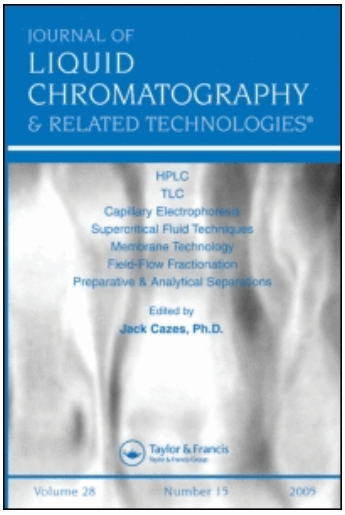

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MEASUREMENT OF TOTAL VITAMIN C ACTIVITY IN CITRUS PRODUCTS BY HPLC: A REVIEW

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ABSTRACT

The reduced form of vitamin C is referred to as L-ascorbic acid (AA), and the oxidized form is referred to as L-dehydroascorbic acid (DHAA). In humans, both forms are biologically active. The total vitamin C activity is defined as the sum of both forms. This review discusses HPLC methodologies for total vitamin C activity with references to their applications mainly in citrus juices and related drink products.

INTRODUCTION

Vitamin C is one of the principle nutrients in citrus and many consumers purchase and consume citrus products because of this vitamin. In citrus, vitamin C is found mainly in its reduced form, L-ascorbic acid (AA). However, its oxidized form, L-dehydroascorbic acid (DHAA), which is often reported, possesses biological activity approximately equal to that of the reduced form of ascorbic acid.[1] Further oxidation of DHAA converts it to the 2,3-diketogulonic acid, which is devoid of biological activity. Methods that assay for only the reduced form of ascorbic acid in biological samples and foodstuffs may provide misleadingly low vitamin C values.[1,2,3]

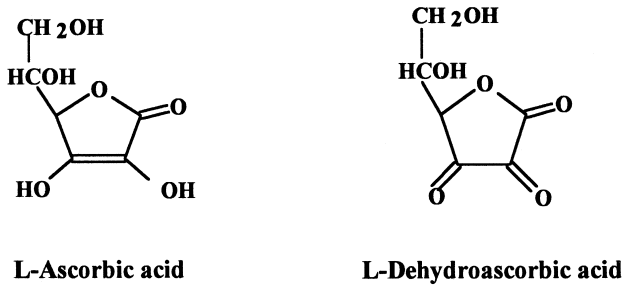

Figure 1. Structures of ascorbic and dehydroascorbic acids.

Thus, the determination of total vitamin C activity in juice, which includes dehydroascorbic acid, is of interest for nutritional labeling and can be a good index for a critical evaluation of processing and storage parameters of citrus products. Furthermore, dehydroascorbic acid may not be a significant fraction of ascorbic acid in citrus products but its importance as a substantial starting material in nonenzymic browning in citrus products has been suggested.[4] Figure 1 shows the structures of ascorbic acid and dehydroascorbic acid.

Thus, the study of the determination of total vitamin C activity in juice which includes dehydroascorbic acid became of interest to claim additional vitamin C activity and to help processors to ensure the vitamin C contents in juice meet the labeling claims throughout its "use by" date.

EXPERIMENTAL

Measurement for total vitamin C activity (AA+DHAA) requires more specific combinations of sample preparation and chromatographic conditions than vitamin C (AA) only. The AOAC official method (AOAC 967.21) for the determination of ascorbic acid is restricted to the measurement of the reduced form of vitamin C only and does not measure total antiscorbutic activity. Also, this method is susceptible to interferences from other compounds in food samples due to the lack of sample preparation steps.

For total vitamin C activity, which includes dehydroascorbic acid, the AOAC microfluorometric method (AOAC 967.22 or 984.26) is specific for total vitamin C and is applicable to products considered to be good sources of vitamin C. However, this method is rather time consuming and is reported to give unrealistically high values. Also, for foods with naturally occurring low levels of vitamin C, a large sample size is required, which increases the likelihood of matrix interference.[5]

Since the most common method of determining vitamin C in fruit products is by high performance liquid chromatography (HPLC), the scope of this article was limited to works with HPLC. HPLC is preferred over traditional techniques of titrimetry and fluorimetry because it is more sensitive and permits more straightforward sample workup procedures.[6] HPLC methodologies for total vitamin C activity include direct measurement, pre- and post-column derivatization, and indirective reduction methods.

Direct Measurements by Simultaneous Analysis of AA and DHAA

Several modes of HPLC including anion exchange, ion-pair reversed phase, and reversed phase are available for total vitamin C in citrus products. The direct measurement of total vitamin C activity (AA+DHAA) using simultaneous analysis of ascorbic acid (AA) and dehydroascorbic acid (DHAA) by high performance liquid chromatography (HPLC) has been the first choice. Several chromatographic conditions were developed to achieve the resolution of ascorbic acid and dehydroascorbic acid.

