

Fate of Vitamin C in Commercial Fruit Juices

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institute of higher learning,

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Abstract

Vitamin C occurs in relatively high concentrations in fresh and processed fruits and vegetables but is found to a lesser extent in animal tissues and animal-derived products. Nearly 90 % of vitamin C in the human diet is obtained from fruits and vegetables but this can be indirect by way of commercially prepared fruit juices. These juices are often enriched with vitamin C which has been synthetically prepared. There is a wide range of such juices on the New Zealand market, and they are a significant source of dietary vitamin C for many in the population. The focus of this research is on the Keri range of juice products.

The present study monitors the fate of vitamin C during storage of Keri juices up to the best-before date, and under a range of other storage and consumption situations.

Two methods were adopted for determining ascorbic acid (AA, the chemical identity of vitamin C). These were the titrimetric method, which is based upon the reduction of the dye 2,6-dichlorophenolindophenol by AA in acidic solution, and liquid chromatography, which is used to separate AA from its immediate oxidation product dehydroascorbic acid. In the latter method these two analytes can be measured independently. The liquid chromatography was less successful than the simpler titrimetric method, so most of the work was done by titration. However, the concentration of dehydroascorbic acid, which has vitamin C activity *in vivo*, remained uncertain.

Moreover, the titrimetric method could not be applied to juices with high purple anthocyanin concentrations, like blackcurrant, because the colour change at the titration end point could not be detected. pH adjustment to change colour was ineffective, and decolourisation with charcoal led to the rapid and complete destruction of AA.

The concentration of AA in Keri juices at the time of manufacture were always much higher than claimed on the labels. Storage for up to nine months at room temperature resulted in a loss in AA of between 37 and 68 %, depending on the juice and exposure to fluorescent light. However, the time of storage was a much more dominant factor than light exposure. The kinetics of loss, straight lines, were most easily explained by an aerobic model of AA degradation from oxygen diffusing across the polyethylene tetrathalate bottle wall. Overall, the label claims made were defensible in terms of the best-before date, because it took at least 100 days of storage before the AA concentration in the most susceptible juices fell below the claimed value. This is because these drinks are fast moving consumer goods and storage

beyond 100 days is unlikely. (Nonetheless, the supplier (Keri Juice Company) has since adopted its new unitised method of formulating juice. This has resulted in an initially higher concentration of vitamin C as compared to the juices under investigation.)

In the nine months storage experiment there was some evidence for the presence of dehydroascorbic acid in blackcurrant drinks, but not in another three juices.

Pasteurisation during preparation of these drinks resulted in up to 7 % loss of AA, probably due to oxygen dissolved in water, and accelerated by heat of pasteurisation. Higher temperatures in later storage also accelerated losses. Progressive exposure of juice to air during simulated consumption of 3 L bottles over a week also accelerated losses. Finally, exposure to sunlight in a diurnal temperature environment accelerated losses five-fold higher than in total darkness. Filtration of ultraviolet light approximately halved the loss due to sunlight.

Overall however, it can be concluded that AA in the Keri range of juices is very resistant to degradation of AA.

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Chapter 1

Introduction

History of vitamin C in human health

Vitamins are organic compounds required in small amounts for metabolism in the body. They are not source of energy to the body. Their function is to promote a wide variety of biochemical and physiological processes necessary for life. Thirteen vitamins are recognised in human nutrition and these are divided into two groups on the basis of their solubility – fat soluble and water soluble. Vitamin C is a water soluble vitamin.

Vitamin C is chemically the simplest of the vitamins and, perhaps for these reasons, was first to be isolated, characterised and to have its structure determined.

Although the name and identity of vitamin C was not recognised until 1919, its effects have been felt in history through the disease called scurvy, or scurfy (Old English). The name was perhaps derived from the Scandinavian terms, *skjoerberg* and *skorbjugg*, meaning rough skin, among other names. Scurvy is a disease characterised by abnormal bone growth, joint pains, bleeding gums, and tiny blue scars on skin. If untreated it is eventually fatal. Scurvy can be called a disease of imperialism because countries like Britain, Portugal and Spain had to develop strong navies to extend their empires and to defend their far-away colonies. Scurvy was a major problem for these navies, and, as is well established now, is caused by vitamin C deficiency. Indirectly this was caused by the unbalanced diets aboard ships. Vegetables and fruits are the principal sources of vitamin C but these did not keep well nor were popular, and sailors were nourished instead on a diet of cured meat, cheese, biscuits, and rum.

The impact of scurvy was severe. For example, Vasco da Gama lost 100 of 150 companions on his way to India, when he first rounded the Cape of Good Hope between 1497-1499. One half of the original 60 colonists of Plymouth died of scurvy in 1628 and scurvy continued to be a scourge even to the start of the 20th century (Rucker, Suttie, Donald, & Machlin, 2001). It has been estimated that over a million seamen have died of scurvy during the 17th and 18th centuries alone (Sauberlich, 2000). It was described in the Ebers Papyrus of

around 3500BP and by Hippocrates. It is said that Crusaders lost more men through scurvy than killed in battle. In 1772 Captain James Cook, proved that long voyage could be successful without scurvy development if the sailors were provided with fruits and vegetables including fermented cabbage (Groff, Gropper, & Hunt, 1995). In the mid 18th century, James Lind, a British navy surgeon, conducted controlled experiments with sailors' diets and published his findings in *A Treatise of the Scurvy* (Lind, 1753). He found that foods like lemons and oranges helped in recovery from scurvy and recommended they be carried on ships. These findings were not widely accepted by the rest of the world and scurvy continued to lead to widespread death throughout the 19th century (Groff et al., 1995). Starting from 1795, one Sir Gilbert Blane, a physician to the British navy, made lemon juice a regular issue to seafarers. This is the origin of the term 'limies', a colloquial term applied to British seamen of the time. In short, scurvy at sea was conquered because of the activities of Cook, Lind and Blane and probably others and by the end of 1800; the connection between scurvy and diet was established.

In 1912, Casimir Funk of the Lister Institute in London proposed that diseases like beri-beri, dropsy, scurvy and pellagra could be cured by nitrogen-containing compounds, specifically amines. He referred to them as 'vital amines' abbreviated to vitamins. The 'e' was later dropped and the compound class is now universally called vitamins (Davies, Austin, & Partridge, 1991). Sir William Drummond (1919), also at the Lister Institute, described antiscorbutic factor as 'water soluble C'. As the term 'vitamin' was adopted for essential factors, so antiscorbutic factor was designated vitamin C (Bucci, 1998). Albert Szent-Gyorgyi, the famous biochemist, who was interested in oxidation-reduction reactions occurring at the molecular level in living systems, isolated an off-white crystalline substance from the adrenal cortex of cattle. He first named it 'ignose' then 'Godnose' and finally 'hexuronic acid', which gave colour tests characteristic of sugars (Coultate, 2002). Later he isolated this compound in much greater quantities from paprika and renamed it 'ascorbic acid' because it prevented scurvy. Later work by Hayworth, a leading structural organic chemist, finally led to synthesis of vitamin C in the laboratory in 1933 (Bucci, 1998). This work paved the way to large scale commercial production of vitamin C that continues to this day. Current world production is estimated at approximately 80,000 tonnes per year with a worldwide market in excess of U.S. \$600 million (Competition Commission, 2001).

Biological effects of vitamin C

According to Stone (1972), an ancient genetic mutation left the primate virtually alone among animals in not producing ascorbic acid. The mutation was the loss of the ability to synthesise L-gluconolactone oxidase, which if active would catalyze the conversion of L-glucono- γ -lactone to L-ascorbic acid (Rucker et al., 2001). Humans, other primates, guinea pigs and fruit bats are unable to synthesise L-ascorbic acid. Therefore, they require it in their diet (Navarra & Lipkowitz, 1996). (In Stone's opinion, by treating consumption of vitamin C as a minimum daily requirement instead of the crucial enzyme deficiency it really is, humans are living in a state of sub-clinical scurvy whose symptoms have been attributed to other ailments.)

Vitamin C is present in all living tissues (Sardesai, 1997). No particular organ acts as storage reservoir for vitamin C but its concentration is higher in certain tissues as the pituitary and adrenal glands, eye lens and leucocytes (Basu & Dickerson, 1996). Table 1 shows concentration ranges in some human tissues.

Table 1. Concentration ranges of vitamin C in several human tissues			
Tissue	Vitamin C (mg.100 g ⁻¹)	Tissue	Vitamin C (mg.100 g ⁻¹)
Pituitary gland	40-50	Brain	13-15
Adrenal gland	30-40	Liver	10-16
Leucocytes	35	Lungs	7
Eye lens	25-31	Plasma	0.4-1.0
From Basu and Dickerson (1996) p.130			

The symptoms of scurvy develop after prolonged deficiency of vitamin C intake that results in defective collagen synthesis, tissue repair, and synthesis of lipids and proteins. If vitamin C is completely withdrawn from the diet it takes between 100 and 160 days for scurvy symptoms to develop. Mild scurvy symptoms appear when the body's vitamin C content is less than 300 mg (Basu & Dickerson, 1996). Vitamin C functions both as a reducing agent and as an antioxidant, and is required for many physiologic functions, including metabolism of iron and folic acid, resistance to infection, and integrity of blood vessels (Laumann & Wong, 2005).

Vitamin C is a cofactor required for the function of several hydroxylases. In scurvy, the absence of vitamin C reduces prolyl hydroxylase activity. This enzyme is required to form hydroxyproline, an amino acid found in collagen. Hydroxyproline in collagen stabilises the collagen triple-helix structure by forming interstrand hydrogen bonds. Collagen lacking hydroxyproline is fragile and contributes to the clinical manifestations of scurvy, including haemorrhage beneath the skin due to fragility of blood vessel walls.

Collagen contributes about one fourth of total protein mass and is the most abundant protein in human body (Wildman & Medeiros, 2000). It is a fibrous protein which accounts for the toughness and tensile strength of connective tissue and any changes in connective tissue leads to great affect on health and integrity of the rest of the body (Elliott, 2002). The structure of blood vessels, bones, skin, teeth, cartilage, tendons and ligaments are based on collagen. Given the ubiquity of collagen in the body it is not surprising that the effects of vitamin C deficiency are manifest in many tissues, at many stages of life, and is implicated in a range of degenerative diseases and conditions (Warner, 1987). For example, whereas ageing is inevitable, adequate vitamin C intake is purportedly useful at slowing ageing through its effects of skin and bone, two collagen-rich tissues. One current theory of ageing (random damage theories) implicates oxidative damage caused by free radicals as the key driver of degeneration (Warner, 1987). Given the antioxidative role of vitamin C, it is again not surprising that it is implicated in ageing phenomena. For example, vitamin C helps in delaying age-related macular degeneration (Murray, 1996).

Cancers become more common as the human body ages. Vitamin C's role in reducing the risk of carcinogenesis includes acting as an antioxidant, blocking formation of nitrosamines and fecal mutagens, enhancing immune system response and aiding detoxification by liver enzymes (Gaby, Bendich, Singh, & Machlin, 1991).

Thus whereas the symptoms of scurvy are the most obvious effects of vitamin C deficiency, the vitamin has a broad range of effects throughout the body.

Absorbance and metabolism of ascorbic acid

Absorption occurs readily in the buccal mucosa, stomach and small intestine by means of a sodium and energy-dependent transport system (Sardesai, 1997). After absorption, it is distributed throughout the water phase of the body by diffusion. The kidney participates with the intestine in maximizing vitamin C conservation in the body. Vitamin C circulates in the

bloodstream mainly in the free (nonprotein bound) and reduced form. Only 5 % of the circulating vitamin C is represented by dehydroascorbic acid (Ball, 1998), an oxidation product to be discussed later. The efficiency of absorption decreases as intake increases. When absorbed in quantities that exceed the body's requirements it is excreted in the urine (Basu & Dickerson, 1996).

Though iron is abundant in many foods only about 10 % is absorbed from a typical Western diet (Coultate, 2002). In New Zealand for example it is estimated that 3 % of young people and 6 % of adults are subclinically or clinically iron deficient (Ministry of Health, 2003). This is considered a significant health issue. In its reduced ascorbic acid form, vitamin C aids iron absorption from the gut. The reduced form of iron, $\text{Fe}(\text{H}_2\text{O})_6^{2+}$, is more soluble in the alkaline environment of the small intestine than $\text{Fe}(\text{H}_2\text{O})_6^{3+}$. Acting as a reductant, ascorbic acid thus improves iron absorption (Wardlaw, 1999).

Structure and properties of vitamin C

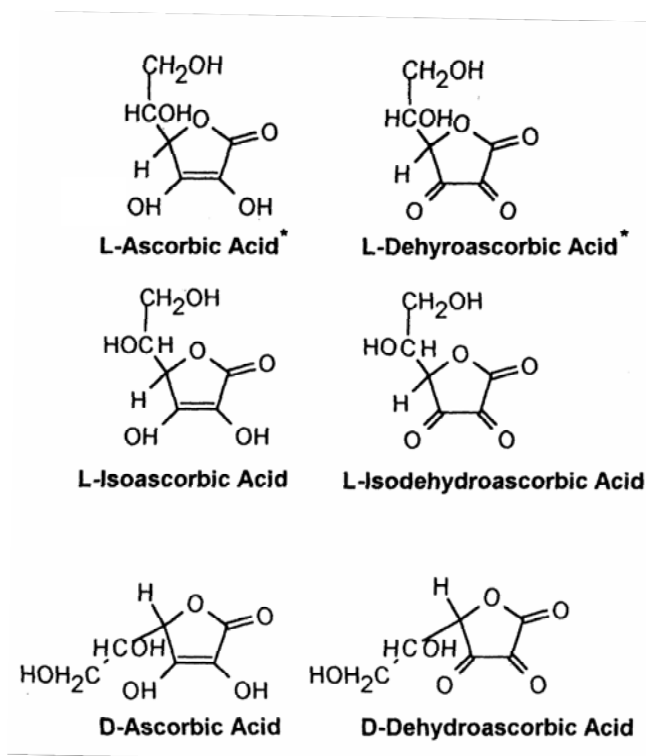


Figure 1. Structures of L-ascorbic acid and related compounds. The two compounds marked with an asterisk have vitamin C activity. From (Gregory, 1996)

Vitamin C comprises the compounds which show the biological activity of authentic L-ascorbic acid (Figure 1). These include salts and esters of ascorbic acid, and its oxidised derivative L-dehydroascorbic acid.

The oxidation of L-ascorbic acid to L-dehydroascorbic acid is freely reversible, so both forms are the primary biological dietary sources of vitamin C. However, in the reductive environment of the body (the partial pressure of oxygen is typically 2 mm Hg) vitamin C is in its reduced form. Its stereoisomer about carbon 5, L-isoascorbic acid, commonly called erythorbic acid, has little vitamin C activity but still behaves as an effective reductant and is used in this role in many foods (Eittenmiller & Landen, 1999).

