Quantitative determination of ascorbic, dehydroascorbic, isoascorbic, and dehydroisoascorbic acids by HPLC in foods and other matrices

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Introduction

The method presented here for the determination of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in foods and biological materials evolved over a number of years.[1-5] After several years of intensive analysis of samples, it has developed into a rugged and stable procedure. AA and DHAA are extracted with metaphosphoric acid and separated and quantitated by isocratic reverse phase high performance liquid chromatography. Detection is based on the post column derivatization of DHAA with o-phenylenediamine (OPD) to give a fluorescent quinoxaline derivative–a reaction introduced into vitamin C methodology by Deutsch and Weeks.[6]

The method does distinguish AA and DHAA from their corresponding diastereoisomers, isoascorbic acid (IAA) and dehydroisoascorbic acid (DHIAA). This is a necessity in nutrient composition studies because, in some processed foods, IAA is used rather than AA as an antioxidant with a subsequent loss of vitamin activity. The quantitation is direct because the concentrations of the compounds are not determined by difference. In most biological systems, IAA can be added initially as an internal standard. This has the great advantage of having the same redox potential as AA, and thus any appearance of DHIAA in the chromatogram of a sample indicates that oxidation is occurring during the extraction and analytical procedures and proper precautions can then be taken. Conversely, when IAA is used as an anti-oxidant in processed foods, AA can be used as an internal standard in the absence of endogenous AA. If both AA and IAA are present, an external standard method can be used for quantitation. The lower limits of detection are ~3 ng, and the procedures have been

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successfully used on a large variety of foods and animal tissue and even a few selected plasma samples.

Because the method can be applied to many diverse types of samples, in the interest of clarity a list of the equipment and reagents will be given prior to a discussion of the general features of the method. Extraction procedures for different types of samples are then discussed followed by a description of the chromatographic procedures that are common to all samples. Formulas for the calculation of response factors and concentrations are presented prior to a final discussion on the overall method.

Materials and Methods

Equipment

Extractions. Blender: Osterizer Touchmatic-14 (Osterizer Corp., Milwaukee, WI USA) or Waring Custom 150 (Dynamic Corp. of America, Hartford, CT USA). Polytron homogenizer: Model PT 10/35 (Brinkman Instrument Co., Westbury, NY USA). Vortexer: Polytron Model M37615 (Thermolyne, Dubuque, IA USA). Centrifuges: Beckman Models JJR and J2-21 (Beckman Instruments, Columbia, MD USA). Filters: 0.45mm, Acrodisc LC13PVDF (Fisher Scientific, Columbia, MD USA).

Chromatography. Two PLRP-S (100A, 5 μm) columns in series: 4.6 mm × 150 mm + 4.6 mm × 250 mm (Polymer Lab, Amherst, MA USA) together with the recommended PLRP-S guard cartridges. PLRP-S resins are polystyrenedivinyl benzene polymers with no active side groups. Three pumps: One Hewlett Packard model 1050 with 100 μL injection valve (Hewlett Packard, Rockville, MD USA) and two Beckman Model 110B (Beckman Instruments Co.). Detector: Perkin Elmer Model LS40 fluorometer set at 350/430 nm (Perkin Elmer Instrument Co., Norwalk, CT USA). Integrator: Shimadzu Model CR501 (Shimadzu Instrument Co., Columbia, MD USA). Stainless steel tubing: 0.25 mm i.d. (Alltech Assoc., Deerfield, IL USA). Teflon tubing: 0.40 mm id (Penntube Plastic Co., Clifton Heights, PA USA) and two Cheminert Tee Connectors (Benchmark Tech. Sales, Lewes, DE USA).

Chemicals, solvents, solutions

Extraction. Metaphosphoric acid (HPO_3), glacial acetic acid, EDTA (disodium salt), n-butanol, and hexane can all be purchased from Fisher Scientific. All chemicals are reagent grade.

Extraction solution #1 for foods: 30 g HPO_3, 0.5 g EDTA dissolved in 500 mL H_2O and 80 mL glacial acetic acid and diluted to 1 L. Extraction solution #2 for animal tissue: 10% (wt/vol) HPO_3.

Solutions of isoascorbic acid used for internal standards are made up in either extraction solution #1 or #2.

Chromatography. Mobile phase 0.2 M NaH_2PO_4 (adjusted to pH = 2.14 with H_3PO_4); oxidizing stream 0.5 mmol/L $CuCl_2$ in H_2O; fluorophor reagent 3.1 mmol/L OPD in H_2O (prepared daily). All required chemicals were obtained from Fisher Scientific. In earlier work, $HgCL_2$ was used as an oxidant but is now replaced by $CuCl_2$ for environmental reasons.

