale brown plastic
Thiamine Mononitrate Mixture	no change	very pale brown	very pale brown darker brown mottling
Mannitol Mixture	no change	no change	no change
Sodium Hydrogen Carbonate Mixture	brown	dark brown	very dark brown moist powder, odour of burnt sugar
L-Ascorbic Acid	no change	no change	no change

Kollidon only and the kollidon mixture both set into a soft plastic material.

At 55°C only the formulations with Cu^{2+} and saccharin sodium discoloured. After one week the Cu^{2+} formulation was a very pale brown colour with brown flecks. The colour darkened slightly after two weeks and slightly more at three weeks storage. The saccharin formulation after one week was a very pale straw colour which darkened to a pale straw colour after two and three weeks.

4.4.5 HPLC Degradation Screen

Initial Samples

All the initial samples exhibited no impurities present.

Three weeks at 37°C/75% RH

Degradation peaks were found in formulations containing 1000ppm Cu^{2+}, kollidon and sodium hydrogen carbonate. The formulation containing sodium hydrogen carbonate was found to have degraded the most, Figure 72, with most degradation products being more polar than L-ascorbic acid. Figures 73, 74 and 75 illustrate the extent of degradation with time and temperature for formulations with Cu^{2+}, sodium hydrogen carbonate and the control solution (wavelength of 245 nm) respectively. Figure 76 illustrates the need to monitor at the three different wavelengths chosen. The major degradation product(s) in the formulation containing sodium hydrogen carbonate eluted as a broad tailing peak centred on 2.1 minutes with other minor peaks superimposed upon it. The main degradation peak at 2.1 minutes integrated at 21.7% of the total area and the L-ascorbic acid was present at 70.59%, no response factors were applied as none were known at the time. This high value for L-ascorbic acid, 70.6%, did not agree with the assay figure of 5.69%. There are three reasons that could account for this descrepency:

a) high response factors need to be applied to the degradation products
b) not all degradation products are being detected at the wavelength used
c) not all the degradation products are being chromatographed

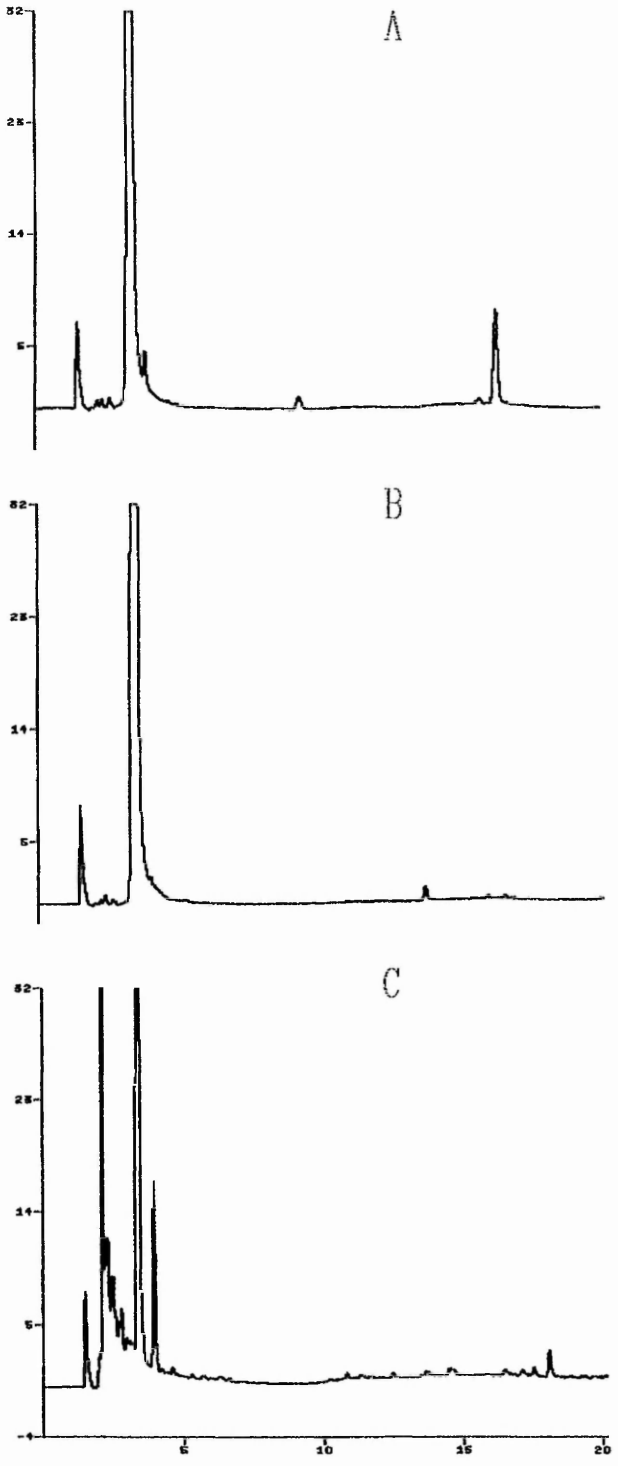


Figure 72: **Degraded Formulations, A = with Cu^{2+}, B = with Kollidon, C = with Sodium Hydrogen Carbonate**

Very minor degradation peaks were found in the other two degraded formulations Figure 73, at <0.5% of the total area. These values agree with the assay results for the kollidon formulation but not for the formulation with Cu^{2+} ions where a 10% reduction in L-ascorbic acid was recorded. Two of the degradation peaks in the formulation with Cu^{2+} ions had the same retention time as two in the sodium hydrogen carbonate mixture. It is possible that one or both of these peaks may have a low UV absorbance which requires a very high response factor applying for a correct determination of the level to be made at the wavelength used.

Three weeks at 55°C

All samples including the formulation with sodium hydrogen carbonate, exhibited no degradation peaks. These findings are in keeping with the HPLC results where samples assayed slightly higher than initial samples after storage at 55°C for 3 weeks.

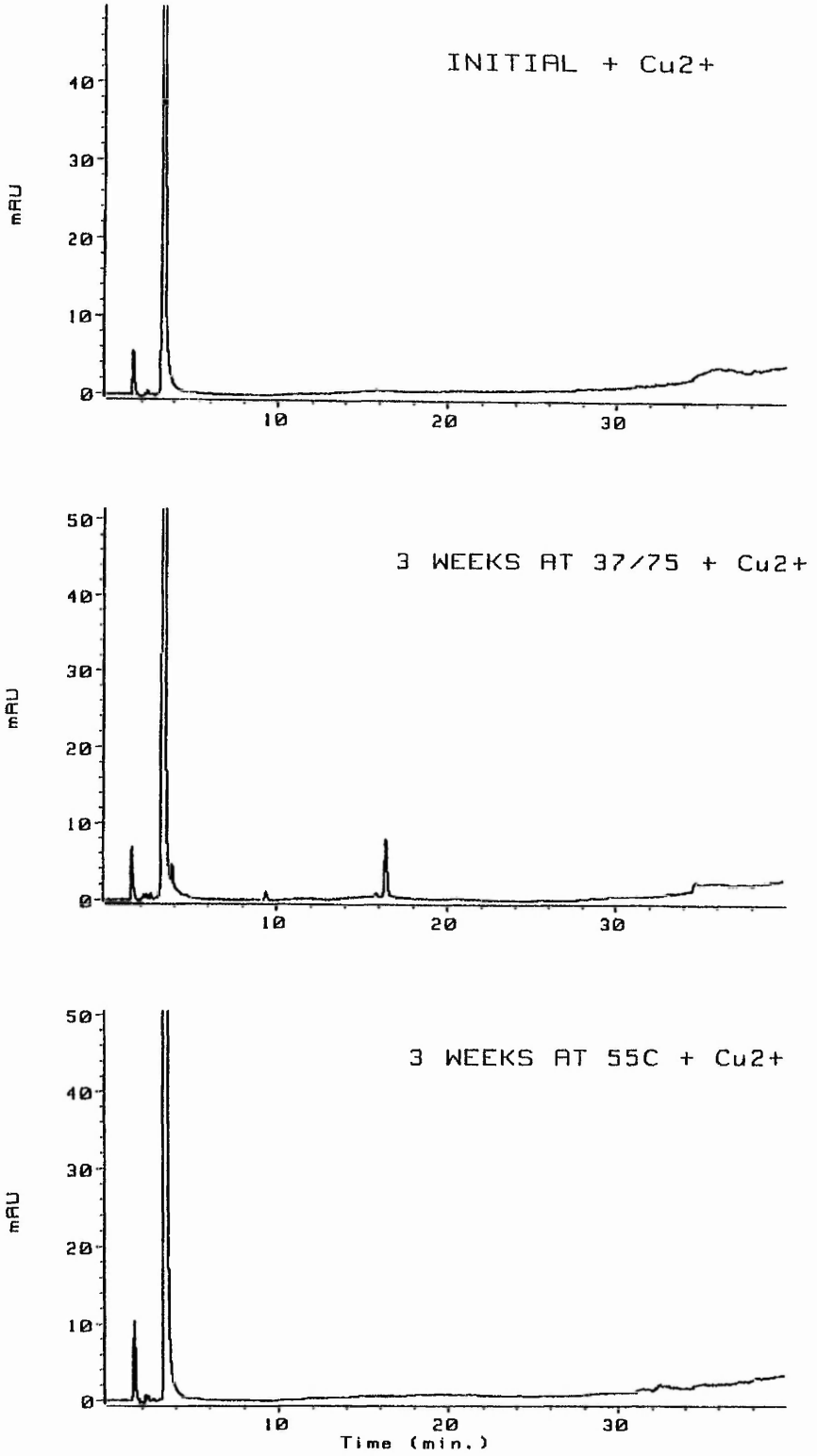


Figure 73: Degradation Profile of Solid Formulation with Cu^{2+} Ions

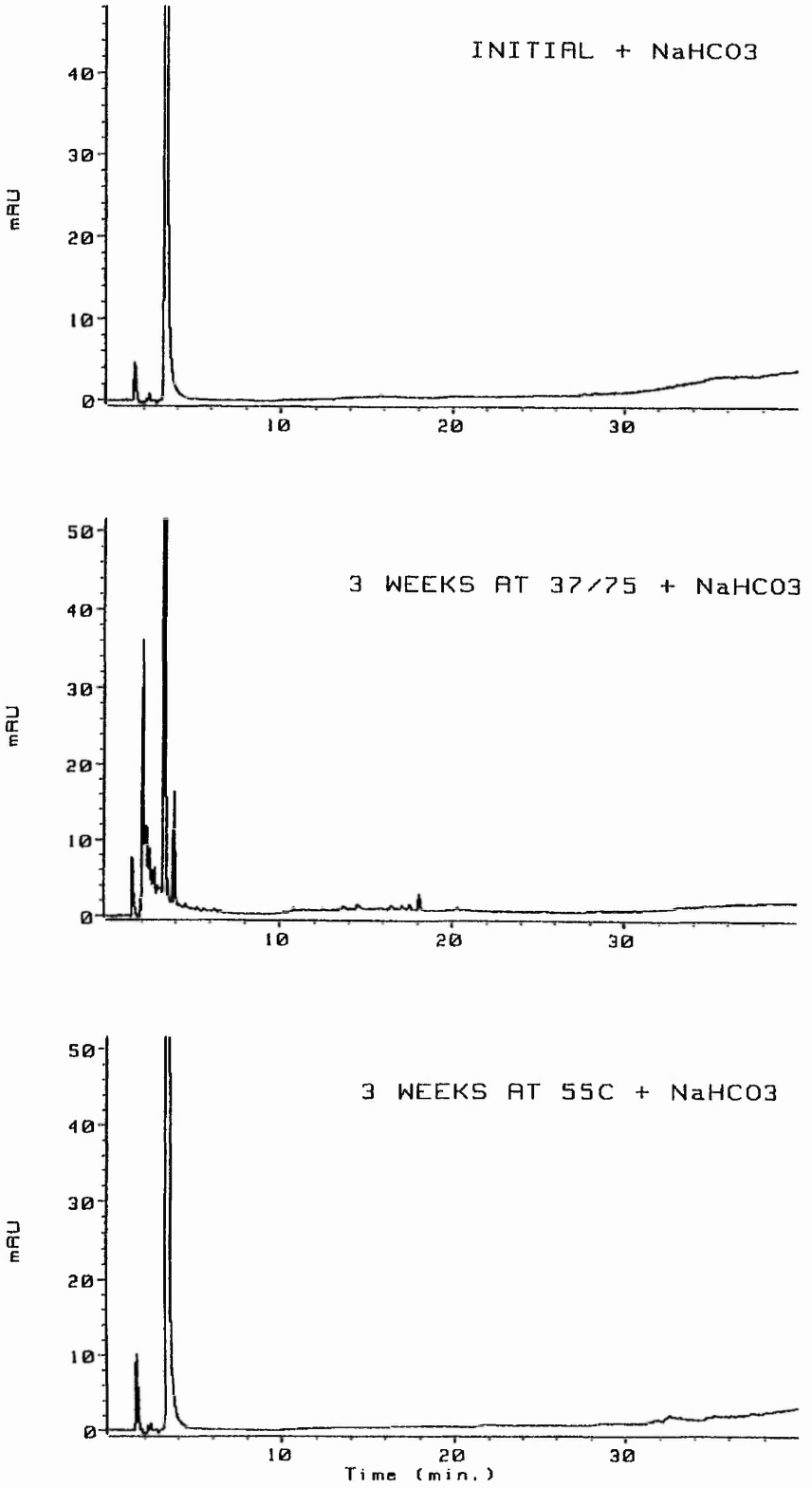


Figure 74: Degradation Profile of Solid Formulation with Na_2CO_3

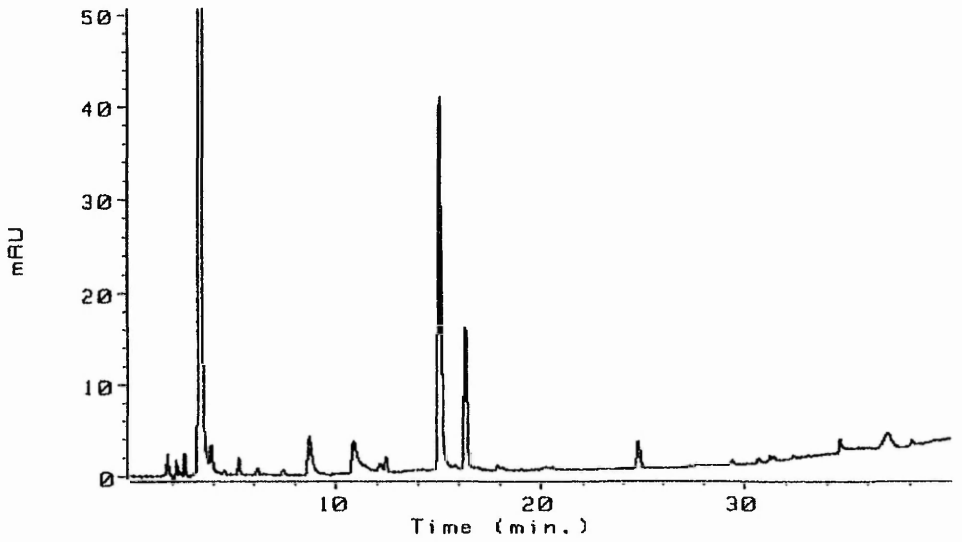


Figure 75: Degradation Profile of Control Solution at 245 nm

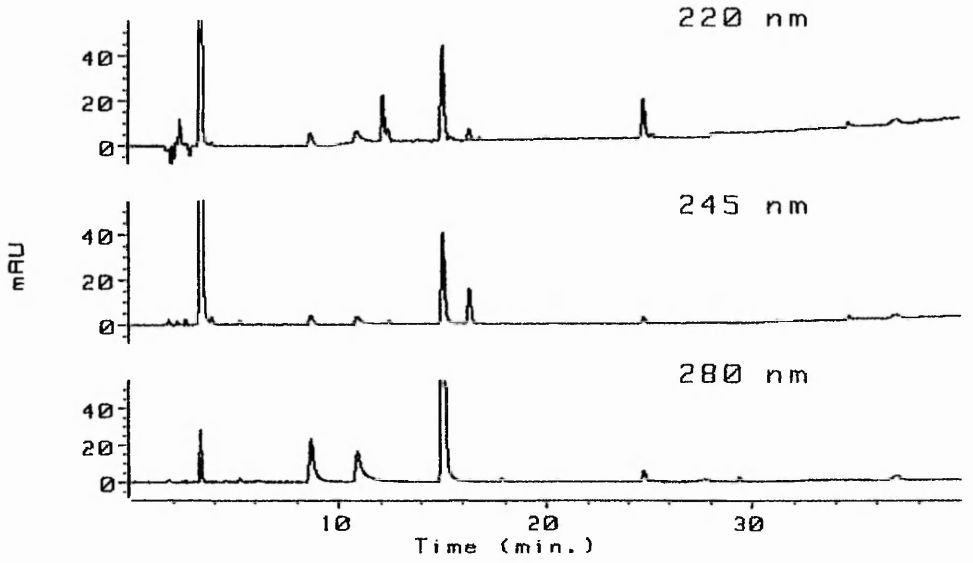


Figure 76: Degradation Profile of Control Solution at 3 Wavelengths

4.4.6 Conclusions

DSC

The formulations made with Cu^{2+}, sucrose, mannitol and sodium hydrogen carbonate were predicted to be incompatible. These predictions were confirmed by the HPLC assay results and to a lesser degree by the HPLC degradation screen.