HPLC with reversed-phase mode has often been used with two analytical columns connected in series to improve the resolution, and eluted with mobile phase with ion-pairing agents. In reversed-phase analysis with μBondapak C_{18} column for ion-pair RP-HPLC, tri-n-butylamine (0.7 ml/L) in 40 mM phosphate buffer, pH 4.3,[7] aqueous methanol containing 0.25 mM hexadecyltrimethyl ammonium bromide, or 40 mM tridecylammonium formate as ion-pair reagents[8] were used as mobile phases. In the ion-pairing mode, ammonium compounds or tertiary amines are added to the mobile phase to control the retention of the compound. In a simultaneous estimation of ascorbic acid and dehydroascorbic acid, ascorbic acid and dehydroascorbic acid were detected at 254 nm and 210 nm, respectively, by two UV detectors.[7,9]

For HPLC in the anion-exchange mode, phosphate-buffered, aqueous acetonitrile solutions were used as the mobile phases in combination with a Zorbax NH_2 analytical column,[10] Lichrosorb-NH_2[11] or μBondapak/carbohydrate[12] as the stationary phases. Using a Dionex strong anion exchange resin, Vanderslice and Higgs[13] were also able to separate dehydroascorbic acid from ascorbic acid itself. The efficiency of the column, however, was very low. Figure 2 shows the HPLC chromatogram of ascorbic acid and dehydroascorbic acid under the same condition detected at 245 nm. Ascorbic acid and dehydroascorbic acid can be separated by HPLC with an amine column and quantitated by absorption at UV 245 nm or at 210 nm.

However, a major drawback with the direct HPLC method for total vitamin C activity was a lack of sensitivity for DHAA. The dehydroascorbic acid poorly absorbs UV light at the same wavelength as ascorbic acid, has no defined absorption spectra in the near UV,[14] and is electrochemically inactive.[15] In order to improve the sensitivity, Wimalasiri and Wills[12] and Finley and Duang[7] used

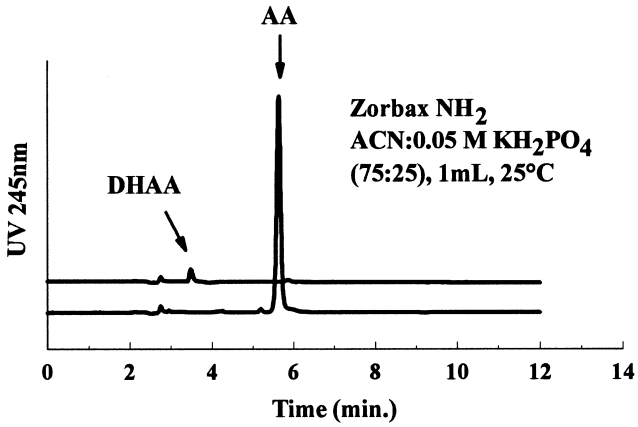

Figure 2. HPLC for AA and DHAA.

UV detection at 245 nm and 210 nm to measure ascorbic acid and dehydroascorbic acid, respectively. Wimalasiri and Wills[12] described that detection limit of 100 ng for dehydroascorbic acid at 214 nm which is significantly high when compared to ascorbic acid of 4 ng at 254 nm.[16] However, when the concentration of dehydroascorbic acid is low, UV detection at 210 nm is not sensitive enough to accurately measure dehydroascorbic acid because of interference from the solvents at low wavelengths. Furthermore, the coelution of an interfering substance makes it difficult to measure both vitamin C compounds in citrus juices simultaneously.[17] A combination of UV and refractive index detectors was also applied to simultaneously measure AA and DHAA for total vitamin C activity in orange juice[10] but this technique did not provide adequate resolution of DHAA from other substances and adequate sensitivity.

Therefore, most of the HPLC analytical procedures used for total vitamin C activity are based on indirect methods including pre-column or post-column derivatization of dehydroascorbic acid with complexing agents. Total vitamin C can be measured indirectly after converting DHAA back to ascorbic acid with reducing agents or after the native ascorbic acid has been oxidized to dehydroascorbic acid.[2]

Derivatization Methods

A complicated derivatization procedure for total vitamin C activity in citrus juice is based on the production of derivatives of dehydroascorbic acid with either 2,4-dinitrophenyl hydrazine (DNP) or *o*-phenylenediamine (OPDA) to enhance the poor UV-absorbing capacity of dehydroascorbic acid. Most

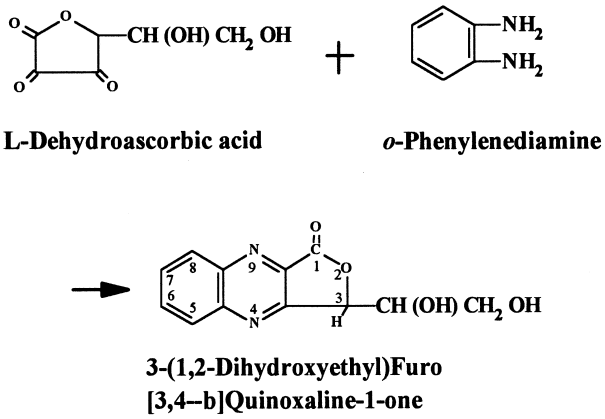

Figure 3. Formation of a fluorescent compound.

