AN ABSTRACT OF THE THESIS OF

Shenhsiu Tuan for the degree of Master of Science in Food Science and Technology presented on March 18, 1986.

Title: THE EFFECT OF ERYTHORBIC ACID ON THE DETERMINATION OF ASCORBIC ACID LEVELS IN SELECTED FOODS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SPECTROPHOTOMETRY.

Abstract approved: _____
Allen F. Anglemier

A high performance liquid chromatography (HPLC) procedure using a LiChrosorb-NH_2 column and a eluant buffer of 75:25 (v/v) of acetonitrile:0.05 M KH_2PO_4, pH 5.95, was developed for the successful separation and determination of ascorbic and erythorbic acids in selected food samples.

Application of the method, which is sensitive, rapid and simple, for the analysis of ascorbic and erythorbic acids in frozen apples, potato products, fruit and vegetable concentrated juices, frozen juices, natural and artificial flavor drink mixes, and Hi-C drinks gave satisfactory results. Dehydroascorbic and dehydroerythorbic acids in these samples could also be determined after reduction with dithiothreitol.

It was verified by HPLC that the presence of erythorbic acid affected the determination of ascorbic acid levels by the spectrophotometric method by causing elevated absorbance

readings and hence, abnormally high values. Erythorbic acid seriously affects the true determination of ascorbic acid contents in food samples by the spectrophotometric method.

The use of HPLC is recommended for the routine analysis of ascorbic acid of those samples containing both ascorbic and erythorbic acids.

THE EFFECT OF ERYTHORBIC ACID ON THE DETERMINATION OF ASCORBIC ACID LEVELS IN SELECTED FOODS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SPECTROPHOTOMETRY

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Completed March 18, 1986

Commencement June, 1986

APPROVED:

Professor of Food Science and Technology in Charge of Major

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Date thesis is presented: March 18, 1986

Typed by Shenhsiu Tuan

ACKNOWLEDGEMENTS

First, I wish to express my gratitude to Dr. Allen F. Anglemier for his guidence, assistance and encouragement throughout the course of this investigation after my major professor left. His invaluable amount of help, support, direction and constructive criticism during the preparation of this manuscript are greatly appreciated.

Special thanks are extended to the members of my graduate committee: Dr. Ronald E. Wrolstad, Dr. Donald L. MacDonald, Dr. Ellen K. Pikitch. I extened my gratitude to Dr. Richard A. Scanlan for making his high performance liquid chromatography apparatus available for use, to Dr. David A. Heatherbell for making his IBM PC available for typying the thesis, to Diane MaCbride for providing technical assistance in the operation of the high performance liquid chromatography equipment, to Geoffrey wang, Johnny Chung, Tracy Mitzel, San-Lang Young, Shelly Burger, and Robbert Hsu for giving friendly helpfulness in this study.

Finally, I wish to express my gratitude to my parents, husband Hei-Min, and son James for their patience, moral support and constant helpfulness.

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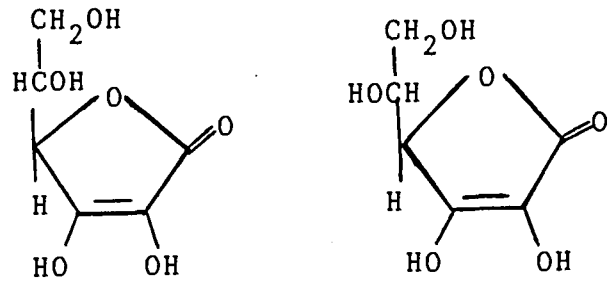
INTRODUCTION

Recently, sodium bisulfite was shown to cause serious allergic reactions in some consumers. The National Restaurant Association has asked its members to stop using sodium bisulfite as a food quality protective agent to inhibit the browning reaction (Andres, 1983). Water-soluble food antioxidants such as erythorbic acid, sodium erythorbate, or ascorbic acid in combination with citric acid have been recommended as alternatives to sulfite (Rice, 1983; Labell, 1983).

Erythorbic acid is an stereoisomer of L-ascorbic acid (vitamin C) (Figure 1). The structure of these two compounds differ only in the spatial configuration of the hydroxyl group at carbon-5. Erythorbic acid has the same acidic and reducing properties as those of ascorbic acid. Because of its reducing properties and lower cost, erythorbic acid is being used at an increasing rate in various foods and beverages as an antioxidant (Pfizer, 1977). However, ascorbic acid is used as both a nutrient and an antioxidant (Youngs, 1984).



L-Ascorbic acid D-Erythorbic acid

Figure 1. The structure of ascorbic and erythrobic acids.

It is generally recognized that the vitamin C activity of erythorbic acid for guinea pigs is one-twentieth of that of ascorbic acid (Hornig and Weiser, 1976). Some studies have shown that the co-administration of erythorbic acid with ascorbic acid reduces the availability of ascorbic acid in guinea pigs (Pelletier, 1969). From the nutritional viewpoint, it is very important to clarify whether erythorbic acid can partly substitute for ascorbic acid or if it functions in another manner in humans. Research on this aspect has shown little progress because no simple and reliable method for the separate determination of ascorbic and erythorbic acid levels has been available. For reasons of food quality assurance and nutrition, it is essential that an effective method be developed to quantify ascorbic and erythorbic acids levels, individually. Most chemical assays for either compound are based on reaction with the enediol group within the lactone ring. Thus, it is difficult to distinguish erythorbic acid

from ascorbic acid in foods by chemical methods or enzymic assays (Thompson, 1982). Methods published to date that differentiate between ascorbic and erythorbic acids include paper and glass fiber chromatography, alternating current polarography, isotachophoresis, and high performance liquid chromatography (Geigert et al., 1981). However, just a few reports are found in the literature that used these procedures on food samples.

Since high performance liquid chromatography (HPLC) has been shown to be a versatile technique for the rapid and effective separation of various biological compounds (Arakawa et al., 1981), experiments reported herein were designed to evaluate HPLC methodology for separating erythorbic and ascorbic acids and some of their oxidative products, and to compare these results with those obtained from food samples by the traditional chemical method. In addition, the effect of erythorbic acid interference upon the determination of ascorbic acid by the chemical method was also investigated.

LITERATURE REVIEW

ASCORBIC ACID

Chemical and Biological Properties

Ascorbic acid, often referred to as vitamin C, is widely distributed in the animal and plant kingdoms (Rosenberg, 1945; Chaudhuri and Chatterjee, 1969). It occurs in most fruits and vegetables at levels between 20 ug and 3 mg/g and in lower amounts in animal sources such as kidney (100 ug/g), liver (300 ug/g), and milk (10 ug/ml) (Thompson, 1982).

Ascorbic acid (L-threohexono-1,4-lactono-2-ene) is clasified as a carbohydrate (Pachela et al., 1985) that has powerful reducing properties (Cort, 1982; Erdman and Klein, 1982). It is very soluble in water (1 g/3 ml of water) but rapidly decomposes in alkali and light. It is relatively stable in acid or in a dry state. Ascorbic acid is very sensitive to metal contamination being easily degraded at an a_w of 0.65 or higher in the presence of iron or copper or zinc ions (Dennison and Kirk, 1982).

Ascorbic acid is easily oxidized to dehydroascorbic acid (Doner, 1983) which has about 80% of the biological activity of vitamin C (Thompson, 1982). Hydrolysis of

dehydroascorbic acid occurs outside a narrow acidic pH range (near pH 7) to yield diketogulonic acid, which has no vitamin C activity (Roe and Barnum, 1936; Mill et al., 1949; Huelin, 1949; Roe, 1957 ; Hegenauer et al., 1972 ; Finley and Duang, 1981).

The undissociated form of ascorbic acid has a maximum absorbance in the ultraviolet region at 245 nm at pH values below 1.5, while the maximum of the dissociated form shifts to 265 nm at pH 6.8 (Rosenberg, 1945; Thompson, 1982; Fung and Luk, 1985).

The best known role for ascorbic acid in humans is for the biosynthesis of collagen (Tuderman et al., 1984). Clinical deficiency of vitamin C leads to an unstable collagen peptide and eventualiy the disease state known as scurvy (Rosenberg, 1945; Dyke, 1965; Chatterjee, 1978; Doner, 1983; Woodruff, 1975). This is a potentially fatal disease characterized by a deterioration of collagenous structures which results in extensive capillary hemorrhaging (Hornig, 1975). Marginal vitamin C deficiency results in behavioral changes, reduced drug metabolism, and reduced immunocompetence (Brin, 1982), thereby affecting social behavior and physiological functions. Primates cannot synthesize ascorbic acid, thus they depend on food sources to satisfy their requirements (Brin, 1982).

The Role and Uses of Ascorbic Acid in Food and Nutrition

Ascorbic acid is used by food processors as a functional food additive to improve the qualities of certain foods (Aderiye, 1985). It can be added to bread as dough improving agent to increase loaf volume, to cured meats and soft drinks to enhance both color development and stability, to oils and fats to extend shelf-life via its antioxidant effect, and to fruits for inhibiting enzymic browning during processing. Occasionally, it is used as a nutritional additive in dehydrated foods and fruit juices to replace vitamin C lost during processing (Cort, 1982; Bauernfeind, 1982; Youngs, 1984; Pensabene et al. 1985).

The formation of N-nitrosamine, a carcinogen, during the heating of cured meat products is reduced when ascorbate is present (Lee et al., 1984; Youngs, 1984). Shorter drug metabolism times are reported to be associated with high ascorbate levels (Brin, 1984).

Ascorbic acid has been implicated in the hydroxylation of proline in the formation of collagen (Barnes, 1975), and vitamin C deficiency is associated with impaired wound healing (Schwartz, 1970). Ascorbic acid may also be involved in the metabolic reactions of amino acids such as tyrosine (La Du and Zannoni, 1961), in the synthesis of

epinephrine and anti-inflammatory steroids by the adrenal gland (Stone and Tounsley, 1973), in folic acid metabolism (Stokes et al., 1975), and in leukocyte functions (Shilotri, 1977). The absorption of non-heme iron is enhanced by dietary ascorbic acid when the two nutrients are ingested simultaneously (Schnefp and Satterlee, 1985). High levels of blood ascorbate appear necessary for stimulation of the immune system and for antioxidant effects by removing destructive free radicals from the body (Youngs, 1984).

In earlier times, some physiologists suggested that a daily intake of 75-100 mg of ascorbic acid was necessary to maintain normal body functions (RDA, 1953). However, the Recommended Daily Allowances (RDA, 1980) of ascorbic acid for the normal healthy adult is currently set at 45-60 mg/day.

ERYTHORBIC ACID

Chemical and Biological Properties

Erythorbic acid (D-erythrohexono-1,4-lactono-2-ene), an epimer to ascorbic acid at C-5, is used as a common preservative, better known as isovitamin C or araboascorbic acid (Pelletier and Godin, 1969; Thompson, 1982).

Erythorbic acid is product of organic synthesis that does not naturally occur in biological tissues. D-Xyloascorbic acid is formed when erythorbic acid is heated with excess base in aqueous methanol (Brenner, 1964). In the dry crystalline state, erythorbate readily reacts with atmospheric oxygen and other oxidizing agents to form dehydroerythorbic acid. The latter hydrolyzes readily and irreversibly to D-diketogluconic acid. It is this property that makes them so valuable as antioxidants. Erythorbic acid shows greater stability under acidic than under more neutral pH conditions (Pfizer, 1977).

Erythorbic acid possesses little or no vitamin C activity (Reiff and Free, 1959; Doner, 1984). Bui-Nguyên (1980) reported that the biological activity of erythorbic acid is twenty times less than that of ascorbic acid. Reynold and Lorenzo (1974) stated that erythorbic acid has only 2-5% of the potency of ascorbic acid. Erythorbic acid has been described as an antagonist of ascorbic acid (Hornig and Weiser, 1976; Omaye et al., 1980), although Turnbull et al. (1979) reported that it was not. Hughes and Hurley (1969) found that ascorbic acid was deposited in tissues of guinea pigs whereas erythorbic acid was not.

Although the acidic and reducing properties and chemical behavior of erythorbic acid are very similar to those of ascorbic acid (Aradawa et al., 1981), it is important to remember that erythorbic acid has little nutritional value for either humans or animals.

The Role and Uses of Erythorbic Acid in Food

Because of its particular oxidation-reduction properties and being less expensive than ascorbic acid (Doner, 1983) , erythorbic acid is widely used as an antioxidant in processed foods. Doner and Hicks (1981) estimated that the price of erythorbic acid was about one-half of that of ascorbic acid.

Erythorbic acid is "generally recognized as safe" (GRAS) for use as a food preservative and has been listed as such by the Food and Drug Administration (FDA) since 1960. The use of erythorbic acid or sodium erythorbate in certain meat and poultry products is also permitted by the United States Department of Agriculture (USDA) (Pfizer, 1977).

Currently, erythorbic acid is used in meat processing to promote the curing reaction by helping to reduce met-myoglobin to myoglobin and to accelerate the conversion of nitrite to nitric oxide. These conditions enhance the development and stabilization of the cured meat color. It

is added in frozen fruits to retard discoloration and off-flavor development and thus, significantly prolongs storage life. Erythorbic acid has been added as an antioxidant to other products such as beer, table wine, carbonated beverages, frozen fish, and processed potatoes. (Pfizer, 1977). Lillard et al. (1982) found that erythorbic acid and its derivatives initiated sulfhydryl-disulfide interchange reactions in wheat flour bread dough to improve viscosity characteristics.

QUANTITATIVE METHODOLOGY OF ASCORBIC ACID AND ERYTHORBIC ACID

Over the last decade, spectroscopic, electrochemical, enzymatic and chromatographic methods of analysis (Mayer, 1966) have been used to quantify the ascorbic acid contents of food products, pharmaceuticals,and biological samples. Each method has its own specificity to certain samples. Because some samples are very complicated biological materials, color and other components in the sample can interfere with the determination of ascorbic acid. The two most popular methods for determining ascorbic acid contents are spectroscopic and chromatographic methods. Although high performance liquid chromatography (HPLC) is one of the more selective and accurate instruments in the field of chemical analysis, the spectrophotometric method for the

determination of ascorbic acid still has its place and remains widely used (Pachla et al., 1985).

Erythorbic acid can be quantified by the same chemical methods as for ascorbic acid, so long as the latter is absent from the sample.

Spectrosocopic Methods

These methods can be divided into three general catagories: redox reactions, derivatization reactions, and difference spectra.

Redox Reactions

The standard redox reagent used for ascorbic acid analysis in a variety of sample types is 2,6-dichloroindophenol (DCIP) (AOAC,1980). DCIP is a dye that is blue in alkali and pink in acid (Lugg, 1942). It is reduced by ascorbic acid to the colorless leucoform. Titration procedure is used to determine residual DCIP (Roe, 1957). This method was used to determine the concentration of ascorbic acid in serum, urine and other biological materials by Pelletier (1968), in orange and grapefruit juices by Hoffman et al. (1970), and in fortified grain products and beverages by Egberg et al. (1973). Many reducing substances such as phenols,

sulfhydryls, triose reductones, ferrous, cuprous or sulfite can reduce DCIP and cause severe interference problems (Roe, 1957). The limitations encountered with DCIP methods result from poor specificity and dye instability (Pachla et al., 1985). The detection limit of the method is 6 ug/ml.

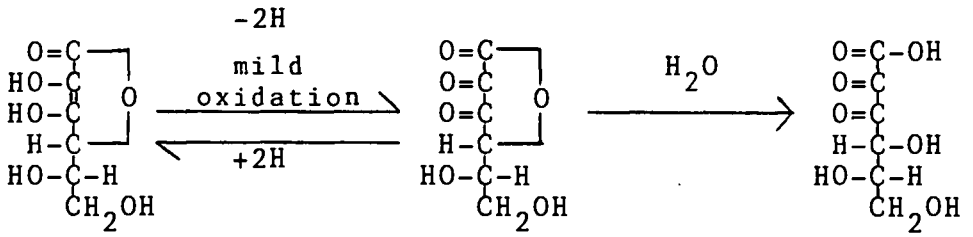
A colorimetric redox reaction involves the reduction of a metal ion. Iron (III) is reduced by ascorbic acid to iron (II), and an intensely colored complex is formed after addition of a chelating agent such as α, α'-dipyridine, 2,4,6-tripyridyl-s-triazine, and ferrozine (Pachla et al., 1985). A specific method for the determination of total ascorbic acid in blood plasma and urine with α,α'-dipyridyl was developed by Okamura (1980, 1981). Jaselskis and Nelapaty (1972) developed a method using ferrozine as the chelating agent. This method was suitable for the quantification of ascorbic acid in citrus fruit. In general, redox metal ion methods have the same disadvantage as DCIP methods. Because ascorbic acid is measured indirectly, the presence of other reducing agents can positively bias the results.

