

ANALYSIS OF ASCORBIC ACID AND RELATED COMPOUNDS IN FLUIDS AND TISSUES

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I. INTRODUCTION

A. Occurrence of Ascorbic Acid

L-Ascorbic acid (AA) is regarded to be a normal cellular constituent of all higher plants and animals. Higher plants all have the capacity to synthesize AA,

but certain animals, including primates, lack this ability (Chatterjee, 1978; Chaudhuri and Chatterjee, 1969). These animals depend on plant sources to satisfy their requirement for ascorbic acid, and for them AA can be referred to as vitamin C. Animals that require dietary AA lack the enzyme L-gulonolactone oxidase, which is required for the terminal step in AA biosynthesis (Burns, 1957).

B. Functions of Ascorbic Acid

Much remains to be learned about the functions of AA in both plants and animals. While specific coenzyme functions have been established for the B group of vitamins, roles for vitamin C at this level have not been established. Instead, vitamin C functions have been examined mainly at the physiological rather than biochemical level. Research results point to the reducing properties of AA and its interaction with destructive free-radical derivatives of oxygen as central to its biological functions.

The best-known role for AA in humans is in the biosynthesis of collagen, where it is involved in the posttranslational formation of hydroxyproline and hydroxyserine residues in the protein. Clinical deficiency of vitamin C leads to an unstable collagen polypeptide and the disease state known as scurvy. Numerous other beneficial effects of the vitamin have been suggested; these and other topics have been discussed in two published volumes on AA (Counsell and Hornig, 1981; Seib and Tolbert, 1982). There is persuasive evidence that AA is associated with respiratory processes in plants (Loewus, 1980) and animals. An important role of AA in plants is in the formation of hydroxyproline-containing proteins in the plant cell wall (Arrigoni *et al.*, 1977)—a role analogous to collagen formation in animals. Other functions in plants include involvement in the hydroxylation of other organic compounds, and protection against accumulation of peroxides and the superoxide radical in the chloroplast during photosynthesis. This latter function also applies to animals, since the findings of Sawyer *et al.* (1982) confirm that AA, its anion, and dehydroascorbic acid are unique in their ability to destroy the superoxide radical without production of reactive intermediate radicals.

C. Biosynthesis of Ascorbic Acid

The AA biosynthetic pathway is more firmly established in animals than in plants. In both, however, AA occurs as the L rather than the D enantiomer. In animals, tracer studies establish that chain inversion occurs, and C-1 of glucose becomes C-6 of AA (Isherwood and Mapson, 1962; Loewus, 1980). The following scheme is proposed: D-glucose \rightarrow D-glucuronolactone \rightarrow L-gulonolactone \rightarrow 2-keto-L-gulonolactone \rightarrow L-ascorbic acid. In this scheme, the conversion of D-

glucuronate to L-gulonolactone involves reduction of C-1 to an alcohol. While Isherwood and Mapson (1962) support the view that the pathway is similar in plants and that uronic acids and their lactones are key intermediates, Loewus and Helsper (1982) have presented compelling evidence that no chain inversion of glucose occurs. They propose that aldonic rather than uronic acids are the intermediates, and that epimerization of a D-gluco compound at C-5 is required for the formation of the L-gulo configuration.

D. Need for Specific Methods of Analysis

Before AA deficiency results in symptoms of scurvy, other metabolic disorders usually occur. The diagnostic procedures used to detect such disorders require methods of determining AA levels in various tissues. Determination of serum AA levels tends to reflect only recent AA intake rather than stores in tissues. In order to understand the distribution and functions of AA among plant and animal tissues, as well as its biosynthesis, it is essential that selective and precise procedures be available for its measurement. Many colorimetric, chromatographic, and enzymatic procedures have been reported. In this article, procedures that appear to be best suited for determining AA and related compounds in a variety of mixtures will be emphasized. It is clear that a variety of methods are required, since extracts from various sources contain different interfering substances. Other reviews on analysis of AA have been published (Cooke and Moxon, 1981; Omaye *et al.*, 1979; Sauberlich *et al.*, 1982).

II. CHEMICAL PROPERTIES OF ASCORBIC ACID AND ITS OXIDATION PRODUCTS

A. Chemical Structures

AA and L-dehydroascorbic acid (DHAA) are the biologically active forms of vitamin C, and throughout this article the combined levels of AA and DHAA will be referred to as vitamin C. The outstanding chemical characteristics of the AA-DHAA system involve its redox properties and the conversion of DHAA to other forms. Since several of the analytical methods for AA and DHAA exploit these properties, they will be briefly discussed.

Figure 1 shows the reaction sequence from AA through DHAA to its hydrolysis product diketogulonic acid (DKGA), which possesses no vitamin C activity. The equilibrium between AA and DHAA is controlled by a number of factors (enzymes, cations, temperature, light), as is the formation of DKGA from DHAA.

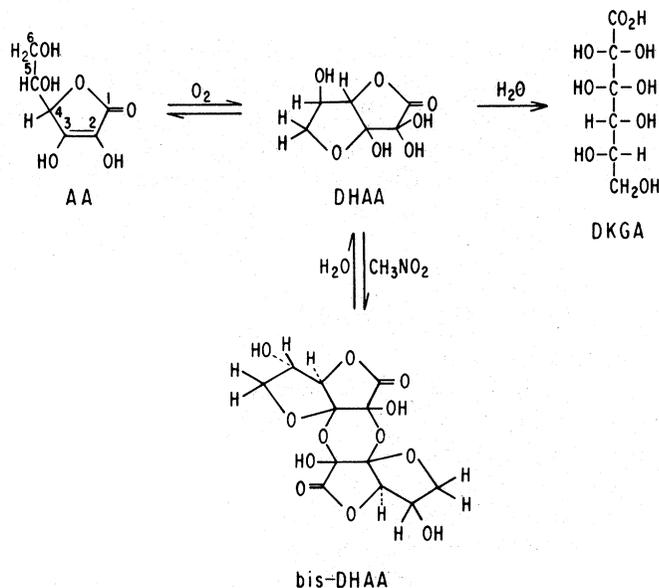


Fig. 1. Predominant structures in aqueous solution of L-ascorbic acid (AA), L-dehydroascorbic acid (DHAA, hemiketal hydrate), L-diketogulonic acid (DKGA, dihydrate), and bis-L-dehydroascorbic acid (bis-DHAA).