reports were based on precolumn HPLC with derivatization of dehydroascorbic by OPDA[8,13,18,19] and DNP[20,21] and postcolumn HPLC with on-line derivatization by OPDA. The principle of total vitamin C measurement by derivatization is based on oxidation of ascorbic acid to dehydroascorbic acid, and the conversion of dehydroascorbic acid into a fluorophore with *o*-phenylenediamine (OPDA), and fluorescence detection of the resulting quinoxaline derivative by HPLC. Neither ascorbic acid nor dehydroascorbic acid is naturally fluorescent, thus, the reaction of dehydroascorbic acid with OPDA to give a fluorescent quinoxaline has been used as the basis of an assay to determine microgram quantities of total vitamin C activity.[18] Oxidation of ascorbic acid to dehydroascorbic acid could be done by mercuric chloride[22] or Norit.[5]

Derivatization techniques using pre-column or post-column systems can enhance the sensitivity of dehydroascorbic acid for total vitamin C measurements by UV or fluorescence detection. Fig. 3 shows the formation of a fluorescent compound from reaction of dehydroascorbic acid with OPDA. Dehydroascorbic acid condenses with OPDA to form highly fluorescent quinoxaline derivatives[8,13,18,19] to enhance the sensitivity, and can then be quantified by fluorometry at 348 nm. Keating and Haddad[8] produced a dehydroascorbic acid-OPDA derivative for increased sensitivity in analyzing orange juice. A high sensitivity can be obtained when the quinoxaline derivative is synthesized prior to injection and assayed directly after elution from the column.[8]

Vanderslice and Higgs[13] proposed an HPLC method with fluorometric detection and post-column derivatization involving oxidation of ascorbic acid to dehydroascorbic acid followed by reaction with OPDA to form a fluorescent product. Later, Kacem et al[19] modified previous work of Vanderslice and

Higgs[13] to obtain an estimation that includes ascorbic acid and dehydroascorbic acid as a separate value, using a single injection and omitting the oxidation step of ascorbic acid. Ascorbic acid was detected at 245 nm in the weak anion exchange chromatographic mode whereas dehydroascorbic acid was derivatized with OPDA using a post-column reaction system and detected fluorometrically. Vanderslice and Higgs[23] used anion-exchange chromatography with post-column oxidation of ascorbic acid to dehydroascorbic acid followed by derivatization with OPDA and fluorescence detection required. A specialized two-step post-column reaction system was required but was applicable to several food products.[23] This method resulted in significant peak broadening and a long run time of more than 30 min.

Later, this method was further modified and automated for extraction and quantitation steps.[22] Total vitamin C was quantitated by oxidizing ascorbic acid to dehydroascorbic acid with mercuric chloride, reacting the total dehydroascorbic acid then present with *o*-phenylenediamine (OPDA) to form a fluorescent derivative.

A comparison of the total vitamin C activities obtained by three methods (AOAC, AOAC-modified, and FIA) for different foods including orange juice has been reported, and the mean values agree reasonably well.[22] Vanderslice and Higgs[24] also reported post-column derivatization which involved oxidation with $HgCl_2$ and subsequent reaction with OPDA. Two PLRP-S reversed-phase columns are connected in series and maintained at 4°C. Zapata and Dufour[25] reported pre-column derivatization of dehydroascorbic acid and ion-pair HPLC method for simultaneous determination of ascorbic acid, dehydroascorbic acid, and isoascorbic acid. Later, Dodson et al.[5] reported an improved AOAC microfluorometric method which is based on oxidation of ascorbic acid with Norit, followed by reaction with *o*-phenylenediamine (OPDA) to form fluorescent derivatives. This method claims to overcome the effects of matrix interference, and elimination needed for the blank corrections as well as additional cleanup steps.

In the derivatization, the concentration of OPDA is most important. Increasing OPDA concentration can accelerate the derivatization reaction, however, there was also a tremendous negative effect on both intensity and stability.[25] Concentration of 85 mg OPDA/100 mL and 37 min reaction time were selected as optimum condition for derivatization of dehydroascorbic acid. The pH range between 2.2 and 2.45 was considered as another factor for derivatization, and side reactions can occur at higher pH values.[25] Post-column derivatization of DHAA for HPLC could also be accelerated with the presence of a complexing agent, such as sodium borohydride.[26] Both derivatization reagents (OPDA and DNP) are known to react with other reductones, including diketogulonic acid, and this can lead to measurement errors in a single chromogenic assay.[27] Further problems with the stability of the derivatives in aqueous solutions were often experienced.

Total Vitamin C by Reduction of Dehydroascorbic Acid to Ascorbic Acid

The reductive method is the most commonly used for the determination of total vitamin C activity in citrus products.[3,6,9-11,28-38] In the reductive method, DHAA is first reduced to AA by reducing agents before analysis, thus, total ascorbic acid content (which includes the oxidized form of ascorbic acid, dehydroascorbic acid) can be measured directly by HPLC. The reduction of dehydroascorbic acid back to ascorbic acid can be accomplished using sulfhydryl compounds such as Homocysteine, D,L-dithiothreitol (DTT) or sodium hydro sulphide (NaSH).

Among these thiols, D,L-homocysteine[3,9,11,28-30] and D,L-dithiothreitol[6,10,31-36] are most frequently applied in foods due to their favorable redox potentials and are known for specifically reducing dehydroascorbic acid. Reduction method requires little additional sample manipulation and diketogulonic acid is unaffected by D,L-dithiothreitol (DTT).[27] However, reducing agents can interfere with the detection method itself, since most of the detection methods are based on oxidation-reduction reactions with ascorbic acid.[37] Interference by reducing agents can be circumvented by separation from ascorbic acid prior to its detection.

