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Research Article

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Ascorbic Acid Determination in Vegetables and Fruits: Comparison of Colorimetry with High Performance Liquid Chromatography

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Abstract

Ascorbic Acid (AsA) is included in various fruits and vegetables and its quantity correlates with quality after harvest. The quantity of AsA is usually evaluated by colorimetry but as this technique uses reduction of iron, it is easy for colorimetry to be influenced by reducing agents such as AsA. In this study, we compare colorimetry with high performance liquid chromatography (HPLC) in measurement of AsA in fruits and vegetables. We also evaluated the correlation between colorimetry and antioxidant and iron reduction ability. As a result, the quantity of AsA measured by colorimetry was higher than that with HPLC for reduction materials except for AsA. There was poor correlation between colorimetry and antioxidant ability but good correlation between colorimetry and iron reduction ability. We conclude that colorimetry is not suitable for accurate evaluation of the quantity of AsA in vegetables and fruits but it is suitable for evaluation of iron reduction.

Keywords

Ascorbic Acid, HPLC, Antioxidant ability, Iron reduction ability

Abbreviations

FRAP: Ferric Reducing Antioxidant Power; HPLC: High Performance Liquid chromatography; AsA: Ascorbic Acid; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; TPTZ: 2,4,6-Tri(2-Pyridyl)-1,3,5-Triazine

Introduction

The consumption of the species defined as functional foods in the medical terminology due to their outstanding phytochemical properties is growing owing to the increasing public awareness of the issue [1, 2]. The importance of berry fruits in a healthy and balanced diet is being increasingly emphasized by dieticians and health care professionals because of their various, rich, and effective phytochemical content [3]. Vitamin C (Ascorbic Acid, AsA) in green vegetables decreases with time [4] but it is used for quality evaluation [5]. Therefore, the accurate measurement of the quantity of AsA in vegetables and fruit is needed. At present colorimetry, titrimetry, enzymatic methods, or HPLC are used [6]. The HPLC method can measure AsA most accurately, but is expensive. There are two methods of colorimetry: (1) AsA reacts with 2, 4-dinitrophenyl-hydrazine directly; (2) Fe^{3+} is reduced to Fe^{2+} by AsA and Fe^{2+} reacts with a color coupler and color appears. The hydrazine method has a high specificity but is slow [7]. The iron reduction method is easy and quick but is affected by other reduction materials and so is more often used. There are three methods of iron reduction colorimetric

measurement of AsA, namely, α, α'-dipyridyl method [7], o-phenanthroline method [8], and ferrozine method [9]. All these methods are often used in determination of AsA in body fluids or tissues in animals [10] but less commonly in fruits and vegetables [6].

Colorimetry of AsA uses the reduction capability of AsA. Therefore other reduction materials present can affect colorimetry results strongly. At present, the determination of the reduction ability of food is often assessed by the ferric reducing antioxidant power (FRAP) method [11]. In addition, it is important to evaluate the antioxidant ability of vegetables and fruits. Colorimetry can also be used to evaluate antioxidant ability in fruits and vegetables using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, an easy and stable method [12]. As colorimetry of AsA using reduction of iron is part of both FRAP and DPPH methods [12], the colorimetry of AsA is more likely to reflect both reduction ability and the antioxidant ability. Therefore, in our study, we compare the quantity of AsA in various fruits and vegetables as measured by colorimetry with that measured by HPLC and evaluate whether we can use colorimetry to evaluate the iron reduction ability and antioxidant ability of various vegetables and fruits.

Materials and Methods

Sample preparation

We purchased oranges, pineapples, lemons, bananas, broccoli, grapefruits, mandarin oranges, and green peppers at the stores near the university. We used strawberries, kiwis, oranges, ponkans, Chinese cabbages, Jabuticabas, raspberries, mangos, apples, passion fruits, artubuses, green soybeans, and tomatoes which were harvested in our university. AsA was extracted by placing 5 g of tissues from fruits and vegetables in 25 mL of 5 % trichloroacetic acid; the mixture was shaken vigorously. The mixture was centrifuged (11,509 ×g, 10 min, 5 ˚C) and the supernatant was used as sample.

