〈Brief Note〉

# Simple and rapid determination of vitamin C content in various fruits using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl and *o*-phenylenediamine dihydrochloride

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**Summary**  Vitamin C (ascorbic acid: AsA) is an essential nutrient in our bodies. Because humans are unable to synthesize AsA, we must obtain AsA from foods (vegetables and fruits). Although fruits are rich sources of AsA, determination of their AsA content by current methods requires the use of expensive types of apparatus or is cumbersome and time-consuming. Thus, simple, rapid, and cost-effective methods are required. Here, we presented a manual procedure for the determination of total AsA content (i.e., the sum of AsA and dehydroascorbic acid) in various fruits using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl and *o*-phenylenediamine dihydrochloride. We determined total AsA content in fruits using a two-step end point assay. The test took only 20 min to perform and cost 2.0 US$ per 100 tests. The observed total AsA content in various fruits was significantly correlated with the total AsA content reported in the "Standard Tables of Food Composition in Japan".

## 1. Introduction

Vitamin C (ascorbic acid: AsA) is known as the antiscurvy vitamin, and like vitamin E (α-tocopherol), AsA also has antioxidant activity. AsA acts as a hydrophilic antioxidant, while α-tocopherol is a hydrophobic antioxidant. When AsA exerts its antioxidant activity, it is converted to dehydroascorbic acid (DAsA), which has not an antioxidant activity[1]. Humans must obtain AsA from foods (i.e., vegetables and fruits) in the forms of

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AsA and DAsA, because we are unable to synthesize AsA *de novo* in the body[2]. AsA is absorbed to intestinal epithelial cells via sodium-dependent vitamin C transporter type 1 (SVCT1); however, DAsA is absorbed via glucose transporter 1 (GLUT1) because of its similar structure to glucose. Absorbed DAsA is reduced to AsA, and then exerts its physiological functions[2].

Currently, the AsA and DAsA in vegetables and fruits are measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) and the classical 2,4-dinitrophenyl-hydrazine (DNPH) method. However, both of these methods have disadvantages; specifically, HPLC requires the use of expensive apparatuses, and the DNPH method requires lengthy heating of the sample under strongly acidic conditions[3]. We previously reported an automated method for measuring total AsA concentration (i.e., the sum of AsA and DAsA) in serum and urine using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and *o*-phenylenediamine dihydrochloride (OPDA)[4]. The cost per test using this automated method is 0.01 US$. Therefore, in this study, we adapted the automated method to a manual procedure, and used the manual procedure to measure total AsA content in various fruits.

## 2. Materials and methods

### 2.1. Specimens

Twelve different kinds of fruits, i.e., apple, banana, dragon fruit, grapefruit, Japanese lemon (*Citrus junos*), kiwifruit, lemon, mango, oleaster, pineapple, satsuma mandarin (*Citrus unshiu*) and strawberry were used for analysis of AsA and DAsA contents. All fruits except oleaster were purchased from local grocery stores. Oleaster was harvested from a tree growing in our schoolyard garden.

Ten volumes of 4.0% metaphosphoric acid (MPA) were added to one part flesh of the fruits (some contained the peel) or squeezed juice of the fruits (w/v). AsA is stable in acidic solution. After homogenization, samples were centrifuged at 6,200 rpm (2,000 x *g*) for 5 min to spin down the pellet and obtain a clear supernatant. The supernatant was applied to the following experiments.

### 2.2. Reagents

TEMPO solution was prepared by dissolving 20 mg of TEMPO (Sigma-Aldrich Co. LCC., MO, USA) in 100 mL of 0.067 mol/L phosphate buffer, pH 6.4. OPDA solution was prepared by dissolving 5 mg of OPDA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 10 mL of the same buffer, and then stored in a refrigerator (4°C) in the dark. MPA solution (4%) was prepared by dissolving 4 g of potassium metaphosphate (Wako Pure Chemical Industries, Ltd.) in 100 mL of distilled water. AsA standard solution was prepared by dissolving 10 mg of AsA (Japanese pharmacopoeia reference standard, Pharmaceutical and Medical Device Regulatory Science Society of Japan, Tokyo, Japan) in 100 mL of distilled water.

### 2.3 Assay procedure

Because OPDA reacts exclusively with DAsA, the AsA in specimens was first oxidized to DAsA by the addition of TEMPO[4]. Subsequently, DAsA originally present in the specimens and that derived from AsA were both condensed with OPDA to form a fluorescent product measurable by spectrophotometry at 340 nm[1].