Sybilska et al.[38] achieved the fast separation of ascorbic acid from cysteine and glutathione (GSH) by using RP HPLC with Lichrosorb RP-18 and elution with 0.067 M phosphate-buffered 0.25% oxalic acid (pH 2.9). HPLC with weak anion exchange mode improved resolution from reducing agents and is most commonly used. The eluent was composed of acetonitrile and a phosphate buffer. Farber et al.[16] reported the separation of ascorbic acid from DTT with Whatman Partisil 10SAX stationary column and 7 mM KCL/ 7 mM KH_2P0_4 (pH 4.0) as the mobile phase. Table 1 summarizes various HPLC methods for the determination of total vitamin C activity in citrus juices and other related products.

Fig. 4 shows the principles of reduction of dehydroascorbic acid by DTT into ascorbic acid. In a typical reaction with DTT developed in our laboratory with citrus juices (Fig. 5), 9 mL of stock DTT solution (2.3 mM DTT in 0.1 M K_2HPO_4, pH 7.0) was added to 1 mL citrus juice sample to bring the final DTT concentration to 2.1 mM. The reduction of neutralized orange juice was accomplished by centrifuging the juice for 10 min at room temperature (ca. 23°C), and then 1 mL of clear supernant was diluted with 9 mL of 2.5% metaphosphoric acid solution prior to filtering (Nylon, 0.45 μm) and injection (Fig. 5). It was necessary to dilute the citrus juices a further 10-fold to obtain suitable peak shapes. For homocysteine, which was also tested in our laboratory with orange juice (Fig. 5), 9 mL of stock homocysteine solution (53 mM in 0.1 M K_2HPO_4, pH 7.0) were mixed with 1 mL juice (final homocysteine concentration, 47.7 mM). The same procedures were then followed as with DTT. Fresh stock DTT and homocysteine solutions were prepared in phosphate solution (0.1 M K_2HPO_4, pH 7.0).

Table 1

HPLC Analysis of Total Vitamin C in Citrus and Other Food Products

Type of Food Analyzed	Extraction Conditions	HPLC Columns and Mobile Phase	Detection Conditions	Reference
Vegetable juice, applesauce, instant mashed potatoes, nectarine, grape drink, frozen peas, tomato, broccoli, green pepper, orange juice	Extracted with sulfuric acid and DHAA reduced by DTT	Anion exclusion Ion Guard cartridge with anion exclusion/HS column (sulfonated polystyrene/divinyl-benzene) (100 x 4.6 mm), 20 mM sulfuric acid solution.	Electrochemical detector with Pt electrode with oxidation potential of 0.6 or 0.8 V vs Ag/AgCl reference electrode was used for detection of AA. Sensitivity range was 100 nA full scale.	33
Satsuma mandarin juice, tomato juice and fruit	Reduction with 0.2 M Na_3PO_4 and 100 mg% DTT-ethanol, incubate 30 min @ 30°C. Add 2% HPO_3, 3.5 mL.		Measure difference spectra @ 243 nm.	32
Cocoa, chocolate, canned juices (orange juice and drinks),vacuum packed meats, powdered drinks, cereals, canned vegetables	AA is oxidized to DHAA with activated charcoal and reacted with o-phenylene-diamine to produce a fluorescent compound.	Reagents: 0.05% N-Bromosuccinimide 0.05% O-Phenylenediamine dihydro-chloride, 5% Boric acid, 0.5% Oxalic acid.	Fluoronephelometer equipped with 365 nm excitation filter and 435 nm emission filter.	43
Fruit juices (orange, lemon, lime, grapefruit), vegetables, beer	MECC: Add 3% m-phosphoric acid, blend, filter. Add D-erythorbic acid in DTT, dilute with DTT and mix. Pass through C18 SepPak cartridge, filter.	Fused silica capillary column (65 cm x 75 μm for fruit analysis, 40 cm long). Buffer: 0.05 M sodium de-oxycholate, 0.01 M sodium borate and 0.01 M potassium dihydrogen orthophosphate.	Electropherograph @ 254 nm at 0.2 AUFS	35