Other redox reagents such as methylene blue (White and Fitzgerald, 1972), O-toluidine (Berek and Berda, 1975), phosphotungstic acid (Kyaw, 1978), and Folin phenol (Jagota and Dani, 1982) were adapted to analyze the ascorbic acid contents in fruit juices, standard ascorbate solution, serum, and biological samples, respectively.

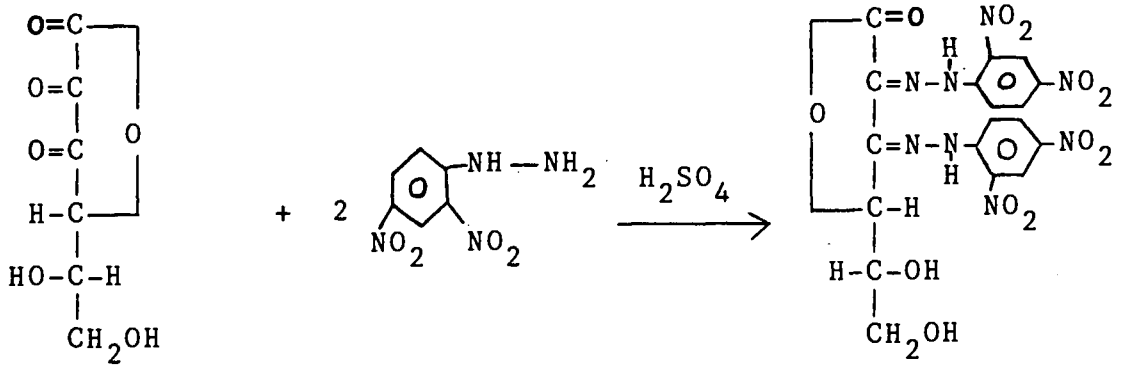
Neither DCIP nor metal ion methods can be used to quantify dehydroascorbic acid content of samples.

Derivatization Reactions

The best known derivatization method (Roe and Kuether, 1943) involves measuring the absorbance produced when 2,4-dinitrophenylhydrazine (DNPH) couples with the oxidized form of ascorbic acid by the following reactions:



L-Ascorbic acid — L-Dehydroascorbic acid — L-Diketogulonic acid



L-Dehydroascorbic acid — 2,4-DNPH — Bis-2,4-dinitrophenylhydrazone

Roe and Oesterline (1944) determined dehydroascorbic acid and ascorbic acid in plant tissue by this method with a detection limit of 0.1 µg ascorbic

acid/g. Mills and Roe (1947) did a critical study of proposed modifications of the Roe and Kuether method (1943) and found that the omission of thiourea caused large errors in the determination of ascorbic acid. The specificity of this method was determined by doing the coupling reaction at different temperatures (Roe, 1961). The derivation conditions of 3-4 hrs. at 37°C, or 1.5 hrs. at 60°C were found to be optimal.

The DPNH method combined with other techniques such as thin layer chromatography (TLC) was used to analyze the ascorbic acid contents of buttermilk (Beljaars et al., 1974), and evaporated, fortified sterilized milk (Toothill et al., 1970). The DPNH method was reported to be more accurate than the DCIP method due to the absence of sulfhydryl groups and reductones that can interfere with the determination. The DNPH method has been automated and applied successfully for analysis of liver tissue samples (Aeschbacher and Brown, 1972). A serious disadvantage of Roe's method (1957) is that dehydroascorbic acid and diketogulonic acid are also measured as ascorbic acid. An in-depth investigation of the automated experimental parameters was conducted with food stuffs by Pelletier and Brassard (1977). At the present, investigative parameters such as minimization of osazone formation by sugars in the sample, and optimization of color development have been established.

The specificity of this method is attributed to the following factors: 1) color is produced more easily with 2,4-dinitrophenylhydrazine derivatives of 2- and 3- carbon, sugar-like compounds; 2) the rate of coupling is much faster with dehydroascorbic acid than with other carbohydrates; and 3) the measurable chromogen formation from non-ascorbic acid substances is minimized by carrying out the reaction at low temperatures (Pachla et al., 1985).

Mills and coworkers (1949) pointed out that dehydroascorbic acid and diketogulonic acid can be determined by difference by reducing dehydroascorbic acid to ascorbic acid with H_2S without reducing diketogulonic acid. However, this is a very time consuming step and it is difficult to control the reducing reaction.

Until now, the DNPH method of Roe (1957) has been widely used because of its accuracy and specificity for the analysis of total ascorbic acid of food samples. Erythorbic acid also can be quantified by this method when the sample does not contain ascorbic acid .

The other derivatization method, diazotization, involves the reaction of ascorbic acid with diazotized 4-methoxy-2-nitroaniline. The diazotization product has maximal absorbance at 570 nm in alkaline solution with the absorbance being proportional to the original ascorbic acid concentration (Schmall et al., 1953, 1954). This method is appropriate for pharmaceutical stability studies (Pachla et

al., 1985). Smith (1972) used diazotization in an ovarian ascorbic acid depletion assay. P-Nitroaniline and diazotized P-aminobenzoic acid also have been used as the redox-derivatization reagent.

Another commonly used method is fluorescent derivatization. It involves the formation of quinoxaline products derived from dehydroascorbic acid and o-phenylenediamine (OPDA) (Deutsch and Weeks, 1965; Kirk and Ting, 1975). Although this method is more specific and faster with less restriction to sample type (Deutsch, 1967), a major drawback is that naturally occurring fluorescing plant components may interfere with fluorometry measurements. Will et al. (1983) used the microfluorometric method as described in AOAC (1980) to determine vitamin C in fresh fruit and vegetables. Visser (1984) reported a ten-fold increase in fluorescent sensitivity when a metaphosphoric acid / acetic acid solvent system was used to replace trichloroacetic acid in this method for determining vitamin C.

Difference Spectra

Difference spectra over the wave length region from 220 to 300 nm before and after oxidation of ascorbic acid by absorbate oxidase were measured using a recording spectrophotometer by Tono and Fujita(1982). A linear

relationship between the peak height of difference spectra and the concentration of ascorbic acid was found. The ascorbic acid content of foods of plant and animal origin (Tono and Fujita, 1982) and of fruit and vegetable juices (Tono and Fujita, 1983) was evaluated by this method.

The catalytic action of the enzyme was approximately the same for both ascorbic acid and erythorbic acid. Sugars give little interference even at a relatively high concentration. The presence of disodium ethylene-diaminetetraacetate (EDTA-2Na), sulfhydryl compounds and diphenols did not seriously influence ascorbic acid estimation. Cupric and ferric ions, which catalyze the autooxidation of ascorbic acid, did not interfere up to 0.1 uM. Tono and Fujita (1982) concluded that the spectrophotometric method based on difference spectra is a simple, rapid and sensitive method for the estimation of ascorbic acid, and it is also possible to determine dehydroascorbic acid and total ascorbic acid in foods. However, this method is not as popular as DPNH derivatization and DCIP titration methods because of interference problems caused by other components such as amino acids.

All of the above methods are based on the chemical properties of compounds. They do not distinguish erythorbic acid from ascorbic acid, thus they can not quantitate ascorbic acid and erythorbic acid in food samples simultaneously.

Chromatographic Methods

Chromatographic methods are commonly employed to increase separation of compounds in food samples. Three kinds of chromatographic methods are available for determining ascorbic acid, erythorbic acid and their oxidative products.

Paper and Thin Layer Chromatography

Paper chromatography is the oldest chromatographic technique for separating compounds. De Ritter (1965) reviewed many paper chromatographic methods which had been used for qualitative and quantitative analysis of ascorbic acid. The first qualitative separation of ascorbic acid and dehydroascorbic acid was done by Partridge (1948). Cold ammonium silver nitrate was used as a visualizing reagent. In 1949, Mapson and Partridge proposed a method to separate ascorbic acid and erythorbic acid, using Whatman NO. 1 filter paper with an acidified potassium cyanide developing solvent. Mitchell and Patterson (1953) used phenol-acetic acid in combination with metaphosphoric acid-impregnated paper to eliminate the hazardous solvent combination. Ascorbic and erythorbic acids could be measured by the 2,4-dichloroindophenol or 2,4-diphenylhydrazine method

after isolating each compound from the filter paper. Kadin and O'Sadca (1959) modified Mitchell and Patterson's method with 2% instead of 1% metaphosphoric acid to stabilize the isomers during the separation of ascorbic and erythorbic acids in urine. Glass-fiber paper chromatography was used (Hornig, 1972) to separate ascorbic acid and several related compounds.

Thin layer chromatography is more useful than paper chromatography for the quantification of the compound. This technique was used to determine ascorbic acid content in pharmaceutical syrup (Kouimtzis and Papadazannis, 1979). The advantages of this separative technique include increased sensitivity and resolution, ability to run several chromatograms in parallel, and the capability of using densitometric and fluorimetric detection (Pachla et al., 1985).

Gas Chromatography

Gas chromatography (GC) was used to analyze trace organic compounds for a period of time. The sensitivity and selectivity of this method was higher than paper or thin layer chromatography. However, polar compounds such as ascorbic and erythorbic acids, were not easily analyzed by this technique. The most popular detectors were thermal conductivity and flame ionization detectors.

The first gas chromatographic analysis of ascorbic acid was done by Sweeley et al. (1963). Trimethylsilylether derivatives were synthesized to increase volatility. Semnello and Argoudelis (1969) applied GC procedures to quantify ascorbic acid, pyridoxine and nicotinamide in multivitamin preparations. This technique was also used to determine the ascorbic acid content in orange drink (Koeppen, 1973), food products and pharmaceuticals (Schlack, 1974).

Liquid Chromatography

The application of liquid chromatography for the analysis of ascorbic acid has been extensively developed, although research on the separation of ascorbic acid and erythorbic acid from the same samples has been emphasized only in recent years (Thompson, 1982). Liquid chromatography combines high selectivity and sensitivity with rapid sample analysis and does not require derivatization. Several reviews of liquid chromatography methods for the determination of ascorbic acid (Thompson, 1982), and both ascorbic acid and erythorbic acid with their oxidative products have recently appeared (Doner, 1983).

Electrochemical Detection

The early studies on the measurement of ascorbic acid by HPLC used electrochemical detection. Kissinger et al. (1974) pioneered the analysis of ascorbic acid in urine with the SAX resin column 0.05 M acetate buffer at pH 4.6 as the mobile phase. Later, Pachla and Kissinger (1976) applied another procedure for the analysis of ascorbic acid in infant foods, fruits, fruit juices, fruit drinks, fortified cereals, milk products, pharmaceuticals and body fluids with the electrochemical detector. The sample extracts were diluted with cold 0.05 M perchloric acid. They also analyzed ascorbic acid by liquid chromatography with amperometric detection in 1978. Amperometric detectors have also been used to measure ascorbic acid in human serum, plasma, and leucocytes (Tsao and Salimi, 1981; Lee et al., 1982). In these instances, samples were deproteinized with 6% trichloroacetic acid, and ascorbic acid concentrations were quantified between 2 and 30 ug/ml.

In 1974, Thrivikraman et al. reported a simple and accurate method of analysis for brain ascorbate of mouse, rat, and guinea pig at nanogram levels. This method employed anion exchange resin HPLC with an electrochemical detector . A routine procedure for measuring ascorbic acid in plasma and urine was described by Mason et al. (1980) which employed a Bondapak-NH_2 bonded-phase column with an electrochemical detector.

Carr and Neff (1980) determined ascorbic acid in tissues from various marine invertebrates using an anion-exchange column and an electrochemical detector. Liquid chromatography with electrochemical detection and some chemical methods for determining ascorbic acid in animal tissues was done by Carr et al. (1983). An ultrafiltration clean up procedure (Green and Perlman, 1980) and C-18 Sep-Pak cartridge (Grun and Loewus, 1983) were used to improve liquid chromatography electrochemical detection selectivity. Recovery was more than 99%. Various sugars, sugar acids, and lactones did not interfere. Tsao and Salimi (1982) used an ion-pair technique to measure ascorbic and erythorbic acids in orange juice, rat plasma, urine, liver and brain tissues. A number of counter ions were investigated with decylamine providing the best separation.

Thompson (1982) reported that the results of early studies using an electrochemical detector did not confirm that it might be useful for the routine analysis of foods because of poor reproducibility and high maintenance costs of the detector.

Spectroscopic Detection

Williams et al. (1973) did the first application of ultraviolet detection at 254 nm for determining ascorbic acid in orange juice. A silica-based anion-exchange column was used. Liebes and coworkers (1981) reported that the results of their analysis of ascorbic acid in human lymphocyte extracts were in good agreement with those of Roe and Kuether (1943). Another method for determining ascorbic acid in food products was done by Floridi et al. (1982). Linearity ranged from 5 to 30 μg/ml, and the agreement was very good for the HPLC method in comparison with those of three chemical methods. Ashoor et al. (1984) used an Aminex HPX-87 LC column with 0.009 N H_2SO_4 as solvent at 245 nm to analyze ascorbic acid in a variety of fresh and frozen fruits and vegetables, fresh and fortified canned juices, and powdered drinks. This procedure compared favorably with the AOAC method (1980) and has the advantage of being more accurate for samples with interfering pigments. Rizzolo et al. (1984) estimated ascorbic acid contents in fresh and processed fruits and vegetables by using anionic pellicular column with 0.1 M sodium acetate eluting buffer at pH 4.25. The amount of ascorbic acid was estimated by adding benzoic acid as an internal standard. Lookhart et al. (1982) used μ-Bondapak-carbohydrate 10-μm particle columns with UV detector at 245 nm to determine the ascorbic acid contents in wheat flours, bread dough conditions and commercial vitamin C tablets. Shaw and Wilson (1982) adapted a C-18

column with 2% $NH_4H_2PO_4$ at pH 2.8 to analyze the ascorbic acid content of some tropical fruit products.

Watada (1982) used reverse phase liguid chromatography to investigate the vitamin in a variety of fruit juices and vegetables. Recoveries of more than 96% were reported, and the method was linear up to 7 ug ascorbic acid.

The first ion-pair liquid chromatography procedure for determining ascorbic acid in foods and multivitamin products was described by Sood et al. (1976). Several counterions were evaluated, with tridecylammomium formate (1 mM) providing the best results. Various foods were extracted with 6% metaphosphoric acid, and detection limits at 254 nm were 5 ug/ml. Augustin et al. (1981) modified this method to analyze ascorbic acid in potatoes and potato products. Recoveries were near 100%, and contents as low as 0.02 ug ascorbic acid could be determined. Lam et al. (1984) determined ascorbic acid in multivitamin-mineral preparations at 254 nm using 0.5% triethylamine at pH 3.6 as ion-pair chromatography with reverse phase C-18 cartridge.

Rose and Nahrwold (1981) reported the direct measurement of ascorbic acid and dehydroascorbic acid in extracts of tissues and foods using an amine column and two different detectors at 254 nm and 210 nm, respectively. The sensitivity of the method for dehydroascorbic acid was poor, however, and the conversion to ascorbic acid before

chromatography was recommended. Finley and Duang (1981) used two reversed-phase columns in series, with a mobile phase of water containing tri-n-butylamine as an ion-pair reagent and quantified ascorbic acid and dehydroascorbic acid at 254 nm and 210 nm, respectively. Wimalasiri and Will (1983) simultaneously analyzed ascorbic acid and dehydroascorbic acid in fruit and vegetables. Column effluents were monitored by UV detectors set at 254 nm and 214 nm. Both compounds were also determined in orange juice by Keating and Haddad (1982) using dual UV detectors. The selectivity of the quinoxaline derivatization reaction was used to enhance the UV absorptivity of dehydroascorbic acid. Dennison et al. (1981) reported a single wavelength (244 nm) method in which ascorbic acid was determined directly while dehydroascorbic acid was estimated by the difference between ascorbic acid and total ascorbic acid after the addition of a reducing agent (DL-homocysteine). U-Bondapak-NH column with a 50:50 (V/V) methanol-0.25% KH_2PO_4 buffer (pH 3.5) was used to analyze ascorbic acid in selected beverages. Will et al. (1977) used μ-Bondapak C-18 and/or μ-Bondapak-NH_2 columns to determine seven water soluble vitamins. The comparison of the separation of ascorbate on μ-Bondapak-NH_2 10 μm (Waters Associates), Econosphere-NH_2 5 μm (Alltech), and Hibar II Lichrosorb 10 μm (E.M. Merk) with a UV detector (254 nm) and a mobile phase of a 50:50 mixture of acetonitrile : 0.68 g/l KH_2PO_4

buffer was done by Rose and Koch (1984). Recently, HPLC methods for ascorbic acid analysis were applied to a wide variety of samples that also included analysis of erythorbic acid and related compounds. A weak anion-exchange column with detection at 265 nm for the separation of ascorbic acid and erythorbic acid was reported by Bui-Nguyên (1980). The mobile phase was 75% acetonitrile in a 0.005 M solution of potassium dihydrogen phosphate. Geigert et al. (1981) separated ascorbic acid and erythorbic acid in various fruit juices with a similar method of eluting with acetonitrile-water mixture. Detection limits of 20 ng for each acid were reported.

