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Impact of Non-Thermal Processing on Antioxidant Activity, Phenolic content, Ascorbic Acid content and Color of Winter Melon Puree

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Abstract

In the last decade, non-thermal processing for inactivating microorganisms has been developed in response to the worldwide interest for more fresh and improved quality of food products. Winter melon is a very perishable fruit, hence, processing into puree is a necessity. However application of heat in the production of puree could affect the nutritional values, thus, application of non thermal treatment in combination with preservation method is significant for this fruit. This study was conducted to evaluate the effect of non-thermal processing in combining with preservation method on antioxidant activity, level of key antioxidant groups (total phenolic and ascorbic acid content) and the color of winter melon puree. Total phenolic content (TPC) was measured using Folin-Ciocalteu reagent. Ascorbic acid (AA) was determined using 2,6-dichlorophenol-indophenol titration method. Antioxidant activity were determined using four antioxidant assays namely Ferric Reducing Antioxidant Potential (FRAP), Oxygen Radical Absorbance Capacity (ORAC), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and β-Carotene Bleaching Assays. For the TPC, puree with pH 3 (28.5±1.3 GAE/g fresh weight) exhibited high in TPC as compared to puree with pH 3.5 and unprocessed puree. In contrast, unprocessed puree contains significantly high ascorbic acid (AA) content (35.9 ± 1.8 mg/100 g fresh mass) as compared to pH 3.0 and pH 3.5 purees. In general, antioxidant activity for all assays of pH 3.0 and pH 3.5 purees were significantly higher ($p<0.05$) than unprocessed puree. Color changes (ΔE) were not significant between puree with pH 3.0 and pH 3.5. Therefore, processed winter melon puree using non-thermal processing in combination with chemical preservation method could be an efficient method to preserve the quality of winter melon puree more superior than unprocessed puree.

Introduction

Nowadays consumers are demanding on high quality and convenient products with natural flavor, taste and greatly appreciate the fresh appearance of minimally processed food (Oey *et al*., 2008). Non-thermal processing is a method for achieving microbial inactivation without exposing to heat whilst extending product shelf life and retaining their freshness in terms of physical, nutritional and sensory quality (Ademowaye *et al*., 2001). In the last decade, non-thermal processing for inactivating microorganisms have been developed in response to the worldwide interest especially scientists, manufacturers and consumers because they exert a minimal impact on the nutritional and sensory properties of food and also extend the shelf life by inhibiting microorganisms. In other word, foods processed in this way are capable to keep its original freshness and minimal changes in flavor, taste and color. Furthermore, non-thermal processing is gentler than thermal processing and its application is a huge advantage for local fruits which often have stronger taste and aroma.

Winter melon or its botanical name is *Benincasa hispida* is one type of fruit that is widely consumed in Chinese and Indian households. It is prized for its very pleasant, sub acid, aromatic and juicy flesh. However, it softens very rapidly during ripening and becomes mushy upon storage and making it difficult to consume in fresh form. Therefore, winter melon can become a potential source of raw material for puree. Puree is a thick, soft dish that is prepared by processing the fruit through a sieve or mixing them by using food processor. Processing winter melon into puree form will make it become more convenient to use by consumers for applications in food product

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development.

Due to degradation of fresh flavor and highly perishable characteristics, winter melon was processed with non-thermal process to maintain its beneficial properties such as antioxidative and also other medicinal properties. Thermal processing has been shown in many studies to reduce the antioxidant capacities of foods (Dewanto *et al*., 2002; Zhang and Hamauzu, 2004). The role of antioxidant compound helps in reducing the risk of many chronic diseases such as cancer, coronary heart disease, declining of immune system (Kaur and Kapoor, 2001) and several studies have demonstrated a relationship between consumption of fruits and a lower incident of degenerative diseases. Therefore there is a need for non-thermal processing which can increase not only microbiological stability, but also preserving their nutritional and physical characteristics. Despite the fact that non-thermal processing has been used commercially, its effect on antioxidant activity and on different antioxidant group (phenolic and ascorbic acid) has not yet been investigated in winter melon puree. Hence the objective of the present work was to study the effect of non-thermal processing on antioxidant activity, TPC, AA and color of winter melon puree with the aim to preserve the quality even better that its fresh form.