	Add 3% m-phosphoric acid, blend, filter. Add D-erythorbic acid in DTT, dilute with DTT and mix. Filter	C18 NovaPak Radial Pak cartridge with C18 precolumn. 0.2% aqueous orthophosphoric acid, flow rate of 1.0 mL/min.	UV @ 254 nm, at 0.2 AUFS.	35
Wine, fruit juice (orange juice and drinks), beer	MECC: SDS buffers-2 mL wine, fruit juice or 5 mL of beer were added to 0.5 mL nicotinic acid sol'n. (1 mg/mL) and diluted to 10 mL with DTT solution (2 mg/mL) and filtered through a 0.45 µm cellulose acetate filter.	SDS: 100 cm x 75 µm i.d. fused-silica capillary column, 75 cm long, buffer: 0.05 M (1.44 g SDS in 100 mL 0.005 M dipotassium hydrogen orthophosphate, pH 9.2).	MECC: Electropherograph, 254 nm @ 0.01 AUFS.	6
	Sodium deoxycholate buffer-2 mL wine, or 5 mL of beer were added to 0.5 mL nicotinic acid solution (1 mg/mL) and diluted to 10 mL with DTT solution (2 mg/mL). For fruit juices, 0.5 mL was added to 1 mL D-erythorbic acid solution (300 µg/mL) and diluted to 10 mL with DTT (2 mg/mL). Apple juice was also diluted with 3% m-PO_4 solution before being mixed with internal standard and diluted with DTT.	Sodium deoxycholate buffers: 75 cm x 75 µm i.d. fused-silica capillary column, 50 cm long, buffer: 0.05 M (2.16 g sodium deoxycholate in 100 mL of a 1:1 mixture of 0.02 M potassium di-hydrogen orthophosphate and 0.02 M sodium tetraborate, pH 8.6).		6
	CTAB buffers: 2 mL wine and 0.4 mL fruit juice were added to 1 mL D-erythorbic solution (300 µg/mL) and diluted to 10 mL	CTAB: 100 cm x 75 µm i..d. fused-silica capillary column, 75 cm long, buffer: 0.05 M (1.83 g CTAB in 100 mL of a 1:1 mixture of 0.02 M potassium di-		6

(continued)

Table 1 (Continued)

Type of Food Analyzed	Extraction Conditions	HPLC Columns and Mobile Phase	Detection Conditions	Reference
	with DTT (2 mg/mL). Reduced with DTT for 12 hr.	hydrogen orthophosphate and 0.02 M sodium tetraborate, pH 8.6). Buffers were filtered through a 0.45 μm cellulose acetate filter and changed daily.		
	Fruit juices and wines were diluted 5x with 3% m-PO_4 and further diluted 2x with DTT (2 mg/mL). Beer was diluted 2 x with DTT (2 mg/mL).	C18 NovaPak Radial Pak column with precolumn. Mobile phase: 0.2% aqueous orthophosphoric acid, flow rate 1.0 mL/min.	UV @254nm @ 0.2 AUFS.	6
Orange juice and wine	AA converted to DHAA by stirring in ethanol containing activated charcoal with air bubbling through solution or reduction with DTT.	Zorbax NH_2 column (4.6 x 250 mm) in a weak anion-exchange mode. Aceto-nitrile: 0.05 M KH_2PO_4 (75:25,w/w), flow rate of 1.5 mL/min.	UV @ 268 nm for AA, RI for DHAA or DHAA by UV after reduction with DTT.	10
Orange juice, lemonade, soft drink infant formula, pea puree, pear puree, cranberry juice, apple juice	Reduction by DTT in dark for 2 hrs.	C18 (250 x 4 mm) with guard column, 0.5 % KH_2PO_4 and 0.1% DTT. Flow rate of 0.4 mL/min.	UV detection @ 254 nm	36
Tomato, Kiwi, orange juice	Sep Pak C18 cartridge clean-up. Added OPDA solution, pH adjusted to 2.20-2.45 after oxidation of OPDA. Filtered, placed in dark for 37 min.	μ-Bondapak C18, 10 μm (30 cm x 3.9 mm) with C18 precolumn. Methanol-water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate, pH 4.59. Flow rate of 1.8 mL/min.	UV detection @ 348 nm for DHAA (DHQ), and changed to 261 nm for AA and IAA.	25

Tomatoes, green peppers, orange juice	Extract with 3% metaphosphoric acid and 8% acetic acid, homogenize, filter, reduce with homocysteine (pH 6.8), add metaphosphoric acid, filter.	Nucleosil 5 C18, 5 x 10^3% (m/v) tetrahexylammonium hydrogen sulfate plus 6 x 10^3% (m/v) acetic acid, to pH 5 with sodium hydroxide.	UV 264 nm or electrochemical with glassy carbon electrode at 800 mV versus Ag/AgCl electrode.	47
Pharmaceuticals, beverages, (orange drink, pineapple-grapefruit drink, pineapple-orange drink, frozen concentrated orange juice), liquid weight control diets, infant formula, whole wheat cereal	Extracted with metaphosphoric acid-acetic acid. AA is oxidized to DHAA with Norit and reacted with o-phenylenediamine di-hydrochloride for 35 min.	Microfluorometric assay	Fluoro-microphotometer with lamp No. F4T4/BL, primary filters-Corning Nos. 7380 and 5860, secondary filters-Corning Nos. 5113 and 3389.	18
Beverages, (orange juice)	AA only: Dilution with water, filtration. DHAA: Addition of homocysteine, pH adjusted to 7.0 with 45% K_2HPO_4 buffer, hold 15 min, filter and assay.	μ-Bondapak NH_2, 4 mm x 30 cm. Methanol-0.25% KH_2PO_4 buffer (50:50, v/v), pH 3.5, flow rate 0.8 mL/min.	UV @ 244 nm	29
Citrus Juices	AA only: Mixed with ethanol and 8%metaphosphoric acid, centrifuged. DHAA: Mixed with ethanol and 8% metaphosphoric acid, centrifuged, mixed with 0.3 M sodium phosphate and 1.2% NaSH, incubated for 20 min, diluted with 8% metaphosphoric acid and filtered	Cosmosil 5 C18 (4.6 mm x 150 mm) at 40°C. 0.2% metaphosphoric acid, flow rate of 1.0 mL/min.	UV @ 243 nm	48