Dehydroascorbic acid and dehydroerythorbic acid could also be measured indirectly following reduction with H_2S. Otsuka et al. (1981) used this method to measure ascorbic and erythorbic acids in rat liver, adrenals, spleen, heart and kidney tissues. An amine column in conjunction with a mixture of acetonitrile, acetic acid and water was selected. No interference problems were observed.

Doner and Hicks (1981) reported a combination of UV and refractive index detectors to measure ascorbic acid, erythorbic acid and related acids in orange juice and urine. In later research, they found that the refractive index could separately determine ascorbic acid, erythorbic acid, dehydroascorbic acid, dehydroerythorbic acid, diketogulonic acid, and diketogluconic acid in standard mixtures (Doner and Hicks, 1984).

Garcia-Castineiras and coworkers (1981) applied derivatization procedures to increase the sensitivity of liquid chromatographic methods. They used the 2,4-DNPH method and detected the resulting compound at 254 nm. Ascorbic acid concentration was taken as the difference between direct sample derivatization and derivatization following sample oxidation. This method also was used in measuring ascorbic acid in beer (Van Boekel and Meeuwissen, 1983).

Recently, a fluorescent detector coupled with HPLC was used to determine ascorbic acid in blood by Speek et al. (1984). They also did fluorometric determination of total ascorbic acid and erythorbic acid in foodstuffs and beverages by HPLC with precolumn derivatization. This assay consisted of derivatization of ascorbic acid to the high fluorescent quinoxaline derivative after oxidation with ascorbic acid oxidase. Total ascorbic acid and erythorbic acid were determined in concentrations as low as 0.2 μg/g. Dehydroascorbic acid and dehydroerythorbic acid in foodstuffs and beverages could be determined separately by the same procedure with omission of enzyme oxidation. Vanderslice and Higg (1984) reported a similar fluorescent assay with HPLC to analyze ascorbic acid in fruit juices, vegetables and fruits.

METHODS AND MATERIALS

SPECTROPHOTOMETRIC DERIVATIZED METHOD

Roe's method (1957) was used for the total ascorbic acid determination. Ascorbic acid was oxidized to dehydroascorbic acid by treatment with acid-washed carbon (Norit). Dehydroascorbic acid undergoes spontaneous transformation into diketogulonic acid, slowly in mildly acid solution, and very rapidly in solution at a pH below 1.0 and in a neutral or alkaline medium. 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. Then as the bis-2,4-dinitrophenylhydrazone of dehydroascorbic acid or diketogulonic acid is treated with 85% H_2SO_4, the derivative undergoes a molecular rearrangement and a highly stable, reddish brown product is formed which can be measured spectrophotometrically at 540 nm. This method will be referred to hereafter as the spectrophotometric procedure.

Preparation of Reagents

Extractant: A 5% metaphosphoric acid-10% acetic acid (glacial) solution was used as the extracting medium. This solution is stable for 7 days when stored between 0-5°C.

Dinitrophenylhydrazine-thiourea reagent: This reagent is toxic and light sensitive. It was prepared by dissolving 2 g of dinitrophenylhydrazine in 100 ml of 9N H_2SO_4. Then 4 g of thiourea was added, stirred until dissolved and filtered immediately through Whatman No. 41 filter paper into a dispensor covered with foil to exclude light. This reagent should be freshly prepared every 2-3 days.

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

Standard stock solutions: Ascorbic acid (Baker Chemical Co.) and erythorbic acid (Sigma Chemical Co.) powders were dried over P_2O_5 in the dark for 12 hr after which, 100 mg of each powder was dissolved separately in 100 ml of 5% metaphosphoric-acetic acid solution (extractant) to yield stock solutions containing 1000 ppm.

All chemicals used in this procedure were reagent grade.

Procedure of Determination

An ascorbic acid working standard was prepared and oxidized along with the samples. A 50 ml (5 µg/ml ascorbic acid concentration) aliquot of either standard or sample solution was mixed with 1 g acid washed Norit and shaken for 10 min and then filtered through Whatman No. 42 paper into brown bottles.

For color development, 4 ml of filtrate and 1.0 ml of dinitrophenylhydrazine-thiourea reagent were mixed in a test tube and incubated 3 hr at 37°C. The tube was then placed in an ice bath and 5 ml of 85% H_2SO_4 were added dropwise. After standing 30 min at ambient temperature, absorbance was read at 540 nm. Appropriate blanks were run with each series of determinations.

Preparation of Ascorbic and Erythorbic Acids Standard Curves

The 0.2, 2.5 ,5, 8, and 10 ppm ascorbic acid and the 0.2, 2.5, 5, 8, and 10 ppm erythorbic acid working standard solutions (50 ml) were freshly prepared from the 1000 ppm ascorbic acid and erythorbic acid stock solutions by a series of dilutions with the extractant under dark conditions. Each of these solutions was oxidized in the presence of Norit and carried through the spectropho-

tometric procedure. Standard curves were prepared by plotting absorbance values against concentration.

Recovery Test of Ascorbic Acid Determination

Tomato was chosen as the sample material for the ascorbic acid recovery studies. Sufficient macerated tissue was weighed to yield an estimated 4-6 ug/ml dehydroascorbic acid in the extracted volume. This material was placed in a Virtis blending jar, covered immediately with 50 ml of extractant and blended for 2 min. Then 50 ml of homogenate was mixed with 1 g Norit and shaken for 10 min and then filtered through Whatman No. 42 paper. This filtrate was designated as the control sample.

Different levels of the standard ascorbic acid solution were added (spiked) to certain macerated tomato samples to achieve final concentrations of 5-45 mg per 100 g tomatoes. The extractant was added and the contents were blended for 2 min. Fifty ml of homogenate was oxidized with 1 g Norit and shaken for 10 min and then filtered (Whatman No. 42 paper). These filtrates were known as the ascorbic acid spiked samples.

Ascorbic acid contents of both of these solutions were determined by the spectrophotometric method described previously.

Effect of Erythorbic Acid on the Ascorbic Acid Determination

The preparation of the control sample was the same as that of the recovery test described above. Different levels of the standard erythorbic acid solution were added to the tomato homogenate to yield final concentrations of 6-72 mg per 100 g tomatoes. The other separatory procedures were the same as that of the recovery test to make erythorbic acid spiked samples.

The ascorbic acid contents of both of these solutions were determined by the spectrophotometric procedure.

Sample Preparation

Thirty-eight food samples divided into seven categories were analyzed for ascorbic acid contents: frozen apples, potato products, fruit and vegetable concentrated juices, frozen fruit juices, natural and artificial flavored drink mixes, Hi-C drinks, and cured meat products. The frozen fruit samples were provided by a food processing company. All of the other samples were purchased from local supermarkets.

The analytical work was conducted under reduced light conditions. Samples of frozen fruit , potato products and meat products were blended separately. Enough well-mixed

sample was weighed to give an estimated 4-6 µg/ml of dehydroascorbic acid in 50 ml of the metaphosphoric-acetic acid extractant. This was homogenized by the Virtis blender for 2 min, then 50 ml of homogenate was oxidized by 1 g acid-washed Norit for 10 min and filtered with Whatman No. 42 paper. The filtrate was used for determining the ascorbic acid content by the spectrophotometric procedure.

Almost all of the fruit and vegetable concentrated juices, frozen fruit juice, and Hi-C drinks had nutrient labels on the package. These were expressed as percentage of RDA (Recommended Daily Allowances) of vitamin C per serving size or per bottle. The content of ascorbic acid in these drinks was calculated from the label imformation before doing ascorbic acid analysis. Each of the samples was completely mixed before opening the containers. Sufficient material was withdrawn to contain 4-6 µg/ml dehydroascorbic acid after diluting with the metaphosphoric-acetic acid extractant fluid to 50 ml volume. This mixture was treated with acid-washed Norit for 10 min and then filtered with Whatman No. 42 paper. The filtrate was analyzed for ascorbic acid content by the spectrophotometric procedure.

Natural and artificial flavored drink mixes were in powder forms. These packages also contained nutritional information on the labels. An amount of each powder which was calculated to contain 4-6 µg/ml dehydroascorbic acid in

50 ml of extractant was weighed, oxidized by Norit for 10 min and filtered through Whatman No. 42 paper. The filtrate was analyzed by the spectrophotometric method for ascorbic acid content.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

A Spectra Physics 8000 liquid chromatograph equipped with a Model SP8700 solvent delivery system, a Model SP8000 auto injector, a two channel SP4000 datasystem, and a SP4010 disc memory module computer system was used for this research. An analytical LiChrosorb-NH_2 column (25 cm x 4 mm i.d.) (E. Merk) 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. The HPLC was connected with a Bio-Sil Amino 5S micro guard column (4 cm x 4.6 mm i.d.) (Bio-Rad) which is essential to prolong life of the anion exchange resin (Atwood et al., 1979).

Chromatographic Conditions

A 75:25 (v/v) acetonitrile:0.05 M KH_2PO_4 buffer (pH 5.95) was used as the mobile phase to separate ascorbic and erythorbic acids. Acetonitrile was added gradually

while stirring to warmed 0.05 M KH_2PO_4 to avoid precipitate formation. The flow rate was 1.5 ml/min. Sample injection volume was 10 µl.

Ascorbic Acid Calibration Curve Preparation

Reagent grade ascorbic acid (0.1132 g) was diluted with 10% acetic acid to 100 ml to make a 1132 ppm ascorbic acid stock solution. Serial dilutions containing 2.5, 25, 45, 90, 135, 226, 340, 453, and 566 ppm were prepared by diluting from the stock standard solution with deionized water. Aliquots (10 ul) of these solutions were injected into the HPLC system, and the resulting peak areas were plotted against concentration for the calibration curve.

Erythorbic Acid Calibration Curve Preparation

Reagent grade erythorbic acid (0.1207 g) was diluted with 10% acetic acid to 100 ml to make a 1207 ppm erythorbic acid stock solution. Serial dilutions containing 2.5, 24, 60, 120, 240, 480, and 965 ppm were prepared from the stock solution by diluting with deionized water. Standard curves were prepared by plotting ascorbic acid concentration against peak height.

Internal Standard Curve Preparation

L-Phenylalanine was purchased from Sigma Chemical Company. A stock solution containing 13,530 ppm was prepared by dissolving 0.67 g of phenylalanine in 50 ml deionized water. A series of dilutions were done to obtain phenylalanine solutions containing 75, 150, 305, 610, 1530, 3180 and 7655 ppm .

Spiked Internal Standard to Ascorbic Acid Calibration Curve Preparation

Ascorbic acid standard solutions of 20, 69, 138, 414, 690, and 1103 ppm each containing 1000 ppm phenylalanine as an internal standard were freshly prepared before injection.

Spiked Internal Standard to Erythorbic Acid Calibration Curve Preparation

Erythorbic acid standard solutions of 24, 48, 120, 240, and 480 ppm, each containing 1000 ppm phenylalanine as internal standard were freshly prepared for doing standard curve.

Each of the above prepared solutions was passed through 0.45 um Millipore filter (Gelman Science, Acrodisc®-CR). Aliquots (10 ul) of these solutions were injected into the

HPLC system. The resulting peak areas were plotted against concentration for the calibration curve.

Dehydroascorbic and Dehydroerythorbic Acid Determination

Dehydroascorbic acid and dehydroerythorbic acid were prepared from ascorbic acid and erythorbic acid, respectively, by a slight modification of an earlier procedure (Ohmori and Takagi, 1978) and a more recent procedure (Doner and Hick, 1984).

Five grams of ascorbic acid was stirred for 3 hr at room temperature in 225 ml ethanol containing 6 g activated carbon. Air was continuously bubbled through the solution. Erythorbic acid (5 g) was stirred for 16 hr at 40°C in 300 ml ethanol containing 5 g activated carbon, with air being bubbled through the solution continously. The activated carbon and ethanol were removed by filtration and vacuum, respectively. Dehydroascorbic and dehydroerythorbic acids were isolated as pure forms for doing HPLC analysis.

One of the duplicate samples had dithiothreitol (DTT), a reducing agent, added to it at a final concentration of 1 mg/ml. The solution was filtered through 0.45 μm Millipore filter before injected (10 ul) onto column. The dehydroascorbic acid and dehydroerythorbic acid levels were indirectly determined by noting the increase in absorbance of ascorbic and erythorbic acids after UV detection.

Sample Preparation

The same food samples as those analyzed for ascorbic acid by the spectrophotometric method were also analyzed for the same component by the HPLC method.

A. Frozen apples were macerated in a plastic food processor (Sunbeam) under reduced light. Ten grams of the macerated fruit was diluted with deionized water to 50 ml and then homogenized with the Virtis blender for 2 min.

B. Fruit and vegetable concentrated juices and frozen fruit juices were pipetted by quantitative pipet after completely mixing the contents of bottle, can or carton. Deionized water was used to dilute the concentrated juices.

C. Natural and artificial flavor drink mixes were diluted directly with deionized water to an acceptable level so that ascorbic acid could be accurately measured.

D. Hi-C drink powders were weighed by an electronically analytical balance (Mettler H80), then diluted with deionized water to make an adequate concentration for doing analysis.

E. Potato products were blended in a plastic food processor. Three kinds of potato products were each mixed 1:1 with ethanol (Augustin et al., 1981) in the Virtis blender for 2 min and then centrifuged.

F. Meat products were first ground in a plastic food processor, then an appropriate amount was weighed, mixed with deionized water, and homogenized for 2 min in Virtis blender.

Thick suspensions of A, B, E, and F were filtered through Whatman No. 41 and No. 42 papers to remove large particles . Solutions C and D were filtered once through Whatman No. 42 paper. Further purification was achieved by passing all sample solutions through Sep-Pak C-18 cartridge (Waters Associates) prior to passing through 0.45 μm Millipore filter. Ten μl of these clarified solutions were immediately injected into the HPLC system.

STATISTICAL ANALYSIS

A linear regression equation for each standard curve of the spectrophotometric method and the HPLC calibration curve was obtained by linear regression analysis (Gunst and Mansom, 1980) of the raw data. Correlation coefficients were obtained simultaneously by statistical calculation.

The quantity of total ascorbic acid in the samples as determined by spectrophotometric method and the quantities of ascorbic acid, erythorbic acid and their dehydro-forms compounds measured by the HPLC method were expressed as an average ± standard deviation.

Since the design of the experiments of the recovery and interference tests utilized paired comparison, data of those two tests were analyzed by the paired t-test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

SPECTROPHOTOMETRIC DERIVATIZED METHOD

Ascorbic Acid Standard Curve

The proportionality of the color developed in this reaction followed Beer's Law in the range from 0.1 to 10 ppm (Figure 2). A linear regression equation of $Y=0.0345X+0.0001$ ($r=0.9995$) was obtained. The absorbance of the ascorbic acid solution deviated from linearity when the concentration exceeded 10 ppm. These data, in part, matched those of earlier investigations (Roe and Kuether, 1943; Roe, 1957), even when the concentration of ascorbic acid was as low as 0.1 ppm. However, it should be emphasized that the earlier reports only presented standard curves ranging from 0 to 5 ppm. The ascorbic acid standard curve shown in Figure 2 was used to quantify the ascorbic acid contents in food samples of this research.