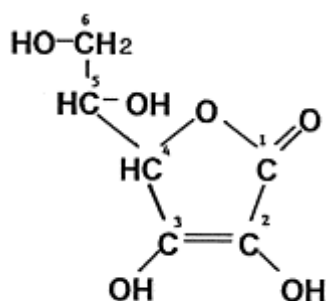


Figure 2. Structure of L- ascorbic acid. From Deutsch (1998)

Ascorbic acid ($C_6H_8O_6$), now abbreviated to AA, is a common name for vitamin C. Depending on the nomenclature used it has a number of formal systematic names, for example L-3-ketothreohexuronic acid γ -lactone (Belitz & Grosch, 1999). AA is a white, crystalline, highly polar compound, which is highly soluble in water at ambient temperature (330 g.L^{-1}) (Eittenmiller & Landen, 1999), but insoluble in non-polar solvents. AA is a hexose derivative and is nominally a carbohydrate. Acidic carbohydrates are generally thought of as containing a carboxylic acid group, but no free carboxylic group is present in AA (Figure 2). A carboxyl group has been lost to the lactone structure. Rather, the hydroxyl groups on carbons 2 and 3 are ionisable. In particular, the hydroxyl group on carbon 3 has a pK_a of 4.04 which is in the pH range of food products. The pK_a for the hydrogen linked to the oxygen atom on carbon 2 is 11.4.

AA is added to food as the free acid or the neutralised sodium salt, or as an ester of AA such as ascorbyl palmitate, which is understandably useful in food systems containing fat. In

that ester, the hydroxyl group on carbon 6 forms the ester. AA is very stable in the dry crystalline form. It is also relatively stable in acid solution, but very prone to oxidation in alkaline solution (Eittenmiller & Landen, 1999). The most susceptible form of AA is the ascorbate dianion (on carbons 2 and 3), which forms under strongly alkaline solution, and the least susceptible is the fully protonated form. In most foods susceptibility is governed by the ratio of the monoanion to the fully protonated form (Buettner, 1993). AA is sensitive to oxidation on exposure to heat and particularly in the presence of Cu^{2+} and Fe^{3+} , so food cooked in copper and non-stainless iron utensils loses ascorbic acid quickly.

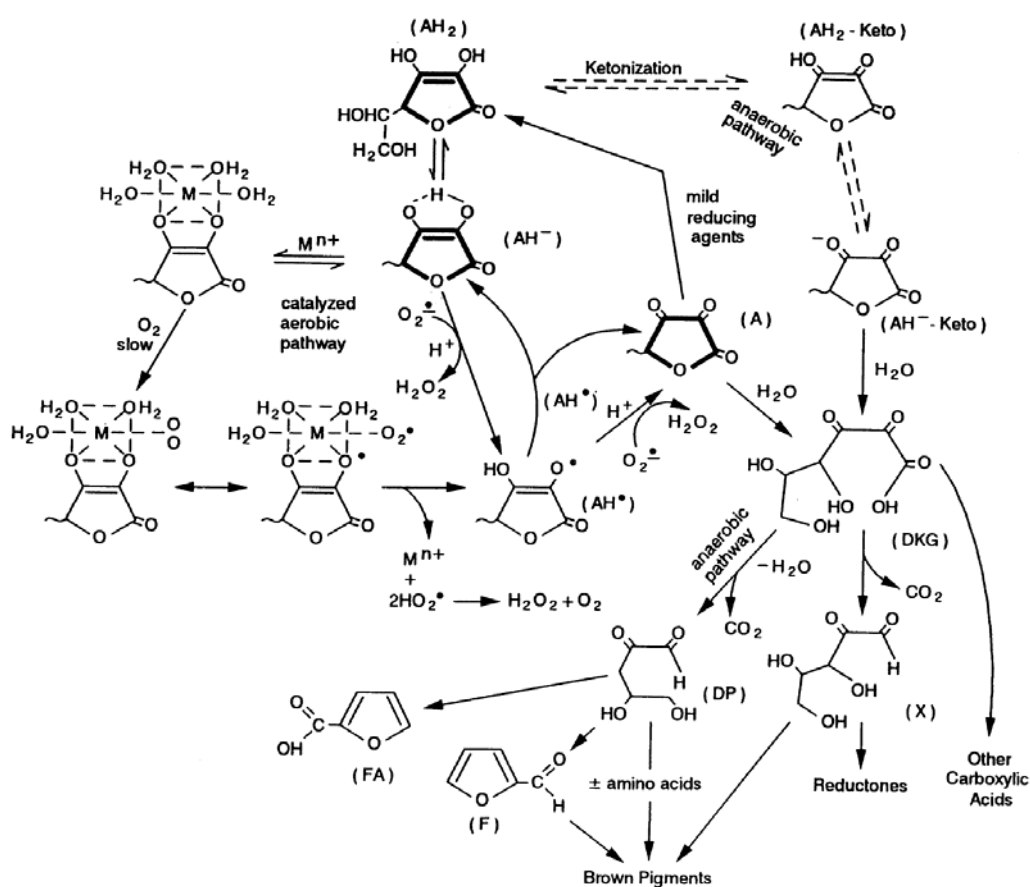


Figure 3. Mechanism for the oxidative and anaerobic degradation of ascorbic acid. Structures with bold line are primary sources of vitamin C activity. Abbreviation: AH_2 , fully protonated ascorbic acid; AH^- , ascorbate monoanion; AH^* , semidehydroascorbate radical; A, dehydroascorbic acid; FA, 2-furoic acid; F, 2-furaldehyde; DKG, 2,3-diketo-1-gulonate; DP, 3-deoxypentosone; X, xylosone; M^{n+} , metal ion catalyst; HO_2^* , perhydroxyl radical. From (Gregory, 1996)

According to a number of authorities (Eittenmiller & Landen, 1999; Jackson, Knize, & Morgan, 1999), the oxidation of AA can be catalysed by metal ions, using molecular oxygen as the substrate, or by superoxide radical generating an intermediate semidehydroascorbate radical (Figure 3). The metal catalysed path is by far the fastest, by several orders of magnitude (Gregory, 1996). Cu^{2+} is roughly 80 times more active as a catalyst than Fe^{3+} . A second superoxide radical completes the oxidation process yielding the triple ketone cyclic product dehydroascorbic acid (DHAA), which as noted before still has vitamin C activity because mild reducing agents are capable of reforming AA.

However, further reaction of DHAA involves irreversible hydrolysis of its lactone yielding 2,3-diketo-1-gulonic acid (DGK) (Figure 4). This is a conventional carboxylic acid. Neither DGK nor its subsequent degradation products – which include Maillard reaction products – have any vitamin C activity. The hydrolysis of DHAA to DGK is favoured by alkaline conditions.

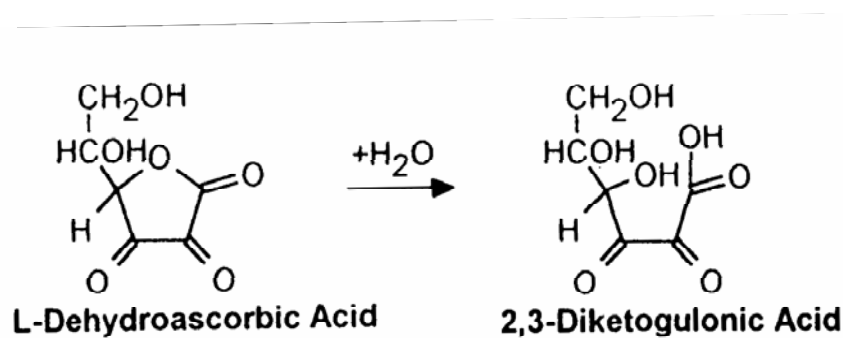


Figure 4. The structure of 2,3-diketo-1-gulonic acid (DKG). From (Gregory, 1996)

Another pathway of AA degradation proceeds anaerobically (Gregory, 1996), but understanding of this pathway is more limited. According to Gregory, cleavage of the 1, 4-lactone bridge to DKG occurs without an intermediate DHAA step. The details are unimportant here except that anaerobic degradation occurs maximally around pH 3 to 4, which clearly contrasts with the aerobic pathway. This pH range may reflect the effects of pH on the opening of the lactone ring and on the relatively high concentration of an intermediate anion in this pH range. Gregory notes that the rate constant for the anaerobic pathway is between 100 and 1000 times slower than that for the oxidative pathway.

Once the ring structure is opened on DKG formation (Figure 3), the way is clear to generate a number of subsequent breakdown products. The degradation of sugars and ascorbic acid are similar (Gregory, 1996) which is perhaps not surprising considering that DKG is a sugar acid that by decarboxylation yields aldehydes that can take part in the Maillard reaction, ultimately leading to the formation of brown pigments and other compounds in, for example, orange juice (Zerdin, Rooney, & Vermue, 2003). As is well known for the Maillard reaction, the exact pattern of breakdown depends on pH, composition, water activity and temperature (Lopez-Nicolas, Nunez-Delicado, Sanchez-Ferrer, & Garcia-Carmona, In press).

Sources of vitamin C in foods

Vitamin C is widely distributed in nature, particularly in the plant kingdom. It occurs in relatively high concentrations in fruits and vegetables but to a much lesser extent in animal tissues and animal-derived products. Nearly 90% of vitamin C in the human diet is obtained from fruits and vegetables (Zee, Carmichael, Codere, Poirier, & Fournier, 1991).

Table 2. Concentration of vitamin C in common fruits and vegetables

Fruit	Typical vitamin C concentration (mg.100 g ⁻¹)	Vegetable	Typical vitamin C concentration (mg.100 g ⁻¹)
Blackcurrants	200	Parsley	150
Orange	50	Broccoli	110
Strawberries	60	Cauliflower	60
Pineapple	25	Cabbage	40
Bananas	11	Peas	25
Peaches	6	Carrot	6
Plums	3		

From Coultate (2002) p. 282

As discussed earlier, humans cannot synthesise vitamin C so the requirement must all come from diet. Vegetables and fruits are the primary sources (Table 2). As might be expected from basic biological experience, the concentration of vitamin C in plant foods depends upon various positional, developmental, environmental, post harvest factors and even chlorination of water. For example a broccoli head contains about 160 mg.100 g⁻¹ whereas its stem contains a lower 110 mg.100 g⁻¹, but on cooking for 10 minutes stem losses are 20 %

whereas head losses are more than 40 % (Basu & Dickerson, 1996).

Concentration of vitamin C in freshly picked potatoes in summer is about 30 mg.100 g⁻¹ whereas in potatoes stored for nine months contain about 8 mg.100 g⁻¹. Sliced cucumber loses 50 % of vitamin C in first three hours (Murray, 1996).

Cereal grains are a poor source of vitamin C (Indian Gyan, 2000). Animal foods are also relatively poor sources except for some organ meats, liver and brain being good examples (Table 1). In freshly secreted cow's milk vitamin C is predominantly in the form of ascorbic acid but human milk has a similarly low concentration, around 5 mg.100 g⁻¹ (Clark, 2005).

Whenever plant foods are eaten raw rather than cooked, the availability of vitamin C is generally higher because losses are accelerated on cooking. Because vitamin C is water soluble it is easily extracted and lost during washing and cooking of vegetables in water. Cooking losses of vitamin C depend upon degree of heating, surface area exposed to water, oxygen, pH and presence of transition metals (Moser & Bendich, 1990).

Synthetic ascorbic acid is available in a wide variety of diet supplements, for example tablets, capsules, chewable forms, crystalline powders, effervescent tablets, and liquid forms. Buffered ascorbic acid and esterified forms of ascorbic acid as ascorbyl palmitate are also available commercially. Naturally occurring and synthetic ascorbic acid are chemically identical and there is no known differences in their biological activities or bioavailability.

Many breakfast cereals and other foods are fortified with vitamin C. For example, in the New Zealand retail market Kellogg's Corn Flakes claims 20 mg.100g⁻¹ and Kellogg's Frosties, Coco Pops and Fruit Loops each claim 33.3 mg.100 g⁻¹. The cocoa-based drink powder Milo (Nestlé) contains 90 mg.100 g⁻¹. The motive for adding vitamin C to foods in general is probably two-fold. First it is a reductant useful in maintaining food quality on storage but is also useful for making implicit or explicit health claims.

Recommended daily allowances and sources in the Western diet

The recommended daily allowance (RDA) varies internationally. Thus for an adult male in the U.K., the RDA is 30 mg.day⁻¹ whereas in the U.S.A. it is 60 mg.day⁻¹ (Basu & Dickerson, 1996). In New Zealand it is 45 mg.day⁻¹ for adults. Within-country RDA varies according to age and physical condition of the body. Adults have a higher requirement than children (Whitney & Rolfes, 1992). During pregnancy, the RDA is 60 mg.day⁻¹ and increases

to $85 \text{ mg} \cdot \text{day}^{-1}$ during lactation (Ministry of Health, 2003). Smoking is a stressor that dictates a higher vitamin C intake.

The vitamin C all comes from diet. The concentration in human milk is $3 \text{ to } 6 \text{ mg} \cdot 100 \text{ g}^{-1}$, so a New Zealand neonate feeding on mother milk requires less than 1 L per day to achieve its RDA. On the other hand cow milk contains only $0.5 \text{ to } 2 \text{ mg} \cdot 100 \text{ g}^{-1}$ (Rucker et al., 2001). It cannot fulfil the infant's ascorbic acid requirement and thus needs to be supplied from other sources. The powder formulas used in New Zealand contain added vitamin C. For example, Nutritica Karicare Gold and Nutritica Karicare Goat contain 112 mg and $75 \text{ mg} \cdot 100 \text{ g}^{-1}$, respectively.

In older humans the sources of vitamin C are very much more complex. Many children, (and adults) are averse to eating vegetables and fruit, but potatoes, milk and meats do provide a base level. Certainly scurvy is extremely rare, so any deficiency must be subclinical at worst. However, as proposed by Stone (1972) and discussed earlier, humans may be living in a state of subclinical scurvy where other ailments may be scurvy-related.

The major food contributors of vitamin C in the Western (principally American) diet are oranges, orange juice, grapefruit and grapefruit juice, tomatoes and tomato juice, fortified fruit drinks, tangerines and potatoes (Wardlaw, 1999). Supplement consumption is common. More than 40 % of older people in the U.S.A. take vitamin C supplements, as do almost 25 % of all adults take vitamin C. In spite of the widespread consumption of vitamin C supplements, one third of all adults in the U.S.A. get less vitamin C from their diet than is recommended by the National Academy of Sciences, and 1 out of every 6 adults gets less than half the amount recommended.

According to the Life in New Zealand Research Unit's 1999 *National Nutrition Survey* (as cited in Ministry of Health, 2003), the principal sources of vitamin C in New Zealand diet are vegetables and non-alcoholic beverages (both 26 %), fruits (23 %) and potatoes and kumara (13 %). The non-alcoholic beverages are fruit juices and fruit drinks that contain either naturally occurring vitamin C and or added vitamin C. Apart from receiving useful amount of vitamin C, fruit juices are of great value to people on low sodium diets as they have relatively low sodium and high potassium content.

Industrial production of vitamin C

Current world production is estimated at approximately 80,000 tonne per year. Vitamin C production is much higher than for any other vitamin.

It is synthesised both biologically and chemically from D-glucose. The Reichstein chemical process developed in 1930s uses a single fermentation step followed by a purely chemical route. The more recent two-stage fermentation process, developed in China in early 1960s (Competition Commission, 2001), uses additional fermentation to replace a later part of the Reichstein process.

In both processes the first step involves oxidation of sorbitol (sorbitol itself is made by reducing glucose at high temperature) to sorbose by fermentation with the bacterium *Acetobactor xylinium*.