(continued)

Table 1 (Continued)

Type of Food Analyzed	Extraction Conditions	HPLC Columns and Mobile Phase	Detection Conditions	Reference
Orange juice, tomato juice, green pepper, spinach	Reduction of 2,6-dichloro-phenolindophenol. Reaction with 2,4-dinitrophenyl-hydrazine with 2,3-diketo-gulonic acid.	Two μ-Bondapak C18 columns (30 cm x 3.9 mm), Disodium phosphate at conc. of 0.004-0.04 M, addition of counter-ion reagents (PIC-A or 0.7 mL, tri-n-butyl amine/L, filtration, pH adjusted with 4 M H_3PO_4, flow rate of 0.7 mL/min.	UV @ 210 nm and 254 nm	7
Orange juice	Extraction witb 1% citric acid containing 0.05% EDTA in 50% methanol. AA: determined by addition of juice to extraction solution and filtered. DHAA: determined by adding juice and homocysteine and adjusting to pH 7 with 45% K_2HPO_4. Reaction time was 15 min and extraction solution was added and solution filtered.	Zorbax NH_2, 5 μm, 4.6 mm x 25 cm. Methanol: 0.25% potassium dihydrogen orthophosphate KH_2PO_4 buffer (60:40), pH 3.5.	UV detection @ 244 cm.	3
Vegetable juice, green pepper, orange juice, tomatoes, apples, bananas, carrots	Activated charcoal technique	Fluorometric analysis, post column tubing: 40 mm x 32 cm, reaction tubing: 38 m, cooling tubing: 3 m. Carrier stream: 0.1 M citrate buffer, pH 4, containing 2.5 mM $HgCl_2$, Flow rate was 1.0 mL/min. Oxidizing stream: 2.5 mM $HgCl_2$ in water, flow rate 0.75 mL/min. Fluorophor reagent: 3.1 mM OPD in water, flow rate 0.75 mL/min.		22

Broccoli, orange juice, fruit punch, orange drink, parsley, tomato, strawberry, banana	Mixed with 3% metaphosphoric acid and SepPak elution.	Altech NH_2 in weak anion-exchange mode. Postcolumn derivitizaiton, post column tubing: 0.40 mm, reaction length: 20 m, cooling length: 2 m. 75% Acetonitrile in 0.05 M monobasic potassium phosphate, pH 5.9, flow rate 1.5 mL/min	UV @ 254 nm. Flourometric detector	19
Canned corn, canned potatoes,infant formula, medical food, potato chips, cereal, green beans, cranberry juice, cranapple juice, multi-vitamins, orange juice, tomato juice, broccoli	3% metaphosphoric acid in 8% acetic acid. Add acid wash Norit Addition of sodium acetate and methanol, dilute with water. Add OPDA solution, dilute with mobile phase, shake, stand for 60 min.	µ-Bondapak C18, 30 cm x 3.9 mm Methanol-water, 55/45, filter	Fluorescence, sensitivity @ 1.0, slit @ 7 mm, excitation-350 nm, emission-430 nm, flow rate1.0 mL/min.	5
Orange, grapefruit, and lemon juices	Reduction with mercaptoethanol	Isotachophoresis		49

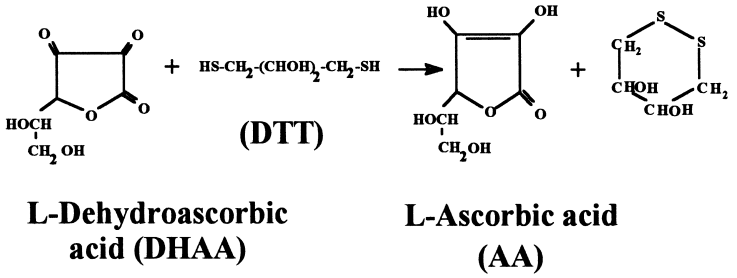

Figure 4. Principles of total AA determination by reducing agent.

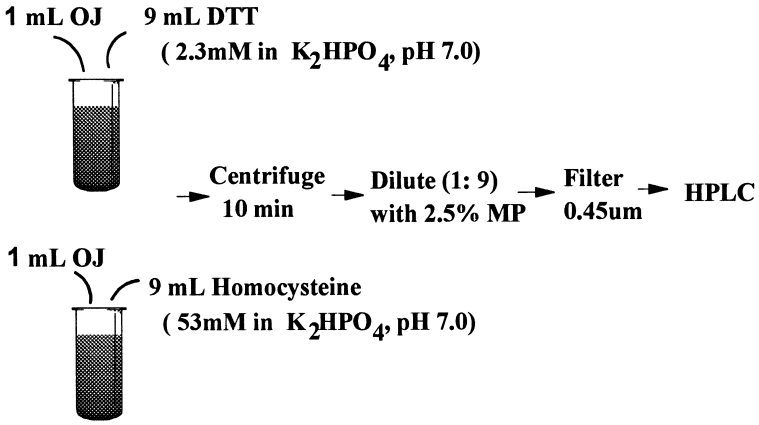

Figure 5. Procedures for total AA (AA + DHAA) determination by HPLC.

