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Thermal Stability of Ascorbic Acid and Ascorbic Acid Oxidase in African Cowpea Leaves (*Vigna unguiculata*) of Different Maturities

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ABSTRACT: Cowpea, an African leafy vegetable (*Vigna unguiculata*), contains a high level of vitamin C. The leaves harvested at 4−9 weeks are highly prone to vitamin C losses during handling and processing. Therefore, the purpose of this research was to study the effect of thermal treatment on the stability of ascorbic acid oxidase (AAO), total vitamin C content (L-ascorbic acid, L-AA), and dehydroascorbic acid (DHAA) and L-AA/DHAA ratio in cowpea leaves harvested at different maturities (4, 6, and 8 weeks old). The results showed that AAO activity, total vitamin C content, and L-AA/DHAA ratio in cowpea leaves increased with increasing maturity (up to 8 weeks). Eight-week-old leaves were the best source of total vitamin C and showed a high ratio of L-AA/DHAA (4:1). Thermal inactivation of AAO followed first-order reaction kinetics. Heating at temperatures above 90 °C for short times resulted in a complete AAO inactivation, resulting in a protective effect of L-AA toward enzyme-catalyzed oxidation. Total vitamin C in young leaves (harvested at 4 and 6 weeks) was predominantly in the form of DHAA, and therefore temperature treatment at 30−90 °C for 10 min decreased the total vitamin C content, whereas total vitamin C in 8-week-old cowpea leaves was more than 80% in the form of L-AA, so that a high retention of the total vitamin C can be obtained even after heating and/or reheating (30−90 °C for 10 min) before consumption. The results indicated that the stability of total vitamin C in situ was strongly dependent on the plant maturity stage and the processing conditions applied.

KEYWORDS: vitamin C, AAO, thermal processing, L-AA/DHAA ratio

INTRODUCTION

Cowpeas (*Vigna unguiculata*) are tropical leafy vegetables from the family Fabaceae (known as well as Leguminosae). They are widely grown and consumed as a basic diet in the African region.[1] The leaves are harvested by plucking them from the stalk in a period from 4 weeks after emergence of seedlings to the onset of flowering (between 6 and 9 weeks depending on the cultivar).[2] After harvest, the leaves are usually washed and cut into pieces for subsequent meal preparation. The preparation of leaves into a meal entails heating them for about 8−15 min either by frying, steaming followed by frying, or boiling them together with tomato and/or onion in a small amount of water. The leaves are consumed as a side dish for *ugali* (a paste prepared from maize meal) and to a lesser extent as part of a main meal when they are cooked with maize kernels, legumes, green bananas, or potatoes. Due to their widespread consumption in Africa, cowpea leaves contribute to the daily diet as sources of vitamins such as vitamin C and folates, fiber, and minerals.[3−7] Hence, a large part of the Kenyan population relies on these vegetables as a source of vitamin C.

Vitamin C in vegetables comprises L-ascorbic acid (L-AA) and dehydroascorbic acid (DHAA). L-AA is the principal biologically active form of vitamin C, whereas DHAA, an oxidation product, also exhibits biological activity but to a lesser extent. Because DHAA can be easily converted into L-AA in the human body, it is important to measure both L-AA and DHAA in vegetables as total vitamin C activity.[8] During the processing of vegetables, it is important to maintain vitamin C in the form of L-AA because L-AA acts as a scavenger of reactive oxygen species; it is effective against superoxide radical anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen,[9,10] whereas DHAA has no radical scavenging activity.[11] Also, the degradation of DHAA to 2,3-diketogulonic acid (DKGA) is not reversible,[12,13,8] as opposed to the degradation of L-AA to DHAA. During cooking, vitamin C losses can be due to enzymatic and chemical degradation, heating, or leaching.[14] In vegetables, when cell disruption occurs, L-AA oxidation to monodehydroascorbic acid (MDHAA) is accelerated by ascorbic acid oxidase (AAO). MDHAA is an unstable radical and rapidly disproportionates to yield DHAA. AAO (EC 1.10.3.3) is a multicopper oxidase[15] glycoprotein widely distributed in higher plants and microorganisms, the main sources being members of the Cucurbitaceae family.[16] It catalyzes one-electron oxidation of L-AA with concomitant reduction of molecular oxygen to water.[17] Under aerobic conditions, thermal degradation of L-AA can be described by apparent first-order kinetics. L-AA is oxidized to form DHAA followed

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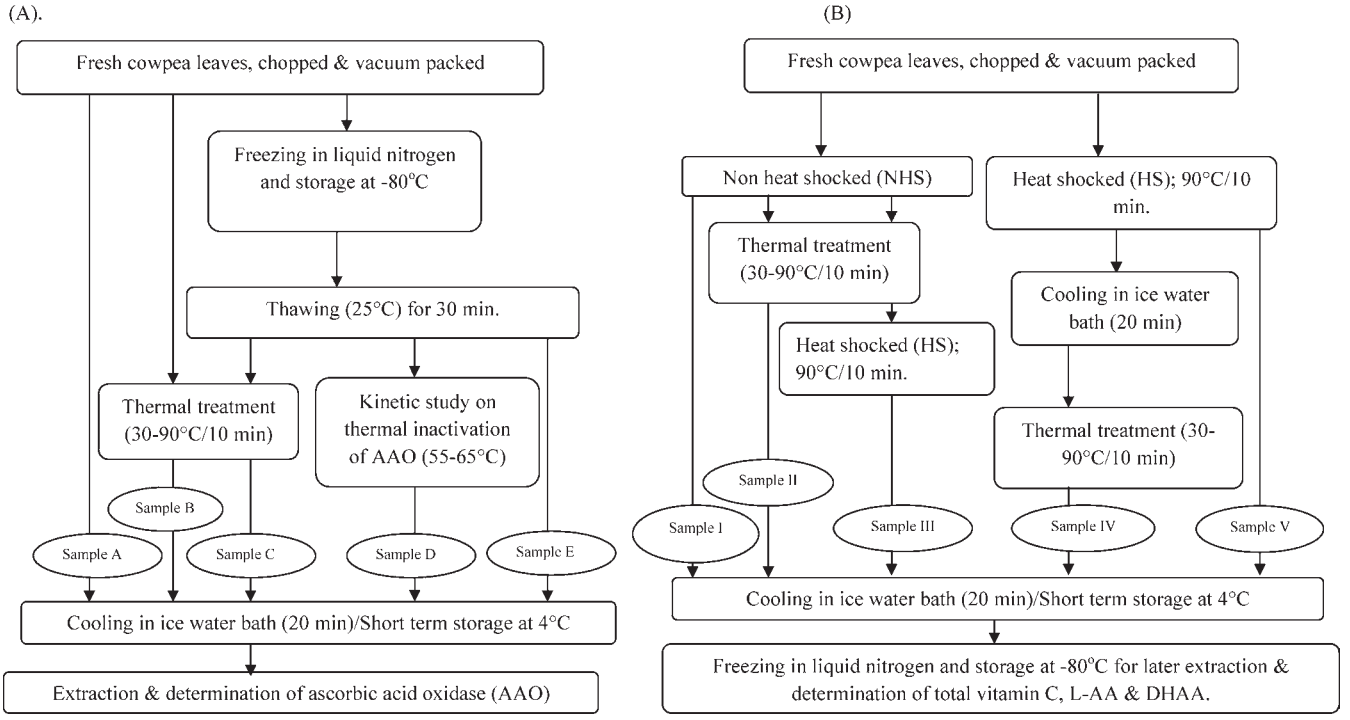