AA is acidic due to the conjugated lactone–enediol structure. The hydroxyl protons at C-3 and C-2 possess pK values of 4.26 and 11.64, respectively (Hvoslef, 1968). DHAA is not a true organic acid, as it contains no readily ionizable protons. The lactone group at C-1 in AA is quite stable in acid or alkali, but this functionality in DHAA is stable only over a rather narrow acidic pH range (Tolbert and Ward, 1982). Nuclear magnetic resonance (NMR) results (Pfeilsticker *et al.*, 1975) reveal that in an aqueous solution DHAA exists in a bicyclic hydrated form (Fig. 1) rather than as the 2,3-diketo compound. These carbonyl functions are also hydrated in DKGA (Fig. 1). A convenient preparation of DHAA from AA is accomplished by bubbling O_2 into a stirred methanolic solution of AA containing charcoal (Ohmori and Takage, 1978). Ethanol has been shown to be a more suitable medium for this oxidation process (Tolbert and Ward, 1982). DHAA is not known as a crystalline compound but is readily converted to its crystalline dimer bis-DHAA (Fig. 1) after dissolution in nitromethane (Dietz, 1970). From the dimer, DHAA is regenerated in water. The ^{13}C NMR spectrum of AA is shown in Fig. 2 (Paukstelis *et al.*, 1982); additional discussions of ^{13}C , 1H , and 2H NMR for AA, DHAA, and related compounds are found elsewhere (Berger, 1977; Hvoslef and Pederson, 1981; Pfeffer *et al.*, 1980; Radford *et al.*, 1979; Tolbert and Ward, 1982).

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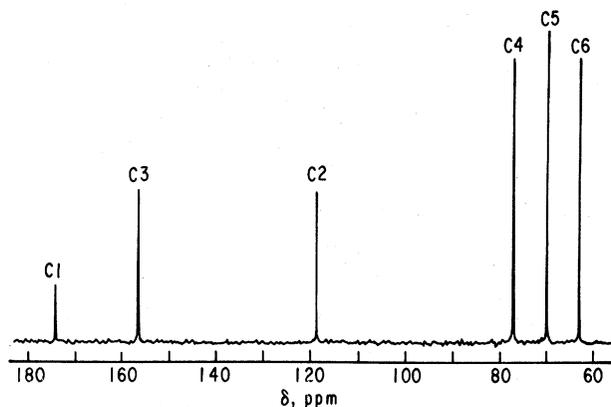


Fig. 2. Proton-decoupled ¹³C NMR spectrum of L-ascorbic acid in H₂O; pH 2.0, 33°C. [Reprinted with permission from American Chemical Society and Paukstelis *et al.* (1982).]

B. Free Radicals of Ascorbic Acid

The two-step redox processes of AA, with free-radical intermediates, have been reviewed by Bielski (1982). Described are the electron paramagnetic resonance (EPR) characterization of AA radicals and the kinetics of their formation and disappearance in enzymatic and nonenzymatic processes. The unusual biological protective properties of AA against free-radical damage were ascribed to its efficiency as a radical scavenger and the stability of the radical. The ascorbate radical reacts preferentially with itself, thus ending the process. These results have been confirmed in a thorough kinetic study (Sawyer *et al.*, 1982). It is one of the few species observed by EPR in studies of tissues.

III. EXTRACTING ASCORBIC ACID AND DEHYDROASCORBIC ACID

The levels of AA and DHAA and the ratios of AA to DHAA vary among tissues and fluids. In selecting one of the many extracting procedures to use, the analyst must consider whether it is necessary to determine individual or total (vitamin C) levels. Table I (Omaye *et al.*, 1979) lists levels of AA in various human tissues; these levels are affected differently by recent dietary intake of the vitamin. Variability also exists among plant parts, where level variations are caused by factors such as light and seasonal changes.

Compatibility with the analytical procedure to be used must also be taken into account when selecting an extracting medium. If levels of both AA and DHAA

TABLE I

Ascorbic Acid Content of Adult Human Tissues^a

Tissue	Ascorbic acid (mg/100 g wet tissue)
Adrenal gland	30-40
Pituitary gland	40-50
Liver	10-16
Spleen	10-15
Lungs	7
Kidneys	5-15
Testes	3
Thyroid	2
Heart muscle	5-15
Skeletal muscle	3-4
Brain	13-15
Pancreas	10-15
Eye lens	25-31
Plasma	0.4-10.0
Saliva	0.07-0.09

^aReprinted with permission from Academic Press and Omaye *et al.* (1979).

are desired, extracting conditions should not affect the equilibrium between them. In some cases, it is desirable to denature proteins during extraction, both to provide clear solutions and to eliminate enzyme activities. Extractions should be conducted rapidly at cold temperatures and in subdued light. While vitamin C seems to be quite stable in the solid form and under certain physiological conditions in living systems, in solution it can be readily oxidized.

A. Metaphosphoric and Trichloroacetic Acids

Most solvent systems for extracting AA and DHAA are aqueous solutions of a variety of acids. Metaphosphoric acid (HPO₃, 3-6%) was first recommended in 1935 (Bradley *et al.*, 1973) and it, along with trichloroacetic acid (3%), remains the most widely used. The acid solutions must be prepared frequently and with pure water; contaminants such as cupric and ferric ions will oxidize AA to DHAA. When such ions are present in the material being analyzed, it has been proved (Archer, 1981; Jager, 1948) helpful to add ethylenediaminetetraacetic acid (EDTA), which will chelate divalent cations and protect AA from oxidation. Both metaphosphoric and trichloroacetic acids provide a medium below pH 4, where AA and DHAA are stable by virtue of stabilizing effects on the lactone rings. The advantages of these and other extracting solutions have been reviewed (Cooke and Moxon, 1981). AA can be air oxidized during extraction and prior to

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analysis (Barker and Mapson, 1959), therefore extracting media should be deoxygenated by bubbling in nitrogen before use.