The reduction of dehydroascorbic acid by DTT and homocysteine are known to be pH dependent,[28,31] reaching a maximum at pH 6.5-8.0. Okamura[31] showed that optimal pH for the reduction was 6.5-8. The pH of the juice needs to be maintained at near-neutral pH. Fig. 6 shows the effects of pH on reduction of dehydroascorbic acid into ascorbic acid, which are adopted from a previous study of Okamura.[31] The range effective for the reduction of dehydroascorbic acid by homocysteine is narrower than that by DTT.[31]

Reduction efficiency can also be affected by temperature and potential concentration of dehydroascorbic acid in juice and concentration of reducing agents used.

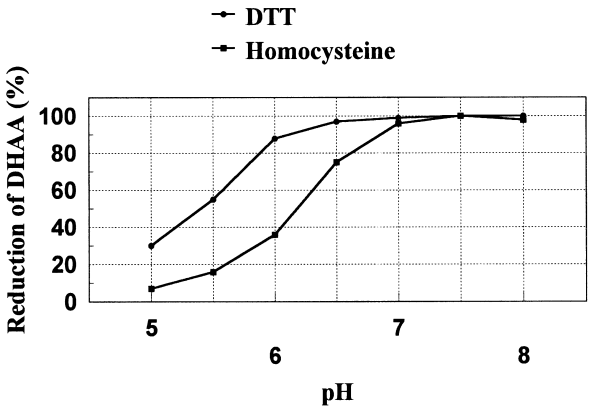

Figure 6. Effects of pH (adapted from Ref. 31).

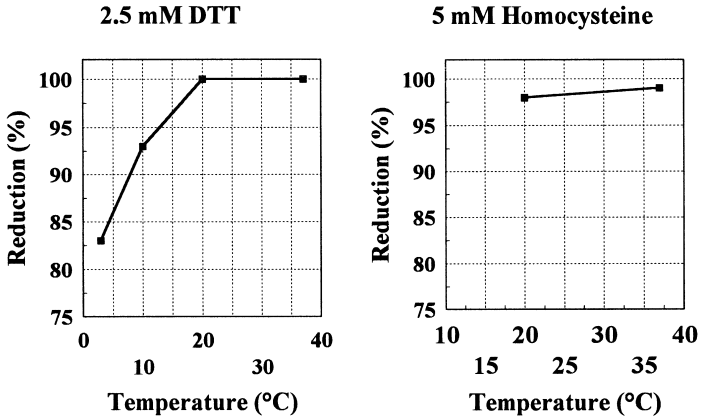

Figure 7. Effects of temperature (adapted from Ref. 31).

Okamura[31] indicated that reduction efficiency with DTT increased slightly as the temperature increased from 3 to 37°C at 2 min incubation time as shown in Fig. 7. There were no significant differences between 20 and 37°C with homocysteine.

Reduction conditions and efficiencies can also vary with various reducing agents. In the test with 0 to 10 mM of DTT,[31] the percentage of reduction of dehydroascorbic acid increased from 21% to 99% as the concentration of DTT increased up to 1.0 mM, but there were no significant differences reported from

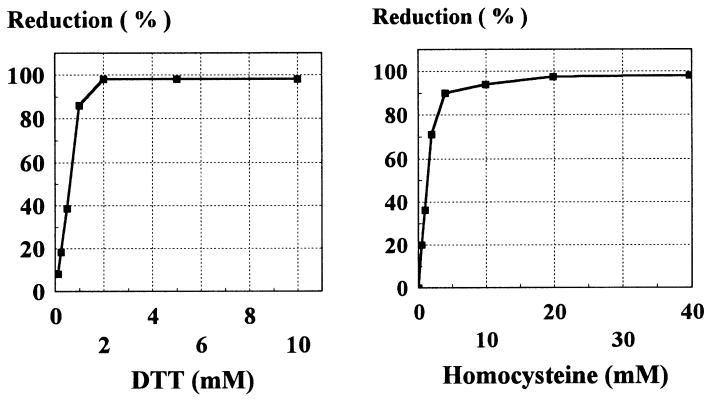

Figure 8. Effects of concentration.

DTT concentration higher than 1 mM with 0.57 mM of dehydroascorbic acid. The 2.5 mM of DTT was sufficient for the reduction of 0.57 mM dehydroascorbic acid. Okamura[31] showed that reduction of dehydroascorbic acid by both DTT and homocysteine is complete within 2 min at 20°C and a similar result was obtained by Kim.[33] The reduction was further tested and no further change was obtained during the following 80 min.[27] The potential impurity in commercial dehydroascorbic acid was considered to be responsible for low reduction efficiency[33] of dehydroascorbic acid, and disparity in fluorometric responses between dehydroascorbic acid and ascorbic acid.[22]

Reduction efficiency of both reducing agents, DTT and homocysteine, was verified later using standard dehydroascorbic acid in our laboratory and was found to be effective for the rapid reduction of dehydroascorbic acid to ascorbic acid within 10 min,[39,40] which is in good agreement with previous works.[28,31,33] However, a higher concentration of homocysteine was required to reach the same level of reduction efficiency as DTT at 23°C, pH 7.0 (Fig. 8). These concentrations of reducing agents could provide a several ten-fold stoichiometric excess of the micromolar dehydroascorbic acid concentrations that are present in citrus juices. Previously, a minimum 40:1 mole excess of homocysteine to dehydroascorbic acid was suggested for nearly complete reduction at room temperature.[28,29] Acar and Gokmen[36] reported a recovery rate less than 90% for vitamin C from orange juices using reductive HPLC method with DTT.