Selection of the methods used and comparison of the quantity of AsA measured by the colorimetry and HPLC method in fruit and vegetables

We compared linearity of the 0-50 mg/L standard curve of AsA by the α, α›-dipyridyl method [10], the o-phenanthroline method [8] and the ferrozine method [9]. In the α,α'-dipyridyl method, the reaction mixture consisted of 150 μL of sample, 10 μL of phosphorus acid, 80 μL of 1% dipyridyl solution, 10 μL of 3% ferric chloride 10 μL to sample 150 μL. The reaction mixture was incubated at 30 ˚C for 15 minutes and measured at 520 nm. In the o-phenanthroline method, the reaction mixture consisted of 250 μL of sample, 10 μL of 3 % ferric chloride, 50 μL of ammonium solution, 50 μL of acetic acid buffer solution (pH 4), and 125 μL of water. The reaction mixture was incubated at room temperature for 40 minutes and centrifuged (12000×g, 5 min, 5 ˚C) and measured at 520 nm. In the ferrozine method, the reaction mixture consisted of 250 μL of sample, 20 μL of 3 % ferric chloride, 50 μL of 5.7 % ferrozine solution, 200 μL of acetic acid buffer solution (pH 4), and 480 μL of water. The reaction mixture was incubated at room temperature for 10 minutes and measured at 546 nm. We measured some samples by the best of these colorimetry methods (i.e., with the best linearity of standard curve and measuring range) and by HPLC and compared the results of the two techniques. The HPLC details were: pump, LC-10AD (Shimazu, Kyoto, Japan); column, COSMOSIL(R) 5C18-MS-II Packed Column 4.6 mmI.D.x150 mm (NACALAI TESQUE, INC, Kyoto, Japan); mobile phase, 2% NaH_2PO_4 (pH 2.8); flow rate, 0.7 mL/min; temperature, 40 ˚C; detector, Shimazu SPD-10A; wavelength, 250 nm.

Comparison of the quantity of AsA measured by colorimetry with antioxidant ability via the DPPH method

We used the DPPH method to measure antioxidant ability [13]. To make DPPH solution, 8 mg of DPPH was dissolved in 50 mL of ethanol and 50 mL of water was added. 3.6 mL of DPPH solution was added to 0.4 mL of sample and incubated at room temperature for 30 minutes and measured at 520 nm. We used the blank 50% of ethanol instead of sample and calculated DPPH radical scavenging ability. We compared the quantity of AsA by colorimetry with antioxidant ability by the DPPH method in samples made as above.

Comparison of the quantity of AsA measured by the colorimetry method with the iron reduction ability

We used the FRAP method to measure iron reduction ability [14]. To make 2, 4, 6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution, 31 mg of TPTZ was dissolved in 10 mL of 40 mM hydrochloric acid. FRAP reagent mixture is obtained by mixing acetic acid buffer solution (pH 3.6) 10 with TPTZ solution 1 with 20 mmol/L $FeCl_3$ 1 in a mixing ratio. We added 1.0 mL of FRAP reagent mixture to 100 μL of sample and incubated at 37 ℃ for 4 minutes and measured at 570 nm. We showed Fe^{2+} production (μmol/L) using $FeSO_4 \cdot 7H_2O$ as a control and compared the quantity of AsA measured by colorimetry method with iron reduction ability by the FRAP method in samples made as above.

Statistical analysis

To compare the content of AsA in vegetables and fruits determined using colorimetry and HPLC, T-tests were used for pairwise comparisons. $P < 0.05$ was considered statistically significant. The relationship between colorimetry and antioxidant ability as well as that between colorimetry and iron reduction ability were evaluated via correlation.