Materials and methods

Chemicals and preparation of fruit purees

β-Carotene, linoleic acid, Tween 20, butylated hydroxytoluene (BHT), butylated hydroxyannisole (BHA), 2,2-Diphenyl-1-PicrylHydrazyl (DPPH), ascorbic acid and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Folin-Ciocalteu reagent, sodium carbonate, sodium acetate, glacial acetic acid, ferric chloride ($FeCl_3.6H_2O$), ferrous sulphate ($FeSO_4.7H_2O$) and absolute ethanol were purchased from Merck (Darmstad, Germany); chloroform was from Fisher Scientific (Loughborough, UK); fructose and citric acid (food grade) were purchased from Danisco.Co, Malaysia.

Winter melon was obtained from a local market in Selayang, Selangor, Malaysia. After washing and dicing, sample was blended in a mechanical food processor (Panasonic, MK-5080M). Sample puree was packed in polyethylene plastics and sealed using impulse foot sealer (Singar Sega. Co. Ltd.) and stored at -21°C prior to non-thermal processing.

Non-thermal processing

After thawing at 4°C the packed sample puree (300g) was placed in a container and then stabilised through slight aw reduction (0.96) by adding Fructose (food grade). pH of the purees was than adjusted by using citric acid to pH 3.0 and 3.5 and 1000 ppm potassium sorbate were added to increase the shelf life of the purees. The purees were then homogenized for 1 minute at 24,000 rpm using Ultra-Turrax homogenizer.

Antioxidant estimations

Total phenolic content (TPC)

TPC was determined by Folin-Ciocalteu method, which was adapted from Singleton and Rossi (1965). 0.1 ml of an aliquot of the extract was added to 0.5 ml Folin-Ciocalteu reagent in a test tube and then mixed well using a vortex. The mixture was allowed to react for 3 minutes and 1.5 ml of 7.5 % w/v Na_2CO_3 and 7.9 ml distilled water were then added and mixed well. The solution was incubated at room temperature (23°C) in the dark for 2 hours. After 2 hours incubation at room temperature, the absorbance was read at 765 nm. The amount of the TPC was expressed in mg of gallic acid equivalent using the standard curve.

Ascorbic acid (AA) content analysis

AA content was determined using the 2,6-dichlorophenol-indophenol titration with meta-phosphoric acid (MPA) solution. AA was used to prepare a standard solution (1 mg/mL). The AA was calculated by comparison with the standard and expressed as mg AA/100 g fresh mass.

Antioxidant Activity Determination

2,2-Diphenyl-1-Picrylhydrazyl radical scavenging (DPPH) assay

The DPPH free radical scavenging activity of each sample was conducted according to the method described by Braca *et al*., (2001). A solution of 0.1 mM DPPH in ethanol was prepared. The butylated hydroxytoluene (BHT)/butylated hydroxyannisole (BHA) combination and AA were used as standards. The concentration of extracts and standards were prepared in the range of 200 to 1000 µg/mL. An aliquot of 0.6 ml of each concentration of extracts and standards were added to 4.5 ml of ethanolic DPPH solution. The mixture was shaken vigorously and left to stand in the dark for 20 minutes at room temperature. Absorbance was read using a spectrophotometer at 517 nm. IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of extracts which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50% (Azizah *et al*., 2007).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to method of Benzie and Strain (1996). The stock solutions

included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$, $3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before used. The extracts (150 mL) were allowed to react with 2850 µL of the FRAP solution for 1 hour in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was linear between 100 and 500 mM Trolox. Results were expressed in mM TE/g extract weight.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was conducted by an automated plate reader (KC4, Bio Tek, USA) with 96-well plates (Prior *et al*., 2003). Analyses were conducted in phosphate buffer pH 7.4 at 37°C. Peroxyl radical was generated using 2, 2'-azobis (2-amidino-propane) dihydrochloride which was prepared fresh for each run. Fluorescein was used as a substrate. Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm. The standard curve was linear between 0 and 50 mM Trolox. Results were expressed as mM TE/g extract weight.