Moisture

At 37°C/75% RH the moisture level of all samples increased. The majority of samples only had a slight increase in moisture but the formulation with Cu^{2+} had a ten fold increase. It was impossible to determine how much moisture uptake in the sodium hydrogen carbonate formulation had occurred. Using loss on drying (at 105°C) liberation of carbon dioxide gave an erronously high result. The saccharin sodium formulation was unusual in that the moisture level dropped from 7.27% w/w to 3.18% w/w. The Karl Fischer analysis method for moisture determination could not be used for any L-ascorbic acid formulations as iodine in the reagent oxidizes L-ascorbic acid to dehydroascorbic acid and an erronously high value is obtained. The recommended method for moisture determination in L-ascorbic acid and its formulations is either loss on drying at 105°C, as used in this study, or gas chromatography with thermal conductivity detection. At the time of this study a thermal conductivity detector was not available.

At 55°C/ambient RH the moisture level in most samples was virtually unchanged from initial values. The saccharin sodium formulation again lost moisture from the initial value along with the kollidon formulation. Moisture was heavily implicated in the stability of the formulations. At 55°C/ambient RH all mixtures were stable. As soon as moisture was introduced, by allowing samples to equilibriate with the atmosphere at 37°C/75% RH degradation occured with reduced levels of L-ascorbic acid being found in formulations with Cu^{2+}, sodium hydrogen carbonate, mannitol and sucrose.

Assay

At 37°C/75% RH the formulation with Cu^{2+} had lost 10% w/w of the L-ascorbic acid content and the sodium hydrogen carbonate formulation had undergone a nine fold reduction. Smaller reductions in the assay of L-ascorbic acid were found in the mannitol and sucrose formulations. The other formulations showed no significant difference from the initial assay values. These assay results confirmed the DSC predictions.

Only the kollidon formulation stored at 55°C showed a change in the assay value from the initial value. This sample was diffficult to sample as it had set into an amorphous plastic material and a homogeneous sample was impossible to obtain. All the other samples showed no significant difference in assay for L-ascorbic acid between values and those when stored at 55°C for 3 weeks.

Degradation Screen

Of the samples stored at 37°C/75% RH formulations with Cu^{2+}, kollidon and sodium hydrogen carbonate were found to show the presence of degradation peaks. The presence of degradation peaks in the formulations with Cu^{2+} and sodium hydrogen carbonate confirmed DSC predictions that these formulations would be incompatible. No degradation peaks were detected in formulations with sucrose and mannitol which DSC had also predicted to be incompatible. The absence of degradation peaks in these two formulations was not unexpected as the degradation screen was not satisfactory, the area percent report gave an artificially high sample purity when compared to the assay result. It would appear that the HPLC assay method resolved L-ascorbic acid from any degradation compounds present but the degradation screen either did not resolve it from some degradation products, did not detect all degradation products or not all were chromatographed, i.e. some were retained on the column.

Appearance

Samples which discoloured noticibly when stored at 37°C/75% RH were those predicted to be incompatible, they had a low assay and showed major degradation peaks, i.e. formulations with Cu^{2+} and sodium hydrogen carbonate. Most other formulations were either unchanged or only slightly discoloured. Noticibly the formulation with saccharin sodium did show more significant discolouration but assay results were unchanged from the initial value. The discolouration of this sample could have been due to degradation of the saccharin sodium as saccharin sodium alone did not discolour. If the degradation of saccharin sodium was catalyzed by L-ascorbic acid it would not have shown up in the HPLC assay for L-ascorbic acid and probably not in the HPLC degradation screen as these chromatographic methods were not designed for the analysis of saccharin sodium. Some mottling or brown flecks were present in some formulations, e.g. the formulation with Ti^{4+}. This was probably due to isolated, very small pockets of degradation.

At 55°C again the formulation with Cu^{2+} and saccharin sodium had discoloured albeit only very slightly. Noticibly the formulation with sodium hydrogen carbonate showed no discolouration indicating the presence of moisture to be a key factor in the discoloration/degradation of L-ascorbic acid formulations.

Some conflicting results were obtained in this study. The moisture content increased from 0.25% w/w to 2.03% w/w for the formulation with Cu^{2+}, from 0.20% w/w to 0.45% w/w with sucrose and from 0.18% w/w to 0.37% w/w with mannitol. The moisture values associated with degraded samples were often lower than the moisture levels in stable formulations e.g. L-ascorbic acid alone, from 0.09% w/w to 0.39% w/w, with Mg^{2+} from 0.22% w/w to 1.27% w/w, with kollidon from 2.75% w/w to 7.38% w/w and from 0.24% w/w to 0.82% w/w with thiamine mononitrate.

The stability of L-ascorbic acid in a formulation at a slightly acidic was dependent

on the excipient present. The pH of the mixture could be almost identical with one mixture, e.g. with sucrose, pH 5.75, being unstable but that with Mg^{2+}, pH 5.50, being stable. It is possible that the mannitol and sucrose assay values, which were only slightly lower at 37°C/75% RH than initial values, i.e. 1.5 and 2.5% w/w lower respectively, were not truely representative of the bulk and were probably unchanged from initial values. This theory is supported by the absence of degradation peaks. The grossly degraded formulations were those with markedly reduced assay values, showed the presence of degradation peaks and were chemically incompatible e.g. Cu^{2+} is an oxidizing agent and sodium hydrogen carbonate has a high pH, 8.12 for the formulation which would probably cause opening of the lactone ring.

Very low levels of degradation, i.e. not detected by a reduction in assay or the presence of degradation peaks were detected by discolouration of the formulation. Monitoring for colour change should give an early indication of the onset of degradation.

The presence of moisture appeared to be a significant factor in the degradation of the formulations. The effect of moisture alone on the stability of L-ascorbic acid was investigated along with the use of tristimulus colorimetry for the monitoring of colour change (Chapter 5).

CHAPTER 5

Moisture Induced Solid Phase Degradation

5.1 Introduction

L-Ascorbic acid has been found to degrade in the solid phase under the influence of moisture [209, 210]. De Ritter and co-workers studied the effect of silica gel on the stability of L-ascorbic acid [211]. They found that the loss of L-ascorbic acid was directly proportional to the amount of unbound moisture. No workers to date have identified the degradation products formed under the influence of moisture alone in the solid phase.

Studies carried out during the work reported herein (Chapter 4) implicated moisture as being a significant factor in the degradation of L-ascorbic acid. This study investigated the effect of moisture only on the stability of L-ascorbic acid. It was designed to generate kinetic data on the rate of degradation of L-ascorbic acid under the influence of moisture and air, to identify whether tristimulus colorimetry could be used as a rapid and non-destructive means of monitoring for the degradation of L-ascorbic acid in the solid phase and to carry out a chemical investigation of the degradation products.

Moisture induced degradation was always characterized by discoloration. Therefore monitoring for colour change could form the basis of an alternative method of measuring the rate of degradation of solid L-ascorbic acid. Vemuri and co-workers [178] have used tristimulus colorimetry to measure the colour stability of L-ascorbic acid tablets but did not confirm their findings with an assay for L-ascorbic acid. In this study use was made of tristimulus colorimetry to monitor the extent of discolouration and the results were compared to those obtained by quantitative high performance liquid chromatography (HPLC) of L-ascorbic acid.

5.2 Kinetic Study

Experimental

Methods

1) Sample Preparation

The effect of moisture on the stability of L-ascorbic acid was investigated at levels between 0 and 10% v/w moisture. Samples of L-ascorbic acid, 10 g, were triturated with deionized water to produce samples with moisture levels of 0.5, 1, 2, 5 and 10% v/w. A 10 g sample containing no added moisture was used as a control. The samples were placed in Pierce Reactivials and sealed with a Teflon coated septum and screw cap. Samples with 5 and 10% v/w moisture were also placed on storage under an atmosphere of nitrogen to exclude oxygen. The samples were placed on storage at 50°C and additional samples containing 5% v/w moisture were stored at 15°C, 30°C and 60°C. The samples were analyzed when prepared and then removed for analysis after 7, 14, 28 and 42 days storage. After removing from storage samples were allowed to equilibriate to ambient temperature and approximately 500 mg removed for analysis. The samples were then returned to storage. Those stored under nitrogen were sampled under nitrogen into sample tubes flushed with nitrogen. The reaction vials were thoroughly purged with nitrogen before being replaced on storage.

2) Analysis

Samples were analyzed for the following;

i) Total ascorbic acid, the sum of the oxidized and reduced forms of L-ascorbic acid, i.e. dehydroascorbic acid and L-ascorbic acid;
ii) L-ascorbic acid;
iii) Colour, tristimulus colorimetry

i) Assay for total L-ascorbic acid and dehydroascorbic acid

Approximately 100 mg of each sample was accurately weighed into a series of 100 ml volumetric flasks and dissolved in 1% w/v aqueous metaphosphoric acid solution to produce 1 mg/ml sample solutions. These sample solutions (1 mg/ml) were diluted five fold with 1% w/v aqueous metaphosphoric acid solution to produce 0.2 mg/ml L-ascorbic acid solutions for quantitative analysis. A 10 µl aliquot of each of the 0.2 mg/ml solutions was chromatographed isocratically in duplicate using reverse-phase HPLC with spectroscopic detection at 245 nm. The sample solutions were assayed initially for total L-ascorbic acid (dehydroascorbic acid and L-ascorbic acid) using the automated precolumn sample preparation system developed for this work and then for L-ascorbic acid only against an external standard solution of 0.2 mg/ml L-ascorbic acid. Samples were freshly prepared and analyzed in small batches for total L-ascorbic acid in order to minimize the loss of dehydroascorbic acid as it is unstable in solution for extended periods of time.

Chromatography was achieved on a Lichrospher 100 RP 18e, 25 x 0.46 cm, 5µm column at 40°C using a mobile phase of 0.1% v/v trifluoroacetic acid, aqueous at a flow rate of 1.5 ml/minute. Detection was carried out at 245 nm and the run time was 4 minutes.

ii) Assay for L-ascorbic acid

Chromatography conditions were identical to those used for total L-ascorbic acid except the automated precolumn sample preparation system was bypassed and 5 µl aliquots of the same sample and standard solutions were chromatographed.

iii) *Colour (Tristimulus colorimetry)*

Tristimulus colorimetry was used in the reflectance mode and colour measurements taken using the $L^* a^* b^*$ scale. On this scale the L axis is the lightness axis and has

limiting values of 0 (black) and 100 (white). Hue and chroma are expressed as a^* and b^* respectively and represent two colour axes with a^* the red-green axis and b^* the yellow-blue axis. A negative a^* value tends to green and a positive a^* tends to red. A negative b^* value tends to blue and a positive b^* value tends to yellow. Using these three coordinates the colour tone of an object can be expressed numerically.

The colour change of an object is the magnitude of the resulting vector of the three component differences ΔE^*ab and is given by Equation 18.

$$\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \qquad \text{Equation 18}$$

The equation assumes that colour space is Euclidean and calculates ΔE^*ab as a straight line between coordinates of the object and a standard.

Reagents/apparatus

Trivector Tristimulus Colorimeter, model CL6000, fitted with reflectance head sample cup, diameter 10 mm and 10 mm deep

Colour measurement

Approximately 100 mg of each sample was pressed into a sample cup and colour readings taken using the reflectance head. Four readings were taken from each sample by rotating the cup through 90° between each reading. The mean of four values was recorded. The readings were recorded as $L^*a^*b^*$ values and calculated as colour differences ΔE^*ab using Equation 18. The visual appearance and colour of each sample was also noted.

Results and Discussion

i) Assay for L-ascorbic acid and dehydroascorbic acid

Assay results for both total ascorbic acid and L-ascorbic acid only were within ± 1% of each other and no evidence was found for the presence of DHAA.

The presence of air and moisture was found to accelerate the degradation of L-ascorbic acid, Figure 77.

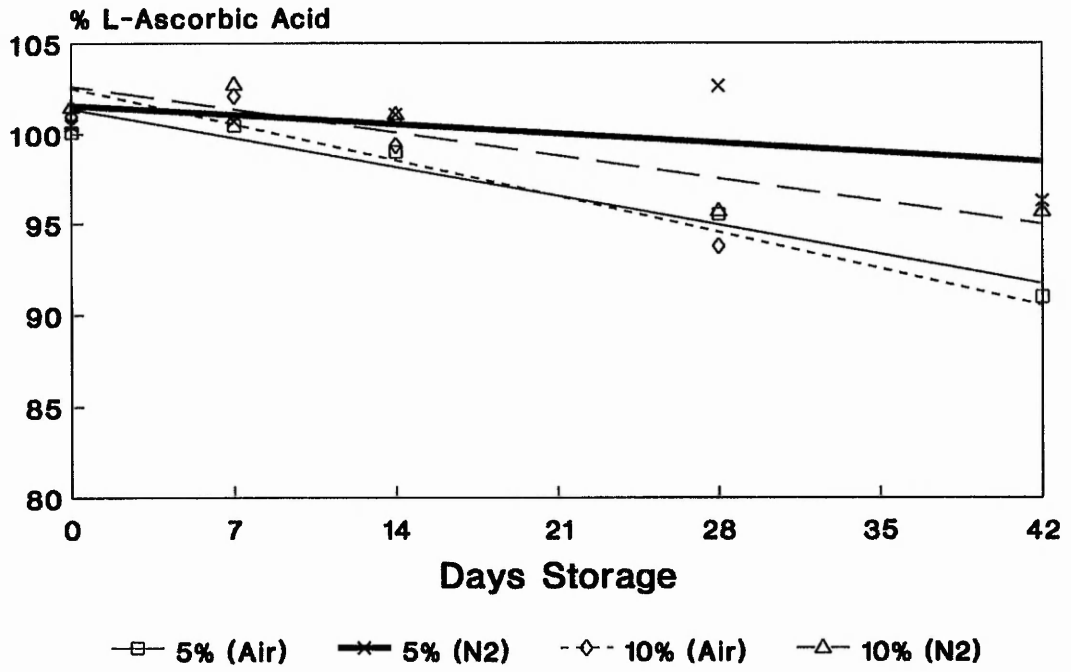


Figure 77: Effect on Assay of Excluding Air from Moist L-Ascorbic Acid

The rate of degradation also increased with increasing moisture content and with elevated temperatures, Figures 78 and 79.

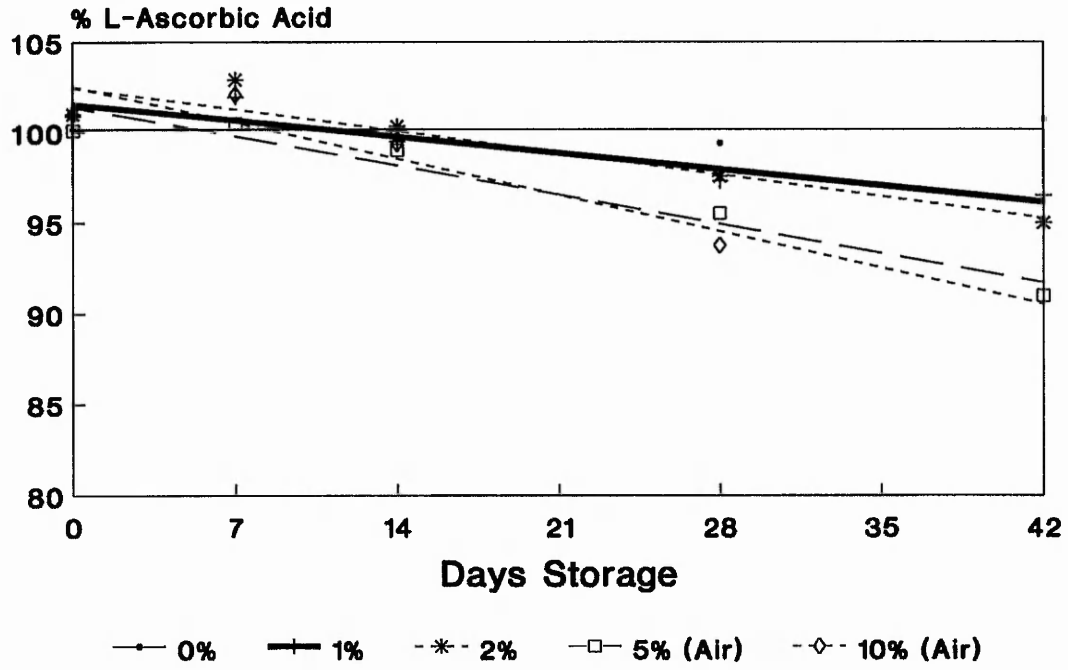


Figure 78: Effect of Moisture on the Assay of L-Ascorbic Acid at 50°C

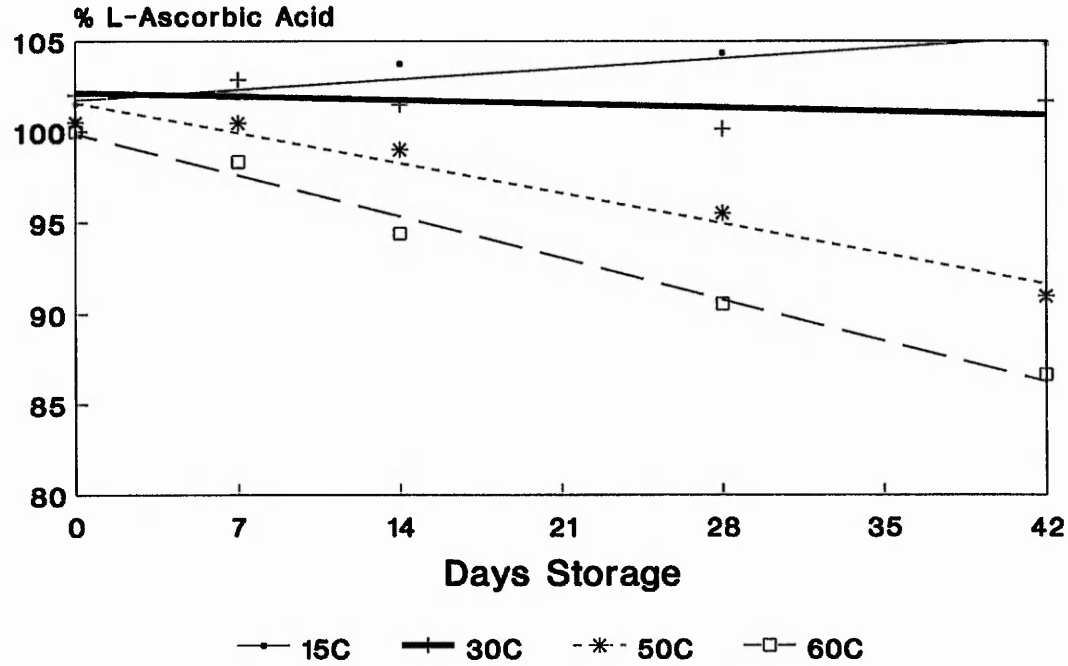


Figure 79:: Effect of Temperature on the Assay of L-Ascorbic Acid in the Presence of 5% v/w Moisture

From the slopes of the plots of total L-ascorbic acid against time the rate constant, k, for each condition was calculated, Table 30. Since the plots were linear the order of reaction was zero order.