Figure 1. Flowchart of sample preparation for determination of AAO stability and vitamin C content in cowpea leaves.

by hydrolysis and oxidation to form DKGA and oxalic acid. Under anaerobic conditions, L-AA undergoes ketonization to form the intermediate keto-tautomer (keto-ascorbic acid), which is in equilibrium with its anion (keto-monoanion ascorbic acid), which by further delactonization forms DKGA.[18,19]

DHAA and AAO play important roles in plant growth. Total vitamin C content and L-AA/DHAA ratio in leaves change at different stages of maturity. Young leaves likely contain a lower L-AA/DHAA ratio than older leaves. Several mechanisms whereby AAO controls cell growth have been proposed.[20,21] In plants, AAO occurs as free or cell wall bound enzyme.[22,23] The bound enzyme occurs tightly adsorbed to cell walls, whereas the free enzyme is found as a soluble protein in the cytosol.[24,25] Furthermore, AAO exists as different isoenzymes.[26] Under stress conditions during plant growth such as pathogen attack, chemical exposure, or adverse weather conditions, the AAO content in leaves increases.[25]

Hereto, the main objective of this study was to analyze the effect of thermal treatment on the stability of AAO and vitamin C in cowpea leaves at different growth stages (maturities). Detailed kinetics on thermal inactivation of AAO in situ were also investigated. This integrated study from farm to processing provided different strategies to achieve high vitamin C content with a high L-AA/DHAA ratio after thermal processing.

MATERIALS AND METHODS

Growth Condition of Cowpea Vegetables. Cowpea (*V. unguiculata*) seeds were planted indoors (Katholieke Universiteit Leuven greenhouse, Heverlee, Belgium) in the period from April 2008 to May 2009. The parameters for growth conditions such as temperature, relative humidity, and light inside the greenhouse were automatically controlled and monitored. From 7:00 a.m. to 11:00 p.m., the temperature averaged 25.5 °C and the relative humidity averaged 80%. From 11:00 p.m. to 7:00 a.m., the temperature averaged 20 °C and the relative humidity, 60%. Artificial light automatically switched on at any time between 6:00 a.m. and 7:00 p.m. when the light was <200 W/m^2. The plants were grown in perforated pots rested in a shallow trough. Watering was done once a day by flooding the trough, thus enabling uptake by the plants through the pot perforations. In this study, no commercial fertilizer was used, and there was no sign of nutrient deficiency. The plants begun to pod from the ninth week after planting; therefore, the leaves were harvested at the ages of 4, 6, and 8 weeks (as usually used for consumption in Kenya).

Sample Preparation after Harvest. Cowpea leaves were packed and cooled immediately after harvest to slow respiration and transpiration during transport from the field to the research laboratory. On arrival, the leaves were rinsed with water, chopped into pieces (simulated as normally done in African cuisines), and packed (approximately 10 g) in plastic pouches (140 × 200 × 40 mm, DaklaPack United Kingdom, Chiswick, London, U.K.). The packages were vacuumized at 34 mbar (Multivac C200 Vacuum Chamber, Sepp Haggenmueller GmbH & Co., Wolfertschwenden, Germany). Fresh leaves were then subjected to different treatments to study vitamin C stability and determination of AAO content (Figure 1), whereas the rest of the fresh leaves were frozen in liquid nitrogen and stored at −80 °C for moisture content determination and AAO thermal stability studies (Figure 1).

In Situ Study on Thermal Stability of AAO in Cowpea Leaves. *Thermal Treatment.* The effect of freezing (in liquid nitrogen and storage at −80 °C) and vegetable maturity on AAO activity for material harvested at all stages of maturity was initially investigated using fresh (Figure 1A, sample A) and thawed cowpea leaves (Figure 1A, sample E). After study on the effect of freezing on AAO activity, the in situ thermal stability of AAO in fresh and thawed frozen leaves was studied at different predefined temperatures for all stages of maturity.

In the case of fresh leaves, approximately 10 g (unfrozen) of fresh leaves at the age of 6 weeks was vacuum packed in plastic pouches and afterward treated at temperatures between 30 and 90 °C for 10 min (Figure 1A, sample B) in a thermostated water bath (Memmert water bath WBU 45, Memmert GmbH & Co. KG, Schwabach, Germany). To stop thermal inactivation, the samples were immediately immersed in an ice−water bath for 20 min, and the residual AAO activity was determined. The control samples (nontreated samples) were used to identify the initial enzyme activity (A_0).

In the case of frozen (fresh samples were initially frozen in liquid nitrogen and then immediately stored at −80 °C) samples, plastic pouches each filled with 10 g of frozen cowpea leaves that had been harvested at all stages of maturity were thawed at 25 °C (30 min) in a thermostated water bath (Memmert water bath WB 22, Memmert GmbH & Co. KG). After thawing, the plastic pouches were heated for 10 min at a predefined temperature from 30 to 90 °C (Figure 1A, sample C) in a thermostated water bath (Memmert water bath WBU 45, Memmert GmbH & Co. KG). Pouches containing leaves that were not subjected to any thermal treatment after thawing were used to assess the initial enzyme activity (A_0). After the heat treatment, the leaves were cooled in an ice−water bath for 20 min, and the residual AAO activity was determined.

Kinetics Study on Thermal Inactivation of AAO. Plastic pouches each filled with 10 g of frozen (in liquid nitrogen and stored at −80 °C) cowpea leaves that had been harvested at 6 weeks were thawed at 25 °C in a thermostatic water bath (Memmert water bath WB 22, Memmert GmbH & Co. KG). The samples were heated at predefined temperatures between 55 and 65 °C for different time intervals (Figure 1A, sample D) in a thermostated water bath. Leaves that were not subjected to any thermal treatments after thawing were used as blank (initial enzyme activity, A_0). After heat treatment, the leaves were cooled in an ice−water bath for 20 min and then subjected to AAO extraction.

Extraction of AAO from Cowpea Leaves. The procedure of AAO extraction was initially optimized. AAO in thawed cowpea leaves harvested at 6 weeks was extracted at different concentrations of sodium chloride (0−1.4 M) and pH levels (pH 4−7). The extraction was done using sodium phosphate buffer (Na_2HPO_4, 4 mM) with different molar concentrations of sodium chloride and at different pH values.

