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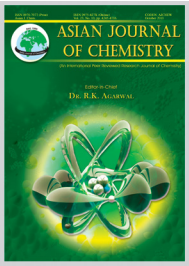

Determination of Ascorbic Acid in Common Fruits, Herbal Tea by Using Cuprac and Cerac Methods

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The method used to determine the ascorbic acid (Vitamin C) is based on the oxidation of ascorbic acid to dehydroascorbic acid with the cupric ion reducing antioxidant capacity (CUPRAC) and ceric-reducing antioxidant capacity (CERAC) reagent of total antioxidant capacity assay. The antioxidant capacity indicates the degree of protection of a certain organism against oxidative damage provoked by reactive oxygen and nitrogen species. These methods were applied to a number of commercial fruits and blackberry herbal tea. The findings of cupric ion reducing antioxidant capacity method are statistically compared to those of the ceric-reducing antioxidant capacity method, which is a relatively new one. In addition to this, we were also investigated the kinetic degradation of the ascorbic acid in a certain type of fruit juice.

Key Words: Ascorbic acid, Ceric-reducing antioxidant capacity, Cupric ion reducing antioxidant capacity, Antioxidant, Kinetics.

INTRODUCTION

Free radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress, which is defined as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage[1]. Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation[2]. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. Reactive oxygen species are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins[3]. occur which increases risk of more than 30 different disease processes[4]. Antioxidants are substances, which counteract free radicals and prevent the damage caused by them. These can greatly reduce the adverse damage due to oxidants by crumbling them before they react with biologic targets, preventing chain reactions or preventing the activation of oxygen to highly reactive products except for anaerobes, oxygen is vital for all the living systems. However, the paradox of aerobic life is that oxidative damage occurs at the key biological sites, threatening their structure and function. Oxygenic threat is met by an array of antioxidants that evolved in parallel with our oxygenic atmosphere. Our body implements various antioxidants, some of which are dietary-derived antioxidants to help restrain potential free radical damage that could occur in our bodies. If one looks back into the evolution of human diet, it can be observed that in the Paleolithic age human intake of plant-derived antioxidants is considered to have been many times higher than current intake[5]. Antioxidants fall in two major groups *viz.*, those preventing the initiation and those slowing down the progression of a peroxidative chain reaction[6-8].

Among several chemical, physical and biological mechanisms of prevention of initiation, the most relevant appear the chemical or enzymatic reduction of hydroperoxides and the chelation of transition metals, which produce initiating radicals from oxygen or, in more favourable reactions, from hydroperoxides[6].

The second group is accounted for by chain breaking antioxidants, which, competing with unsaturated lipids for the reaction with peroxidation driving peroxy radical, slow down the rate of the oxidative process[6] and literature cited therein.

The most direct, although empirical, approach for measuring an antioxidant effect, which accounts for both mechanisms, evolves from the simplest concept of antioxidant *i.e.*, a molecule which delays, by a different mechanism, the onset of massive

oxidative degradation of target molecules endowed in their specific matrix-from rubber polymers[6] to foods[9] to plasma lipoproteins[10]. To analyze antioxidant capacity, a sample, containing both the antioxidant and the molecule to be protected, is most frequently challenged with an accelerator of the free radical oxidation[9-11]. Then, the antioxidant capacity is measured as period of protection. Ascorbic acid, commonly known as vitamin C, is one of the most important water-soluble vitamins in the human diet, because it helps the body in forming connective tissue, bone, teeth, blood vessel walls and assists the body in assimilating iron and amino acids[12]. Vitamin C prevents scurvy and lowers the incidence of and mortality from cardiovascular disease and cancer[13]. The essential supply of humans with vitamin C is through dietary uptake. Vitamin C is present naturally in a wide range of natural food comprized of fruits and vegetables and in many formulations of food supplements and pharmaceuticals[12]. Humans cannot synthesize ascorbic acid (vitamin C)[14], so it must be provided exogenously in the diet and transported intracellularly. Until recently, it was not known how ascorbic acid is transported into human tissues[15]. Recent studies have shown that ascorbate and DHA are transported into human neutrophils by two distinct mechanisms[16]. Other studies performed with the HL-60 cell line that served as a model for human myeloid cells have shown that these cells transport ascorbic acid only in the form of DHA[17,18]. Once inside the cell, DHA is reduced to ascorbic acid, a non-transportable moiety. Ascorbic acid is known to accumulate in tissues[15,16] and is crucial to health[19-21]; however, it is not clear how the vitamin functions intracellularly in most cell types. For example, ascorbic acid is necessary for normal leukocyte function, but the precise role it plays in leukocyte biology is uncertain.