A plot of ln k against 1/T for the 5% v/w moisture samples stored at 15°C, 30°C, 50°C and 60°C gave a straight line plot, the activation energy was calculated at 14.2 kcal/mol. This value compared favourably with those obtained by Vemuri *et al.*

The samples stored under nitrogen showed significantly lower rate constants than when no precautions were taken to exclude oxygen. When compared with the rate constants obtained from tristimulus colorimetry $L^*a^*b^*$ results good agreement was achieved.

ii) Colour (Tristimulus colorimetry)

Colour differences, ΔE^*ab, for the sample were calculated using Equation 18 and plotted against time to obtain rate constants, Table 30. Rate constants by both quantitative HPLC assay and tristimulus colorimetry were in good agreement for this type of work. A plot of ln k against 1/T for the 5% v/w moisture stressed samples stored at 15°C, 30°C, 50°C and 60°C gave a straight line plot and the activation energy was calculated at 7.96 kcal/mol. This value was slightly lower than that obtained by quantitative HPLC assay.

Table 30: Reaction Constant, k, for Moisture Stressed L-Ascorbic Acid

Storage Condition (% v/w moisture,temperature,°C)	Technique / Rate	
	HPLC/day	$L^*a^*b^*$/day
1, 50	0.1141	0.5768
2, 50	0.1556	0.6684
5, under air, 50	0.2281	0.6412
5, under N_2 , 50	0.0659	0.2994
10%, under air, 50	0.4694	0.6869
10%, under N_2 , 50	0.1584	0.5541
5, 15	0.0298	0.1290
5, 30	0.0524	0.2725
5, 50	0.2281	0.6412
5, 60	0.3238	0.9387

Appearance of Samples

Samples were visually assessed for colour and rated using a numerical scale from 1 to 5, Table 31. The control sample, <0.1% w/w moisture, remained unchanged throughout the study. The 5% v/w sample stored at 15°C and under nitrogen discoloured the least with the others turning a brown or dark brown colour with time. The sample stored at 60°C rapidly discoloured and turned a very dark brown colour after 14 days and further darkening was difficult to quantify visually.

Table 31: Visual Assessment of Sample Colour Change.

Key: 1= white, 2 = pale straw, 3 = yellow, 4 = brown, 5 = dark brown

Storage Condition (% v/w moisture, temperature,°C)	Storage Time (days)			
	7	14	28	42
0, 50	1	1	1	1
1, 50	2	3	4	5
2, 50	2	3	4	5
5, under air, 50	3	3	4	5
5, under N_2, 50	2	3	3	3
10, under air, 50	3	4	5	5
10, under N_2, 50	2	3	3	4
5, 15	2	2	2	3
5, 30	2	3	3	4
5, 50	2	3	4	5
5, 60	4	5	5	5

Conclusion

L-Ascorbic acid was found to degrade in the solid phase in the presence of moisture alone. The degradation was found to cause severe discolouration, the rate of which could be monitored by tristimulus colorimetry. The reaction was found to be zero-order.

Exclusion of air reduced the rate of reaction infering the degradation occured via an oxidative route although no evidence was found for the presence of dehydroascorbic acid.

Rate constants and the activation energy were determined for L-ascorbic acid stressed with moisture by quantitative HPLC and tristimulus colorimetry and were found to be in close agreement with each other and compared favourably with values quoted by other workers.

Rate constants determined by tristimulus colorimetry were consistantly higher than those determined by HPLC.

An automated and rapid precolumn reduction reaction was developed for the determination of total L-ascorbic acid (L-ascorbic acid and dehydroascorbic acid). A reaction time of only 60 seconds at 50°C was required for the reduction of dehydroascorbic acid to L-ascorbic acid.

5.3 Isolation of the Discoloured Degradation Products

Moisture induced degradation of L-ascorbic acid has been found to give rise to a discolouration of the samples, the most severely degraded samples were almost black in appearance although over 68% w/w of the L-ascorbic acid remained. The appearance of this discolouration occurs before any noticeable decrease in purity as measured by chemical or chromatographic means. A chromatographic procedure is required to detect both the early stages of solid state degradation, as shown by a light brown discolouration, and for gross degradation as shown by an extremely dark brown discolouration.

Previous workers [107, 205] have shown the effect of tablet lubricant and glidants on the stability of L-ascorbic acid in the solid phase but did not investigate the reaction route of the degradation or products formed.

The first degradation stage of L-ascorbic acid in solution is the formation of dehydroascorbic acid. Von Euler and Hasselquist [212] found that by heating L-ascorbic acid with 1M sodium hydroxide solution at 100°C under an atmosphere of nitrogen the lactone ring opened. By heating L-ascorbic acid solutions at different pH values below 5.5 under anaerobic conditions Finholt and co-workers demonstrated that carbon dioxide, furfural and xylose were formed [213]. It is feasible that carbon dioxide and other volatile compounds may be evolved during solid state degradation.

The isolation of ten furan type compounds, two lactones, three acids and 3-hydroxy-2-pyrone was reported by Tatum, Shaw and Berry when L-ascorbic acid was heated in aqueous solution [106]. Coggiola found 2,5-dihydro-2-furoic acid to be a product of the anaerobic degradation of L-ascorbic acid in aqueous solution at 100°C [214]. In order to determine whether furan type compounds are a product of the solid state degradation of L-ascorbic acid a HPLC screening procedure was developed capable of resolving furan and related compounds that have previously been identified as degradation products of solution chemistry.

Löwendahl and Petersson have investigated the degradation pathway of L-ascorbic acid in neutral and alkaline aqueous solution [146] and found that dehydroascorbic acid converts to 2-(threo-1, 2, 3-trihydroxy-propyl) tartronic acid. Niemelä [104] used GC-MS to detect over 50 compounds, of which 32 carboxylic acids could be identified during oxidative and non-oxidative alkali-catalysed degradation.

The purpose of this study was to monitor any degradation products formed in the solid phase during storage.

Experimental

Methods

1) Sample Preparation

The sample preparation was described previously, Section 5.1.

2) Analysis

This study investigated the sample analysis for the following degradation products;

i) carbonyl compounds;
ii) furan related compounds;
iii) coloured compounds;
iv) evolution of gases/volatile compounds.

Equipment

Hewlett Packard HP1090M with diode array detection and ChemStation.
Lichrospher 100 RP C18e (25 x 0.46 cm, 5 μm) column (Merck).
Hewlett Packard 5890 GC fitted with a Carbowax 20M WCOTcapillary column (30 m x 0.22 mm, 0.15 μm film) and FID detector.

Carlo Erba HRGC Mega 2 fitted with a GS-Q megabore fused silica column (30 m x 0.538 mm, thick film) and thermal conductivity detector

i) Carbonyl compounds

Any carbonyl compounds formed during the degradation of L-ascorbic acid were converted into their 2,4-dinitrophenylhydrazones and were separated by reverse-phase HPLC using a water-acetonitrile gradient.

Derivatizing Reagent

The reagent was prepared as a solution of 5 g 2,4-DNP in 60 ml orthophosphoric acid, 85%, and 40 ml ethanol [198].

Chromatography and Derivatization Conditions

Conditions were those described in Section 3.3.3.

Sample Preparation

The sample, 50 mg, was weighed into a 25 ml volumetric flask and dissolved in 1% w/v metaphosphoric acid, 10 ml, the reagent added, 1 ml, and mixed thoroughly. The flask was heated at 50°C for 3 hours then cooled to room temperature. Any precipitate was dissolved by the addition of the minimum volume of acetonitrile, typically 10 ml, the solution made to volume with deionized water and thoroughly mixed. A 5 µl aliquot was chromatographed on a Lichrospher RP 100 18e, 25 x 0.46 cm, 5 µm column with a flow rate of 1.5 ml/minute and detection at 350 nm. Separation of the derivatives was achieved using linear gradient elution from 25% acetonitrile acidified with 0.1% TFA to 65% acetonitrile over 20 minutes.

ii) Furan related compounds

Approximately 100 mg of each sample was accurately weighed into a series of 100 ml volumetric flasks and dissolved in 1% w/v aqueous metaphosphoric acid solution to produce 1 mg/ml sample solutions. A 5 µl aliquot of each solution was chromatographed using reverse-phase HPLC at a flow rate of 1.0 ml/minute on a Lichrospher RP 100 RP 18e, 25 x 0.46 cm, 5 µm column with gradient elution from 0% acetonitrile to 40% acetonitrile, acidified with 0.1% TFA, in 40 minutes and spectroscopic detection at 3 wavelengths.

The gradient was optimized by using a standard mixture of potential furan and furan type degradation products of L-ascorbic acid. The elution order and their retention times are listed, Table 32, Figure 80 illustrates a chromatogram.

Table 32: Elution Order and Retention Times of Some Furan and Furan Type Degradation Products of L-Ascorbic Acid

Peak No.	Compound	Retention Time (minutes)
1	L-Ascorbic acid	3.5
2	2 (5H) Furanone	6.6
3	2,5-Dihydrofuran	6.8
4	2,3-Dihydrofuran	7.1
5	γ-Butyrolactone	8.3
6	Furfuryl alcohol	13.9
7	Furfural	15.6
8	2-Furoic acid	16.6
9	Furoin	19.8
10	2-Acetylfuran	21.7
11	Furil	38.1

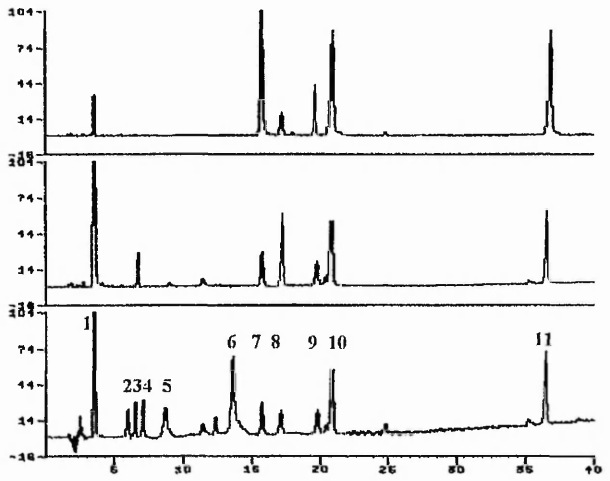


Figure 80: Mixed Furans and Furan - Type Compounds

iii) Coloured compounds

Approximately 50 mg of sample was accurately weighed into a 10 ml volumetric flask and dissolved in 10 ml 1% w/v metaphosphoric acid. A 10 µl aliquot was chromatographed on a Lichrospher RP 100 RP 18e, 25 x 0.46 cm, 5 µm column using linear gradient elution at 1.0 ml/minute. The mobile phase consisted of a propan-2-ol/water mixture with a gradient from 0% propan-2-ol to 40% in 5 minutes. The column eluent was monitored at 245 and 280 nm.

iv) Evolution of Gases/Volatile Compounds

Samples of L-ascorbic acid were triturated with water to produce samples at 5% v/w water. The samples were placed in Pierce Reactivials and each vial sealed with a PTFE septum and screw cap. The vials were weighed and placed on storage at 60°C for 42 days. After removal from storage and equilibriation to room temperature the vials were connected to a manometer and the volume of gas produced on storage measured. A 100 µl aliquot of the head space above the samples was sampled using a gas syringe and chromatographed on a GS-Q, 30 m x 0.538 mm column at 30°C with a thermal conductivity detector at 200°C. After

release of pressure the vials were reweighed in order to determine the weight loss on storage. A calibration graph was constructed for carbon dioxide and found to be linear over the range 0.045 μmol to 4.5 μmol.

Results and Discussion

i) Carbonyl compounds

No extra peaks were observed in the chromatograms for any sample at all time points (0, 7, 14, 28 and 42 days). There was no evidence of any compounds being formed which would derivatize with 2,4-DNP for any sample. All the chromatograms were similar for all samples and conditions at all time points.

ii) Furan related compounds

No additional peaks were observed in the chromatograms for any sample and any time point.

iii) Coloured compounds

Eight different degradation products were detected in the most severely degraded samples, Figure 81.

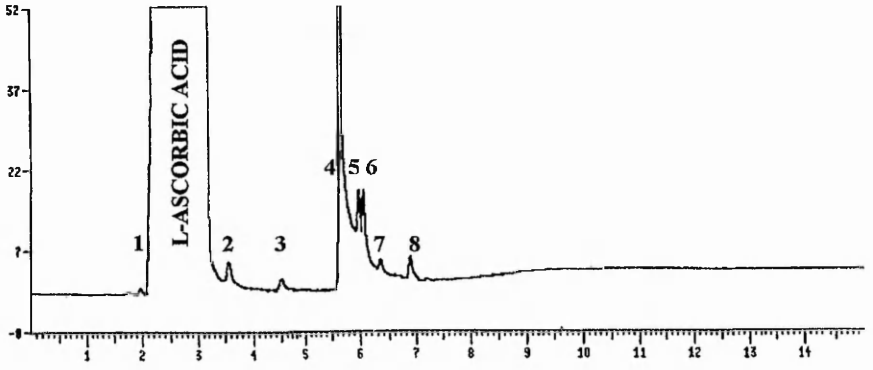


Figure 81: Chromatogram of L-Ascorbic Acid with 5% v/w Moisture Stored at 60°C for 42 Days

A minor peak eluted prior to the main L-ascorbic acid peak and the other components within eight minutes run time. Samples that were visually only slightly discoloured exhibited the peak (4) eluting at 5.5 minutes and the two peaks eluting on its downslope (5, 6). Results for the samples stored for 42 days at 50°C and 60°C are given in Table 33. The effect of the presence of moisture on the formation of the coloured degradation compounds can be seen with an absence of all peaks in the sample with no moisture present and a steady increase in levels with increasing moisture content. The influence of temperature is also illustrated with the 5% moisture samples stored at 50°C and 60°C. The total coloured impurities, expressed as a percentage of the total peak area, correlate closely with assay results for L-ascorbic acid. A relative response factor of 5.47, with respect to L-ascorbic acid at 280 nm, was calculated for the coloured compound, peak 4, using a known weight of the purified main coloured compound. This response factor was applied to all the eight peaks detected by this chromatographic screen.

Table 33: Comparison of Levels of Coloured Compounds and Assay of L-Ascorbic Acid

Peak Number	Moisture (% v/w) / Storage Temperature					
	0% 50°C	1% 50°C	2% 50°C	5% 50°C	10% 50°C	5% 60°C
1	-	-	0.03	0.02	-	0.08
2	-	-	0.06	0.32	0.50	0.57
3	-	-	-	-	-	0.24
4	-	2.40	4.55	11.59	17.11	29.47
5	-	0.10	0.12	0.17	0.05	1.46
6	-	0.10	0.15	0.49	0.59	2.38
7	-	-	-	0.02	0.02	0.15
8	-	-	-	0.04	0.10	0.64
Total	-	2.60	4.91	12.65	18.37	34.99
Assay	101.37	98.68	97.27	93.97	80.44	67.65

The UV spectrum was similar for all peaks and characterized with a λ_{max} around 280 nm and a shoulder at 350 nm with additional absorbance to 600 nm, Figure 82.