β-Carotene bleaching assay

The β-carotene bleaching assay was conducted by the spectrophotometric method at 470 nm adapted from method of Velioglu *et al*. (1998). This assay was carried out to measure the % antioxidant activity of extracts of each part. In this method combination of BHA/BHT at 200 µg/ml was used as a standard. 0.2 mg/ml of β-carotene was dissolved in chloroform, 0.02 ml linoleic acid and 0.2 ml Tween 20 were transferred into a 50 ml round bottom flask. Chloroform was then removed at room temperature under vacuum at reduced pressure using a rotary evaporator (R-20, Buchi, Switzerland) for 10 minutes. After evaporation, 50 ml of distilled water was added to the mixture and shaken vigorously to form an emulsion. 2 ml aliquots of the emulsion was pipetted into 0.2 ml of extract, standard and distilled water (as control) and immediately placed in a water bath (Memmert, Kuala Lumpur, Malaysia) at 50°C. The absorbance was read at 20 minutes intervals for 2 hours at 470 nm and the rate of bleaching of β-carotene was recorded to calculate the % antioxidant activity of each part of winter melon extracts.

Measurement of color

The colors of the samples were measured using a Hunter-Lab colour meter (Hunter Lab DP-9000 colour difference meter, Hunter Associates Laboratory, Virginia, USA) fitted with a 2.5 cm diameter aperture. The instrument was calibrated using the white tile provided. Colour was expressed in Hunter Lab units L*(Lightness/ darkness), a*(redness/greenness) and b*(yellowness/blueness). Samples of purée were filled into a plastic petri dishes (i.d. 50 mm) taking care to exclude air bubbles and placed under the aperture of the colour meter. Three readings were taken for each measurement and results were then averaged. In addition, hue angle and total colour difference (ΔE) were calculated using the following equations, where L_0, a_0, b_0 are the control values for unprocessed purées.

$$\text{Hue angle} = \tan^{-1}(b/a) \quad (1)$$

$$\Delta E = [(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2]^{1/2} \quad (2)$$

Statistics

Each measurement was carried out in triplicates. All data were expressed as mean ± standard deviation. Data were analysed using SAS (9.1) (2008). P-values<0.05 were considered statistically significant.

Results and discussion

Total phenolic and ascorbic acid content

TPC and AA content before and after non-thermal processing are presented in Table 1. TPC in unprocessed (fresh) and processed winter melon puree ranged from 26.6–28.5 mg GAE/g fresh weight, while ascorbic acid (AA) content in winter melon puree ranged from 33.4–35.9 mg/100g fresh mass. The values of TPC and AA contents for unprocessed winter melon purees complies with those reported by previous authors (Nurul *et al*., 2010; Huang *et al*., 2004). Results indicate that levels of AA content in samples for pH 3 and pH 3.5 were lower than unprocessed puree. According to Ajibola *et al*., (2009), addition of fructose in processed winter melon puree (pH 3 and pH 3.5) can cause AA breakdown. In other words, AA will reacts with carbonyl group of fructose to decrease ascorbic acid concentration. The higher the fructose content in processed winter melon purees, the greater the loss of AA will be. However, higher levels of citric acid were able to stabilize the loss of AA content (Padayatty *et al*., 2003).

The results for TPC were in contrast with that of AA content where TPC of processed winter melon purees (pH 3 and pH 3.5) showed significantly slight increment as compared to unprocessed winter melon puree. This may be related to an increased in extractability of some antioxidant components in the processed purees where citric acid that was added to treat puree might be one of the reason for the increment

Table 1. TPC and AA Contents of unprocessed winter melon puree and processed purees (pH 3 and pH 3.5)

Treatment	Total Phenolic Content (mg GAE/g fresh weight)	Ascorbic Acid Content (mg/100 g fresh mass)
Unprocessed puree	26.6 ± 1.5^{A}	35.9 ± 1.8^{A}
Processed purees		
pH 3 puree	28.5 ± 1.3^{B}	34.6 ± 3.9^{B}
pH 3.5 puree	26.6 ± 1.5^{C}	33.4 ± 2.8^{C}

Values are expressed as mean ± standard deviation (n = 3). Means with different letters within a column were significantly different at the level p<0.05.

