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Fluorometric Determination of Total Vitamin C and Total Isovitamin C in Foodstuffs and Beverages by High-Performance Liquid Chromatography with Precolumn Derivatization

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A reliable and sensitive high-performance liquid chromatographic (HPLC) method is described for the simultaneous determination of total vitamin C (*l*-ascorbic acid, AA, plus dehydro-1-ascorbic acid, DHAA) and its C-5 epimer, total isovitamin C (erythorbic acid, EA, plus dehydroerythorbic acid, DHEA), in foodstuffs and beverages. After extraction AA and EA are oxidized enzymatically to DHAA and DHEA with the aid of ascorbate oxidase (EC 1.10.3.3). The latter compounds are condensed with *o*-phenylenediamine (OPDA) to their highly fluorescent quinoxaline derivatives. These derivatives are separated on a reversed-phase HPLC column and detected fluorometrically. Total vitamin C and isovitamin C can be determined in concentrations as low as 0.2 μg/g. The amounts of DHAA and DHEA present in foodstuffs and beverages can be determined separately by the same procedure with omission of the enzymatic oxidation.

Vitamin C, a water-soluble vitamin, consists of AA and its oxidized form DHAA. Both forms are equally biologically active (Procházka, 1964). The C-5 epimer of AA, erythorbic acid, which is much less biologically active (Procházka, 1964; Pelletier and Godin, 1969), may be used as an antioxidant in foods and beverages.

Numerous methods for the analysis of vitamin C and/or isovitamin C in foodstuffs have been described. These include the indicator-dye reduction method with dichlorophenolindophenol (Hiromi et al., 1980), the ketone derivatization method with dinitrophenylhydrazine (Bourgeois and Mainguy, 1974; Pelletier and Brassard, 1977), fluorometric methods by condensation of DHAA with OPDA (Kirk and Ting, 1975; Egberg et al., 1977), an enzymatic method using ascorbate oxidase (Beutler and Beinstingl, 1980), and HPLC methods with UV detection (Geigert et al., 1981; Bui-Nguyên, 1980; Dennison et al., 1981; Keating and Haddad, 1982) and electrochemical detection (Pachla and Kissinger, 1976; Rückemann, 1980).

However, the indicator-dye reduction method, the ketone derivatization method and the fluorometric methods are not very specific and do not differentiate between AA and EA. Furthermore they have the drawback that blank values have to be determined owing to chemical interference in the color-inducing reaction that can be a source of error. The enzymatic method is difficult to perform in large-scale routine analysis and also does not differentiate between AA and EA.

HPLC methods with electrochemical detection only allow the determination of the reduced forms, AA and EA. Dennison et al. (1981) described an HPLC method for the analysis of total vitamin C in beverages by UV measurement of AA after reduction of DHAA with homocysteine. Keating and Haddad (1982) and Wimalasiri and Wills (1983) described HPLC methods with UV detection for the simultaneous determination of AA and DHAA. However, the authors mentioned did not consider possible interference by isovitamin C.

Therefore, we developed an HPLC method for the simultaneous determination of total vitamin C and isovitamin C. After enzymatic oxidation of AA and EA to DHAA and DHEA, the latter, having themselves insufficient UV absorptivity, are condensed with OPDA to their highly fluorescent 3-(1,2-dihydroxyethyl)furo[3,4-*b*]-quinoxalin-1-ones (DFQ and IDFQ, respectively). These derivatives are separated on a reversed-phase HPLC column and detected fluorometrically.

Furthermore, by omission of the enzymatic oxidation step, the concentrations of DHAA and DHEA in foodstuffs

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and beverages can be determined.