Standards. AA and IAA were obtained from Sigma Chemical Co. (St. Louis, MO USA). Commercial samples of DHAA or DHIAA are impure[3,7] or unattainable and must be prepared as needed. The acid-washed activated-charcoal method of Deutsch and Weeks[6] has been found to be satisfactory. Two hundred g of activated charcoal (Darco G-60, J. T. Baker, Inc., Phillipsburg, NJ USA) is added to 1 L of 10% (vol/vol) concentrated HCl in H_2O. The mixture is heated to boiling, cooled, and filtered with suction through a Buchner Funnel using Whatman No. 2 filter paper. The cake of acid-washed charcoal is transferred to a large beaker and 1 L of

water is added with stirring. After filtration, the washing and filtration are repeated again. The acid-washed charcoal is dried overnight at 110–120° C.

To obtain DHAA or DHIAA from AA or IAA, 2 g of the acid-washed charcoal is added to a 10–20 mL solution of 1 mg/mL of AA or IAA, made up in the MPA/HAc extraction solution. This is necessary not only to stabilize AA and IAA, but acetic acid is necessary to quantitatively oxidize AA to DHAA with the activated charcoal.[8] The standard solution is then mixed vigorously for 1 min and filtered through Whatman No. 2 paper. One hundred percent conversion to the dehydro forms can be expected.[3]

It is convenient to prepare solutions of standards at concentrations of approximately 1 mg/mL. Dilutions of 1:10 and then 2:50 are made to give approximately 4 μg/mL, which is a good working concentration for most samples.

Outline of method

The overall method has the usual major features, i.e., sample selection, extraction, clean up, chromatographic analysis, and calculations for quantitation of the analytes in an unknown sample. Details of the different steps are presented.

Sample selection, extraction, and cleanup. The extraction and clean up procedures include treatment with metaphosphoric acid, centrifugation, filtering, and dilution of sample if necessary. Variations will occur in these operations depending on sample type.

Solid foods. The nutrient concentration normally varies throughout the edible portion of a given sample. Thus, a representative sample for a given food would be the entire edible portion of that food; for example, the normally chosen part of a stalk of broccoli or a whole cabbage with the core removed. In a recent study on foods,[5] sample sizes ranged from ~10–200 g. In general, 10 mL of extraction solution #1/g sample is added to the sample prior to blending. In cases in which the expected concentration of the vitamin is high, 20 mL extraction solution #1/g sample can be used. It is convenient to have the expected vitamin C levels to be ~1–5 μg/mL in the solution to be injected onto the analytical column. For some samples this may necessitate dilution after extraction and prior to injection. Sufficient volume of the internal standard IAA solution is added prior to blending to make the final concentration of IAA be ~1–5 μg/mL prior to injection on the analytical column.

The extraction procedure for foods is outlined in *Figure 1*. The sample and the extraction solution and the internal standard are blended until smooth (≥30 sec). Aliquots of approximately 10 mL of solution are withdrawn from the blended mixture. For a non-fat and low starch sample, the sample is centrifuged at 1200*g* at 4° C for 5 min. After filtration through the 0.45 μm filter, any dilutions needed are made and 100 μL are injected onto the analytical column. For low starch samples with fat (the majority of samples) 10 mL of hexane are added to 10 mL of the blended solution and vortexed for 1 min prior to centrifugation at 1200*g*. The separated aqueous layer is then filtered prior to injection onto the column. For high starch foods, 10 mL of n-butanol is added to 10 mL of the homogenized sample, vortexed for 1 min and centrifuged at 48,400*g* at 4° C for 10 min. After filtration, 100 μL of the separated aqueous layer are injected onto the analytical column.

Liquid foods. Generally 0.5–1.0 mL are withdrawn from the sample while it is being stirred and 10 mL of extraction solution #1 are added. The amount of liquid is chosen to give a concentration of ~1–5 μg/mL of analyte in the solution. The internal standard is added and subsequent extraction steps are the same as for solid foods.