The antioxidant properties of ascorbic acid have been emphasized previously[22,23]. Ascorbic acid, however, can act as an antioxidant or a pro-oxidant, for example, in the Fenton reaction with iron[24].

EXPERIMENTAL

Chemicals, herbal samples and instrumentation: Ethyl alcohol (96 % EtOH) from Riedel; Ce(IV) sulfate tetrahydrate, sulfuric acid (98 %), potassium dichromate, sodium thiosulfate, iodine, KI, starch, ammonium acetate, copper(II) chloride and ascorbic acid, neocuprain hemihydrate were purchased from Merck. Herbal tea and fruit used were supplied from the local supermarkets in Edirne and Tekirdag, west part of Turkey. A cerium(IV) sulfate solution containing $2 \times 10^{3-}$ M Ce(IV) was prepared by dissolving 0.0809 g of $Ce(SO_4)_2 \cdot 4H_2O$ in 25 mL distilled water, adding 17 mL concentrated H_2SO_4 and thoroughly mixing by the aid of a magnetic stirrer until total dissolution at room temperature. This solution was totally transfered to a 100 mL-flask and diluted to the mark with distilled water. The Ce(IV) stock solution was standardized iodometrically by adding excess KI and titrating the liberated iodine (triiodide complex) with a standard solution of sodium thiosulfate (which was previously standardized against potassium dichromate) using starch as indicator. $1.0 \times 10^{2-}$ M copper(II) chloride solution was prepared from $CuCl_2 \cdot 2H_2O$ (0.4262 g) dissolved in H_2O and diluted to 250 mL with additional water. Ammonium acetate buffer at pH = 7.0 was prepared by dissolving ammonium acetate (19.27 g) in water and diluting to 250 mL. Neocuproine solution (7.5×10^{-3} M) was prepared by dissolving neocuproine (0.039 g) in 96 % EtOH and diluting to 25 mL with ethanol.

All absorbance measurements were made at the prespecified wavelength of the selected spectrophotometric method using a Shimadzu UV-1601 VIS spectrophotometer using a pair of matched quartz cuvettes of 1 cm thickness (light path).

Plant materials and extraction: The fruit samples and herbal tea (weighing 1 g) was pounded into small parts with a porcelain mortar. The samples were extracted with distilled water and were mixed with magnetic stirrer for 0.5 h. The extract was filtered through a Whatman filter paper into a 25 mL-flask and diluted to the mark with distilled water. The aqueous extract of nettle was prepared just before the experiments so as to prevent any undesired degradation reactions.

Ceric-reducing antioxidant capacity measured by spectrophotometric method: The method is based on the oxidation of antioxidants with cerium(IV) sulphate in dilute sulphuric acid at room temperature. The Ce(IV) reducing capacity of the sample is measured under carefully adjusted conditions of oxidant concentration and pH such that only antioxidants and not other organic compounds would be oxidized[25]. The spectrophotometric determination of the remaining Ce(IV) was performed after completion of reaction with antioxidants. This method had been chosen because this methods is low cost, have few reaction step, is made device not specific and this method's repeating is high. To a test tube were added 1 mL of $2 \times 10^{3-}$ M Ce(IV), x mL of fruit or herbal tea samples and (10-x) mL of water were added (total volume = 10 mL) and mixed well. Absorbance against a reagent blank was measured at 320 nm after 0.5 h. Since the molar absorptivity of Trolox in the ceric-reducing antioxidant capacity method is $\varepsilon = 12950$ L mol^{-1} cm^{-1}

