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Title: THE STABILITY OF ASCORBIC ACID IN BOTTLED CRANBERRY-BASED BEVERAGES UNDER HOME-USE CONDITIONS.

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Commercially bottled, vitamin C-fortified, cranberry juice cocktail and cranberry-apple (cranapple) juice drinks were stored at 5°C under simulated home-use conditions. Ascorbic acid (AA) and dehydroascorbic acid (DHA) levels were evaluated over a period of seven days by high performance liquid chromatography (HPLC). Total vitamin C content was also determined by a classical spectrophotometric method.

The HPLC data revealed a significant decrease of AA during the storage of both juice drinks resulting in losses of 35% and 27% in the cranberry and cranapple drinks, respectively. The loss of ascorbic acid was offset by the simultaneous increase of DHA (a biologically active vitamin) so that the sum of AA+DHA decreased 13% and 0% in the cranberry and cranapple drinks, respectively. Thus ascorbic acid retention was apparently influenced by the nature of the juice system.

The spectrophotometric results indicated no significant loss of total vitamin C activity during the storage of either juice drinks. The observed differences between the spectrophotometric and HPLC results were attributed to interference problems associated with the spectrophotometric method.

THE STABILITY OF ASCORBIC ACID IN BOTTLED CRANBERRY-BASED BEVERAGES UNDER HOME-USE CONDITIONS

by

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Ephesians 3: 20-21.

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INTRODUCTION

Ascorbic acid (vitamin C) is essential for normal body functioning. An ascorbic acid deficiency disease called scurvy is characterized by weak muscles and bleeding gums. Ascorbic acid has many roles in the body with some of these roles still being discovered and understood. Among these functions are collagen biosynthesis, immune system interactions, hormone metabolism, and nutrient interactions (Brin, 1982; Zapsalis and Beck, 1985).

Ascorbic acid is naturally present in a wide variety of food sources. However, high concentrations may be found in foods such as kale, broccoli, and black currant. More commonly, citrus fruits such as lemon, orange, and grapefruit are important dietary sources (Zapsalis and Beck, 1985; and Nagy, 1980).

Altough vitamin C is very important to the human body and is readily found in the food supply, it is the least stable of all the vitamins. It is easily destroyed during the processing and storage of foods. The destruction of vitamin C in food systems may be very complicated

involving many pathways and factors. These are discussed in detail by Tannenbaum et al. (1985) and Nagy (1980).

Ascorbic acid under the presence of oxygen degrades to dehydroascorbic acid (DHA), which is a biologically active vitamin (Bissett and Berry, 1975). DHA can further oxidize and break the ring system in an irreversible reaction to form diketogulonic acid which does not have vitamin C activity. The diketogulonic acid can further oxidize to other forms, including furfural (Pachla et al., 1985; Zapsalis and Beck, 1985).

Recognizing the potential losses due to ascorbic acid instability, it is important to know how much vitamin C is actually present in the foods we consume. Since citrus juices historically have been a major source of vitamin C, the factors which influence ascorbic acid stability in these juices have been studied rather extensively. Though the factors are many, among the more significant ones are seasonal variations, processing operations, and juice constituents including the presence of oxygen, metal catalysts, enzymes and organic acids (Nagy, 1980).

Some of the more critical factors, however, may well be storage time and temperature. Results of many citrus juice studies have indicated that at a given single temperature, more ascorbic acid (AA) was lost with increased storage time. Also, the higher the storage temperature, the greater the AA loss for a given time

period. The results indicated that at cool to moderate temperatures, high levels of AA were retained in some packaged containers even after 90 days. However, these studies were completed on unopened containers (Bissett and Berry, 1975; Smoot and Nagy, 1980).

Studies also have been conducted after the juice beverage containers have been opened. For example, after the consumer takes the juice home, opens it, and puts it in the refrigerator, how well is the AA retained? Some of these home-use retention studies have involved orange juice which have shown that even after 8 days of storage, relatively high levels of vitamin C were still present (Bissett and Berry, 1975; Horton and Dickman, 1977).

By contrast, studies involving vitamin C enriched apple juice did not show good retention. After 8 days of storage in the refrigerator, less than 50% of the original vitamin C was present. The researchers concluded that this was due to inherent differences in the juice systems (Noel and Robberstad, 1963).

It is, therefore, of concern as to how well other fortified juices will retain vitamin C under home use conditions. For purposes of this research, the fortified juices analyzed were cranberry juice cocktail and cranberry-apple juice drinks.

The objectives of this study were to determine the effects of refrigerated, home-use conditions on levels of

ascorbic and dehydroascorbic acids in vitamin C fortified cranberry juice cocktail and cranberry-apple juice drinks. DHA was included for analysis because it is biologically active and has nutritional significance. Juices were analyzed over a period of 7 days for AA and DHA contents by high performance liquid chromatography. For comparison, total ascorbic acid activity (AA+DHA) was also analyzed by a classical chemical method.

LITERATURE REVIEW

ANALYTICAL METHODOLOGY FOR ASCORBIC AND DEHYDROASCORBIC ACID

A considerable amount of attention has been directed to the determination of ascorbic and dehydroascorbic acids over the past decade. Many reliable assays are in existence and each has its appropriateness for a given situation. Since some samples are complicated biological materials, colored species as well as other components in the sample can interfere with ascorbic acid analyses. Two very common methods for determining ascorbic acid and its oxidative products are spectroscopic and chromatographic procedures (Tuan, 1986). Many situations exist in food analysis where the classic colorimetric and titrimetric methods are adequate to achieve the desired goals. However, in some cases these methods are inadequate in detection limits. Interference problems may also be present (Kissinger and Pachla, 1987). Ascorbic acid methodology has been reviewed by many workers (Pachla et al., 1985; Doner, 1984; Bui-Nguyen, 1985).

High Performance Liquid Chromatography Method

In recent years high performance liquid chromatography (HPLC) has received much attention due to its performance, accuracy, speed, and detection limits. Separations have generally fallen into three categories; reverse phase, strong anion exchange, and weak anion exchange. Detection has generally fallen in the categories of optical absorbance detection, electrochemical detection, and fluorescence detection (Doner, 1984; Pachla et al., 1985; Tuan, 1986).

Weak Anion Exchange Methodology

Several investigators have used weak-anion exchange methodology to separate various isomers of ascorbic acid (AA) including its oxidative compounds. Such compounds include erythorbic acid (EA), dehydroascorbic acid (DHA), diketogulonic acid (DKG), dehydroerythorbic acid (DHEA), and diketogluconic acid (DKGl). Separation has commonly been accomplished through the use of the amino-propyl columns. Such column packings are applicable to ascorbic acid because it exists as an anion in mildly acidic conditions. Though amino-propyl columns have usually had more application in the separation of sugars, they can

function in the weak anion-exchange mode when appropriate buffers are used (Doner, 1984).

Bui-Nguyen (1980) was among the first researchers to separate ascorbic acid from isoascorbic acid on a weak anion exchange (LiChrosorb-NH_2) column with ultraviolet (UV) detection at 268 nm. The mobile phase consisted of 75% acetonitrile (ACN) in 0.005M solution of potassium dihydrogen phosphate(pH 4.4-4.7) at a flow rate of 3 ml/min.

Arakawa and co-workers (1981) applied HPLC to separate AA and EA using a LiChrosorb-NH_2 column. They pretreated the column with 0.1M ammonium monophosphate solution. The mobile phase consisted of acetonitrile, acetic acid, and water (87:2:11 v/v). DHA and DHEA were determined using H_2S as a reducing agent.

Otsuka et al. (1981) developed a method for the separation and determination of AA and EA in animal tissues using a LiChrosorb-NH_2 column and a mobile phase containing acetonitrile, acetic acid, and water. DHA was be measured by difference after reduction with H_2S.

Doner and Hicks (1986) used a Zorbax-NH_2 analytical column with a mobile phase of 0.05M KH_2PO_4: acetonitrile (75:25, w/w). Their system was able to separate ascorbic acid, erythorbic acid, dehydroascorbic acid, dehydroerythorbic acid, diketogulonic acid, and diketogluconic acid in model systems.

Rose and Nahrwold (1981) also used a LiChrosorb-NH_2 column to separate and quantitate ascorbic and dehydroasorbic acids. The technique was applied to analyze foods, plasma, vitamin pills and physiological buffer solutions.

In later work, Rose and Koch (1984) discussed the applications of amino columns. They tested prefilled amino colums from three manufacturers to determine their usefulness in separating low quantities of C^{14}-labeled AA from degradation products like C^{14}-DHA and C^{14}-DKG. They found that the ascorbic acid retention on some amine columns varied with the levels of AA injected onto the column. They observed that on certain columns a considerable amount of radioactivity did not elute with the typical AA fraction unless there was also nonlabeled AA in the system. The unpredictability was not explained though they speculated that some amine columns may have catalytic sites which may oxidize AA.

Kacem and co-workers (1986) used anion-exchange chromatography with a NH_2 column to determine AA and DHA simultaneously. The mobile phase was acetonitrile-0.05 M KH_2PO_4(75:25,v/v), pH 5.9. AA was quantified at 254 nm while DHA was detected with fluorescence after postcolumn derivatization using o-phenylenediamine(OPDA).

Dennison et al. (1981) described a HPLC method using a u-Bondapak-NH_2 column with a 50:50(v/v) methanol-0.25% KH_2PO_4 buffer (pH 3.5) to quantify total ascorbic acid (AA plus DHA). Their method was employed successfully to determine the levels of ascorbic acid in four commercial beverages.

Reversed-phase Methodology

Much research has been completed using reverse phase methodology, most commonly with C-18 silica packings. Since ascorbic acid is a small hydrophilic acid, it cannot perform in the complete range of retention control normally associated with reverse phase liquid chromatography. In these procedures the AA is often ion-paired with various cationic detergents so that the AA may be retained longer on the column (Kissinger and Pachla, 1987).

Sood et al. (1976) used tridecylammonium formate as the counterion in a HPLC reversed phase ion-pairing method to effectively determine AA in foods and multivitamin products.

Augustin et al. (1981) developed a quantitative procedure for the determination of AA in potato products by using reverse-phase, ion-pairing HPLC with a C-18

column. The results of the experiment showed the method to be effective.

Finley and Duang (1981) studied the use of paired-ion chromatography for the simultaneous assay of ascorbic, dehydroascorbic and diketogulonic acids. They used two wavelengths (210 and 254 nm) and two C-18 columns in series to successfully analyze the vitamin C compounds. The mobile phase contained disodium phosphate and counter-ion reagents such as tri-n-butyl amine or tetrabutyl ammonium phosphate. Attempts were made to isolate isoascorbic acid and successful separation was obtained when the mobile phase pH was above 3.5.

Keating and Haddad (1982) described a method to separate and quantify ascorbic and dehydroascorbic acid by reversed-phase ion-pair HPLC. The dehydroascorbic acid was precolumn derivatized with 1,2-phenylenediamine. Ion pair reagents used were tridecylammonium formate or hexadecyltrimethylammonium bromide. They applied the method to analyze orange juice and reserved the evaluation for a later communication.

Reverse phase systems involving C-18 columns have also been used by some workers without ion-pairing modifiers. Wimalasiri and Wills (1983) used HPLC to simultaneously analyze AA and DHA in fruit and vegetables. They used a carbohydrate column and UV detection at 254 and 214 nm.

The mobile phase consisted of acetonitrile-water (70:30,v/v) with 0.01M ammonium dihydrogen phosphate and pH adjusted to 4.3 with orthophosphoric acid. Wills et al. (1983) applied this method in the determination of ascorbic acid in fresh fruits and vegetables. This work also compared the liquid chromatographic (LC) method with microfluorometry and the 2,6-dichloroindophenol dye-titration methods. The results showed that the LC and dye-titration method yielded similar levels of ascorbic acid except in cases where the colored products made the titration end-point difficult to detect. The microfluorometric and the LC methods were also in good agreement except that the microfluorometric values were higher for total vitamin C in about one-third of the samples. In later work, Wills et al. (1984) effectively applied the LC method to study DHA and AA levels of fresh fruits and vegetables purchased from retail markets and held at 20^{o}C for a maximum of 12 days.

Shaw and Wilson (1982) used a C-18 column to successfully analyze the ascorbic acid content of guava, mango, papaya, and orange processed products. The mobile phase was 2% $NH_4H_2PO_4$ aqueous solution at a wavelength of 254 nm. Several of the products contained relatively low levels of AA as compared with levels in fresh fruit.

Watada (1982) used a HPLC method for determining ascorbic acid content of fresh fruits and vegetables. Extraction was done with 6% metaphosphoric acid and the separations were effectively carried out on a C-18 column with 1.5% $NH_4H_2PO_4$ as the mobile phase.

Wilson and Shaw (1987) studied the HPLC determination of AA in aseptically packed orange juice measured with ultraviolet and electrochemical detectors on a C-18 column. The method was effective and the resulting AA values did not significantly differ from those obtained by a potentiometric titration method.

Strong Anion Exchange Methodology

The use of strong anion exchange methodology does not seem as widespread as other modes but it was used successfully by Pachla and Kissinger (1976) on certain foodstuffs, body fluids, and pharmaceuticals. The columns used were strong anion exchange packings. However, they encountered a problem that when the column had not been used for some time, the first sample injected always gave a value too low by 5-10%. Once the column was pretreated with the first sample or a standard, the quantitation of the following injections was accurate and precise. They did not offer an explanation for this observation.