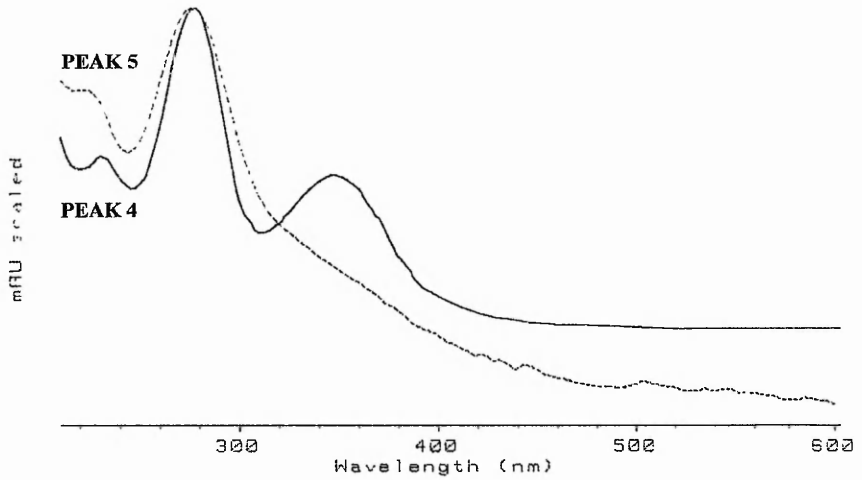


Figure 82: Spectra of Coloured Degradation Products of L-Ascorbic Acid

iv) Evolution of Gases/Volatile Compounds

Only carbon dioxide was detected by GC and the volume evolved during degradation was equivalent to 1 mole/mole L-ascorbic acid. An HP Ultra 1 12 m x 0.2 mm fused silica capillary column and FID detection was also used to detect volatile compounds. None were detected.

Conclusion

The effect of moisture on the solid phase degradation of L-ascorbic acid produced discolouration of the samples. Suprisingly, the samples failed to form derivatives with 2,4-DNP indicating an absence of carbonyl compounds. Furans and furan related compounds were undetected using a HPLC screen developed for the detection of these compounds. This is in contrast to heat degradaded solutions of L-ascorbic acid which degrade to furans which are detected on the HPLC screen.

Coloured compounds were detected using a water/propan-2-ol based reverse phase HPLC system. This chromatographic system could be used as an early indicator of the onset of degradation. The UV spectra of the compounds suggested they were carbonyl in nature although they failed to react with 2,4-DNP. The shoulder with UV spectrum at 350 nm and further absorbance down to 600 nm would also indicate that the compounds are highly conjugated systems.

Carbon dioxide was evolved during the degradation equivalent to a 1:1 molar ratio which may suggest that some degradation products could be based on a five carbon skeleton (cyclic) compound.

This work demonstrates that degradation of L-ascorbic acid in the solid state is significantly different to that in solution as furans were not detected or other volatile compounds produced. Carbon dioxide was evolved during the solid state degradation in stoichiometrically different proportions to that produced when degraded in solution.

5.4 Identification of the Discoloured Degradation Products

The influence of moisture on the solid phase degradation of L-ascorbic acid and the chemical characteristics of the degradation products has been investigated previously, Section 5.2 and 5.3. Moisture induced degradation in the solid phase leads to severe discolouration. A reverse-phase HPLC screen resolved up to eight different degradation compounds that could be responsible for the discolouration of degraded samples. The major degradation compound resolved by the screen was present at 29%. This degradation product was isolated and chemical and elemental analysis performed as described in this section.

Periodate consumption was measured in order to determine the ease at which it is oxidized and to estimate the number of hydroxyl groups present per moiety. The cleavage of α-glycol groups by sodium or potassium metaperiodate in aqueous solution produces two aldehyde groups. Other groups are also oxidized and include α-hydroxyaldehydes and α-hydroxyketones. Compounds containing three hydroxyl groups on adjacent carbon atoms consume two moles of periodate and are cleaved to yield two aldehyde groups and one mole of formic acid. If a primary alcohol group is oxidized formaldehyde is produced which may be estimated and used to determine the number of primary hydroxyl groups present. For cyclic compounds, *cis* α-glycols react more rapidly than trans isomers and α-glycols with the hydroxyl group in the diaxial position are not oxidized at all.

The hydroxyl group content was estimated by first acetylating the free hydroxy groups and then determining the acetate content by the Kuhn-Roth method. The carbonyl content was similarly determined after first reducing the carbonyl groups to hydroxyl, the difference between the two values is a measure of the carbonyl content.

Pyrolysis at two temperatures followed by gas chromatography of the pyrolysates was used to gain an insight into the backbone structure of the compound. The compound(s) maybe high molecular weight substances which can be fragmented by

pyrolysis into smaller repeating units which are easier to identify than the parent compound.

This study investigated the isolation of the degradation products responsible for the discolouration and their chemical characterization. A possible structure and degradation pathway is proposed.

Experimental

1) Isolation of Coloured Degradation Compound

The preparation of solid state degraded samples of L-ascorbic acid has previously been described, Section 5.2. The sample exhibiting the most extensive degradation, that with 5% v/w moisture stored at 60°C for 42 days, was chosen for the isolation of the major coloured degradation compound.

Undegraded L-ascorbic acid and water soluble degradation products were removed by repeated extraction with water until the aqueous extract was colourless. The aqueous extract was chromatographed to confirm the absence of L-ascorbic acid. The dried water insoluble compounds were extracted with DMSO until the extracts were colourless. The remaining solid material was washed with dichloromethane to remove residual DMSO then dried under vacuum overnight at 50°C. Yield was 1.7 g from 10.0 g starting material.

2) Analysis

The isolated coloured compound was subjected to the following analyses:

i) periodate consumption;
ii) estimation of hydroxyl and carbonyl group content;
iii) pyrolysis at 300°C and 500°C;
iv) further methods of analysis.

Equipment

Perkin Elmer DSC7 differential scanning calorimeter.
Shimadzu GC-17A with AOC-1400 autosampler and AOC-17 autoinjector fitted with a Carbowax 20M WCOT (25 m x 0.22 mm, 0.15 μm film) or HP1 WCOT (30 m x 0.53 mm x 0.365 μm film).
Carlo Erba CHNS-O EA 1108 Elemental Analyzer.
Perkin Elmer FT-IR, model PE System 2000 FTIR.
Metrohm 684 KF Coulometer Karl Fischer instrument.

i) Periodate Consumption

The sample (35 mg) was accurately weighed into a 200 ml volumetric flask and suspended in ethanol (80 ml). Water (80 ml) was added and the mixture left to stand at room temperature overnight. An aqueous solution of sodium periodate (0.5 M, 40 ml) was added and the mixture diluted to volume (200 ml) with ethanol. A blank reaction was performed omitting the sample. The periodate content was determined initially and at intervals for up to 7 days titrating a 10 ml aliquot against sodium arsenite solution (0.05 M). Any free acid liberated was determined by titrating a 20 ml aliquot against 0.01 M NaOH with phenolphthalein as indicator. An aliquot of sample and blank solutions was chromatographed by GC and any formaldehyde and formic acid determined.

ii) Estimation of hydroxyl and carbonyl group content

The hydroxyl group content was evaluated by first acetylating the sample then determining the acetyl group of the acetate product by the Kuhn-Roth method. The sample (50 mg) was dissolved in an equimolar mixture of acetic anhydride in pyridine (6 ml) and reacted for 30 days at 25°C. The reaction was quenched by the addition of water (200 ml) and extracted with chloroform (3 x 15 ml). The chloroform extract was washed with water (3 x 20 ml) and dried over anhydrous sodium sulfate. Evaporation yielded the acetate which was quantified using the

Kuhn-Roth method. This method involves refluxing the acetate with chromic acid followed by distillation of the free acetic acid. Titration of the acid with dilute aqueous sodium hydroxide solution using phenolphthalein indicator enables the acetate content to be calculated.

The carbonyl group content was determined by the same method but first reducing any carbonyl group present by hydrogenation. The sample (50 mg) was dissolved in 50% aqueous ethanol (30 ml) and the solution adjusted to pH 8-9 by the addition of pyridine (3 ml). Sodium borohydride was added (200 mg) and the mixture reacted for 18 hours at room temperature. The mixture was evaporated to dryness and the residue suspended in water, separated by centrifugation and dried. Acetylation of the reduced compound was carried out as per the estimation of the hydroxyl group. The difference between acetyl groups before and after reduction correspond to the number of carbonyl groups present.

iii) Pyrolysis at 300°C and 500°C

A 200 mg sample was pyrolyzed at 300°C and 500°C for 30 minutes under a stream of nitrogen using a Perkin Elmer DSC7 differential calorimeter. Any volatile compounds evolved were trapped in a series of three traps, an empty tube cooled in dry ice/propan-2-ol followed by a cold water trap (2 ml) and finally an ice cold dichloromethane trap (2 ml). The weight loss at each temperature was recorded. After pyrolysis the volume of water condensed in the empty tube was measured and the volume adjusted to 2 ml with water before chromatography. Chromatography of the pyrolysates was achieved on a Carbowax 20M WCOT column with a temperature ramp from 50°C to 250°C at 10°C/minute.

iv) Further Methods of Analysis

a) Determination of the carbon, hydrogen and oxygen content using a Carlo Erba elemental analyzer.

b) Measurement of the pH of a 1% w/v suspension in carbon dioxide free water.

c) Thermal analysis including TGA in order to determine the type of weight loss and DSC to monitor for any transitions occuring during heating.

d) Acid and base hydrolysis followed by HPLC analysis using the chromatographic screen described in Section 5.2.

e) Infra-red spectrum obtained as a KBr disk.

f) Moisture determination using a coulometric Karl Fischer instrument.

g) Attempts were made to obtain proton and ^{13}C nuclear magnetic spectra in both DMSO and NaOD (solvents in which the solid was most soluble).

Results

i) Periodate Consumption

Periodate consumption was observed to increase slowly for 22 hours followed by a rapid acceleratory period then a gradual uptake to 166 hours, periodate consumption was still occuring beyond 166 hours. The initial slow consumption was possibly caused by the slow dissolution followed by reaction of the material as the initial suspension slowly dissolved followed by rapid oxidation of the dissolved material. Titration against 0.01 M NaOH solution consumed 2.0 ml more than a blank reaction solution. GC chromatography failed to detect any formaldehyde or formic acid in the sample solution. The absence of these compounds suggests that any alcohol groups present are not on adjacent carbon atoms or primary alcohols.

ii) Estimation of hydroxyl and carbonyl group content

Carbonyl group content was calculated assuming complete conversion of any carbonyl groups to hydroxyl groups, Table 34.

Table 34: Hydroxyl and Carbonyl Content of Degradation Product

Sample	Acetyl, % w/w	Hydroxyl / unit	Carbonyl / unit
Reduced	25.48	0.43	0.24
Unreduced	10.99	0.19	-

iii) Pyrolysis at 300°C and 500°C

The weight loss was 30.34% w/w at 300°C and almost doubled to 59.02% w/w at 500°C. Water condensed in the first trap, a dry condensing trap, and was found to be 200 µl at 300°C and 100 µl at 500°C. GC of the pyrolysates at both temperatures were very similar with a higher concentration of components found in the 500°C traps. All further studies were carried out on the 500°C pyrolysates. The dry trap gave eight peaks due to the condensate eluting between 12 minutes and 19.0 minutes, Figure 83. The two major peaks (peaks 4 and 5) had retention times of 14.4 and 15.1 minutes respectively. These peaks were also present in the water trap. In total the water trap condensate contained nine components of which two were major (peaks 2 and 5) eluting at 2.9 minutes and 14.4 minutes, Figure 84. The dichloromethane trap contained no additional peaks to those present in a blank injection. Much more volatile material condensed on the inside of the DSC pan heater. This condensate was dissolved in a mixture of 1:1 methanol / dichloromethane (2 ml) and when chromatographed was found to consist of seven major higher boiling components not present in the three pyrolysate traps, Figure 85, none of which were identified.

