ESTIMATION OF ASCORBIC ACID IN ORANGE JUICE BY A CHRONOMETRIC METHOD

Bongwoo Roe and Joseph H. Bruemmer
U. S. Citrus and Subtropical Products Laboratory1
Winter Haven

Abstract. The redox couple between ascorbic acid (vitamin C) oxidation and reduction of the semiquinoid form of p-phenylenediamine (PPDA) is the basis of a new chronometric assay of vitamin C. Commercial horseradish peroxidase or citrus peroxidase isolated from seeds catalyzes the hydroperoxidation of PPDA through a reversible semiquinoid form to a colored condensation product. Ascorbic acid interrupts formation of the colored product by coupling its oxidation to reduction of the semiquinoid. After all the ascorbic acid is oxidized, oxidation of the semiquinoid proceeds to the dye form. By using a standard peroxidase preparation, ascorbic acid content of orange juice was shown to be inversely proportional to the time lag from initiation of the reaction to first appearance of colored product. A rapid “yes-no” method for testing juices for labeling compliance and a simple method for quantitating ascorbic acid levels in citrus juices were developed.

After December 31, 1974, all prepared and packaged orange juices must comply with the Nutrition Labeling Regulations, if the manufacturer claims nutritive value for their product (3, 4). Under these regulations the percentage per serving of the United States Recommended Daily Allowance (U. S. RDA) of vitamin C (60 mg) and seven other nutrients must be listed on the label. Vitamin C content of canned orange juice averages 40 mg per 100 gms of juice. Reconstituted juice from orange concentrate contains about 11% more than single-strength canned juice (13). Thus, both canned orange juice and juice reconstituted from concentrate would provide more than 100% of the U. S. RDA per 6 fl oz serving (124 and 140% respectively).

For compliance with the regulations vitamin C content of the product must not fall more than 20% below the labeled value. A quick method of testing orange juice products for label compliance is needed. In the Official Method of the Association of Official Agricultural Chemists, vitamin C is titrated with the redox dye, 2,6-dichloroindophenol (7). Introduced by Tillmans (12) and modified by others (6, 10), the method recommends 3 aliquots of each sample be titrated with reproducibility of better than 99%.

We have developed a fast sensitive method of determining vitamin C in orange juice and modified this procedure so that only a few minutes are required to test samples for labeling compliance.

Materials and Methods

Horseradish Peroxidase, grade II, 50 U/mg (HR Per) was obtained from Boehringer Mannheim Corporation. All other chemicals were from Fisher Scientific Company. HR Per assay of Luck (8) was modified to measure the time lag of p-phenylenediamine (PPDA) oxidation by ascorbic acid. The reaction was measured by following product formation with the Beckman recording spectrophotometer model DB at 485 nm. The sample cell contained 0.1 to 0.5 μg HR Per, 0.2 ml orange juice serum, 0.1 ml 2% PPDA, 2 ml 0.1 M phosphate buffer, pH 7.0 and 1 ml 3 x 10⁻³M H₂O₂. The reference cell contained the juice blank, the same constituents except that H₂O replaced H₂O₂. The reaction was initiated by adding H₂O₂ lastly.

Orange juice was used as stabilizing solvent for standard solutions of ascorbic acid. Standards were prepared containing 0.2, 0.3, 0.4 and 0.5 mg/ml added ascorbic acid.

Lag times and rates of color formation were charted on a 5-in. recorder attached to the spectrophotometer.

Results and Discussion

Peroxidase-H₂O₂ Oxidation of PPDA: Interference by Ascorbic Acid

Oxidation of PPDA by HR Per H₂O₂ results in polymerization to a colored product. Under low enzyme concentration the rate of color formation is proportional to enzyme concentration and linear with time for at least 5 min (8). Peroxidase in orange juice was assayed by this method (2).
However, substance(s) in the juice interfered with the assay by causing a time lag in formation of the colored product without affecting the rate after the product started to form. Ascorbic acid was shown to be the major and probably the only interfering substance.

Ascorbic acid also interrupts product formation in the HR Per-H₂O₂ reaction. It prevents color development or stops it after the reaction has started. The time lag for color development is proportional to ascorbic acid concentration and inversely related to enzyme activity in the reaction mixture (Figure 1). The proportionality constant for the lowest enzyme concentration (activity: change of 0.26 Absorbance per min) is equivalent to 1.5 sec per µg ascorbic acid. These standard curves for ascorbic acid added to orange juice were used to estimate the ascorbic acid concentration of the original juice. The following values were obtained: 84.4, 82.0, 81.0 and 84.0 µg of ascorbic acid per cuvette, equivalent to 42.2, 41.0, 40.5 and 42.0 mg ascorbic acid per 100 ml original juice. The concentration of ascorbic acid in the juice determined by the 2,6-dichloroindophenol method was 42.0 mg per 100 ml. These results suggested that the time lag was an accurate measure of the ascorbic acid content of the juice.