Metaphosphoric and trichloroacetic acids are effective protein denaturants, so they are especially useful for extracting materials which contain proteins. Whichever extracting medium is selected, it is necessary to conduct the analysis within a short time—if not possible, samples should be stored in the dark, frozen at -65°C . In selecting among the many extracting solutions which have been reported, and in deciding what proportion to use relative to the sample, it is best to refer to the literature for guidance from others studying similar systems. For example, in the colorimetric analysis of McGown *et al.* (1980), trichloroacetic acid was found suitable, but metaphosphoric acid inhibited the assay. As an alternative to using acidic denaturants, Green and Perlman (1980) showed that plasma could be deproteinized by ultrafiltration, a useful procedure when protein precipitants interfere with AA analysis.

B. Stabilizing Ascorbic Acid with Sulfhydryl Compounds

The reduction of DHAA to AA with hydrogen sulfide or thiourea was studied by Roe *et al.* (1948); later Hughes (1956) showed that DL-homocysteine also efficiently accomplishes the conversion. The presence of such a sulfhydryl reagent in the extracting solution, or its addition after extraction, allows the determination of the vitamin C (AA + DHAA) level. The approach using DL-homocysteine was employed by Dennison *et al.* (1981) to determine vitamin C levels in beverages. Lookhart *et al.* (1982) extracted various materials in the presence of dithiothreitol; extraction efficiency was not affected and vitamin C was readily determined. Others (Arakawa *et al.*, 1981; Doner and Hicks, 1981; Okamura, 1980) have determined AA before and after dithiothreitol addition, providing the basis for indirectly determining DHAA after its reduction.

Several variations of the extracting solutions have been demonstrated to restrict losses of vitamin C to 5% or less during the time of extraction and analysis. Cooke and Moxon (1981) state with regard to selecting an extracting medium: "Unfortunately, different workers have come to a variety of conclusions and it is not possible by studying the literature to select the solvent system which has the best performance for stabilizing all vitamin C extracts."

IV. DETERMINATION OF ASCORBIC ACID, DEHYDROASCORBIC ACID, AND DIKETOGULONIC ACID

Because AA and DHAA are the compounds with vitamin C activity, the principle and procedures for their determination will be emphasized in this section. In a later section, the analysis of some related natural compounds and synthetic derivatives will be described. While over 700 references to the deter-

mination of AA and DHAA have been given in chemical abstracts since 1972, most of the methods still widely used are variations of procedures described as early as 50 years ago. Exceptions are the chromatographic methods—high-pressure liquid chromatography (HPLC) separation and detection methods have been developed quite recently. It will become apparent that the chemical methods, based on reactions of either AA or DHAA, remain suitable for many analyses, especially when interfering compounds are present only at low levels.

A. Biological Methods

Vitamin C is the only water-soluble vitamin for which a microbiological assay is not available—no microorganism has been identified which has a requirement for the vitamin. In addition, the usual laboratory test animal, the rat, can synthesize AA, so it cannot be used. The guinea pig requires vitamin C to prevent scurvy; some early procedures (Key and Elphick, 1931; Sherman *et al.*, 1922) assayed vitamin C activity by determining the protective effects that various mixtures offered against scurvy in guinea pigs. These methods are time consuming, imprecise, and expensive; details of such bioassays and problems inherent to them have been reviewed (Olliver, 1967). The chemical and physical methods for AA determination are superior, and today's bioassays are limited to testing the antiscorbutic activity of various AA analogs.

B. Direct Spectrophotometric Methods

Applications of direct UV absorption spectrophotometry for the determination of AA have been described. At a neutral pH value, AA absorbs maximally at 265 nm with an extinction coefficient (ϵ) of 16,500; at pH 2, UV_{max} is 244 nm and ϵ is reduced to 10,500 (Hewitt and Dickes, 1961). DHAA is transparent in these regions of the spectrum, but absorbs weakly at 210 and 300 nm (Mattock, 1965). Hewitt and Dickes (1961) developed a UV method to estimate AA and DHAA in leaf tissue. AA oxidase (Tono and Fujita, 1981) and AA peroxidase (Kelly and Latzko, 1980) have been used in developing UV-difference spectral methods for AA. These latter approaches may prove to be quite selective for AA. Generally, however, the direct spectral procedures are subject to interferences from many other substances.

C. Chemical Methods

The colorimetric methods for analysis of AA and DHAA, along with a fluorometric procedure, continue to be most widely used. Here the emphasis is on the three most popular methods, which utilize either the reducing property of AA or condensation reactions of its oxidation product DHAA. Related methods using

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these properties will be described more briefly, mainly to refer the reader to pertinent papers. All colorimetric methods appear to be subject to some limitations; since 1980, most reports of new methods for vitamin C have utilized HPLC, and the trend likely will be toward wider applications.

1. Methods Based on Reductions by Ascorbic Acid

a. *Determination by Reduction of 2,6-Dichlorophenol-indophenol (2,6-DCPIP)*. The capacity of AA to reduce the blue dye 2,6-DCPIP to its leuco form was first reported by Tillmans (1927); the first application to quantitative analysis of AA was reported by Harris and Ray (1933). The reaction scheme is given in Fig. 3, and experimental details have been reviewed by Omaye *et al.* (1979). This titrimetric method is especially useful for analyzing samples that contain no other compounds (sulfhydryl compounds, certain cations, and some plant pigments) that can reduce 2,6-DCPIP. Interference by sulfhydryl compounds has been shown to be blocked when *p*-chloromercuribenzoate is included in the assay system (Owen and Iggo, 1956). Procedures to overcome interferences by other compounds have been reviewed (Cooke and Moxon, 1981). A novel stopped-flow procedure has been described (Hiromi *et al.*, 1980; Karayannis, 1975) which prevents interferences by materials such as reductones, which are present in many food products. The titration endpoint in the 2,6-DCPIP assay is difficult to visualize when the extract being analyzed is colored or when interfering compounds are present. A potentiometric endpoint determination is useful in such cases (Spaeth *et al.*, 1962). If one wishes to determine vitamin C, a preliminary reduction of DHAA to AA is required prior to the assay; 2,3-dimercaptopropanol has been used for this purpose (Gero and Candido, 1969). Many procedures for determining AA and DHAA first call for AA to be determined in one of a pair of replicate samples, and determined in the other sample