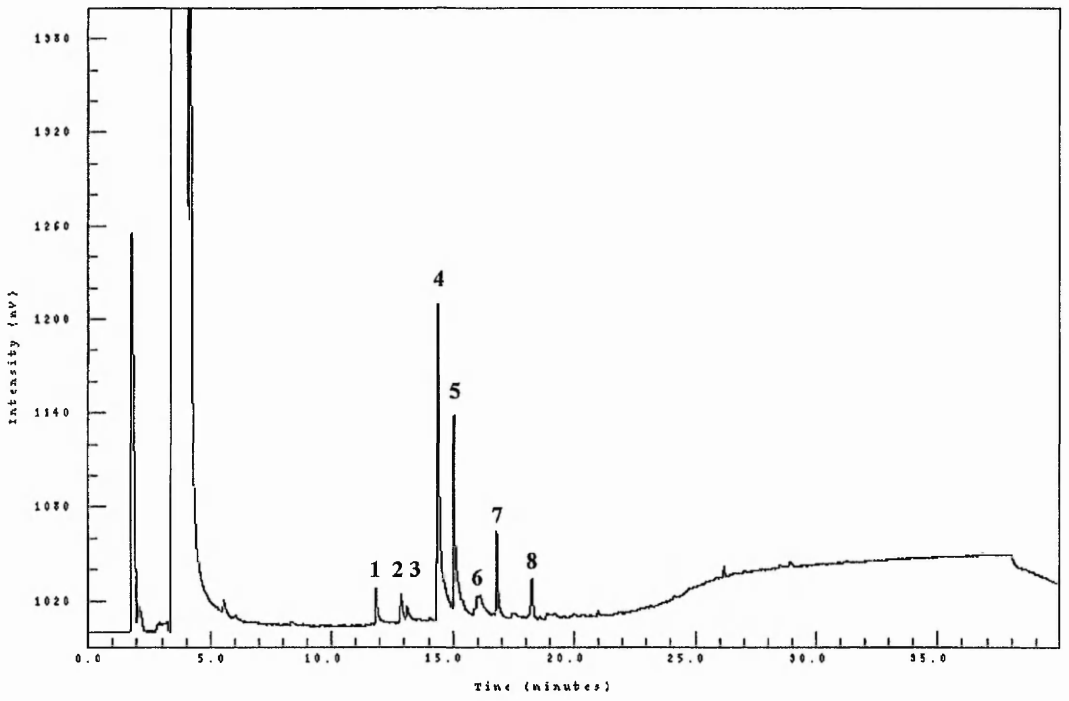


Figure 83: Chromatogram of Pyrolysate in Dry Trap

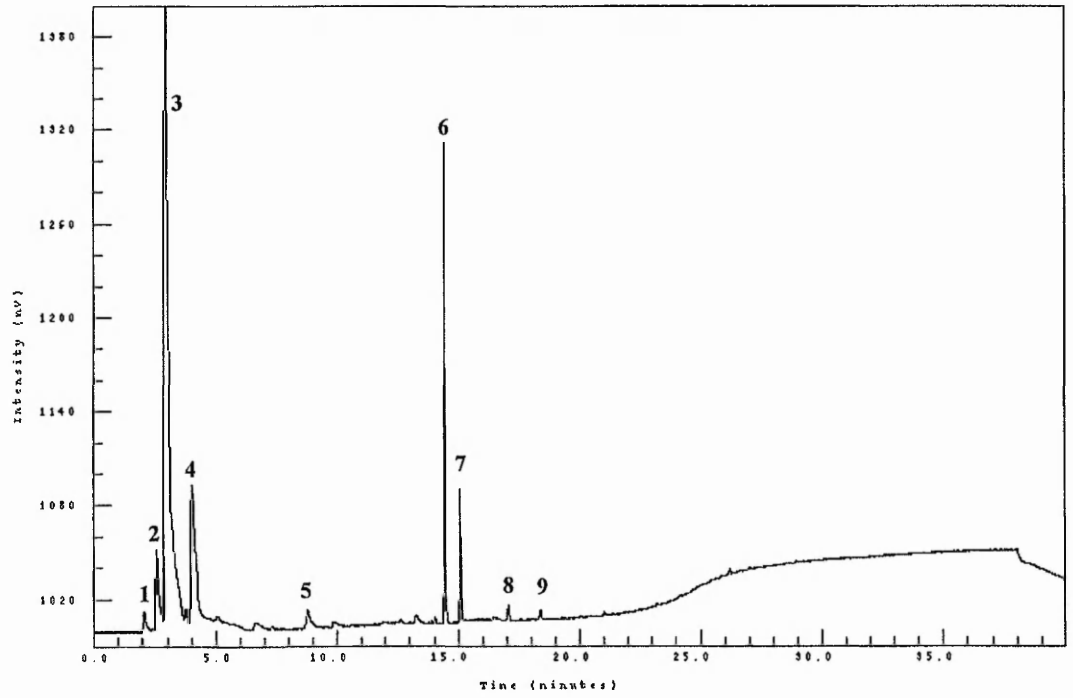


Figure 84: Chromatogram of Pyrolysate in Water Trap

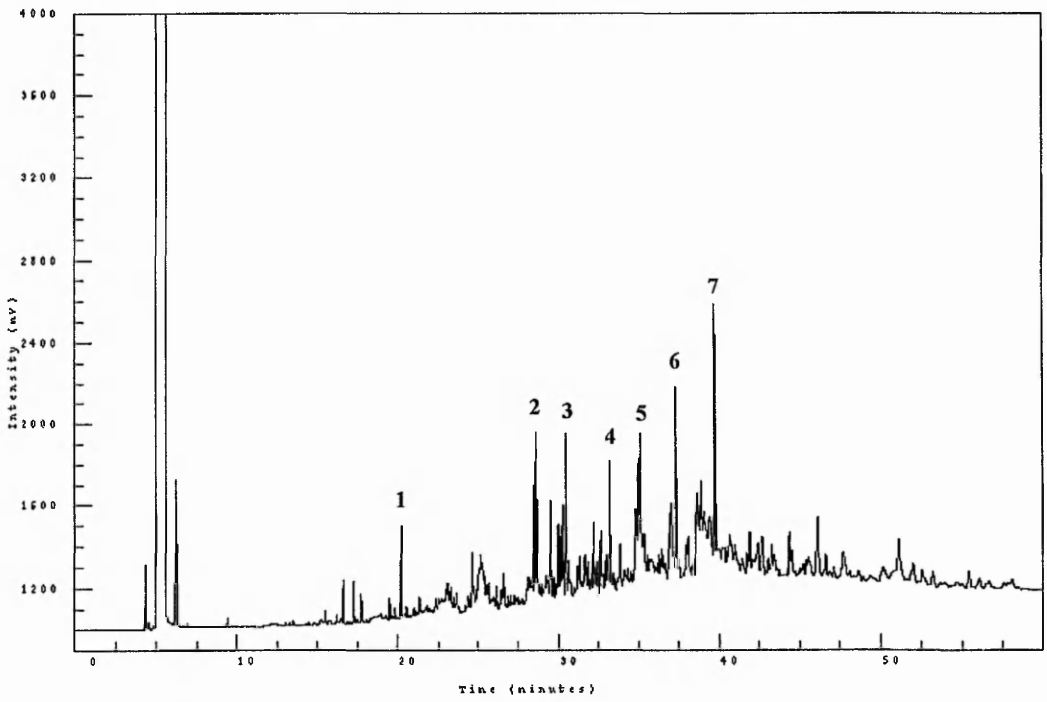


Figure 85: Chromatogram of Condensate from Pyrolysis

GC-MS of the main peaks in the dry trap identified peak 1 as 2-furancarboxylic acid, peak 4 as 1,1'-(1-methyl-1,2-ethenediyl)bis-benzene, peak 5 as 2-phenyl-2,3-dihydroindene and peak 8 as 1-methyl-2-(2-phenylethenyl)benzene. The first three peaks in the water trap were due to the solvent and, peak 4 was identified as 2,3-dimethylbutane, peak 5 as 3-methylpentane, peak 6 as 1-(2-furanyl)-ethanone, peak 7 as 2-furancarboxaldehyde and peak 8 as 1-(2-furanyl)-1-propanone.

The presence of furan related compounds in the pyrolysates can be accounted for by dehydration and decarboxylation giving rise to 2-furancarboxaldehyde [105]. Once formed the 2-furancarboxaldehyde could react as a conjugated diene and undergo a Diels-Alder reaction involving the 1,4-addition of an unsaturated furan (dienophile) to a conjugated diene. This reaction between two furan compounds would form an oxo-bridged six membered ring which upon pyrolysis would eliminate water to produce a benzene ring, Figure 86.

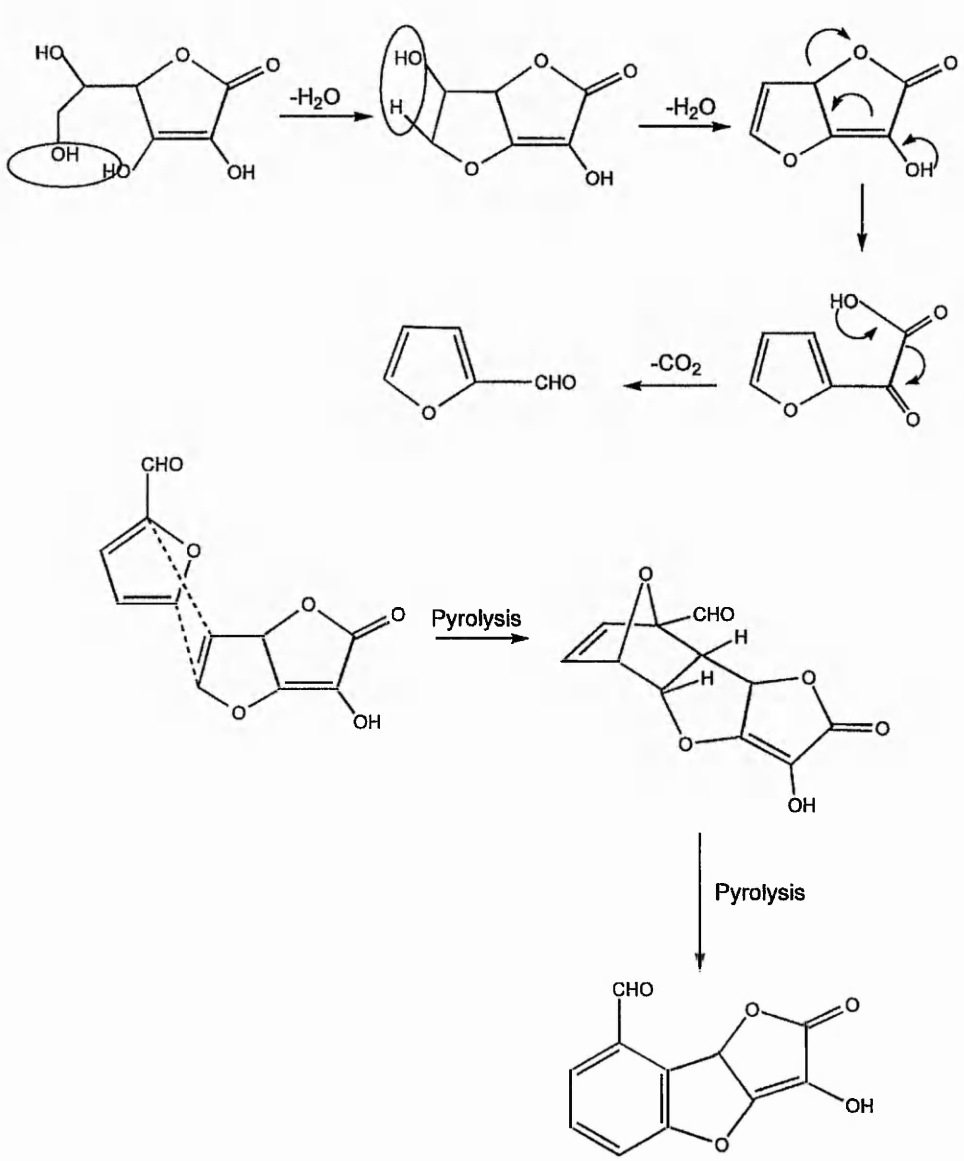


Figure 86: Possible Reaction Scheme for the Formation of Six Membered Aromatic Rings

iv) Further Analysis of the Main Coloured Degradation Product

Elemental analysis of the isolated main coloured degradation product gave 46.26% carbon, 5.42% hydrogen and 48.32% oxygen (by difference to 100%), empirical formula CH_2O. Moisture was determined at 2.61% w/w.

The pH of a 1% w/v aqueous suspension was found to be 2.78, identical to that for L-ascorbic acid.

Proton and ^{13}C NMR were inconclusive due to lack of signal. Proton NMR in dimethylsulphoxide gave very weak quartets at 6.7 ppm and 8.0 ppm which could be due to a heterocyclic aromatic ring.

Thermal analysis by TGA showed a steady loss between 30°C and 150°C with a plateau to 200°C (total loss 9.60%) followed by a rapid loss presumably due to decarboxylation, Figure 87.

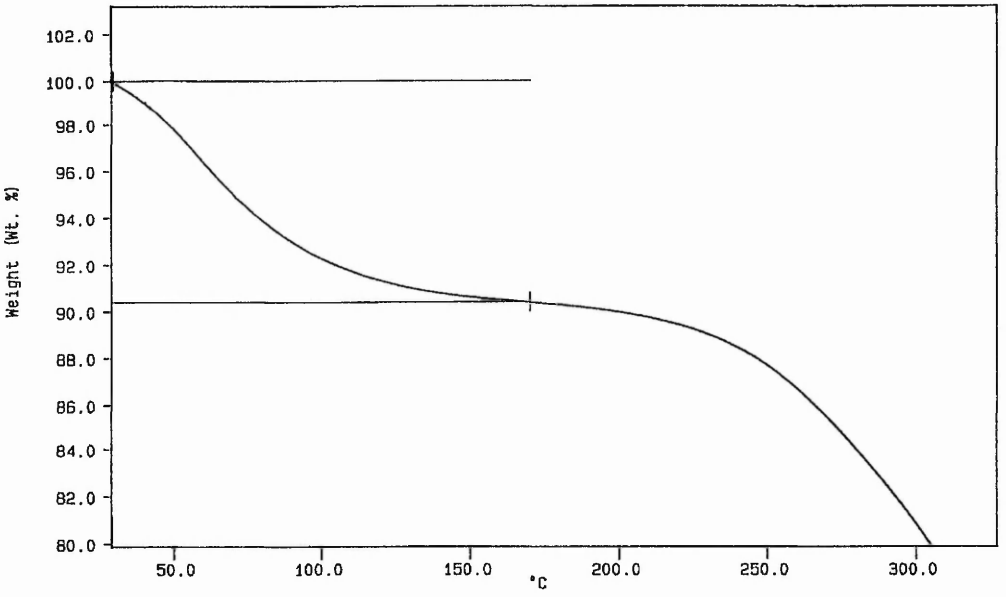


Figure 87: TGA Thermogram of Coloured Degradation Product

DSC analysis gave one major endotherm at 137.9°C to 195.8°C with a maximum at 141.5°C, ΔH = 135.41J/g. Small endothermic events were observed prior to the major endotherm between 121.4°C and 136.4°C with a maximum at 129.5°C, ΔH = 3.60 J/g, Figure 88.

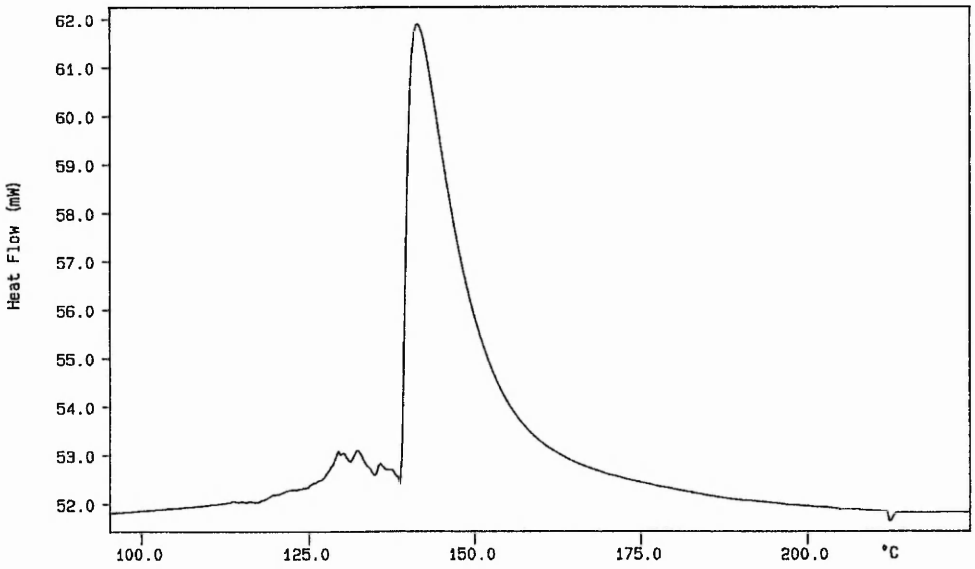


Figure 88: DSC Thermogram of Coloured Degradation Product

When dissolved in 0.2M NaOH solution and chromatographed four peaks eluted which were poorly resolved. All peaks had similar spectra (λ_{max} at 280 nm with a shoulder at 340 nm), Figure 89. Two were major peaks with 53.0% and 37.3% of the total area and the other two were minor peaks at 7.1% and 2.6%. After isolating these components by semi-preparative HPLC and using the same HPLC system as above each individual component eluted at the same retention time indicating that interconversion to the same compound could have taken place.

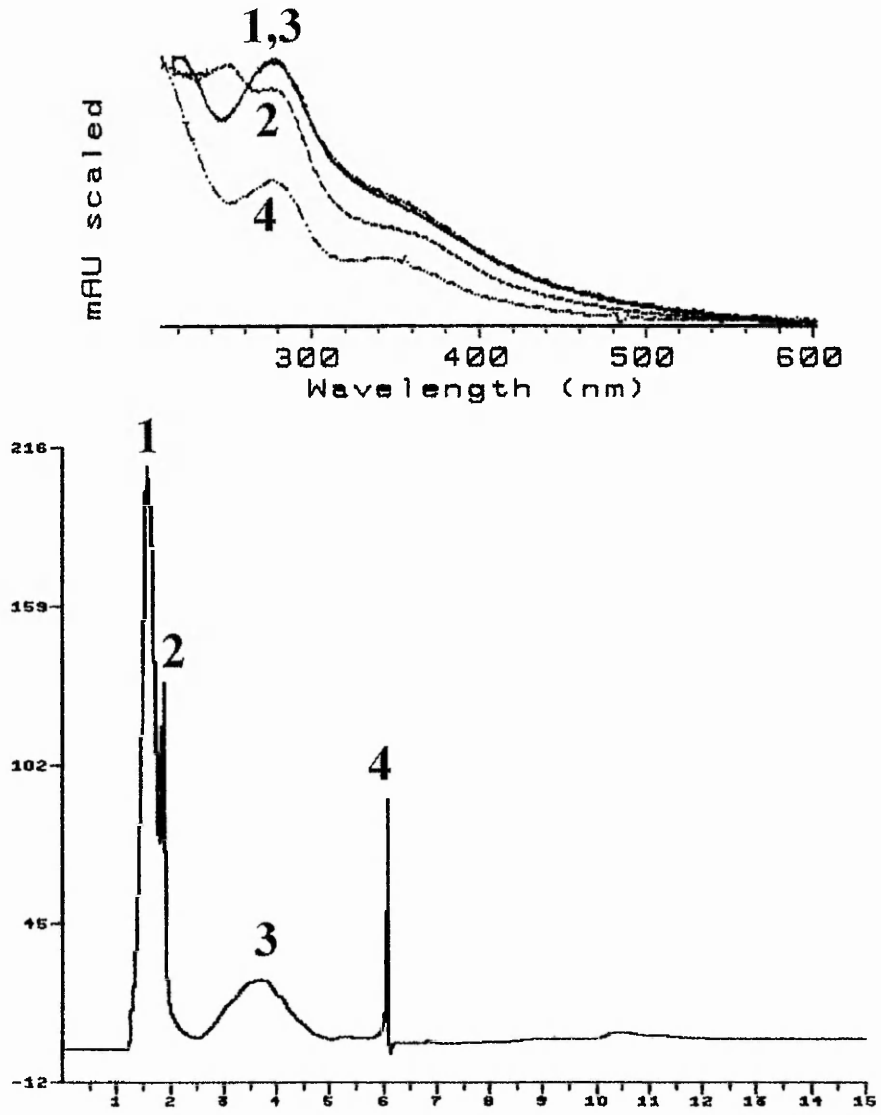


Figure 89: Chromatogram of Coloured Degradation Product after Base Hydrolysis

The infra-red spectrum, Figure 90, shows a strong broad band at 3428 cm^{-1} which may be due to O-H stretch. Noticibly absent are the four bands between 3220 and 3525 cm^{-1} which are present in L-ascorbic acid and caused by the high frequency hydrogen bonded O-H stretching bands on C-5 and C-6. A sharper band at

2924 cm^{-1} is assigned to C-H stretch of alkyl groups. The region between 1800 and 1600 cm^{-1} is different from that in L-ascorbic acid with the strong absorption at 1754 cm^{-1} in L-ascorbic acid attributed to C = O stretching of the lactone ring now coupling with C = C stretching vibration. The fingerprint region in the coloured degradation product is ill defined compared to L-ascorbic acid. The absence of detail may be attributed to loss of the hydroxy group on C-2 of L-ascorbic acid which gives rise to the strong, sharp band at 1320 cm^{-1} and 1275 cm^{-1} and the absence of a strong band at 1140 cm^{-1} may be due to loss of C-5 O stretch.