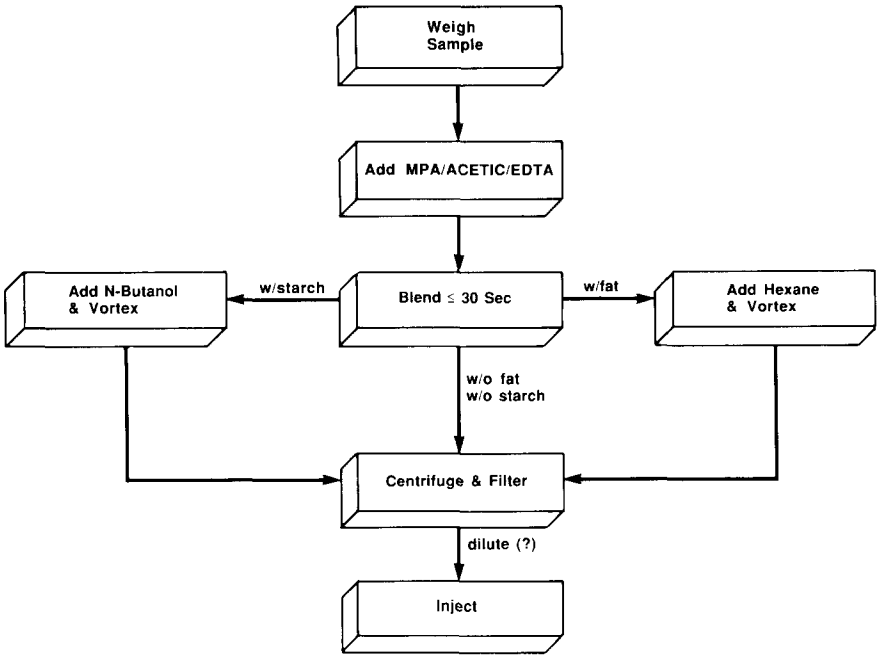

Figure 1 Block diagram of extraction procedure for foods.

Rat tissue. Four types of tissue have been studied: liver, kidney, adrenal gland, and large intestine. Tissues are excised immediately after sacrificing and dropped into liquid nitrogen and stored at −40° C for up to 6 weeks prior to analysis. Approximately 1–3 g of liver and kidney, ~0.05 g of adrenal gland, and ~0.2 g of large intestine were used for analysis. The frozen tissue was dropped into 10 mL of extraction solution #2, to which 10 mL of hexane was added, along with an internal standard in an amount comparable to the expected vitamin content. The samples were then blended in a polytron for 1 min. The mixture was centrifuged at 48,400*g* at 4° C for 5 min prior to filtration of the aqueous layer. Prior to injection, the filtrate is diluted with 1–5 mL deionized water for liver, kidney, and adrenal gland samples and 2–5 mL in the case of intestine.

Chromatographic system. The chromatographic analysis remains the same for all samples. It involves column separation, post column oxidation to convert all compounds to the dehydro forms, reaction of the dehydro forms with OPD to obtain fluorescent derivatives, and finally detection and quantitation with either internal or external standards. A block diagram of the chromatographic system is shown in *Figure 2*. Prior to the column, the tubing consists of 0.25 mm i.d. stainless steel. Post column it is 0.40 mm i.d. teflon tubing.

The initial mobile phase (~0.8 mL/min) passes through 2 PLRP-S columns held at 4° C. The eluate from the columns is first mixed in a tee with an oxidizing stream ($CuCl_2$) and then through a tee with a reaction stream (OPD), both at a flow rate of ~0.4 mL/min. (Flows are chosen to yield reasonable elution times. The flow rates of the oxidizing and reactions stream are normally set at 50% of the initial stream. Slight adjustments of these flow rates are usually made to assure maximum response.) The oxidation path is 32 cm, while the reaction (70° C) and cooling coils (20° C) were 45.7 m and 1.5 m, respectively. Cooling the stream after reaction and prior to detection enhances the fluorescence and gives a more uniform response.

Operation. It is necessary to determine the fluorescent response factors of AA and DHAA relative to IAA on a daily basis when IAA is being used as an internal standard. Several solutions of varying concentrations (in the