Table 2. Antioxidant activity of various parts of wax gourd extracts as determined by the DPPH (IC_{50}), FRAP, ORAC and β-carotene bleaching (% Inhibition) assays

Treatment	Antioxidant Activities			
	DPPH (IC_{50})[a]	ORAC [b]	FRAP[c]	B-Carotene bleaching assay[d]
BHA/BHT (Standard)	120.0 ± 0.2^{A}	Not applicable	Not applicable	94.9 ± 4.5^{A}
Ascorbic Acid (Standard)	104.0 ± 0.2^{A}	Not applicable	Not applicable	Not applicable
Unprocessed puree	294.8 ± 24.1^{A}	985.3 ± 21.7^{A}	0.26 ± 0.009^{A}	57.4 ± 2.9^{D}
Processed purees				
pH 3 puree	210.0 ± 1.2^{B}	1034.0 ± 8.4^{A}	0.32 ± 0.009^{A}	50.9 ± 0.5^{B}
pH 3.5 puree	205.6 ± 5.9^{B}	1025.0 ± 24.4^{A}	0.30 ± 0.009^{A}	42.8 ± 0.1^{C}

Values are expressed as mean ± standard deviation (n=3). Means with different letters within a column were significantly different at the level p<0.05.
[a]Scavenging activity IC_{50} of various part of wax gourd extracts on DPPH radicals in μg/ml.
[b]ORAC assay result expressed as μM Trolox equivalent (TE)/g fresh weight.
[c]FRAP assay result expressed as mM TE/g fresh weight.
[d] Inhibition (%) by β-carotene linoleate bleaching assay

of TPC values. This is because citric acid is an organic acid that can be used as synergists and study done by Jay *et al*. (2001) revealed that organic acid act as food antioxidant and also as organic polyvalent (phenolic compound) that increase phenolic content in the food. This study was supported by Pujimulyani *et al*.,(2010), who reported that white saffron showed an increment in TPC when the extracts contained 0.05% citric acid.

Chemical indices of antioxidant activity

2,2-Diphenyl-1-Picrylhydrazyl radical scavenging (DPPH) assay

The antioxidant activity as determined by DPPH assay for standards (BHA/BHT and AA) unprocessed and processed winter melon purees are shown in Table 2. IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of each part of wax gourd extracts, which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% where the lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH scavengers (Azizah *et al*., 2007). Table 2 shows that there was a significant difference between IC_{50} of unprocessed puree with both processed purees at different pH. The result also revealed that unprocessed puree extract had the lowest ability to act as DPPH scavengers due to high IC_{50} as compared to processed puree. The correlation between TPC and AA content with IC_{50} is shown in Table 3, and negative correlation was found between TPC and unprocessed and processed winter melon purees while strong positive correlation was found between AA content. Study by Norshazila *et al*. (2010) found negative correlation between scavenging activity and TPC of selected Malaysian tropical fruits. This is because the activity is not limited to phenolic compound but also from the presence of other antioxidant secondary metabolites presence in the purees such as vitamins, carotenoids and volatile oil (Javanmardi *et al*., 2003). Hence it can be assumed that AA content in unprocessed and processed winter melon purees contributes to strong ability to act as DPPH scavengers.

Ferric reducing antioxidant power (FRAP) assay

In FRAP assay, antioxidant activities are expressed as reducing potential of an antioxidant reacting with ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex. The reducing properties are associated with the presence of compounds in the extracts breaking the free radical chain through donating a hydrogen atom (Duh *et al*., 1999). Table 2 shows that there are no significant difference between FRAP values of unprocessed and processed winter melon purees. However, there was slight increment after the application of combined preservation treatments (non-thermal processing) to the processed winter melon purees. This might be due to high in TPC in both processed purees and there was a positive correlation between TPC and FRAP values. This result was in agreement with Benzie and Stezo (1999) who found a positive correlation between TPC and FRAP assay. In addition, Pujimulyani *et al*., (2010; 2012) reported that addition of citric acid in white saffron increased its antioxidant activity. This supported the results obtained from this study where addition of citric acid in processed winter melon purees slightly increased the FRAP values.