Detection

UV detection has been used by a majority of the ascorbic acid researchers, including those applying weak anion exchange or reverse phase chromatography. Direct UV detection is possible for AA, DHA and related compounds although in certain situations the selectivity is not very good, particularly when interfering compounds are present. (Kissinger and Pachla, 1987). Bui-Nguyen (1980) reported that 268 nm was the maximum absorption of AA. Doner and Hicks (1986) also used 268 nm to quantify AA, while other workers successfully used 254 nm (Rose and Nahrwold, 1981).

The quantification and separation of DHA is more complicated and DHA detection has been performed in a variety of ways. Wills et al. (1984) and Finley and Duang (1981) used UV at 214 nm while some workers used homocysteine to convert DHA back to AA and calculate the difference (Rose and Nahrwold, 1981; Dennison et al., 1981). Rose and Nahrwold (1981) discussed the problems inherent in using 210 nm detection. Although it was possible to detect DHA at 210 nm, they concluded that the use of homocysteine to convert DHA to AA was a better method to detect low levels of DHA. Arakawa et al.(1981)

used H_2S as did Otsuka et al. (1981), to reduce DHA but Doner and Hicks (1981) advocated the use of dithiothreitol.

Other detection methods of ascorbic acid compounds have involved derivatization to fluorescent compounds. Speek et al. (1984) employed fluorometric determination of total vitamin C and total isoascorbic acid in foodstuffs and beverages by HPLC with precolumn derivatization. In this method, the AA and EA are oxidized to their dehydro forms by the use of ascorbate oxidase. These were then condensed with o-phenylenediamine and the levels determined by fluorescence detector. Dehydro forms could be determined separately by omitting the enzymatic oxidation.

Vanderslice and Higgs (1984) also developed a HPLC procedure with fluorometric detection of AA and DHA in food systems. Separation was carried out with anion exchange and the detection accomplished with post-column, in-line chemistry involving oxidation of ascorbic acid to dehydroascorbic acid and then reacting with o-phenylene-diamine to make a fluorescent product. Kacem et al. (1986) also used postcolumn derivatization with o-phenylenediamine to quantify AA and DHA.

Electrochemical (EC) detection methods have been improved recently and have been used successfully by several workers (Pachla and Kissinger, 1976; Wilson and

Shaw, 1987; Tsao and Salami, 1982). In comparing AA detection methods, Wilson and Shaw (1987) found that values obtained by UV and EC detection procedures were not significantly different from those derived by the potentiometric titration technique. The EC method was 100 times more sensitive than the UV detector in the measurement of AA. However, DHA cannot be measured with the EC detector at the voltage setting used for AA.

Pachla and Kissinger (1976) developed a liquid chromatographic analysis for ascorbic acid with electrochemical detection. When compared with titration or colorimetric redox procedures, the EC method provided straightforward sample preparation and better sensitivity and selectivity. The electrochemical method was not directly suitable for detecting dehydroascorbic acid although it may be reduced to ascorbic acid and then analyzed.

Within a single injection system, however, Kissinger and Pachla (1987) stated that they preferred to use ultraviolet in conjunction with electrochemical detection because it offers optimum flexibility and included peak ratios between detector channels to eliminate interferences. Conversely, Ziegler et al. (1987) showed the possibility of interferences occurring due to the reduction of DHA by dithiothreitol in the electrochemical detection method.

Spectroscopic Methods

The most common spectroscopic methods are divided into two general types. The first type includes those which use a redox indicator in its oxidized form since this method utilizes the reducing property of AA (Doner, 1984). Secondly are those methods which involve chromagen formation by derivatization. These utilize the property of DHA to participate in condensation reactions (Pachla et al., 1985; Doner, 1984).

Redox Indicator Methods

2,6-Dichlorindophenol (DCP) is the standard redox reagent used for the first type of ascorbic acid analysis (Pachla et al., 1985). This titrimetric procedure is especially useful when samples to be analyzed do not contain other compounds (certain cations, some plant pigments, sulfhydryl compounds) which may reduce the DCP (Doner, 1984). Such interfering compounds would cause the titration end-point to be difficult to determine (Doner, 1984; Roe, 1954). In the DCP procedure, DHA is difficult to measure unless it is converted to AA. Cooke and Moxon (1981) have described procedures that can be used to overcome interferences caused by other compounds. In view of the various interferences, the DCP procedure has been

successfully applied to a variety of foods as shall be discussed later (Pachla et al., 1985).

Spaeth et al. (1962) developed a potentiometric method to circumvent interference problems. Modifications of this method were employed by others (Nagy and Smoot, 1977; Wilson and Shaw, 1987).

Nagy and Smoot (1977), using a modified potentiometric method, found it to be more precise and reproducible than the standard DCP method which may have had errors due to end-point recognition. These workers advocated the use of this method for highly colored fruit juices including citrus and grape. In studies by Wilson and Shaw (1987), resulting values from the potentiometric method compared favorably with HPLC techniques.

The DCP method has been successfully applied to a variety of ascorbic acid studies in citrus fruit products. The method has been employed in the vitamin C determination of grapefruit juice, orange juice, and orange juice products (Heikal et al., 1967; Kanner et al., 1982; Trammel et al., 1986).

The application of the DCP method has been useful for anthocyanin/ascorbic interaction studies involving fruit juices such as cranberry, strawberry, raspberry, and blackcurrant (Starr and Francis, 1968; Beattie et al., 1943; Hooper and Ayres, 1950), as well as in anthocyanin model systems (Poei-Langston and Wrolstad, 1981;

Shrikhande and Francis, 1974). Conversely, Timberlake (1960) studied model systems resembling blackcurrant juice and found that solutions containing anthocyanins could not be visually titrated and consequently used a modified potentiometric procedure. Clegg and Morton (1968) also reported pigment interference problems in the DCP method for blackcurrant juice analysis.

Derivatization Methods

Some of the other chemical methods make use of the condensation reactions of the ascorbic acid oxidation product, DHA. A common reagent used is dinitrophenylhydrazine (DNPH).

Roe (1954) described a procedure for total vitamin C (including AA, DHA, and DKG). He reported that a major difficulty with the DCP method was the interfering effect of metallic ions (e.g., Cu^{++}, Cu^{+}, Fe^{+++}, Fe^{++}, and Sn^{++}) which may catalyze the oxidation of ascorbic acid or may reduce the dye. Roe claimed that the metal ions do not have any effect on the DNPH method. In the same report, Roe also presented a differential method for selectively determining AA, DHA, and DKG. This method uses H_2S to reduce DHA to AA but under the prescribed conditions it does not reduce DKA. One aliquot is reduced with H_2S, and DKG can then be determined by coupling.

The second aliquot is left untreated which allows for the measurement of DHA and DKA. The third aliquot is oxidized with bromine for determining AA, DHA, and DKG. In this method it is necessary to use a 6-hour coupling period. Other studies by Roe before and after this study, also discuss various aspects of this method (Roe and Kuether, 1943; Roe et al., 1948; Roe, 1961).

In a paper estimating the DHA in blood of diabetic patients, Chatterjee and Bangejee (1979) used a modification of a method by Roe et al. (1948). They used homocysteine instead of H_2S to reduce the DHA since the H_2S method is laborious and lengthy. They found that homocysteine reduced DHA more efficiently than H_2S.

Cooke and Moxon (1981) reviewed many procedures designed to minimize the problems of the DNPH assay. These problems included interferences from other osazone forming compounds such as sugars, yields which were less than quantitative, and time-consuming analyses. Bourgeouis and Mainguy (1975) also discussed limitations of the DNPH method.

Pelletier and Brassard (1977) developed a method involving DNPH and applied it in the AA determination of fruit products. Their proposed method compared favorably with a modified Roe procedure (1961) in almost all of the

samples. Increased sensitivity of the new method was cited to explain incidences in which the values did not match well.

Otsuka et al. (1981) compared their HPLC method with the dinitrophenyhydrazine method. They concluded that the HPLC method seemed to be the more sensitive and practical procedure.

The DNPH method or modifications of it have been successfully employed to assay a variety of food products, including citrus juice and orange juice (Smoot and Nagy, 1980; Hooper and Ayres, 1950; Lopez et al., 1967).

Fluorometric Methods

This assay makes use of the property of AA to condense and form a fluorescent derivatized product. Mokady (1984) used condensation with o-phenylenediamine to determine AA plus DHA in fruits and fruit blends. Comparisons of liquid chromatography (LC), microfluorometry and dye-titration methods were completed by Wills et al. (1984). Their findings were discussed previously.

ASCORBIC ACID STABILITY IN FRUIT JUICES

Many studies have been done concerning the stability of ascorbic acid in fruit juices under a variety of

packaging and storage conditions. Pertinent reviews of these topics have been published by several workers (Pollard and Timberlake, 1970; Shrikhande, 1976; Mapson, 1970; Henshall, 1981; Nagy, 1980). Because of their significant contribution of vitamin C to the diet, citrus products have undergone a vast amount of study related to storage and processing effects.

Home-use Studies

Several workers have conducted studies involving AA retention under home-use conditions. Bissett and Berry (1975) studied the retention of ascorbic acid in reconstituted commercial orange juice concentrates. Juices were reconstiututed to 13^{o} Brix and the initial levels of AA determined. Portions of juice were placed in 64-oz glass or plastic containers. The containers were three-quarters filled and kept closed or open at storage temperatures of 4.4, 10 and 21.1^{o}C. Periodic AA determinations were performed to record the rate of change under conditions likely to occur in the home. It was expected that consumers would use the reconstituted concentrates within a period of seven days. Thus they ran their studies for seven days.

Their results showed that after 7 days, the reconstituted orange juice had 80-85% retention regardless

of storage temperature studied (4.4, 10 or 21.1°C). At 4.4 and 10°C, closed containers had about 1-2% better retention than opened. At 21.1°C the closed containers had a 3-5% better retention than open. Differences between retention in plastic or glass containers were less than 1% in all cases. They found that 90% or more of the original AA was present during the first 4 days at 4.4 or 10°C. However, samples at 21.1°C began to ferment after 3-4 days (Bissett and Berry, 1975).

Horton and Dickman (1977) also did a study of the AA stability of reconstitued frozen orange juice. They observed that the total AA+DHA was very stable over a 2-week period at 4°C and at room temperature. They also noted that aerating the juice by a blendor did not effect the stability. The conversion of AA to DHA and DHA to DKG occurred faster at room temperature than at 4°C, and the results showed that DKG accumulated slowly at 4°C but it quickly decreased after 20 days at 22°C. Interestingly, the AA in phosphate buffer at pH 3.95 at 4°C was not as stable as in orange juice at pH 4.0. The authors concluded that orange juice must contain factors which protect AA from oxidation other than pH. The presence of citrate, a chelator of heavy metal ions, may have had a beneficial effect. The study did not mention container types or whether the containers were open or closed.

Pelletier and Morrison (1965) studied the content and stability of AA in dry and liquid fruit drink preparations and canned fruit juices during normal conditions of use. Canned juices included grapefruit, tomato, and orange juices stored in covered glass jars at 40°F. The liquid drinks were also held at 40°F in original closed glass or plastic containers. Five of the six dry premixes contained at least 90% of the AA content stated on the label after 3-4 weeks of dry storage. For the liquid drinks, 13 of the 20 samples initially assayed showed AA levels below the label claims. The AA values dropped gradually over the time for normal usage. After three days of normal use only seven of the twenty liquid samples contained at least 90% of the AA listed on the label. The ascorbic acid content in the natural juices decreased only slightly during the seven day-test period. The AA in natural juices was found to be more stable than the AA which had been added to the fruit drinks. Part of the reason for this finding was attributed to the natural antioxidants present in canned juices which tend to protect ascorbic acid from oxidative losses. Comparisons were also made for loss due to containers of glass, plastic, or lacquered can. Similar results were obtained with the three types of containers.

Lopez et al. (1967) completed a home-use type of study on the influence of time and temperature on AA stability

in several orange juice preparations; including fresh, frozen, and canned, and some chemical mixtures containing AA stored at room and refrigerated temperatures for 24, 48, 72 hr and eight days. Other similar samples were boiled for 10 minutes. Samples were stored in tightly covered glass containers. All samples showed good stability of AA even when stored at room temperature for eight days. They found no difference in the stability of the naturally occuring AA and the artificially added AA products. When tap water was compared with distilled water, the stability was unaffected by the water type. After boiling for 10 minutes, there was still considerable AA stability in that no more than 25% of AA was lost.

Noel and Robberstad (1963) also did a type of home-use study on the stability of AA in canned apple juice (AA added) and orange juice under refrigerated conditions. They concluded that in open containers in the refrigerator, AA was inherently more stable in the orange juice than in the supplemented apple juice. Containers were compared in order to show differences between open tin, closed milk bottle, open beaker, and closed plastic containers. Some minor effects due to type of container were noted although the greatest difference was between juices.