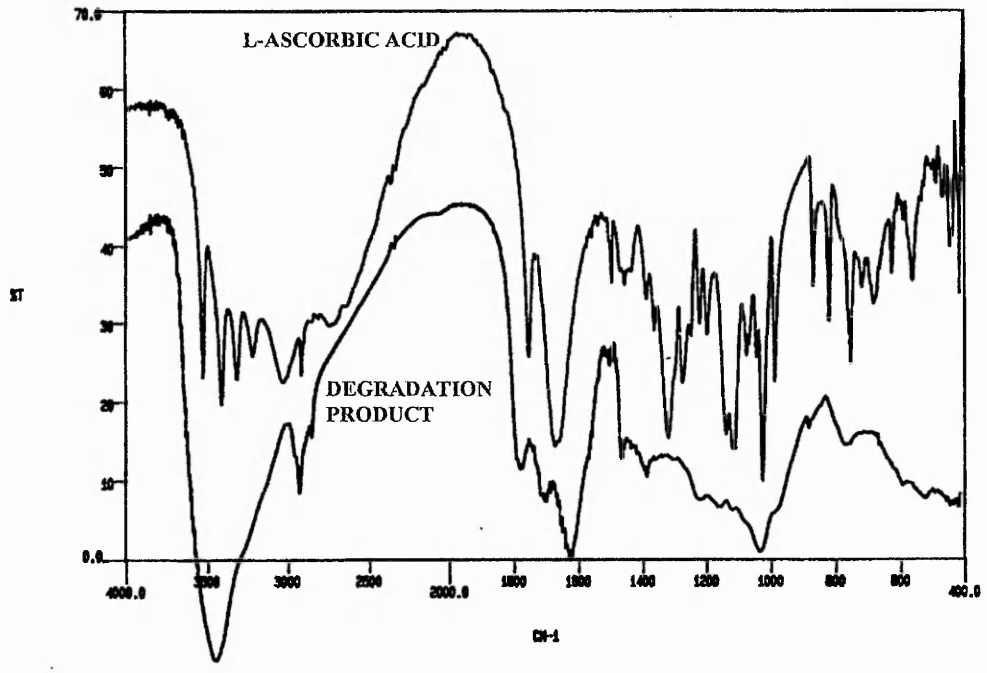
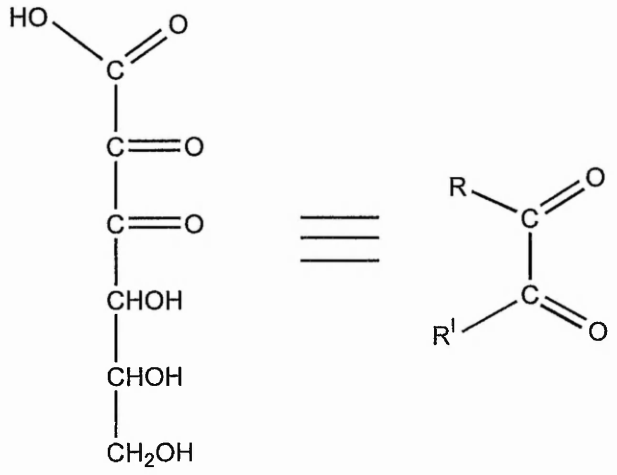


Figure 90: Infra-Red Spectrum of L-Ascorbic Acid and the Coloured Degradation Product

No dehydroascorbic acid was found during the solid phase degradation of L-ascorbic acid, Section 5.2 . Due to the chemical instability of dehydroascorbic acid it may have been formed and immediately ring opened to give 2,3-diketogulonic acid (2,3-DKGA) which then reacted as a diene with L-ascorbic acid, Figure 91. This would account for the apparent absence of dehydroascorbic acid.



2,3-DKGA

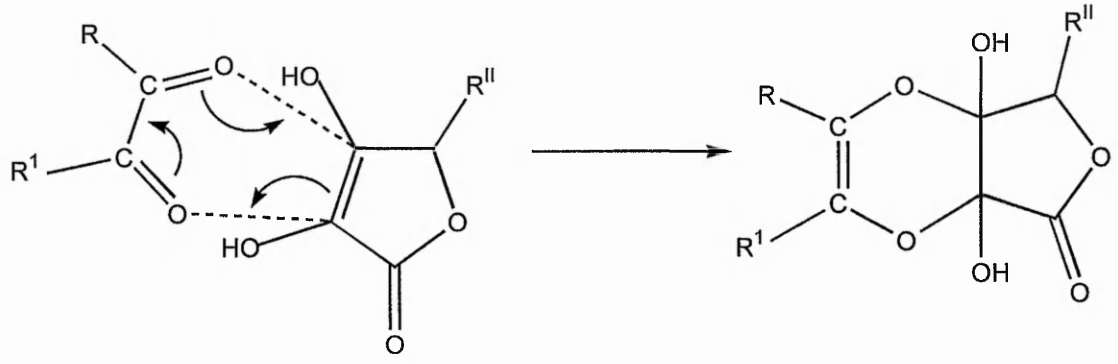


Figure 91: Possible Reaction Scheme for Formation of Six Membered Aromatic Rings

From the chemical and spectral information obtained from this work it is suggested that the coloured degradation product is possibly polymeric and the structure made up of units of polymeric furan related compounds, Figure 92, polymerized with the structure in Figure 91. Structures A and B arise from electrophilic attack at α and β possitions respectively.

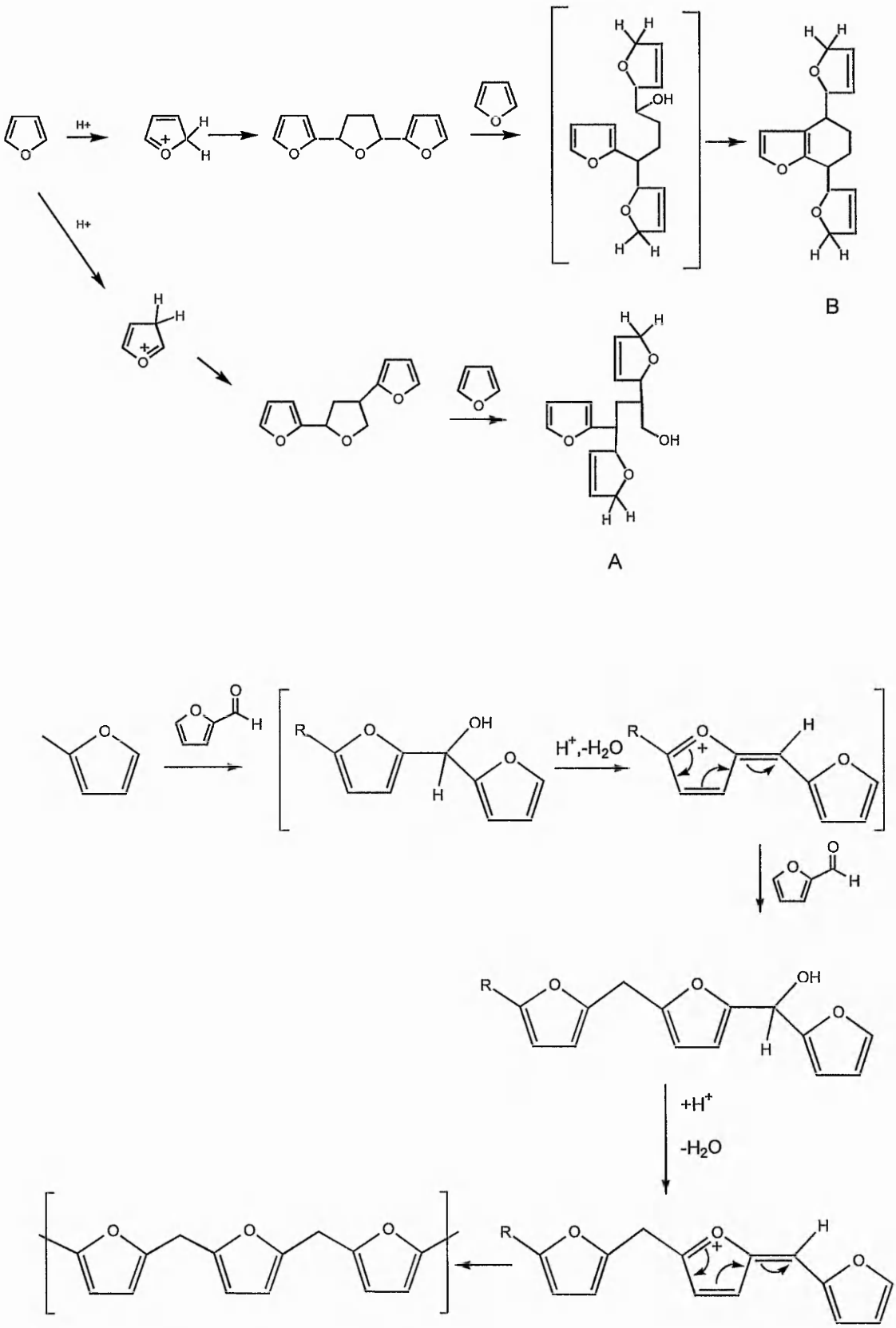


Figure 92: Possible Furan Polymeric Units

Conclusion

Eight different degradation compounds were found to be present in a severely discoloured sample, Section 5.2. Three of the compounds were present at levels above 1% of the total peak area with the major degradation peak present at 29% in a sample with 5% v/w moisture present stored at 60°C for 42 days. The major impurity was isolated 98.7% pure and was found to exhibit a λ_{max} at 280 nm with further absorbances to 600 nm.

The material was pyrolyzed at 300°C and 500°C. The volatile pyrolysates furfural, 2-furancarboxylic acid, 1-(2-furanyl)-ethanone and 1-(2-furanyl)-1-propanone were tentatively identified along with certain aromatic six membered ring compounds.

The periodate consumption was examined and the products of the reaction investigated. Estimation of hydroxyl and carbonyl group content by acetyl group determination of the acetate and reduced acetate found that for every five repeating units there was one hydroxyl group and for every four repeating units there was one carbonyl group.

Elemental analysis gave 46.26% carbon, 5.42% hydrogen and 48.32% oxygen, the empirical formula was CH_2O.

CHAPTER 6

The Stabilization of L-Ascorbic Acid in the Solid Phase

6.1 Introduction

L-Ascorbic acid has been found to degrade in the presence of moisture. The degradation could be catalyzed by metal ions such as iron and copper impurities present as impurities in trace amounts. If degradation occurs via a carbonyl fuctional group i.e. oxidation leading to the formation of aldehydes and ketones then addition of the metabisulfite anion should form bisulfite addition compounds thus arresting further degradation [215].

The use of chelating agents to effectively remove any impurity cations acting as catalysts and the metabisufite anion were investigated in this study to improve the stability of L-ascorbic acid solid in the presence of moisture. Only chelating agents and stabilizing agents suitable for use in foods and pharmaceutical products were studied.

6.2 Analysis of L-Ascorbic Acid for Cations

In order to ascertain the level of cations in typical samples of L-ascorbic acid samples were purchased from three different suppliers and analysis carried out by inductively coupled plasma (ICP) for 28 elements.

Experimental

Samples were purchased in different grades of chemical purity from three different suppliers;

Sigma Chemical Company, as analytical reagent grade;
Aldrich Chemical Company, as British Pharmacopæia grade;
Fisher Scientific (UK), as analytical reagent grade.

Sample Preparation

Approximately 1g of each sample was accurately weighed and dissolved in purified water (10 ml). A sample of the water was also submitted for analysis.

Analysis

Analysis was carried out on an ARL instrument, model 3580 ICP which can determine up to 28 elements simultaneously. The sample solutions were aspirated directly into an Argon plasma at 8000°C. Mean values for the water control were subtracted from the sample results and quoted in parts per million (ppm), Table 35.

Results and Discussion

Table 35: Results of ICP Analysis for Three Batches of L-Ascorbic Acid (ppm)

Element	Sigma	Aldrich	Fisher
Ag	0.0128	0.0090	0.0130
Al	0.7392	0.7922	0.8354
As	0.0975	0.0692	0.0696
Ba	0.0144	0.0061	0.1824
Ca	1.6185	0.4326	0.8635
Cd	0.0150	0.0152	0.0153
Co	0.0318	0.0321	0.0323
Cr	0.0327	0.0330	0.0332
Cu	0.1115	0.0711	0.0621
Fe	0.1754	0.1981	0.1017
Hg	0.0292	0.0294	0.0296

Table 35 Continued

Element	Sigma	Aldrich	Fisher
K	0.8188	0.3547	0.7897
Li	0.0124	0.0054	0.0078
Mg	0.2276	0.0639	0.2033
Mn	0.0118	0.0095	0.0017
Mo	0.0219	0.0221	0.0222
Na	4.3656	1.0649	7.1217
Ni	0.0468	0.0473	0.0476
P	0.8694	0.9233	0.7635
Pb	0.2209	0.2230	0.2245
S	0.0707	0.0714	0.1539
Se	0.5213	0.5264	0.5299
Si	0.0530	0.0535	0.0539
Sn	2.7261	2.8436	2.7452
Sr	0.0104	0.0047	0.0116
Ti	0.0408	0.0412	0.0377
V	0.0390	0.0105	0.0106
Zn	0.0053	0.0054	0.0093

The only elements present at > 1 ppm were calcium, sodium and tin, none of these elements are known catalysts for the degradation of L-ascorbic acid. All samples were very similar in impurity profile with only sodium being significantly different.

Conclusion

It may be possible that transition metals, even in the insignificant quantities found,

could catalyze the degradation of L-ascorbic acid. Therefore a study was undertaken to determine the effect of formulating L-ascorbic acid with chelating agents to effectively remove any metal ion from the reaction.

6.3 Stabilization with Chelating Agents and Sodium Metabisulfite

Chelating agents can be used to form complexes with metal ions and effectively prevent them from taking part in any further reactions. Transition metals are particularly good at forming complexes but certain chelating agents, such as diamino-ethanetetra-acetic acid, sometimes referred to as ethylenediaminetetraacetic acid (EDTA) can form complexes with almost every metal ion including sodium and lithium.

Chelating agents have the ability to form very stable complexes with most metal ions with the metal ion bound in a ring structure. Five or six-membered rings are the most stable and therefore the most useful chelating reagents are those which favour the formation of the maximum number of rings and fully saturate the co-ordination requirements of the ions in a one step reaction. EDTA is such a compound, Figure 93.

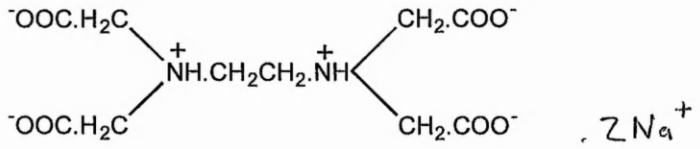


Figure 93: Structure of EDTA

Nitrilotriacetic acid (NTA) is probibly the next most important chelating agents after EDTA. The presence of a third acetic acid group renders NTA, Figure 94, more reactive towards cations in general than most other chelating agents.
NTA forms an appreciable complex with lithium and a strong one with sodium.

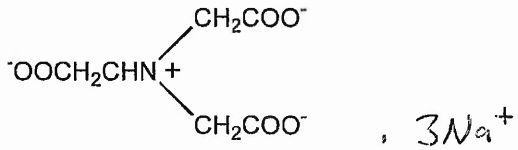


Figure 94: Structure of NTA

This investigation studied the use of these two powerful chelating agents alone and in combination with sodium metabisulfite. Sodium metabisulfite was also used formulated without a chelating agent to determine whether bisulfite addition to any carbonyl group was sufficient alone to reduce the extent of degradation and discoloration.

Experimental

The following solid phase formulations were prepared by triturating L-ascorbic acid with the reagent followed by the addition of water, triturating again in an agate mortar, to produce a formulation containing moisture at 5% v/w;

i) L-ascorbic acid + 0.1 M EDTA
ii) L-ascorbic acid + 1 M EDTA
iii) L-ascorbic acid + 0.1 M NTA
iv) L-ascorbic acid + 1 M NTA
v) L-ascorbic acid + 0.1 M sodium metabisulfite
vi) L-ascorbic acid + 1 M sodium metabisulfite
vii) L-ascorbic acid + 0.1 M sodium metabisulfite + 0.1 M EDTA
viii) L-ascorbic acid + 0.1 M sodium metabisulfite + 0.1 M NTA
ix) L-ascorbic acid as control

The colour of the samples was measured using a Trivector tristimulus colorimeter using the same procedure as used in Section 5.1. The mean value of four $L^*a^*b^*$ readings was taken and the total magnitude of colour difference, ΔE^*ab, calculated using Equation 18. A visual assessment of the sample appearance was also noted,

Tables 36 to 39. Each sample was placed in a Pierce Reactivial and sealed then placed in an oven at 60°C. The samples were removed from storage after 5, 10, 15 and 20 days and colour readings taken along with a visual assessment, Tables 36 to 39.