**Optimum Conditions for Assaying Ascorbic Acid in Orange Juice with PPDA Oxidation by HR Per-H₂O₂.**

Oxidation of PPDA proceeds through a reversible free radical semiquinone form (9) to a non-reversible colored product. Ascorbic acid reacts with the semiquinone as quickly as it is formed in the HR Per-H₂O₂ reaction and reduces it back to the diamine. When all ascorbic acid is oxidized through this redox couple, the oxidation of PPDA proceeds at the peroxidase controlling rate. This redox couple between ascorbic acid and PPDA semiquinone is similar to the reaction of ascorbic acid with o-toluidine semiquinone in the peroxidase assay described by Purr (11) and with benzidine semiquinone in the chronometric method for peroxidase described by Gregory (5). Because the rate of PPDA semiquinone formation by HR Per-H₂O₂ controls the time lag for the oxidation of ascorbic acid, the enzyme reaction was optimized to obtain accuracy and reproducibility in the ascorbic acid assay.

**pH.** The optimum pH for HR Per-H₂O₂ oxidation of PPDA is 5.8 (Figure 2). Luck (8) recom-
mended pH 7.0 in his assay using 0.02 M phosphate buffer and 10 mg PPDA. The higher molarity (0.1 M) required to buffer the acidic juice and higher PPDA (20 mg) used in the ascorbic acid assay might explain the lower pH optimum for the enzyme in our system.

**PPDA Concentration.** The rate of color development approached a plateau as the concentration of PPDA was increased to 3%. However, the reaction blank at 2% was less than at 3% so the lower concentration was selected for the assay.

**Hydrogen Peroxide Concentration.** Final concentration of $1 \times 10^{-2} \text{M} \ H_2O_2$ supported the optimum rate of color development at pH 5.8 and 2% PPDA.

**Temperature.** Reaction temperature of 30°C was selected as optimum on the basis of the ease of control, and enzyme activity and stability.

**Proposed Method for Vitamin C Content of Orange Juice.**
Reagent concentration, pH of the buffer and order of addition were adjusted to permit optimum control of pH and temperature dependent reaction rate.

**Reagents.**

1. **Citrate-phosphate buffer, 0.1 M, pH 6.4:** Sol. A: dissolve 192.1 g citric acid (M.W. 192.1) in 1000 ml deionized water. Sol. B: dissolve 268 gm of dibasic sodium phosphate heptahydrate (M.W. 268) in 1000 ml deionized water. Buffer prepared by diluting 30.8 ml of sol. A and 69.2 ml Sol. B to 1000 ml with deionized water. Store at 4°C; prepare fresh monthly.

2. **Horseradish peroxidase solution:** Stock solution I: dissolve 10 mg HR Per in 10 ml deionized water. Stock solution II: dilute 3.5 ml of stock solution I to 50 ml with 0.1 M citrate-phosphate buffer, pH 6.4. Working solution: dilute 0.1 ml of stock Sol. II to 100 ml with 0.1 M citrate phosphate buffer pH 6.4. Stock solutions are stored at 20°C. Working solution is prepared fresh daily and protected from light at 4°C.

3. **p-Phenylenediamine, 2% solution:** Dissolve 2 g of PPDA in 100 ml of deionized water. Store in brown bottle at 4°C. Prepare fresh weekly.

4. **Hydrogen peroxide, $3 \times 10^{-2} \text{M}$:** Dilute 0.33 ml 30% H$_2$O$_2$ to 100 ml with deionized water. Store at 4°C; prepare fresh weekly.

5. **Standard vitamin C solution:** A vitamin C standard is prepared by dissolving exactly 100 mg desiccator-stored ascorbic acid in 250 ml of an orange juice sample. The vitamin C standard and the standard juice blank (250 ml aliquot of the same orange juice sample without vitamin C added) are stored at 4°C, in 50 ml brown bottles without headspace or under N$_2$ atmosphere. Standards and blanks are prepared fresh weekly.