Miller and Marsteller (1952) studied the storage life of frozen orange juice concentrate from the standpoint of

the consumer. The concentrate was thawed and stored in glass quart jars with glass tops at 25, 5, and 0°C for various periods of time. Portions of the concentrate were reconstituted and also stored at the various conditions. The storage at 25°C was discontinued after 24 hours because of adverse flavor changes. Concentrate thawed and stored at 0°C for 4 days retained 95% of its original ascorbic acid content as did the corresponding reconstituted juice. At the end of 8 days of storage at 0°C the AA retention was still high (91-92.5%) with no significant difference between concentrate and reconstituted juice. At 5°C, AA retention at 4 days for both concentrate and reconstituted was relatively high (92.3-92.7%). But after 8 days the difference between the two forms became pronounced with the concentrate and the reconstituted juice retaining 91.2% and 85.6%, respectively, of their initial AA contents. They also found that AA stability was higher in juice reconstituted with distilled water than with tap water.

Effects of Oxygen Levels

Some workers have studied the effects of oxygen levels on ascorbic acid retention. Trammell et al. (1986) studied the effects of oxygen on ascorbic acid loss of high temperature-short time (HTST) pasteurized, single

strength orange juice. Oxygen was dissolved at levels of 0.6, 1.8, 6.5, and 10.1 ppm and the juices pasteurized and stored for five months at 22°C. Ascorbic acid loss was linearly related to initial oxygen concentration.

Kefford and co-workers (1959) studied the effects of oxygen on juice quality and ascorbic acid retention in canned and frozen orange juices. They were able to place 3.5% to 0.002% initial oxygen in the cans. They found that in frozen juices stored at least one year at 0°, slow oxidative loss of AA occurred. In pasteurised juices stored at 86°F the first few days showed oxidative destruction of AA due to the availability of free oxygen. For the following periods, the ascorbic acid loss continued at one-tenth of the initial rate due to the absence of free oxygen and, therefore, under anaerobic conditions.

Eison-Perchonok and Downes (1982) studied the kinetics of ascorbic acid loss as a function of dissolved oxygen concentration and temperature in model systems. Their data showed that ascorbic acid oxidation was dependent on dissolved oxygen concentration. It seemed that the rate of oxygen dissolving into the ascorbic acid solution was dependent on temperature and headspace oxygen levels.

Time/Temperature Studies in Various Containers

Heikal et al. (1967) did AA retention studies with grapefruit juice. The juice was heat-treated in glass bottles for at least one minute at 190-192°F and then tightly closed, and stored at either 32° or 40°F. They found that heating destroyed about 11% of the original AA content. The processed juice stored at 32° and 40°F retained between 91 and 95% of the original ascorbic acid. In some samples sulfur dioxide or sodium benzoate was added to the juice in small concentrations but did not affect the ascorbic acid retention significantly.

Smoot and Nagy (1980) reported the effects of storage temperature (10, 20, 30, 40, and 50°C) and time on the total AA content of single strength commercially canned grapefruit juice. DHA and DKG were determined in addition to AA. At the end of 12 weeks the total AA loss ranged from less than 3% at 10°C to more than 68% at 50°C. During storage, AA decreased continuously with rate of loss increasing with higher storage temperatures. The levels of DHA and DKG remained fairly constant during the 12-week storage period. Since large levels of DHA and DKG were not found, they concluded that AA breakdown proceeded by an anaerobic pathway. During this storage period, they also noted that the pH and citric acid content of the

samples at 50°C did not change. The sum of the DHA and AA values showed the same pattern of retention as the AA alone. During any storage time, the AA activity due to DHA was only 1.6 to 3.1% with the exception of storage at 50°C and time greater than 3 weeks. Their conclusions about the anaerobic pathway were based on the data of Kefford et al. (1959) and Nagy and Smoot (1977) which indicated that the free oxygen in the juice and headspace of the closed container was depleted within the first two weeks of storage.

Bisset and Berry (1975) studied the ascorbic acid retention in orange juice as influenced by container type. Single strength orange juice packed in 7 oz glass bottles retained approximately 90% of the original AA for a period of 4 months and 87% for 1 year at 4.4°C. AA retention was progressively less at 10° and 15.6°C (84 and 79%, respectively) at the end of one year. At 26.7°C retention decreased to 67% after 8 months, at which time this part of the study was discontinued due to unacceptable juice flavor and appearance. AA retention in 64-oz polyethylene bottles was much less than that stored in glass. Retention at -6.7°C and 1.1°C was 82% and 38.5%, respectively, after six months. AA retention was only 32% after 3.5 months storage at 10°C in polyethylene bottles. Retention was even lower in the 4 oz polyststyrene containers than in polyetheylene or glass.

At -6.7°C storage for six weeks retention was 80.5% and 21% for that held at 1.1°C. AA retention of 37% was found in juice stored for 3 weeks at 10°C. However, all samples retained 90% or more for one week. AA retention in 64-oz cardboard cartons was similar to that of the small polystyrene bottles. AA retention in cartons stored at -6.7°C for 6 weeks was 81.5% while for polystyrene it was 80.5%. For the same time period at 1.1°C the cartons retained 39%. After 3 weeks at 10°C, retention in the cartons was only 26.5%. Bissett and Berry (1975) concluded that crown-capped glass bottles provided the best AA protection of the four types of containers used even at higher temperatures. They attributed the differences to the thin walls of the non-glass containers and the nature of the package material.

Nagy and Smoot (1977) studied the effects of temperature and storage on AA retention in canned single-strength orange juice. Results showed that at storage temperatures of 29.4°, 37.8°, and 46.1°C for 12 weeks the log % vitamin C retention was linearly related with time at 29°C but not at 38° or 46°C. When considering the storage of the juice over 12 weeks at a temperature range of 4.4° to 48.9°C, they found that the rate of ascorbic acid retention decreased significantly at temperatures greater than 27°C. AA decreased rapidly in the first two weeks of storage at temperatures

of 29.4°, 37.8°, and 46.1°C. Based on their data and those of Kefford et al. (1959), they concluded that the initial loss was due to oxidation by the residual air layer trapped within the can during processing, and the loss after two weeks resulted from the anaerobic degradation of ascorbic acid. Hence, Nagy and Smoot (1977) also concluded that the mechanism of ascorbic acid degradation was not the same at all temperatures.

Feaster et al. (1950) studied the influence of storage conditions on ascorbic acid content and the quality of canned (metal containers) orange juice. They found that changes in ascorbic acid during the period that stacks of canned juice were cooling were minor when compared to the changes taking place at ambient temperatures in warehouse storage for seven to twelve months. They stated that the optimum temperature for canned orange juice from the perspective of cost and the retention of quality and ascorbic acid content ranges between 40°-50°F.

ASCORBIC ACID-ANTHOCYANIN INTERACTIONS

Cranberry Anthocyanins

Starr and Francis (1968) investigated the effect of oxygen and AA on the relative stability of anthocyanin pigments in cranberry juice. Three concentrations of AA

and three levels of headspace oxygen were studied. Pigment losses increased as both of these factors increased. Losses were the greatest when the highest values of both oxygen and AA were in the same sample. Headspace oxygen decreased more rapidly as AA levels increased. Conversely, AA decreased faster with higher levels of headspace oxygen during the first eight weeks of storage. A slow rise in AA was noted during storage which was attributed to a possible interfering component. They thought that pigment loss in the juice was due, in part, to reactions with AA or the degradation products which were present in the original juice.

Starr and Francis (1973) studied the effect of metal ions on pigment content and color in cranberry juice cocktail. They found that the presence of metallic ions had a small protective effect on the pigments. However, when AA was added to the systems, these systems suffered greater losses of pigment than in the samples to which no AA was added. In addition, there was reduced pigment loss due to AA when the pH was at 2.2 as compared with pH 2.7.

Blackcurrant Anthocyanins

Timberlake (1960a, b) studied the oxidation of AA in blackcurrant juice. He reported that copper ions naturally present in the juice catalyze the oxidation of

AA in the presence of air by a largely non-enzymatic mechanism. The oxidation was enhanced by the presence of anthocyanins and iron naturally present in the juice. AA oxidation was greatly increased if the juice was contaminated by added copper. Timberlake also studied the oxygen uptake and the loss of ascorbic acid in model systems which were similar to blackcurrant juice. In the presence of copper, ascorbic acid oxidation was greatly increased by the addition of small amounts of iron, though iron by itself had little effect. Anthocyanins increased the metal-catalyzed oxidation of ascorbic acid while glucose reduced the oxidation.

Beattie et al. (1943) were among the first investigators to associate loss of color to direct or indirect ascorbic acid interactions with pigment compounds. They found that ascorbic acid decreased progressively during storage and that red and yellow colors declined at a rate similar to ascorbic acid loss. They thought that their results pointed to the reaction of oxidizable ascorbic acid with reducible pigments. If the concentration of ascorbic acid or its isomers increased, the rate of color change and ascorbic acid loss also increased.

Hooper and Ayres (1950) studied the inhibitory effects of substances occuring in black currants on the enzymatic oxidation of ascorbic acid. To facilitate the study, the

authors added to the juice system oxidative enzymes derived from apples in order to enhance enzymatic oxidation. They reported that the black currant contains at least two substances which are able to inhibit the oxidation of ascorbic acid in the presence of the oxidizing enzymes. One of the components was thought to be associated with the red pigments while the other was a yellow material, probably a flavonone.

Clegg and Morton (1968) studied the stability of AA in citrate buffer containing some of the phenolic compounds (flavonols and anthocyanins) associated with blackcurrant juice. They found that the flavonols used, especially quercitin, gave positive protective effect to ascorbic acid whether in the presence or absence of added copper. When copper was absent the anthocyanins accelerated the oxidation of AA, but when copper was present, the anthocyanins had a slight protective, antioxidant effect. The pigments degraded faster, though, in the copper-containing systems. When both quercetin and anthocyanin glycosides were present ascorbic acid was protected and the degradation of anthocyanin was retarded in comparison with anthocyanin as the sole additive. Clegg and Morton (1968) also compared the natural stability of ascorbic acid in lemon juice and blackcurrant juice with model systems of similar ascorbic acid-citric acid. Without added Cu^{++}, blackcurrant juice was more protective than

the model systems; in lemon juice, though, the AA oxidized faster than in the model system. Such findings provided further evidence for the high stability of AA in blackcurrant juice.

Harper et al. (1969) studied the mechanism of ascorbic acid oxidation in model systems and its inhibition by flavonoids present in black currants. They found that certain flavonoids displayed antioxidant properties in the absence of added copper ions with quercetin having the most activity. Quercetin showed increased protective activity in the presence of added copper ions. The antioxidant activity seemed to be related to the ability of the flavonoids to act as free radical acceptors, which is the property attributed to them in their role as lipid antioxidants. They thought that the ability of flavonoid compounds to chelate metal ions would not contribute to the antioxidant activity of low pH fruit juices since the high acidity and sequestering ability of the fruit acids would prevent complex formation. These workers were not able to demonstrate any complexing between either quercitin or anthocyanins and cupric ions.

Strawberry Anthocyanins

Meschter (1953) investigated the effects of carbohydrate and other factors on fruit color loss of

strawberry products. Ascorbic and dehydroascorbic acid increased the rate of pigment loss. The rate could be further increased by the additional presence of iron or copper.

Pratt et al. (1954) also studied color stability of strawberry juice from the context of interactions among ascorbic acid, riboflavin and anthocyanin pigments. Their results confirmed the conclusion of others (Beattie et al., 1943; Meschter, 1953) that ascorbic acid and anthocyanin pigments interact to result in the destruction of the pigments. The results also indicated that riboflavin contributes to the instability of anthocyanin pigments. The greatest loss of ascorbic acid and riboflavin occurred when samples contained ascorbic acid, riboflavin, and anthocyanin pigments. The ascorbic acid loss was minimized when it was stored alone or in mixtures containing riboflavin.

Sondheimer and Kertesz (1952) studied the kinetics of strawberry anthocyanin oxidation by hydrogen peroxide. Hydrogen peroxide can be formed in the aerobic oxidation of AA in the presence of cupric ions. Several experiments were conducted which showed that dehydroascorbic acid was not the actual oxidizing pigment compound. They indicated that dehydroascorbic acid per se was not capable of oxidizing the anthocyanins at a rate which would account for the velocity of the reaction. They found two possible

reaction pathways for hydrogen peroxide with one being catalyzed by ferrous ions. Results of their studies show that hydrogen peroxide could be responsible for some of the color loss in strawberry products.

In later work, Sondheimer and Kertesz (1953) studied the participation of ascorbic acid in the destruction of strawberry anthocyanin in model systems. They found that maximum anthocyanin loss occurred in the presence of ascorbic acid, particularly when conditions favored the aerobic oxidation of ascorbic acid. They considered it noteworthy that the anthocyanin in strawberry juice was destroyed at a much higher rate than in the model system even if the AA content and pH were identical. Therefore, they thought their studies pointed to an indirect ascorbic acid-induced destruction of the strawberry anthocyanin and concluded that the rate of anthocyanin loss was influenced by the rate of ascorbic acid loss.