MATERIALS AND METHODS

Reagents. Ascorbate oxidase spatula was obtained from Boehringer, Mannheim, FRG, catalog no. 736.619. *o*-Phenylenediamine (OPDA) was from Merck, Darmstadt, FRG, catalog no. 9721. HPLC mobile phase containing 0.08 M KH_2PO_4 and 20% (v/v) methanol, pH 7.8, flushed with a stream of helium gas for 10 min before use, was used. *l*-Ascorbic acid (Merck, catalog no. 500577)/erythorbic acid (Sigma Chem. Co. St. Louis, MO, catalog no. I-0502) stock standard solution containing about 400 mg/L of both compounds in 1% (w/v) metaphosphoric acid was used. This solution was stored in the dark at 4 °C and was stable for at least 14 days. *l*-Ascorbic acid/erythorbic acid working standard solution was prepared daily by diluting 1 mL of the stock standard solution with water to 25 mL.

HPLC Apparatus. High-performance liquid chromatography was performed using a system incorporating a Gilson Model 302 constant-flow pump (Meyvis and Co, Bergen op Zoom, The Netherlands), a Perkin-Elmer ISS 100 automatic injector with cooling tray (Perkin-Elmer Nederland, Gouda, The Netherlands), and a Kratos FS 950 Fluoromat fluorescence spectrophotometer (Kipp Analytica, Delft, The Netherlands) equipped with a mercury light source (type FSA 110), an excitation interference filter of 365 nm (type FSA 401), and an emission cutoff filter of 418 nm (type FSA 426).

A Hyperchrome SC stainless steel column (125 × 4.6 mm i.d.) equipped with a 2-cm precolumn RP-18 cartridge (Bischoff GmbH, Leonberg, FRG) was home packed with ODS-Hypersil 3 μm (Shandon Southern Products, Ltd., Astmoor, U.K., catalog no. 580 × 24) by the balanced-density slurry technique using a Haskel pump type MCP 110 (Ammann Technik, Stuttgart, FRG). Elution profiles were displayed on a Kipp BD-8 recorder (Kipp Analytica, Delft, The Netherlands).

Extraction and Derivatization Procedures. *For the Determination of Total Vitamin C and Isovitamin C.* A portion of 5 g of foodstuffs was blended for 1 min with 25 mL of 0.3 M trichloroacetic acid (TCA). Thereafter the total volume was adjusted to 50 mL with 0.3 M TCA. A 5-g portion of beverages was diluted with 0.3 M TCA to 50 mL. The foodstuff extract and the diluted beverage were filtered through medium pore size filter paper. The filtrate was diluted with 0.3 M TCA to obtain a vitamin concentration of 1–40 μg/mL. Three milliliters of the diluted filtrate was brought into a 10-mL plastic tube containing 0.4 mL of 4.5 M sodium acetate buffer, pH 6.2. After an ascorbate oxidase spatula was added, the tube was placed in a water bath at 37 °C for 5 min with once mixing after 2 min. Thereafter, 0.5 mL of a freshly prepared 0.1% (w/v) OPDA solution was added. After the contents were mixed, the tube was wrapped with aluminum foil to screen from daylight and placed in a water bath at 37 °C for 30 min. It was then stored in the dark at 4 °C for HPLC analysis within 12 h.

For the Determination of DHAA and DHEA. The treatment of samples for the analysis of DHAA and DHEA was performed as mentioned above but without using the ascorbate oxidase spatula and without subsequent heating for 5 min at 37 °C.

Working Standard Solution. The working standard solution was derivatized by adding 1 mL of it to a 10-mL tube containing 4 mL of 0.3 M TCA and 1.2 mL of 4.5 M sodium acetate buffer, pH 6.2, adding an enzyme spatula and proceeding as described for samples.

High-Performance Liquid Chromatography. HPLC analysis of the derivatives DFQ and IDFQ was carried out by injecting 30 μL of the derivatized sample extract into the ODS-Hypersil column. The tray of the automatic injector was thermostated at 4 °C. The column was eluted isocratically with the mobile phase at a flow rate of 1.0 mL/min. The effluent was monitored with the fluorescence spectrophotometer with the recorder set at 10 mV full scale. Duration of the chromatographic run was about 5 min/sample.