Results and Discussion

Selection of the methods used and comparison of the quantity of AsA measured by the colorimetry method and the HPLC method in fruit and vegetables

Each correlation and standard curve in the α,α'-dipyridyl, o-phenanthroline, and ferrozine methods are showed in table 1. From Table1, the standard curves and correlation coefficients were: $y = 0.027x+0.083$ and $R^2 = 0.99$ for the α,α'-dipyridyl method; $y = 0.019x+0.16$ and $R^2 = 0.90$ for the

o-phenanthroline method; and y = 0.031x+0.092 and R^2 = 1.0 for the ferrozine method. Thus the ferrozine method showed the best correlation and was used in subsequent comparisons in our study.

Table 1: Correlation and standard curve.

	R^2	standard curve
α,α'-dipyridyl	0.99	y = 0.027x+0.083
o-phenanthroline	0.90	y = 0.019x+0.16
ferrozine	1.00	y = 0.031x+0.092

The quantities of AsA in fruit and vegetables measured by the ferrozine method and by the HPLC method are shown in table 2. The quantities of AsA by the ferrozine method (0.70-1.0 mg/g) were higher than by HPLC (0.010-0.59 mg/g) in all examined sample ($p<0.05$). The largest difference between the methods was seen in raspberries (0.92 mg/g), while the smallest difference was in oranges (0.023 mg/g). The correlation of the quantities of AsA measured by the two methods was high (R^2 = 0.92) (Figure 3).

Table 2: Quantity of AsA in fruit and vegetables measured by the ferrozine method and by the HPLC method.

	Ferrozine	HPLC
Japanese oranges	0.31 ± 0.042	0.19 ± 0.022
Oranges	0.070 ± 0.0091	0.050 ± 0.0066
Ponkans	0.37 ± 0.00075	0.26 ± 0.0014
Raspberries	0.94 ± 0.013	0.020 ± 0.000017
Jabuticabas	0.70 ± 0.060	-
Mandarin oranges	0.30 ± 0.0013	0.082 ± 0.0032
Tomatoes	0.14 ± 0.077	0.023 ± 0.00046
Bananas	0.66 ± 0.029	0.0059 ± 0.00033
Broccolis	0.058 ± 0.0049	0.013 ± 0.00097
Kiwis	0.49 ± 0.0020	0.29 ± 0.016
Chinese cabbages	0.10 ± 0.0010	0.045 ± 0.00019
Green peppers	0.15 ± 0.0020	0.0068 ± 0.0012
Lemons	0.47 ± 0.0087	0.28 ± 0.015
Grapefruits	0.62 ± 0.033	0.27 ± 0.052
Strawberries	0.80 ± 0.076	0.58 ± 0.0015
Pineapples	1.0 ± 0.049	0.59 ± 0.026

Values represent the mean of ten independent determinations (n = 10). Standard deviations are given in ± values.

Our results suggest that as the colorimetry method uses strong iron reduction ability, we can use that method when a sample has little reducing material except for AsA [15]. Many fruits and vegetables have reducing materials other than AsA but the quantity of AsA in orange, lemon, pepper and tomato is measured using 2, 2-bipyridyl [16]. On the other hand, as the reducing materials affect the measurement of AsA in tomato, AsA was measured using HPLC [17]. The quantity of AsA by the ferrozine method was higher than that by HPLC in fresh and freezing vegetables, and this may be caused by polyphenol in fruits and vegetables [13]. Fruits and vegetables have much more polyphenol than AsA, plums have 167 -250 mg/100 mL of polyphenol and 2.3-4.0 mg/100 mL of AsA [18], and persimmon leaves have 6.3×10^2 mg/100 g of polyphenol and 87 mg/100 g of AsA [19].