The extraction of AAO was done on cowpea leaf samples, fresh or thawed, depending on the experiment as illustrated in Figure 1A. The extraction of AAO was done in a mixer (magic bullet blender, J-26, Ningbo Vanguard Import & Export Co, Ningbo, China) by mixing 10 g of vegetable sample with 20 mL of sodium phosphate buffer (Na_2HPO_4, 4 mM, pH 5.6, containing 1 M NaCl). After mixing, the extract was filtered through sieves with 1 mm^2 pores, with the aid of a vacuum pump. The filtrate was then centrifuged (Beckman J2-HS, Palo Alto, CA) at 13300*g* and 4 °C for 20 min. AAO crude extract was obtained by 80% ammonium sulfate precipitation at 4 °C for 30 min. The mixture was then centrifuged at 17700*g* and 4 °C for 20 min (Beckman J2-HS). The supernatant was discarded and the residue dissolved in sodium phosphate buffer (Na_2HPO_4, 4 mM, pH 5.6). This crude extract solution was afterward frozen in liquid nitrogen and stored at −80 °C until AAO assay.

Determination of AAO activity. A polarographic assay (Strathkelvin model 78 1 B oxymeter, Glasgow, Scotland) based on oxygen consumption due to AAO reaction was used to determine AAO activity. The assay was performed at 25 °C in a reaction cell (4 mL) by homogenizing a mixture of 2.9 mL of air-saturated phosphate buffer (4 mM and pH 5.6 containing 0.5 mM EDTA), 100 μL of substrate solution (0.5 mM L-ascorbic acid dissolved in sodium phosphate buffer (4 mM, pH 5.6), and 750 μL of cowpea leaf AAO extract/concentrate. All solutions were thermostated at 25 °C before the enzyme assay. The decrease in oxygen concentration was followed, and the AAO activity (expressed in units) was calculated on the basis of eq 1

$$\text{AAO activity (units)} = \frac{\Delta[O_2]}{t} \times \frac{1}{32} \times V \qquad (1)$$

where $\Delta[O_2]$ is the change in oxygen consumption due to oxidation, in parts per million (ppm) over reaction time (t in seconds); the value of 32 is the molecular weight of oxygen, and V is the total reaction volume (liters). One unit of AAO activity is the amount of enzyme needed to catalyze the oxidation of 1 μmol of L-AA per second at 25 °C and pH 5.6.

Study on Vitamin C Stability. *Sample Preparation.* Freshly harvested (4, 6, and 8 weeks old) leaves, rinsed with water, chopped into pieces, and vacuum packed (approximately 10 g) in plastic pouches, were divided into two types of samples: non-heat-shock samples (NHS samples) and heat-shock samples (HS samples) as illustrated in Figure 1B. NHS samples were not subjected to any thermal treatments after harvest while in plastic pouches (approximately 10 g per package) (Figure 1B, sample I). HS samples were prepared by boiling fresh cowpea leaves at 95 °C for 10 min and followed by immediate cooling for 20 min in an ice−water bath to inactivate AAO prior to further investigations (Figure 1B, sample V). These samples were stored at 4 °C before further treatments for less than 24 h after harvest.

Thermal Treatment of Cowpea Leaves. To investigate the thermal stability of vitamin C and the effects of AAO activity on vitamin C content and L-AA/DHAA ratio, different strategies of thermal treatment were adopted. The L-AA/DHAA ratio was monitored using NHS (in the presence of AAO activity) and HS (in the absence of AAO activity) samples. In this setup, plastic pouches containing approximately 10 g of the leaves (NHS) were subjected to thermal treatment at 30−90 °C for 10 min (Figure 1B, sample II) and then cooled in an ice−water bath for 20 min, frozen in liquid nitrogen, and stored at −80 °C before analysis. In addition, by using the NHS and HS samples, effects of different strategies on total vitamin C content and L-AA/DHAA ratio were studied. The study was conducted by heating NHS samples at 30−90 °C for 10 min and immediately followed by heat shock in a boiling water bath for 10 min (Figure 1B, sample III). To stop thermal treatment, the samples were cooled in an ice−water bath for 20 min, frozen in liquid nitrogen, and stored at −80 °C until analysis. HS samples were used to validate the thermal stability of vitamin C, L-AA, and DHAA. This was done by heat shocking the samples in a boiling water bath for 10 min, followed by thermal treatment at 30−90 °C for 10 min (Figure 1B, sample IV). To stop the reaction, the samples were cooled in an ice−water bath for 20 min, frozen in liquid nitrogen, and stored at −80 °C until analysis. Thermal treatments were carried out in a thermostated water bath (Memmert water bath WBU 45, Memmert GmbH & Co. KG). During the thermal treatments, the time to achieve the desired temperature (nonisothermal condition) was followed and monitored for each experiment.

Vitamin C Extraction. Frozen (in liquid nitrogen and stored at −80 °C) cowpea leaf samples were thawed in a water bath at 25 °C (30 min) (Memmert water bath WB 22, Memmert GmbH & Co. KG). The extraction of vitamin C was done under subdued light by mixing 10 g of sample with 50 mL of cold extraction buffer (NaH_2PO_4 solution (20 mM, pH 2.1 acidified with 1 N HCl) containing 1 mM EDTA) in a mixer (magic bullet blender, J-26, Ningbo Vanguard Import & Export Co.) for 30 s. Subsequently, the extract was centrifuged (Beckman J2-HS) at 4 °C and 17700*g* for 30 min. The supernatant was filtered using a 1.2 μm membrane filter paper (Whatman, Maidstone, U.K.) with the aid of a vacuum pump. The pH of the supernatant was brought to 4 by adding NaOH (1 N) or HCl (1 M). The volume of NaOH and HCl solution was determined and incorporated in the further quantification. The supernatant was frozen in liquid nitrogen and stored at −80 °C until the HPLC analysis of L-AA, DHAA, and total vitamin content.

Determination of Vitamin C Content. The supernatant obtained from the extraction procedure above was thawed in a water bath at 25 °C (Memmert water bath WB 22, Memmert GmbH & Co. KG) and divided into two portions for determination of L-AA and total vitamin C content. To determine the content of L-AA, the supernatant was filtered using a 0.45 μm cellulose acetate syringe filter (Macherey-Nagel, Düren, Germany) and brought to the autosampler (4 °C) for HPLC analysis. Total vitamin C content was determined by performing a precolumn reduction of DHAA to L-AA using a reducing agent, tris(2-carboxyethyl)phosphine-HCl/TCEP solution (2.5 mM dissolved in NaH_2PO_4 solution (20 mM, pH 3.5) containing 1 mM EDTA). Two parts of TCEP solution were added to one part of the supernatant and incubated at 25 °C for 30 min. This solution was filtered using a syringe filter, 0.45 μm cellulose acetate syringe filters (Macherey-Nagel) and brought to the autosampler (4 °C) for HPLC analysis.