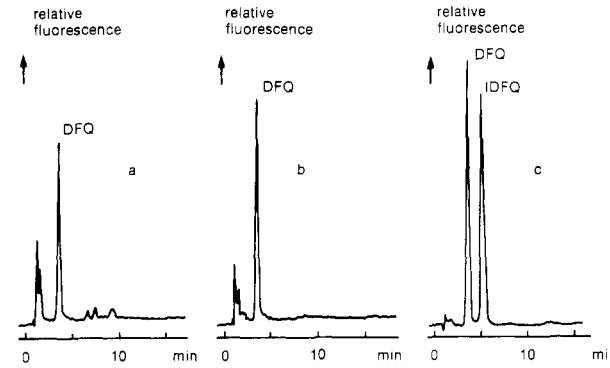

Figure 1. Typical elution profiles of a derivatized fresh green paprika extract (a), of a diluted orange juice (b), and of the working standard solution (c).

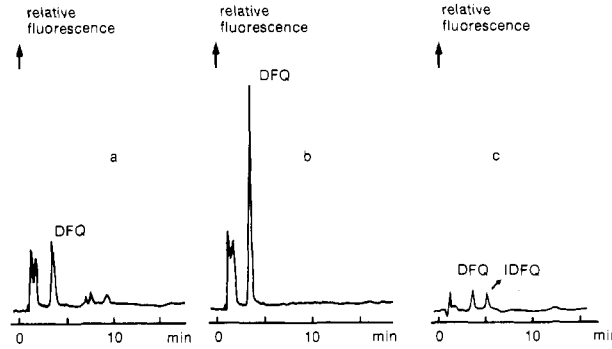

Figure 2. Elution profiles of a fresh green paprika extract (a), of a diluted orange juice (b), and of the working standard solution (c). The extracts were the same as in Figure 1. Both extracts as well as the working standard solution were derivatized as described under Materials and Methods with omission of the enzymatic oxidation.

The vitamin C and isovitamin C concentrations of the original sample were calculated from peak heights with the working standard solution as the reference. In routine analysis this solution was run before each series of five samples.

Selection of Fluorometric Parameters. Under the chromatographic conditions employed DFQ as well as IDFQ had their excitation maximum at a wavelength of 355 nm and their emission maximum at a wavelength of 425 nm. The light source and filters for the fluorescence detector were chosen in accordance with this wavelength pair. Both the excitation and the emission maxima proved to be pH independent over the range 3.5–8.5. A pH of 7.8 was chosen as a result of an optimization procedure for best HPLC separation.

RESULTS

Characteristics of the Method. Typical elution profiles of a derivatized fresh green paprika extract, a diluted orange juice extract, and the working standard solution are shown in Figure 1. In blank experiments only a solvent peak was recorded. The HPLC procedure affords an excellent separation of DFQ, IDFQ, and other extract components. Figure 2 shows the elution profiles of the same extracts and working standard solution derivatized

Table I. Total Vitamin C and DHAA Concentrations in Some Vegetables, Fruit, and Fruit Juices

product[a]	concentration, μg/g, of total vitamin C	DHAA	remarks
green paprika	1000	359	
red paprika	2622	621	
Brussels sprouts 1	823	549	
Brussels sprouts 2	907	739	not fresh
parsley	1428	857	
lettuce	74	19	
cauliflower	761	233	
lemon juice	472	270	freshly pressed from a lemon
orange juice	666	597	freshly pressed from an orange
orange juice	390	390	packed as juice
green peas	170	41	
green cabbage	850	226	
kale	639	186	
avocado	134	134	

[a] The products were bought at a local greengrocer.

with the omission of the enzymatic oxidation. In the orange juice investigated vitamin C seems to be present exclusively as DHAA. The AA standard seems to be oxidized partly to DHAA.

As far as has been investigated the fluorescence response was linear for concentrations corresponding to 0.2 μg/g up to 400 mg/g for total vitamin C as well as for (added) total isovitamin C. Assuming the signal to noise ratio should be at least three, the detection limit of the method described in this paper corresponds to a concentration of 0.2 μg/g for vitamin C as well as for isovitamin C.