Oxygen radical absorbance capacity (ORAC) assay

ORAC values for unprocessed and processed winter melon purees showed no significant difference among each other (Table 2) where the values ranged from 985.3-1034.0 μM TE/g fresh weight but slight increment in ORAC values for processed winter melon purees. A positive correlation was found between TPC and ORAC assay and the possible reasons for the increased in values might be due to high TPC in both processed winter melon purees. This study is supported by Conner *et al*. (2002), where they revealed high correlation in blueberry fruit due to high TPC. Positive correlation between TPC and ORAC could be due to secondary metabolites like phenolic compounds that are most abundant in fruit

Table 3. Pearson's correlation coefficients (r^2) of antioxidant activities (AOA), Ascorbic Acid (AA) and Total Phenolic Content (TPC)

Trait	TPC	AA	DPPH (EC_{50})	% Inhibition	FRAP
AA	-0.604				
DPPH (IC_{50})	-0.928	0.857			
% Inhibition	0.986	-0.460	0.581		
FRAP	0.255	-0.989	0.581	0.984	
ORAC	0.940	-0.296	-0.996	-0.852	-0.925

Table 4. Instrumental colour parameters of unprocessed and non-thermal processed (pH 3 and pH 3.5) winter melon purees

Treatment	*L** (Lightness/ Darkness)	*a** (redness/ greenness)	*b** (yellowness/ blueness)	Total colour change (ΔE)	Hue angle
Unprocessed puree	42.6 ± 0.62^{A}	-3.67 ± 0.14^{A}	1.38 ± 0.39^{A}	0	-20.61 ± 0.23^{A}
pH 3 puree	42.4 ± 0.62^{A}	-3.57 ± 0.09^{A}	1.34 ± 0.15^{A}	39.8 ± 0.01^{A}	-20.57± 0.41^{A}
pH 3.5 puree	42.6 ± 0.62^{A}	-3.8 ± 0.25^{A}	1.33 ± 0.08^{A}	40.0 ± 0.02^{B}	-19.29± 0.42^{A}

Values are expressed as mean ± standard deviation (n=3). Means with different letters within a column were significantly different at the level $p<0.05$

(Macheix *et al*., 1990). ORAC assay takes a longer time to perform and requires expensive equipment but it is the only method that takes free radical action to completion and uses the area under the curve (AUC) technique for quantitation, thus it combines both inhibition percentage and length of inhibition time of the free radical action by antioxidants in single quantity (Prior *et al*., 1999). Another advantage of ORAC assay is that it is more significant as it uses a biologically relevant radical source (Prior *et al*., 2003) where it reduce free radicals protecting cells from oxidative damage. The rationale of the ORAC assay is antioxidants (phenolic compounds and vitamins) react with pro-oxidants (peroxyl radicals) in the presence of fluorescein, a marker molecule of reaction development.

β-Carotene bleaching assay

Table 2 shows the comparative percent inhibition of β-carotene and the results indicates that there was a significant difference ($p<0.05$) between standard (combination of BHA/BHT) and unprocessed and processed winter melon purees. The percent inhibition of β-carotene was in the order of: BHT/ BHA>pH 3 processed puree> pH 3.5 processed puree>unprocessed puree. The possible reason for the high percent inhibition of processed puree pH 3 could be due to high TPC where positive high correlation was found between TPC and β-carotene bleaching assay. Another reason is due to high in FRAP value, and this is supported by positive high correlation between FRAP and β-carotene bleaching assay. Moreover, better extractability of antioxidant components could contribute to the high percent inhibition of β-carotene.

Instrumental color parameters

Instrumental color parameters of unprocessed and processed winter melon purees as affected by combined preservation method (non-thermal processing) are illustrated in Table 4. The rectangular coordinates of an early opponent color system (Hunter) labeled the three dimensions of a color as L,a,b. L*a*b* is an opponent-type color space with rectangular coordinates similar to Hunter L,a,b. The L* indicates lightness (0 to 100), the a* indicates redness (+) and greenness (–), and the b* indicates yellowness (+) and blueness (–). The limits for a* and b* values are around –80 and +80. The two color scales do not correlate and a color located in L,a,b space will be in a different location in L*a*b*. The non-thermal processing by combined preservation method did not cause any significant decrease in a* values compared to unprocessed and processed winter melon purees. On the other hand, lightness L* and yellowness b* values of processed purees were slightly increased after the application of combined preservation treatment. However, the increment was not significant as compared to unprocessed puree. There was a significant in total color change (ΔE) between processed puree at pH 3 and at pH 3.5.

Conclusions

This study concluded that application of non-thermal processing in combination with preservation method in winter melon puree increased in the TPC and antioxidant activity, but reduced in AA content. Application of non-thermal processing in combination with preservation method was also found to retain the color of purees similar to the unprocessed puree.

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