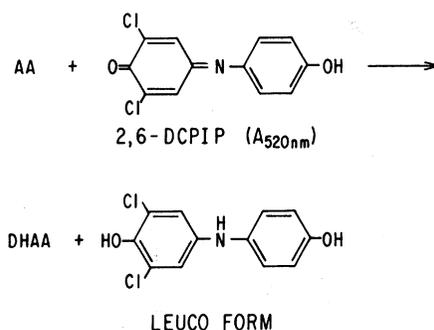


Fig. 3. Reaction for determination of L-ascorbic acid (AA) by reduction of 2,6-dichlorophenol-indophenol (2,6-DCPIP).

after reduction. This allows the determination of DHAA by difference. Such procedures are especially important in light of suggestions that low DHAA/AA ratios or DHAA levels are sensitive indicators of pathogenic conditions in cells (Banerjee, 1977; Edgar, 1979; Warden *et al.*, 1972). Automated procedures for determining AA in blood serum based on reduction of 2,6-DCPIP have been reported (Garry and Owen, 1968; Koch *et al.*, 1980; Pelletier and Brassard, 1973; Sauberlich *et al.*, 1976).

b. Determination by Reduction of Ferric Ion. Several procedures involve determination of ferrous ion after reduction by AA. The most widely used is the α, α' -dipyridyl reaction, illustrated in Fig. 4 and first described by Sullivan and Clark (1955), who showed that ferrous ion interacts with α, α' -dipyridyl to give a red-orange complex. The method was later improved and extended to analysis of AA in animal tissues (Maickel, 1960; Zannoni *et al.*, 1974). Okamura (1980, 1981) has enhanced the utility of the assay by demonstrating that interfering substances in tissue extracts can be removed with activated carbon. Prior to analysis, DHAA was reduced to AA with dithiothreitol, thus giving vitamin C levels.

Compounds other than α, α' -dipyridyl have been used to monitor reduction of ferric ion by AA, and some are more sensitive. Whereas the complex ferrous ion- α, α' -dipyridyl absorbs at 525 nm with an ϵ of 8650, the complex with ferrozine absorbs at 562 nm with a much higher ϵ , 27,900. Ferrozine was first used for AA analysis by Stookey (1970), and it since has been applied to determining AA levels in citrus fruits (Jaselskis and Nelapaty, 1972) and urine (Butts and Mulvihill, 1975). By deproteinizing extracts, McGown *et al.* (1982) have extended the utility of the ferrozine method to animal tissues. They demonstrated the ease and sensitivity of the method and obtained AA levels in various tissues comparable to those found using a more lengthy procedure.

Another assay procedure based on ferric ion reduction by AA was reported by Day *et al.* (1979), which involves formation of 2,4,6-tris(2-pyridyl)-s-triazine

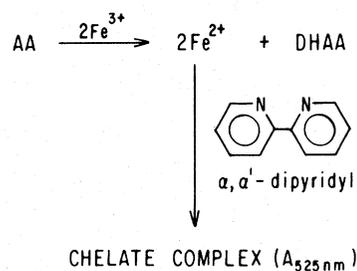


Fig. 4. Determination of L-ascorbic acid (AA) by reduction of ferric ion and formation of ferrous- α, α' -dipyridyl complex.

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(Fe^{2+}) from the corresponding Fe^{3+} complex; the reduction product absorbs at 593 nm ($\epsilon = 22,140$). Liu *et al.* (1982) employed AA oxidase, rendering the procedure much more specific for AA in blood serum and plasma, and no deproteinization was required.

Additional methods based on ferric ion reduction include colorimetry with either ferricyanide (Rukmini *et al.*, 1981) or 4,7-diphenyl-1,10-phenanthroline (Arakawa *et al.*, 1981), or by enthalpimetry with hexacyanoferrate (Bark and Kershaw, 1975).

c. Other Methods Based on Reductions by Ascorbic Acid. Readily reducible compounds which have been used in colorimetric assays for AA include methylene blue (White and Fitzgerald, 1972), dimethoxyquinone (Eldawy *et al.*, 1975), phenolphthalein (Shahine and Mahmoud, 1980), 2,3-diphenyl-3-thiazoltetrazolium chloride (Wassileva-Alexandrova and Nejetscheva, 1982), and tetrachlorobenzoquinone (Pandey, 1982). Procedures based on reduction of halogen-containing compounds were reviewed by Krishna Murty and Rama Rao (1979), and more recent applications were reported by Puzanowska-Tarasiewicz *et al.* (1980) and Wang and Freiha (1982). Wang and Freiha employed an amperometric titration to follow reduction of iodine to iodide ion. Reduction of metallic cations other than iron by AA has been the basis of additional methods. Analysis of AA based on reduction of cupric ion was reported by Faye (1966), Kidani *et al.* (1981), Shahine (1980), and Shieh and Sweet (1979). Turbidimetric assays for selenium after reduction of selenium oxide have been reported (Ralls, 1975; Sarwar *et al.*, 1980). Finally, the reduction of ammonium molybdate by AA has been used to determine AA levels in fruits and vegetables (Bajaj and Kaur, 1981).

Few of the procedures described in this section have been demonstrated to perform with the selectivity or sensitivity of the methods based on reduction of ferric ion, and are not so widely used.

2. Methods Based on Reactions of Dehydroascorbic Acid: A Fluorometric Procedure

Condensation of DHAA with *o*-phenylenediamine forms a quinoxalinyllactone (Erlbach and Ohle, 1934; Hensecke and Ditttrch, 1959), as shown in Fig. 5. This product fluoresces at 423 nm when irradiated at 348 nm (Archibald, 1945; Ogawa, 1953); this has provided the basis for a sensitive procedure to determine AA (Deutsch and Weeks, 1965). Vitamin C (AA + DHAA) is determined, since a preliminary oxidation step is involved. A number of oxidizing agents have been used, including activated charcoal (Deutsch and Weeks, 1965), 2,6-DCPIP (Ziegenhagen and Zobel, 1969), and *N*-bromosuccinimide (Roy, 1976). A parallel blank determination for distinguishing DHAA from interfering substances was introduced by Deutsch and Weeks (1965). This method is quite useful

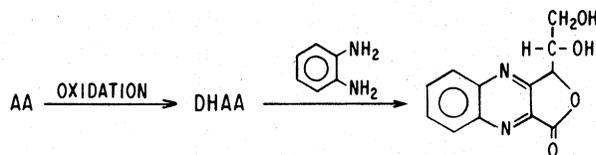


Fig. 5. Reaction for fluorometric determination of L-ascorbic acid (AA), based on condensation of L-dehydroascorbic acid (DHA) with *o*-phenylenediamine. Excitation, 348 nm; emission, 423 nm.

because boric acid forms a complex with DHA in the blank—the complex does not condense with *o*-phenylenediamine. The procedure is sensitive and widely used; the results compare well with a variety of other procedures.