A number of vegetables and fruit juices was analyzed for total vitamin C and DHAA. The method was found to give an adequate resolution of DFQ and other extract components. The results of the vitamin C analyses are given in Table I. The levels of total vitamin C and DHAA are on the order of those expected from literature. No EA was observed (<0.2 μg/g).

Derivatization. *Reaction Periods.* The minimum periods needed for complete oxidation of AA and EA to DHAA and DHEA, respectively, and for complete derivatization of these to DFQ and IDFQ were determined for a prepared complete meal sample (a homogenized mixture of meat, vegetables, and potatoes) to which EA has been added as well as for the working standard solution.

Therefore, 3-mL portions of a complete meal extract with added EA and 1-mL portions of the working standard solution were derivatized as described under Materials and Methods, the reaction period for the oxidation step being varied from 2 to 8 min in 2-min steps. A second set of portions of the same complete meal extract and of the working standard solution was derivatized as described with the reaction period for the condensation reaction with OPDA ranging from 5 to 40 min in 5-min steps. An aliquot of 30 μL of the derivatized extract was injected into the ODS-Hypersil column immediately after termination of the reaction period for the condensation reaction.

Results indicated that the minimum reaction period needed for complete oxidation of both AA and EA was 4 min for a complete meal extract as well as for the working standard solution. The minimum reaction period needed for complete derivatization of both DHAA and DHEA with OPDA to DFQ and IDFQ, respectively, was 25 min for a complete meal extract as well as for the working standard solution. No decrease in peak height of DFQ and IDFQ was observed in tests with reaction periods of 8 and 40 min, respectively, as compared to the peak heights observed in tests with reaction periods of 4 and 25 min. The reaction periods of the assay were chosen in accordance with these findings.

Ascorbate Oxidase Activity. In order to determine whether the activity of one ascorbate oxidase spatula employed under the oxidation conditions described sufficed for complete oxidation of the AA and EA, increasing amounts of AA and EA were added to various portions of a complete meal extract, the concentrations of both compounds being varied in the range 1–40 μg/mL. Three-milliliter aliquots of the extracts were derivatized and analyzed for DFQ and IDFQ as described under Materials and Methods.

The results indicated that the activity of ascorbate oxidase from one spatula under the oxidation conditions described sufficed for the simultaneous oxidation of at least 120 μg of AA and 120 μg of EA. From this it was judged that the sample extract has to be diluted with 0.3 M TCA to a concentration per vitamin lower than 40 μg/mL.

Stability of DFQ and IDFQ. Portions of a derivatized extract of a complete meal with added EA were stored under daylight exposure at ambient temperature and in the dark at 22, 4, and −20 °C. A derivatized working standard solution was stored in the dark at 4 °C. After the storage period DFQ and IDFQ in the extract were determined by HPLC. The results are given in Table II. As can be seen from this table DFQ and IDFQ deteriorated rapidly under daylight exposure and in the dark at 22 °C whereas they were fairly stable in the dark at 4 and −20 °C for at least 12 h. Therefore, practical conditions to carry out large-scale routine analysis of vitamin C in foodstuffs and beverages are storage of the derivatized extracts in the dark at a temperature between 0 and 4 °C with HPLC analysis within 12 h.

Precision and Recovery. In order to determine the precision of the method, several portions of one and the same complete meal with and without the addition of AA and EA were analyzed. The results are given in Table III. As can be seen from this table, the within-assay coefficient of variation for AA is sufficiently low. Furthermore, the recovery of AA as well as of EA added to foodstuffs is good.

Table II. Stability of DFQ and IDFQ in a Derivatized Complete Meal Extract and Working Standard Solution[a]

storage period, h	complete meal extract								working standard, dark, 4 °C	
	daylight, 22 °C		dark, 22 °C		dark, 4 °C		dark, −20 °C			
	DFQ	IDFQ	DFQ	IDFQ	DFQ	IDFQ	DFQ	IDFQ	DFQ	IDFQ
0	100	100	100	100	100	100	100	100	100	100
3	63	67	90	-	99	102	100	102	98	98
6	54	55	87	82	99	100	101	100	98	98
12	36	38	70	72	97	97	99	98	97	98
24	12	12	-	-	84	80	90	92	88	88

[a] The stability is expressed as the percentage of the obtained peak height compared with the peak height measured at zero storage time.