Table 36: Total Magnitude of Colour Change, ΔE*ab, for Formulations after 5 Days Storage at 60°C

Sample	ΔE*ab	Appearance
i	37.82	Brown powder
ii	32.41	Pale light brown powder
iii	35.98	As i
iv	32.04	Slightly paler than i & iii
v	30.54	Yellow hard foam
vi	14.36	Very pale straw colour, hard foam
vii	31.62	Brown/yellow soft foam
viii	30.04	Similar to v
ix	27.94	Pale brown, slightly darker than ii

Table 37: Total Magnitude of Colour Change, ΔE*ab, for Formulations after 10 Days Storage at 60°C

Sample	ΔE*ab	Appearance
i	40.83	Dark brown powder
ii	37.61	Light brown powder
iii	43.66	As i
iv	38.18	Slightly paler than i & iii
v	32.87	Yellow/brown hard foam
vi	17.33	Pale yellow hard foam
vii	37.69	Brown foam
viii	40.39	Similar to i
ix	33.25	Brown powder

Table 38: Total Magnitude of Colour Change, ΔE*ab, for Formulations after 15 Days Storage at 60°C

Sample	ΔE*ab	Appearance
i	41.47	Dark brown powder
ii	44.67	Slightly lighter than i
iii	47.30	Slightly darker than i
iv	43.13	As i
v	37.77	Yellow/brown hard foam
vi	21.77	Pale yellow hard foam
vii	40.83	Brown hard foam
viii	43.76	Very dark brown moist powder
ix	33.68	As ii

Table 39: Total Magnitude of Colour Change, ΔE*ab, for Formulations after 20 Days Storage at 60°C

Sample	ΔE*ab	Appearance
i	40.95	Dark brown powder
ii	46.17	Slightly lighter than i
iii	46.77	Very dark brown moist powder
iv	44.77	As iii
v	39.05	Yellow/brown hard foam
vi	24.56	Pale yellow hard foam
vii	41.13	Dark brown hard foam
viii	44.33	Similar to iii
ix	29.45	As ii

Discussion

There was good agreement between the visual appearance of the samples and the Δ E*ab value i.e. the more discoloured the sample the higher the Δ E*ab value. Plots of Δ E*ab values against time were variable with those formulations with 0.1 and 1 M sodium metabisulfite, v and vi, being linear, Figures 95 and 96.

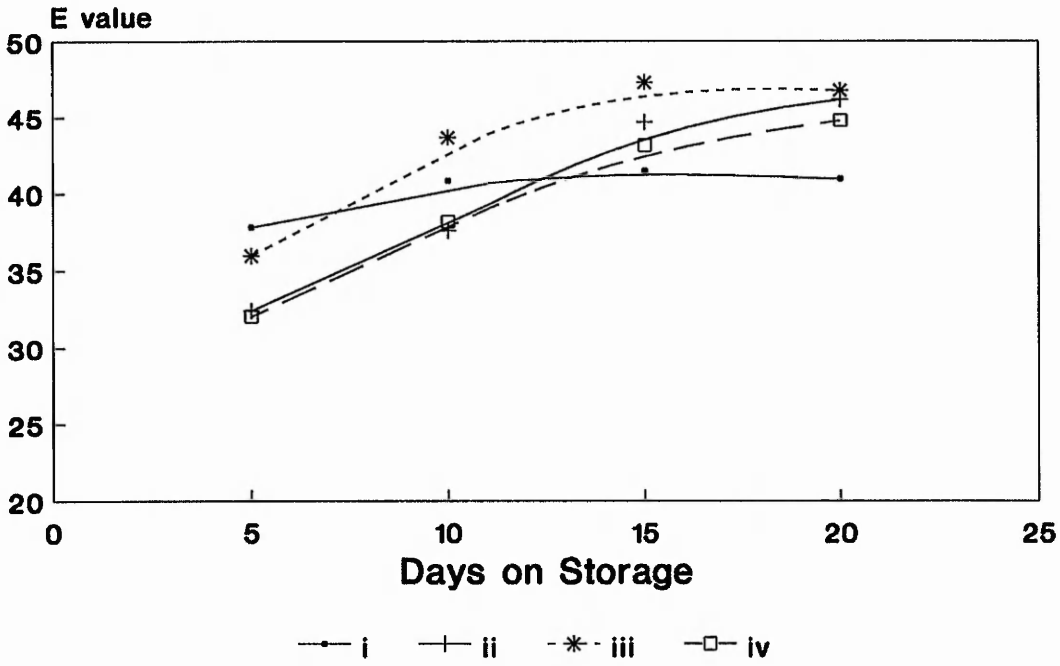


Figure 95: Curves for Formulations with EDTA and NTA

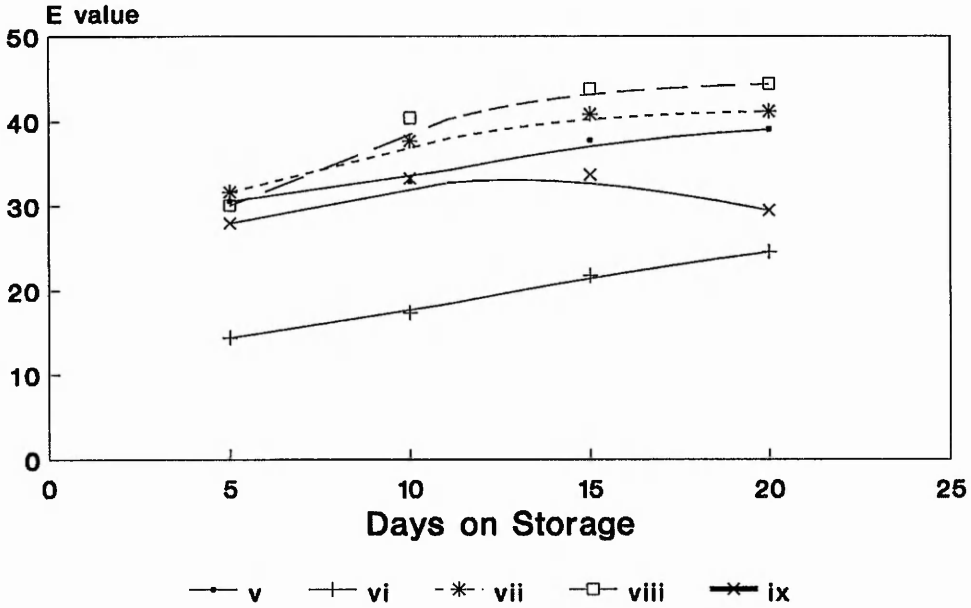


Figure 96: Curves for Formulations with Sodium Metabisulfite and Control

Changes in the magnitude of total colour difference were plotted against time and from the slopes of the curves rate constants, k, calculated, Table 40.

Table 40: Rate Constant for Formulations

Sample	k
i	0.2599
ii	0.4323
iii	0.8512
iv	0.5254
v	0.1152
vi	0.0133
vii	0.5770
viii	0.9528
ix	0.1224

Formulations containing either chelating agent (i to iv) discoloured considerably and were more discoloured than the control sample (ix) as shown by the higher rate constants. That containing 1 M NTA (iv) produced a hard foam indicating a gas or gases were evolved on storage.

Those formulations containing chelating agent and sodium metabisulfite (vii and viii) discoloured to a similar degree as those formulated with chelating agent only.

Formulations with 0.1 and 1 M sodium metabisulfite (v and vi) both produced a foam and smelled of sulfur dioxide. The formulation with 1 M sodium metabisulfite (vi) discoloured the least, shown by the lowest rate constant. This formulation was assayed for remaining L-ascorbic acid by HPLC which showed only 23.3% remained of the original level. When chromatographed on a reverse-

phase stationary phase using gradient elution from 0 to 65% v/v acetonitrile for degradation and any bisulfite adducts (λ at 320 nm) no additional peaks to that for L-ascorbic acid were observed.

Conclusion

The presence of the chosen chelating agents increased the extent of discoloration over that of a control as shown by higher rate constants.

Sodium metabisulfite did reduce the extent of discoloration but severe loss of L-ascorbic acid was recorded. When chromatographed on a HPLC gradient no additional impurity peaks were observed. It was conceivable that the L-ascorbic acid had degraded to such an extent that only oxalic acid remained which would not have been detected using the chromatographic conditions used. Carbon dioxide was almost certainly liberated, along with sulfur dioxide which could be smelled, but was lost during storage.

The value of tristimulus colorimetry was demonstrated for quantifying the extent of discoloration and determining rate constants.

Summary of Conclusions

Insignificant levels of transition metals were found in three batches of L-ascorbic acid from three different suppliers.

Chelating agents increased the rate of discoloration of L-ascorbic acid containing 5% v/w moisture.

Sodium metabisulfite decreased the rate of discoloration but the level of L-ascorbic acid was much reduced.

Tristimulus colorimetry was found to be a rapid, non-destructive means of monitoring the rate of discoloration and determination of rate constants.

Alternative means of stabilizing L-ascorbic acid in the presence of moisture must be sought. The alternative is to ensure moisture levels are reduced significantly. This could be achieved by examining the manufacturing and packaging processes, storage conditions and tablet presentation for sales.

CHAPTER 7

Conclusions and Suggestions for Future Work

7.1 Conclusions

The purpose of this project was to study the solid phase degradation of L-ascorbic acid. The initial work concentrated on non-chromatographic methods used for the assay of L-ascorbic acid thus titrimetric and colorimetric methods were investigated and compared using aqueous solutions of the acid. Results obtained by both methods were found to be in good agreement and proved to be accurate and reproducible. Although these methods are satisfactory for analyzing L-ascorbic acid they are not specific and are therefore unsuitable for samples containing other analytes and for degradation studies, the subject of this work. Chromatographic methods, in particular HPLC, were developed.

Detailed investigations into the use of different HPLC techniques for the isocratic assay of L-ascorbic acid were carried out by studying aqeous solutions. Suppressed ionization reverse-phase chromatography was studied in detail using a wide range of different stationary phases. An end-capped C18 stationary phase was choosen based on the selectivity between L-ascorbic acid and dehydroascorbic acid.

The precision of the choosen isocratic HPLC assay was determined. Linearity and the limit of detection were demonstrated using both variable wavelength and diode array detectors.

The stability indicating properties of the assay method were investigated using degraded solutions of L-ascorbic acid and found to be specific for L-ascorbic acid. The stability of L-ascorbic acid was studied and its stability in aqueous metephosphoric acid solution confirmed.

Comparison of HPLC with a titrimetric assay, using N-bromosuccinimide, was

shown to be excellent using degraded aqueous solutions and solid formulations of L-ascorbic acid.

HPLC methods for the detection of the degradation products of L-ascorbic acid were investigated. Various reverse-phase stationary phases and mobile phases were studied using gradient elution to resolve degradation products in solutions and solid samples of L-ascorbic acid. The best resolution and peak symmetry was obtained using a trifluoroacetic acid buffered mobile phase and a C18 end-capped stationary phase. Polar degradation products were not sufficiently retained to be resolved from each other. Data were conclusive in showing different degradation products were produced in solution to that in the solid phase.

The response characteristics of a conductivity detector was studied with respect to the detection of weakly chromophoric species in degraded samples. The detector demonstrated excellent response to polar impurities having a low UV response. The practical application of the detector coupled to a reverse-phase stationary phase was discounted due to the poor retention and separation of the polar impurities.

High performance ion chromatography coupled to a conductivity detector was investigated with a view to obtaining better separation of the polar degradation products. The technique suffered from poor chromatography due to the lower efficiencies of the stationary phases compared to reverse-phase HPLC. However, the technique did show that chemical degradation was occuring by different pathways in solid and in solution formulations.

The use of derivatizing agents for reverse-phase HPLC was studied to both retain and resolve derivatized degradation products and to enhance their detection by UV absorbance. The use of 1,2-diaminobenzene was discounted due to L-ascorbic acid degrading under the reaction conditions. The reagent 2,4-dinitrophenylhydrazine was adopted as L-ascorbic acid was found to be stable with the reagent. Further, reverse-phase HPLC of derivatized solid formulations showed differencies between hydrolysis and oxidative degradation.

Excellent chromatography was demonstrated using GC with BSTFA derivatized formulations of L-ascorbic acid degraded in the solid phase. It was demonstrated that moisture was responsible for the formation of the coloured degradation products of interest to this work. Solid L-ascorbic acid degraded in the presence of moisture alone, although severely discoloured and showing a much reduced amount of L-ascorbic acid failed to show any additional degradant peaks when chromatographed by GC or when derivatized with 2,4-DNP.

Normal-phase TLC was found to be a suitable screening method for resolving and detecting the discolored degradation products. The spot due to the discoloured degradation product was found to consist of more than one polar component.

DSC was used to predict the stability of solid formulations containing L-ascorbic acid and typical tablet excipients. Predictions made were confirmed by a HPLC assay for L-ascorbic acid and HPLC gradient elution. Samples that were discoloured did not always exhibit low assay values for L-ascorbic acid nor were any degradation peaks detected. A change in colour of a formulation was found to give an early indication of the onset of degradation. The presence of moisture was found to be a significant factor in the degradation of the formulations.

Moisture induced degradation is characterized by discolouration. Tristimulus colorimetry was therefore investigated as a method of measuring the rate of degradation of L-ascorbic acid. The degradation of L-ascorbic acid in the solid phase under the influence of moisture was studied and allowed kineteic constants to be calculated. Rate constants and activation energy were determined by tristimulus colorimetry and a quantitative assay for L-ascorbic acid and found to be in close agreement with each other. The activation energy was calculated by applying the Arrhenius equation to the experimental data. The value was of the same order as that calculated by other workers. The reaction was found to be zero order.

Exclusion of air reduced the rate of reaction infering the degradation occurred via

an oxidative route although no evidence was found for the presence of dehydroascorbic acid using an automated pre-column sample reduction method. This study was novel in demonstrating that L-ascorbic acid does not degrade via dehydroascorbic acid in the solid phase.

Differences in degradation of L-ascorbic acid in the solid phase and solution were demonstrated. Unlike in solution, solid phase degradation was not characterized by the formation of furan-type compounds or carbonyl compounds. Coloured degradation compounds formed during moisture induced solid phase degradation were detected by HPLC. This chromatographic procedure can be used to detect the onset of degradation in the solid phase. This study demonstrated for the first time that excipients were not required to cause the degradation of L-ascorbic acid.

Carbon dioxide was evolved during the degradation in a 1:1 molar ratio.

The major coloured degradant was isolated and chemical analysis performed. Spectroscopic analysis using NMR and MS failed to yield spectra due to the insolubilty and involatility of the compound.

Pyrolysis of the coloured degradant followed by GC-MS yielded important information on the carbon skeletal backbone of the compound structure. It is postulated that the compound is polymeric and made of units of furan related compounds.

Attempts at stabilizing L-ascorbic acid to moisture induced solid phase degradation failed. Formulations containing chelating agents discolored to a greater degree than a control. Sodium metabisulfite did arrest the discoloration but much reduced levels of L-ascorbic acid were recorded.

7.2 Suggestions for Future Work

Moisture alone has been identified as the primary source of degradation of

L-ascorbic acid in the solid phase. Means of stabilizing L-ascorbic acid against the effect of moisture are required. Encapsulating L-ascorbic acid could be a method of isolating L-ascorbic acid from moisture in a solid dosage form. Different compounds in which to encapsulate L-ascorbic acid would be worthy of investigation. A study of hydrophobic and hydrophylic encapsulators may demonstrate that a hydrophobic shell would produce greatest stability.

Towards the end of the study attempts were made to synthesize deoxy compounds of L-ascorbic acid. Derivatives of L-ascorbic acid in which one or more of the hydroxy groups on C-2, C-3, C-5 or C-6 are substituted by atoms or groups other than hydroxy are described as deoxy compounds. By virtue of their deoxy structure these compounds may exhibit enhanced stability whilst still retaining antiscorbutic activity.

Attempts were made to synthesize L-ascorbate-2-phosphate, chloro and fluoro analogs and the carbonate.

Considerable effort was expended in performing the reactions, product isolation, development of chromatographic screens to assess product purity and analysis to confirm product identity.

Due to difficulty in isolating the phosphate, chloro and fluoro analogs no product was isolated to study. Only the carbonate was isolated in high purity but due to lack of quantity no work could be carried out.

It is of considerable interest that deoxy compounds, such as those mentioned above, are synthesized and their chemical stability assessed in a similar study to this project. Their antiscorbutic and antioxidant properties should be studied as these compounds may be of sinnificant biological importance.