Modifications of the fluorescence procedure have been reported, some employing substituted *o*-phenylenediamines (Dunmire *et al.*, 1979; Egberg *et al.*, 1977; Szepesi, 1973). The remaining references to analyses based on reactions of DHA involve the formation of colored products with glycine (Brunet, 1968; Müller-Mulot, 1969), and pyrrole and cuprous ions (Kochi and Kaneda, 1970).

3. Methods Based on Derivatization of Diketogulonic Acid with 2,4-Dinitrophenylhydrazine

Roe and Kuether (1942, 1943) demonstrated that DKGA reacts with 2,4-dinitrophenylhydrazine (2,4-DNPH) in acidic solutions to form an osazone, which upon treatment with concentrated sulfuric acid produces a red chromophore that absorbs at 520 nm. This finding provided a method for vitamin C determination which is still widely used; the general reaction scheme is given in Fig. 6. Cooke and Moxon (1981) reviewed the many efforts to minimize the problems of this assay, which include interferences from other osazone-forming

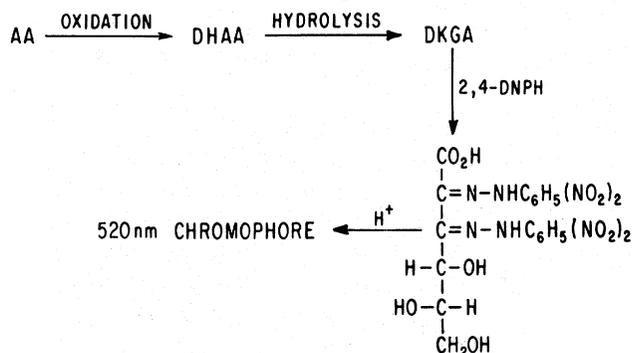


Fig. 6. Determination of L-ascorbic acid (AA) after conversion to osazone derivative by condensation with 2,4-dinitrophenylhydrazine (2,4-DNPH).

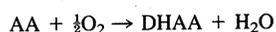
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compounds (i.e., sugars), less than quantitative yields, and lengthy analysis times.

Pelletier (1968) refined the 2,4-DNPH method; automated procedures were developed later, as reported by Aeschbacher and Brown (1972) and Pelletier and Brassard (1977). Behrens and Madere (1979) modified the automated methods to accommodate smaller samples. Applications of the 2,4-DNPH procedure to animal studies have been reported (Chatterjee and Banerjee, 1979; Evans *et al.*, 1980; Odumosu, 1982; Tillotson and McGown, 1981).

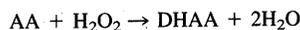
D. Enzymatic Methods

Two enzymes known to have activities toward AA have been used in assay procedures. Ascorbate oxidase (AAO) catalyzes the following reaction:



Procedures for isolating the enzyme from plant sources and the details of its properties have been described by Lee and Dawson (1979). AAO requires copper as a cofactor and is specific for substrates possessing an enediol structure adjacent to a carbonyl group (Dawson, 1966). The enzyme is commercially available and considerable attention has been given to its application for AA determination.

Ascorbate peroxidase has been isolated (Kelly and Latzko, 1979) and used to determine AA. It catalyzes the following reaction:



1. Ascorbate Oxidase

Marchesini *et al.* (1974) assessed the properties of the enzyme AAO and found that it is inhibited to some extent by compounds such as reductones and sulfur dioxide, and also by ferric ion. Procedures were developed to minimize such interferences (Marchesini *et al.*, 1974) so that AAO can be applied in determining AA. The conversion of AA to DHAA is monitored by following the concomitant consumption of oxygen, either with an oxygen electrode (Marchesini *et al.*, 1974; Schindler *et al.*, 1978) or by other means (Henniger, 1981; Schenk *et al.*, 1982). The results of Schenk *et al.* (1982) were obtained from AA analysis of brain tissue, and the method was rapid and sensitive. Measurements of amperometric responses of oxidizable species other than AA were accounted for before addition of AAO. Immobilization of AAO on a Clark oxygen electrode offers the possibility for a continuous method of AA analysis (Macholan *et al.*, 1981; Matsumoto *et al.*, 1981). List and Knechtel (1980) immobilized AAO on a polyamide powder and found that the half-life of the enzyme is significantly longer than that of the enzyme in solution. Liu *et al.* (1982) used AAO to account

for interference by reducing agents other than AA in their procedure, which is based on reduction of a ferric ion complex. One of a pair of replicate samples was treated with AAO, then both were reacted with the ferric complex; the difference in absorption at 593 nm was linearly related to AA concentration.

2. Ascorbate Peroxidase

The procedure reported by Kelly and Latzko (1980) used only partially purified ascorbate peroxidase; nonetheless the method shows promise. Ascorbate peroxidase may have a narrower substrate specificity than AAO, and has the advantage that the reaction can be initiated by addition either of hydrogen peroxide or of ascorbate peroxidase.

E. Chromatographic Methods

Recent developments in column, detector, and data-handling technology continue to expand the applications of HPLC to analysis of AA, DHAA, and related compounds. The selectivity for individual compounds is greater than most, if not all, other available analytical procedures. Most attention in this section is on HPLC; reports describing separations and analysis by other separation methods, including paper, thin-layer, and gas-liquid chromatography and isotachopheresis are also discussed.

