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Rapid High-Performance Liquid Chromatographic Determination of Ascorbic Acid and Combined Ascorbic Acid–Dehydroascorbic Acid in Beverages

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A simple HPLC method is described for the rapid estimation of ascorbic acid and dehydroascorbic acid, the physiologically active forms of vitamin C. Isocratic separation of ascorbic acid was accomplished in 6 min on a μBondapak NH_2 column using a 50:50 (v/v) methanol–0.25% KH_2PO_4 buffer (pH 3.5) solvent. Total ascorbic acid is determined by first reducing the dehydroascorbic acid to ascorbic acid by treatment with DL-homocysteine. This reaction is complete in 15 min and total ascorbic acid analysis is performed immediately. This HPLC procedure has been applied to the analysis of ascorbic acid in selected beverages.

Three classic methods have been developed for the estimation of both ascorbic acid (AA) and dehydroascorbic acid (DAA). Roe et al. (1948) determined DAA by condensation with 2,4-dinitrophenylhydrazine and total ascorbic acid (TAA) by oxidation of AA to DAA and subsequent osazone formation. The procedure of Tillmans et al. (1932) was based on the titration of AA with 2,6-dichloroindophenol. Reduction of DAA followed by titration permitted the estimation of TAA. Finally, DAA was treated with *o*-phenylenediamine which yielded an easily detected fluophor (Deutsch and Weeks, 1965). TAA could be determined after oxidation of AA. Each of these methods has been adapted for semiautomated continuous flow analysis of ascorbic acid in pharmaceutical and food products by Pelletier and Brassard (1975), Garry and Owen (1968), and Kirk and Ting (1975). However, these procedures are nonspecific for ascorbic acid, require the preparation and analysis of blanks, and are time consuming.

Several HPLC methods have been developed for the analysis of AA. These assays utilized various column packings and solvent compositions, and detection is based on the UV absorbance or the electrochemical properties of AA (Sood et al., 1976; Pachla and Kissinger, 1976). No HPLC method has been reported which allows the determination of the combined physiologically active forms of vitamin C, that is, ascorbic acid and dehydroascorbic acid. This paper reports an HPLC procedure for the rapid estimation of AA and TAA in beverages based on the observation of Hughes (1956), who showed that DL-homocysteine could rapidly and completely reduce DAA to AA.

METHODS AND MATERIALS

High-Performance Liquid Chromatography. Separation of ascorbic acid was achieved with a Waters Associates liquid chromatograph, ALC 202, equipped with a Model 6000A pump and a Model U6K injector. Column effluents were monitored at 244 nm, the UV absorbance maximum for AA in the solvent system described, with a Tracor Model 970A UV–vis variable-wavelength detector. A μBondapak NH_2 packed column, 4 mm i.d. × 30 cm (Waters Associates), efficiently separated AA isocratically by using a 50:50 (v/v) methanol–0.25% KH_2PO_4 buffer (pH 3.5) mobile phase. The flow rate was 0.8 mL/min. Sample injection volume was 20 μL.

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Table I. Percent Reduction of DAA to AA by DL-Homocysteine

initial AA,[a] mg/mL	AA, mg/mL, after homocysteine treatment	% reduction
0.500	0.481	96.2
0.500	0.495	99.0
0.500	0.457	91.4
0.500	0.502	100.4
0.500	0.472	94.4
0.500	0.480	96.0
0.500	0.520	104.0
0.500	0.482	96.4
0.500	0.497	99.4
0.500	0.446	89.2
0.500	0.478	95.6
0.500	0.493	98.6
		av: 96.7
		SD: 3.98

[a] AA oxidized with activated charcoal to DAA and then treated with homocysteine.

Sample Preparation. Samples were diluted with distilled water so as to provide an estimated ascorbic acid concentration of 10–100 μg/mL, filtered, and assayed immediately for AA. The analysis of TAA was performed by adding 2.0 mL of a 0.8% DL-homocysteine solution (Research Organics, Inc.) to 0.5 mL of the sample extract (a minimum 40:1 mole excess of homocysteine to dehydroascorbic acid). The pH was adjusted to 7.0 with a 45% K_2HPO_4 buffer. After 15 min, the sample was filtered and assayed immediately. The retention time and response factor for AA were found to be independent of sample pH. Care was taken to exclude light during all steps of sample handling.

If the sample contained proteinaceous material, 15 mL was treated with 5 mL of a 12.5% trichloroacetic acid solution, centrifuged, and filtered, and the filtrate assayed for AA. TAA analysis was then performed on the filtered extract described above, taking care to ensure that the filtrate pH was adjusted to 7.0.