Table III. Precision of the Method for Determination of Total Vitamin C and Total Isovitamin C in a Complete Meal

	within-assay precision		recovery test[a]	
	total C	total iso-C	total C	total iso-C
n	6	6	6	6
mean	91.7 μg/g	< 0.2 μg/g	97.1%	99.3%
SD	1.9 μg/g		4.7%	4.7%
CV	2.1%			

[a] The amounts added were 63 μg/g AA and 67 μg/g EA, respectively.

DISCUSSION

The method described in this paper allows a relative fast and sensitive simultaneous determination of total vitamin C and total isovitamin C in foodstuffs and beverages. Also, DHAA and DHEA can be determined simultaneously with the same procedure with omission of the oxidation by ascorbate oxidase. The method is easy to perform and is suited for large-scale routine analysis. The linearity range and sensitivity permit determinations of total vitamin C and total isovitamin C in a concentration as low as 0.2 μg/g. The precision of the method and the recoveries of AA and EA are good.

Keating and Haddad (1982) described a method for the simultaneous determination of AA and DHAA in foodstuffs by HPLC with UV measurement. The UV absorptivity of DHAA was enhanced by using the same derivatization procedure as described in this paper. We observed, however, that fluorometric detection of the derivative highly improved the sensitivity. Although Keating and Haddad also observed an increased sensitivity using fluorescence detection, this advantage was largely offset in their method by increased background signal due to fluorescent impurities that were not separated from DFQ by their HPLC system. The HPLC system with fluorescence detection incorporated in our method provided well-separated DFQ and IDFQ peaks.

Registry No. AA, 50-81-7; EA, 89-65-6; DHAA, 490-83-5; DHEA, 5959-82-0; ascorbate oxidase, 9029-44-1.

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Oligosaccharide Content of Ten Varieties of Dark-Coated Soybeans

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Dry ripe beans of 10 varieties of black- or brown-coated soybeans [*Glycine max* (L.) Merril] were evaluated for oligosaccharide content. Oligosaccharides were extracted with 80% ethanol and analyzed qualitatively and quantitatively by thin-layer chromatography. An average of 3.88% sucrose, 0.79% raffinose, and 3.37% stachyose, on a dry weight basis, was found, with an approximate proportion of 5:1:4 for the three sugars. This ratio was of 4.5:1:4 for the yellow-coated soybeans, widely produced in this region and investigated here for comparison. On a dry weight basis, the sum of raffinose plus stachyose represented 15.84% of the carbohydrates present in dark-coated beans and 16.32% of those present in yellow-coated beans. Due to the similar content of nondigestible sugars, commonly accepted as the cause of flatulence, none of the varieties studied (black-, brown-, or yellow-coated beans) is preferable to any of the others.

The main oligosaccharides present in soybeans (*Glycine max*, L., Merril) are sucrose, raffinose, and stachyose. Verbascose has also been reported to be present, although in minimal amounts (Smith and Circle, 1972). These sugars represent approximately 15% of the dry bean weight and are important sources of energy for embryo development during germination and initial growth (Pazur et al., 1962). The level of oligosaccharides in legume seeds is known to differ among varieties and lines and also according to the degree of ripening (Hymowitz et al., 1972). Hardinge et al. (1965) stated that among the legume seeds studied by them (dry soybeans, dry black mung beans, dry green mung beans, cowpea, field bean, chickpea, horse gram, lentils, canned lima beans, and pigeon pea) soybeans had the highest level of raffinose (1.9%) and stachyose (5.2%). These sugars are even more abundant in ripe and dry beans, with sucrose, raffinose, and stachyose occurring at an approximate ratio of 4:1:2 (Pazur et al., 1962). Kawamura (1967) observed that more than 90% of the sugars present in ripe soybeans consist of sucrose, raffinose, and stachyose.

Soybean oligosaccharides are generally considered undesirable because they represent one of the causes of fla-

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