1. High-Performance Liquid Chromatography

The first applications of HPLC for AA analysis were reported by Nelson (1973) and Williams *et al.* (1973), who used commercially available silica-based anion-exchange packings. Sauberlich *et al.* (1982) reviewed papers relating to AA separations through 1979. Anion-exchange packings have been used by a number of researchers, and are applicable to AA analysis because AA exists as an anion in mildly acidic solutions. Additional reports have utilized silica-based packings which are derivatized with aminopropyl groups. These columns are more generally applied to sugar separations, but function as weak anion exchanges for AA separations when eluted with appropriate buffers. Reverse-phase HPLC, normally with C₁₈-silica packings, has been used; in such cases, AA is ion-paired with various amphiphilic cations so that it is retained by the column. The separation process for these packings involves partitioning of the AA-ion pair between the mobile phase and the hydrophobic stationary phase. References relating to AA separations are listed in Table II, which indicates the column packing, detection method, and the compounds analyzed. For monitoring the elution of AA and related compounds from HPLC columns, both UV and amperometric detections have been used; one report describes the use of a chemiluminescent detector (Veazey and Nieman, 1980). Various wavelengths have been used for UV monitoring, since the UV_{max} for AA varies with the pH value

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TABLE II
HPLC Separations of Ascorbic Acid and Related Compounds

Silica bonded phase	Detection	Compounds analyzed ^a	Reference
Reverse phase			
Corasil C ₁₈	Amperometric	Water-soluble vitamins	Stillman and Ma (1974)
μ-Bondapak C ₁₈	UV, 254 nm	AA in food	Sood <i>et al.</i> (1976)
μ-Bondapak C ₁₈	UV, 254 nm	Water-soluble vitamins	Wills <i>et al.</i> (1977)
μ-Bondapak C ₁₈	UV, 254 nm	AA in urine	Wagner <i>et al.</i> (1979)
μ-Bondapak C ₁₈	UV, 254 nm	AA (2,4-DNPH derivative)	Garcia-Castineiras <i>et al.</i> (1981)
μ-Bondapak C ₁₈	UV, 210 and 254 nm	AA, DHAA, DKGA	Finley and Duang (1981)
μ-Bondapak C ₁₈	UV, 254 nm	AA in potatoes	Augustin <i>et al.</i> (1981)
LiChrosorb RP-8, 18	UV, 264 nm	AA and EA	Coustard and Sudraud (1981)
μ-Bondapak C ₁₈	UV, variable	AA and DHAA (OPD derivative)	Keating and Haddad (1982)
Ultrasphere ODS	Amperometric	AA in brain	Dozier <i>et al.</i> (1982)
LiChrosorb RP-18	UV, 245 nm	AA in fruits	Shaw and Wilson (1982)
μ-Bondapak C ₁₈	Amperometric	AA in lymphocytes	Lee <i>et al.</i> (1982)
Anion exchange (strong)			
Permaphase AAX, Zipax SCX, Zipax SAX	UV, 254 nm	Water-soluble vitamins	Williams <i>et al.</i> (1973)
Zipax SAX	UV, 254 nm	Food additives	Nelson (1973)
Zipax SAX	Amperometric	Vitamins, food, serum, urine	Pachla and Kissinger (1976, 1979)
Nucleosil SB-10	Amperometric	AA	Brunt and Bruins (1979)
Partisil-10 SAX	Amperometric	Marine animal tissues	Carr and Neff (1980)
Bondapak AX/Corasil	UV, 254 nm	Ascorbyl sulfates and phosphates	Mauro <i>et al.</i> (1980)
SAX-801	UV, 260 nm	AA, reductones in fluids	Obata <i>et al.</i> (1980)
Partisil-11 SAX	Chemiluminescence	AA, glucose, creatinine, glucuronic acid	Veazey and Nieman (1980)
Partisil-11 SAX	UV, 254 nm	AA in lymphocytes	Liebes <i>et al.</i> (1981)
Zipax SAX	Amperometric	AA in serum, plasma, leukocytes	Tsao and Salimi (1981)
Aminex A-14	UV, 254 nm	AA in foods	Floridi <i>et al.</i> (1982)

TABLE II (Continued)

Silica bonded phase	Detection	Compounds analyzed ^a	Reference
Anion exchange (weak)			
μ -Bondapak NH ₂	UV, 254 nm	Water-soluble vitamins	Wills <i>et al.</i> (1977)
Altex-NH ₂ bonded phase	Amperometric	AA in plasma	Mason <i>et al.</i> (1980)
μ -Bondapak NH ₂	UV, 255 nm	AA and EA in foods	Archer (1981)
LiChrosorb-NH ₂	UV, 270 nm	AA and amino acids	Schuster (1980)
LiChrosorb-NH ₂	UV, 268 nm	AA and EA in juices	Bui-Nguyen (1980)
LiChrosorb-NH ₂	UV, 254 nm	AA and EA from animal tissues	Otsuka <i>et al.</i> (1981)
μ -Bondapak NH ₂	UV, 244 nm	AA and DHAA in beverages	Dennison <i>et al.</i> (1981)
Zorbax NH ₂	UV, 268 nm; refractive index	AA, DHAA, EA, DHAA, DHEA, DKGA, DKGIA	Doner and Hicks (1981)
μ -Bondapak NH ₂	UV, 265 nm	AA and EA in juices	Geigert <i>et al.</i> (1981)
LiChrosorb-NH ₂	UV, 210, 240, 254 nm	AA and DHAA	Rose and Nahrwold (1981)
μ -Bondapak NH ₂	UV, 254 nm	AA in vitamins, bread	Lookhart <i>et al.</i> (1981)

^aAA, L-Ascorbic acid; DHAA, L-dehydroascorbic acid; DKGA, L-diketogulonic acid; OPD, *o*-phenylenediamine; 2,4-DNPH, 2,4-dinitrophenylhydrazine; EA, D-erythorbic acid; DHEA, D-dehydroerythorbic acid; DKGIA, D-diketogluconic acid.

of the eluent. AA is readily oxidized at +0.45 V (versus Ag/AgCl electrode), allowing for convenient amperometric detection at +0.70 V.

a. Ion-Exchange Separations with UV Detection. AA was separated from a mixture of 20 amino acids by Schuster (1980), using UV detection with time program capability. Bui-Nguyen (1980) accomplished the separation of AA from D-erythorbic acid (EA), a closely related compound which differs from AA only in configuration at C-5. AA and EA were efficiently resolved and detected at 268 nm. This separation was also accomplished by others (Geigert *et al.*, 1981; Otsuka *et al.*, 1981; Vuilleumier and Pongraoz, 1976). It is essential in HPLC of AA that both the sample solvent and the eluting solvent be solvents that stabilize AA. Archer (1981) found that even in the presence of cupric ion, EDTA (sodium salt) is effective for this purpose.