An aliquot (0.125 mL) of the above supernatant ("measurement") or AsA standard solution ("calibration") was mixed with 1.0 mL of TEMPO solution and incubated at 37°C for 5 min. Next, 0.425 mL of OPDA solution was added to the mixture, and the absorbance ($A_{340}$) of the reaction mixture was measured using a Hitachi 7012 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) for 15 min. AsA content (mg/L) in the supernatant was obtained from the $A_{340}$ of "measurement" as compared with that of "calibration", and AsA content in fruits was expressed in terms of mg AsA per 100 g of fruit. For the measurement of DAsA, TEMPO solution was replaced by 0.067 mol/L phosphate buffer, pH 6.4, followed by incubation and addition of OPDA solution in the same manner as with the AsA measurement in the absence of

Table 1.  AsA and DAsA contents in various fruits.

| | Our analysis | | | STFCJ |
|---|---|---|---|---|
| | Total AsA (mg/100 g) | DAsA (mg/100 g) | DAsA/Total AsA ratio (%) | Total AsA (mg/100 g) |
| Apple | 18.1 | 6.3 | 34.8 | 6 |
| Banana | 10.0 | 9.4 | 94.0 | 16 |
| Dragon fruit | 3.4 | 2.9 | 85.3 | 7 |
| Grapefruit | 40.6 | 9.6 | 23.6 | 36 |
| Japanese lemon | 53.4 | 8.3 | 15.5 | 40 |
| Kiwifruit | 101.6 | 13.0 | 12.8 | 69 |
| Lemon | 52.7 | 9.4 | 17.8 | 50 |
| Mango | 18.6 | 3.2 | 17.2 | 20 |
| Oleaster | 8.1 | 7.2 | 88.9 | 5 |
| Pineapple | 59.6 | 10.8 | 18.1 | 27 |
| Satsuma manda | 46.9 | 7.2 | 15.4 | 32 |
| Strawberry | 78.3 | 11.3 | 14.4 | 62 |

AsA: ascorbic acid; DAsA: dehydroascorbic acid.
STFCJ: Standard Tables of Food Composition in Japan (2015).

TEMPO.

## 3. Results

With the addition of OPDA, $A_{340}$ (i.e., λmax of DAsA-OPDA conjugates) began to increase and reached maximum at 12 min, and thereafter remained unchanged for 30 min. Thus, we set the incubation period as 15 min in the manual procedure. The increase of $A_{340}$ was linear from 0.0 mg/L up to 100 mg/L for AsA content, but the limit of quantitation (LOQ) was 1.0 mg/L. Although this procedure covered a wide range of AsA contents, the experimental samples were diluted with 4% MPA solution or distilled water if the $A_{340}$ of the original sample exceeded this range. Nevertheless, in the manual procedure, within-day variation for AsA (10.0±0.2 mg/L) was found to have a coefficient of variation (CV) of 2.0% (six measurements). Between-day variation for the same samples over ten successive days exhibited a CV of 4.7%. Meanwhile, pressed apple juice spiked with 10.0 mg/L AsA showed a recovery of 13.1 mg/L.

Total AsA and DAsA contents in the various fruits were assayed (Table 1). The highest total AsA content was observed in kiwifruit, and the lowest in dragon fruit. Total AsA content in the twelve fruits was significantly correlated with those reported in "Standard Tables of Food Composition in Japan"[5] (Fig. 1). Although the DAsA content was highest in kiwifruit and lowest in dragon fruit, the DAsA/total AsA ratio was inversely related to the total AsA content in these fruits (Fig. 2).



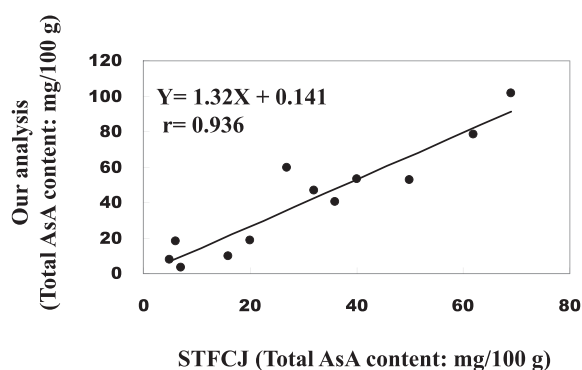$$Y = 1.32X + 0.141$$
$$r = 0.936$$

Fig. 1   Comparison of total ascorbic acid (AsA) content in various fruits as measured by our manual procedure to the total AsA content reported in the "Standard Tables of Food Composition in Japan" (STFCJ).
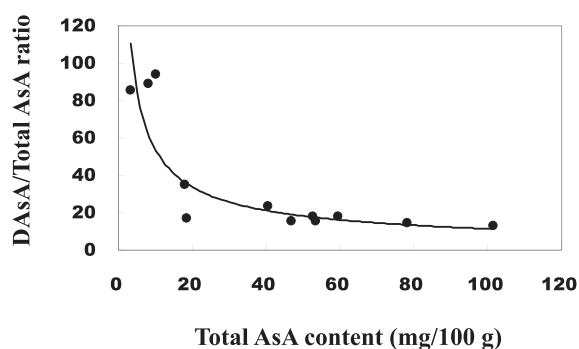
Fig. 2    Relation between DAsA/total AsA ratio and total
AsA content in various fruits.
DAsA: dehydroascorbic acid; AsA: ascorbic
acid.