Poei-Langston and Wrolstad (1981) studied the color degradation in an ascorbic acid-anthocyanin-flavonol model system. Anthocyanins were derived from strawberries and the flavonol used was catechin. This study provided evidence of anthocyanin and AA degradation through a direct condensation mechanism. In discussing their work, the authors described two different mechanisms which have been proposed for the destructive effect of ascorbic acid on anthocyanin pigment. According to one mechanism,

ascorbic acid oxidizes to form hydrogen peroxide which, in turn, oxidizes the anthocyanin pigment (Sondheimer and Kertesz, 1952; Harper et al., 1969). The other pathway proposes the direct condensation of AA with the anthocyanin (Jurd, 1972). The data of Poei-Langston and Wrolstad (1981) showed that anthocyanin stability in samples containing AA was greater in oxygen-treated samples than in corresponding nitrogen-treated samples. They concluded that the reverse would be true if an oxidative mechanism had been in effect; thus, a direct condensation was implicated. Their data also showed the expected effect of oxygen on AA loss was statistically significant. Oxygen-treated samples showed a rapid loss of ascorbic and dehydroascorbic acids and a 7-day induction period for brown pigment formation which then occurs very quickly. The study demonstrated the varied roles that AA plays in the deterioration of color. AA increases anthocyanin pigment loss in environments of oxygen and nitrogen causing the solutions to lose redness. AA enhances polymer pigment formation and increases browning in oxygen and nitrogen environments though by different mechanisms.

METHODS AND MATERIALS

Two methods, a traditional chemical method and the other involving high performance liquid chromatography (HPLC), were used to monitor the stability of ascorbic acid in this study. Only reagent grade chemicals were used in both methods.

CHEMICAL METHOD

The chemical method of Roe (1954) was used to determine total ascorbic acid (AA). The latter was oxidized to dehydroascorbic acid by treatment with acid-washed carbon (Norit). Dehydroascorbic acid (DHA) is converted spontaneously to diketogulonic acid in acid solution at a pH below 1.0. When treated with 2,4-dinitrophenylhydrazine, dehydroascorbic acid and diketogulonic acid form a derivative, a bis-2,4-dinitrophenylhydrazone, in which the 2,4-dinitrophenylhydrazine is coupled to carbon atoms 2 and 3. When the bis-2,4-dinitrophenylhydrazone of dehydroascorbic acid or diketogulonic acid is treated with 85% H_2SO_4, the derivative undergoes a molecular rearrangement to form a highly stable, reddish-brown product which can be measured

spectrophotometrically at 540 nm. This method is referred to hereafter as the chemical or spectrophotometric procedure.

Preparation of Reagents

Extactant: A 5% metaphosphoric acid-10% acetic acid (glacial) solution was prepared and used to extract ascorbic acid and dehydroascorbic acid from the beverages. This solution is stable up to 7 days when stored at 5°C or less.

Dinitrophenylhydrazine-thiourea solution: This reagent is light sensitive and toxic. It was prepared by dissolving 1.32 g of dinitrophenylhydrazine in 68 ml of 9N H_2SO_4. Then 2.66 g thiourea was added, stirred until dissolved and immediately filtered through Whatman No. 41 filter paper into a glass dispensor covered with foil to exclude light. This reagent is stable for 3 days.

Acid-washed Norit: 200 g of Norit and 1 L 10% HCl were mixed in a large flask. The solution was heated to boiling then vacuum filtered with Whatman No. 42 paper. After the flask was rinsed and the cake returned, 1 L deionized water was added and the solution filtered. This procedure was repeated once more and the Norit was dried overnight in an oven at 110-120°C.

Standard stock solutions: Ascorbic acid (J.T. Baker Chemical Co.) powder was dried for at least 12 hr over P_2O_5 in the dark. Then 10 mg of the ascorbic acid powder was dissolved in 100 ml of 5% metaphosphoric-acetic acid solution (extractant) to yield stock solutions containing 100 ppm ascorbic acid.

Procedure of Determination

An ascorbic acid working standard was prepared and oxidized along with the samples. Two-50 ml aliquots of the ascorbic acid standard solution containing different AA concentrations between 1-10 ug/ml were prepared. Alternately, 50 ml aliquots of the sample solutions of juice containing between 1-10 ug/ml AA were prepared by diluting with the extractant. Each of these solutions was mixed with 1 g acid-washed Norit and shaken at room temperature for 10 minutes using an automatic shaker. Solutions were then filtered through Whatman No. 42 paper into brown bottles.

For color development, 4.0 ml of filtrate and 1.0 ml of dinitrophenylhydrazine-thiourea reagent were mixed in a test tube and incubated for 3 hr at 37°C. The tube was then cooled in an ice bath and 5 ml of 85% H_2SO_4 were added dropwise to the tubes with swirling. After standing

30 min at ambient temperature, absorbance was read at 540 nm in a Bausch & Lomb (Spectronic 20) spectrophotometer. Duplicate samples and appropriate blanks were prepared and analyzed with each series of determinations.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

The instrument used for this research was a Spectra Physics 8000 liquid chromatograph equipped with a Model SP8700 solvent delivery system, a Model SP8000 auto injector, a two channel SP4000 data system, and a SP4010 disc memory module computer system. An analytical LiChrosorb-NH_2 column (25 cm x 4 mm i.d.) (E. Merck) was used for ascorbic acid separation and quantification. Column effluents were monitored at 268 nm by a SP8440 UV/VIS detector. Peak areas were electronically integrated by the data system. A Bio-Sil Amino 5S micro guard column (4 cm x 4.6 mm i.d.) (Bio-Rad) was connected to the instrument to prolong the life of the analytical column.

Chromatographic Conditions

Separations were achieved by using a LiChrosorb-NH_2 column in a weak anion-exchange mode with a mobile phase

of 75:25 (v/v) acetonitrile: 0.05 M KH_2PO_4 buffer (pH 5.95) with a flow rate of 1.5 ml/min. All solvents were vacuum filtered through a 0.2 micron mesh nylon filter with a Millipore apparatus and later degassed using helium. The sample injection volume was 10 microliters. Attenuation was set at 0.08 absorbance units full scale (AUFS). DHA was detected by UV at 268 nm after reduction to AA by reaction with dithiothreitol (DTT).

Sampling Procedures and Storage Conditions

Bottled cranberry juice cocktail and cranberry-apple juice drink were purchased from their respective cases from a local supermarket. The bottles were stored in the dark in a refrigerated room at 5^{o}C. Each bottle contained 32 fluid ounces of beverage. Sampling was taken from a single bottle on days 0,1,3,5, and 7. After one bottle was completely assayed, another bottle was assayed using the same procedure. Both the HPLC and chemical methods were performed on the same bottle of juice on a given day of sampling. A total of 3 bottles of cranberry juice cocktail and 5 bottles of cranberry-apple juice drinks were analyzed. In general, HPLC analysis was done in duplicate on the samples, morning and afternoon, for both AA and AA+DHA. Ascorbic acid standards were run

between these two sets and the chemical method assay was begun during the initial runs of the second HPLC set.

The following is the procedure for a given day of analysis. First, sampling was done for AA only. In a refrigerated room, the bottle was swirled for 30 seconds. The cap was removed and 50 ml of juice was transferred to a graduated cylinder and the bottle was recapped. The total time a bottle was uncapped was 30 seconds per sampling. The sample was taken to the lab and allowed to warm to ambient temperature in the dark for 3 min. For sample clean-up, a C-18 Sep-Pak cartridge (Waters Associates) was activated with 5 ml of acetontrile and then with 5 ml of H_2O to rinse. To the Sep-Pak was first added 1 ml of juice to rinse away any residual water. This filtrate was discarded. Then 2.5 ml of the juice was added to the Sep-Pak and allowed to filter slowly into a test tube. An aliquot of 2.0 ml of filtrate was pipetted into a 25 ml volumetric flask and diluted to volume using the mobile phase as the diluent. The solution was filtered through a Durapore 0.45 micron hydrophobic filter held in a 13mm Millipore Swinnex Filter Unit. This filtrate was injected into the HPLC five successive times and the average value calculated.

Second, sampling was done for AA+DHA. A juice aliquot was placed in a cylinder as described for the AA assay, without any swirling. Steps were repeated as described

earlier for sample clean-up with the Sep-Pak. About 3.5 ml of juice was filtered so there were at least 3 ml of filtrate to be reacted with DTT. DTT (2.0 mg/ml for cranberry and 3.0 mg/ml for cranberry-apple) was placed in a test tube and 3 ml of sample solution was added and mixed briefly. This solution was allowed to react at ambient temperature for 15 min. The sample was then filtered through the Durapore filter and then injected into the HPLC 4-6 times and the average value calculated.

Thirdly, the ascorbic acid standard curve was derived. Into a volumetric flask was accurately weighed 180 mg of AA. It was diluted to 100 ml with the mobile phase (stock solution). Aliquots were diluted from this to make standard solutions in the range 18-45 ppm AA. Three solutions were prepared and assayed starting with the most dilute ones. The stock solution was kept in the refrigerator until needed for another dilution. The levels of dilutions were maintained within the limits found by the test for linearity of the column.

Fourthly and fifthly, after the standards were injected, replicate measurements of AA and AA+DHA were repeated following the above steps.

Sixthly, the sampling procedure was done for the chemical method. No swirling of the bottle took place. Ten ml of juice was poured into a cylinder and the bottle resealed after a period of 30 sec. The juice was allowed

to warm at ambient temperature for 5 min. An aliquot of 2 ml was pipetted into a 100 ml volumetric flask and diluted with extractant. A second aliquot was placed in another volumetric flask and thus samples were run in duplicate according to the spectrophotometric procedures described earlier.

STATISTICAL ANALYSIS

For each standard curve of the spectrophotometric method and HPLC calibration curve, a linear regression equation was obtained by linear regression analysis of the raw data (Neter, 1983). By statistical computer calculation, correlation coefficients were obtained simultaneously.

The data was subjected to an analysis of variance (ANOVA) (Peterson, 1985). Results of the ANOVA were applied in calculating Newmann-Keulls multiple comparisons (Snedecor, 1967).

RESULTS AND DISCUSSION

PRELIMINARY TRIALS AND CONSIDERATIONS

Samples were obtained directly from a supermarket where they had been stored under normal warehouse conditions at approximately 21°C. Bottles of the same juice type were taken from the same case. It was assumed that since the bottles were from the same case they would have similar initial ascorbic acid contents. After acquisition, the bottles were stored in a refrigerated room (5°C) in the dark. All sampling was also performed in this room.

Home-use conditions were simulated by swirling and by taking out the same amount of fluid each sampling period. The swirling was also done to facilitate homogeneity in the bottles during sampling.

It was assumed that the average consumer would consume a bottle of juice in seven days (Bissett and Berry, 1975), which was the time interval chosen for this study. Ideally, all tests should be run simultaneously using several HPLC's along with the chemical method to minimize sampling variation. However, restrictions of equipment and time caused the study to be performed as presented. The assumption was made that though the bottles were

assayed at different times during the month, the AA and DHA contents between bottles would be similar and that the pattern of AA degradation after the bottles were opened would also be similar. In part, this assumption was confirmed at the end of the experiment by an analysis of variance (ANOVA) which showed that the bottle to bottle variation was small in comparison with other effects. With this in mind the results of the bottles within each juice type could be averaged and conclusions drawn from the averages as will be discussed later.

The original research plan called for 3 bottles to be assayed of each juice type. Three bottles were assayed for the cranberry juice; however, because of the unexpected variation between the bottles of cranapple juice, 5 bottles of this juice were assayed in order to clarify conclusions.

Chemical Method

Since the chemical/spectrophotometric method of Roe (1954) had been used previously in our laboratory (MacBride, 1983; Tuan, 1986) to study the AA content of foods, it was considered appropriate to use it in this research to take advantage of the experience and knowledge thus gained with this procedure.

The ascorbic acid standard curve for the chemical method is shown in Figure 1. The proportionality of the colored species formed in this reaction followed Beer's Law from a range of 0.9 to 10.4 ppm. A linear regression equation of Y=0.0260X+0.0037 (r=.999) was obtained which is similar to that reported by Tuan (1986). Standards were run simultaneously with the juice samples. A linear regression was performed using two dilutions of AA standard and assuming that the regression line passed through the point of origin. The linear regression was generally calculated with the r>0.95. Both MacBride (1983) and Tuan (1986) found that the reaction deviates from linearity when the AA concentration exceeds 10 ppm.

High Performance Liquid Chromatographic Method

Initial studies (Tuan, 1986) in our laboratory involved the separation and quantification of ascorbic and erythorbic acids using the HPLC amino column procedure published by Bui-Nguyen (1980) and Doner and Hicks (1981).