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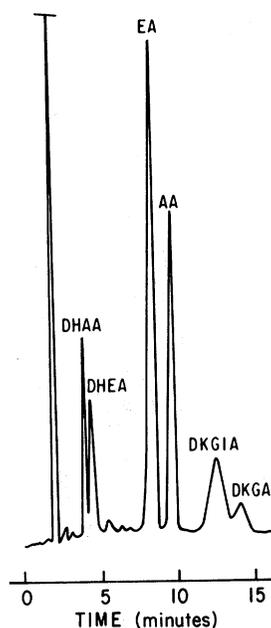


Fig. 7. HPLC chromatogram; the separation of L-dehydroascorbic acid (DHAA), D-dehydroerythorbic acid (DHEA), D-erythorbic acid (EA), L-ascorbic acid (AA), D-diketogluconic acid (DKGIA), and L-diketogulonic acid (DKGA). Conditions: column, Zorbax NH₂, 4.6 mm × 25 cm; mobile phase, CH₃CN/0.05 M KH₂PO₄ (75:25, w/w); refractive index detection (×8 attenuation); flow rate, 1.5 ml/min; chart speed, 8 in./hr. [Reprinted with permission from Academic Press and Doner and Hicks (1981).]

Doner and Hicks (1981) reported the separation of AA, EA, DHAA, dehydroerythorbic acid (DHEA), DKGA, and diketogluconic acid (DKGIA) on a Zorbax NH₂ column (Fig. 7). Refractive index (RI) detection was required to monitor the compounds, since only AA and EA were detected at 268 nm. The dehydro forms of AA and EA could, however, be determined by differences after reduction with dithiothreitol. DL-Homocysteine was used as reductant by Denison *et al.* (1981). Using UV and RI detectors in tandem, it was possible to determine AA (UV) and fructose, glucose, and sucrose (RI) in orange juice (Fig. 8) (Doner and Hicks, 1981). In studies with human lymphocytes, Liebes *et al.* (1981) reported that AA can be resolved efficiently in mixtures with nucleotides. Rose and Nahrwold (1981) developed a procedure for direct HPLC determination of AA and DHAA by detecting DHAA at 210 nm and AA at 254 nm.

b. Reverse-Phase Separation with UV detection. Finley and Duang (1981) resolved AA, DHAA, and DKGA in less than 8 min by using tri-*n*-butylamine as an ion-pairing reagent in the mobile phase. The method was applied to determin-

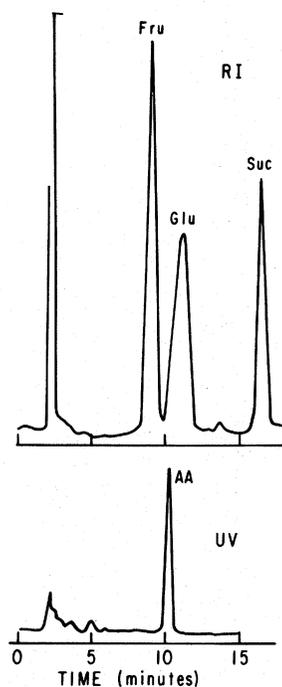


Fig. 8. HPLC chromatogram of orange juice monitored by ultraviolet (UV, 268 nm) and refractive index (RI) tandem detection. UV displays L-ascorbic acid (AA), and RI displays the sugars fructose (Fru), glucose (Glu), and sucrose (Suc); other conditions as in Fig. 7. [Reprinted with permission from Academic Press and Doner and Hicks (1981).]

ing these compounds in various foods. Coustard and Sudraud (1981) employed paired-ion chromatography to resolve AA and EA.

Two reports describe methods of determining AA after forming precolumn derivatives. Garcia-Castineiras *et al.* (1981) converted DHAA to its osazone after oxidation of AA and separated the product from other compounds in aqueous humor. Keating and Haddad (1982) condensed DHAA with *o*-phenylenediamine and used ion-pair reverse-phase HPLC to separate the product from AA. The derivative was monitored at 348 nm and the separation was accomplished in less than 3 min.

c. Amperometric Detection. High-pressure liquid chromatography with electrochemical detection (LCEC) was demonstrated to be both sensitive and selective for AA by several workers (Brunt and Bruins, 1979; Pachla and Kissinger, 1976). The principles and procedures for LCEC analysis have been reviewed (Pachla and Kissinger, 1979), and the procedure is compatible with ion-

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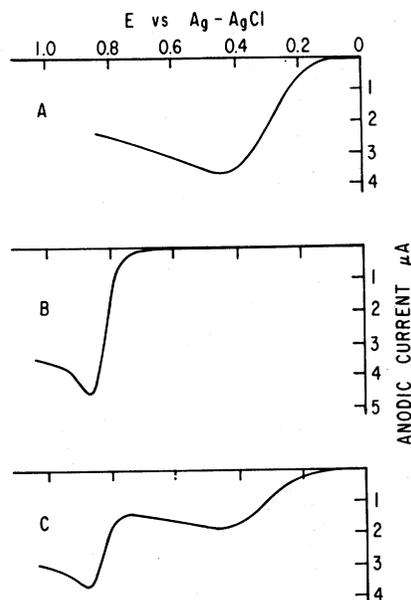


Fig. 9. Linear sweep voltammetry (0.03 V/sec) at carbon paste electrode in 1.0 M acetate buffer (pH 5.25): (A) 1 mM ascorbic acid; (B) 1 mM ascorbic acid 2-sulfate; (C) 0.5 mM ascorbic acid and 0.5 mM ascorbic acid 2-sulfate. [Reprinted with permission from Academic Press and Pachla and Kissinger (1979).]

exchange and reverse-phase HPLC for determining AA and other easily oxidized or reduced compounds.