HPLC analysis (Agilent 1200 Series, Agilent technologies, Diegem, Belgium) was conducted to identify and quantify L-AA using a reverse phase C18 column (Prevail C18, 250 mm × 4.6 mm, 5 μm particle size, Grace, Deerfield, IL) at 25 °C equipped with a DAD (G1315B, Agilent Technologies) at 245 nm. The separation was done isocratically using an ammonium acetate solution (10 mM, pH 3.0, 1 mM EDTA) with an elution rate of 0.8 mL/min for a total elution time of 30 min. The injection volume was 40 μL. Quantification was done by comparing the peak area/height of L-AA in the sample with known concentrations based on an external calibration curve of standard solutions, prepared on the day of use (500 μg/mL L-AA dissolved in NaH_2PO_4 (20 mM, pH 4.0, 1 mM EDTA)). The content of DHAA was calculated by subtracting the content of L-AA after and before TCEP reduction. The vitamin C content was expressed as micrograms or milligrams of L-AA per gram of dry matter (μg or mg L-AA/g DM).

Measurement of Moisture Content. Moisture content of cowpea leaves was determined gravimetrically on the basis of the AOAC method.[27]

Data Analysis To Estimate Kinetics Parameters for AAO Inactivation. AAO inactivation was described by a first-order kinetics model (eq 2), and the temperature dependence of the inactivation rate constant was estimated by Arrhenius equation (eq 3).[28]

$$A = A_0 \exp(-k_t t) \quad (2)$$

$$K_t = K_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (3)$$

A is AAO activity (in units) after heat treatment for time (t) in minutes at a given temperature, whereas A_0 is the initial AAO activity (in units). k_t is the inactivation rate constant (min^{-1}), k_{ref} the inactivation rate constant (in min^{-1}) at reference temperature, E_a the activation energy (in kJ/mol), R the ideal gas constant (0.008314 kJ/mol.K), T the actual treatment temperature (in K), and T_{ref} the reference temperature (in K, in this study T_{ref} = 60 °C). The k values were estimated on the basis of nonlinear regression analysis. The E_a value was estimated by integrating the kinetics models considered (eq 2) with the Arrhenius equation (eq 3), that is, substituting k_t in eq 2 with eq 3.

D values, the time required to reduce the AAO content by 90%, and z values, the temperature required for a 90% decrease in D value,[29] were estimated as

$$A = A_0 \times 10^{(-t/D)} \quad (4)$$

$$A = A_0 \times 10^{\left(\frac{-t}{D_{ref} \times 10^{((T_{ref} - T)/Z)}}\right)} \quad (5)$$

where D_{ref} is the D value at temperature T_{ref}.

The standard errors at 95% confidence intervals were used to assess the precision of the estimates, whereas the fitting of the model was evaluated by randomness and normality of residuals. SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for nonlinear regression analysis and ANOVA analysis procedures (using least-squares estimation and Levenberg−Marquardt method, for minimizing the sum of squares of the deviations between experimental values and the ones predicted by the mathematical model).

RESULTS AND DISCUSSION

Study on Cowpea Leaves AAO. *Preliminary Study on Enzyme Extraction and Storage.* The enzyme extraction yield was affected by several factors including the maturity stage of the vegetables, the environment under which the extraction was done (with or without NaCl), and the storage conditions (frozen, refrigerated, or ambient temperature conditions). First, the extraction procedure of AAO from cowpea leaves harvested at different maturity stages was optimized. It was observed that NaCl addition and the pH of the extraction buffer affected the AAO extraction yield. Increasing the pH to 5.4−5.8 and increasing the NaCl concentration to 1 M led to an increase in enzyme extraction yield. Extraction in the absence of NaCl led to partial extraction, whereas NaCl addition, up to 1 M, led to an increase in the AAO activity by up to 45% due to extraction of both free and membrane-bound enzyme. This could indicate that the proportions of free and membrane-bound AAO were almost evenly distributed, as also observed in cabbage by Hallaway et al.[30] Optimum cowpea leaf AAO activity was found at pH around 5.4−5.8, similar to the optimum pH of AAO in other plants such as cabbage (5.5−6.6), as observed by Tauber et al.,[31] and pH of 5−6 in star fruit as observed by Saari et al.[32]

Second, the optimal storage conditions for AAO were studied. In this experiment, the storage conditions (quick freezing under liquid nitrogen followed by storage at −80 °C) did not lead to

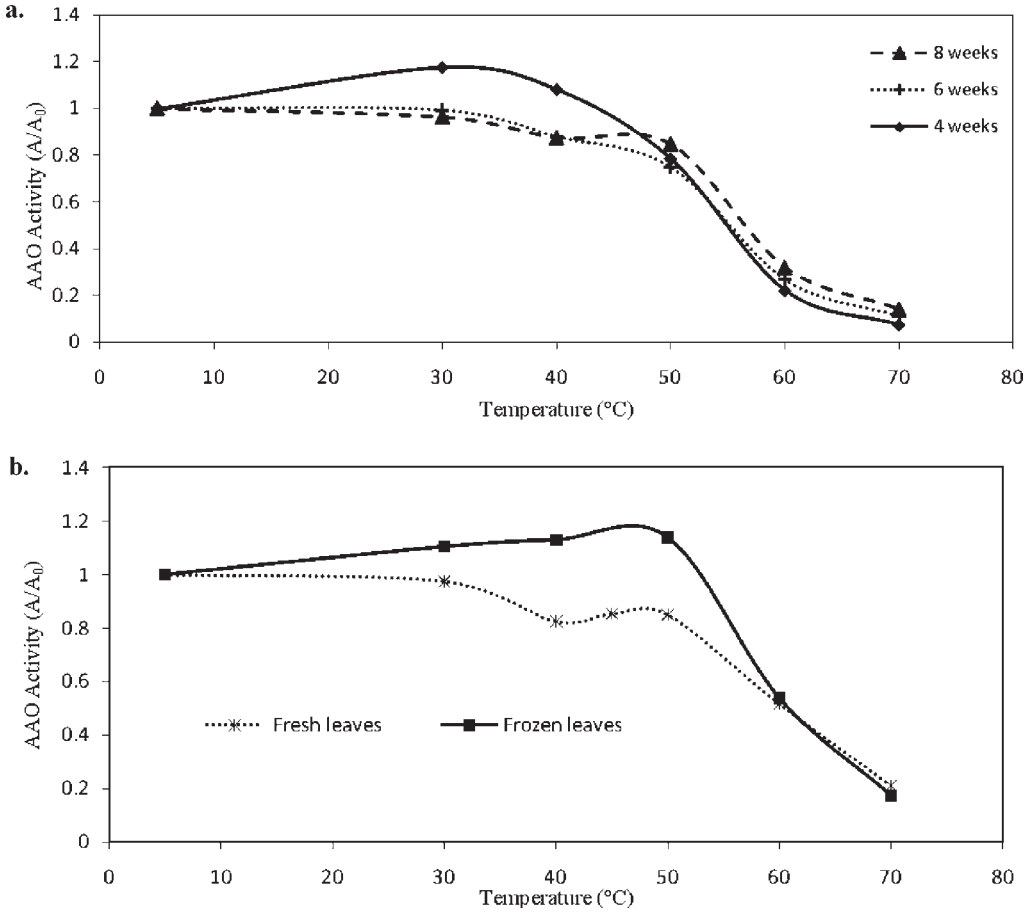

Figure 2. Temperature stability of AAO in cowpea leaves freshly harvested at 4, 6, and 8 weeks (a) and harvested at 6 weeks followed by freezing (b). Thermal treatment for 10 min was conducted.