Tuan (1986) found that ascorbic and erythorbic acids could be separated effectively by HPLC using a Zorbax-NH_2 column. Unfortunately, the Zorbax-NH_2 column deteriorated during her research and required replacement. Because of costs involved, the less expensive LiChrosorb-NH_2 column was selected which Tuan

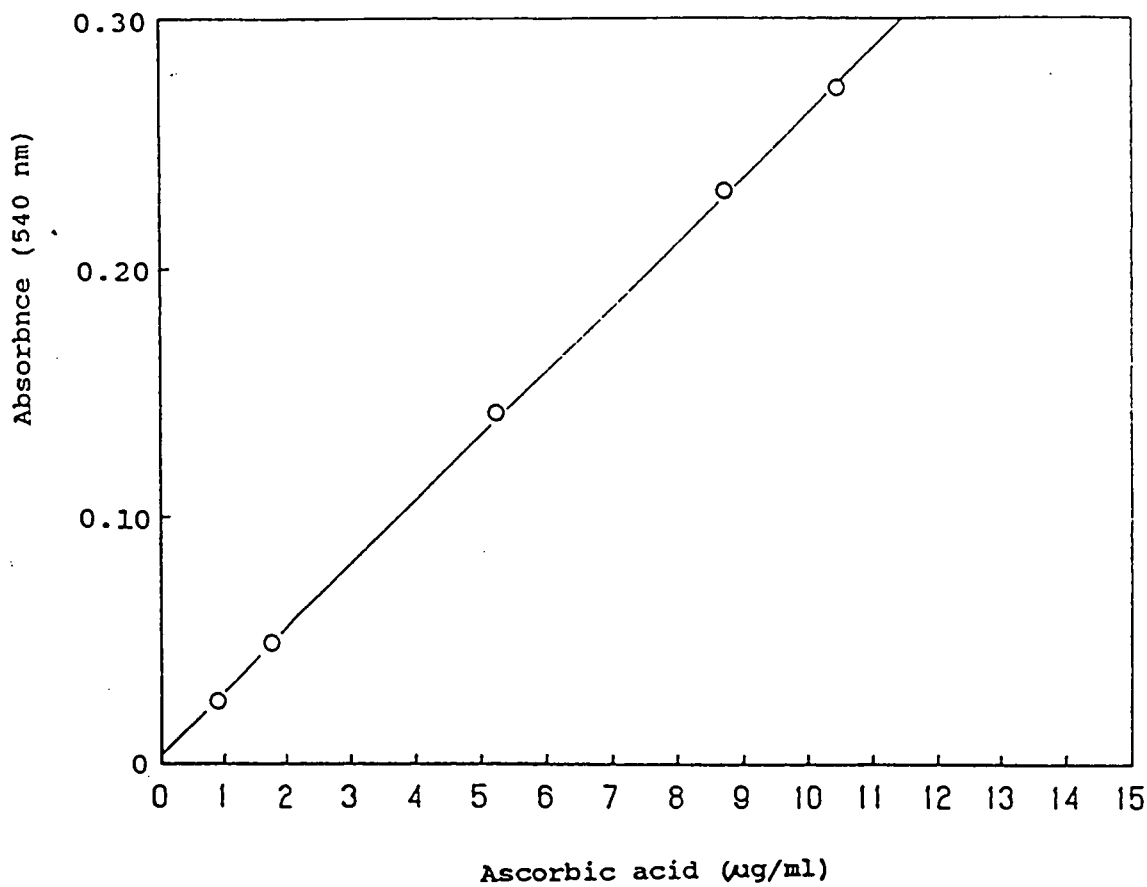

Figure 1. Ascorbic acid standard curve for spectrophotometric method.

(1986) found to be effective in the separation of ascorbic and erythorbic acids. Although the present study did not involve erythorbic acid the LiChrosorb system was used to quantitate ascorbic acid. The ascorbic acid peak in cranberry juice was well-resolved with a sharp peak being eluted at about 6 min after injection (Figure 2). The ascorbic acid in the cranapple drink was similarly well-resolved though there was a peak (labelled ?) eluting about 5 min later than AA which was not present in the cranberry beverage (Figure 3).

The detection of AA was direct and there were no interfering compounds co-eluting with AA that absorbed at 268 nm. Preliminary studies were conducted to test for interfering compounds in the juice by oxidizing the AA with Norit and then injecting the filtered sample. Only a very small peak was eluted near that for AA. When AA was present, this peak was seen as a small shoulder which did not significantly change in size upon reaction with dithiothreitol; thus, it was considered negligible. Similar methods for determining the presence of interfering compounds were followed by Wilson and Shaw (1987) in dealing with AA analysis in foods.

Replicate injections were made in succession and each injection needed approximately 5-7 minutes to be completed. With the cranapple juice, a few more min were needed in order to complete a run because of the peak

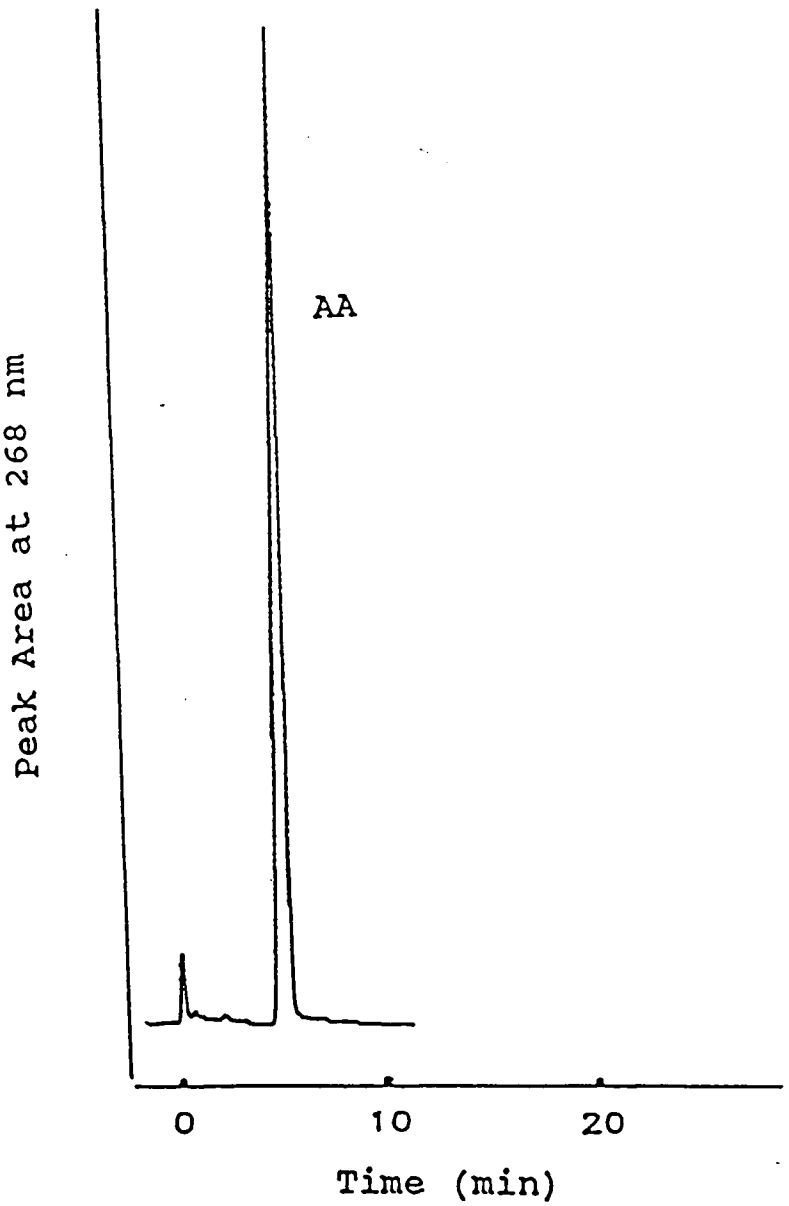

Figure 2. HPLC separation of ascorbic acid (AA) in cranberry juice cocktail. Operating condition: column, LiChrosorb-NH_2; mobile phase, 3:1 acetonitrile/0.05M KH_2PO_4; flow rate, 1.5 ml/min; UV detection at 268 nm; 0.08 AUFS.

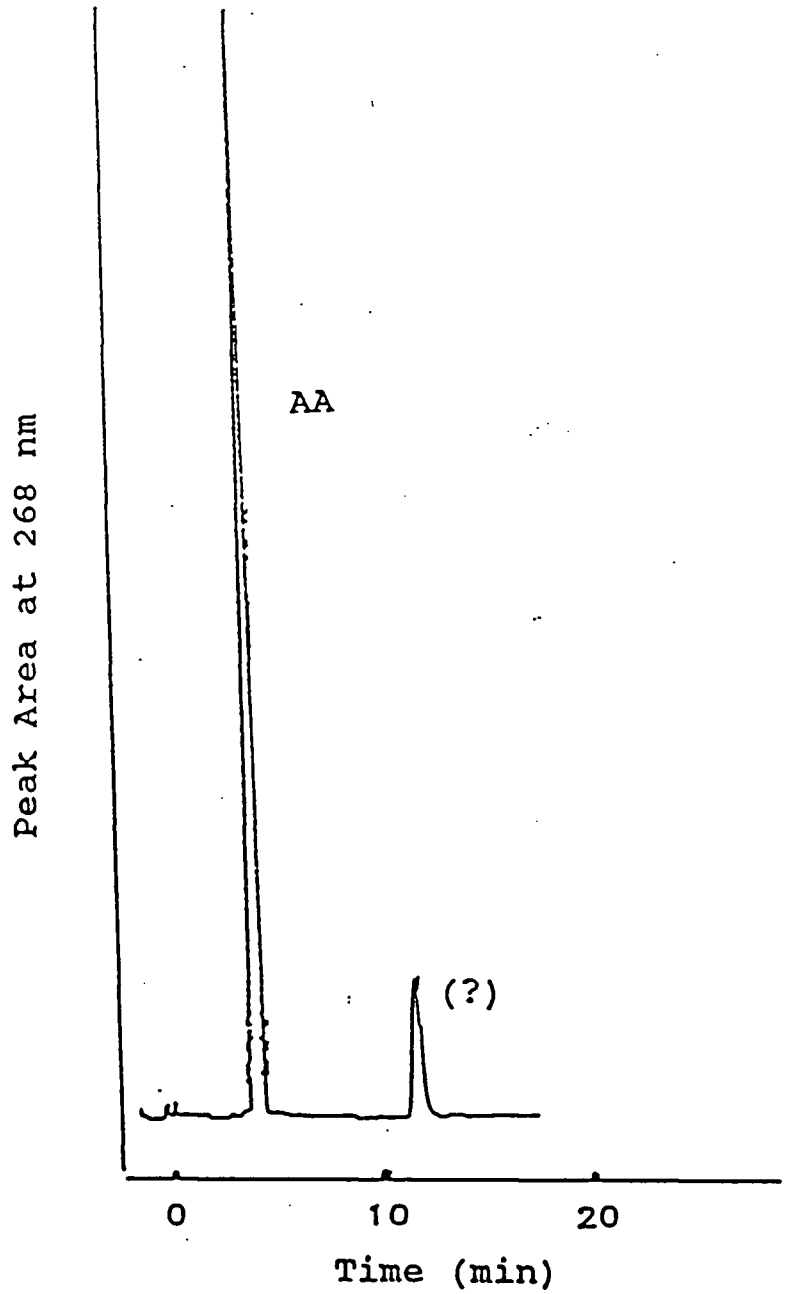

Figure 3. HPLC separation of ascorbic acid (AA) in cranapple juice drink. Operating condition: column, LiChrosorb-NH_2; mobile phase, 3:1 acetonitrile/0.05M KH_2PO_4; flow rate, 1.5 ml/min; UV detection at 268 nm; 0.08 AUFS.

which eluted later than the AA. About 4-5 values were taken and the results averaged. This averaging procedure was repeated for the AA as well as the AA+DHA.

A Sep-Pak cartridge was used in a sample clean-up procedure to remove pigments and also other compounds that could potentially interfere with AA detection. Other workers have also employed the Sep-Pak in their methodology for sample clean-up purposes (Finley and Duang, 1981; Shaw and Wilson, 1982; Tuan, 1986; Wills et al., 1984). Results of preliminary experiments done on this system showed that only small percentages (<2%) of ascorbic acid were retained in the Sep-Pak. Because AA was not significantly retained on the Sep-Pak, it was assumed that dehydroascorbic acid also would not be retained. The Sep-Pak cartridge was very effective in removing red pigments from both juices, resulting in a colorless filtrate.

The procedure of Bui-Nguyen (1980) was initially followed to clean-up the HPLC analytical LiChrosorb-NH_2 column daily. This involved a washing with purified water for at least three hours, though occasionally the column was washed longer than that. However, during the course of the study, it was found that the column manufacturer's instructions stated that large concentrations of water should not be used in the column for any extended period of time because of potential damage. Since high levels

of water in the cleaning procedure could damage the column, the washing procedure was altered to a solution of 70:30 (v/v) acetonitrile:H_2O for overnight rinsing. This altered procedure appeared to improve column performance and thus, the cleaning procedure was altered early in the cranapple trials. The cranberry trials did not go through this modified cleaning procedure.

In respect to column care and performance, it was found that after the analytical column was cleaned as described above, it needed to be primed by ascorbic acid standards prior to analytical runs. This priming involved the injection of standard ascorbic acid solutions at the beginning of the day to get more consistent AA peak areas for later samples. An explanation for this may be the presence of some catalytic sites opened during cleaning of the column which must be neutralized or reacted with before the column performs in a consistent manner. The presence of such catalytic sites was postulated by Rose and Koch (1984).