Figure 9 illustrates the electrochemical behavior of AA and its metabolite AA-2-sulfate. The product of electrochemical oxidation of both compounds is DHAA and the reaction is irreversible. While AA is oxidized at +0.45 V, a much higher potential (+0.88 V) is required for AA-2-sulfate. Several recent reports have demonstrated the versatility of LCEC for analysis of AA in a variety of biological fluids. Mason *et al.* (1980) determined AA in human blood plasma and urine, while Carr and Neff (1980) applied LCEC to the determination of AA in tissues of marine invertebrates. Tsao and Salimi (1981) determined AA in human plasma and obtained levels consistently lower than those found by the colorimetric 2,4-DNPH procedure—suggesting LCEC is more selective. Dozier *et al.* (1982) showed that the method can determine extracellular AA levels in brain, and Lee *et al.* (1982) determined AA in leukocytes. Lee and colleagues used *N*-octylamine as the ion-pairing reagent in reverse-phase separations and also used an internal standard (IS; 3,4-dihydroxybenzylamine hydrobromide) in order to calculate AA levels. The IS and AA had retention times of 3.5 and 5.5

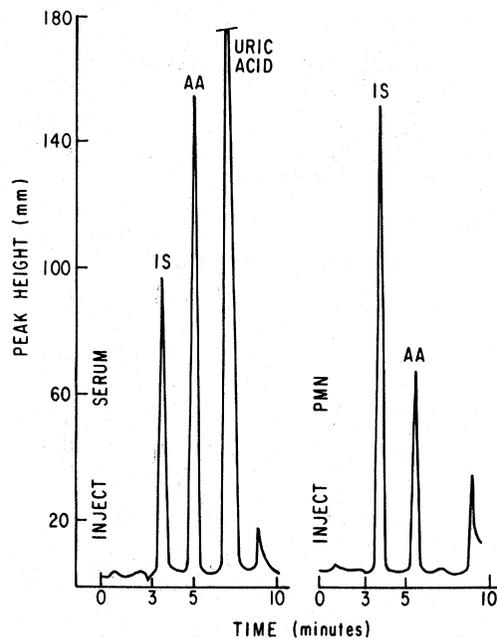


Fig. 10. Ascorbic acid (AA) LCEC chromatograms of preparations from serum and polymorphonuclear (PMN) leukocytes. The internal standard (IS) is 3,4-dihydroxybenzylamine hydrobromide. μ -Bondapak C_{18} column and amphoteric detection were used. [Reprinted with permission from American Association for Clinical Chemistry and Lee *et al.* (1982).]

min, respectively. Figure 10 shows LCEC chromatograms of AA in serum and polymorphonuclear lymphocytes (Lee *et al.*, 1982).

2. Other Chromatographic Methods

Glass-fiber paper chromatography was used (Horn, 1972) to separate AA and several related compounds. The R_f values for AA, DHAA, and DKGA were 0.64, 0.85, and 0.54, respectively. Thin-layer chromatography on cellulose plates was used to separate AA (R_f , 0.52) from EA (R_f , 0.62); spots were detected by spraying the plates with a reducible quinone. Gas-liquid chromatography (GLC) has been used (DeWilt, 1971; Pfeilsticker and Marx, 1974; Schlack, 1974) to separate AA from related compounds. These chromatographic procedures require more time than HPLC, and in the case of GLC, sample derivatization.

An electrophoretic separation procedure, isotachopheresis, has been the subject of two reports (Baldesten *et al.*, 1978; Rubach and Breyer, 1980) which suggest its utility for determining various organic acids, including AA, DHAA, and EA.

F. Other Methods

Several amperometric titration procedures use electrodes to monitor the oxidation of AA. The dropping mercury electrode was applied (Owen and Smith, 1975) for determining AA in foods; this reverse-sweep cathode ray polarographic method requires little sample preparation and is sensitive. Further refinement is needed before it can be widely applied, however, because it is subject to interference from other oxidizable substrates. Carbon electrodes have been used in amperometric determinations of AA (Falat and Cheng, 1982; Lechien *et al.*, 1982; Plotsky, 1982; Wang and Dewald, 1982; Wang and Freiha, 1982). Advantages over the dropping mercury electrodes were outlined, and in one case (Wang and Dewald, 1982) a stopped-flow procedure was applied. The methods were demonstrated to distinguish AA from other readily oxidizable compounds, such as catecholamines in brain tissue (Plotsky, 1982). These amperometric methods may develop into extremely rapid and sensitive procedures for AA.

One atomic absorption spectrophotometric procedure has been reported (Kidani *et al.*, 1981). A Cu^{2+} -neocuproine chelate was reduced by AA to Cu^+ -neocuproine chelate; the reduced form was extracted from the solution into chloroform in the presence of nitrate ion. Copper determination by atomic absorption spectrophotometry allowed the indirect determination of AA.

V. DETERMINATION OF COMPOUNDS RELATED TO ASCORBIC ACID

Procedures for determining EA were detailed in the previous section on HPLC separations. This closely related analog of AA (epimeric at C-5) is as effective an antioxidant as AA; since it is somewhat less expensive than AA, EA is commonly used as a food additive. EA possesses little or no vitamin C activity, however, and may be an antagonist of vitamin C (Hornig and Weiser, 1976; Omaye *et al.*, 1980; Turnbull *et al.*, 1978). For reasons of food quality assurance and nutrition, it is essential that effective methods be available to distinguish EA from AA.

Ascorbyl palmitate is a synthetic derivative of AA (the C-6 ester) and is marketed as an antioxidant for fats and oils. Several colorimetric procedures have been developed and were reviewed by Cooke and Moxon (1981), who reported that no single procedure is preferred. HPLC has not been applied to the determination of ascorbyl palmitate. Another synthetic derivative of AA, L-ascorbate-2-phosphate, has been resolved from AA and related compounds by ion-exchange HPLC on Bondapak AX/Corasil, and detected at 254 nm (Mauro *et al.*, 1980).

Physiological metabolites of AA include 2-O-methyl-AA and AA-2-sulfate,

both of which have been identified in human urine. In studies of the enzymatic methylation of AA by catechol-*O*-methyltransferase, 2-*O*-methyl-AA was efficiently separated from synthetic 3-*O*-methyl-AA by HPLC on a μ -Bondapak C₁₈ column (Bowers-Komro *et al.*, 1982). Bigler and Kelly (1975) and Mauro *et al.* (1980) have successfully applied HPLC to the resolution of AA-2-sulfate in mixtures with closely related compounds. Baker *et al.* (1973) reported that AA-2-sulfate interferes with the 2,4-DNPH colorimetric method, and Terada *et al.* (1978) used a variation of the procedure to determine AA-2-sulfate in the presence of AA. Pachla and Kissinger (1979) demonstrated that LCEC can be used for determination of AA-2-sulfate.

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