Methods of producing vitamin C

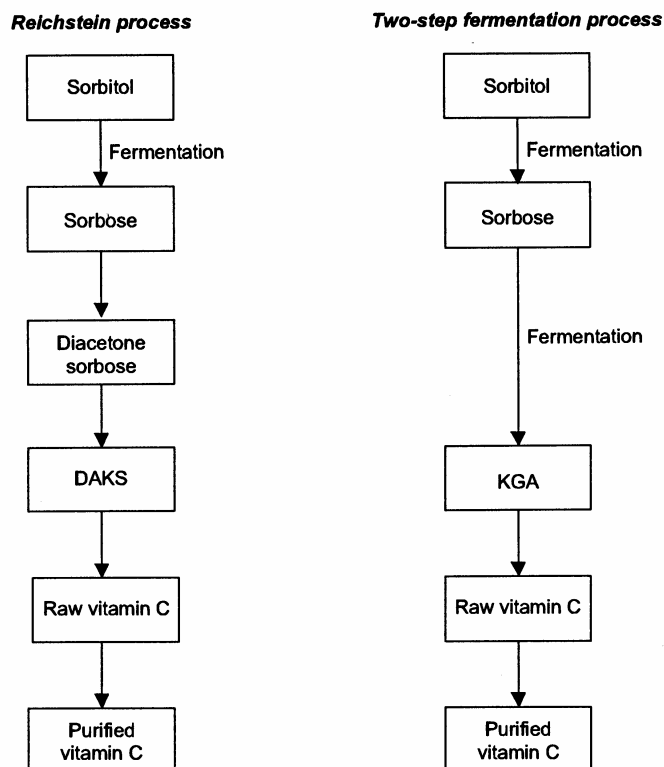


Figure 5. Schematic of industrial production of vitamin C. DAKS is diacetoneketo-1-gulonic acid and KGA is 2-keto-1-gulonic acid. (Competition Commission, 2001)

In the Reichstein process, still used by the chemical giants Roche, BASF and Takeda, sorbitose is transformed into diacetoneketogulonic acid (DAKS) in a two stage chemical process. It is first converted to diacetone sorbose by addition of acetone, and then oxidised with chlorine and sodium hydroxide to produce DAKS. Subsequently, DAKS is rearranged with an acid catalyst to form vitamin C. Figure 5 summarises the steps.

In the two-stage fermentation process, sorbose is fermented to 2-ketogulonic acid (KGA). Finally KGA is processed chemically (Thomas, Powell, & Grant, 2001) to produce vitamin C. Process development continues with a focus on biotechnological processes.

Industrial vitamin C is used in a very wide range of food products among them non-alcoholic drinks. Subsequent text in this thesis will usually refer to vitamin C as ascorbic acid (AA).

Ascorbic acid in non-alcoholic drinks

Ascorbic acid (AA) is used commonly in the beverage industry, especially those made from fruit juices. The technical reasons are to restore the AA lost value during processing, and to contribute to the product's appearance and shelf life. (The addition is also exploited to make implicit health claims in promotion of these drinks, as is obvious from advertising literature.) In the manufacture of fruit juices from fruit such as apple and peach, AA may be added during crushing, straining and processing to prevent enzymatic browning and flavour deterioration (Kacem, Marshall, Shiremean, Cornell, & Matthews, 1987). In these roles it is acting as a reductant, reversing the primary reaction of polyphenol oxidase, semiquinone formation.

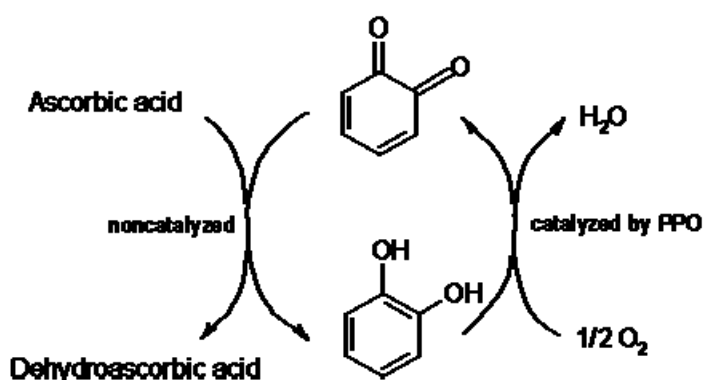


Figure 6. Mechanism of prevention of colour formation by ascorbic acid. From (Marshall, Kim, & Wer, 2000)

AA also reduces oxygen concentration in the headspace of drink containers to minimise future oxidation of the product. About 3.3 mg of AA will remove the oxygen in 1 mL of headspace (Takeda USA Inc., n.d.).

Vitamins and minerals are no longer classified as additives under the food standard code (Food Standards Australia New Zealand), but AA has a declaration code number (E¹) because it also acts as an antioxidant. AA can be added in a number of forms: as the free acid E 300; as sodium ascorbate E 301, calcium ascorbate E 302; and fatty acid esters of ascorbic acid E 304, e.g. ascorbyl palmitate and ascorbyl stearate.

There is a wide range of commercial fruit juices on the New Zealand market. These are usually made from frozen concentrated juice and puree bases. One family of brands is the Keri, Thexton and E2 range of juices. These juices generally have a higher AA concentration than freshly squeezed or not-from-concentrate (NFC) juice.

The claimed concentrations of AA in some common New Zealand juices are listed in Table 3 and 4.

Table 3. Claimed concentration of AA in the Keri family of brands in New Zealand

Product name	AA concentration mg.100 mL ⁻¹	Product name	AA concentration mg.100 mL ⁻¹
Thexton's Blackcurrant	45	Keri Original Apple	32
Thexton's Cranberry	7.5	Keri Original Orange	32
Thexton's Red Grape	7.5	Keri Original Orange Extra	32
Thexton's Apple Raspberry	6	Keri Tomato	32
Keri Original Grapefruit	32	Keri Spiced Tomato	32
Keri Original Pineapple	32	Keri Traditional Apple	32

¹ E numbers are code numbers for different food additives on the label to avoid the confusion caused by some additives having more than one name, or additives having similar names. 'E' refers to the fact that the food ingredient list is suitable for the European Union market and potentially other markets.

Table 4. Claimed concentration of AA in some other popular juices in New Zealand

Product name	AA concentration (mg.100 mL ⁻¹)	Product name	AA concentration (mg.100 mL ⁻¹)
Freshup Apple Nectar	35	Simply Squeezed Berry fruit	40
Freshup Apple Orange	35	Simply Squeezed Orange	35
Freshup Orange	35	Simply Squeezed Tropical	30
Just Juice Cranberry	35	Simply Squeezed Grapefruit	25
Just Juice Passionfruit	35		

The issue

As discussed in the Section *Structure and properties of vitamin C*, AA is stable under acidic conditions so in theory AA should be stable in fruit juices. These are always acidic. However, a substantial literature shows that AA does degrade in stored drinks. Johnston and Bowling (2002) claimed a decline in AA on storage of a range of orange juices. Loss of AA on storage for four months was studied by Kabasakalis, Siopidou and Moshatou (2000), who found a loss of 29 to 40 % of AA in four months. As temperature rises the rate of loss of AA in citrus juice concentrates increases (Burdurlu, Koca, & Karadeniz, 2005). Residual oxygen in the headspace has shown to be the primary cause for AA reduction in fruit juices (Kirk, Dennison, Kokoczka, & Helman, 1977; Riemer & Karel, 1978). Thus, there is no shortage of literature on the stability of vitamin C in fruit juices.

Both water soluble and fat soluble vitamins are variously included in Keri products. The company has no product specific information on the fate of vitamins during storage or shelf life as predicted by the best-before dates.

For technical and legal reasons, the company wanted to know the fate of vitamin C in its formulated fruit juices. It is aware that literature on the stability of vitamin C in fruit juices exists, but clearly there is no substitute for direct determination of bioavailable AA in its products at all stages between production and consumption. In particular, it wanted to be sure that the stated concentration of vitamin C is maintained up to the acceptable level through its supply chain (manufacture-customer-consumption). Also there is no information for a range of other storage and consumption situations, for example, seasonal effects and slow consumption of large 3L bottles of juices. This research sets out to fill those gaps in knowledge.

Chapter 2

Basic Methodologies

Introduction

In this chapter the basic methods used to measure the concentration of AA and bioavailable vitamin C (AA plus DHAA) in juices are described, along with a summary description of the experiments performed on the selected juices. Details specific to each experiment are presented in the relevant chapter.

Titrimetric Method

Principle

Vitamin C is chemically both a reductant and a weak acid. The titrimetric method for determining ascorbic acid is based upon the reduction of the dye 2,6-dichlorophenolindophenol (DCPIP) with AA in acidic solution. As DHAA is not estimated by this method, it does not determine the bioavailable vitamin C activity of a food. According to Nisperos-Carriedo, Busling and Shaw (1992) the concentration of DHAA with respect to AA in commercial orange juice ranges from 0 to 0.2 % which is negligible. If these authors are correct, the error in assessing bioavailable vitamin C activity by DCPIP will also be negligible.

In its oxidised form, DCPIP is purple-blue in neutral or alkaline solution, and pink in acidic solution. The reduced form is colourless at all pHs, so the end point is detected by loss of colour (Figure 7). A persistent light pink signals the presence of excess unreacted dye and therefore the end point. The titration is performed rapidly in the pH range 3 to 4. In the absence of interfering substances, the capacity of the sample to reduce the dye is directly proportional to the ascorbic acid content. The most common substances to interfere in the vitamin C contents in the sample are the reductants sulphhydryl compounds (e.g. glutathione and cysteine), phenols, sulphites and certain metal ions (Eittenmiller & Landen, 1999).

Titration is performed in the presence of a phosphoric acid/acetic acid solution to maintain acidity and to avoid direct oxidation of ascorbic acid at higher pH.

In this procedure detection of an end point will obviously be hard to detect when the drink sample is blue, red or purple. Thus, Thexton's Blackcurrant juice was to be a problem to analyse. (This issue is addressed in Chapter 3.)

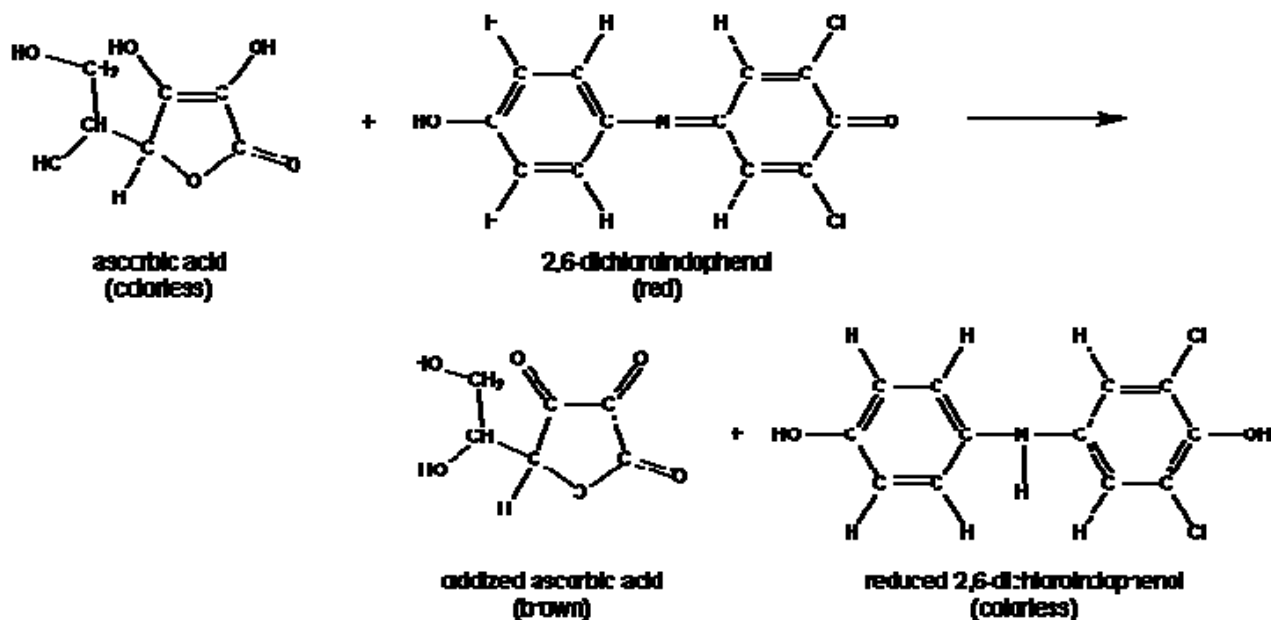


Figure 7. Redox reaction between AA and 2, 6-dichlorophenolindophenol (DCPIP) in acid solution. From (Marshall, Kim, & Wei, 2000)

Reagents

All reagents were analytical grade and were obtained from common suppliers of chemistry laboratory materials. Metaphosphoric/acetic acid solution (acid buffer) was prepared by dissolving 15 g of (solid) metaphosphoric acid in 40 mL of acetic acid, the mixture is gently stirred with a magnetic bar and finally diluted to 500 mL with distilled water. Ascorbic acid standard solution was prepared by dissolving 100 mg of ascorbic acid (BDH Ltd., U.K.) in 100 mL of water. Aqueous 2,6-dichlorophenolindophenol solution was prepared by dissolving 50 mg of desiccated 2,6-dichlorophenolindophenol, sodium salt dihydrate (Merck, Darmstadt, Germany), in 50 mL of water to which 42 mg of sodium bicarbonate had been added. The solution was diluted to 200 mL, filtered through Whatman No. 1 paper and stored protected from light.

Procedure

Aliquots (2.0 mL) of a standard ascorbic acid dilution series were placed in 50 mL Erlenmeyer flasks containing 5.0 mL of acid buffer solution. For each flask, the mixture was gently stirred with a magnetic bar. Titration was done rapidly with DCPIP solution from a burette until the light pink colour of the reduced dye persists at least for five minutes. A standard curve was constructed by titrating different concentrations of AA with DCPIP as shown in Figure 8.

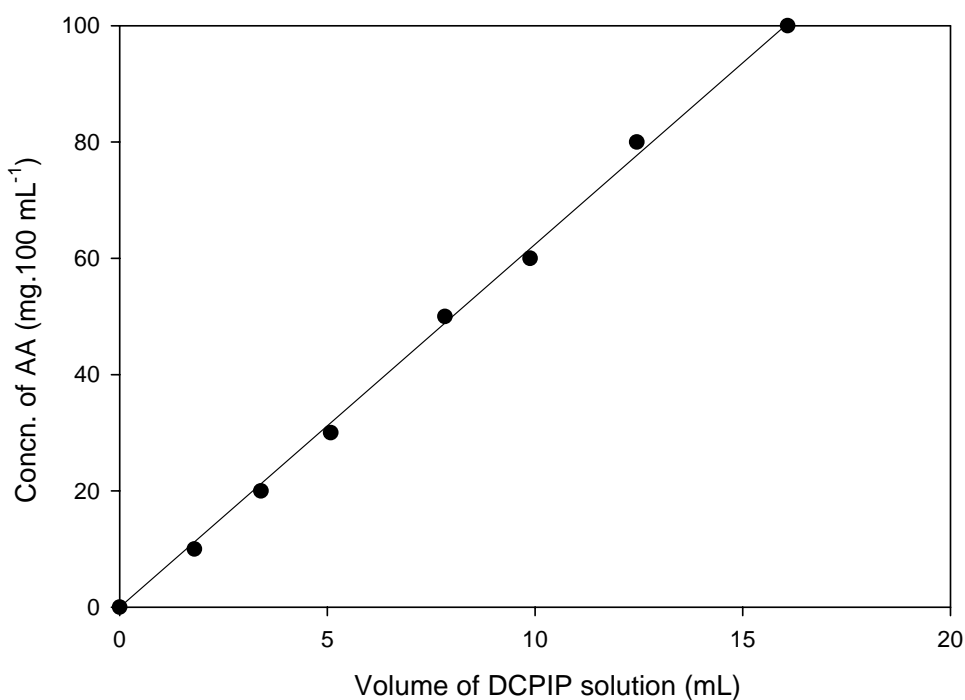


Figure 8. Typical calibration curve for the titrimetric method of AA determination

Sample preparation

Before opening a juice bottle for determination of AA, it was shaken to uniformly distribute any suspended components. Titration was replicated with quadruplet 2.0 mL aliquots of juice as done above with standard ascorbic acid solution. Concentrations were calculated by reference to the standard curve.