loss of enzyme activity. This can be attributed to fast freezing in liquid nitrogen because fast freezing is associated with the formation of small crystals that do not damage cell compartmental structure by the ice crystals, leading to less leaching of enzyme on thawing.[33] Apart from fast freezing, losses due to leaching were minimized by carrying out the treatments of the leaves in plastic bags, and the extraction was done by emptying all of the contents in the mixer.

Effect of Leaf Maturity on Thermal Stability of AAO. AAO plays a role in plant growth by controlling cell growth.[20,21] In this study, AAO activity increased during maturation. Parallel to this investigation, the moisture content of cowpea leaves at different stages of maturity was followed. The moisture content slightly decreased during maturation, that is, from 84.75 ± 1.27 (4 weeks old) to 82.76 ± 1.11 (6 weeks old) to 81.81 ± 0.80 (8 weeks old).

Figure 2a illustrates the thermal stability of AAO in cowpea leaves at different stages of maturity (4, 6, and 8 weeks). The results indicate that AAO thermal stability was not affected by harvesting age (4, 6, and 8 weeks old). AAO remained stable up to temperatures around 50 °C. This is similar to AAO in star fruit, which is stable up to 45 °C as indicated by Saari et al.[32] AAO was largely inactivated by elevating the temperature above 50 °C. Due to the detection limit of the polarographic assay used in this study, enzyme activity after heating at temperatures above 70 °C was not detected. The residual enzyme activity after heating >70 °C was 5−7% of the initial activity at 50 °C. High amounts of interfering substances (minerals, chlorophyll, and other enzymes such as polyphenol oxidase and lipoxygenase) that involve depletion of oxygen in the enzyme reactor cell resulted in a high baseline for the detection of AAO. Vegetables harvested at 6 weeks old were used to investigate the effect of freezing on the thermal stability of AAO (Figure 2b). The results indicated that in 6-week-old fresh leaves the thermal stability of AAO was lower ($p < 0.05$) than in thawed cowpea leaves (fast freezing done in liquid nitrogen prior to storage at −80 °C) (Figure 2b). AAO activity in fresh leaves gradually decreased when the heating temperature was increased to 50 °C and afterward depleted significantly at temperatures above 50 °C, as also observed in thawed cowpea leaves. Therefore, further kinetics studies on thermal inactivation of AAO were carried out in the temperature range between 50 and 70 °C using frozen leaves.

In situ thermal inactivation of AAO in cowpea leaves followed first-order kinetics with an Arrhenius dependence. The model fitting of a one-step nonlinear regression analysis based on integrating eq 3 into eq 2, the relationship between predicted and measured enzyme retention, and a linear Arrhenius plot between inactivation rate constants (ln (k)) and temperature ($1/T$) are depicted in Figure 3. The estimated kinetics parameters of AAO inactivation (k, D, z, and E_a values) are summarized in Table 1. AAO inactivation was enhanced by increasing heating temperature. From the kinetics studies, a complete inactivation of AAO occurred at temperatures of >80 °C (based on the detection limit of the assay used in this study). AAO inactivation in cowpea leaves (E_a = 238.4 ± 11.2 kJ/mol) is comparable to that in other