Calibration of HPLC Standard Curve

Figure 4 shows a representative calibration curve of the peak area of ascorbic acid at 268 nm versus the concentration of ascorbic acid from 15 to 46 ug/ml. One column tested gave linearity over the range of 15 to 46

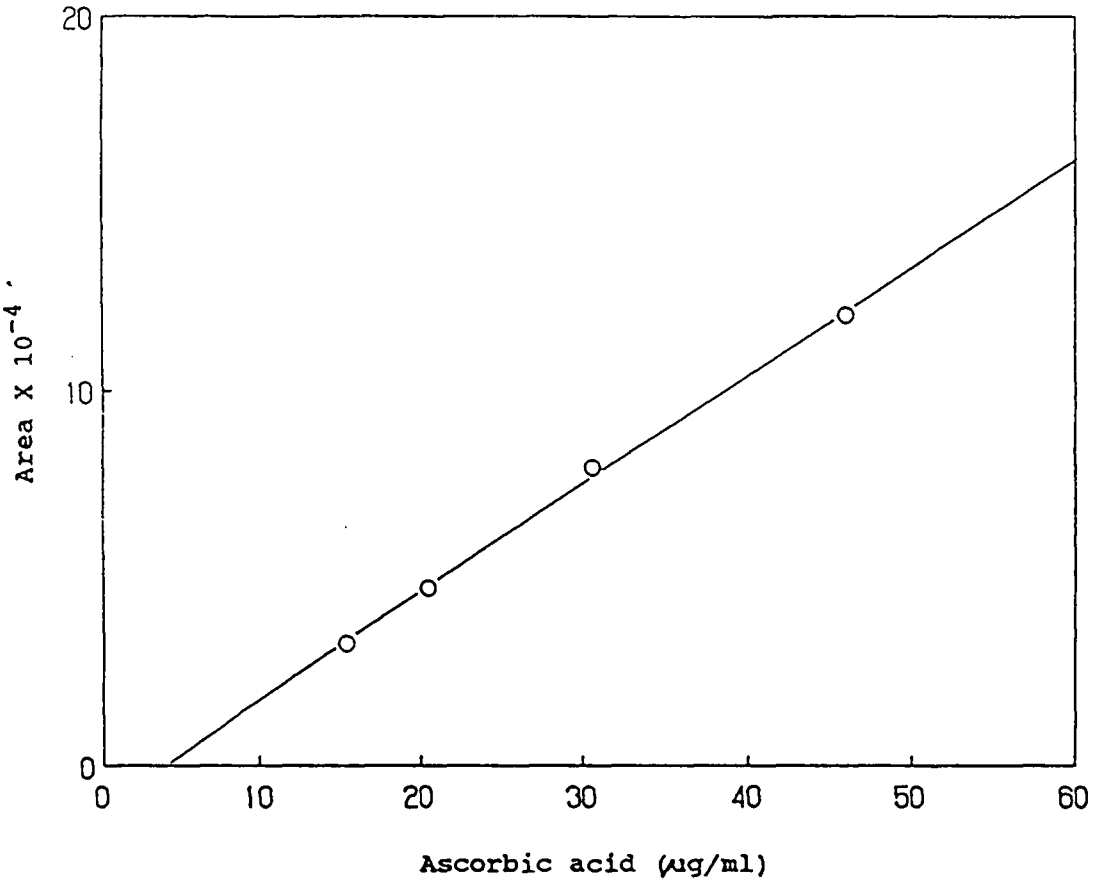

Figure 4. HPLC calibration curve of ascorbic acid. Chromatographic conditions were the same as those given in Figures 1 and 2.

ug/ml with a regression coefficient of 0.999 while the other two columns had a linear range of 17 to 46 ug/ml with regression coefficients of 0.999. A total of three columns were used in this study. This graph demonstrates a linear relationship of Y=2880.5X-11427.8 with r=0.999. Standard curve measurements were performed on a daily basis with regression coefficients usually greater than 0.95. With the same system, Tuan (1986) found that levels as low as 23 ng ascorbic acid could be determined by using 0.005 absorbance unit full scale (AUFS). Internal standard measurements were considered since Tuan (1986) used phenylalanine and Doner and Hicks (1981) used tyrosine, but in this research it was considered equally appropriate and more convenient to use the external standard method as described in the Methods and Materials section. Although standards were run each day, they were completed in one block of time rather than at intervals throughout the day as would be ideal. The reason why this procedure was chosen was due to the instability of the ascorbic acid standard solution. If standards were run at intervals, a new standard would need to be prepared each time which could affect linearity if the points were to be analyzed together. Alternatively, a full curve could have been prepared in both morning and afternoon but this would have been very time-consuming.

Dehydroascorbic Acid Determination

Since dehydroascorbic acid was analyzed by difference, a standard curve per se was not neccesary. In preliminary experiments, attempts were made to synthesize dehydroascorbic acid by the procedure of Doner and Hicks (1981); however, those efforts were not successful due to contaminating compounds in the DHA syrup and the relative ease of dehydroascorbic acid to further oxidize to diketogulonic acid.

Following the method of Doner and Hicks (1981), dithiothreitol was used to reduce DHA to AA. Levels of 2 mg/ml for the cranberry juice and 3 mg/ml for the cranapple juice were used in this study although Doner and Hicks (1981) used only 1 mg/ml. Increased DTT levels were used in this study to insure complete conversion of DHA to AA. Also, preliminary trials indicated that the cranapple juice needed more than 1 mg/ml DTT to effect the conversion to AA and stabilize it. Reasons for this could be the presence of other chemical species which the DTT can reduce other than the DHA. A 15 min incubation time was used as proposed by Doner and Hicks (1981), and used by Tuan (1986). Preliminary studies were conducted with standards which indicated that DHA levels equivalent to 39% of the total AA (AA+DHA) were converted back to AA by DTT at levels of 1 mg/ml. Since the DHA content of most

juices is usually less than 40% of the total AA content (Wills et al., 1984), it was assumed that DTT levels of 2-3 mg/ml, as used, would exceed the minimum required to complete the conversion.

Replicated injections for AA+DHA determination were made in succession. Four to five values were taken and averaged. The data show that a maximum peak area among sample injections occurred after 15 min from the first injection. The occurrence of maximum peak areas when using DTT was also reported by Tuan (1986).

CHANGES IN ASCORBIC ACID AND DEHYDROASCORBIC ACID IN CRANBERRY JUICE COCKTAIL UNDER HOME-USE CONDITIONS

Changes in Ascorbic Acid Measured by HPLC

Results of the AA analysis of cranberry juice cocktail by HPLC are given in Table 1 and also shown graphically in Figure 5. Initial values of AA on day 0 ranged from 36.2 to 40.3 mg% (mg AA/100 ml juice) with a mean value of 37.8 mg%. AA levels gradually decreased over the 7-day storage period to final levels ranging from 22.5 to 26.5 mg% and a mean value of 24.4 mg%.

An analysis of variance (ANOVA) was performed on the AA values to determine if the values over days were significantly different from one another. These results

Table 1. Levels of ascorbic acid (AA) and dehydroascorbic acid (DHA) in cranberry juice cocktail determined by spectrophotometric (SP) and HPLC methods.

Day	SP[a] (mg%)	HPLC[a] (mg%)		
	Total AA	AA	AA+DHA	DHA[b]
0	39.5±1.4^{c}	37.8±2.7^{c}	41.9±5.1^{c}	4.1±3.3^{c}
1	41.7±3.8^{c}	36.3±3.1^{cd}	40.9±5.4^{c}	4.6±3.2^{c}
3	40.7±1.2^{c}	34.0±2.8^{d}	40.4±4.1^{c}	6.4±2.3^{c}
5	42.2±1.7^{c}	29.5±3.2^{e}	39.0±3.5^{c}	9.5±2.0^{d}
7	40.0±3.6^{c}	24.4±2.8^{f}	36.5±2.3^{d}	12.1±2.0^{e}

[a]Values are means±S.D. of six determinations.

[b]Values were calculated as the difference between AA+DHA minus AA.

Means in columns having different superscripts (c,d,e, or f) are significantly different ($p<0.05$).

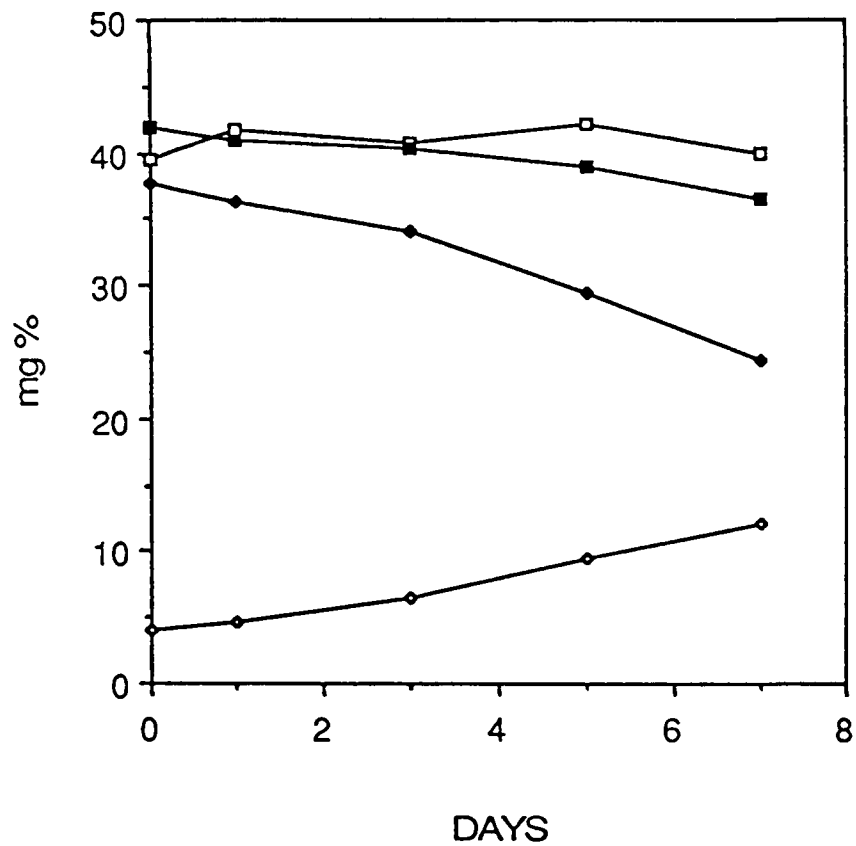

Figure 5. Retention of ascorbic acid (AA) and dehydroascorbic acid (DHA) in cranberry juice cocktail determined by spectrophotometric (SP) and HPLC methods.

are summarized briefly in Table 2. In summary, the ANOVA shows there was a day effect ($p<0.001$), meaning that a statistical difference existed between the AA values among days. The Newman-Keulls multiple comparison test was done on the AA values over days to determine which days were different. These results indicated that a simple linearity does not exist. Results of the multiple comparison test (Table 1) show that the AA content at day 0 was similar to that of day 1. The AA level at day 1 was similar to day 3, but day 3 was significantly ($p<0.05$) lower than day 0. Day 5 was significantly ($p<0.05$) lower than day 3, and day 7 was significantly ($p<0.05$) lower than day 5. The ANOVA for the data analysis was made on the assumption that the variance of each day was similar. The mean square error (MSE) used the same pooled variance term.

Results of AA degradation followed trends similar to those reported by others (Pelletier and Morrison, 1965; Noel and Robberstad, 1963) for other types of juices (orange and apple) studied under home-use conditions. In summary, the AA decreased from 38 to 24 mg% over 7 days which was a 35% loss.

A closer examination of the data reveals that the morning and afternoon values for a given day followed a rather parallel relationship (Table 3). For example, the average morning and afternoon AA values for cranberry

Table 2. One-way ANOVA of ascorbic acid in cranberry juice as determined by HPLC.

Source	df	Mean Square	F-Value
Bottle	2	7.54	
Day	4	88.96	38.91***
Error	8	2.29	

*** Significance $p<0.001$.

Table 3. Comparison of morning and afternoon ascorbic acid values in cranberry juice measured by HPLC.

Day	Ascorbic Acid (mg%)	
	Morning	Afternoon
0	39.2	36.3
1	38.9	33.7
3	36.1	32.0
5	31.7	27.3
7	26.2	22.6

juice cocktail on day 1 were 38.9 and 33.7 mg%, respectively. But on day 3, the average morning and afternoon values were 36.1 and 32.0 mg%, respectively. The data show that the morning AA values were almost always higher than the afternoon values of the same day. Also the morning AA levels of the next sampling day were almost always higher than those of the afternoon of the previous day. The two data sets were shown by ANOVA (Table 4) to be statistically different from one another without any interaction.

Some experts in the field of ascorbic acid chemistry and analysis were consulted through personal communication about probable sources of this problem. Dr. Peter Kissinger of Purdue University did not think that the problem was related with the chemistry. He indicated there might be some changes in temperature occurring from morning to afternoon which might cause differences in peak areas (Kissinger, 1987). However, evidence against this is that with temperature changes, retention times should also change, but upon re-examination of the chromatograms, this was not evident. Kissinger also indicated that instrumentation might be a factor. He suggested the possibility of O_2 absorbing through the Teflon tubing into the mobile phase; however, it seems difficult to explain such large differences between morning and afternoon AA values only by this factor.

Table 4. Two-way ANOVA of ascorbic acid levels in cranberry juice as determined by HPLC; comparison of morning and afternoon values.