Reproducibility and recovery test for AA in selected juices

A known amount of AA was added to several juices, and recovery was tested using the titration method with eight replicates of each fruit juice. The results showed excellent reproducibility and recovery (Table 5).

Table 5. Reproducibility and recovery of AA spiked into selected Keri juices, using the titration method

Fruit juice	Determined mean value (mg.100 mL ⁻¹) (and CV %) of unspiked juice*	Determined mean value (mg.100 mL ⁻¹) (and CV %) of juice spiked with 100 mg of AA per 100 mL	Recovery (%)
Keri Original Apple	34.7 (1.00)	135.1 (0.27)	100.4
Keri Original Orange	84.5 (0.37)	185.0 (0.26)	100.4
Keri Crush Tropical	27.1 (1.28)	127.9 (0.24)	100.8

*Of eight replicates for all determinations. CV = coefficient of variation

Chromatographic method

As explained in the Introduction, both AA and DHAA are bioavailable. Thus measurement of AA alone is theoretically insufficient in terms of claims. The titrimetric method measures only AA so a method to measure AA and DHA had to be employed. Liquid chromatography can be used to separate AA from DHAA and these analytes can be measured independently.

In its analytical applications, liquid chromatography is usually called high pressure liquid chromatography (HPLC). Whether in the gaseous (gas chromatography) or liquid state (HPLC), the capacity of chromatography to separate one analyte from others depends on the relative affinity of each analyte for a stationary phase (the packing in a column) and a mobile gaseous or liquid phase or phases. There are a number of well-described HPLC methods that can simultaneously measure AA and DHAA.

The two analytes have been separated on a number of phase including reversed phase C18 (Gennaro & Abrigo, 1981), bonded-phase amine (Arakawa, Otsuka, Kurata, & Inaka, 1981), ion-pair reversed phase C18 (Madigan, McMurrough, & Smyth, 1996), and anion exchange (Williams, Baker, & Schmit, 1973). Vanderslice and Higgs (1984) separated AA

and DHAA with an anion-exchange resin column, while fluorescence detection was achieved through post-column in-line derivatisation involving oxidation of AA to DHAA followed by reaction of the latter with o-phenylenediamine to form a fluorescent product. Kall and Andersen (1999) described a related post-column derivatisation method, with direct absorbance detection of AA at 247 nm, and indirect fluorescent detection of DHAA after a post-column derivatisation. Ziegler, Meier, & Sticher (1987) estimated DHAA directly in addition to AA, by separating the two compounds using reversed-phase HPLC with ion suppression, and reducing DHAA to AA with dithiothreitol in a post-column in-line system. With the facilities available at AUT the method of (Furusawa, 2001) was the most attractive.

Furusawa's method claims to provide a simple and fast isocratic (unchanging mobile phase) method for qualitative and quantitative estimation of AA and DHAA in commercial fruit juices. This method requires minimum sample preparation with no derivatization procedures. Two chromatographic runs are required. The first measures AA as present in the juice and the second measures AA after any DHAA is reduced to AA with the powerful reductant dithiothreitol. Thus AA and bioavailable AA can be measured.

As explained in *Titrimetric Method* above, blackcurrant and other similar anthocyanin pigments render the titrimetric method nearly useless for such juices. The chromatographic method avoids this problem. However, any pigment that coelutes with AA or DHAA would cause problems if it absorbed at the detection wavelengths of AA and DHAA.

HPLC apparatus

The HPLC pump (LC-10AD, Shimadzu, Japan) has a dynamic range of 0.1 mL. min⁻¹ to 9.9 mL. min⁻¹. The stainless steel column was a Synergi Polar-RP80A (Phenomenex, California), measuring 250 mm x 4.6 mm and packed with a 4 µm particles of stationary phase. This column is designed to resolve, among other analytes, polar acidic and basic compounds in the range pH 2 to pH 7. A guard column (Nova-Pak 4 µm 60A C18, Waters, Ireland) was placed immediately upstream of the Synergi column to protect it from foreign particles.

The detector was a SPD-M10AV (Shimadzu) diode array detector. Two wavelengths are used, 220 nm for DHAA and 243 nm for AA. Samples were introduced automatically with a SIL-10A injector (Shimadzu) maintained at 20°C.

Reagents and standards

Mobile phase was prepared by dissolving 5 mL of metaphosphoric acid in a final volume of 500 mL of water. This was mixed with 500 mL of methanol (HPLC grade, BDH Ltd., U.K.). AA standard solution was prepared as described for the titrimetric method. As well as sourcing purportedly authentic DHAA from Alltech, USA, it was also prepared from the standard AA solution. Fifty millilitres of standard AA solution was titrated with 0.1 % (w/v) iodine until the stirred solution had a constant light yellow colour. To reduce the surplus iodine from the solution, two crystals of sodium thiosulphate were added. The final volume was made 100 mL.

1,4-Dithiothreitol (Fluke, Switzerland) solution was made by dissolving 17.5 mg in a final volume of 10 mL with deionised water. This was 11 mM.

Sample preparation and chromatography

Before opening, juice bottles were shaken to uniformly distribute any suspended components. The juices were filtered through a 0.45 μm nylon syringe filter (Bonnet, Australia), diluted to suitable concentration and mixed 1:1 (v/v) with double-strength mobile phase. The injection volume was always 20 μL . Samples for injection were prepared just before analysis to minimise any decomposition of AA.

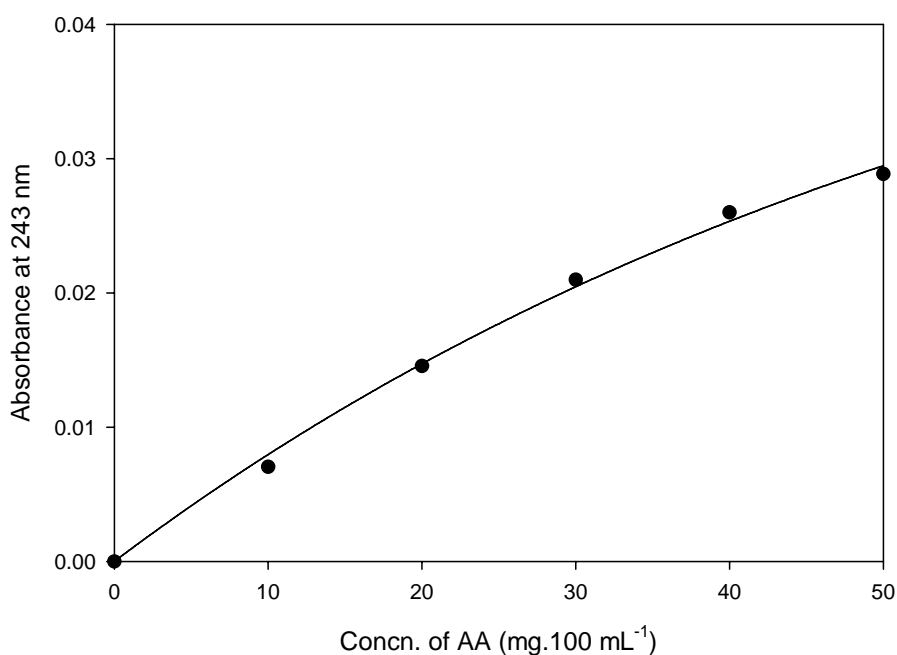


Figure 9. Typical calibration curve for the HPLC method of vitamin C determination.

Helium gas was continually bubbled through all solutions to remove dissolved air. Before analysis, the column was cleaned with acetonitrile followed by methanol. The flow rate of mobile phase was increased slowly starting from 0.25 to 1.0 mL min⁻¹, the chosen flow rate for chromatography.

The injection needle was automatically cleaned with methanol between samples. After passing from the column, the separated solutes were sensed by the in-line diode array detector. Runs typically took 20 minutes. A standard curve was constructed by eluting different concentrations of AA under standard conditions (Figure 9).

Precision of the chromatographic method

Precision was tested on replicate samples prepared from Keri Original Apple in two ways. In the first, 1 mL samples of filtered juice were placed in beakers to which was added 1 mL of double-strength mobile phase. This tested precision of replicate subsamples. These were directly injection (20 µL as always) and the AA content determined (Table 6). In the second, a single 1 mL sample with 1 mL of double-strength mobile phase was repeatedly injected. This tested precision of injection (Table 6).

Table 6. Precision of the chromatographic method		
Fruit juice	Determined mean value (mg.100 mL ⁻¹) (and CV %) for replicate subsamples*	Determined mean value (mg.100 mL ⁻¹) (and CV %) for replicate injections*
Keri Original Apple	56.6 (3.2)	57.9 (0.5)
*Of 10 replicates. CV = coefficient of variation		

The precision of automated replicate injections was much better than the precision attained in replicated subsamples, presumably arising from pipetting errors. However, the latter precision was considered adequate for the work in hand.

Chapter 3

Methods Refinement

Introduction

This chapter reports a comparison of the two analytical methods testing the juices, developmental work on the chromatographic method, and experiments to overcome the colour problem that prevents detection of the dichloroindophenol endpoint in the titrimetric method.

As discussed in Chapter 1, bioavailable vitamin C includes AA and DHAA. The simpler titrimetric method detects only AA, so reducing agents can be used to regenerate AA from DHAA for accurate determination of vitamin C. Possible reducing agents of value include L-cysteine and dithiothreitol (DTT) that are added directly to juices before chromatography.

Comparison of the titrimetric and chromatographic methods

Table 7 compares the values obtained with four juices as determined by the two methods. Due to an oversight, replicate determinations were obtained for the titrimetric method but not the chromatographic method.

Table 7. Direct comparison of the titrimetric and chromatographic methods.

Fruit juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Determined mean value of AA (mg.100 mL ⁻¹) (and CV %) by titration*	Determined mean value of AA by chromatography (mg.100 mL ⁻¹)
Keri Original Apple	32	55.2 (2.4)	56.0
Keri Original Orange	32	70.1 (0.0)	79.0
Keri Crush Tropical	24	41.6 (0.0)	47.9
Keri Crush Apple Orange	24	43.8 (1.5)	49.1

*Of four replicates. CV = coefficient of variation

Recognising that no replication was applied in the chromatographic method, the results appear similar with a hint that the latter may yield higher values. In future work the two methods were never mixed so comparison within experiments will be valid.

Effect of cysteine and DTT on AA concentration in Keri Original Apple

Methods

A sample of Keri Original Apple was filtered through a 0.45 μm nylon filter. To 1 mL of filtered juice was added 1 mL of 0.1 M cysteine or dithiothreitol followed by 2.0 mL of double-strength mobile phase solution. The final concentration of reductant was 0.025 M, which would exceed the maximum possible DHAA concentration by about 50-fold based on the claimed concentration of 32 mg of vitamin C.100 mL⁻¹ in this juice. This would be the situation where all vitamin C in the juice was in the oxidised form, DHAA. Two other samples were prepared, one where 1 mL of water was substituted for juice and the other where 1 mL of water was substituted for reductant. The six treatments were analysed by chromatography in duplicate.

Results

Table 8 shows that cysteine eluted very shortly before AA and as a result overlapped the AA peak (not shown). In contrast, dithiothreitol was well resolved. Dithiothreitol was chosen as the reductant for subsequent experiments.

Table 8. Elution times of AA, cysteine and dithiothreitol on the chromatographic column

Compound	Elution time (min)
AA	3.13
Cysteine	3.00
Dithiothreitol	5.28

Effect of dithiothreitol on DHAA prepared from AA solution

Methods

DHAA was prepared from 100 mg.100 mL⁻¹ AA solution by using iodine as an oxidant as described in Chapter 2. The final DHAA concentration was 50 mg.100 mL⁻¹.

Subsequently, the effect of a 10-fold molar excess of dithiothreitol on DHAA was tested under standard dilution conditions (1 volume of DHAA, 1 volume of dithiothreitol or water, 2 volumes of double-strength mobile phase). This procedure was duplicated and retested in later work, reported in Chapter 5.

Results

Table 9 shows area data for 243 nm (optimum for AA) and 220 nm (DHAA) at the elution times of interest. There was no significant decrease in the concentration of DHAA nor an increase in the concentration of AA. Under these conditions it appeared that the 10-fold excess of dithiothreitol was incapable of reducing DHAA back to AA.

Table 9. Effect of dithiothreitol on prepared DHAA

Treatment	Area x 10 ⁻⁶ (and CV%)* under curve at 243 nm, 3.20 min (best for AA)	Area x 10 ⁻⁶ (and CV%) under curve at 220 nm, 3.25 min (best for DHAA)
DHAA (50 mg.100 mL ⁻¹)	12.6 (1.4)	34.4 (1.0)
DHAA (50 mg.100 mL ⁻¹) + dithiothreitol	12.9 (2.1)	34.7 (0.6)
*CV % of three replicate preparations, single injection		

Experiments with commercially-sourced DHAA

With the failure of DTT to reduce laboratory-prepared DHAA to AA, it was decided to repeat the experiment with purportedly authentic DHAA (Sigma catalogue DB132).

Methods

Solutions of 50, 100 and 500 mg of DHAA per 100 mL were prepared. After dilution (1 volume of DHAA, 1 volume of excess dithiothreitol or water, 2 volumes of double-strength mobile phase) aliquots were analysed by liquid chromatography.

Results

At all concentrations, absorbance by the purportedly pure DHAA was insignificant (Table 10) as compared to pure AA and DHAA prepared in the laboratory by the iodine oxidation method (Table 9).

Table 10. Effect of dithiothreitol on commercially-sourced DHAA

Treatment	Area* x 10 ⁻⁶ under curve at 243 nm, 2.94 min (best for AA)	Area x 10 ⁻⁶ under curve at 200 nm, 2.98 min (best for DHAA)
Purported DHAA (100 mg.100 mL ⁻¹)	0.02	0.04
Purported DHAA (500 mg.100 mL ⁻¹)	0.03	0.10
Purported DHAA (100 mg.100 mL ⁻¹) + 10 fold excess of dithiothreitol	No peak except for dithiothreitol	No peak except for dithiothreitol
Purported DHAA (500 mg.100 mL ⁻¹) + 10 fold excess of dithiothreitol	No peak except for dithiothreitol	No peak except for dithiothreitol
Expected area for authentic DHAA (100 mg.100 mL ⁻¹)	At least 12.6 (see Table 9)	At least 34.4 (see Table 9)
*Mean of duplicates		

It is clear from Table 10 that the commercial preparation of DHAA was completely impure, probably through oxidation, and of no value to this project. The powerful reductant dithiothreitol was incapable of restoring whatever was in the bottle to DHAA.