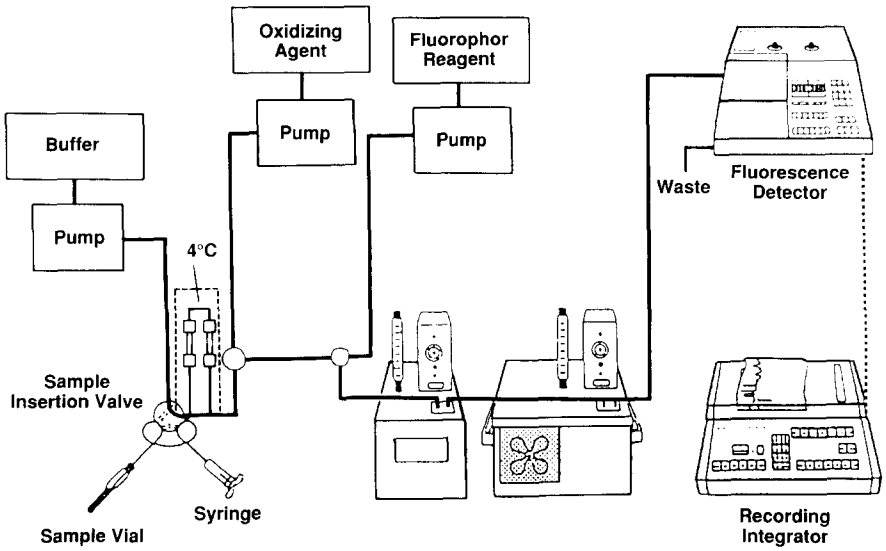

Figure 2 Block diagram of chromatographic system. The buffer, oxidizing agent, and flurophor reagent are described in text.

5 μg/mL range) of AA, DHAA, and IAA are injected onto the column to check linearity and the corresponding peak areas measured so that the response factors can be calculated as described in the following section.

Extracts of food or tissue samples are then injected onto the column and the concentrations in the unknown sample calculated as in the next section.

Typical traces of approximately 22 min are shown for standards, foods, and tissues in *Figure 3*. In most foods there is no appreciable (or detectable) amount of DHIAA found, while in rat tissues conversion of IAA into DHIAA partially occurred. This problem will be addressed further in the calculation and discussion sections.

Response factors and calculations. The necessary mathematical expressions are presented for the preferred internal standard method for the determination of AA and DHAA in samples. Here IAA is used as the internal standard. In the case of processed foods where IAA is used as an antioxidant, the roles of IAA and AA are reversed. In all food samples analyzed in our laboratory so far, either IAA or AA was present but not both. If, however, both are present, then an external standard calibration is necessary.

To determine the amount of AA or DHAA in a given sample by the internal standard method, it is necessary to know two response factors, f_{AA} and f_{DHAA} defined by Equations 1 and 2.

$$(AA) = f_{AA}\left(\frac{A_{AA}}{A_{IAA}}\right)(IAA) \tag{1}$$

Where A_{AA}, A_{DHAA}, and A_{IAA} are the peak areas for AA, DHAA, and IAA.

$$(DHAA) = f_{DHAA}\left(\frac{A_{DHAA}}{A_{IAA}}\right)(IAA) \tag{2}$$

The amounts of AA, DHAA, and IAA will normally be in μg. f_{AA} and f_{DHAA} are determined daily from standards.

It is important to note that Equations 1 and 2 apply where there is no significant (<1%) conversion of IAA to DHIAA during the extraction and analytical procedures. Such is normally the case with food samples. (Composite diets are likely to have a slightly higher conversion.) With rat tissues and blood, significant conversion of IAA and DHIAA is observed and a

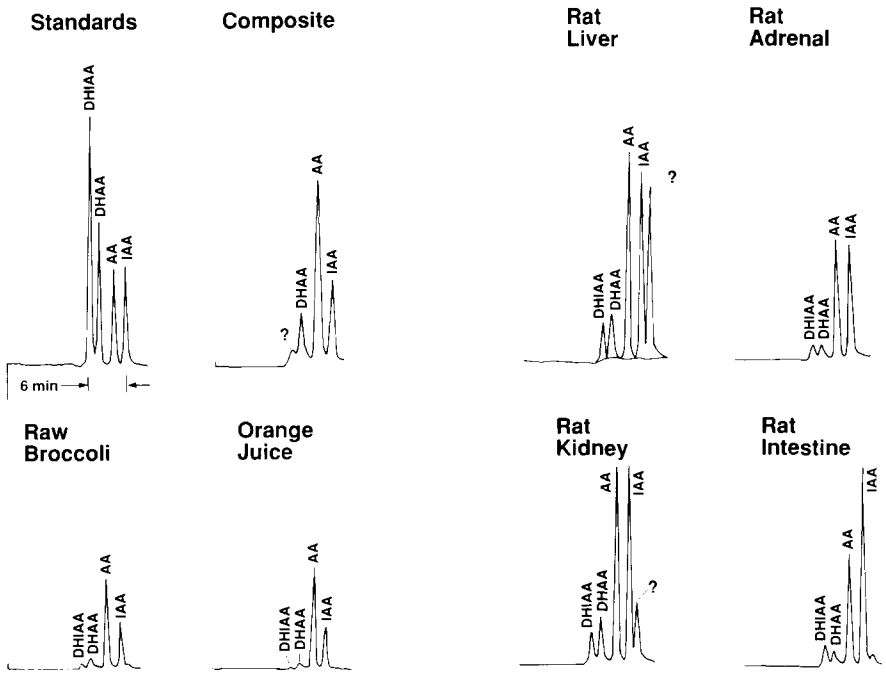

Figure 3 Chromatograms of standards, food samples, rat tissue, and plasma. The peaks for rat kidney and liver marked with a question mark are unidentified peaks.