Erythorbic Acid Standard Curve

The color development in this reaction was similar to that of ascorbic acid although lower absorbance values were obtained when the same concentrations of erythorbic

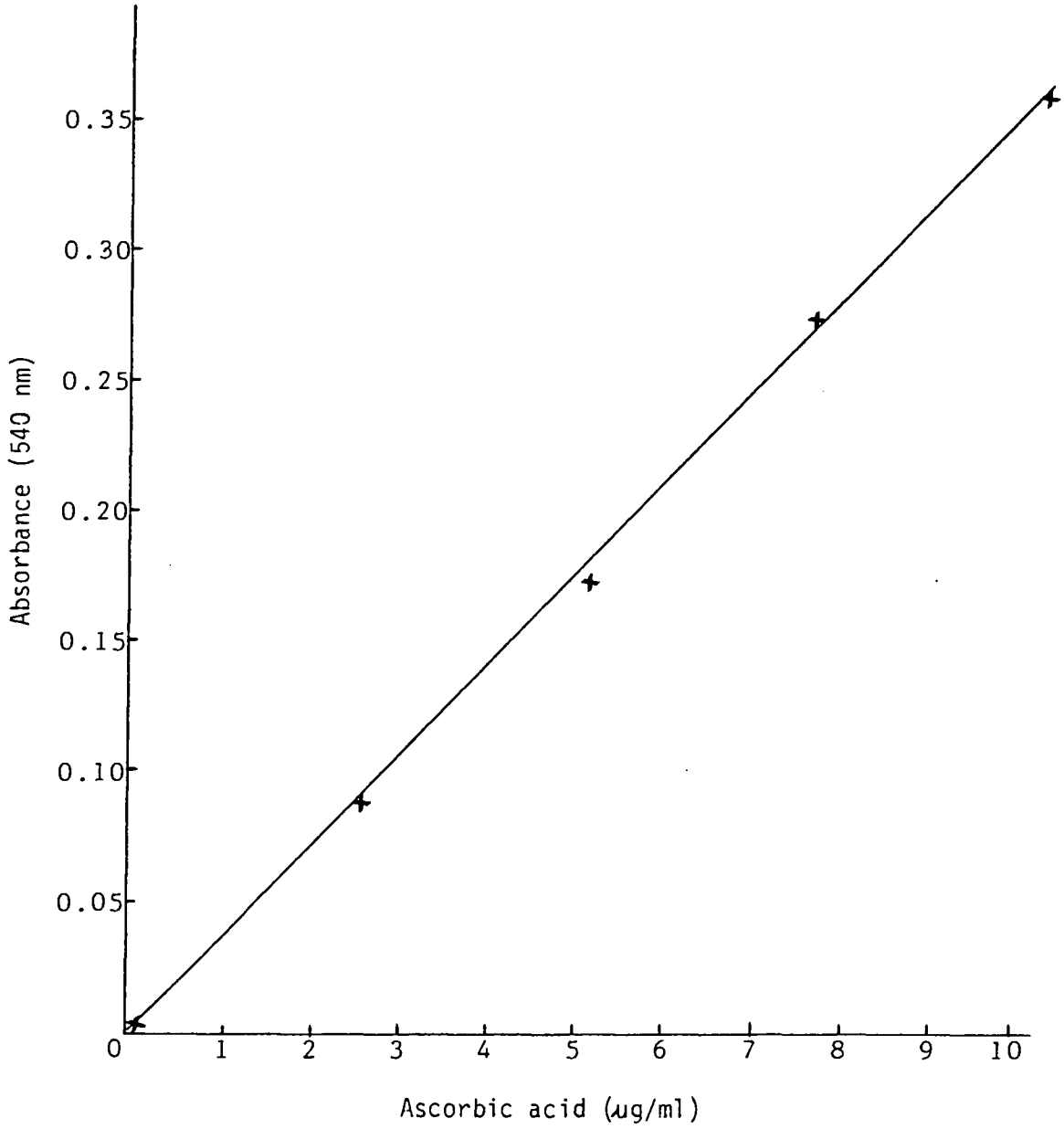


Figure 2. Ascorbic acid standard curve.

acid were measured in comparison to ascorbic acid. This curve obeyed Beer's Law and yielded a linear regression equation of Y=0.0312X+0.0011 (r=0.9999). The standard curve for erythorbic acid at concentrations ranging from 0.1 to 10 ppm is shown in Figure 3. These results showed that erythorbic acid coupled with 2,4-dinitrophenylhydrazine to produce a derivative as did ascorbic acid.

Recovery Test of Ascorbic Acid Determination

The percentage recoveries of ascorbic acid added to the tomato samples in which ascorbic acid occurred naturally are shown in Table 1. The average initial ascorbic acid content was 18.4 mg%. The ascorbic acid spiking levels ranging from 5.37 to 44.68 mg% were designed to match the linear range of the ascorbic acid standard curve. Recoveries of 90 to 105% and a range of ±0.02 to ±0.19 standard deviations of the measured values showed a high efficiency of recovery by the spectrophotometric method (Roe, 1957) for the determination of ascorbic acid. These values and range of standard deviations were comparable to those obtained in earlier studies completed in our laboratories by Macbride (1983) on tomato sauce. There was no significant (α=0.01) difference in ascorbic acid contents between the empirical and theoretical values as determined by paired comparison statistical analysis.

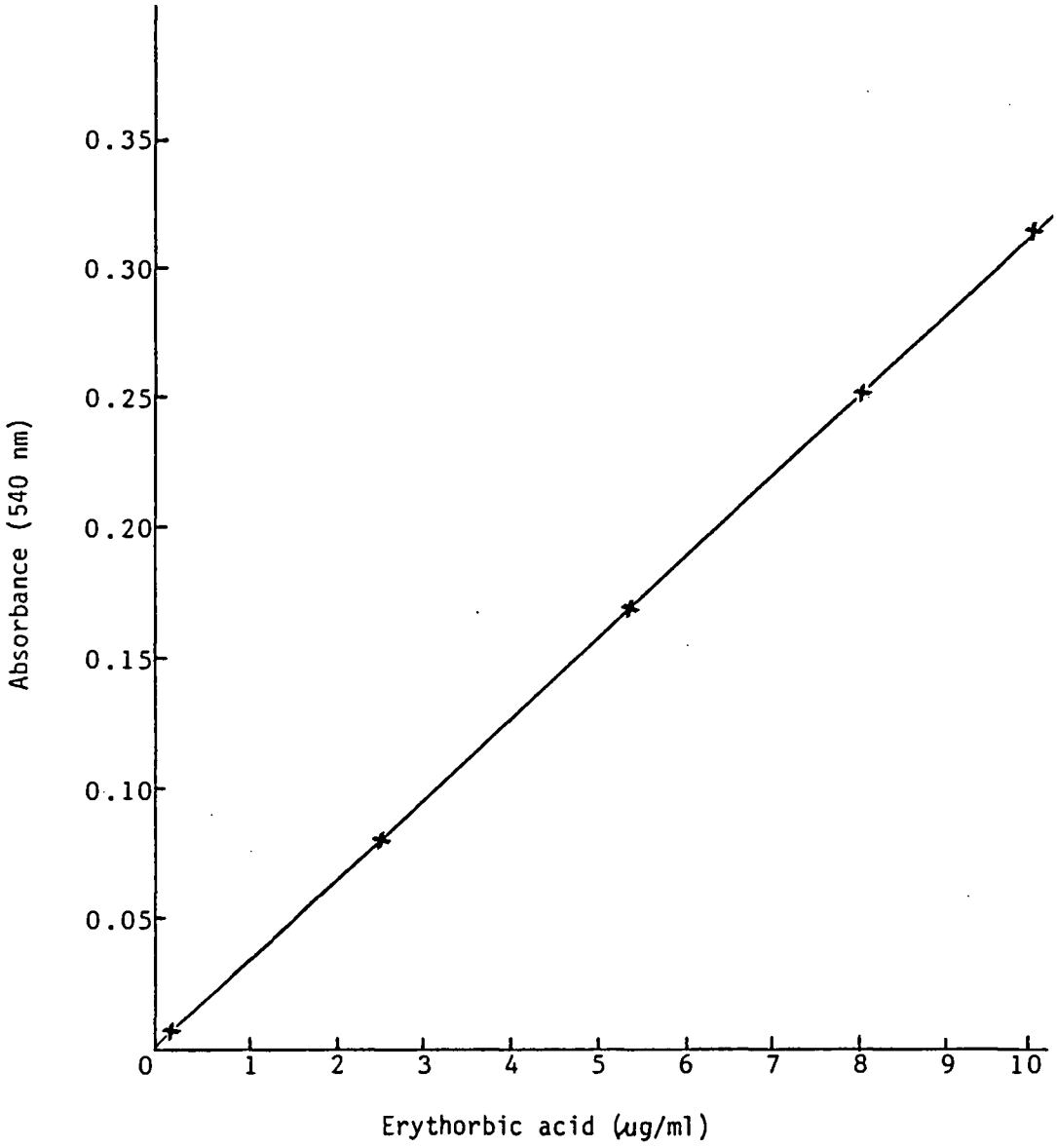


Figure 3. Erythorbic acid standard curve.

Table 1. Recovery of ascorbic acid (AA) added to tomato test solutions.

Samples	AA Spiked (mg%)	AA Found[a] (mg%±S.D.)	Recovery of AA (%)
Tomato I	0.00	18.30±0.01	-
	5.37	24.88±0.02	105.1
	10.47	28.69±0.04	98.8
	16.11	33.27±0.07	96.7
	21.48	37.09±0.10	93.2
Tomato II	0.00	19.18±0.05	-
	11.17	29.60±0.19	97.5
	22.34	38.07±0.09	91.7
	44.68	57.63±0.12	90.2
Tomato III	0.00	17.09±0.07	-
	11.17	27.86±0.15	98.6
	22.34	38.21±0.08	94.5
	44.68	57.86±0.07	93.7

[a] Each value is the mean of five determinations.

S.D.= Standard deviation.

Effect of Erythorbic Acid on the Ascorbic Acid Determination

No reports were found in the literature concerning the use of the spectrophotometric method to measure ascorbic acid in samples to which erythorbic acid had been added. Thus this aspect was examined. The addition of 6.26 to 71.67 mg% of erythorbic acid to tomato samples which contained 18.74 mg% ascorbic acid showed significant (α =0.01) increases in ascorbic acid levels (Table 2). The recoveries of ascorbic acid ranged from 129 to 283%. The percentage recoveries increased with increasing additions of erythorbic acid.

These data showed that the presence of erythorbic acid seriously influenced ascorbic acid estimation by the spectrophotometric method. It is interesting to note that the ascorbic acid levels did not increase linearly when erythorbic acid was added at levels of 58.64 to 71.67 mg% (Figure 4) even though the quantities of erythorbic acid were not beyond the linear range of Beer's Law. The influence from some components of tomato could be the reason for this result.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

It was found in preliminary experiments that ascorbic

Table 2. Effect of erythorbic acid (EA) added to tomato test solutions on ascorbic acid (AA) determinations.

Samples	EA Spiked (mg%)	AA Found[a] (mg%±S.D.)	Recovery of AA (%)
Tomato I	0.00	18.30±0.01	-
	6.26	23.64±0.06	129.2
	12.53	24.41±0.12	133.4
	18.79	28.22±0.09	154.2
	25.06	30.81±0.14	168.4
Tomato II	0.00	19.18±0.05	-
	13.03	26.86±0.09	140.0
	26.06	34.53±0.04	180.0
	39.09	42.40±0.07	220.0
	52.12	49.88±0.06	260.0
	58.64	52.68±0.04	274.7
	65.25	54.18±0.01	282.5
	71.67	54.18±0.02	282.5

[a] Each value is the mean of five determinations.

S.D.= Standard deviation.

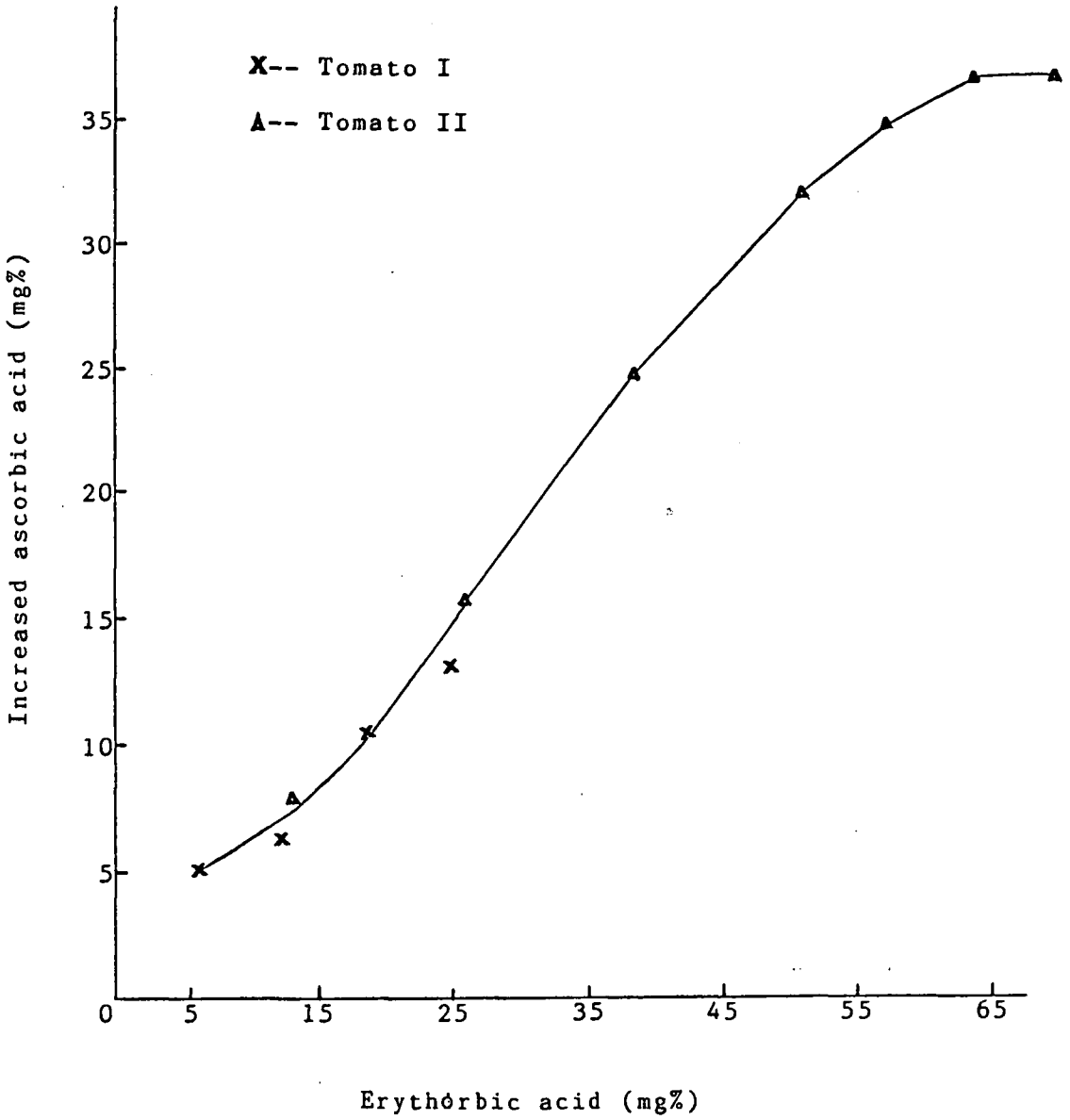


Figure 4. Effect of erythorbic acid additions on ascorbic acid levels.

and erythorbic acids could be separated effectively by HPLC using a Zorbax-NH_2 column with a mobile phase of acetonitrile:0.05 M KH_2PO_4 buffer (75:25) (v/v). These acids could also be quantified by adding tyrosine as an internal standard and monitoring the eluant by a UV detector at 268 nm. Unfortunately, the Zobax-NH_2 column deteriorated during the trial studies and had to be replaced. Because of the costs involved, a less expensive LiChrosorb-NH_2 (LC) column was purchased. This column proved to be effective in separating erythorbic acid from ascorbic acid (Figure 5). This figure also shows that the results could be quantified by adding phenylalanine as an internal standard. Tyrosine could not be used as an internal standard for the LC column because it had a retention time very similar to that of ascorbic acid. The chromatographic characteristics of the LC column are listed in Table 3.

Since the resolution (Rs) (Johnson and Stevenson, 1978) between all adjacent pairs was more than 1.0, the three compounds (phenylalanine, erythorbic and ascorbic acids) could be accurately quantified. The column capacity from 1.77 to 5.12 verified that the efficiency of the LC column is greatest for these three compounds according to the theories of previous studies (Majors, 1974). If the flow rate increased, ascorbic and erythorbic acids still could be efficiently separated at retention time of less than 5

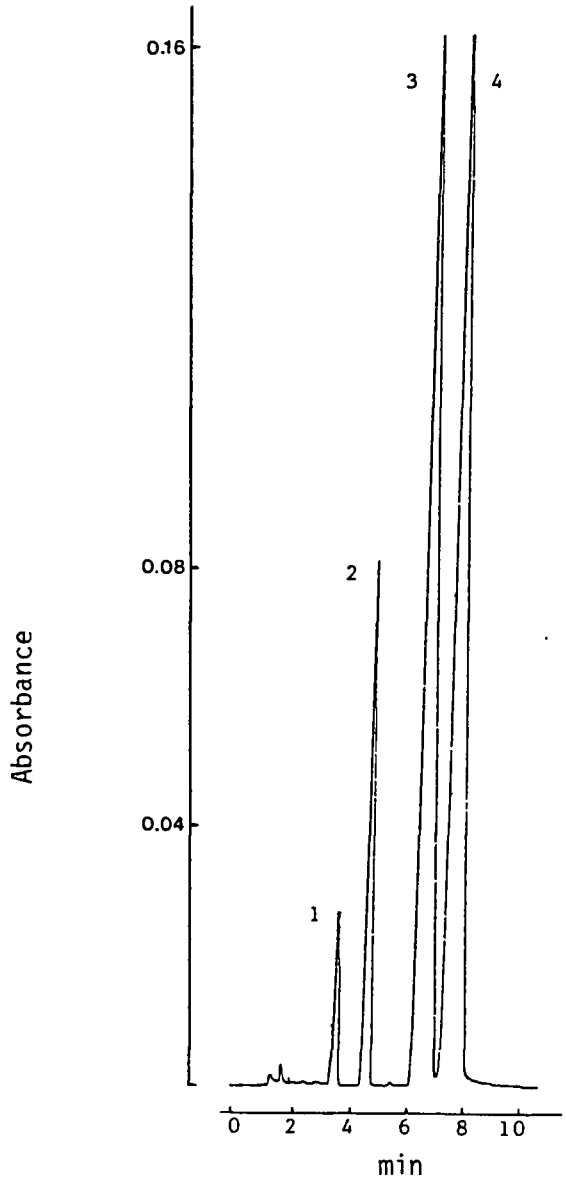


Figure 5. HPLC separation of a standard solution of acetic acid (1), phenylalanine (2), erythorbic acid (3), and ascorbic acid (4). Operating conditions: column, LiChrosorb-NH_2; mobile phase, 3:1 acetonitrile/0.05 M KH_2PO_4; flow rate, 1.5 ml/min; UV detection at 268 nm, 10X attenuation.