**Procedure**

Approximately 10 ml of orange juice is centrifuged for 10 min at 2000 rpm in counter top centrifuge (Model CL, International Centrifuge or similar model), to obtain clear serum. While the juice is being centrifuged, a colorimeter tube containing 3.0 ml of cold peroxidase working solution is warmed to 30°C in a constant temperature water bath. The 0.2 ml serum, and 0.1 ml 2% PPDA (both at room temperature) are added and the contents mixed. The colorimeter or spectrophotometer is adjusted with this solution to give zero absorbance or 100% transmission. The 0.1 ml cold $3 \times 10^{-2} \text{M} \ H_2O_2$ is added and the stopwatch activated. The contents are mixed for 3 sec on a Vortex mixer and the tube is immediately inserted in the precalibrated photometer with 490 filter or spectrophotometer set at 485 nm. The meter needle should register zero Absorbance when the tube is inserted; if not, adjust to zero. The stopwatch is stopped when meter needle reaches 0.1 absorbance. The time to the nearest 0.1 sec shown on the stopwatch is recorded as “vitamin C time” for the juice sample. The vitamin C content of the juice in mg per 100 ml is obtained by multiplying vitamin C time by the reciprocal of the proportionality constant for the enzyme solution. The proportionality constant must be determined daily for the freshly prepared enzyme solution.

**Proporionality constant.** The vitamin C time for standard juice blank is subtracted from the vitamin C time for the standard to give the vitamin C time equivalent to 40 mg ascorbic acid per 100 ml juice. The proportionality constant is equal to vitamin C time per mg per 100 ml juice or 0.025 times (1/40th of) the vitamin C time for the 40 mg per 100 ml standard.

**Proposed Assurance Test for Labeling Compliance.**

Reagent concentration, pH and order of addition were adjusted to give instantaneous color formation at optimum pH.

**Reagents.**

1. **Phosphate buffer, 1.0M, pH 7.0.** Dissolve
55.6 gm of monobasic sodium phosphate and 166.0 gms of dibasic sodium phosphate heptahydrate in 1000 ml deionized water. Prepare fresh monthly, store at 4°C.

2. Horseradish peroxidase solution. Stock solution: dissolve 10 mg HR Per in 1000 ml of 1.0 M phosphate buffer, pH 7. Working solution: dilute 5 ml of stock solution to 50 ml with 1.0 M phosphate buffer, pH 7. Stock solution at —20°C. Prepare working solution fresh daily, store at 4°C.

3. p-Phenylenediamine, 3% solution. Dissolve 3 gm PPDA in 100 ml deionized water. Store in brown bottle at 4°C. Prepare fresh weekly.

4. Hydrogen peroxide, 0.6%. Dilute 1 ml of 30% H₂O₂ to 50 ml with deionized water. Store at 4°C, prepare fresh weekly.

5. Standard U. S. RDA orange juice. Prepared by diluting orange juice with deionized water to contain 35.2 mg vitamin C per 100 ml. Some Florida canners plan to declare 130% U.S. RDA for vitamin C (60 x 130 for 6 fl oz or 177.4 ml = 44 mg/100 ml). Adjusting for 20% deficiency allowance gives 35.2 mg per 100 ml requirement.

Procedure

Warm 5 ml of orange juice containing exactly 35.2 mg vitamin C per 100 ml and 1 ml of peroxidase solution to 30 °C in water bath (10 min). Add 0.5 ml of cold 3% PPDA solution, mix contents, then activate stopwatch when 0.5 ml of cold 0.6% H₂O₂ is added. Mix and watch tube for sudden color change to dark green. Stop the watch when color changes and record time as “assurance time.” Repeat procedure with orange juice samples to be tested. All samples with assurance times the same or longer than the standard would meet the labeling requirement of 130% U. S. RDA in a 6-fl oz serving of juice.

Accuracy and Reproducibility of the Proposed Method and Test.

Interfering substances: Glutathione also reduces PPDA semiquinone which causes a time lag in colored product formation in the oxidation of PPDA. However, orange juice contains less than 6 mg glutathione per 100 ml (1) and about 60 mg per 100 ml is required to produce a perceptible time lag. The other reducing substances in orange juice are present in lower concentration than glutathione. Ferrous ions interfere with the assay at concentrations greater than 6 mg per 100 ml juice. However, the normal concentration in canned orange juice is only 0.4 mg per 100 ml (13).

Enzyme stability. HR Per retains complete activity when stored at 4°C for 8 hrs at 0.1 μg/ml (0.26ΔA/min) in 0.1 M citrate phosphate buffer pH 6.4, or in phosphate buffer, pH 7.0. The pH of the buffers used in the proposed method and assurance test is lowered to final pH of 6.0 by dilution with the acidic (pH 3-3.4) juice. Activity declines about 15% after 24 hrs at 4°C. At 30°C this concentration of enzyme in buffer retains complete activity for 2 hrs.

Standard deviations. The mean ± standard deviation of 11 analyses of an orange juice sample for ascorbic acid by the proposed method was 38.0 ± 0.9 μg. This value is equivalent to 42.5 ± 0.5 mg ascorbic acid per 100 ml juice that contained 42 mg per 100 ml by the 2,6-dichlorophenol method.

The mean of six analyses of orange juice for assurance time ± SD by the quick test was 19.3 ± 0.3 sec.

Literature Cited