Source	df	Mean Square	F-Value
Bottle	2	15.08	
Treatment	1	123.02	18.88*
Error 1	2	6.51	
Day	4	177.91	38.91***
Error 2	8	4.57	
Day X Trmt	4	1.08	0.96(NS)
Error 3	8	1.13	

* Significance $p<0.05$.

*** Significance $p<0.001$.

Dr. Paul Seib of Kansas State University suggested the possibilty of some unknown substances which could combine with AA and bind it through the day but release it after completion of analytical runs. Though theoretically possible, he could not recall any actual cases of this happening. He suggested that the analysis of the testing regimen as the first cause rather than the chemistry. Moreover, he was not familiar with any ascorbic acid reactions which would cause such a pattern (Seib, 1987).

Another possible reason for the decrease of ascorbic acid values in the afternoon would be the gradual accumulation of contaminants from the juice sample onto the LiChrosorb-NH_2 column or the guard column during the sampling day. It is possible that such contaminants could have initiated on-column oxidation of AA; however, there was no evidence of this occurring since DHA values did not increase during afternoon determinations. If such oxidation did occur, it would have greatly affected the conclusion of the study by over-estimating the amount of DHA present in the juice samples.

A more probable explanation would be the accumulation of contaminants on the column which were capable of binding increasing portions of ascorbic acid to the column so that decreased levels were actually eluted. During column clean-up procedures at the end of the day, the contaminants were presumably washed out and the column

rejuvenated. Thus, the column washing may be one reason why the AA readings of the following sampling day were higher in the morning than those of the afternoon of the previous day.

It is of concern, therefore, which AA values were correct, those of the morning or the afternoon determinations. The peaks obtained in the morning were probably the true peaks, but since standards were not done until early afternoon, between the morning and afternoon runs, the calculated AA values derived from the morning chromatograms may be too high. In the same manner, the calculated AA values obtained from the afternoon analysis were likely too low. Therefore, what may be the most meaningful evaluation is to average the morning and afternoon values, and to analyze these results by a separate ANOVA. A one-way ANOVA using the average of the morning and afternoon AA values was performed (Table 2). These results showed that the F-values and statistical conclusions for day effects did not differ with those of an ANOVA (Table 4) in which the morning and afternoon AA values were treated separately. Hence, this type of averaging was done for all subsequent ANOVA calculations involving the levels of AA and DHA.

There are many factors contributing to the degradation of ascorbic acid in cranberry juice cocktail. Among these are oxidation from the air in the headspace (Trammel et.

al., 1986; Eison-Perchonok and Downes, 1982). The headspace increased and was also refreshed on each sampling day, meaning that new oxygen was allowed to enter the system. It should be noted that during the course of the 7-day storage period the juices were swirled several times to facilitate proper sample homogeneity but obviously this procedure may have incorporated more air into the system. Other potential oxidation catalysts were the presence of anthocyanins and metal ions in the juices (Starr and Francis, 1969; Timberlake 1960a,b). Storage time and temperature effects may also have major contribution in aerobic AA oxidation (Nagy, 1980). However, these effects indicate nothing about degradation patterns that may have occured during storage or processing before the bottle was opened. A detailed discussion of factors affecting ascorbic acid stability was covered in the Literature Review.

Changes in AA+DHA Measured by HPLC and the Spectrophotometric Method

The same bottles of cranberry juice that were assayed for AA were also assayed for AA+DHA by HPLC. Total ascorbic acid was determined simultaneously by the spectrophotometric method. The data for these determinations are presented in Table 1 and in Figure 5.

For the HPLC determination, initial values of AA+DHA on day 0 ranged from 40.2 to 43.7 mg% with mean value of 41.9 mg%. The level of total ascorbic acid decreased after 7 days of storage to levels ranging from 35.1 to 37.8 mg% and a mean value of 36.5 mg%. For the chemical method, initial values at day 0 ranged from 37.7 to 40.6 mg% with a mean of 39.5 mg%. Final levels at day 7 ranged from 36.0 to 43.9 mg% with a mean of 40.0 mg%.

A two-treatment ANOVA (Table 5) was conducted which compared the average HPLC values on a given day with those obtained by the chemical method. This ANOVA showed that there was day x treatment interaction ($p<0.05$). Because of the interaction, the ANOVA results showing treatment effects and day effects were not applicable. The interaction will be more fully discussed later.

In order to determine day differences of the HPLC values for AA+DHA, it was found that by taking a one-way ANOVA (Table 6) of the HPLC values without consideration of the chemical method data, conclusions concerning the day effect would be more valid. This analysis resulted in a decreased error term which may be attributed to higher variability in the chemical method than with HPLC. The day effect was significant ($p<0.01$) and multiple comparisons (Table 1) with these averages showed that AA+DHA values for days 0,1,3, and 5 were not significantly different from each other, but that day 7 was

Table 5. Two-way ANOVA of ascorbic acid plus dehydroascorbic acid (AA+DHA) levels in cranberry juice as determined by HPLC and spectrophotometric methods.

Source	df	Mean Square	F-Value
Within Cells	15	0.17	
Bottle	2	32.19	
Treatment	1	12.05	1.03 (NS)
Error 1	2	11.72	
Day	4	8.98	0.97(NS)
Error 2	8	9.28	
Day X Trmt	4	11.36	4.88*
Error 3	8	2.33	

* Significance $p<0.05$.

Table 6. One-way ANOVA of ascorbic acid plus dehydroascorbic acid (AA+DHA) levels in cranberry juice as determined by HPLC.

Source	df	Mean Square	F-Value
Bottle	2	0.52	
Day	4	12.94	7.60**
Error	8	1.70	

** Significance $p<0.01$.

significantly ($p \leq 0.05$) lower than the others. Thus, the preferred conclusion for HPLC day effects should be taken from the one-way ANOVA (Table 6) rather than the two-treatment ANOVA (Table 5). It may be observed in Table 1 that the AA+DHA values decreased from 42 to 37 mg% during 7-days of storage which was a loss of 13%. Again, there was the tendency for the morning HPLC values to be consistently higher than the corresponding afternoon values as shown in Table 7.

The two-treatment ANOVA (Table 5) indicated that for the chemical method there was no significant difference among days (Table 1). This conclusion was confirmed by a separate one-way ANOVA (Table 8) performed on data for the chemical method only without consideration of the HPLC values. This result was expected since the variance in this test was larger and fewer differences were noted, and there were no apparent differences from the results of the two-treatment ANOVA. Although graphically (Figure 5) there appears to be considerable variation of chemical results among days, the differences were not statistically different.

Differences in the total ascorbic acid values derived from the HPLC and chemical methods resulted in the day x treatment interaction observed in the two-treatment ANOVA (Table 5). Pairs of treatments on a given day were compared using Newman-Kuells multiple comparison test and

Table 7. Comparison of morning and afternoon ascorbic acid plus dehydroascorbic acid (AA+DHA) values in cranberry juice measured by HPLC.

Day	AA+DHA (mg%)	
	Morning	Afternoon
0	46.2	37.5
1	45.6	36.1
3	43.9	36.9
5	42.0	36.0
7	38.2	34.9

Table 8. One-way ANOVA of total ascorbic acid levels in cranberry juice as determined by the spectrophotometric method.

Source	df	Mean Square	F-Value
Bottle	2	43.38	
Day	4	7.40	0.75(NS)
Error	8	9.91	
Within Cells	15	0.17	

the results showed that values for days 0,1,3, and 5 are similar for both methods but that on day 7, the chemical value was significantly ($p \leq 0.05$) higher than that for HPLC.

The higher value at day 7 for the chemical method may be explained in the following way. As previously mentioned, the chemical method determines the sum of AA+DHA. Unfortunately, the method as performed in this experiment can be affected by diketogulonic acid, an oxidation product of AA, and other related compounds. Thus, results may be influenced by interfering compounds not present in the early sampling days but being formed during later storage time as the AA decomposes. Similar trends of total ascorbic acid retention were seen in studies of reconstituted orange juice by Lopez et al. (1967) using the dinitrophenylhydrazine method. These authors also indicated the possibility of non-active ascorbic acid substances interfering with the assay. Tuan (1986) also postulated the interference of diketogulonic acid when comparing Roe's method (1954) with HPLC methodology for determining total ascorbic acid content of potato products. The results from the present study support the postulation by Tuan (1986) that the HPLC method does not have the same interfering compounds that are apparent with the chemical method. It should be noted that others workers (Cooke and Moxon, 1981; Bourgeouis and

Mainguy, 1975) also have reported interference problems with the chemical method.

Changes in DHA Measured by HPLC

Dehydroascorbic acid (DHA) values were calculated by taking differences between values for AA+DHA minus AA. These values are listed in Table 1 and presented graphically in Figure 5. Initial DHA values ranged from 3.3 to 5.1 mg% with a mean of 4.1 mg%. DHA levels increased gradually over the storage period so that by day 7, values ranged from 10.8 to 14.1 mg% with a mean of 12.1 mg%. Thus the DHA content increased 33% during the 7-day test period. When the average values of the AA and AA+DHA are placed on the same graph (Figure 5) , it is apparent that the curves are not shaped similarly but the AA slope declines more sharply than the AA+DHA. Thus, as the the differences over each day are calculated, the resulting DHA values increase over the 7-day storage period.

ANOVA calculations were done for the DHA data and the results, presented in Table 9, show that there were day effects ($p<0.001$). The multiple comparison (Table 1) among days showed that DHA values for days 0, 1, and 3 were similar while values for days 5 and 7 were significantly ($p\leq0.05$) higher than the values for the

Table 9. One-way ANOVA of dehydroascorbic acid levels in cranberry juice as determined by HPLC

Source	df	Mean Square	F-Value
Bottle	2	5.33	
Day	4	34.66	16.43***
Error	8	2.11	

*** Significance $p<0.001$.

earlier days. Also, the DHA value for day 7 was significantly ($p<0.05$) higher than that for day 5.

The increase of DHA with time was expected since it is an oxidation product of AA. The results, however, do not provide any information about the further oxidation of DHA. It may be assumed that the DHA oxidized further or that the AA was degraded to other forms without being converted to DHA. Again, there was the tendency for the morning values to be consistently higher than the afternoon values as reflected in Table 10.

Table 10. Comparison of morning and afternoon dehydroascorbic acid values in cranberry juice measured by HPLC.

Day	Dehydroascorbic Acid (mg%)	
	Morning	Afternoon
0	7.0	1.2
1	6.8	2.4
3	7.8	5.0
5	10.3	8.6
7	11.9	12.3

CHANGE OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN CRANAPPLE JUICE DRINK UNDER HOME-USE CONDITIONS

Changes in Ascorbic Acid Measured by HPLC

The HPLC data for the AA content of the cranapple beverage are shown in Table 11 and in Figure 6. At day 0, AA levels ranged from 30.5 to 38.0 mg% with a mean of 35.6 mg%. AA levels decreased during the storage period and at day 7, levels ranged from 19.5 to 30.8 mg% with mean value of 25.9 mg%. The results of the ANOVA for AA values determined by HPLC are summarized in Table 12 which shows a statistically significant ($p<0.001$) day effect. Although the highest AA value was observed at day 3, it was not significantly ($p>0.05$) different from the values for days 0, 1, and 5. Table 11 shows that the AA level at day 7 was significantly ($p<0.05$) lower than those for days 0,1,3 and 5. Overall, the AA content of the cranapple beverage decreased 27% during the 7-day storage period as compared to a loss of 35% for cranberry juice stored an equal length of time. Also, the rate of AA loss was greater for cranberry juice than for cranapple because the former showed a significant ($p<0.05$) decrease of AA at days 3 and 5 versus day 7 for the latter.

Table 11. Levels of ascorbic acid (AA) and dehydroascorbic acid (DHA) in cranapple juice drink determined by spectrophotometric (SP) and HPLC methods.

Day	SP[a] mg%	HPLC[a] (mg%)		
	Total AA	AA	AA+DHA	DHA[b]
0	41.3±2.7^{d}	35.6±3.5^{d}	37.8±6.2^{de}	4.0±3.8^{cd}
1	40.8±1.5^{d}	35.0±2.2^{d}	38.3±3.6^{de}	3.3±3.0^{d}
3	40.0±1.5^{d}	36.5±3.9^{d}	41.6±4.2^{d}	5.1±3.3^{d}
5	40.8±1.0^{d}	32.0±1.9^{d}	39.7±3.7^{de}	7.7±2.5^{e}
7	41.0±1.3^{d}	25.9±4.1^{e}	35.7±5.1^{e}	9.8±2.6^{e}

[a]Values are means±S.D. of ten determinations.

[b]Values were calculated as the difference between AA+DHA minus AA.

[c]Value is mean±S.D. of eight determinations.

Means in columns having different superscripts (d or e) are significantly different ($p<0.05$).