Attempts to determine AA in red-blue juices by the titrimetric method

A wide range of Keri juice products are made. For some, the titration method is suitable because it is easy to detect the persistent light pink colour indicating the titration end point. Such juices include Apple, Orange and Tropical. In contrast, Thexton's Red Grape, Thexton's Blackcurrant and Thexton's Cranberry etc. the titration method does not work because the pink colour cannot be seen against the red/blue background. Two methods were tried to solve the problem of detecting end point. These were by decolourisation with activated charcoal and changing the juice pH to alter the colour due to the fruit anthocyanins.

Methods

For decolourisation, 0.5 g of activated charcoal (heated in oven at 80°C overnight to increase its adsorbing capacity) was stirred into 50 mL of Thexton's Blackcurrant and the mixture left for 20 minutes. A slurry of diatomaceous earth was prepared by adding 2 g of Celite powder (545) to 10 mL of distilled water. A Buchner funnel was fitted with a sheet of Whatman No. 1 filter paper and a layer of Celite was developed with a water pump vacuum. The mixture of juice and charcoal was then poured over the Celite layer and finally rinsed with 20 mL of water. The total volume was made to 100 mL. AA in this solution was determined by the standard titrimetric procedure using DCPIP. As one control, 40 mL of AA solution (100 mg.100 mL⁻¹) was mixed with 0.5 g of activated charcoal, filtered through Celite filter in same the way as for Thexton's Blackcurrant, and the AA concentration determined. As another control, an AA solution without charcoal was passed through Celite.

For pH adjustment, aliquots of Thexton's Blackcurrant (40 mL) were titrated with 0.1 M NaOH to increase the pH in steps from 3.25 to 8. The final volumes were made to 100 mL. Each sample of Thexton's Black currant was then scanned in the visible wavelength range with an Ultrospec 2100 spectrophotometer (Amersham Bioscience, U.K.)

Results

Charcoal was extremely effective as a decolourant, because the juice emerged clear and totally colourless. However, the treatment had the unfortunate side effect of catalysing the rapid and complete oxidation of AA to DHAA. This was not due to Celite, because the trial with Celite showed zero loss (Table 11).

Table 11. Titration results for Thexton's Blackcurrant and AA treated with activated charcoal and filtered through Celite

Treatment	Mean volume (mL)* (and CV %) of DCPIP solution to titrate to end point
AA, 100 mg.100 mL ⁻¹	16.2 (0.3)
AA, 100 mg.100 mL ⁻¹ treated with charcoal	0
Thexton's Blackcurrant treated with charcoal	0
AA, 100 mg.100 mL ⁻¹ treated with Celite alone [†]	15.1 (0.3)

*Of four replicates for all determinations. [†] Measured in later experiment

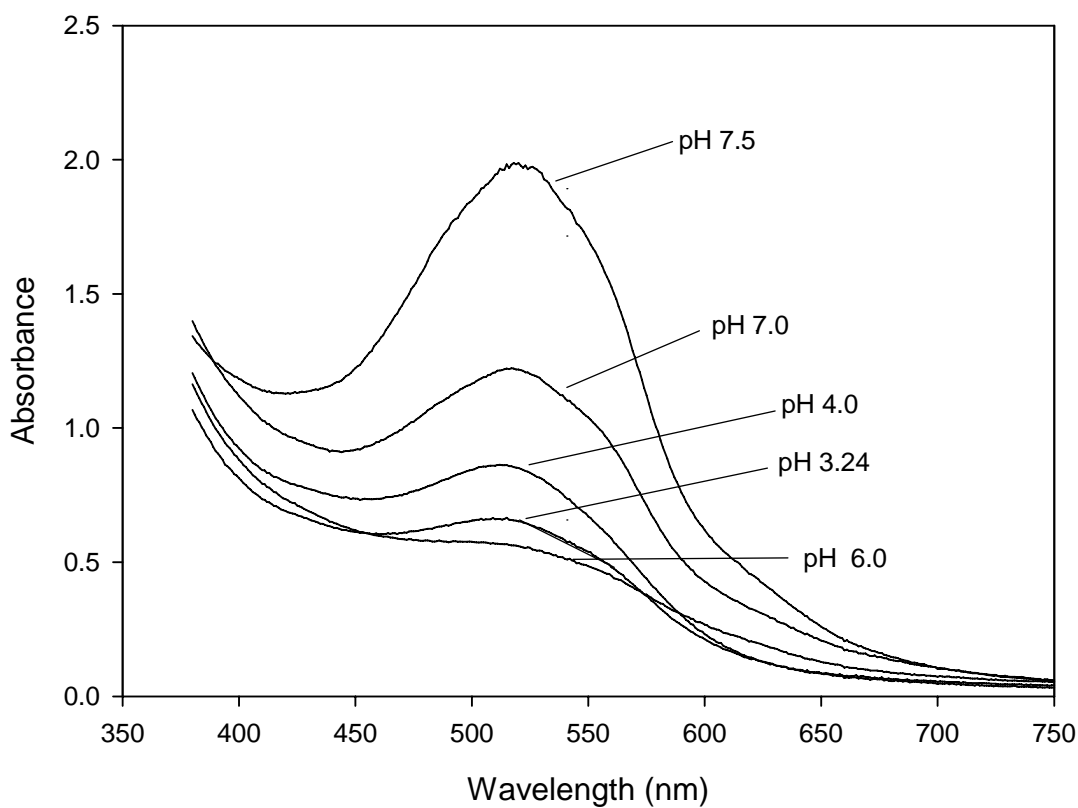


Figure 10. Visible spectra of Thexton's Blackcurrant juice at a range of pH values

The pH of Thexton's Blackcurrant juice was 3.24, and in its native state was characteristically purple. At all tested pH values to 8, some colour was lost (e.g. at pH 6) but generally changing the pH only increased colour (Figure 10). Even if colour had been lost and titration had become possible, there was always the risk that AA would become unstable prior to titration and values would lower than actual.

Discussion

The choice of potential reductant was clear. DTT was well resolved from AA and DHAA under the chromatographic conditions used. However, it failed to perform as described by (Furusawa, 2001). This matter was not pursued because the chromatographic procedure was found to be unsuited to monitoring AA over many months. It was expensive on chemicals, time consuming within the context of part-time enrolment, and above all, the equipment was being used by several operators each with different objectives and analysis

parameters. Therefore it was decided to choose the titrimetric method for regular use though it is not suitable for dark drinks. However, the chromatographic method was used spasmodically to monitor DHAA formation (if any).

In the supply chain from production to consumption, fruit juices are stored and consumed under a variety of conditions, including different exposures to temperatures and light (sunlight and fluorescent), and exposure to air when large containers are opened and consumed after refrigeration over several days. The following experiments were performed.

- **Effect of long term storage under fluorescent light and in darkness.** Juices are displayed in supermarkets under bright fluorescent lights as a merchandising aid, but fluorescent light may affect the stability of AA. Thus a long term storage experiment compared the fate of vitamin C concentration in selected juices stored under fluorescent lights with storage in total darkness. Because the titration method measures only AA, at several time points in this exposure-to-fluorescent-light trial samples were also analysed by chromatography in attempt to determine AA and DHAA concentration. These experiments are reported in Chapter 4.
- **Effect of different temperatures of dark storage.** After bottling, products are stored first in company's warehouses, later in the supermarkets, and finally in customers' homes. The temperature may vary markedly in this supply chain. An experiment was conducted to determine the effect of different temperatures on AA for two months of storage in the dark (Chapter 5).
- **Effect of repeated exposure to air.** Keri juices are available in different size of bottles. The largest size is 3 L Keri bottle, which is economical for families. Generally, 3L bottles are not consumed in one day. After every pour the lost volume is replaced by air, which through its oxygen content, is likely to oxidise AA. An experiment was conducted to determine the effect of repeated opening and pouring in refrigerated 3 L bottles (Chapter 5).
- **Effect of pasteurization.** After industrial batching (mixing ingredients in the correct proportions) juices are pasteurised at temperature between 92 to 100°C and then cooled to 63 to 70°C before bottling. In this experiment, the fate of AA concentration was determined at the time of batching before pasteurisation and after bottling (Chapter 5).

- **Effect of exposure to sunlight.** When juices are exposed to sunlight, ultraviolet (UV) light may affect AA. The impact of direct sunlight and UV-filtered sunlight was therefore examined (Chapter 5).

In the next chapter, the stability of AA was monitored in selected juices under total darkness and fluorescence light.

Chapter 4

Long-term storage effect of total darkness and fluorescent lights

Introduction

In this chapter the fate of AA was examined during the long-term storage of four fruit juices. The storage period was nine months. There were two storage conditions at the same temperature: under fluorescent light such as might be found in a supermarket, and in total darkness. AA was mainly determined by the titration method.

Methods

Comparison was done for juices stored under fluorescent lights to simulate display conditions with storage at the same temperature in darkness (in cartons). The best-before date printed on the bottles is nine months from the date of manufacturing. Vitamin C was determined over 9 months routinely by titration and, occasionally, by chromatography. Routine monitoring by chromatography was discontinued early in the trial due to various factors e.g. long periods to stabilise the column, complexity of sample preparation of, high cost, and most important, the time required to analyse all the samples. However, the chromatographic method was periodically employed in attempt to determine the concentration of bioavailable vitamin C (AA plus DHAA).

Juice formulations

The juices, which were all prepared in February 2005, were Keri Original Apple, Keri Crush Tropical, Keri Crush Apple Orange Mango, and Thexton's Blackcurrant. They are various mixtures of juice concentrates, purées and flavours, citric acid, ascorbic acid, food-approved ingredients, and water. The exact formulations are confidential proprietary information. The labels on all of these juices declare an AA concentration, which can derive from an addition of AA and/or from the juice concentrate or purée.

Storage conditions

The smallest bottle size, 375 mL, was selected to maximise light exposure and surface-to-volume ratio. A high ratio will maximise oxygen transmission across the plastic (polyethylene terephthalate) containers into the juice. The bottles were stored in a room

which was closed on three sides with a louvred window on the fourth side that opened into a large warehouse. It was open at all times. Any exposure to daylight was insignificant. Half the bottles were kept in closed cartons to effect total darkness. The other half were displayed on a table measuring 1.2 m by 2.4 m. The surface was covered with matt white paper.



Figure 11. Display of juices under fluorescent light

Bottles were displayed in column and rows, with a minimum gap of 20 cm between each bottle. The positions of the bottles were changed every week to maintain an equal gap between each. They were also rotated at this time. Three sets of fluorescent tubes were fixed on to the ceiling. The sets were parallel and 1.1 m apart. The average intensity of light at table top was 1443 lux, as measured by a light meter (Model Tes-1330A, ShangHai ZhongXuan Electronic, Shanghai). Four thermometers were placed in four corners of the room so temperature could be monitored several times a week.

Analysis was done weekly when bottles were chosen at random off the light table and sequentially out of cartons. Bottles were shaken to mix any suspended ingredients before opening. AA was estimated immediately after opening, and was always accompanied by titration of a reference AA solution ($100 \text{ mg} \cdot 100 \text{ mL}^{-1}$).

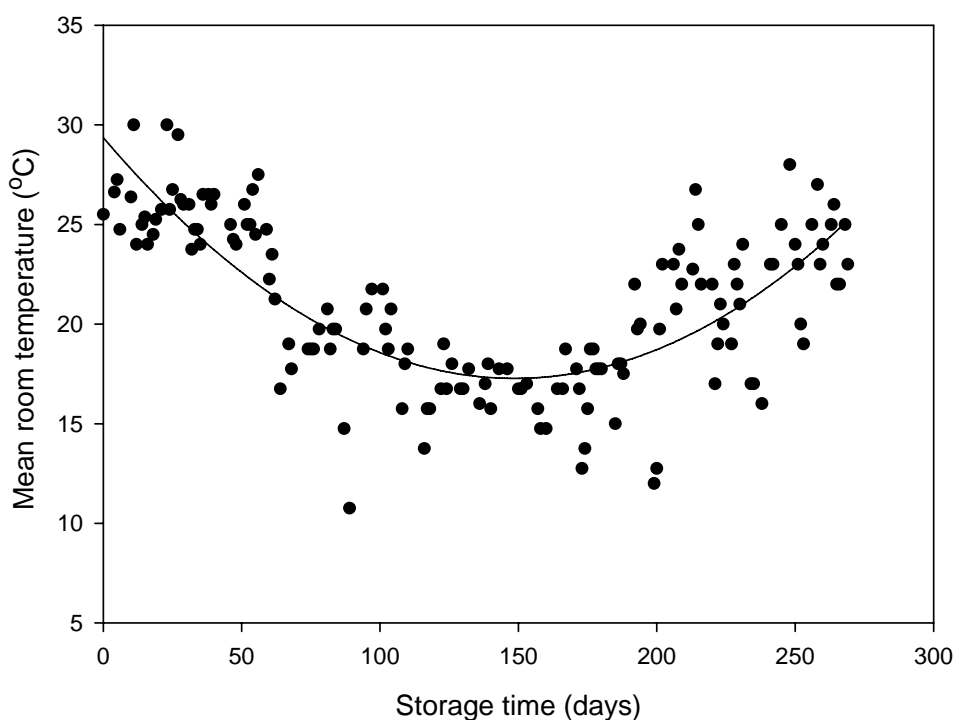


Figure 12. Room air temperature plotted over 270 days. Data points are means on a given day, and the line is a quadratic line of best fit for which R^2 was 0.61

Results

The temperature difference between the four thermometers was never more than 0.5°C . The mean temperature fluctuated between 28°C during summer and 17°C in winter (Figure 12). The highest recorded temperature was 30°C in summer and minimum of 10°C in winter. It is expected that the thermal inertia of the bottles would result in less between-day variation than shown in Figure 12.