correction must be made. The appropriate concentrations are then calculated with Equations 3 and 4.

$$(AA) = f_{AA}\frac{(A_{AA})}{(A_{IAA})}[IAA]\left(1 - \frac{f_{DHAA}\dfrac{(A_{DHIAA})}{(A_{IAA})}}{1 + f_{DHAA}\dfrac{(A_{DHIAA})}{(A_{IAA})}}\right) \quad (3)$$

$$(DHAA) = f_{DHAA}\frac{A_{DHAA}}{A_{IAA}}[IAA] \times \left(1 - \frac{f_{DHAA}\dfrac{(A_{DHIAA})}{(A_{IAA})}}{1 + f_{DHAA}\dfrac{(A_{DHIAA})}{(A_{IAA})}}\right) \quad (4)$$

Where we have used the approximation $f_{DHAA} \sim f_{DHIAA}$ because this is usually the case, and where A_{DHIAA} is the peak area for DHIAA.

After the response factors have been determined, the amount of AA and DHAA in a sample can be determined by Equations 1 and 2 from the known amount of IAA added to the sample, the measured peak areas, and the response factors. The amounts found from equations 1 and 2 divided by the weight of sample in grams yield values with units (μg) unknown/gm sample.

Flow cell clean up. The flow cell of the fluorescent detector tends to get coated in time with OPD, resulting in a loss of detector sensitivity. To prevent this, at the end of a day's run the OPD solution in the reservoir is replaced by distilled water and the system pumped for 20 min. Once a week the cell is flushed with 10 mL of methanol.

Stability of method. The reproducibility of the method over time is conveniently checked by using samples of "name brand" frozen orange juice from the same lot. It has been determined that, if kept frozen, the loss of vitamin C is negligible for at least 6 months and perhaps for a much longer time. Periodic analyses of such samples will serve as a check on the overall stability of the analytical procedure.

Discussion

The methods outlined in this paper have been employed most extensively in the analysis of a wide variety of food samples. They have been shown to give results in good agreement with the classical AOAC procedures of Deutch and Weeks.[1] Recoveries are in the 80–113% range.[1] The methods distinguish between AA, DHAA, and their epimers IAA and DHIAA, which is an important consideration in light of the proposed new food labeling laws and the fact that IAA is found in some processed foods. During the latest studies on food samples, over 2,000 injections were made and the columns had to be cleaned only twice with a mixture of 40% ethanol, 30% hexane, and 30% dichloromethane. Plasma and rat tissue extracts introduced no observable contamination problems.

The fluorescence response is linear over the range from 3–2,000 ng. Therefore, it is convenient to work at the 500 ng response level. With the usual 100 μL inject volume, this corresponds to a concentration of 5 μg/mL.

In a flow injection analysis (FIA) version of this method in which no column is present in the system,[3] the fluorescence response of DHAA and AA are equal. When the columns are incorporated into the system, the response of AA (and also IAA) differs from that of DHAA. When $CuCl_2$ is used as the oxidant, the responses are approximately equal but, in our earlier work, when $HgCl_2$ was used as the oxidant, the response of AA and IAA fell to approximately one-half of DHAA. The reason(s) for this remain obscure.

Once the vitamins have been extracted, they are stable for at least 8 hrs at room temperature. Extraction, however, should be performed as soon as possible on fresh samples. It is important that the sample be homogenized in the presence of the extraction solutions.

As mentioned earlier, DHIAA, when IAA is used as an internal standard, to a significant degree in rat tissues but not in foods. We attribute this to oxidation occurring during sample handling, perhaps due to the presence of endogenous activators. Calculations for the unknown concentrations in these cases are given in equations 3 and 4. The fact that the (DHAA):(AA) and (DHIAA):(IAA) ratios are the same for plasma[9] and tissue[10] indicates to us that DHAA is not present in either type of sample prior to analysis.

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