Table 3. Chromatographic evaluation of compounds separated on LiChrosorb-NH_2 column[a].

compound	Retention time(min)	K'[b]	S[c]	R_s[d]
Phenylalanine	4.7	1.77		
			1.63	5.00
Erythorbic acid	6.4	2.88		
			1.78	1.25
Ascorbic acid	7.7	5.12		

[a] Chromatographic conditions as in Figure 5.

[b] Column capacity.

[c] Column selectivity.

[d] Resolution between adjacent peaks.

min. Quantities injected (Figure 5) were: 40.7 ug phenylalanine, 4.82 ug erythorbic acid and 5.66 ug ascorbic acid. The signals of HPLC analysis of this research were carried out at 10X attenuation.

Ascorbic Acid Calibration Curve

A calibration graph (Figure 6) plotted as the peak area of ascorbic acid at 268 nm against the concentration of ascorbic acid ranging from 2.3 to 566 ppm demonstrates a linear relationship of Y=0.2180X-2.6565 with r=0.9994. The chromatograms (Figure 5) showed that the ascorbic acid peak had a retention time of approximately 8.1 min. Levels as low as 23 ng ascorbic acid could be determined by using 0.005 absorbance unit full scale (AUFS). This result was better than the detection limit of 100 ng ascorbic acid reported by Rose and Nahrwold (1981), who used the same type of column as was used in this study. However, Rose and Nahrwold used a mobile phase of acetonitrile:0.05 M KH_2PO_4 buffer at a ratio of 50:50 (v/v) whereas a 75:25 (v/v) ratio was employed in the current work. This change in the solvent proportion of the mobile phase may be the reason for the improved detection limit reported herein. This result is in accord with the detection limit of 25 ng reported by Doner and Hick (1981) even though they used an Zorbax-NH_2 column rather than the LiChrosorb-NH_2 column.

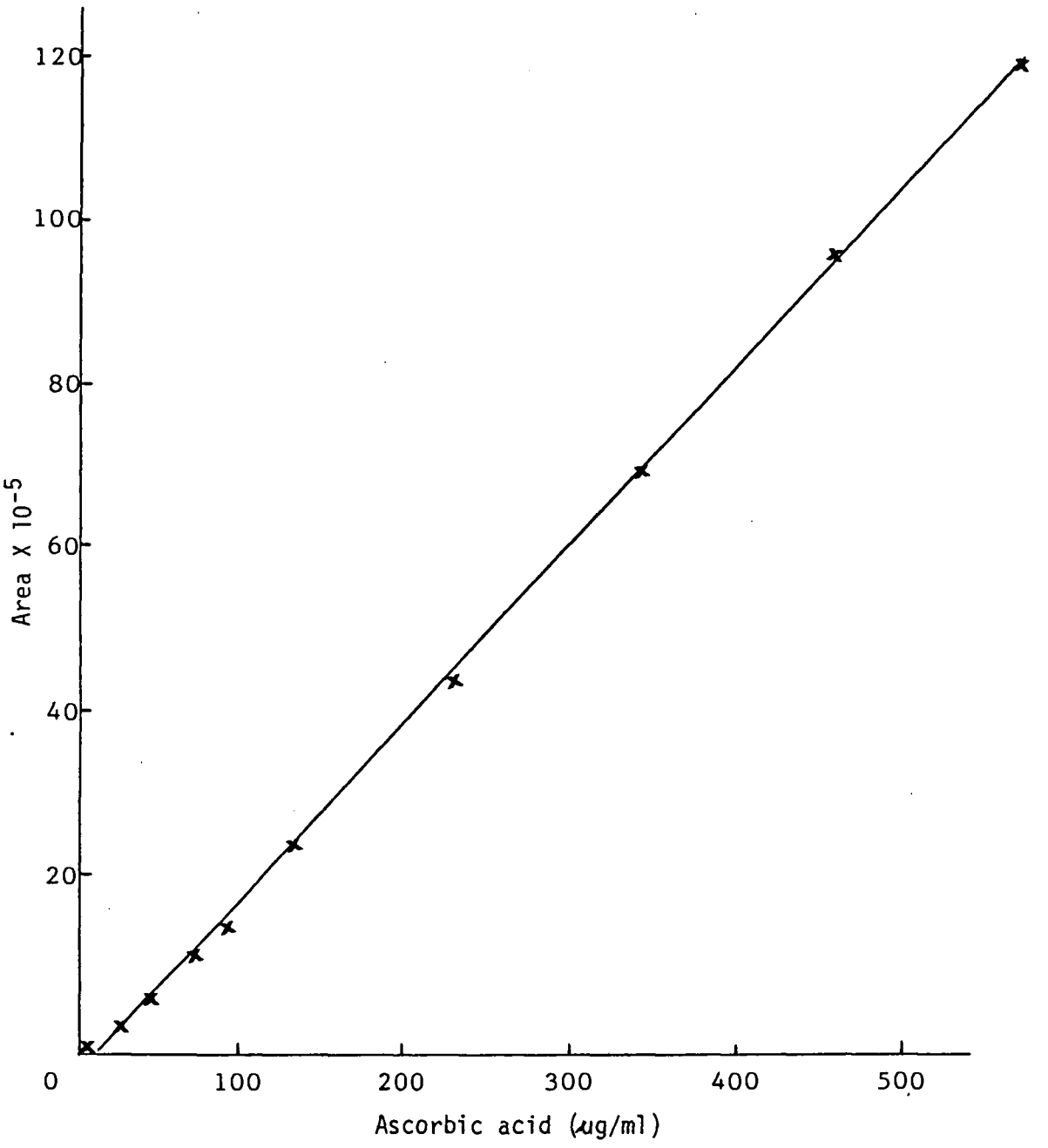


Figure 6. HPLC calibration curve of ascorbic acid. Chromatographic conditions were the same as those given in Figure 5.

Thus, the latter column is appropriate for assaying low levels of ascorbic acid in biological fluids.

Erythorbic Acid Calibration Curve

Figure 7 shows that the peak area of erythorbic acid at 268 nm was proportional to the concentration of erythorbic acid in the range of 2.4 to 966 ppm. The relationship between these parameters follows a linear regression equation of Y=0.2049X-4.3877 (r=0.9990). The limit of detection was 24 ng of erythorbic acid when 0.005 AUFS was used to increase the sensitivity of this method. The peak of erythorbic acid eluted earlier than that of ascorbic acid. The retention time of erythorbic acid was about 6.8 min (Figure 5).

Internal Standard Curve

Phenylalanine was found to be a good internal standard for the quantification of ascorbic and erythorbic acids in this HPLC method. The retention time of phenylalanine was 4.8 min. A linear relationship between the peak area and phenylalanine concentration (Figure 8) was established by the equation of Y=0.0014X-0.0031 (r=0.9992). The concentration of phenylalanine of 76 to 7656 ppm was much higher than expected to plot a standard curve. The AUFS

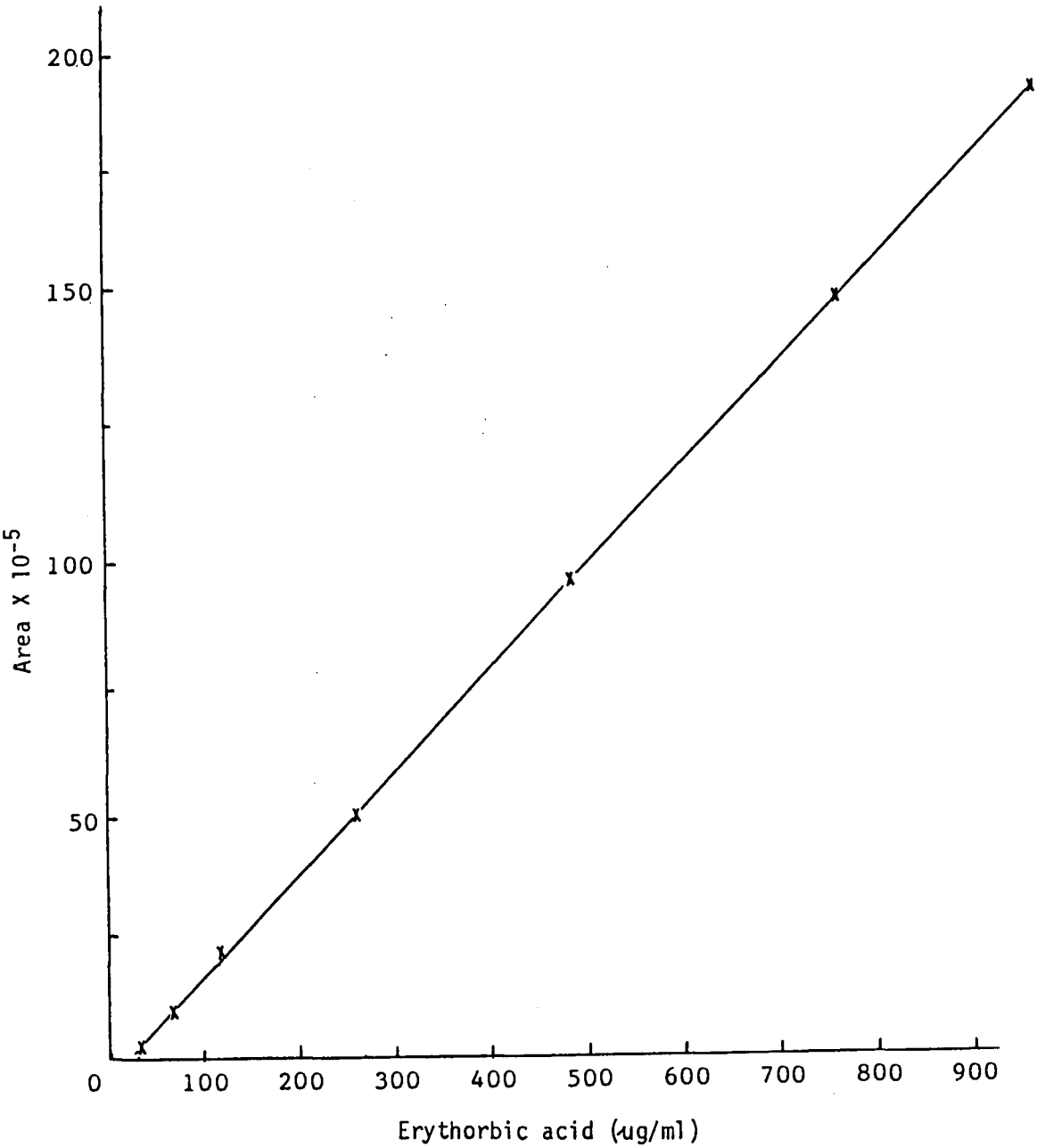


Figure 7. HPLC calibration curve of erythorbic acid. Chromatographic conditions were the same as those given in Figure 5.

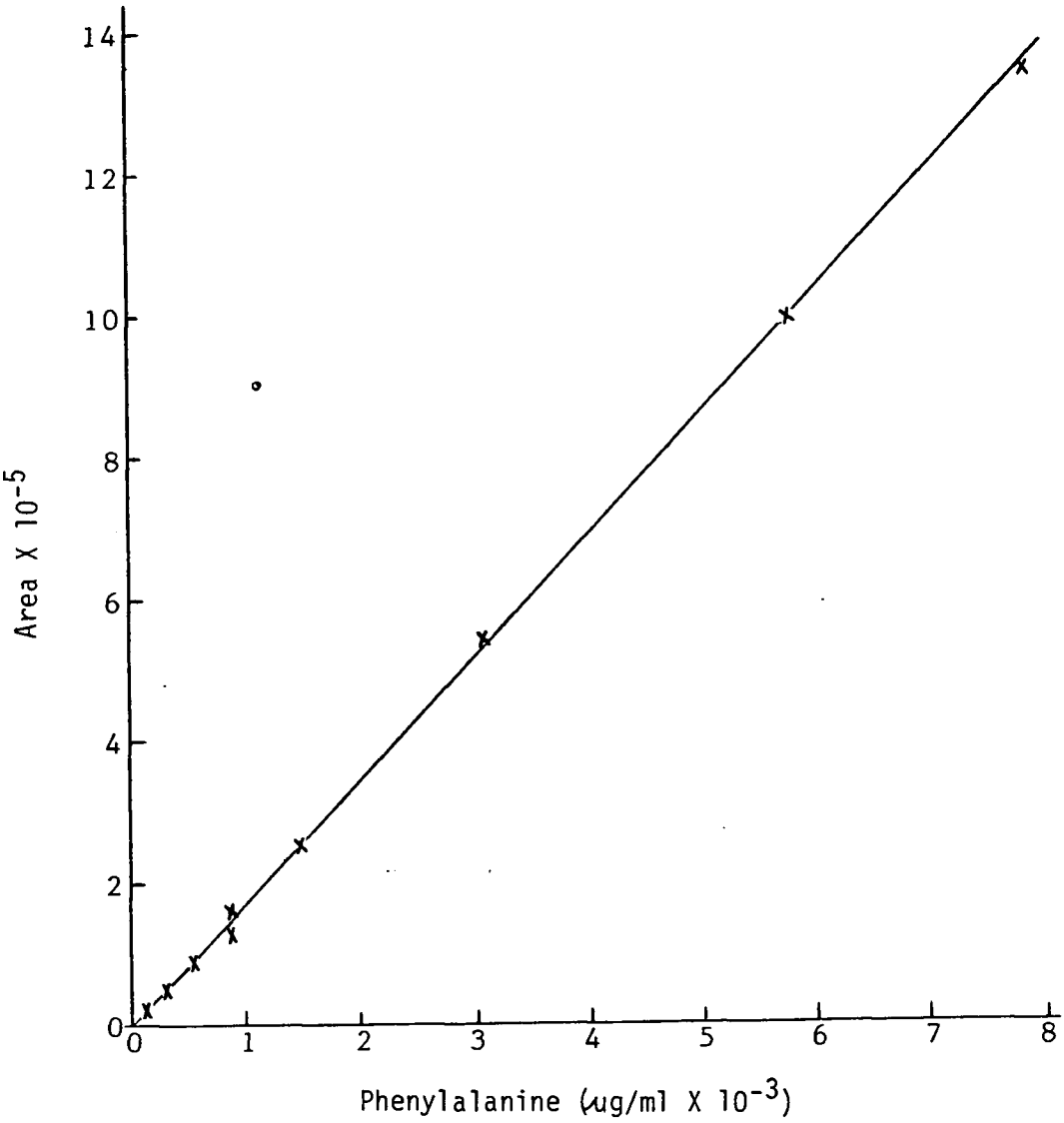


Figure 8. HPLC standard curve of phenylalanine. Chromatographic conditions were the same as those given in Figure 5.

ranging from 0.01 to 0.16 was chosen to determine the absorbance of phenylalanine with varying concentration.

Spiked Internal Standard to Ascorbic Acid Standard Curve

A series of ascorbic acid solutions containing 1000 ppm phenylalanine were analyzed by HPLC. The peak areas of ascorbic acid compared to that of 1000 ppm phenylalanine were constant which allows the development of a ratio. A linear relationship of Y=0.1617X-1.5809 (r=0.9991) between the ratio from the different concentrations of ascorbic acid and the concentrations of ascorbic acid under 700 ppm is shown in Figure 9. Concentrations of ascorbic acid over 700 ppm can not be quantified by this curve.

Spiked Internal Standard to Erythorbic Acid Standard Curve

As shown in Figure 10, the calibration curve of erythorbic acid standard solutions containing 1000 ppm phenylalanine produced excellent linearity of Y=0.1190X-1.5232 (r=0.9997).