Table 12. Overall effect of fluorescent light and storage to 270 days on the retention of AA in fruit juices

Fruit juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Measured concentration* of AA \pm SD (mg.100 mL ⁻¹) at start of storage trial	Approximate percent remaining AA after storage under fluorescent lights	Approximate percent remaining AA after storage in total darkness
Keri Original Apple	32	45.7 \pm 0.1	32	53
Keri Original Orange	32	65.7 \pm 0.3	53	63
Keri Crush Tropical	24	42.6 \pm 0.1	34	46
Keri Crush AOM	24	40.0 \pm 0.1	38	47

*Mean of four replicate titrations

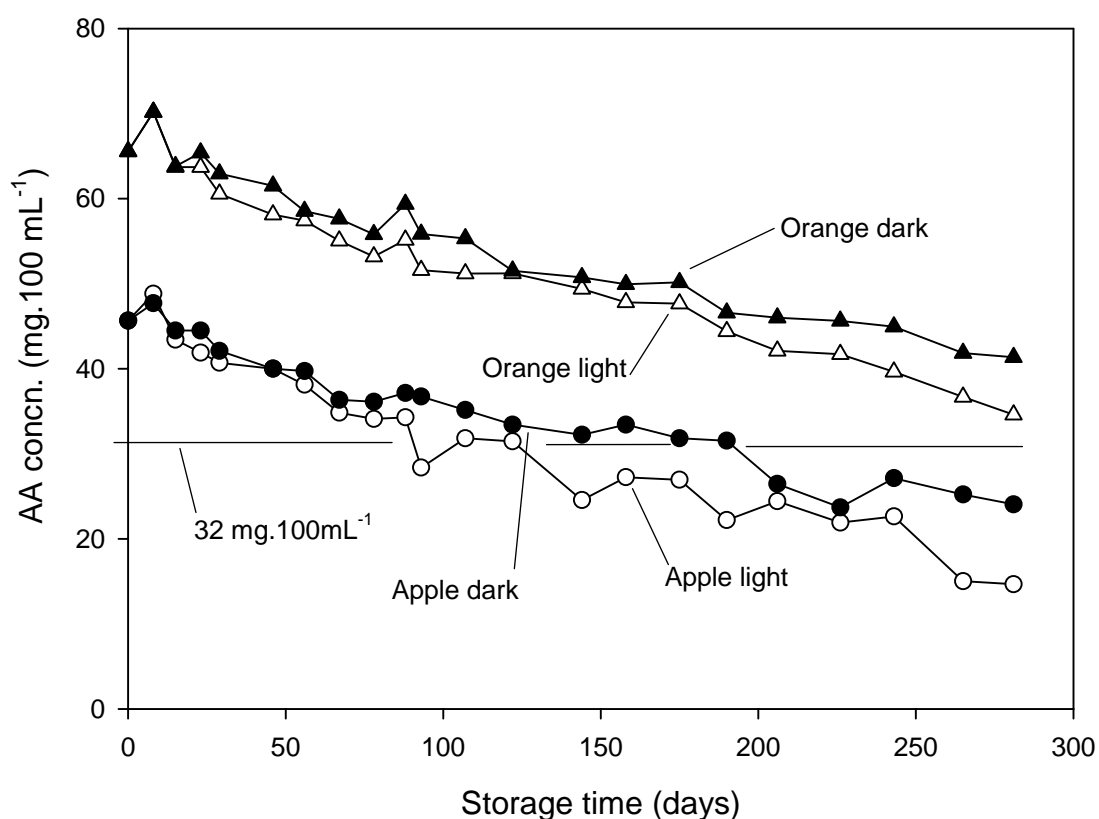


Figure 13. Effect of storage time under light and dark conditions on AA concentration in Keri Original Apple and Keri Original Orange. The horizontal line indicates the claimed concentration of vitamin C

Concentrations of AA remaining after 270 days are shown in Table 12. For Original Apple, the percent remaining was 53 % (dark) and 32 % (light), while the respective figures for Original Orange were 63 % and 53 %. Similar results were obtained for Keri Crush Tropical and Keri Crush AOM. The percent remaining was 34 % (dark) and 46 % (light) for pure Tropical, while the respective figures for mixed Apple Orange Mango were 38 % and 47 %.

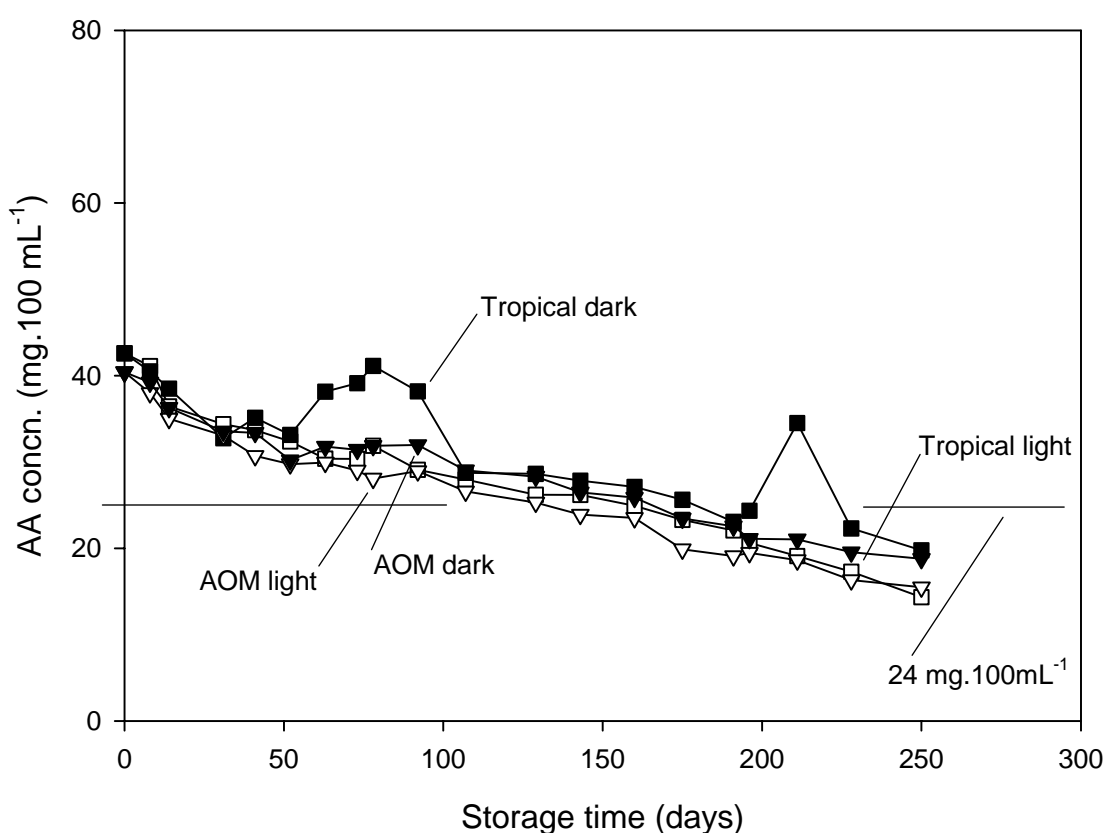


Figure 14. Effect of storage time under light and dark conditions on AA concentration in Keri Crush Tropical and Keri Crush Apple Orange Mango (AOM). The horizontal line indicates the claimed concentration of vitamin C

The claimed AA concentration in juices under investigation was either 24 or 32 mg.100mL⁻¹ of juice. (This is stated as vitamin C on the labels.) The starting concentrations were very much higher, particularly in the case of Keri Original Orange (Table 12) where the initial concentration was about double that claimed.

There was an essentially linear decrease in AA concentration in both conditions (total darkness and fluorescence light) (Figure 13) in all juices. (Linearity is examined in more detail below).

Losses were clearly greater with light exposure but the differences were not important except toward the end of storage. Keri Crush Tropical and Keri Crush Apple Orange Mango were least affected by fluorescence light. This may be due to partially opaque sleeves covering most of the bottle.

In the case of Keri Original Orange, the concentration was above the claimed vitamin C concentration for the entire period, but for Keri Original Apple the concentration fell below 32 mg.100 mL⁻¹ after about 120 days in the light and 180 days (dark), well before the best-before date.

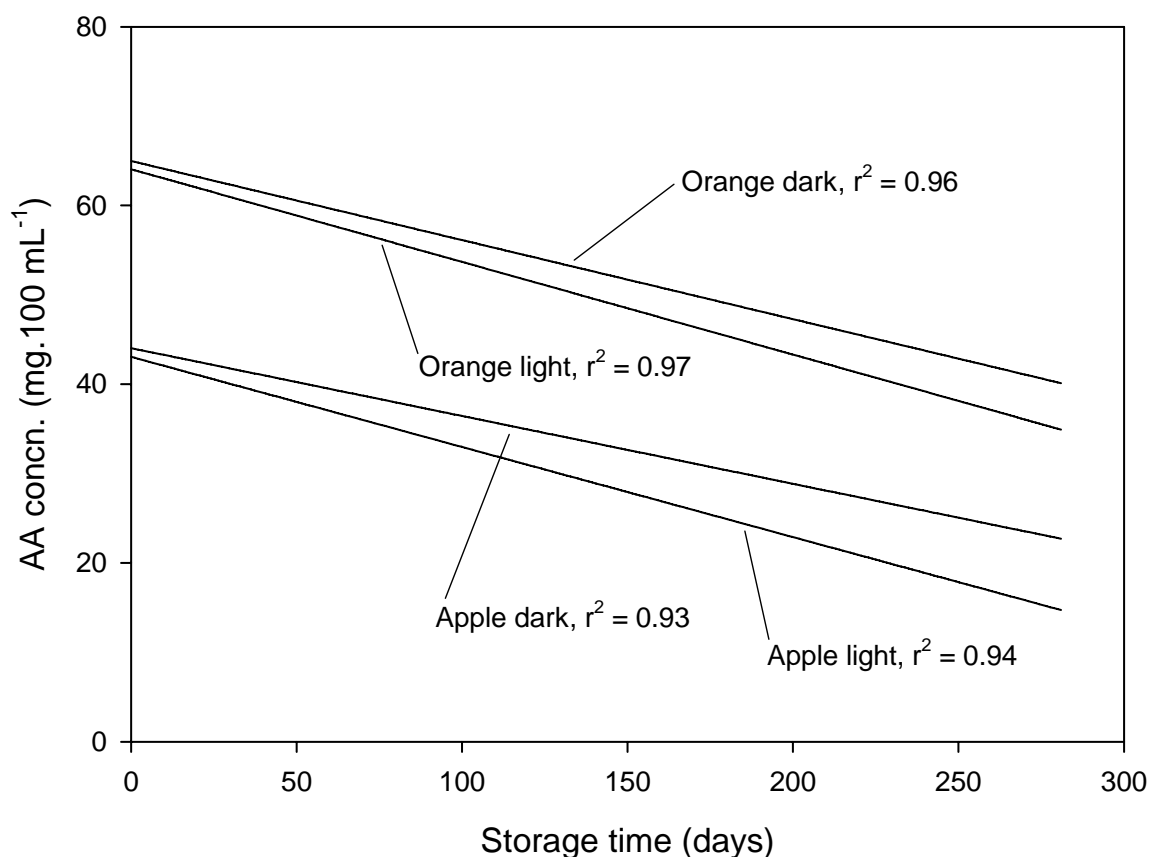


Figure 15 Least squares lines of best fit for Keri Original Apple and Keri Original Orange under light and dark storage conditions

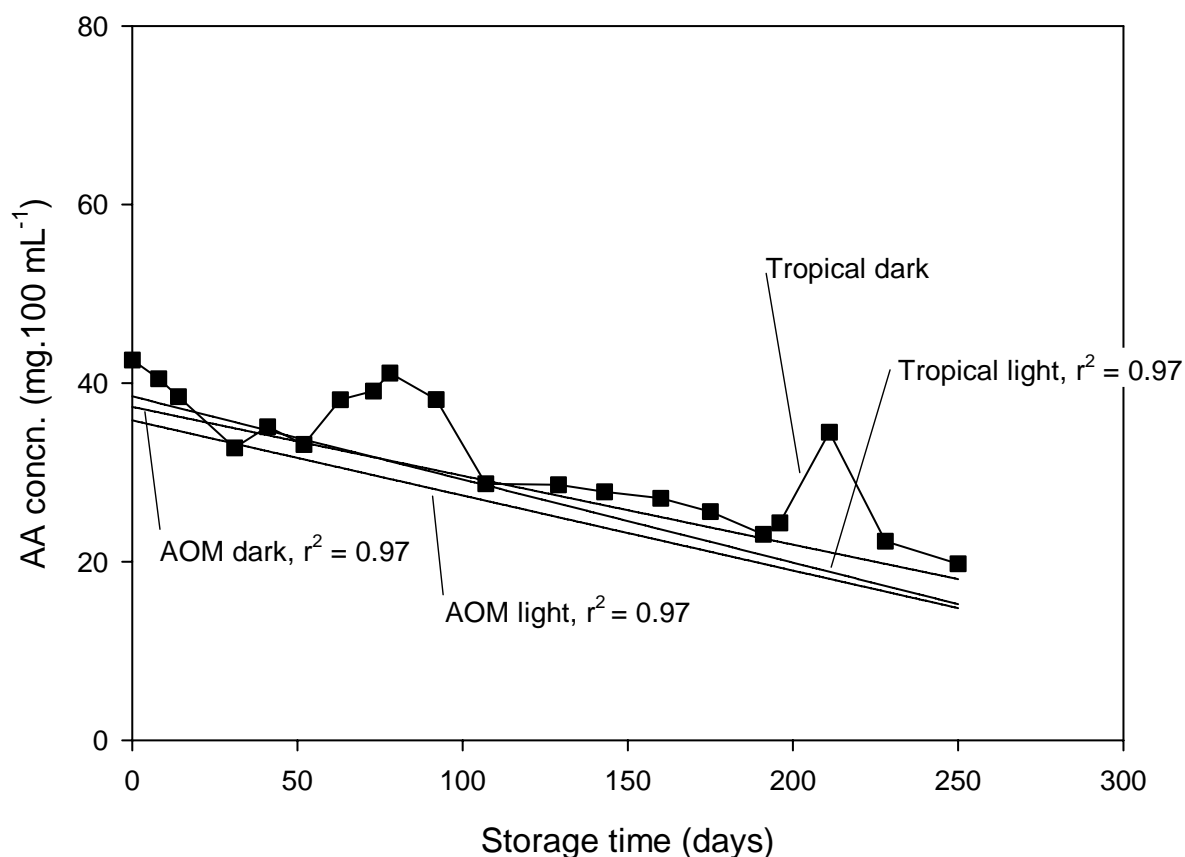


Figure 16. Least squares lines of best fit for Keri Crush Apple Orange Mango for both storage conditions and Keri Crush Tropical for storage in the light

The AA in Keri Apple Orange Mango (AOM) in the light survived to about 140 days, and in the dark to about 175 days. Tropical in the light survived to about 180 days and Tropical in the dark to over 200 days (Figure 14).

There were some apparently spurious results with Tropical dark between 50 and 100 days. There was an apparent increase in titre. It is possible, but not proven, that sequences of bottle in the particular carton being accessed were fermenting. At these four times a second and adjacent bottle was analysed with similar results. This matter was not pursued further.

Table 13. Effect of DTT on the concentration of AA in fruit juices stored under fluorescent light for 70 days

Fruit juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Mean* of AA \pm SD without DTT (mg.100 mL ⁻¹)	Mean* of AA \pm SD with DTT (mg.100 mL ⁻¹)
Keri Original Apple	32	46.4 \pm 0.5	45.7 \pm 1.1
Keri Original Orange	32	62.4 \pm 0.8	61.6 \pm 0.5
Keri Crush Tropical	24	33.7 \pm 0.5	34.0 \pm 0.3
Keri Crush AOM	24	32.1 \pm 1.9	31.6 \pm 0.3
Thexton's Blackcurrant	45	99.3 \pm 0.8	106.8 \pm 2.4

*Of six replicate chromatographic runs. SD = Standard deviation

Table 14. Effect of DTT on the concentration of AA in fruit juices stored under fluorescent light for 101 days

Fruit juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Mean* of AA \pm SD without DTT (mg.100 mL ⁻¹)	Mean* of AA \pm SD with DTT (mg.100 mL ⁻¹)
Keri Original Apple	32	33.0 \pm 1.4	32.4 \pm 4.3
Keri Original Orange	32	58.7 \pm 0.6	59.2 \pm 3.9
Keri Crush Tropical	24	35.3 \pm 2.4	35.1 \pm 3.9
Keri Crush AOM	24	29.4 \pm 6.9	31.6 \pm 6.7
Thexton's Blackcurrant	45	89.3 \pm 0.9	99.6 \pm 0.8

*Of three replicate chromatographic runs. SD = Standard deviation

Table 15. Effect of DTT on the concentration of AA in fruit juices stored under fluorescent light for 241days

Fruit juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Mean* of AA \pm SD without DTT (mg.100 mL ⁻¹)	Mean* of AA \pm SD with DTT (mg.100 mL ⁻¹)
Keri Original Apple	32	30.7 \pm 0.7	31.6 \pm 0.2
Keri Original Orange	32	54.1 \pm 2.4	52.9 \pm 1.2
Keri Crush Tropical	24	23.5 \pm 2.0	21.2 \pm 0.8
Keri Crush AOM	24	20.2 \pm 0.9	18.8 \pm 0.2
Thexton's Blackcurrant	45	77.1 \pm 1.4	75.8 \pm 1.3

*Of six replicate chromatographic runs. SD = Standard deviation

While monitoring the fate of AA by the titration method over 285 days, the chromatographic method of measurement with and without DTT was repeated three times (at 70, 101 and 241 days) in an attempt to determine DHAA – if any. The following results were obtained (Table 13, 14 and 15). It seems likely that DTT had no effect on AA values, although in two situations – both with Thexton's Blackcurrant, there was an indication that some DHAA was present at 70 and 101 days, but not at 241 days. Thus, the AA value was clearly significantly higher in the presence of DTT than in its absence, e.g. 99.6 vs 89.3 mg.100 mL⁻¹ at 101 days.