$$2Ce^{4+} + H_2Asc \rightarrow 2Ce^{3+} + DAsc + 2H^+ \; (pKH_2As = 4.08)$$

$$H_2Asc \leftrightarrow HAsc^- + H^+$$

$$HAsc^- + Ce^{4+} \rightarrow Ce^{3+} + HAsc^{\cdot} + H^+$$

$$HAsc^{\cdot} + Ce^{4+} \rightarrow Ce^{3+} + DAsc + H^+$$

Calculation of ceric-reducing antioxidant capacity by spectrophotometric method: If a herbal infusion (initial volume = V_E) prepared from m grams of plant was dilute (r) times prior to analysis and a sample volume of V_S was taken for analysis from the diluted extract and colour development (after addition of reagent) was made in a final volume of (V_f) to yield an absorbance (A_f), then the ascorbic acid equivalent antioxidant capacity of the herb (in mmol ascorbic acid per gram of plant, or simply mmol ascorbic acid/g) was found using the equation:

$$\text{Capacity (in mmol ascorbic acid/g)} = [A_f/\varepsilon_{AA}] (V_f/V_S) SF (V_E/m)^{24}$$

Cupric ion reducing antioxidant capacity spectrophotometric assay of total antioxidant capacity: Cupric ion reducing antioxidant capacity method is based on the oxidation of ascorbic acid to dehydroascorbic acid with the cupric ion reducing antioxidant capacity reagent of total antioxidant capacity assay, *i.e.*, Cu(II)-neocuproine (Nc), in ammonium acetate-containing medium at pH 7, where the absorbance of the formed bis(Nc)-copper(I) chelate is measured at 450 nm[26]. To a test tube were added 1 mL of $CuCl_2$ solution ($1.0 \times 10^{2-}$ M), 1 mL of neocuproine alcoholic solution ($7.5 \times 10^{3-}$ M) and 1

mL ammonium acetate buffer solution and mixed; (x) mL of herbal extract followed by (1.1-x) mL of water were added (total volume = 4.1 mL) and mixed well[27]. Absorbance against a reagent blank was measured at 450 nm after 0.5 h. Since the molar absorptivity of Trolox in the cupric ion reducing antioxidant capacity method is e = 1.67×10^4 L mol^{-1} cm^{-1} and the calibration curve for Trolox is a line passing through the origin, the Trolox equivalent molar concentration of the herbal extract sample in final solution may be found by dividing the observed absorbance to the e for Trolox. The Trolox equivalent antioxidant capacity may be traced back to the original extract considering all dilutions and proportionated to the initial mass of herbal material taken to find a capacity in the units of mmol Trolox/g dry matter.

Calculation of cupric ion reducing antioxidant capacity by spectrophotometric method: The molar absorptivity of Trolox in the above reference methods were as follows: eTrolox = 1.67×10^4 L mol^{-1} cm^{-1} (cupric ion reducing antioxidant capacity method).

If a herbal infusion (initial volume = V_{cup}) prepared from (m) grams of dried plant was diluted (r) times prior to analysis and a sample volume of V_s was taken for analysis from the diluted extract and colour development (after addition of reagents) was made in a final volume of (V_f) to yield an absorbance of (A_f), then the Trolox equivalent antioxidant capacity of the herb (in mmol Trolox per gram of dried plant, or simply mmol Trolox/g) was found using the equation:

$$\text{Capacity(in mmol Trolox/g)} = (A_f/\varepsilon_{TR})(V_f/V_s)r(V_{cup}/m)$$ [25]

Degradation kinetic models: The degradation kinetics of most biological materials of food system follows the zero-order eqn. (1) or first-order eqn. (2)[28]:

$$C_t = C_0 + k_0 t \quad (1)$$

$$C_t = C_0 \exp(- k_1 t) \quad (2)$$

where, C_t and C_0 are the biological materials content at time t and zero, respectively, k_0 and k_1 are the zero- and first-order kinetic constants, respectively and t is the storage time.

RESULTS AND DISCUSSION

Calibration lines: Fig. 1 shows calibration line of ascorbic acid. The equation for the calibration line of ascorbic acid in the ascorbic acid concentration range 1.5×10^{-5} to 7.5×10^{-4} M was:

$A_{320} = 1.295 \times 10^4 C_{asc.} - 0.9648$, ($R^2 = 0.9983$) yielding a molar absorptivity of 1.295×10^4 L mol^{-1} cm^{-1} for ascorbic acid. Antioxidants were quantified indirectly through their ability to reduce a fixed initial concentration of Ce(IV).