It was ascertained in preliminary trials that no other peaks had the same retention time as phenylalanine in the sample extracts. In case of the presence of peaks similar to phenylalanine, another internal standard would have to be selected (Rizzolo et al., 1984) in order to establish

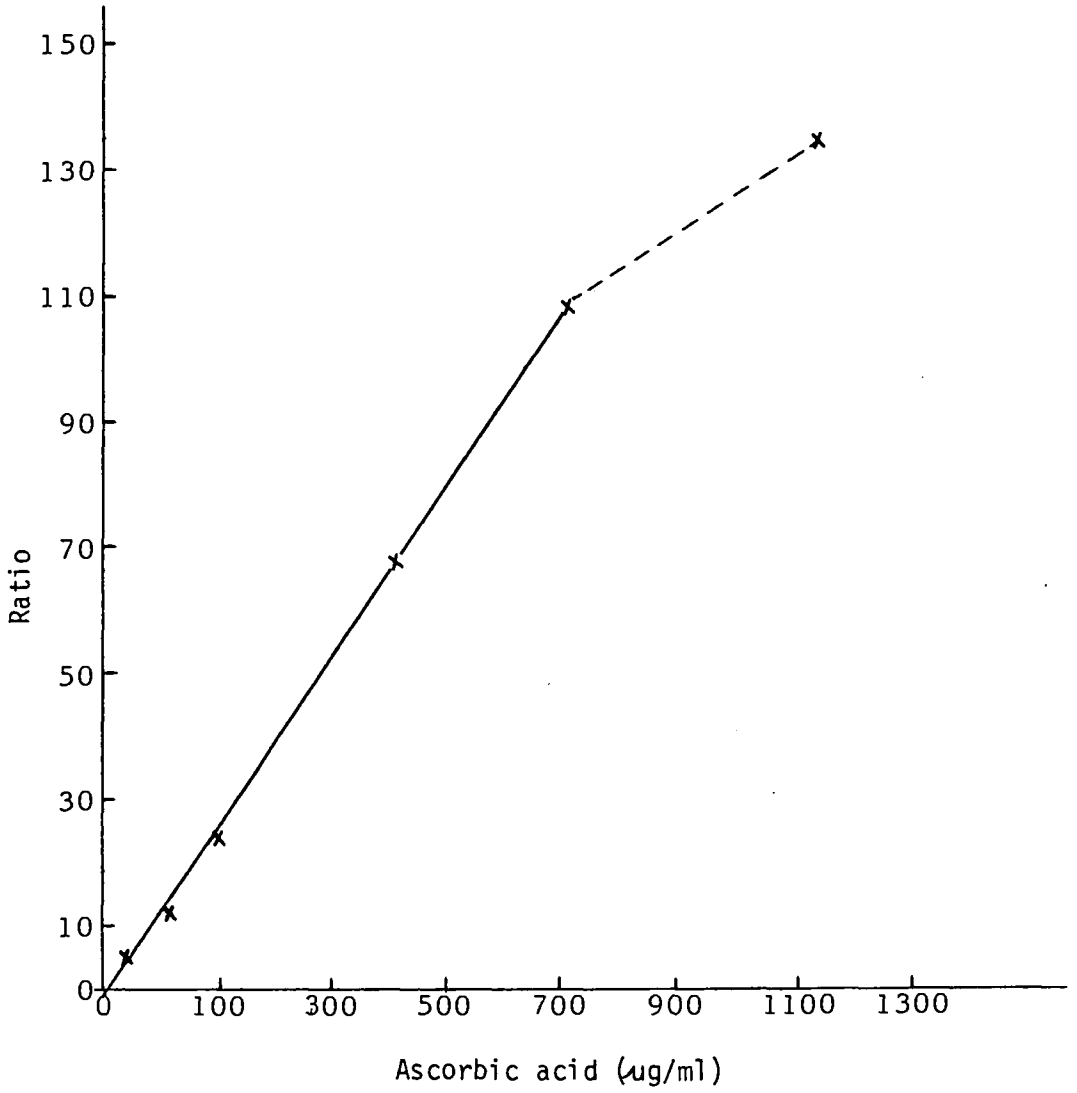


Figure 9. The ratio of chromatographic peak areas of different concentrations of ascorbic acid to that of 1000 ppm phenylalanine versus concentrations of ascorbic acid.

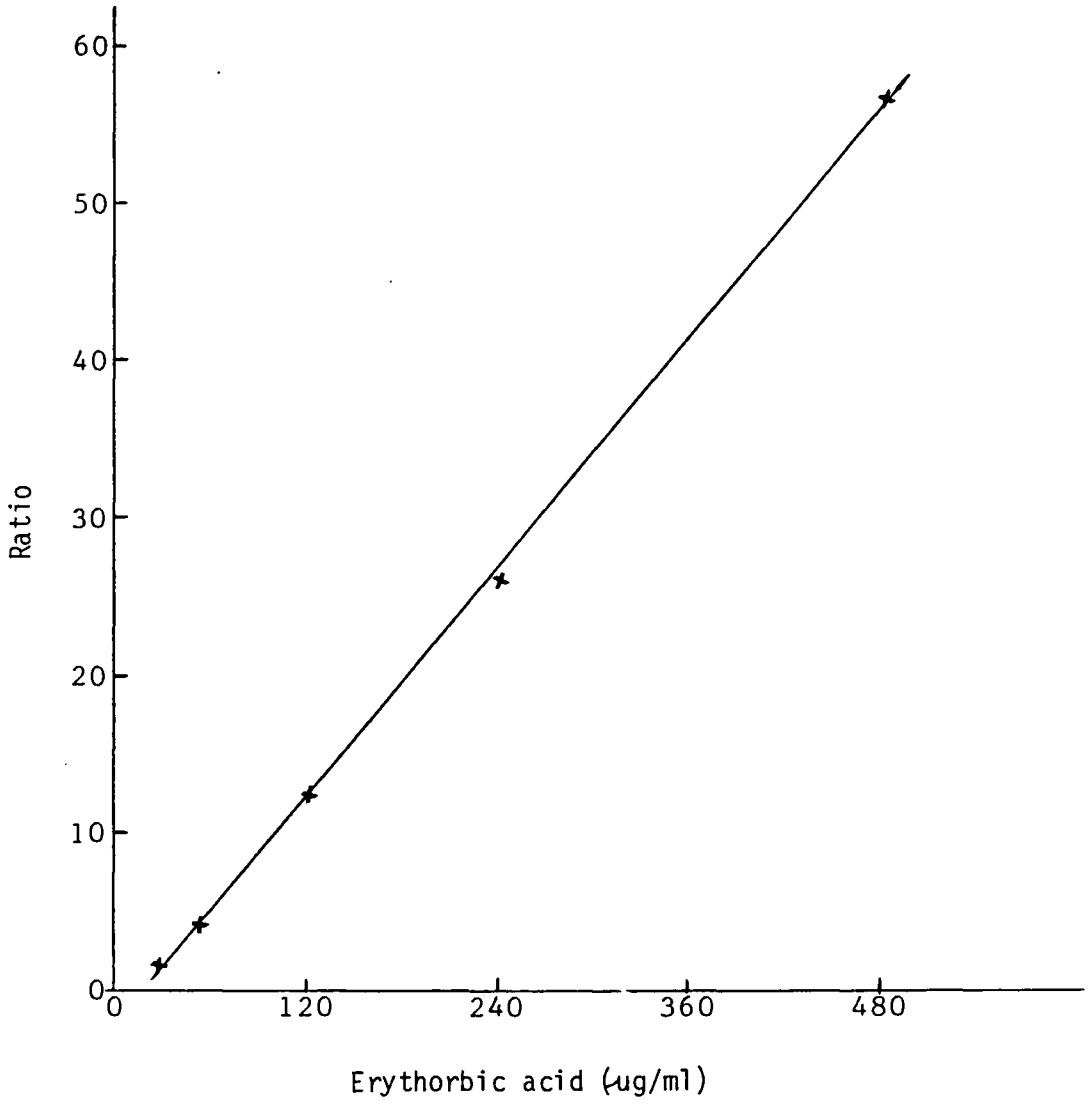


Figure 10. The ratio of chromatographic peak areas of different concentrations of erythorbic acid to that of 1000 ppm phenylalanine versus concentrations of erythorbic acid.

the appropriate calibration curves for ascorbic or erythorbic acids.

Dehydroascorbic and Dehydroerythorbic Acid Standard Curve

Dehydroascorbic and dehydroerythorbic acids could be indirectly quantified after reduction with DTT which was added to the diluent. The peak areas of dehydroascorbic acid and dehydroerythorbic acid plotted against concentration produced good linearity of Y=0.2115X-2.5765 (r=0.9994) (Figure 11) and Y=0.1987X-4.2560 (r=0.9990) (Figure 12), respectively. It was found that DTT quantitatively reduced dehydroascorbic acid to ascorbic acid after 15 min in frozen apple juice I and III (Fig. 13) as did dehydroerythorbic acid in apple juice II.

COMPARISON OF LEVELS OF ASCORBIC ACID, ERYTHORBIC ACID AND THEIR OXIDATIVE PRODUCTS IN VARIOUS FOOD SAMPLES AS DETERMINED BY SPECTROPHOTOMETERIC AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

Levels of ascorbic and erythorbic acids and their dehydro- forms in various food products are given in the following tables; frozen apples (Table 4), potato products (Table 5), fruit and vegetable concentrated juices (Table 6), frozen fruit juices (Table 7), natural and artificial

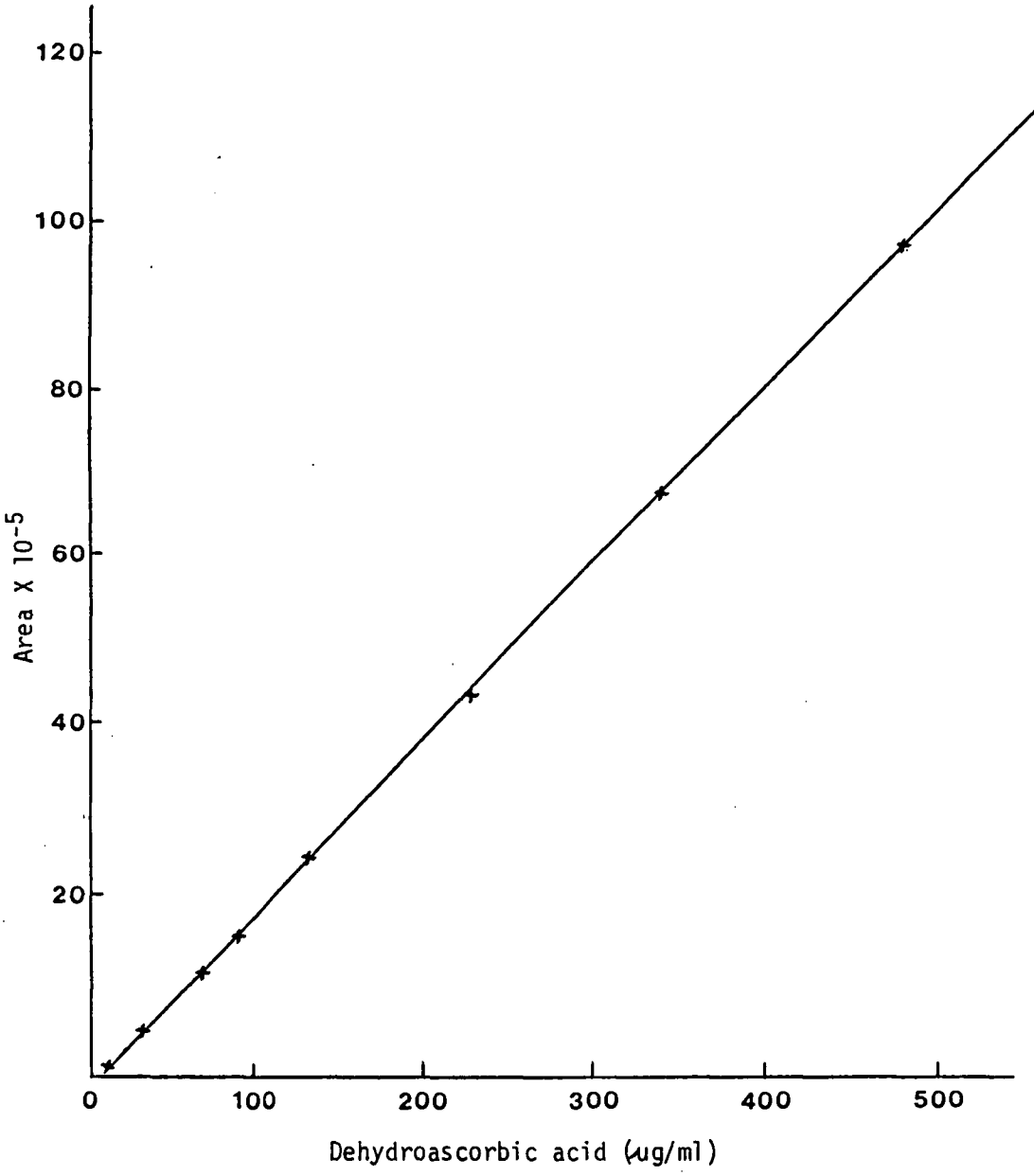


Figure 11. HPLC calibration curve of dehydroascorbic acid. Chromatographic conditions were the same as those given in Figure 5.

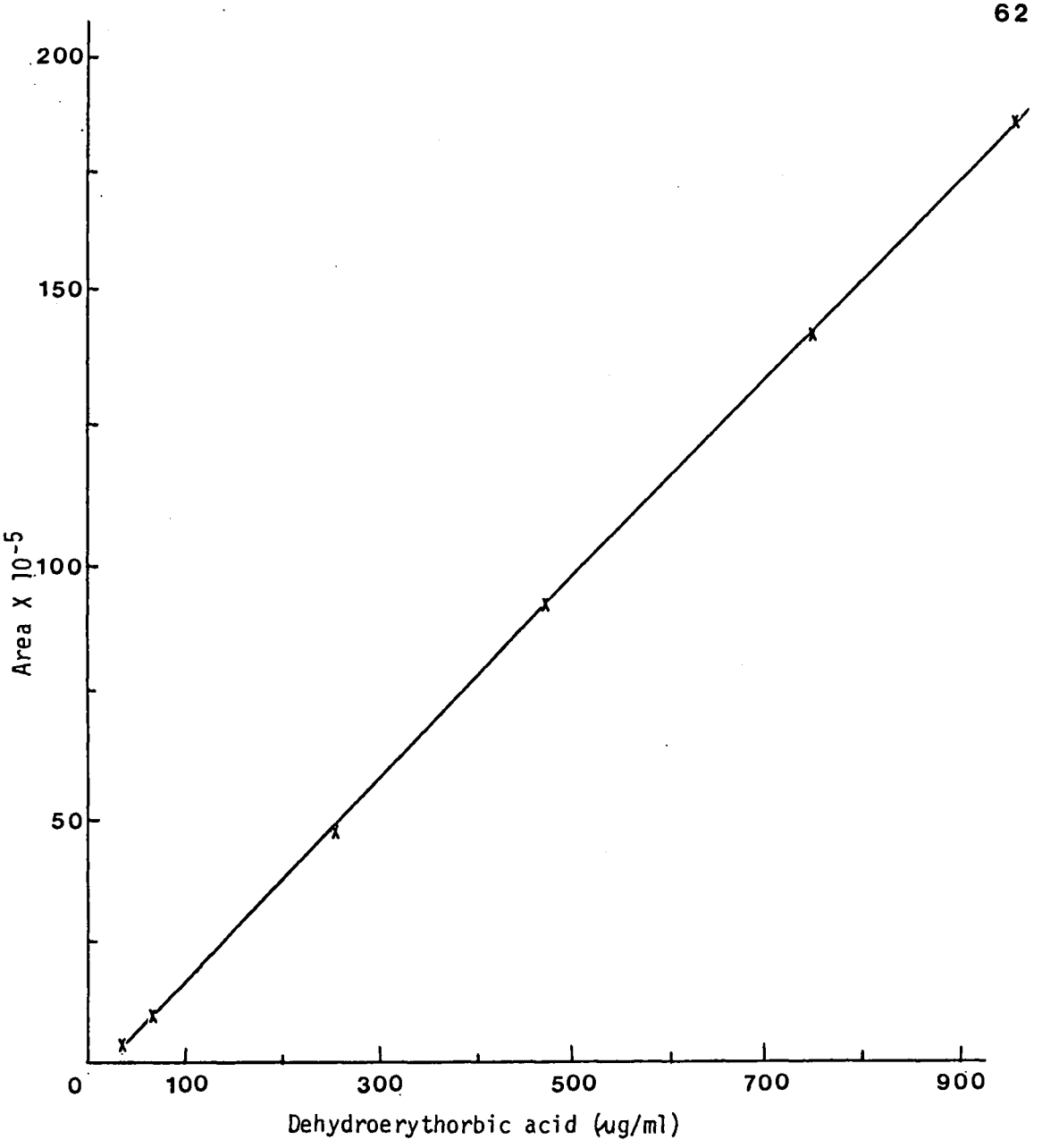


Figure 12. HPLC calibration curve of dehydroerythorbic acid. Chromatographic conditions were the same as those given in Figure 5.