Standardization. Serial dilutions containing 25–100 μg/mL were prepared by dissolving USP reference grade ascorbic acid in the mobile phase. Aliquots (10 μL) of each standard were analyzed, and a standard curve was determined by plotting peak height vs. concentration. Ascorbic acid concentration of the sample extracts was calculated by interpolation on the standard curve, and by application of the dilution factors, the AA concentration of the sample was expressed as milligrams per 100 mL.

RESULTS AND DISCUSSION

Determination of total ascorbic acid by HPLC required a rapid method of converting DAA to AA, since only AA possessed the strong ultraviolet absorption necessary for detection. Hughes (1956) described a simple and convenient procedure for the reduction of DAA to AA with DL-homocysteine. For verification of this method, standard solutions of 0.5 mg/mL AA were prepared and oxidized with activated charcoal to DAA. HPLC analysis of the oxidized solution showed no unreacted AA. The DAA was treated with a minimum 40:1 mole excess of DL-homocysteine. After reaction for 15 min, the solutions were assayed and found to contain 96.7 ± 3.98% of the expected AA (Table I). This confirmed Hughes' results for the efficiency of the reaction and provided a rapid method of reducing DAA for total ascorbic acid analysis.

Typical chromatograms are shown for the elution of a standard solution of ascorbic acid (Figure 1A), AA in a commercial powdered drink (Figure 1B), and the homocysteine-treated powdered drink sample for TAA analysis (Figure 1C). The homocysteine treatment results in a doublet absorbing strongly at 244 nm which is attributed to reacted and unreacted homocysteine but is easily separated from AA.

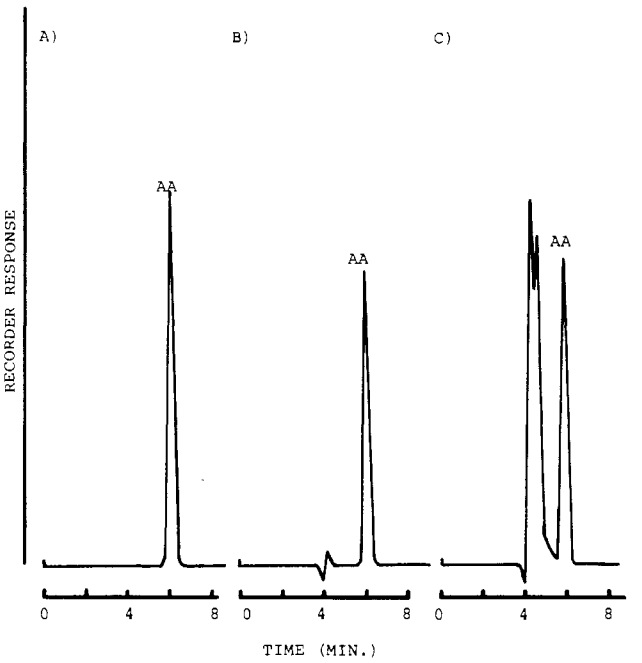

Figure 1. Typical HPLC chromatogram for (A) standard ascorbic acid, (B) ascorbic acid in a powdered drink, and (C) total ascorbic acid in a powdered drink.

Table II. Ascorbic Acid Content of Selected Beverages

beverage, serving size	mg/100 mL ± SD AA	TAA	DAA
orange juice, 6 oz.[a]	60.9 ± 3.9	66.8 ± 5.0	5.9
soft drink, 6 oz.[a]	60.0 ± 3.9	57.9 ± 2.7	
powdered drink, 6 oz.[b]	17.8 ± 1.0	18.4 ± 1.3	0.6
protein-fortified drink, 8 oz.[b]	13.5 ± 0.6	16.4 ± 2.5	2.9

[a] Number of samples = 10. [b] Number of samples = 6.

Table III. Recovery of Added Ascorbic Acid and Dehydroascorbic Acid in Selected Beverages

beverage	% recovery AA	DAA
orange juice[a]	99.8 ± 5.2	103.0 ± 4.8
soft drink[a]	102.2 ± 4.6	99.2 ± 4.6
powdered drink[b]	99.8 ± 7.0	95.0 ± 6.0
protein-fortified drink[b]	104.8 ± 1.8	97.4 ± 4.0

[a] Number of samples = 10. [b] Number of samples = 6.

This rapid HPLC procedure was successfully applied to the analysis of vitamin C in four commercial beverages. The results for ascorbic acid, dehydroascorbic acid, and total ascorbic acid are presented in Table II. The values obtained were in excess of label claims, which is to be expected if the products are in compliance with nutritional labeling regulations. The data in Table III show that both ascorbic acid and dehydroascorbic acid are completely recovered from the food matrices examined.