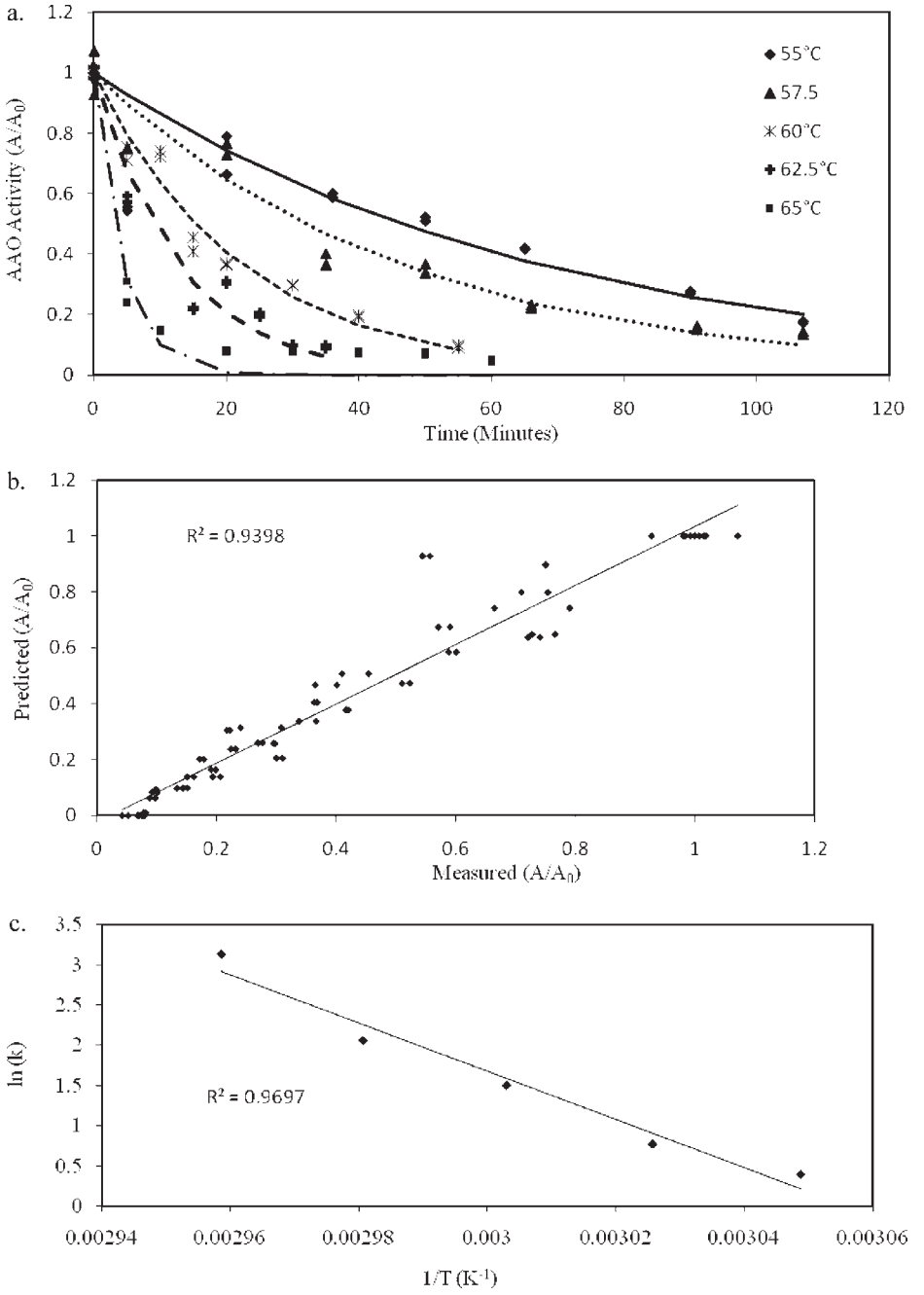

Figure 3. (a) Residual activity of AAO in 6-week-old cowpea leaves as a function of time at different temperatures. Symbols represent experimental values, and lines correspond to individual fittings of the first-order kinetics model to each temperature with an Arrhenius temperature dependence using nonlinear regression analysis. (b) Scatter plot of predicted versus measured residual activity of AAO activity in 6-week-old cowpea leaves. (c) Arrhenius plot between natural logarithm of reaction rate constant versus 1/T for thermal inactivation of AAO in cowpea leaves.

green vegetables such as broccoli as determined by Munyaka et al.[34] at 260−266 kJ/mol.

Effect of AAO on Thermal Stability of Vitamin C. On the basis of the study above, it was clear that AAO was inactivated by heating. Because vitamin C is very heat sensitive, the stability of vitamin C in vegetables during themal inactivation of AAO is questioned. Therefore, an integrated experiment following AAO activity, L-AA content, DHAA content, and total vitamin C content at different heating temperatures was conducted. For this purpose, 8-week-old non-heat-shock (NHS) cowpea leaves were used as illustrated in Figure 1B (sample II) because they contained a higher AAO activity compared to 4- and 6-week-old cowpea leaves.

Total vitamin C content remained stable during thermal inactivation of AAO, even up to 90 °C for 10 min (Figure 4). Most of the vitamin C (>97%) was present in the form of DHAA, especially when AAO was still active even at very low residual enzyme activity. As mentioned previously, AAO activity after heating at temperatures of 80 and 90 °C for 10 min was not able to be measured due to the detection limit of the assay; however, a high proportion of DHAA to L-AA was still found even after complete inctivation of AAO (>80 °C for 10 min, Figure 4). This

is due to the fact that during heating the temperature of the leaves in the package rose to equilibrium, approaching the surrounding temperature (i.e., the temperature of the water bath). In this period, the samples passed through the temperature regimen where AAO still has high stabiltiy and activity (≤50 °C). For example, the leaves that were thermally treated at 60 °C for 10 min experienced nonisothermal condition for 2−10 min before reaching 60 °C (≤60 °C) in which AAO was still active, leading to the enhancement of enzymatic oxidation of L-AA to DHAA.

At high temperatures (e.g., 80 and 90 °C), the temperature gradient between the sample and the surrounding was greater, so that the samples went through the temperature regimen favorable for AAO activity (≤50 °C) more quickly and followed by immediate AAO inactivation. As a result, most of the vitamin C was present in the form of L-AA due to limited enzymatic oxidation of L-AA to DHAA (high L-AA/DHAA ratio). Despite the fact that the vegetables were vacuum packed before thermal treatments, vacuum packing removed only "free" oxygen inside the package and not dissolved oxygen trapped in the plant cells, which also plays an important role in the oxidation reduction process. A similar phenomenon was also observed by Munyaka and others.[34]

Study on Vitamin C Content in Cowpea Leaves. Total vitamin C, L-AA, and DHAA contents in cowpea leaves were followed at different stages of maturity (Figure 5). On average, the amount of total vitamin C (1−1.3 mg/g dry weight) was comparable to total vitamin C in cowpea leaves (1.64 mg/g dry weight) purchased from a local market in Uganda (Africa).[35] At all stages of maturity, the total vitamin C content in cowpea leaves (17−22 mg/100 g fresh weight) was higher than in lettuce (1.6 mg/100 g fresh weight) as determined by Yamaguchi et al.[36] However, the total vitamin C content in the leaves (1−1.3 mg/g dry weight) was less than in brocolli florets (7 mg/g dry weight) or brocolli stalks (12 mg/g dry weight) as reported by Munyaka et al.[34] It is worth noting that the total vitamin C content at the maturity stages of 4 and 6 weeks was mostly in the DHAA form, even though AAO had been inactivated by cooking in boiling water (heat-shocked samples).

In fresh cowpea leaves (NHS samples, Figure 5A), 87−98% of vitamin C was in the DHAA form as also noted by Munyaka et al.[34] in brocolli. The high amount of DHAA can be attributed to the activity of AAO that was released during the extraction process and therefore led to the oxidation of L-AA to DHAA. Cooking the vegetables in boiling water for 10 min (heat shock) did not result in any significant loss of total vitamin C for leaves harvested at 8 weeks (Figure 5B), and most of the vitamin C was in the form of L-AA instead of DHAA. It was also observed that heat shock treatment resulted in a decrease in total vitamin C content, that is, 68% (4-week-old leaves) and 74% (6-week-old leaves) (Figure 5); however, the heat shock pretreatment prior to extraction led to not only AAO inactivation resulting in protection of L-AA but also DHAA degradation. Higher L-AA content after a heat shock treatment was also noted in brocolli florets (from 0 to 83%) and brocolli stalks (from 17 to 93%) by Munyaka et al.[34] The results were in agreement with other findings made by Maeda et al.[37] and Yamaguchi et al.[38] in which cooked vegetables (burdock, lettuce, and brocolli) exhibited higher radical scavenging activity than fresh ones due to inactivation of AAO (by cooking) leading to higher content of L-AA; L-AA has higher radical scavenging activity than DHAA.[11] Even though there is no significant difference in total vitamin C content between the fresh sample (NHS) as shown in Figure 5A and the HS samples (Figure 5B), failure to inactivate AAO in fresh leaves led to a reduced redox state of vitamin C. The high stability of vitamin C in leaves harvested at 8 weeks after heat shock treatment could be explained by high initial content of L-AA compared to 4- and 6-week-old cowpea leaves. When compared with evolution of AAO with maturity of the vegetables (previously discussed), there was no relationship found between the level of AAO activity in the vegetables and the content of total

Table 1. Estimated Kinetics Parameters for Thermal Inactivation of Ascorbic Acid Oxidase (AAO) in Cowpea Leaves Harvested at 6 Weeks[a]

temperature (°C)	parameter estimate, $k(\times 10^{-2}\ \mathrm{min}^{-1})$	parameter estimate, D value (min)
55	1.5 ± 0.2	154.1 ± 19.3
57.5	2.2 ± 0.2	106.1 ± 7.7
60	4.5 ± 0.2	51.0 ± 2.6
62.5	7.9 ± 0.6	29.2 ± 2.1
65	23.1 ± 2.4	10.0 ± 1.0
T_{ref} = 60 °C	$k_{ref}(\times 10^{-2}\ \mathrm{min}^{-1})$ = 4.9 ± 0.2	D_{ref}(min) = 47.1 ± 2.1
	E_a(kJ/mol) = 238.4 ± 11.2	Z value (°C) = 8.9 ± 0.4

[a] The standard errors are defined at 95% confidence level.