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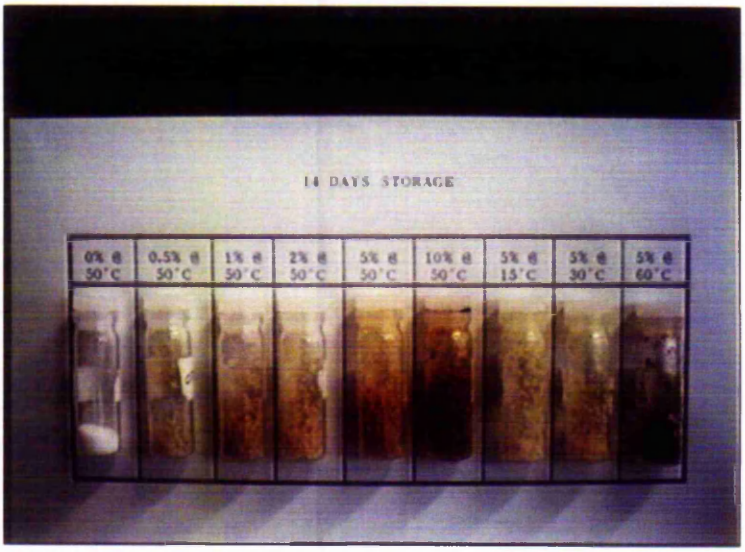
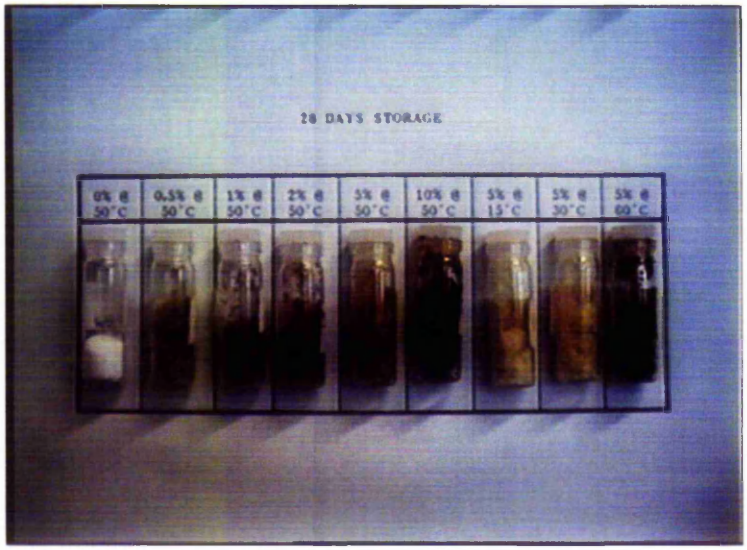
215. Chemistry of Sulphur Dioxide in Foods. B.L. Wedzicha, Elsevier Applied Science.

APPENDIX 1

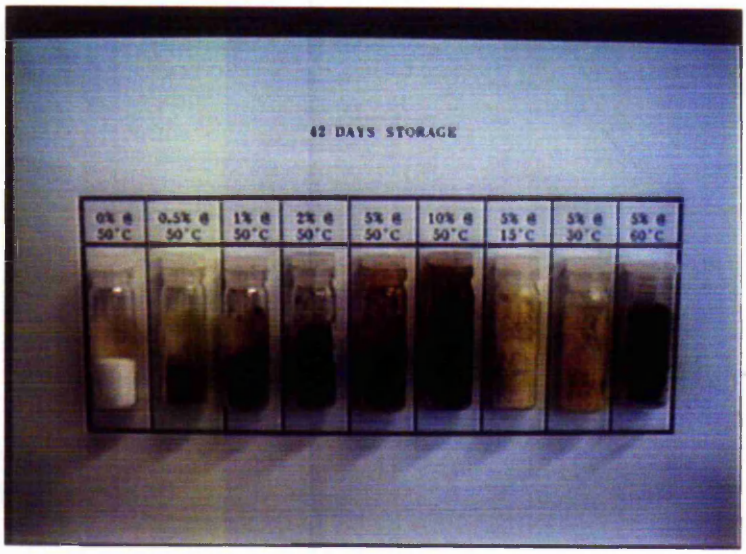
PHOTOGRAPHS OF SAMPLES FROM THE KINETIC STUDY



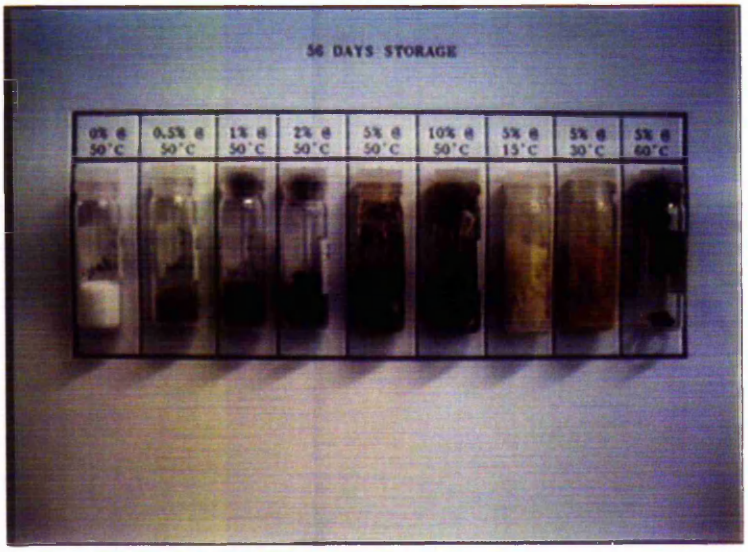
14 DAYS STORAGE



28 DAYS STORAGE



42 DAYS STORAGE

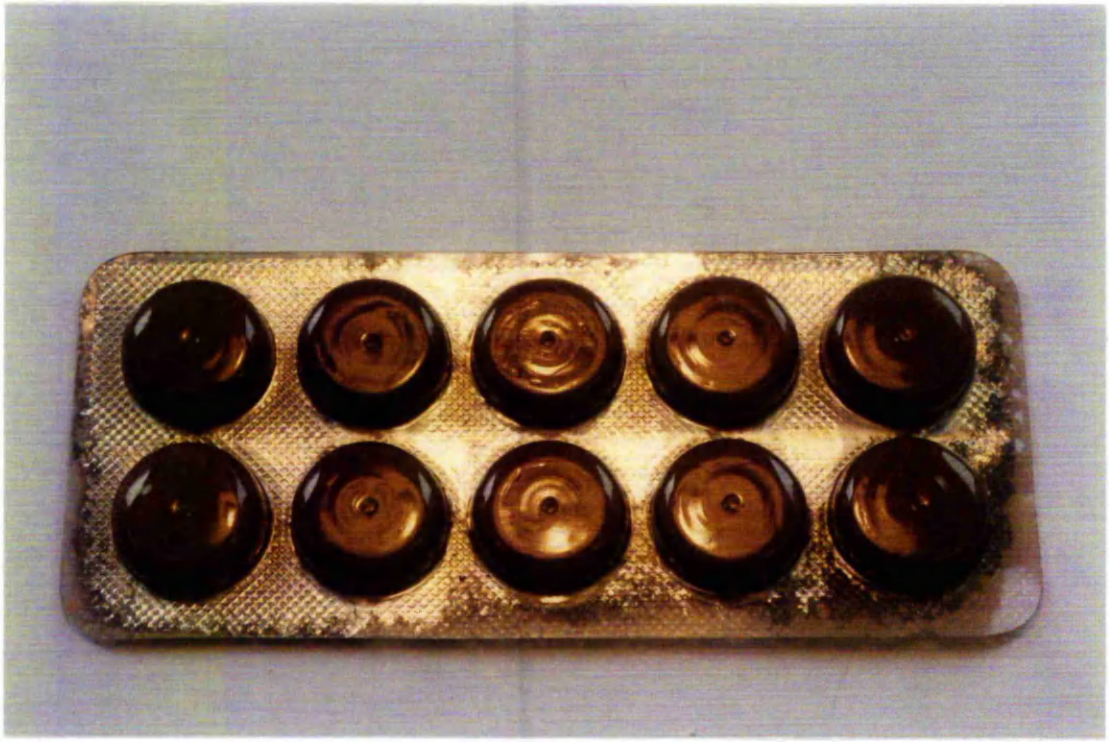


56 DAYS STORAGE

APPENDIX 2

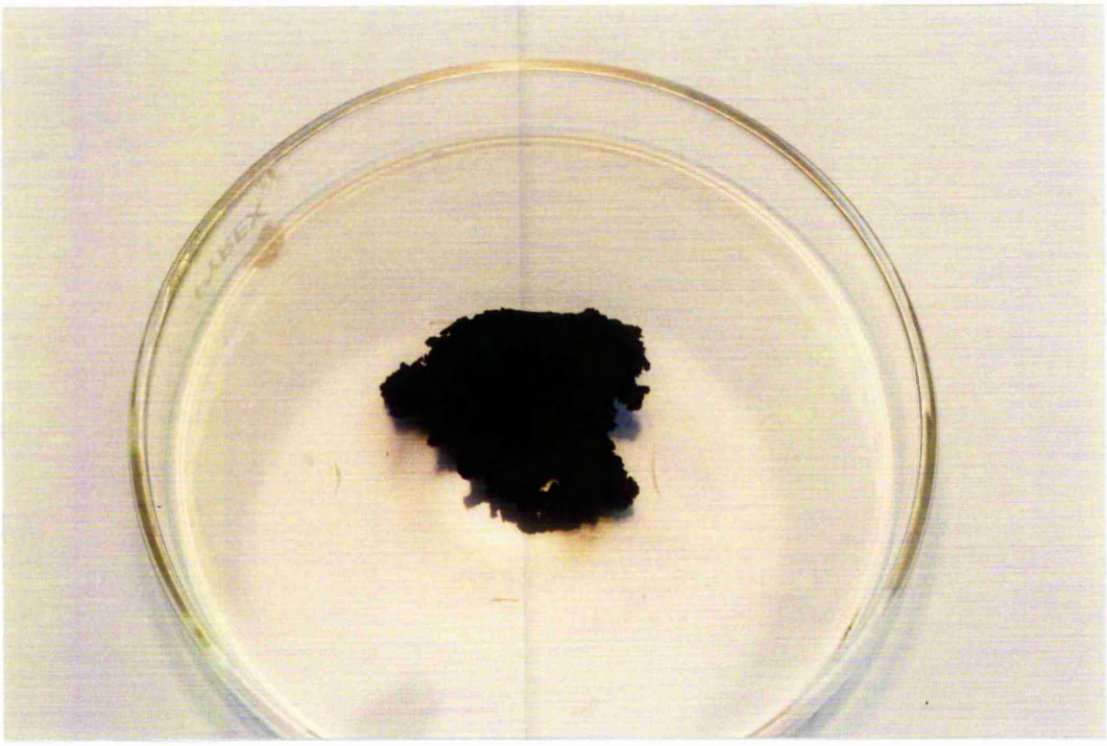
TYPICAL DEGRADED L-ASCORBIC ACID TABLETS

ENTERIC COATED TABLETS



FOIL WRAPPED TABLETS

ISOLATED COLOURED DEGRADATION PRODUCT



GLOSSARY OF CHROMATOGRAPHIC TERMS AND MATHEMATICAL DEFINITIONS

Definitions of Chromatographic Terms

t_o column dead-time.

t_R solute retention time from injection.

t_R corrected solute retention time.

K distribution ratio.

k solute partition ratio (also termed capacity factor).

α relative partition ratio (relative capacity factor).

h height equivalent to a theoretical plate (HETP).

n number of theoretical plates.

N_{eff} number of effective theoretical plates.

N_{req} number of theoretical plates required to effect a separation between peaks.

l column length.

$W_{0.5}$ width of peak at half height.

R_s resolution of two peaks.

A_s peak asymmetry (skew factor).

R gas constant.

T temperature.

STANDARD CHROMATOGRAPHIC PARAMETERS

Parameters	Unit	Symbols *Kirkland et al.**	ASTME E-19**	Chromatographia**
Retention time of an unretained solute	s	t_0	t'_M	t_m
Retention time, measured from the start	s	t_R	t_R	t_{m+s}
Reduced retention time	s	$t'_R = t_R - t_0$	$t'_R = t_R - t_M$	$t_s = t_{m+s} - t_m$
Band width	s	w	y_t	w_b
Capacity factor (Retention factor)	---	$k' = \frac{t'_R}{t_0}$	$k' = \frac{t'_R}{t_M}$	$k = \frac{t_s}{t_m}$
Selectivity factor	---	$\alpha = \frac{k'_2}{k'_1} = \frac{t'_{R2}}{t'_{R1}}$	$r_{ji} = \frac{t'_{Rj}}{t'_i}$	$r = \frac{t''_s}{t'_s}$
Resolution	---	$R_s = 2\left(\frac{t'_{R2} - t'_{R1}}{w_2 + w_1}\right)$	$R_{ji} = 2\left(\frac{t_{Rj} - t_{Ri}}{y_j + y_s}\right)$	$R_s = 2\left(\frac{t''_{m+s} - t'_{m+s}}{w''_b + w'_b}\right)$
Number of theoretical plates	---	$N = 16\left(\frac{t_R}{w}\right)^2$	$n = 16\left(\frac{t_R}{y_t}\right)^2$	$n = 16\left(\frac{t_{m+s}}{w_b}\right)^2$
Number of effective plates	---	$N_{eff} = 16\left(\frac{t'_R}{w}\right)^2$	$n_{eff} = 16\left(\frac{t'_R}{y_t}\right)^2$	$n_{eff} = 16\left(\frac{t_s}{w_b}\right)^2$
Column length	cm	L	L	L
Height equivalent of a theoretical plate (plate height)	cm	$H = \frac{L}{N}$	$H = \frac{L}{n}$	$h = \frac{L}{n}$
Effective plate height	cm	$H_{eff} = \frac{L}{N_{eff}}$	$H_{eff} = \frac{L}{n_{eff}}$	$h_{eff} = \frac{L}{n_{eff}}$
Linear velocity of the mobile phase	cm s^{-1}	$u = \frac{L}{t_0}$	$\bar{u} = \frac{L}{t_m}$	$\bar{u} = \frac{L}{t_m}$
Pressure drop in the column	bar	1 bar = 100 kPa, 1atm = 101.3 kPa, 1 psi = 6.9 kPa		
Particle diameter	µm	d_p	---	---

* Modern Practice of Liquid Chromatography, Ed. J.J. Kirkland, Wiley, New York (1971)
** B. Versino and F. Geib, Supplement in: Chromatographia 3 (1970)

HPLC CALCULATIONS

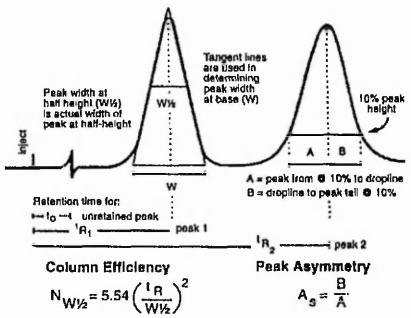
HPLC CALCULATIONS

COLUMN EFFICIENCY: In general, **N** = Number of Theoretical Plates, **a** is a constant depending on method used, t_r = retention time of peak, and **W** = the peak width at a given peak height.

$$N = a\left(\frac{t_r}{W}\right)^2$$

Method	a
Peak Width ½ Peak Height	5.54
Peak Width at 4.4% Peak Height (5s method)	25
Tangential (ca. 13.5%)	16

The peak width at ½ height is the most commonly used method for calculating HPLC column efficiency.



PEAK ASYMMETRY: A_s = B/A at 10% peak height.

CAPACITY FACTOR (also known as RETENTION FACTOR or RELATIVE RETENTION): The Capacity Factor, k', of a sample component is a measure of the degree to which that component is retained by the column relative to an unretained component (such as uracil).

$$k' = (t_r - t_o)/t_o$$

Where t_r is the elution time of retained component, and t_o is the elution time of the unretained sample.

SEPARATION FACTOR (also known as SELECTIVITY): The selectivity parameter, α, is a measure of peak spacing and is expressed as: $\alpha = k_2'/k_1'$

RESOLUTION: Rs, defined as the amount of separation between two adjacent peaks, is given by:

$$Rs = (1/4)\,(\alpha - 1)\,(N)^{1/2}\,[k'/(1 + k')]$$

where k' is the average value for the two peaks.

LECTURES, SEMINARS AND ASSOCIATED STUDIES

Lough, D., University of Sunderland — 24th January 1990
Recent Advances in Chiral Chromatography

Shaw, R., Hichrom — 22nd July 1993
The Comparison of Base Deactivated Silica Gel
Reverse Phase Packing Materials

Bartle, K., University of Leeds — 14th March 1994
Pharmaceutical Chemistry Goes Supercritical

Richie, H., Shandon HPLC — 16th March 1994
The Universal HPLC Column - Fantasy or Fact

Davies, M., Anglia Polytechnic University — 10th May 1994
Vitamin C - A History of Controversy

Rocklin, R., Dionex (USA) — 23rd May 1994
The Use of Pulsed Electrochemical Detection in
Pharmaceutical Research and Development

Barrett, D., University of Nottingham — 14th December 1994
Surface Substituents and the Separation Behaviour
of HPLC Stationary Phases

Hawks, D., Research — 18th January 1995
Capillary and Microbore LC -
Proven Routes to Sensitive Enhancement

Smith, N., Glaxo Group Research — 29th March 1995
Practical Approaches to Electrochromatography

Phillips, D., Waters, USA — 21st July 1995
Strategies for Faster Method Development
for Pharmaceutical Compounds

Lim, C. K., University of Leicester — 26th October 1995
Separation of Anionic and Cationic Compounds of
Biochemical Interest by HPLC on Porous Graphitic Carbon

Cleveland, T., Phenomenex, USA — 22nd April 1996
Chiral HPLC Separations of Racemic Compounds

Ahmed, F., Phenomenex, USA — 22nd April 1996
"All C - 18's are NOT the Same"
Synthesis and Characterization of Stable Bonded Silica Gels

Organic Chemistry for Analysts
A 12 week internal course from December 1993 to May 1994. Presented by the
Organic Chemistry Section,
Department of Chemistry,
Loughborough University of Technology