Discussion

Exposure to fluorescent light significantly affects the stability of AA in these bottles, but the effect is unlikely to be commercially important.

Based on titration and chromatography, which measures only AA, the initial concentration of AA greatly exceeded the claimed value, especially in the case of Keri Original Orange and Thexton's Blackcurrant. For Orange, the concentration based on AA addition was 40 mg.100 mL⁻¹, but the starting value was 66 mg.100 mL⁻¹ for this particular batch. Thus 26 mg.100 mL⁻¹ must have been derived from the concentrate (neglecting any losses from pasteurisation as discussed in Chapter 5). For Blackcurrant, where the claimed value is 45 mg.100 mL⁻¹ and where no AA is added at mixing, the initial concentration must have exceeded about 100 mg.100 mL⁻¹. This is clear from Table 13. For both juices in these particular batches, the concentration of AA after long term storage will exceed the claimed value. Thus the claims on the labels are modest. However, variation in the concentration of AA in the Blackcurrant and Orange concentrates may be variable, so the company would be unwise to increase the claimed value.

The situation is rather different for the other three juices, where juices can and do fall below the claimed concentration before the best-before date. These juices are fast moving consumer goods, and it is unlikely that more than 120 days would be exceeded before consumption. Based on this assumption the claimed values are defensible.

According to my supervisor, Dr Young (personal communication), the loss of AA followed zero-order kinetics after about 25 days, because the rate of reduction in concentration preceded independent of AA concentration. The loss followed a straight line. Thus, at all times AA concentration was not limiting the loss. Again according to Dr Young,

the initially slightly steeper loss at early times may reflect loss of AA by an oxidative and probably first-order reaction (Figure 3) mechanism due to oxygen remaining trapped in the bottle at the time of bottling. Once trapped oxygen is exhausted, what then? According to Gregory (1996), anaerobic AA degradation exhibits a maximum rate between pH 3 and 4, which is the pH range of fruit juices. Thus it is possible that the main pathway of AA degradation in these fruit juices is anaerobic if the rate of oxygen diffusion across the wall of the bottle is very low.

However, a simpler aerobic model that appears to fit the facts is as follows: The concentration of oxygen outside a bottle is a constant because air circulates freely around the bottles. If it assumed that the rate of oxygen diffusion across the bottle wall is low, then the rate of aerobic degradation of AA will be limited by oxygen concentration and thus the constant slow rate at which it diffuses. The AA concentration is considered infinite in this model. Therefore, the reaction will follow zero-order (straight line) kinetics until such time as the AA concentration falls to very low levels when the reaction type should revert to first-order. Thus, the kinetics can be said to follow pseudo zero-order as a special case of the more intuitive first-order. Restated, given sufficient storage time the curves would flatten out as AA became limiting.

In contrast, an anaerobic model would be consistent with most of the results in Table 13, 15 and 16 where the reductant DTT was ineffective in increasing AA concentration in four of the five juices tested. However, the results with Thexton's Blackcurrant at 70 and 101 days – where DTT clearly increased the HPLC peak due to AA – suggest the situation may be complex in some juices.

Whereas the above arguments fit the data obtained in this storage trial, it must not be forgotten that DTT failed to reduce prepared DHAA back to AA (Table 9). The reason for this is not known and has not been pursued.

Chapter 5

The Effects of Shorter Term Treatments on the Fate of Ascorbic Acid in Fruit Juices

Introduction

The previous chapter explored the fate of AA under fluorescent light or total darkness over a period of many months and established that the claims for concentration made by the company were generally robust and defensible. In this chapter the effect shorter term treatments are explored.

The first short term treatment is the effect of pasteurisation. Although it has long been known that high temperatures decrease AA stability (Eittenmiller & Landen, 1999), it would be useful to know the exact losses that occur in the production process for the company's particular formulations. Knowing how much is added from the juice concentrates and how much is added as AA is useful but more useful if the losses during pasteurization are also known. Thus, the concentration of AA in a newly produced batch would be predictable.

The second is storage temperature, the importance of which is obvious. A variation on this is exposure to air at different temperatures, the third treatment examined in this Chapter.

The final treatment is exposure to sunlight. In Chapter 4 it was established that fluorescent light had a small adverse effect on AA stability during long term storage, but one that was commercially unimportant. An experiment with exposure to sunlight was performed out of curiosity. The ultraviolet light component in sunlight is very much higher than in domestic fluorescent light, and this component is implicated in free radical-mediated reactions leading to deterioration of things as diverse as paint, cooking oil and skin (Flexner, 1999).

The juices used in these trials were chosen from Keri Original Orange, Keri Original Apple and Keri Crush Tropical. In all cases AA was estimated by the titration method.

Effect of pasteurisation on AA in fruit juices

Method

In commercial production, fruit juices are prepared by first mixing all the ingredients in a so-called batching tank, which eventually flows through pasteurisation pipes before bottling. Six commercial batches of each of Keri Original Apple, Keri Original Orange and Keri Crush Tropical were mixed according to the company's proprietary recipes. Each was passed through a pasteurisation process achieved with flow through concentric pipes. The inner pipe contains the product flowing at defined rate. The outer contains hot water flowing counter current. The water temperature and flow conditions are such that during the residence time in the pasteuriser, typically 40 seconds, and the juices reach between 90 and 96°C. Subsequently, the juices are rapidly cooled by water spray to between 63 and 70°C prior to labelling. The total time spent by juice in the pasteurisation process to the point of filling is typically four to five minutes. However this increases if there is, for example, a delay in filling due to a mechanical problem downstream.

For this experiment, the before-pasteurisation samples were taken from the batch mixing tanks and the after-pasteurisation samples were taken at the time of carton packing.

Results and discussion

Table 16. Loss of AA during pasteurisation of fruit juices (mg.100 mL⁻¹)

	Keri Original Apple		Keri Original Orange		Keri Crush Tropical	
	Initial	Pasteurised	Initial	Pasteurised	Initial	Pasteurised
Six replicate pasteurisations	63.7	58.3	101.1	99.8	51.3	47.4
	62.3	58.9	98.5	94.5	52.1	48.7
	62.5	59.2	97.3	95.3	51.5	48.4
	66.4	62.3	97.6	97.1	54.7	49.5
	61.8	56.1	97.7	96.9	52.3	48.5
	61.5	55.8	115.3	110.4	53.1	49.1
Mean	63.0	58.4	101.2	99.0	52.5	48.6
SD*	1.8	2.4	7.0	5.9	1.3	0.7
Mean percent loss	7.3		2.2		7.4	

* SD = Standard deviation.

Six batches of each three juices were prepared on different days, so the data give an indication of day-to-day variation in AA concentration. Losses in the very short pasteurisation period were high. In case of Keri Original Apple and Keri Crush Tropical, the losses were about 7.4 %, whereas for Keri Original Orange the loss was only 2.2 % (Table 16). Whether Keri Original Orange is truly more stable than the other two cannot be proven from these data because no record was kept of individual pasteurisation times. However the data do suggest greater stability to pasteurisation.

Effect of temperature during short term storage

Method

To observe the influence of different temperatures of storage, the three selected juices in 375 mL bottles were kept at three different temperatures, 6°C in a chiller, ambient room temperature (~24°C) in the room used for the long term storage experiment (Chapter 4), and 30°C. All bottles were stored in cardboard cartons away from light. Temperatures were monitored regularly. Each time a bottle was removed for analysis it was mixed by inversion. AA was estimated at regular intervals over 64 days by the standard titration method.

Table 17. Overall effect of storage temperature to 64 days on the retention of AA in fruit juices

Juice	Claimed concentration of vitamin C (mg.100 mL ⁻¹)	Measured concentration* of AA (mg.100 mL ⁻¹) at start of temperature trial	Approximate percent remaining AA after storage at three different temperatures		
			6°C	(~24°C)	30°C
Keri Original Apple	32	57.4	83	74	68
Keri Original Orange	32	99.5	88	82	77
Keri Crush Tropical	24	47.2	83	73	68

*Means of four replicate titraions

Results and discussion

As is clear from Table 17 and Figure 17, the loss of AA between 6 and 30°C is not great. This is a 24°C difference. Many chemical reactions show a doubling in rate for every 10°C

rise in temperature. This is clearly not happening in this case. If this common relationship were followed, the percent remaining should be less than 50 percent, which it is not.

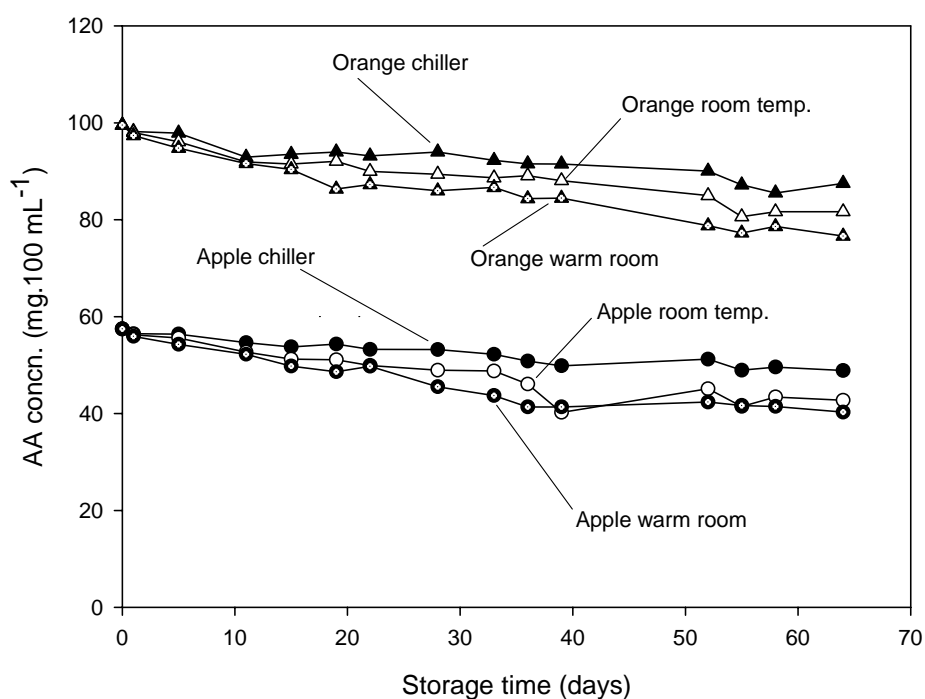


Figure 17. Effect of different temperatures on AA concentration in Keri Original Orange and Keri Original Apple. For clarity, Keri Crush Tropical is not plotted but shows the same kinetics as Apple

Again according to my supervisor, these data suggest that the loss of AA is limited by the rate of oxygen diffusion across the wall of the juice bottle. A 25 μm polyethelene tetraphthalate (PET) film transmits 95 cm^3 of oxygen per m^2 per day per atmosphere (Davidson & Partridge, 2002). Although the surface area of a 375 mL bottle is known (about 0.035 m^2), the juice bottles are much thicker than 25 μm , and there is not a one atmosphere difference in pressure across the bottle wall. Thus the diffusion rate for these particular juices as packaged is not known, nor is the effect of temperature on diffusion.

Effect of deliberate exposure to air at two temperatures

The previous experiments were conducted with 375 mL bottles. These are normally opened and drunk within say 20 minutes. In contrast, larger bottles, 3 L for instance, are often opened and periodically drunk over a period of days, with a headspace of air that increases progressively. This may accelerate loss of AA. In this situation bottles are normally stored in a domestic refrigerator at nominally 6°C, but the increased availability of oxygen in the headspace may lead to excessive losses of AA. This hypothesis was tested at 6°C and ambient room temperature.

Methods

The effect of repeated exposure to air was conducted under two conditions, 6°C and ambient temperature ranging from 17 to 23°C. Three juices were tested, Keri Original Apple, Keri Original Orange, and Keri Crush Tropical. Juice (500 mL) was poured every second day from 3 L Keri bottles thus providing an increasing headspace and some exchange of air between the headspace and the external atmosphere. AA concentration was tracked over 10 days. At each time point a fresh sealed bottle was opened from both conditions (refrigeration and ambient) and AA was determined. These bottles were the controls.

Results and discussion

Exposure to air affected the juices slightly differently. In refrigerated storage, progressive exposure to air caused a decrease in the concentration of AA in Apple by 8 %, whereas it was 11 and 13 % for both Tropical and Orange. In the controls, the respective losses were 4 % for Apple and Orange and negligible (0.2%) for Tropical. At ambient temperature the losses were higher, as expected, but the overall pattern was unchanged. Because the pattern of loss was similar for all three juices only representative data are shown Figure 18.