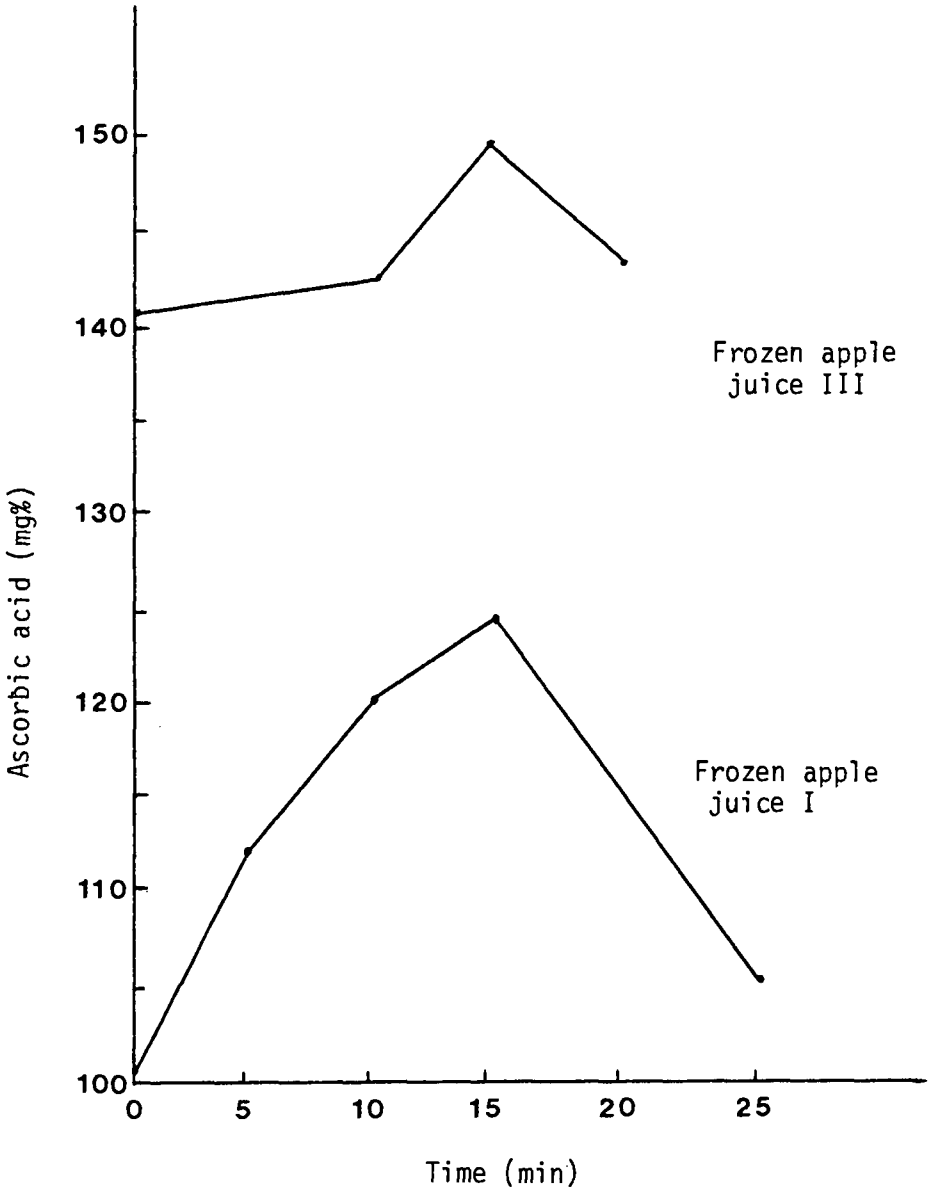


Figure 13. The changes of ascorbic acid concentration in frozen apple juice against time after treatment with dithiothreitol.

flavor drink mixes (Table 8), Hi-C drinks (Table 9) and meat products (Table 10).

The agreement of total ascorbic acid contents between HPLC and spectrophotometric methods of determination is very good as long as the samples did not contain erythorbic acid (Tables 6, 7, and 8).

In Table 4, apples of one sample were peeled and frozen as whole apples while in the other sample, apples were peeled, diced into 1 cm cubes and dipped in a 1% citric solution containing erythorbic acid before being individually quick frozen (IQF). The spectrophotometric method showed high total ascorbic acid contents of 112.6 mg% in IQF apples, however, the results of HPLC analysis (Table 4 and Figure 14) indicated 4.4 mg% ascorbic acid, 0.8 mg% dehydroascorbic acid, 90.9 mg% erythorbic acid and 20 mg% dehydroerythorbic acid in this samples. For frozen whole apples, 4.7 mg% total ascorbic acid was obtained by the derivatized method, but 1.6 mg% ascorbic acid and 2.6 mg% dehydroascorbic acid was determined by HPLC. Table 4 shows that the erythorbic acid content interferes with the spectrophotometric determination of ascorbic acid levels in processed apples, whereas the HPLC method produced more accurate contents of both reduced and oxidized forms of ascorbic acid or erythorbic acid in the samples.

Erythorbic acid was not found in the three potato

Table 4. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in apple samples determined by spectrophotometric (SP) and HPLC methods.

Samples[a]	SP[b]	HPLC[b]				
	mg%	mg%				
	Total AA	AA	DHAA	(AA+DHAA)	EA	DHEA
Frozen apples (whole)	4.7±0.1	1.6±0.0	2.6±0.0	4.2	-	-
IQF apples[c] (3/8" cubes)	112.6±4.5	4.4±0.1	0.8±0.0	5.2	90.9±3.2	20.0±1.1

[a] Commercial samples provided by Tree Top Company.

[b] Each value is the mean±S.D. of five determinations.

[c] IQF= individually quick frozen apple cubes that were dipped in a 1% citric acid solution containing erythorbic acid before freezing.

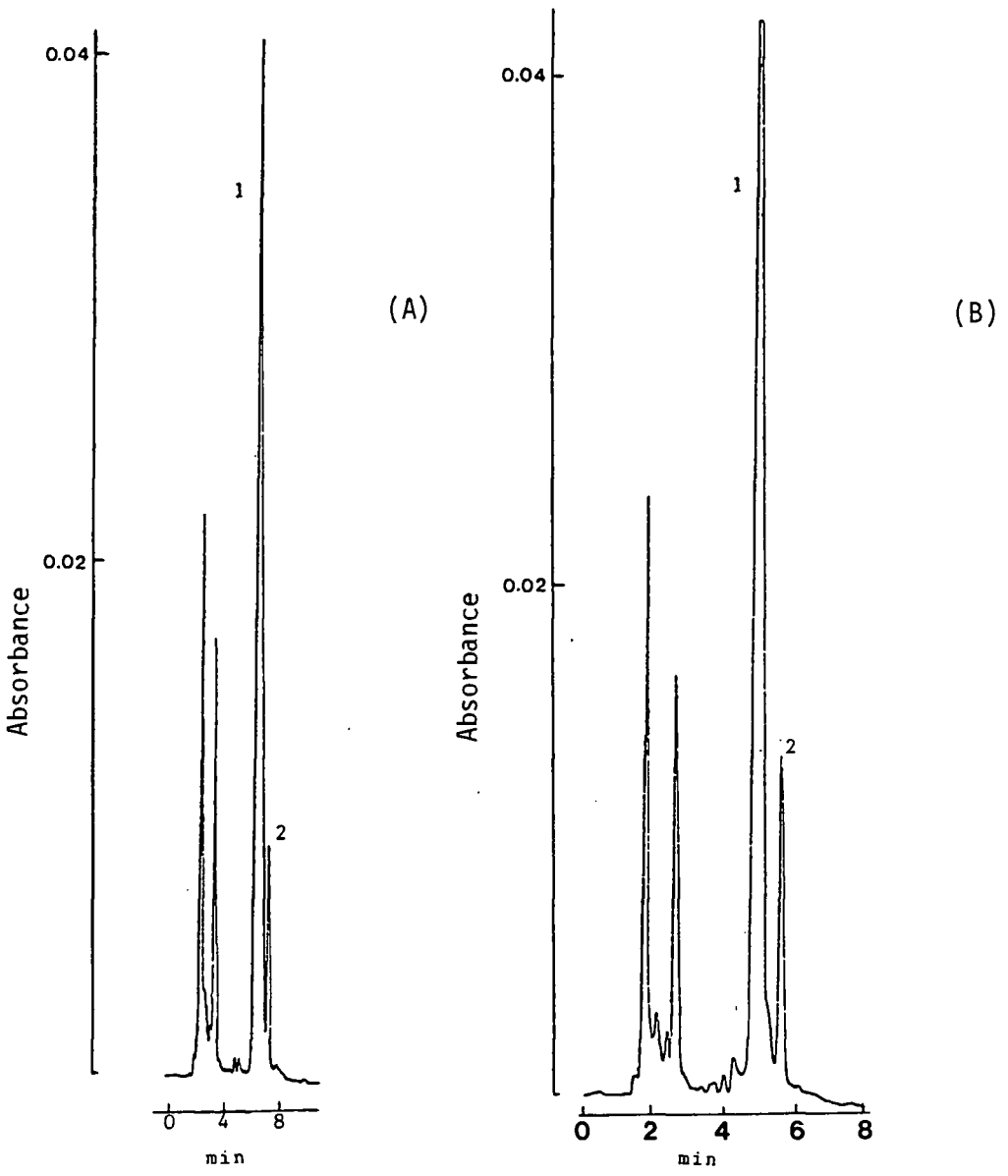


Figure 14. Chromatograms of ascorbic and erythorbic acids in IQF apples. (A) Direct injection of sample solution: erythorbic acid (1), and ascorbic acid (2). (B) After DTT treatment: total erythorbic acid (1), and total ascorbic acid (2).

products listed in Table 5. The total ascorbic acid levels of french fried potatoes, potato puffs and shoestring potatoes as determined by the spectrophotometric method were a little higher than the sum of ascorbic acid and dehydroascorbic acid contents obtained by the HPLC method. Since diketogulonic acid was not detected by HPLC, it would be included in the total ascorbic acid contents measured by the spectrophotometric method. This might be the reason why the spectrophotometric method for the analysis of ascorbic acid reflected higher vitamin C activity.

Table 6 shows the analytical data for the 12 different samples of the fruit and vegetable concentrated juice group. Except for apple juice samples I and II, all samples had container labels that listed the ascorbic acid contents on the basis of one serving size (250 ml). The total ascorbic acid contents measured by the spectrophotometric method compared favorably with the amount of ascorbic acid plus dehydroascorbic acid determined by HPLC in most of the samples. The HPLC data for dehydroascorbic acid contents ranged from 0.1 mg% in pineapple juice I to 10.1 mg% in apple sauce. Thus, dehydroascorbic acid accounted for 8.4% of the total vitamin C activity in apple juice III, 19.1% in mixed vegetable juice, and more than 16.5% in apple sauce. Since dehydroascorbic acid can be a significant contributor to total vitamin C activity (Wills, et al.,

Table 5. Levels of ascorbic acid (AA), dehydorascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in potato products determined by spectrophotometric (SP) and HPLC methods.

Samples	SP[a] mg%	HPLC[a] mg%			
	Total AA	AA	DHAA	(AA+DHAA)	EA
French fried potatoes (golden fries)	7.9±0.2	6.4±0.3	0.3±0.0	6.7	-
Potato puffs (frozen fresh)	7.0±0.1	4.9±0.2	0.9±0.0	5.8	-
Shoestring potatoes (french fried)	10.0±0.2	8.0±0.3	-	8.0	-

[a] Each value is the mean±S.D. of triplicate determinations.

Table 6. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in fruit and vegetable concentrated juices determined by spectrophotometric (SP) and HPLC methods.

Samples[a]	Label mg% AA	SP[b] mg% Total AA	HPLC[b] mg% AA	DHAA	(AA+DHAA)	EA	DHEA
Apple juice I	-	3.9±1.9[c]	1.6±0.3	-	1.6	-	-
Apple juice II	-	2.5±1.9[c]	2.2±0.1	-	2.2	-	-
Apple juice III	34	39.8±1.7	35.8±1.6	3.3±0.2	39.1	-	-
Apple sauce	2	67.3±0.0	51.0±2.3	10.1±0.5	61.1	-	-
Orange juice	40	40.0±0.9	37.7±1.5	-	37.7	1.7±0.0	-
Pink grapefruit juice	34	38.5±0.7	33.3±2.0	3.2±0.1	36.5	3.6±0.1	-
Grapefruit juice	34	37.2±0.7	35.3±2.1	-	35.3	-	-
Pineapple juice I	34	44.4±0.0	43.2±2.4	0.1±0.0	43.3	-	-
Pineapple juice II	34	42.0±1.1	41.7±2.2	1.3±0.0	43.0	-	-
Tomato juice I	12	13.1±0.7	7.6±0.5	0.3±0.0	7.9	-	-
Tomato juice II	9	4.6±1.1	1.0±0.1	0.3±0.0	1.3	-	-
Mixed vegetable juice	15	22.7±0.0	13.1±0.6	3.1±0.0	16.2	2.0±0.0	1.3±0.0

[a] Apple juice III and apple sauce were packaged in glass and the other samples were packaged in metal cans.

[b] Each value is the mean±S.D. of three determinations.

[c] Each balue is the mean±S.D. of five determinations.

1984), the HPLC method of analysis of vitamin C activity should consider the amount of ascorbic acid plus dehydroascorbic acid. Some erythorbic acid was found in orange juice, pink grapefruit juice and mixed vegetable juice. For these samples, slightly higher ascorbic acid levels were determined by the spectrophotometric method then by HPLC. Although no erythorbic acid was found in apple sauce, results of the spectrophotometric method (67.31 mg%) showed ascorbic acid contents 6 mg% higher than that of the HPLC method. The label, however, listed the ascorbic acid contents as only 2 mg%. The agreement was relatively good between total vitamin C activity determined by HPLC and that listed on the container labels for most samples except apple sauce and tomato juices I and II. The ascorbic acid levels in tomato juices were 4 mg% and 8 mg% lower than that of the label of I and II, respectively.

Two frozen grape and three frozen apple juices were analyzed by HPLC and the spectrophotometric method. These results are shown in Table 7. Although frozen apple juice II did not list any ascorbic acid data on the container label, it was found to contain ascorbic acid at a level of 19.7 mg% by the spectrophotometric method while the HPLC data indicated 6.2 mg% of ascorbic acid and 13.4 mg% of erythorbic acid. The presence of the latter apparently caused an overestimation of total ascorbic acid contents

Table 7. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in frozen juices determined by spectrophotometric (SP) and HPLC methods.

Samples	Label mg% AA	SP[b] mg% Total AA	HPLC[b] mg% AA	DHAA	(AA+DHAA)	EA	DHEA
Grape juice I[a]	61	80.9±0.0	77.2±3.1	0.5±0.0	77.7	-	-
Grape juice II[a]	68	37.9±0.8	33.9±1.5	3.9±0.0	37.8	-	-
Apple juice I[a]	135	107.8±3.4	101.7±4.1	23.5±0.2	125.2	-	-
Apple juice II	-	19.7±0.0	6.1±0.1	-	6.2	13.4±0.7	-
Apple juice III[a]	135	146.6±1.7	140.8±3.2	9.3±0.1	150.1	-	-

[a] Vitamin C enriched juice.

[b] Each value is the mean±S.D. of three determinations.

(19.7 mg%) by the spectrophotometric method. The HPLC value of 6.2 mg% seems to be a more accurate assessment of the ascorbic acid level in frozen apple juice II.

Some discrepancies were noted in comparing container label information for ascorbic acid contents of frozen grape juice II and frozen apple juice I with the actual values determined by both the spectrophotometric and HPLC methods of analysis (Table 7). The label for grape juice II listed an ascorbic acid content of 68 mg% versus 37.9 mg% as determined by the spectrophotometric procedure and 33.9 mg% detected by HPLC. However, the actual vitamin C activity of the latter should be considered as 37.8 mg% when the 3.9 mg% of dehydroascorbic acid is added to the 33.9 mg% of ascorbic acid. For apple juice I, the label listed an ascorbic acid content of 135 mg% compared to 107.8 mg% determined by the spectrophotometric method and 101.7 mg% ascorbic acid plus 23.5 mg% of dehydroascorbic acid estimated by HPLC. The sum of the latter two HPLC values for vitamin C activity was 125.2 mg% which is 16% higher than the total ascorbic acid content of 107.8 mg% determined by the spectrophotometric method. However, values obtained by both methods were much less than the 135 mg% ascorbic acid listed on the label. Reasons for explaining the discrepancies between ascorbic acid label contents of grape juice II and apple juice I and the levels determined in this study are not apparent. Perhaps, the

contents were in accord with label information at the time of processing but ascorbic acid may have degraded irreversibly by a way other than to form dehydroascorbic and diketogulonic acids. Hughes (1985) recently postulated a degradation pathway for ascorbic acid leading to oxidation products without forming dehydroascorbic acid. For the other juices (grape juice I and apple juices II and III), the ascorbic acid contents determined by both the spectrophotometric and HPLC methods yielded values higher than those listed on the container labels. Thus, these juices were in compliance with the labelling data.