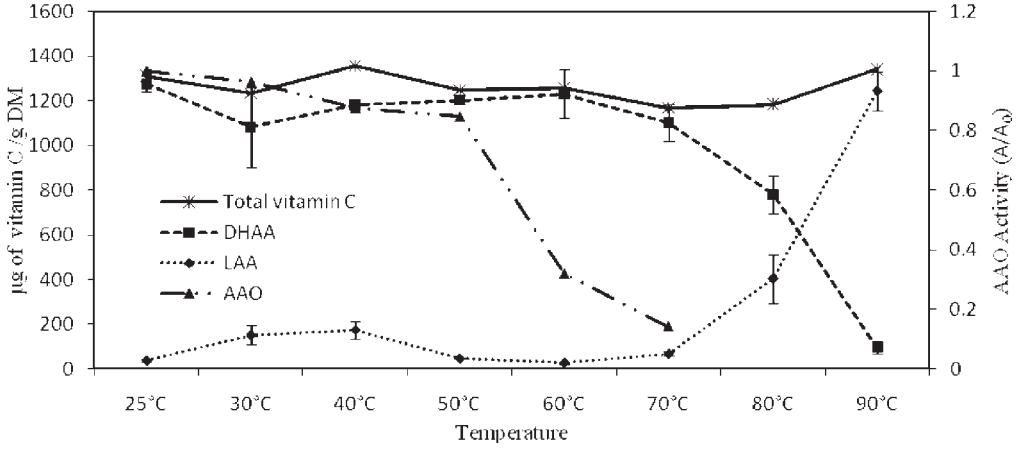

Figure 4. Evolution of the content of total vitamin C, DHAA, L-AA, and AAO residual activity in 8-week-old cowpea leaves not subjected to heat shock as a function of temperature during heating (10 min).

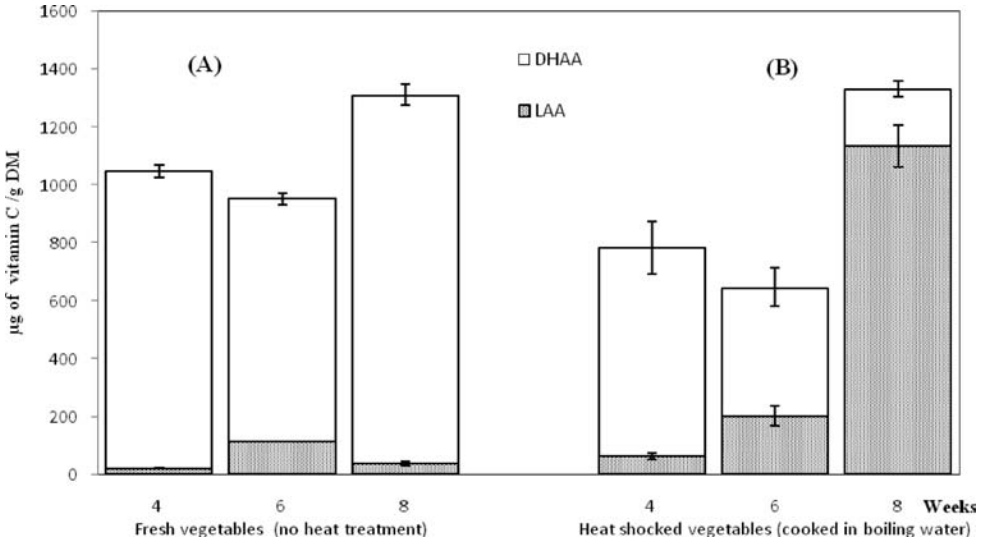

Figure 5. Total vitamin C, L-AA, and DHAA contents in fresh cowpea leaves (A) and heat-shocked (boiled for 10 min) cowpea leaves (B) at different levels of maturity.

vitamin C or the L-AA/DHAA ratio. This is because AAO is majorly linked to cell expansion and plant growth, whereas vitamin C is linked to other oxidative compounds influencing the antioxidant capacity of the vegetable. The high L-AA/DHAA ratio for the older leaves (8 weeks old) showed that the conversion of L-AA to DHAA by AAO occurs during cell disruption, and this can be mitigated by heat shocking. For the younger leaves L-AA could be converted to DHAA by AAO in the apoplast during the plant growth, and hence heat shocking only partially protects the L-AA or the high level of AAO inhibitors could be present in young leaves. The fact that even with total thermal inactivation of AAO there was still some conversion of L-AA to DHAA showed the possible activity of other enzymes that use L-AA as indirect substrate in the presence of oxygen, such as ascorbate peroxidase. Ascorbate peroxidase reduces hydrogen peroxide in the chloroplasts to water, utilizing L-AA as electron donor and in turn leading to the conversion of L-AA to DHAA.[39] The results in Figure 5 indicate that heat shock treatment could be an appropiate pretreatment step prior to extraction to obtain a complete inactivation of AAO with an expectation of protecting L-AA against enzyme oxidation; however, the practice of this pretreatment step should be taken into consideration, especially for young leaf vegetables in which vitamin C is predominantly in the form of DHAA. The heat shock treatment could lead to underestimation of the L-AA, DHAA, and total vitamin C contents because of DHAA degradation.

Strategy To Optimize Vitamin C Content and L-AA/DHAA Ratio in Cowpea Leaves. Blanching is used to inactivate undesirable enzymes and to prolong the shelf life of cowpea leaves after harvest. In this study, it is clear that AAO should be inactivated to achieve a high level of L-AA at the moment of consumption. Different strategies of cowpea leaf preservation and cooking steps on the total vitamin C content and L-AA/DHAA ratio in cowpea leaves at different stages of maturity were examined following the flowchart as illustrated in Figure 1B. Strategy 1 consisted of cooking the fresh vegetables at different temperatures as a single heating step (Figure 1B, sample II), strategy 2 consisted of heating fresh vegetables at different temperatures (30−90 °C, using NHS samples) immediately followed by blanching (95 °C, 10 min) to inactivate the residual AAO activity (Figure 1B, sample III), and strategy 3 consisted of blanching the fresh vegetables (95 °C, 10 min) followed by reheating at different temperatures, 30−90 °C (Figure 1B, sample IV).