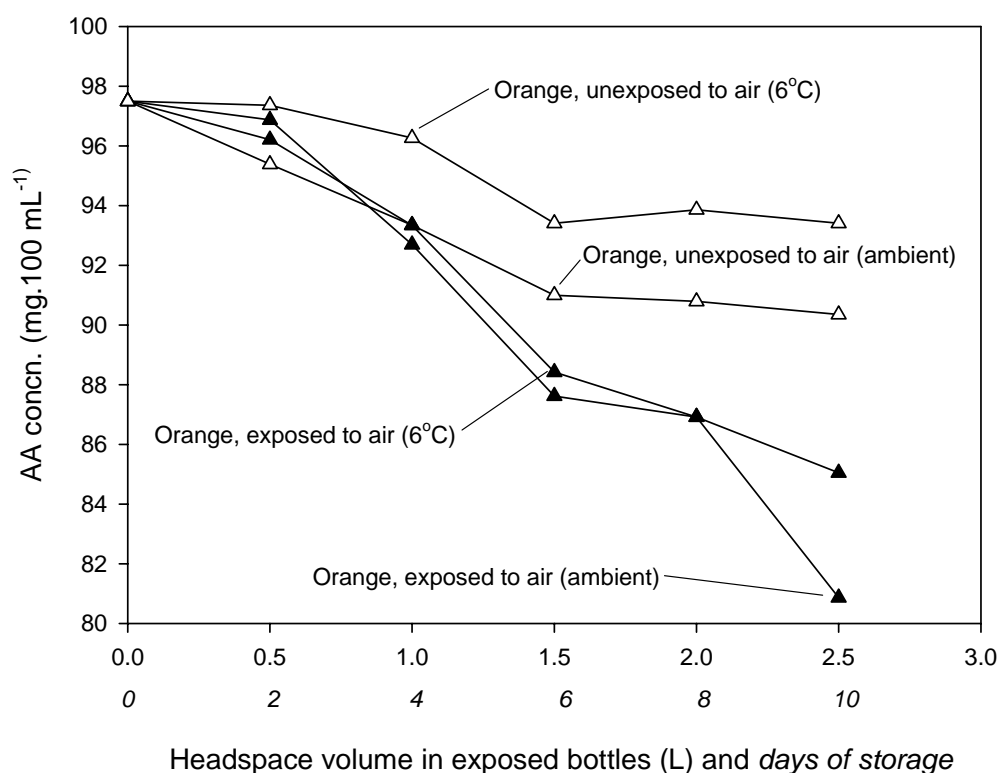


Figure 18. Effect of headspace air on AA concentration in Keri Original Orange stored in refrigeration and ambient room temperature

Effect of two daylight conditions on AA concentration in juices

Methods

The effect of exposure to sunlight (ultraviolet-filtered and unfiltered) was studied with two juices, Keri Original Orange and Keri Original Apple. Bottles (375 mL) of both products were used without labels to maximise the exposure to radiation.

The juice bottles were placed horizontally in the gaps on two pallets. Polycarbonate clear sheet (Palsun UV2, 1.5 mm, from PSP Limited, Auckland) was fixed 40 cm above one pallet, and Acrylic clear from Bunnings Warehouse, Auckland) of same thickness was fixed at the same height above another. The Palsun UV2 is claimed to filter ultraviolet light from both sides of the sheet, but data have been hard to find about performance. No light filtration data was available from the supplier of the acrylic sheet. (Ultraviolet light absorption data

could be obtained with a spectrophotometer, but this idea was not pursued.) Air circulated freely above and below the bottles because the sides were not enclosed.

Both pallets were placed side-by-side in a sunny industrial yard (~50 m by 20 m) in December 2005 and progressively opened for AA determination over 35 days. Obviously exposure to light occurred only during daylight hours, so the treatments are really daylight/darkness treatments. Temperature was unfortunately not monitored.

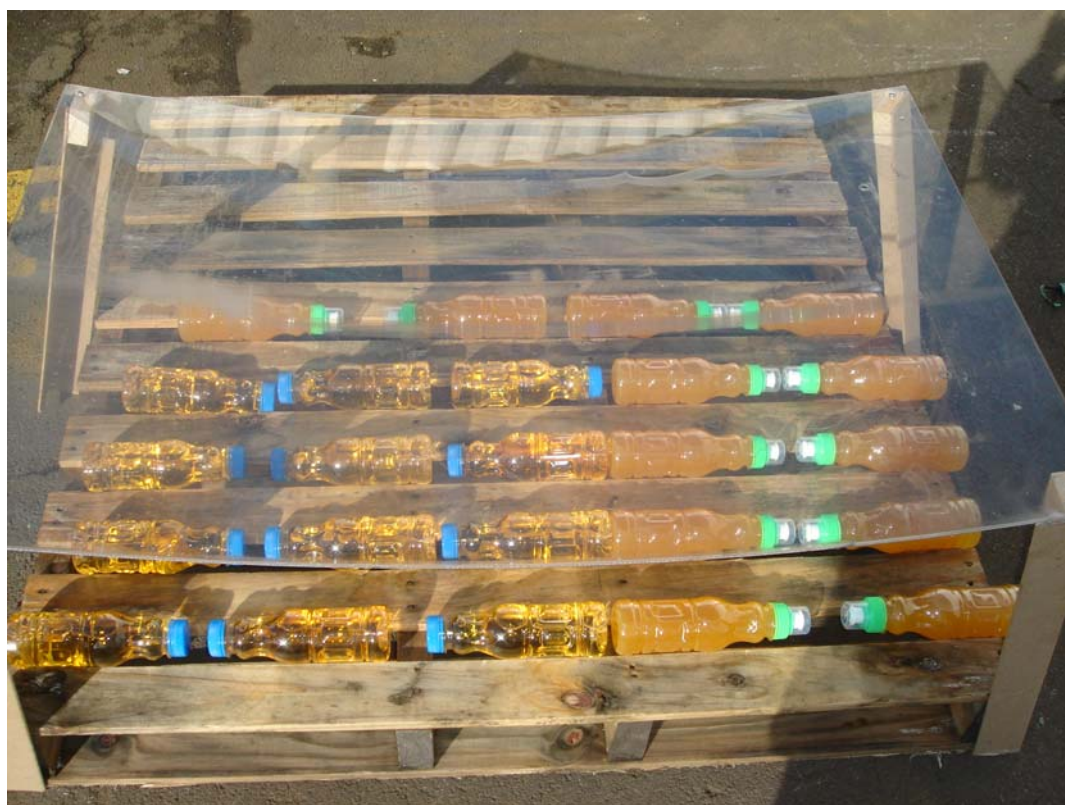


Figure 19. Display of juices exposed to sunlight

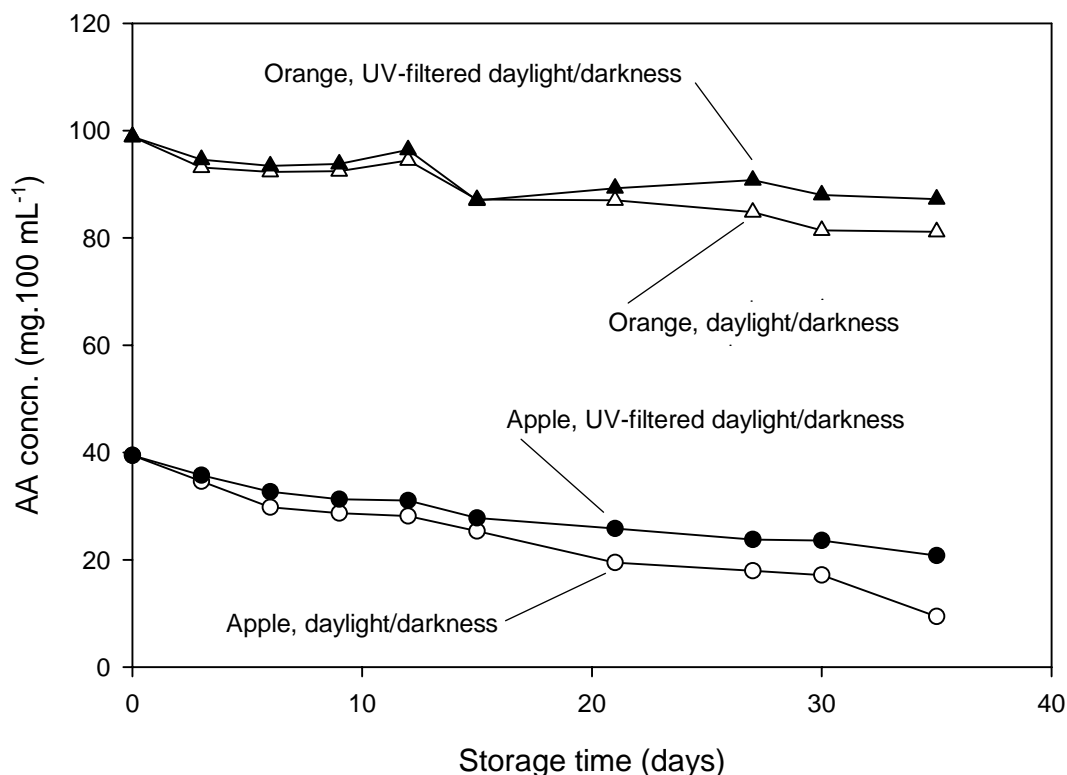


Figure 20. Effect of two daylight conditions on AA concentration in Keri Original Orange and Keri Original Apple

Results and discussion

Figure 20 shows the effect of the two exposure conditions over 35 days. The losses of AA under these strong light conditions are clearly not dramatic and the effect of filtering out an undetermined fraction of the ultraviolet light was relatively minor. (Temperature was unfortunately not monitored in this trial but in December temperatures in daytime would be higher than in the long-term storage trial reported in Chapter 4. Equally they would be lower at night.)

It was instructive to compare the rate of loss of AA in this experiment with the rate in the fluorescent light trial (Table 18). This has been done for Keri Original Orange as an example.

Table 18. Rates of AA loss in Keri Original Orange under the treatments: darkness, fluorescent light, ultraviolet-filtered daylight/darkness, daylight/darkness.

Treatment	Temperature range (°C)	Rate of AA loss [¶] (mg.100 mL ⁻¹ .day ⁻¹)
Darkness	11-30	-0.089
Fluorescent light	11-30	-0.104
Ultraviolet-filtered daylight/darkness	Not recorded	-0.266
Daylight/darkness	Not recorded	-0.459
[¶] Linear least squares regression curves yielded the rates		

Exposure to ultraviolet-rich light (daylight/darkness treatment) caused a roughly five-fold increase in loss compared to storage in total darkness. However, juices are never stored under these conditions in a commercial setting.

Chapter 6

Conclusion

The principal sources of vitamin C in New Zealand diet are vegetables and non-alcoholic beverages both 26 %, fruit at 23 %, and potatoes and kumara at 13 % (Ministry of Health, 2003). Thus the contribution from non-alcoholic beverages like the juices in Keri range is thus highly significant in the New Zealand diet given that consumers of these juices may have otherwise unbalanced diets.

There is a wide range of literature on the stability of AA in fruit juices in general, but this research was aimed at AA in a specific range of juices under a variety of storage conditions.

In the beginning, two methods, liquid chromatography and a titrimetric method, were adopted to determine vitamin C. Liquid chromatography with an ultraviolet absorbance detector is intrinsically the more accurate method because it can identify then quantify individual chemical species, specifically AA plus DHAA, which together represent bioavailable vitamin C. In contrast, the titrimetric method employed here depends on the (non-specific) reduction of 2,6-dichlorophenolindophenol. Any chemical species in a juice that can do this will contributed to the determination of AA, although there are no obvious other ingredients that will perform this way.

The coefficient of variation of automated replicate injections in the liquid chromatography procedure was a low 0.5 % and the ability to measure AA plus DHAA would nominally make it the method of choice. However, the outcome of experiments aimed at determination of AA's immediate oxidation product, DHAA, was poor, and its role in AA loss remains unresolved. Perhaps significantly, the purportedly authentic AA bought from Sigma Chemical Company had deteriorated to the point that there was no absorbance at its characteristic wavelength maximum, 220 nm, in HPLC analyses. This suggests an intrinsic instability. Moreover, Nisperos-Carriedo, Busling and Shaw (1992) reported that the concentration of DHAA with respect to AA in commercial orange juice ranges from 0 to 0.2

% which is negligible. If these authors are correct, the error in assessing bioavailable vitamin C activity by DCPIP will also be negligible.

The matter of DHAA was not pursued, but it is important to note that all subsequent experiments aimed at determining vitamin C can only report AA concentration rather than AA plus DHAA, which together represent bioavailable vitamin C.

The titrimetric method for determining ascorbic acid is based upon the reduction of the dye 2,6-dichlorophenolindophenol (DCPIP) with AA in acidic solution. In its oxidised form, DCPIP is pink in acidic solution. A persistent light pink colour signals the presence of excess unreacted dye and therefore the end point. Excellent reproducibility and recovery was observed (100 %) for titrimetric method which was calculated after adding a known amount of AA to the selected juices with eight replicate of each fruit juice Table 5.

The titration method is particularly suitable for light coloured juices because it is easy to detect the persistent light pink colour indicating the titration end point, but for dark-coloured juices such as Thexton's Red Grape, Thexton's Blackcurrant and Thexton's Cranberry etc. the titration method does not work because the pink colour cannot be seen against the red/blue background. Two methods – decolourisation with activated charcoal and changing the juice pH to alter the colour due to the anthocyanins – were tried to solve the problem of detecting end point. Although charcoal was extremely effective as a decolourant, the treatment had the unfortunate side effect of catalysing the rapid and complete oxidation of AA, presumably to DHAA. The pH of dark-coloured juices was from 3.24 to 3.60, but an increase in pH did not render them colourless to the point that titration could be performed. Moreover AA is unstable at higher pH. Subsequent work focused on juices for which the titration method would work.

A long term storage experiment compared the fate AA concentration in selected fruit juices stored under fluorescent lights with storage in total darkness. Based on the titration method, the initial concentration of AA greatly exceeded the claimed value, especially in the case of Keri Original Orange and Thexton's Blackcurrant. For both juices in these particular batches, the concentration of AA after long term storage exceeded the claimed value. The situation was rather different for the other three juices (Keri Original Apple, Keri Crush Apple Orange Mango and Keri Crush Tropical), where juices do fall below the claimed concentration before the best-before date. In all cases the losses followed zero-order kinetics

(a straight line over time), strongly suggesting that losses were limited by the availability of one reactant. This was presumably oxygen, which has to diffuse across the juice bottle wall.

Several short term experiments were also conducted. Losses in the very short pasteurisation period (minutes) were high. In case of Keri Original Apple and Keri Crush Tropical the losses were about 7.4 % whereas for Keri Original Orange the loss was only 2.2 %. The temperature at the point of ingredients mixing the (batching, Chapter 4) is around 20°C. Based on the reported concentration of oxygen in water at 25°C by Weast (1968), the concentration of oxygen in Keri juices at batching will be around 1.5 mmole.L⁻¹. The analyses showed that AA concentration could be as high as 100 mg.100⁻¹ mL in Keri Original Orange. This equivalent to 5.7 mmole.L⁻¹. Thus there is adequate oxygen to cause high early losses.

The stability of AA in Keri Original Orange, Keri Original Apple and Keri Crush Tropical was also observed for nearly three months at three different temperatures (6°C, ambient and 30 °C). The percentage losses at 30°C were double the losses at 6°C. This is a 24°C difference. Many chemical reactions show a doubling in rate for every 10°C rise in temperature. If this were the case here, the losses should be a minimum of four-fold. The explanation advanced was that the rate of loss of AA was limited by the rate of oxygen diffusion across the wall of the juice bottle, which is unknown information.

The effect of repeated exposure to air was conducted under two conditions, 6°C and ambient temperature. This experiment was intended to replicate the domestic consumption of larger bottles of fruit juice where juice is progressively consumed over a period of days. In refrigerated storage, progressive exposure to air caused a decrease in the concentration of AA in Apple by 8 %, whereas it was 11 and 13 % for both Tropical and Orange. In the controls, the respective losses were 4 % for Apple and Orange and negligible (0.2 %) for Tropical. These data show that nutritionally important concentrations remain even under these nominally poor storage conditions.

Finally the effect of exposure to sunlight (ultraviolet-filtered and essentially unfiltered) was studied with two juices, Keri Original Orange and Keri Original Apple. The losses of AA under these strong light conditions were not dramatic and the effect of filtering out an undetermined fraction of the ultraviolet light was relatively minor.

From a commercial perspective it can first be concluded that the claims for vitamin C concentration on the labels of the Keri range of juices tested here are modest and defensible. Second, it can be concluded that ascorbic acid in these juices is extremely resilient to loss.

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