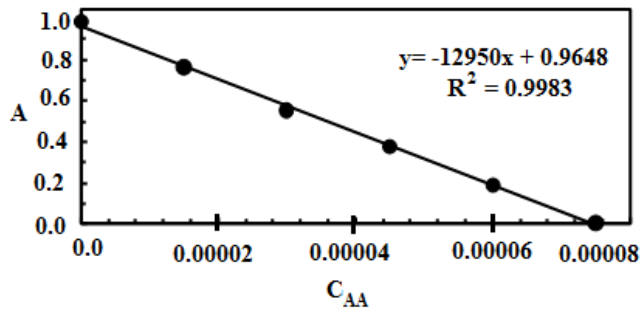

Fig. 1. Calibration line of ascorbic acid

Linear calibration curves: Calibration curves of ascorbic acid in pure aqueous solution and in green kiwi as assayed by the ceric-reducing antioxidant capacity method were parallel lines (Fig. 2), showing that there were no chemical deviations from Beer's law arising from chemical interactions between ascorbic acid and the measured antioxidant constituents of green kiwi.

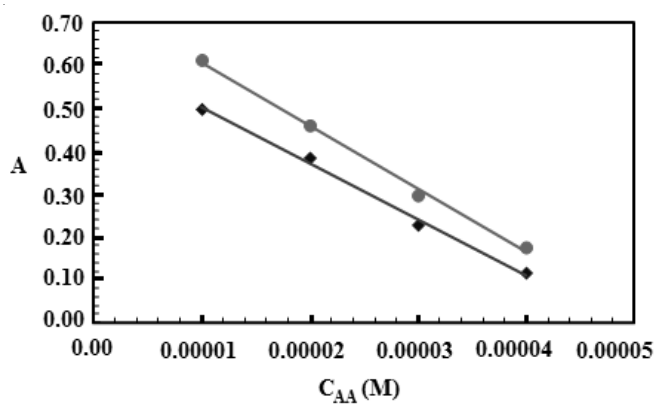

Fig. 2. Calibration curves of ascorbic acid in pure aqueous solution and in green kiwi as assayed by the ceric-reducing antioxidant capacity method. [Calibration curves of ascorbic acid (◆) in pure aqueous solution and in green kiwi (●)]

Radical scavenging activity is very important, due to the deleterious role of free radicals in foods and in biological systems. Diverse methods are currently used to assess the antioxidant activity of plant compounds. Ceric-reducing antioxidant capacity method is a simple, low-cost, sensitive and diversely applicable indirect spectrophotometric method for the determination of total antioxidant capacity of several plants. The method is based on the oxidation of antioxidants with cerium(IV) sulfate in dilute sulfuric acid at room temperature. The Ce(IV) reducing capacity of the sample is measured under carefully adjusted conditions of oxidant concentration and pH such that only antioxidants and not other organic compounds would be oxidized. The spectrophotometric determination of the remaining Ce(IV) was performed after completion of reaction with antioxidants. The concentration of remaining Ce(IV) is measured as an indication of initial antioxidant concentration. Therefore the maximum absorption wavelength selection for Ce(IV) is important and solution acidity should be properly adjusted such that this wavelength should not shift with pH. Considering the hydrolytic equilibria of Ce(IV);

$$Ce^{4+} + H_2O \rightleftharpoons Ce(OH)^{3+} + H^+, \; K_1 = 0.2$$

$$Ce(OH)^{3+} + H_2O \rightleftharpoons Ce(OH)_2^{2+} + H^+, \; K_2 = 0.16$$

The advantage of the ceric-reducing antioxidant capacity method is its simplicity and applicability to conventional laboratories without high-level instrumentation. The cupric ion reducing antioxidant capacity reaction oxidized ascorbic acid to dehydroascorbic acid, while the A_{450} measurement was due to the coloured Cu(I)-neocuproine chelate formed as the result of the redox reaction[27,29]. The cupric ion reducing antioxidant capacity reagent (an outer-sphere electron-transfer agent) is fast enough to oxidize thiol-type antioxidants. The reagent is much more stable and easily accessible and selective, because it has a lower redox potential. The cupric ion reducing anti-