In strategy 1, there was a definite increase in the amount of L-AA (and subsequent reduction in the amount of DHAA) with increasing temperature treatments at all stages of leaf maturity. A profound increase was noted in cowpea leaves harvested at 8 weeks (Table 2). An increase in L-AA content was noted at temperatures of >70 °C. The 8-week-old leaves had 34% of AA in the L-AA form at 80 °C and 92% of vitamin C in the L-AA form at 90 °C, whereas at temperatures of <70 °C maximally only about 12% of vitamion C was present in the form of L-AA. These results indicated that at temperatures of <70 °C the enzyme AAO was still active and, hence, high amounts of DHAA were produced, whereas at temperatures of >70 °C, the gradual increase in L-AA content shows a decrease in AAO activity, as observed previously (Figure 4).

When strategy 2 was applied to preserve cowpea leaves after harvest, the L-AA content was very low for 4- and 6-week-old leaves (sometimes below the detection limit), whereas for the 8-week-old leaves the percentage of L-AA was high and remained stable with increasing temperatures (Table 2). There was also a general decline in the total vitamin C content with increased heating temperature. This was due to the fact that by the time the heat shock treatment was done, AAO had already catalyzed the oxidation of L-AA to DHAA, which is more heat labile than L-AA.

Strategy 3 decreased the total vitamin C content by respectively 50 and 79% but increased the proportion of L-AA (Table 2). There was a marginal loss of total vitamin C content for leaves harvested at 8 weeks and a marginal increase in the percentage of L-AA of the total vitamin C during these thermal treatments. The decline in the total vitamin C in the younger leaves compared to the 8-week-old ones is due to the higher amount of DHAA in the younger leaves compared to the older leaves. DHAA was less heat stable than L-AA; therefore, the younger leaves showed a general decline in the total vitamin C content during reheating. Because a high amount of DHAA was present in the younger leaves (4−6 weeks old), boiling pretreatment for the younger leaves (for AAO inactivation) did not give an advantage to the preservation of L-AA as witnessed in the 8-week-old leaves. The results of this investigation indicated that the

Table 2. Effect of Different Strategies Conducting Thermal Treatments on Total Vitamin C Content and the Percentage of L-AA and DHAA

		strategy 1			strategy 2			strategy 3		
temperature (°C)	maturity (weeks)	total vitamin C (mg/g DM)	L-AA (%)	DHAA (%)	total vitamin C (mg/g DM)	L-AA (%)	DHAA (%)	total vitamin C (mg/g DM)	L-AA (%)	DHAA (%)
30	4	0.96 ± 0.05	0.0	100.0	0.74 ± 0.05	0.0	100.0	0.78 ± 0.07	32.2	67.8
	6	0.94 ± 0.05	1.7	98.3	0.85 ± 0.06	2.4	97.6	0.86 ± 0.01	41.5	58.5
	8	1.23 ± 0.14	12.3	87.7	1.37 ± 0.07	84.3	15.7	1.41 ± 0.00[a]	82.8	17.2
40	4	1.00 ± 0.05	0.0	100.0	0.74 ± 0.05	0.0	100.0	0.61 ± 0.10	38.9	61.1
	6	1.07 ± 0.05	1.9	98.1	0.84 ± 0.05	2.2	97.8	0.67 ± 0.13	54.6	45.4
	8	1.36 ± 0.03	12.9	87.1	1.44 ± 0.00[a]	88.5	11.5	1.43 ± 0.06	72.3	27.7
50	4	1.02 ± 0.00[a]	0.0	100.0	0.79 ± 0.00[a]	0.0	100.0	0.68 ± 0.05	32.8	67.2
	6	1.06 ± 0.00[a]	0.9	99.1	0.78 ± 0.02	2.2	97.8	0.82 ± 0.04	42.6	57.4
	8	1.25 ± 0.02	3.6	96.4	1.38 ± 0.08	87.4	12.6	1.44 ± 0.05	81.6	18.4
60	4	0.77 ± 0.20	0.0	100.0	0.74 ± 0.00[a]	0.0	100.0	0.65 ± 0.04	25.9	74.1
	6	0.97 ± 0.02	0.0	100.0	0.75 ± 0.20	0.0	100.0	0.79 ± 0.14	38.2	61.8
	8	1.26 ± 0.10	2.1	97.9	1.26 ± 0.05	89.6	10.4	1.32 ± 0.09	85.5	14.5
70	4	0.85 ± 0.12	0.0	100.0	0.62 ± 0.04	0.0	100.0	0.65 ± 0.04	34.7	65.3
	6	0.90 ± 0.12	0.0	100.0	0.68 ± 0.12	0.0	100.0	0.78 ± 0.01	46.0	54.0
	8	1.17 ± 0.09	5.7	94.3	1.27 ± 0.01	81.6	18.4	1.18 ± 0.06	91.7	8.3
80	4	0.75 ± 0.00[a]	0.0	100.0	0.62 ± 0.04	2.5	97.5	0.51 ± 0.02	32.0	68.0
	6	0.84 ± 0.00[a]	3.3	96.7	0.63 ± 0.00[a]	0.0	100.0	0.76 ± 0.03	40.7	59.3
	8	1.18 ± 0.02	34.1	65.9	1.19 ± 0.09	85.1	14.9	1.24 ± 0.03	93.3	6.7
90	4	0.66 ± 0.00[a]	0.0	100.0	0.57 ± 0.05	3.0	97.0	0.51 ± 0.01	26.7	73.3
	6	0.77 ± 0.00	2.6	97.4	0.54 ± 0.00	0.0	100.0	0.76 ± 0.04	40.2	59.8
	8	1.34 ± 0.06	92.9	7.1	1.21 ± 0.02	82.7	17.3	1.30 ± 0.07	92.3	7.7

[a] Standard deviation (<5 μg/g DM).

age of cowpea leaves at the time of harvest and the processing/preservation methods determine the nutritional value of cowpea leaves in terms of vitamin C. This integrated study implies that high retention of vitamin C in vegetables before consumption can be obtained by optimizing conditions starting from farm to processing/meal preparation. Harvesting cowpea leaves at the age of 8 weeks (before the development of the pods) is the best harvest time to have a high content of vitamin C in the form of L-AA. Because the overexpression of AAO concurrently takes place during maturity, high AAO activity can jeopardize the stability of L-AA, leading to losses of nutritional value during maturation. With process optimization such as for blanching or thermal processing, vitamin C can be kept in the L-AA form. Therefore, conditions of blanching and/or other thermal treatments should be appropiately designed.

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