Erythorbic acid was not found in the five natural and artificial flavor drink mixes (Table 8). All samples contained much higher vitamin C activity than that shown on the labels by both the spectrophotometric and HPLC methods of determination. The reason for the appearance of the high ascorbic acid levels in theses samples may be that some manufacturers add larger amounts during processing as an insurance factor. The dehydroascorbic acid contents ranged from a 2.3 mg% for the orange flavor mix to a high of 45.2 mg% for the citrus flavor mix. The high value for the latter indicates that processing conditions were less than optimal because a relatively high amount of ascorbic acid was oxidized to dehydroascorbic acid. Results of the HPLC determination of ascorbic acid activity for this group of samples agree well with those obtained by the spectropho-

Table 8. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in natural and artificial flavor drink mixes determined by spectrophotometric (SP) and HPLC methods.

Samples	Label mg% AA	SP[a] mg% Total AA	HPLC[a] mg% AA	DHAA	(AA+DHAA)	EA
Apple flavor mix[b]	376	527±5	521±10	7±0	527	-
Orange flavor mix[c]	57	88±5	88±4	2±0	90	-
Lemon flavor mix	343	585±5	584±10	4±0	588	-
Lemonade flavor mix	282	517±3	518±10	3±0	521	-
Citrus flavor mix	427	481±9	424±8	45±2	469	-
Orange flavor mix	310	398±2	384±5	15±1	399	-

[a] Each value is the mean±S.D. of three determinations.

[b] Vitamin C enriched mix.

[c] Aritificial flavor mixed with less than 5% orange juice.

tometric method. The HPLC profile of natural apple juice (Figure 15A) contains several more peaks (unidentified compounds) than that of the artificial flavor drink mix (Figure 15C).

Analytical data concerning the Hi-C drinks are presented in Table 9. The Hi-C drinks were prepared by adding water to the package contents according to label instructions. These particular products contained 10% fruit juice prior to dehydration and were vitamin C enriched. The total vitamin C activity of this category was higher than that listed on the label. The total vitamin C levels of these four Hi-C samples as determined by the HPLC method were less than those of the spectrophotometric method. However, Mayer (1966) indicated that the formation of diketogulonic acid, reductone, and the presence of sugar could increase the absorbance values resulting from the derivatized reaction of the spectrophotometric method.

Meat products were the last group of food samples to be analyzed for ascorbic and erythorbic acid contents. Results of the spectrophotometric determination of ascorbic acid in six meat products are listed in Table 10. Values ranged from 17.8 mg% of ascorbic acid for the pork and beef wiener to 26.4 mg% for turkey bologna I. Conversely, no ascorbic acid was detected in these samples by the HPLC method (Table 10) although erythorbic acid was found in all

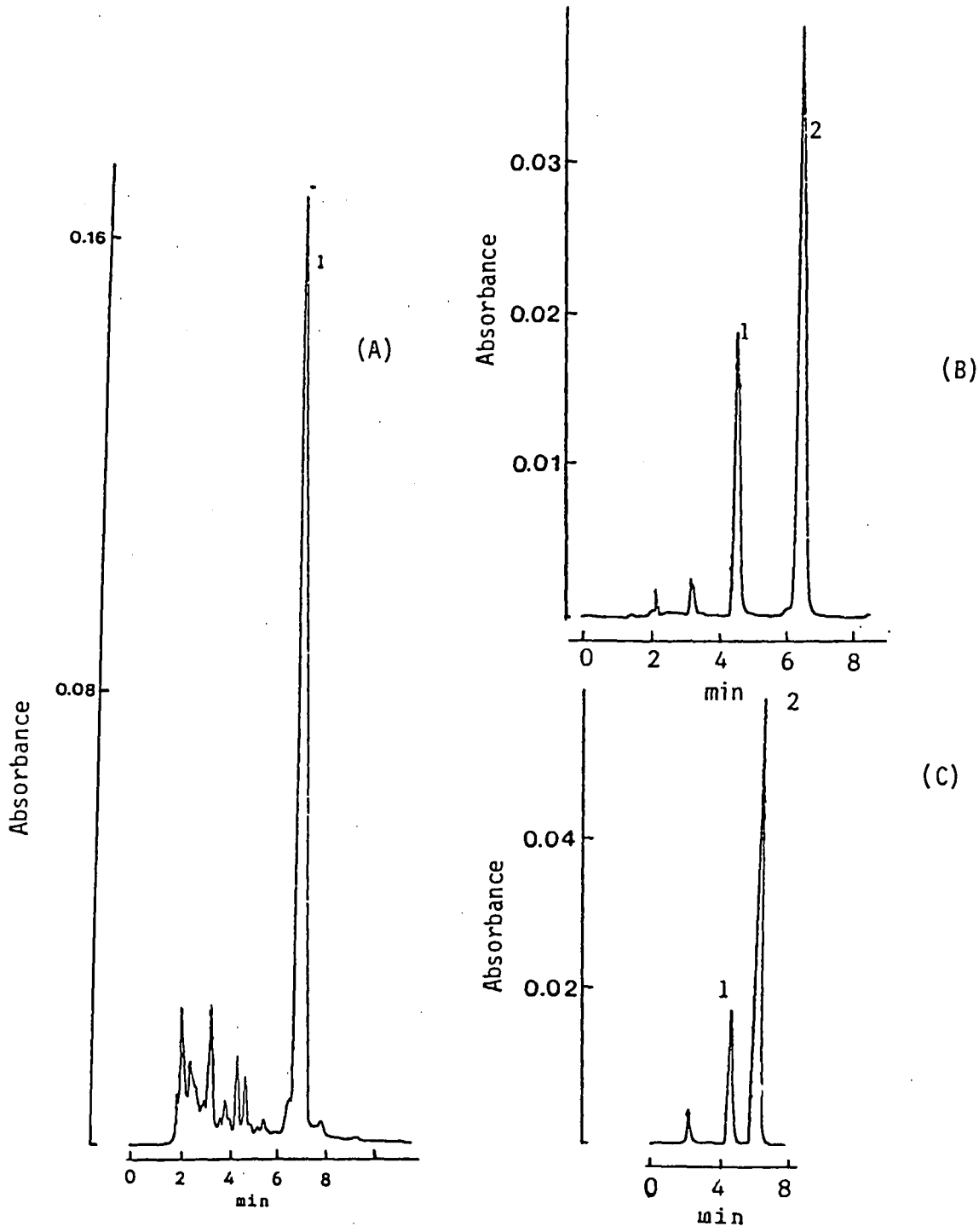


Figure 15. Chromatograms of ascorbic acid in apple products. (A) Apple concentrated juice III: ascorbic acid (1). (B) Frozen apple juice I: phenylalanine (1), and ascorbic acid (2). (C) Apple flavor mix: phenylalanine (1), and ascorbic acid (2).

Table 9. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydroerythorbic acid (DHEA) in Hi-C drinks determined by spectrophotometric (SP) and HPLC methods.

Samples[a]	Lable mg% AA	SP[b] mg% Total AA	HPLC[b] mg% AA	DHAA	(AA+DHAA)	EA
Orange drink	34	50.7±6.8[c]	36.4±3.0	5.0±0.3	41.4	-
Fruit punch	34	68.8±0.0	56.2±2.2	7.3±0.4	63.5	-
Candy apple cooler	34	63.7±1.0	45.8±1.5	11.9±0.4	57.7	-
Cherry drink	34	66.1±2.7	60.9±2.5	2.4±0.1	63.3	-

[a] All samples were enriched with vitamin C and contained 10% fruit juice as follows: orange juice in the orange drink; mixed fruit juice in the fruit punch; apple juice in the apple cooler; and cherry juice in the cherry drink.

[b] Each value is the mean±S.D. of three determinations.

[c] Each value is the mean±S.D. of five determinations.

Table 10. Levels of ascorbic acid (AA), dehydroascorbic acid (DHAA), erythorbic acid (EA), and dehydro-erythorbic acid (DHEA) in meat products determined by spectrophotometric (SP) and HPLC methods.

Samples	SP[a] mg%	HPLC[a] mg%		
	Total AA	AA	EA	DHEA
Ham	22.7±1.2	-	24.0±1.4	5.4±0.0
Beef	20.0±1.0	-	41.6±1.5	-
Turkey bologna I	26.4±0.3	-	69.3±2.7	-
Turkey bologna II	24.7±0.3	-	46.4±1.5	11.3±0.5
Wiener (pork + beef)	17.8±0.5	-	17.7±0.9	1.4±0.0
Beef wiener	17.9±0.3	-	27.0±1.5	0.6±0.0

[a] Each value is the mean±S.D. of three determinations.

samples and DHEA was detected in four of the six samples. Skeletal meats contain little or no ascorbic acid whereas organ meats such as heart and liver contain 5 and 20 mg per 100 g of wet tissue, respectively. Small amounts (5-10%) of heart muscle may be incorporated occasionally in emulsion-type products (bologna, wieners, etc.). Ascorbates and/or erythorbates are added routinely during the preparation of processed meats to enhance the development of the characteristic cured-meat color. Erythorbates, rather than ascorbates, are used almost exclusively by the meat processing industry because they are more stable, more efficient in stabilizing color, and are more economical.

The ascorbic acid data given in Table 10, as determined by the spectrophotometric method, are erroneous and misleading because erythorbic acid which has no vitamin C activity is largely responsible for positive ascorbic acid results obtained with this procedure. The HPLC profile in Figure 16A shows a definite peak of erythorbic acid with a retention time of 4.5 min whereas ascorbic acid has a retention time of about 6.5 min (Figure 15) under the HPLC conditions used.

The HPLC profile of turkey bologna II (Figure 16B) shows a component with a retention time of 5 min which was very close to the 4.5 min for erythorbic acid (Figure 16A). The former peak may have had some influence on the magnitude of the erythorbic acid peak to cause the latter

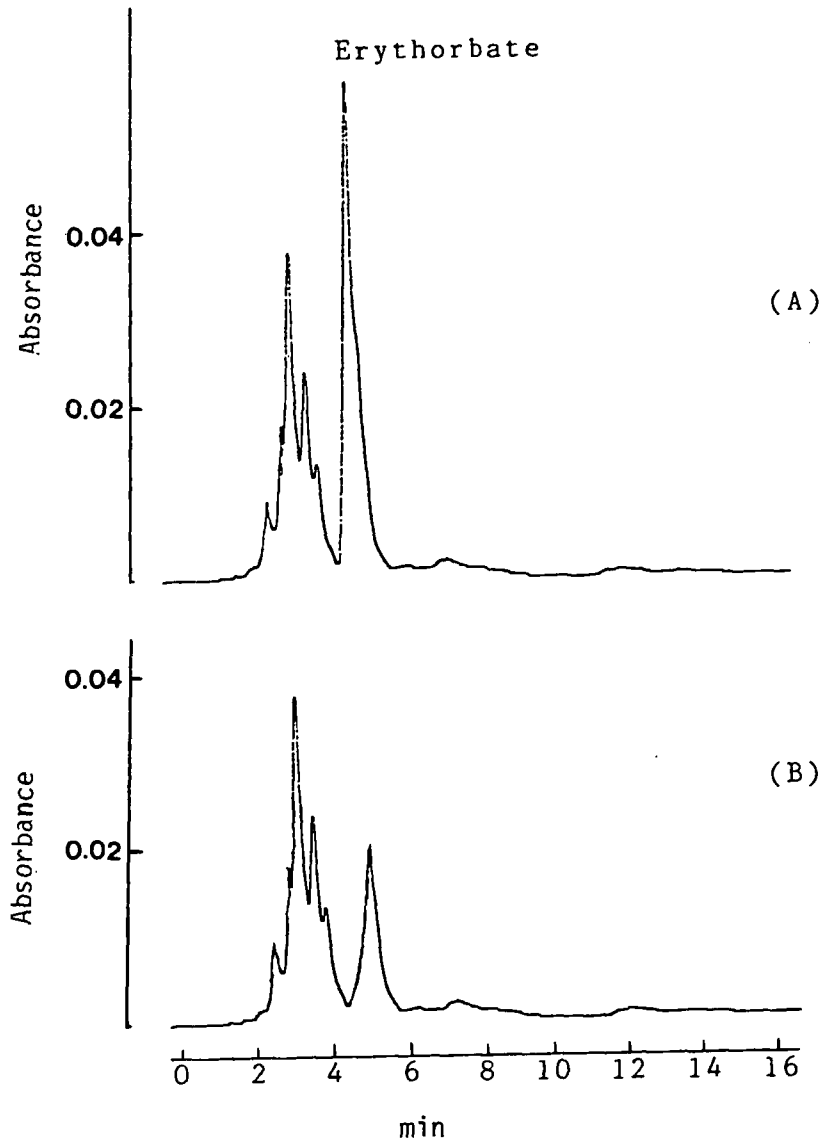


Figure 16. HPLC chromatograms of turkey bologna II extracts containing erythorbate (A) or with no erythorbate (B).

to show higher levels than were actually present. Attempts to improve separation of these two peaks by manipulating chromatogrphic conditions were unsuccessful. Increasing the flow rate of the mobile phase from 1.5 to 1.8 ml per min (the maximal flow rate for the LiChrosorb-NH_2 column is 2 ml/min) did not improve the resolution. Less separation was observed by decreasing the flow rate of the mobile phase. When the ionic strength of the mobile phase was increased from 0.05 M to 0.1 M KH_2PO_4, precipitation occurred. Decreasing the proportion of acetonitrile to 0.05M KH_2PO_4 buffer reduced resolution of these two peaks. Acetonitrile: 0.05 M KH_2PO_4 buffer ratio of 75:25 (v/v) was optimal for separation of these two peaks. Additional studies need to be conducted with other mobile phases and analytical chromatographic columns in order to achieve more efficient separation of erythorbic acid in meat products. Several peaks appeared around the retention time of the phenylalanine peak. Phenylalanine could not be used as an internal standard for quantifying erythorbic acid levels in meat products. Thus the calibration curve (Figure 7) was used to quantify erythorbic acid contents of the meat samples.

The HPLC profile (Figure 16) proved that these meat products contained erythorbic acid but not ascorbic acid. From this view point, the effect of erythorbic acid on the accurate determination of ascorbic acid levels by the

spectrophotometric method was verified. The presence of erythorbic acid caused higher absorbance readings of the spectrophotometric method.

CONCLUSIONS

On the basis of the studies reported herein, it was concluded that:

1. The spectrophotometric method of Roe (1957) has an important place in the determination of total ascorbic acid levels in food samples. Less interference by metal ion contamination and formation of more stable derivatives are some of the advantages of this method over the titration (DCIP) method for quantifying ascorbic acid contents in foods.
2. An HPLC system utilizing an LiChrosorb-NH_2 column, a 75:25 mobile phase of acetonitrile:0.05 M KH_2PO_4 buffer and a UV detector at 268 nm could effectively separate erythorbic acid from ascorbic acid in most food samples. Moreover, the levels of ascorbic and erythorbic acids in food samples could be quantitated by this procedure as well as their dehydro- forms after reduction with dithiothreitol. The high selectivity and specificity are the major advantages of the HPLC method.
3. The spectrophotometric method did not distinguish between ascorbic and erythorbic acids, whereas the HPLC did. The presence of erythorbic acid leads to an overestimation of total vitamin C activity when ascorbic acid levels are determined by the spectrophotometric

method.

4. Since the spectrophotometric method does not accurately reflect the actual ascorbic acid contents in food samples when erythorbic acid is present, the use of HPLC is recommended for analysis of those samples containing both ascorbic and erythorbic acids.

SUMMARY

A method for the separation and determination of ascorbic and erythorbic acids in selected food samples is described. It employs high performance liquid chromatography using a LiChrosorb-NH_2 column in conjunction with a mixture of acetonitrile and 0.05 M KH_2PO_4 buffer as an eluant. Application of the method, which is sensitive, rapid and simple, to the analysis of ascorbic and erythorbic acids in frozen apples, potato products, fruit and vegetable concentrated juices, frozen juices, natural and artificial flavor drink mixes, and Hi-C drinks gave satisfactory results. Dehydroascorbic and dehydroerythorbic acids in these samples could be determined after reduction with dithiothreitol.

It was verified by HPLC that the presence of erythorbic acid affected determination of ascorbic acid levels by the spectrophotometric method by causing elevated absorbance readings.It was proved that samples containing erythorbic acid could not be accurately assessed for ascorbic acid activity by the spectrophotometric method. Erythorbic acid seriously affects the true determination of the quantity of ascorbic acid in food samples.

The HPLC method was shown to be useful for the routine analysis of most food samples but not for processed meat

products. More research is needed concerning the determination of erythorbic acid in meat products by HPLC.

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