## 4. Discussion

Plants synthesize AsA from glucose via the mannose-galactose pathway, and mammals except for guinea pig and primates synthesize AsA from glucose via the glucuronic acid pathway and the catalytic activity of L-gulonolactone oxidase (GLO: EC 1.1.3.8), which is encoded by the *GLO* gene[2,6]. Humans have a non-functional gene (*GLO*) on chromosome 8p21.1 (i.e., one-base deletion in exons 7 and 10, and three-base deletion and one-base insertion in exon 9) for making AsA. *GLO* was inactivated by mutation about 40 million years ago in primates[7]. Therefore, humans must obtain AsA from their diet. The recommended dietary allowance (RDA) for AsA in Canada and the United States is 75 mg/day for adult women and 90 mg/day for adult men[8]. For Japanese adults of both genders, the RDA for AsA is 100 mg/day[9]. Thus, it is necessary to obtain 75-100 mg/day of AsA or more from foods. When the RDA amounts of AsA were obtained, blood concentrations of AsA and DAsA in healthy Japanese adults were 7.0-13.8 mg/L and 0.8±0.3 mg/L, respectively, as determined by our automated method[4]. Urinary excretion of DAsA in the corresponding adults varied from 0.9 to 13.8 mg/L[10].

On the other hand, plants synthesize AsA in order to scavenge active oxygen formed through metabolism, especially in chloroplasts[2,6]. However, the ability to synthesize AsA changes according to the life cycle (i.e., germination, rooting, growing, flowering, and ripening) and environmental conditions (light and temperature). Moreover, AsA content in plants differed among roots, shoots, stems, leaves, and fruits (or berries). In this study, we determined total AsA content in various fruits using a simple and rapid manual procedure. In this study, TEMPO-mediated oxidation of AsA and condensation reaction of furfural was accomplished in 15 min using the two-step end point assay. The cost was 2.0 US$ per 100 tests, including the cost for the AsA standard solution. Observed total AsA content was significantly correlated with the total AsA content reported in the "Standard Tables of Food Composition in Japan (STFCJ)"[5]. We previously reported a significant correlation ($r= 0.981$, $P< 0.001$) on the measurement of total AsA concentrations in serum specimens between TEMPO and HPLC methods[4]. Although not the same sample was assayed, there are some bias in total AsA contents from those reported in STFCJ (assayed by HPLC method) in apple, kiwifruit, and pineapple by TEMPO method (Table 1). In future study, we would like to investigate endogenous substances present in vegetables and fruits that interfere with the TEMPO method.

In this study, DAsA and AsA contents [(DAsA, mg/100 g)/(total AsA, mg/100 g)] were 9.4/10.0 (94.0%) in banana, 9.4/52.7 (17.8%) in lemon, and 6.3/18.1 (34.8%) in apple. In contrast, Mazurek et al.[11] reported these respective contents as 2.24/15.42 (14.5%) in banana and 2.58/73.75 (3.5%) in lemon, while Roe et al.[12] reported 25.0/210.0 (11.9%) in lemon peel and 0.0/4.5 (0.0%) in apple, in which all samples were purchased from a city market. However, Wills et al. reported that DAsA content was not present in fresh fruits of banana and lemon assayed following purchase from local retail markets in Australia[13]. These conflicting results were observed especially in regards to DAsA content between our study and the previous reports.

Many reports on AsA content in vegetables and fruits have been published to date; however, they have not considered the significance of the presence of DAsA. When AsA exerts antioxidant activity, a

decrease in AsA in plant cells is observed with a simultaneous increase of DAsA. In this study, we detected specific amounts of DAsA in fruits, some of which agreed with the previous reports. On the other hand, AsA content in vegetables and fruits following harvest was also decreased with a simultaneous increase in DAsA over time[11]. Finally, DAsA is irreversibly converted to 2,3-diketogulonic acid, which does not react with OPDA. The degradation of AsA to DAsA, and then to 2,3-diketogulonic acid could be the reason for the differences between our results and previous reports. Further, because the various fruits assayed in this study were purchased from a grocery store, we were unable to adjust the post-harvest analysis dates to correspond to the previous reports.

In conclusion, total AsA content (i.e., the sum of AsA and DAsA) in fruits could be easily determined by a manual procedure with the use of TEMPO-mediated oxidation of AsA and the condensation reaction between DAsA and OPDA. However, the accurate determination of DAsA content remains to be further investigated, since DAsA was artificially produced from AsA during the storage of fruits post-harvest. In the context of AsA degradation to DAsA during storage, total AsA represents a practical measure of the actual vitamin C content in fruits.

## Acknowledgments

## Conflict of Interest

The authors have declared that no conflict of interest exists.

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