This HPLC procedure for ascorbic acid is simple, requires a minimum of sample preparation, and provides a method for the rapid determination of physiologically active vitamin C. Further, this HPLC method measured ascorbic acid directly, eliminating the need for time-con-

suming blank determinations. The combined analysis for any one sample may be completed in 25 min. Finally, it is anticipated that this procedure would be applicable to a variety of food products and other biological systems.

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Structure–Taste Relationships among Cyclic Glycols, Levoglucosan, and Methyl Glycopyranosides

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Investigation of the stereochemical requirements for hydroxyl groups in cyclic compounds to elicit a sweet taste showed that, among the diols with known and relatively stable conformations, those that were sweet had oxygen atoms spaced within the range 3.5–5.5 Å. Those that were not sweet had oxygen atoms spaced within the intramolecular hydrogen bonding range (<3 Å) or wider than 5.5 Å. Two synclinal (gauche) vicinal hydroxyl groups in cycloalkanes do not elicit a sweet taste, whereas anticlinal and antiperiplanar conformations of vicinal hydroxyl groups do. In O-heterocyclic glycols and in methyl glycosides, the ring oxygen probably participates with gauche-oriented hydroxyl groups to induce sweetness, because intramolecular hydrogen bonding of an axial hydroxyl group with the ring oxygen reduces sweetness.

The search for nonnutritive sweeteners to replace those of doubtful healthfulness has developed increased interest in establishing a stereochemical basis for sweet taste. Many structural analogues of different classes of sweet compounds have been synthesized to study structure–taste relationships; yet, glycols, the simplest sweet compounds and probably basic for the analysis of sugar sweetness, have not been structurally correlated with taste.

Sugars and sugar derivatives have been correlated by Birch and Lee (1971), Birch et al. (1971), Birch and Lindley (1973), and Dick et al. (1974), peptide analogues by Mazur et al. (1969, 1970), Lapidus and Sweeney (1973), and Van Der Heijden et al. (1978), amino acids, peptides, and proteins by Solms (1969), Lelj et al. (1976), Temussi et al. (1978), and Wieser and Belitz (1977), flavonoid dihydrochalcones by Krbechek et al. (1968) and Horowitz and Gentili (1971), perillartine analogues by Acton et al. (1970) and Unterhalt and Boeschemeyer (1971), phyllodulcin analogues by Yamato et al. (1972, 1977), cyclamate analogues by Unterhalt and Boeschemeyer (1972), and oxathiazinone dioxides by Clauss and Jensen (1973).

Glycols give sweetening powers less than half that of sucrose and obviously do not provide for useful sweeteners. However, the taste of glycols might yield basic information on the primary spacing requirements for electronegative and hydrogen-bonding functional groups in other compounds and in the taste bud receptor site, in view of the molecular theory of Shallenberger and Acree (1967, 1969, 1971) and Shallenberger and Birch (1975). Their theory is distinguished in that it embraces the widest variety of sweet and bitter compounds; moreover, it is sufficiently specific for experimental verification.

The primary objective of this investigation was to determine the spacing requirements for hydroxyl oxygen atoms in glycols of relatively fixed conformations to elicit a sweet taste. Glycols of known or predictable conformations were selected from the cycloalkanediols, anhydro and dianhydro alditols, and a group of methyl 4,6-*O*-methylene-D-glycopyranosides containing only two free hydroxyl groups. Levoglucosan, anhydro alditols, and methyl glycosides also were selected for comparative purposes. Their sweet and bitter tastes were evaluated for intensity by a trained taste panel under controlled environmental conditions.

EXPERIMENTAL SECTION

Cyclic Groups. These compounds were synthesized by published methods or purchased. They were recrystallized, sublimed, or distilled to the established melting or boiling points, and known derivatives were prepared to confirm their identity (Table I). Each compound presented to the taste panel gave only one large symmetrical peak by gas–liquid chromatography (GLC) of the trimethylsilyl (Me_3Si) ether derivatives. The *cis*- and *trans*-1,2- and 1,3-cyclohexanediols were separated by the GLC conditions adopted, but the *cis*- and *trans*-1,4-cyclohexanediols were not (Table I). Identity of the glycols also was confirmed through identity of their infrared absorption bands in carbon tetrachloride (OH stretching) with those published by Kuhn (1952, 1954) and Brimacombe et al. (1958).

Anhydro Sugars and Alditols. These compounds were prepared by well-known methods. The purity was established by GLC. Single, symmetrical peaks were ob-

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