Comparison of the quantity of AsA measured by colorimetry with antioxidant ability in DPPH method

The quantities of AsA by the ferrozine method and antioxidant ability by the DPPH method in fruits and vegetables are shown in table 3. The quantities of AsA measured by the ferrozine method were between 0.058 and 0.62 mg/g, with the highest seen in grapefruits (0.62 mg/g) and the lowest in broccoli (0.058 mg/g). Antioxidant ability measured by the DPPH method was between 25% and 51%, highest in pineapple (51%) and lowest in broccoli (25%). It has been reported that pineapple has several beneficial properties, including antioxidant ability [20]. Furthermore, it has higher antioxidant ability than papaya, blueberry, and olive [21]. In this study, pineapple exhibited the highest antioxidant ability. Thus, fruit and vegetables that have a lot of AsA also have high antioxidant ability but with poor correlation (R^2 = 0.49) (Figure 1). DPPH radical scavenging ability is often assessed in various fruits and vegetables and the main contributing components to DPPH depend on the kind of fruits and vegetables. It was reported that main contributing components are polyphenol in rosemary [22], persimmon leaves [19], pears [23], apples, Japanese pears, peaches (white or yellow pulp), nectarines, Japanese plums, prunes, and grapes [24] and ellagic acid in strawberry [25] and melanoidin in miso [26]. AsA hardly contributes DPPH radical scavenging ability as we confirmed and so colorimetry is not suitable for evaluation of antioxidant ability.

Table 3: Quantities of AsA by the ferrozine method and antioxidant ability by the DPPH method in fruits and vegetables.

	Ferrozine	DPPH
	AsA	Radical scavenging ability %
	mg/g	
Japanese oranges	0.31 ± 0.042	35 ± 2.6
Oranges	0.40 ± 0.036	34 ± 0.58
Painapples	1.0 ± 0.18	51 ± 0.31
Tomatoes	0.14 ± 0.077	41 ± 1.5
Bananas	0.066 ± 0.029	29 ± 12
Broccolis	0.058 ± 0.0048	25 ± 15
Green peppers	0.15 ± 0.0020	46 ± 1.7
Lemons	0.47 ± 0.0087	41 ± 1.9
Grapefruits	0.62 ± 0.033	40 ± 3.8

Values represent the mean of five independent determinations (n = 5). Standard deviations are given in ± values.

Comparison of the quantity of AsA measured by colorimetry method with iron reduction ability

The quantities of AsA measured by the ferrozine method

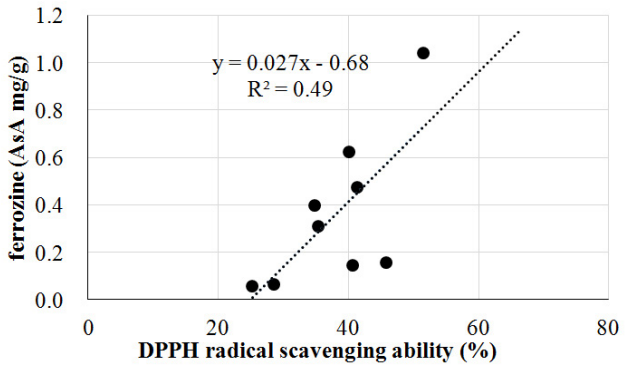

Figure 1: The correlation between quantities of AsA measured by colorimetry method and antioxidant ability.

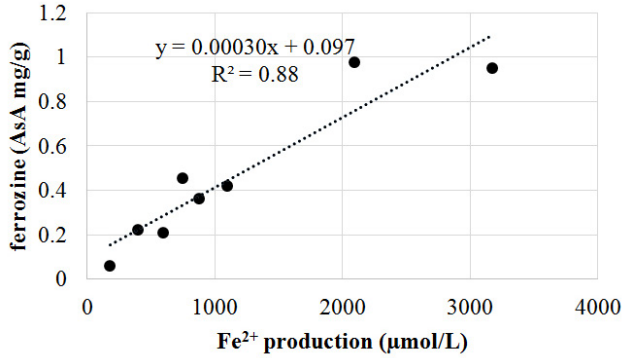

Figure 2: The correlation between quantities of AsA measured by colorimetry method and iron reduction ability.

