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# 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,

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**

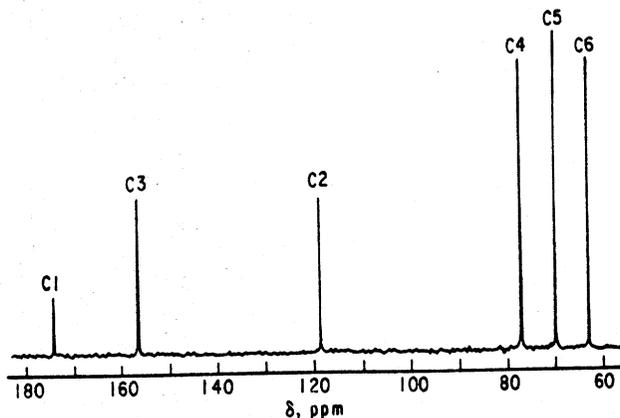
Before AA deficiency results in symptoms of scurvy, other metabolic disorders usually occur. The diagnostic procedures used to detect such disorders require methods of determining AA levels in various tissues. Determination of serum AA levels tends to reflect only recent AA intake rather than stores 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. 2.** Proton-decoupled  $^{13}\text{C}$  NMR spectrum of L-ascorbic acid in  $\text{H}_2\text{O}$ ; pH 2.0,  $33^\circ\text{C}$ . [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

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  $-65^{\circ}\text{C}$ . 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."

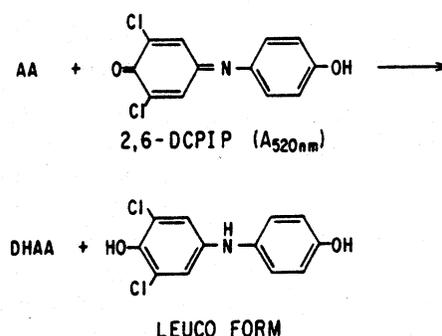
### **IV. DETERMINATION OF ASCORBIC ACID, DEHYDROASCORBIC ACID, AND DIKETOGULONIC ACID**

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-

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).

( $\text{Fe}^{2+}$ ) from the corresponding  $\text{Fe}^{3+}$  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

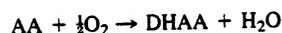
Condensation of DHAA with *o*-phenylenediamine forms a quinoxalanyl lactone (Erlbach and Ohle, 1934; Hensecke and Dittsch, 1959), as shown in Fig. 5. This product fluoresces at 423 nm when irradiated at 348 nm (Archibald, 1945; Ogawa, 1953); this has provided the basis for a sensitive procedure to determine 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

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).

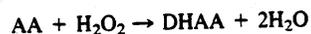
#### D. Enzymatic Methods

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

**TABLE II**  
**HPLC Separations of Ascorbic Acid and Related Compounds**

Silica bonded phase	Detection	Compounds analyzed <sup>a</sup>	Reference
<b>Reverse phase</b>			
Corasil C <sub>18</sub>	Amperometric	Water-soluble vitamins	Stillman and Ma (1974)
μ-Bondapak C <sub>18</sub>	UV, 254 nm	AA in food	Sood <i>et al.</i> (1976)
μ-Bondapak C <sub>18</sub>	UV, 254 nm	Water-soluble vitamins	Wills <i>et al.</i> (1977)
μ-Bondapak C <sub>18</sub>	UV, 254 nm	AA in urine	Wagner <i>et al.</i> (1979)
μ-Bondapak C <sub>18</sub>	UV, 254 nm	AA (2,4-DNPH derivative)	Garcia-Castineiras <i>et al.</i> (1981)
μ-Bondapak C <sub>18</sub>	UV, 210 and 254 nm	AA, DHAA, DKGA	Finley and Duang (1981)
μ-Bondapak C <sub>18</sub>	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 <sub>18</sub>	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 <sub>18</sub>	Amperometric	AA in lymphocytes	Lee <i>et al.</i> (1982)
<b>Anion exchange (strong)</b>			
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)

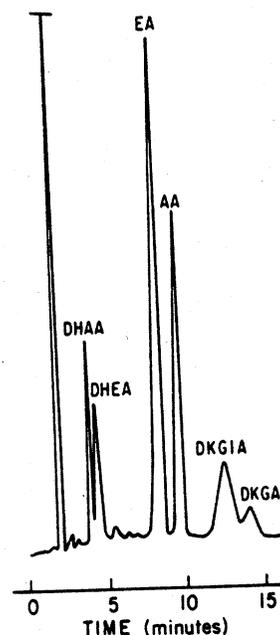
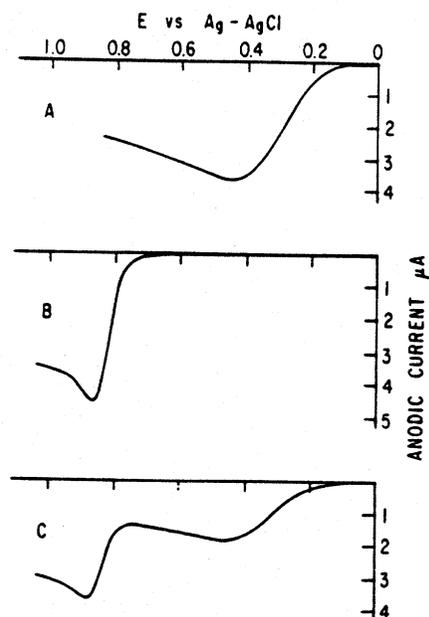


Fig. 7. HPLC chromatogram; the separation of L-dehydroascorbic acid (DHAA), D-dehydroerythorbic acid (DHEA), D-erythorbic acid (EA), L-ascorbic acid (AA), D-diketogluconic acid (DKGIA), and L-diketogluconic acid (DKGA). Conditions: column, Zorbax NH<sub>2</sub>, 4.6 mm × 25 cm; mobile phase, CH<sub>3</sub>CN/0.05 M KH<sub>2</sub>PO<sub>4</sub> (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 NH<sub>2</sub> 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. 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

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  $\text{Cu}^{2+}$ -neocuproine chelate was reduced by AA to  $\text{Cu}^+$ -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,

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