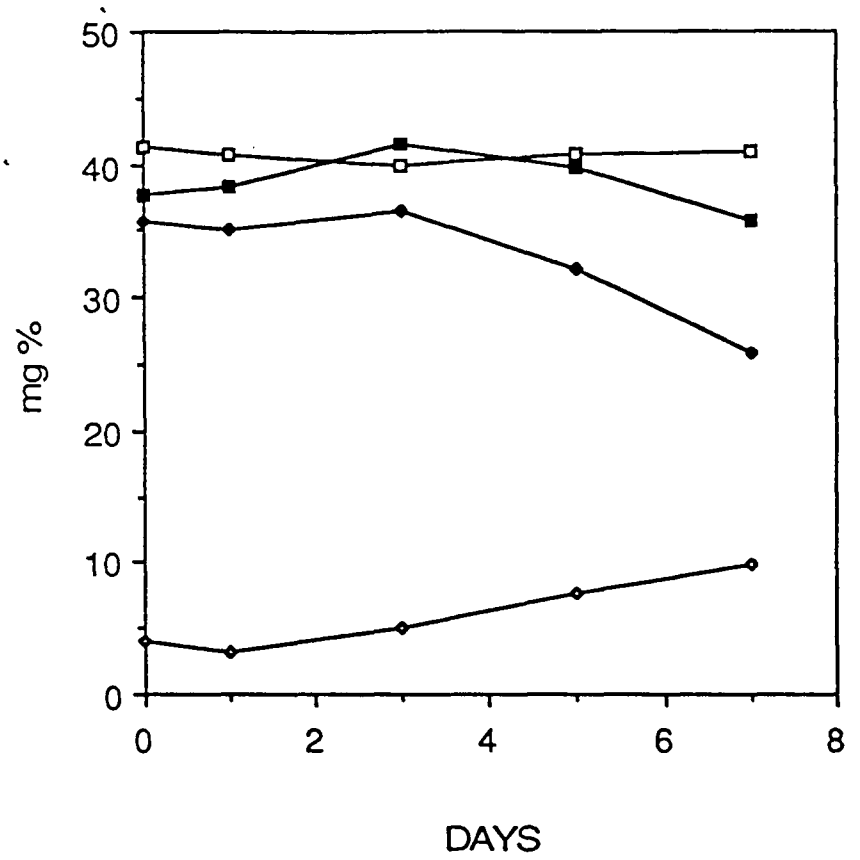

Figure 6. Retention of ascorbic acid (AA) and dehydroascorbic acid (DHA) in cranapple juice drink determined by spectrophotometric (SP) and HPLC methods.

Table 12. One-way ANOVA of ascorbic acid levels in cranapple juice as determined by HPLC.

Source	df	Mean Square	F-Value
Bottle	4	5.53	
Day	4	93.58	10.13***
Error	16	9.24	

*** Significance $p<0.001$.

These results were unexpected and thus 5 bottles were assayed instead of 3 as with the cranberry juice. It was predicted that the AA degradation would occur at a faster rate in cranapple juice since previous studies showed a rapid AA degradation in vitamin C-enriched apple juices (Noel and Robberstad, 1973). Comparisons of AA stability in cranapple and cranberry juices will be discussed later. As was noted for cranberry juice, morning values were consistently higher for the cranapple beverages than the afternoon values, apparently for the same reasons as previously discussed (Table 13).

Table 13. Comparison of morning and afternoon ascorbic acid values in cranapple juice measured by HPLC.

Day	Ascorbic Acid (mg%)	
	Morning	Afternoon
0	36.9	34.3
1	35.3	34.7
3	37.8	35.2
5	33.1	30.9
7	26.7	25.1

Changes in of AA+DHA Measured by HPLC and the Spectrophotometric Method

The cranapple juice was also assayed for AA+DHA. The results are shown in Table 11 and in Figure 6. Initial values of AA+DHA on day 0 for the HPLC method ranged from 32.7 to 42.5 mg% with a mean of 37.8 mg%. Final AA+DHA levels at day 7 ranged from 28.9 to 40.0 mg% with a mean value of 35.7 mg%. For the chemical method, initial values ranged from 38.3 to 44.4 mg% with mean value of 41.3 mg%. Final values at day 7 were similar to the initial results ranging from 40.1 to 43.2 mg% with mean value of 41.0 mg%.

A separate ANOVA was done which compared the average HPLC values with those for the chemical method. These results are summarized in Table 14. The ANOVA showed a the day x treatment interaction ($p<0.05$) when the error terms were pooled, which was justified since the error terms were of the same magnitude.

Figure 6 shows an apparent increase of AA+DHA on day 3 although this increase was not statistically significant ($p>0.05$). The multiple comparison results (Table 11) show that for HPLC, days 0,1,3, and 5 were similar to day 3 and that days 0,1,5, and 7 were similar to day 5. The multiple comparison also shows that values for the chemical method were similar for all sampling days.

Table 14. Two-way ANOVA of ascorbic acid plus dehydroascorbic acid (AA+DHA) levels in cranapple juice as determined by HPLC and spectrophotometric methods.

Source	df	Mean Square	F-Value
Within Cells	25	0.22	
Bottle	4	3.22	
Treatment	1	76.27	4.27(NS)
Error 1	4	17.86	
Day	4	4.03	0.62(NS)
Day X Trmt	4	22.73	3.07*
Error (2&3)	32	7.40	

* Significance $p<0.05$.

When pairs of treatments (HPLC vs chemical) were compared for a given day, the results showed that days 0,1,3,and 5 were similar but the day 7 value for chemical method was significantly ($p<0.05$) higher than that for HPLC at day 7. The day x treatment interaction in the two-treatment ANOVA (Table 14) was attributed to this difference on day 7 . As previously mentioned, the differences may have been due to interferences occurring in the chemical method.

An ANOVA was performed to determine the day effect of the HPLC values without consideration of the chemical method data. These results, listed in Table 15, show no day effect at the 5% significance level. This conclusion differs from the two-treatment test (Table 14) in that the error term had increased. The increase of error term may be due to larger variations between cranapple juice bottles due to the nature of the juice. Therefore, for analyzing the HPLC values, the two-treatment ANOVA was preferred.

A separate ANOVA was also performed on the chemical method only (Table 16) and the conclusion did not differ with that of the two-treatment analysis. The error term decreased which was consistent with the previously mentioned effects.

Table 15. One-way ANOVA of ascorbic acid plus dehydroascorbic acid (AA+DHA) levels in cranapple juice as determined by HPLC.

Source	df	Mean Square	F-Value
Bottle	4	12.91	
Day	4	24.28	2.65(NS)
Error	16	9.15	

Table 16. One-way ANOVA of total ascorbic acid levels in cranapple juice as determined by the spectrophotometric method.

Source	df	Mean Square	F-Value
Bottle	4	8.17	
Day	4	2.48	0.44(NS)
Error	16	5.66	
Within Cells	25	0.22	

The HPLC values ranged from 38 to 36 mg%, with a 5% loss occurring between day 0 and 7, which was not statistically significant. This loss was small compared to the 13% loss observed with the cranberry juice. Again, the higher morning and lower afternoon values followed trends mentioned previously (Table 17).

Table 17. Comparison of morning and afternoon ascorbic acid plus dehydroascorbic acid (AA+DHA) values in cranapple juice measured by HPLC.

Day	AA+DHA (mg%)	
	Morning	Afternoon
0	40.8	34.9
1	40.8	35.9
3	45.1	38.1
5	42.9	36.6
7	38.0	33.4

Change in DHA Measured by HPLC

The DHA values were calculated as before, and the average values are given in Table 8 and shown graphically in Figure 9. At day 0, initial values of DHA ranged from 0.7 to 8.1 mg% with a mean value of 4.0 mg%. DHA values increased during the storage period to final levels ranging from 8.2 to 12.8 mg% with a mean of 9.8 mg%. When the DHA values were plotted the resulting curve (Figure 6) looks similar to the DHA curve for cranberry juice (Figure 5). The values increased from 3 to 9.8 mg% which was slightly lower than the cranberry values. There was an increase of DHA equal to 28% of the total ascorbic acid.

Separate calculations of ANOVA were done and the results are summarized in Table 18. The multiple comparison shows that days 0,1,and 3 were similar but that days 5 and 7 were equal but higher than days 0,1,and 3 as presented in Table 10. This increase was expected as was noted with the cranberry juice. The higher morning and lower afternoon values showed similar trends as before (Table 19).

It should be mentioned that in two of the ten replications on day 0, the AA value was larger than the corresponding AA+DHA values resulting in a DHA value of zero. By following the recommendations of the statistical consultant (Maresh, 1987), the data for these two

Table 18. One-way ANOVA of dehydroascorbic acid levels in cranapple juice as determined by HPLC.

Source	df	Mean Square	F-Value
Bottle	4	2.65	
Day	4	36.02	9.40***
Error	8	3.83	

*** Significance $p<0.001$.

Table 19. Comparison of morning and afternoon dehydroascorbic acid values in cranapple juice drink measured by HPLC.

Day	Dehydroascorbic Acid (mg%)	
	Morning	Afternoon
0	5.6	2.4
1	5.4	1.2
3	7.3	2.9
5	9.8	5.6
7	11.3	8.3

replications were considered unreliable and were not used in the ANOVA calculations nor in the final DHA averages for day 0. Thus the average DHA value of 4.0 mg% for day 0 (Table 11) likely reflects an overestimate of the actual levels of DHA on that day.

It is not known why these unusual results were obtained on day 0. It was possible that during the first few hours when this particular juice was exposed to the air, many changes of equilibrium were taking place causing ratios of AA/DHA to shift dramatically. Errors due to experimental technique and column performance, though unlikely, cannot be dismissed.

PRACTICAL CONSIDERATIONS

Differences were noted when comparing the results of AA contents determined by the chemical method with those obtained by HPLC for both juices. The HPLC method was less labor intensive and less time consuming than the chemical method, as well as being more specific for the various ascorbic acid forms. It was concluded that ascorbic acid oxidation products and other components in the juices caused the interferences that became more pronounced in the later days with the chemical method.

For cranberry juice cocktail, the HPLC data showed a 35% loss of ascorbic acid during 7 days of storage, and a

13% loss in the AA+DHA. The DHA levels increased over time to a maximum value equal to 33.2% of the total vitamin C activity. However, data for the chemical method showed no loss of total vitamin C activity with time.

For the cranapple juice drink, the HPLC data showed a 27% loss of ascorbic acid by the end of 7 days and an insignificant loss of AA+DHA when compared with day 0. The DHA values increased over time to a maximum value of 27.5% of the total vitamin C activity. For the chemical method, the data showed no loss of vitamin C activity.

The differences of AA stability in the two juice systems are also apparent. It is possible that apple juice, in combination with cranberry juice offers a more protective AA environment than the cranberry juice alone. Factors which may contribute to such an effect include interactions of metal ions with flavonoids and anthocyanins, and the antioxidant effects of pectins and proteins.

When the present results were compared with other home-use AA retention studies done on orange and apple juices, the patterns of degradation were similar. The study by Noel and Robberstad (1963) showed that after 7 days of storage the orange juice had a 15% loss of AA while the vitamin C-enriched apple juice showed at least 45% loss of AA. Thus, the AA retention of both of the cranberry beverages in this study fall somewhere between

the stabilities of apple and orange juice. One of the factors for the differing AA retentions in the study of Noel and Robberstad (1963) may be that the pH of the orange juice was lower than that of the apple juice. However, the authors did not report the measurements of pH data in their study.

In both juice systems, as determined by the HPLC methodology, the AA decreased below the manufacturer label claims on the bottle by day 7. The claim was that 6 fluid ounces (177 ml) contain 100% of the USDA Recommended Dietary Allowance (60 mg/day)(Food and Nutrition Board, 1980). In order for this to be true, the concentration in the juice must be 33.82 mg% but the data indicate that the AA contents had decreased below this level by the end of the seventh day of storage under home-use conditions However, when the DHA (a biologically active form of vitamin C) is taken into account, the total vitamin C activity is within the range of the label claims, even at day 7. Conversely, by the chemical method, the levels of total ascorbic acid do not decrease below the label claims. Thus, from the consumer standpoint, the nutritive value of both beverages was not significantly affected by home-use conditions and these drinks could serve as a major source of vitamin C in the diet.

SUMMARY AND CONCLUSIONS

Commercially bottled, vitamin C-fortified, cranberry juice cocktail and cranberry-apple (cranapple) juice drinks were stored at 5°C under simulated home-use conditions. Ascorbic acid (AA) and dehydroascorbic (DHA) levels were evaluated over a period of seven days by high performance liquid chromatography (HPLC). Total vitamin C content was also measured by a classical spectrophotometric method.

The HPLC results showed a significant decrease of AA over the storage time in both juice drinks resulting in a loss of 35% and 27% in the cranberry and cranapple drinks, respectively. The loss of ascorbic acid was offset by the simultaneous increase of DHA (a biologically active vitamin) so that the sum of AA+DHA decreased 13% and 0% in the cranberry and cranapple drinks, respectively.

The spectrophotometric results indicated no significant loss of total vitamin C activity over the storage period in either juice drinks. The observed differences between the spectrophotometric and HPLC results were attributed to interference problems associated with the spectrophotometric method.

The experimental results indicated apparent differences in ascorbic acid retention between the two

juice systems. The cranapple juice showed a greater protective effect upon AA retention than cranberry juice.

Although the AA loss as determined by HPLC was statistically significant on a numerical basis, the actual loss in total vitamin C activity (AA+DHA) was not considered significant from a practical nutritional viewpoint. Even with the AA losses noted, the normal daily intake (6 fluid oz) of either juice would nearly meet the recommended requirements of 60 mg of vitamin C per day without considering dietary vitamin C contributed by other foods consumed daily.

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