TABLE-1
TOTAL ANTIOXIDANT CAPACITY OF SOME SELECTED FRUITS AND HERBAL TEA (IN THE UNITS OF ASCORBIC ACID EQUIVALENT ANTIOXIDANT CAPACITY) AS MEASURED BY CERIC-REDUCING ANTIOXIDANT CAPACITY (CERAC) AND CUPRIC ION REDUCING ANTIOXIDANT CAPACITY(CUPRAC) SPECTROPHOTOMETRIC METHOD

Latin name	Fruit name	CERAC (average mmol/g)	CUPRAC (average mmol/g)
Ficus carica	Fig	0.0155 ± 0.2	0.0122 ± 0.1
Prunus sp.	Plum	0.0111 ± 0.1	0.0085 ± 0.5
MusaX paradisiacal	Banana	0.0197 ± 0.3	0.0131 ± 0.7
Prunus persica	Peach	0.0136 ± 0.8	0.0086 ± 0.4
Mangifera indica	Mango	0.0185 ± 0.4	0.0119 ± 0.2
Ananus comosus	Pineapple	0.0136 ± 0.1	0.0103 ± 0.3
Persea americana	Avocado	0.0272 ± 0.3	0.0246 ± 0.1
Pyrus communis	Pear	0.0056 ± 0.2	0.0030 ± 0.1
Actinidia deliciosa	Green Kiwi	0.0210 ± 0.9	0.0140 ± 0.5
	Blackberry herbal tea	0.2010 ± 0.1	0.1570 ± 0.8
Ribes satidum	Redcurrant	0.0190 ± 0.2	0.0120 ± 0.9
Ziziphus jujuba	Jujube	0.0210 ± 0.4	0.0180 ± 0.2

oxidant capacity method is easily and diversely applicable in conventional laboratories using standard colourimeters rather than necessitating sophisticated equipment and highly qualified operators. It responds equally well to both hydrophilic and lipophilic antioxidants. The redox reaction giving rise to a coloured chelate of Cu(I)-neocuproine is relatively insensitive to a number of parameters adversely affecting radical reagents (*e.g.*, air, sunlight, humidity and pH, to a certain extent)[30].

Measured absorbance values: Fruit and herbal tea samples were measured twice. Results of analysis were showed Table-1 as mmol/gr. Total antioxidant capacity of some selected fruits and herbal tea were calculated from average absorbance values.

Kinetic data: In this study, the fruit juice was stored at 5, 15, 25, 35 and 45 ºC in the dark and determination of ascorbic acid was carried out on three replicates at 12 h interval. Fig. 3(a, b) shows that the residuals from first order kinetic model are closer to zero than other models in this study.

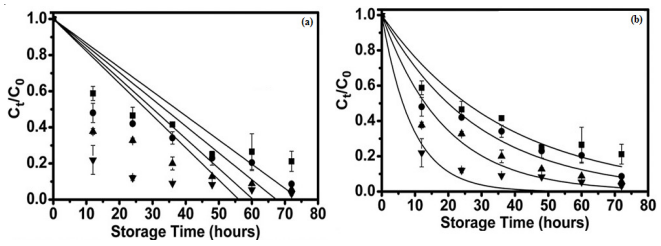

Fig. 3. Effect of storage time and temperature on the retention of ascorbic acid (a) Zero-order kinetic, (b) First-order kinetic [15 ºC (■), 25 ºC (●), 35 ºC (▲), 45 ºC (▼)].

Conclusion

This work reports the antioxidant assay of ascorbic acid in water solvent media using ceric-reducing antioxidant capacity and cupric ion reducing antioxidant capacity methods. This work brings several contributions to food analytical chemistry (specifically to antioxidant assays). The decreasing order of TAC for fruits and herbal tea with respect to the cupric ion reducing antioxidant capacity and ceric-reducing antioxidant capacity methods was: blackberry herbal tea > avocado > banana > mango > fig > peach = pineapple > plum > green kiwi = jujube > redcurrant > pear. The results of this work may be useful in recognizing the nutritive antioxidant values of commercially fruits and herbal tea and comparing their antioxidant capacities for possible health benefits through diet.

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