Lee[39,40] presented the results from a comparison study with two reductive methods for the determination of total vitamin C in citrus juices, and also presented the partition of the oxidized form and reduced form of ascorbic acid in commercially processed Florida citrus products. Two reducing agents, D,L-

homocysteine and D,L-dithiothreitol (DTT), were compared for total vitamin C activity determination in citrus juices. Both reducing agents were effective and reproducible. The total vitamin C contents obtained by the two reductive methods was highly correlated (r=0.9783). The high correlation coefficient indicates that the two methods are comparable, having a strong positive linear relationship. From statistical evaluation of the values obtained by the two reductive methods, there was no evidence of a statistically significant (P=0.05) difference between the methods.[40] Reproducibility between samples was good; coefficient of variation (CV) was below 3% for both reducing agents. These results demonstrated good reproducibility for both methods with citrus juices in our laboratory.

The HPLC procedure using amino column and phosphate-buffered, aqueous acetonitrile in the anion-exchange mode was applied for ascorbic acid analysis in our laboratory. However, in the HPLC analysis, the citrus juice sample prepared with DTT can be analyzed within 7 min but the same sample prepared with homocysteine requires about 5 more minutes than DTT to completely elute excess homocysteine from the column before the injection of the next sample. Thus, the HPLC method with homocysteine required more analysis time compared to the method with DTT. Longer analysis time means more solvent consumption, solvent waste, and will have an immediate effect in increasing analytical costs. Another factor for our preference of DTT over homocysteine is due to the somewhat unpleasant odor of homocysteine.

A rapid micellar electrokinetic capillary chromatographic (MECC) method for total vitamin C was also developed and validated against HPLC for the variety of fruit beverages,[6] and fruits and vegetables.[35] The vitamin C is extracted with 3% metaphosphoric acid and reduced, then stabilized with aqueous 0.2% DTT. In MECC, an ionic surfactant (sodium deoxycholate) is added to the buffer to provide a phase for chromatographic separation. The MECC method performed on fused-silica capillary column (effective length of 50 cm) has the same order of repeatability but was faster and more cost effective than the HPLC procedure.

CONCLUSION

Table 2 is a literature compilation of total vitamin C contents reported in citrus products. Results for the different types of citrus juices ranged from 13.8 - 138.6 mg/100 mL for ascorbic acid, 0.0 - 15.3 mg/100 mL for dehydroascorbic acid, and 14.3 - 148.1 mg/100 mL for total vitamin C. Thus, vitamin C activity in these citrus juices is predominantly in the reduced form of vitamin C but about 14% of vitamin C activity in the juices is in the form of the oxidized form of vitamin C. This suggests that we should assume that vitamin C activity in citrus juices is attributed not only to ascorbic acid but also to dehydroascorbic, which can be reduced biologically to ascorbic acid.

Table 2

Literature Compilation of Total Vitamin C Activity in Citrus Products*

Ascorbic Acid	Dehydroascorbic Acid	Total	Reference
31.0 - 43.1	0.9 - 4.7	33.5 - 45.3	42
38.9 - 78.4	0.0 - 7.9	63.1 - 90.7	41
62.3	1.9	64.2	43
33.6 - 35.8	0.7 - 1.2	34.3 - 37.0	44
60.9	5.9	66.8	29
15.2 - 138.6	0.3 - 9.5	15.5 - 148.1	32
46.0	0.0	46.0	12
13.8	0.51	14.3	45
40.0 - 43.0	N/A	51.0 - 58.0	46
42.7	2.9	45.6	19
62.5	10.1	72.6	33
19.3 - 63.4	0.0 - 3.9	21.5 - 63.7	17
25.4 - 35.9	1.1 - 1.2	26.5 - 37.1	25
32.6 - 40.0	2.0 - 8.7	37.0 - 41.0	24

* mg/100 mL

Most previous work has shown the dehydroascorbic acid contents in citrus products (Table 2) in the range of 0-10.1 mg/100 mL (Table 2), whereas others have demonstrated the absence of it in citrus juices.[12,17,41] This controversy could be the result of the sample processing as well as quality of citrus fruits used as described by Vanderslice et al.[24] In general, dehydroascorbic is relatively more unstable and is most susceptible to degradation during sample pretreatment.

In summary, we have discussed HPLC and HPCE methods that have been used to measure the total vitamin C activity. These involve derivatization, reductive method, and direct simultaneous method.

Significant amounts of dehydroascorbic acid were found in tested commercial citrus juices, which, judging from the literature and our analyses, could provide higher total values for vitamin C, allowing for increased claims for nutritional labeling of citrus juices based on the total vitamin C method.

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