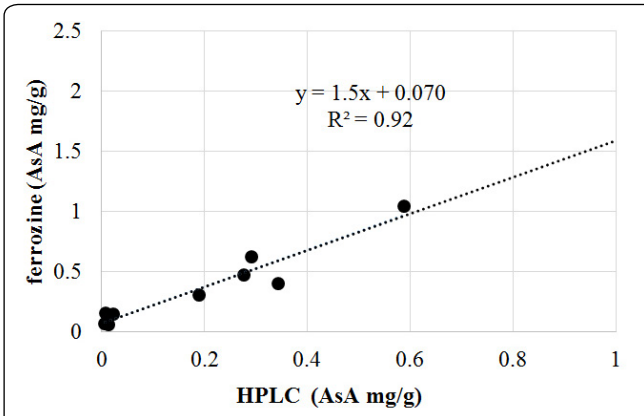

Figure 3: The correlation of quantity of AsA measured by ferrozine method and HPLC.

and the iron reduction ability measured by the FRAP method in fruits and vegetables are shown in table 4. The quantities of AsA were between 0.14 and 0.95 mg/g, with the highest in strawberries (0.95 mg/g) and the lowest in bayberries (0.14 mg/g). The iron reduction ability by the FRAP method was between 1.8×10^2 and 3.1×10^3 µmol/L, with the highest in strawberries (3.1×10^3 µmol/L) and the lowest in mangoes (1.8×10^2 µmol/L). While determining the iron reduction ability of 35 Ugandan fruits and vegetables, it was reported that mangoes had relatively higher values than other fruits and vegetables [27]. Iron reduction ability depends on each fruits and vegetables. There was high correlation between quantity of AsA by colorimetry and iron reduction ability (R^2 = 0.85) (Figure 2).

Table 4: Quantities of AsA measured by the ferrozine method and the iron reduction ability measured by the FRAP method in fruits and vegetables.

	Ferrozine	FRAP
	AsA	Fe^{2+} production
	mg/g	µmol/L
Strawberries	0.95 ± 0.18	3.1×10^3 ± 54
Green soybeans	0.22 ± 0.038	4.0×10^2 ± 10
Tomatoes	0.36 ± 0.0079	8.8×10^2 ± 7.7
Kiwis	0.94 ± 0.013	7.5×10^2 ± 19
Apples	0.70 ± 0.060	5.9×10^2 ± 5.8
Mangoes	0.30 ± 0.0013	1.8×10^2 ± 10
Japanese Plums	0.98 ± 0.19	2.1×10^3 ± 60
Passion fruits	0.66±0.029	1.1×10^3±25

Values represent the mean of five independent determinations (n =5). Standard deviations are given in ±values.

The FRAP method is often used in evaluation of iron reduction ability in various food; strawberry purees [28], avocado, pineapple, banana, papaya, passion fruit, watermelon and melon's different parts (pulp, seed, raw peel and dried peels) [29], some beverages, chocolates, nuts, and seeds [30]. The quantity of Fe^{2+} reduced from Fe^{3+} by samples are measured in the FRAP method. This is the same principal as used in the ferrozine method, but iron reduction ability evaluated using ferrozine method has not been reported in food samples. As the ferrozine method is used in measuring iron reduction ability in the process of birch and pine wood degradation [31] and in dry beans [32], colorimetry is suitable for evaluation of iron reduction ability.

Conclusion

In this study, we evaluated the colorimetry method for AsA measurement in fruits and vegetables. In colorimetry, some reduction components affect AsA measurement in fruits and vegetables and differ from results obtained using HPLC. Therefore, we conclude that colorimetry is not suitable for AsA measurement in fruits and vegetables. The correlation between the ferrozine method and antioxidant ability is low but between the ferrozine method and iron reduction ability is high, so we conclude that colorimetry can evaluate iron reduction ability in fruit and vegetables.

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