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Shivashankar Sanganamoni
Agricultural and Food Engineering Department, IIT Kharagpur, West Bengal, India

Naveen Kumar Mahanti
Agricultural and Food Engineering Department, IIT Kharagpur, West Bengal, India

Srinivasa Rao P
Agricultural and Food Engineering Department, IIT Kharagpur, West Bengal, India

Correspondence
Shivashankar Sanganamoni
Agricultural and Food Engineering Department, IIT Kharagpur, West Bengal, India

Modeling of polyphenol oxidase and peroxidase inactivation in coconut water during thermal treatment

Shivashankar Sanganamoni, Naveen Kumar Mahanti and Srinivasa Rao P

Abstract
The effect of hot water bath temperature (80, 85, 90, 95 ºC) and treatment time (2.5, 5, 7.5, 10 min) on enzymes (*viz*. Polyphenol oxidase and Peroxidase) and nutritional properties (*viz*. Ascorbic acid, Antioxidant activity and Total phenolic content) of tender coconut water (*Cocos nucifera*) was studied using 2 factor and 4 level factorial design. The treatment temperature and treatment time both had significant effect on ascorbic acid, total phenols, antioxidant activity, PPO and POD. The complete inactivation of POD achieved after thermal processing at 95 ºC for 5 min but the complete inactivation of PPO obtained after 10 min, so PPO is more heat resistant than POD. Increase in process temperature and time had negative effect on PPO, POD, total phenols, antioxidant activity and ascorbic acid. The relation between independent variables and individual dependent variable was explained by a quadratic model. The regression coefficients and statistical results of the model were obtained from regression analysis. For all the dependent variables the R^2 value of the quadratic model is higher than 0.9.

Keywords: Tender coconut water, thermal treatment, PPO, POD, total phenols

1. Introduction
Coconut water comes from immature coconuts (tender coconut) consumed as a beverage. The tender coconut water (TCW) is considered a natural health drink due to its unique characteristics (Debmandal *et al*., 2011) [11]. This natural drink is useful in preventing and relieving many health problems, including dehydration, constipation, digestive problems, fatigue, heatstroke, diarrhea, kidney stones and urinary tract infections (Campbell *et al*., 2000) [7]. The market for TCW is increasing considerably due to its medicinal, nutritional and sensory properties. So, there is a huge demand in the market for processed bottled TCW to make it available for in all the locations and throughout the year (Sanganamoni *et al*., 2017) [25]. However, there is a challenge for developing a process to ensure that the product is safe and high quality retention.

Generally, the TCW present inside the fruit is shelf sterile and stable for a few days **(**Yong *et al*., 2009**)** [30], but the shelf life of extracted TCW is very less. The spoilage of extracted TCW is mainly due to the presence of enzymes, belongs to the oxidase family (Polyphenol oxidase and Peroxidase. The oxidative enzymes have high thermal resistance and their activity leads to yellow, brown or even pink coloring during storage, even under refrigeration.

Polyphenol oxidase (PPO) and Peroxidase (POD) are widely detected in many fruits and vegetables and are closely linked to enzymatic color changes with consequently loose on sensorial properties (Campos *et al*., 1996) [8]. According to some food technologists, Polyphenol oxidase is indirectly responsible for fruit and vegetables enzymatic browning**,** it catalyzes two types of oxidative reactions. Such as hydroxylation of monophenols to o-diphenols, and the oxidation of this last one colorless compound to highly colored o-quinones.

Presently thermal treatment is a most commonly applied enzyme inactivation technique for coconut water. Thermal treatment requires less maintenance and low energy consumption. By considering the facts, the present experiment was aimed to study the effect of thermal treatment on bioactive components and enzyme activity kinetics.

2. Materials and Methods

2.1 Procurement of Tender Coconut Water (TCW)

Tender coconuts (6-8 months matured) of around same size contains coconut flesh (jelly like less than 2 mm and without any visible damage on the outside were purchased from the local market at IIT Kharagpur, West Bengal. The surface of coconut husk was properly cleaned with distilled water followed by 1% sodium hypochlorite sanitize solution (Walter *et al*., 2009) [28]. After, the coconuts were placed in a laminar flow UV light chamber for 30 min to make the coconuts free from surface contamination.

TCW was manually extracted from coconut using free washed and sanitized sharp stainless steel knife, and filtered through muslin cloth. The filtered TCW obtained from several coconuts (4-5 coconut fruits having same maturity level) was mixed in a glass beaker. The mixed TCW was filled and packed in LDPE (low-density polyethylene) pouches and immediately stored at -18 ˚C for future study. Whole TCW extracted from coconut was processed on the same day of extraction.

2.2 Chemicals and reagents

All the chemicals and reagents used in this study were analytical grade and procured from Merck, India and Sigma-Aldrich, Germany.

2.3 Experimental design

Thermal treatments were performed in a temperature controlled (± 0.5 ºC) water bath (Ultrasonic cleaner-Memory Quick, Takashi: UD80 SH-3L) at 80, 85, 90 and 95 ºC for 2.5, 5, 7.5 and 10 min. Approximately 50 mL of TCW was filled and packed in EVOH (Ethylene vinyl alcohol copolymer) packing film. The packets were placed in a water bath and the countdown time began when the centre of the sample reached the target temperature. The relative activity of PPO, POD and nutritional properties (*viz.* Ascorbic acid, Total phenolic content and Antioxidant activity) were analyzed to know the effect of treatment on it.

2.4 Measurement of Bioactive Components of TCW

2.4.1 Measurement of ascorbic acid (AA)

Ascorbic acid (AA) content of TCW was determined by a spectrophotometric method based on its ability to decolorize 2, 6-dichlorophenol-indophenol dye solution proposed by Ranganna (1991) [23]. Briefly, take 1 mL of sample and makeup to 5mL with 2% Metaphosphoric acid (HPO_3) solution. Then mix with 10 mL dye solution and measure the absorbance at 518 nm using UV-visible spectrophotometer against a blank (contains 5 ml 2% HPO_3 +10 mL distilled water). Interference was avoided by rapid determination and the corresponding AA content was obtained from a standard curve drawn for pure L-ascorbic acid (Sigma-Aldrich) solution which varied within 0.2 to 1 g·L^{-1}

Standard AA conc. (mg.mL^{-1}) = 0.783 × (absorbance) … (1)

$$\text{AA of coconut water } (mg.mL^{-1}) = \frac{\text{standard AA concentration} \times \text{final make up volume}}{\text{volume of sample taken}} \text{ ..(2)}$$

2.4.2 Total phenols

The methanolic extract of coconut water was used for analysis of total phenols and antioxidant capacity. It was prepared by shaking a solution of 5 mL coconut water with 25 mL 80% methanol in distilled water for 3h at ambient temperature (27 ± 1 °C). Total phenol content was determined using the Folin-Ciocalteu reagent (FCR) assay according to the method of Singleton *et al*. (1999) with slight modifications as described by Wijngaard and Brunton (2010) [29]. The blue color was developed using a Folin–Ciocalteu reagent (FCR) in an alkaline medium (20% sodium carbonate) over 90 minutes and its absorbance was measured at 750 nm in a UV-visible spectrophotometer (Model: UV1700; Make: Shimadzu, Japan). Gallic acid was taken as the standard for the phenolic and total phenolic content was expressed in Gallic acid equivalent.

Standard Phenolic conc. (GAE in mg.mL^{-1}) = 0.2437 × (absorbance)…... (3)

Phenolic concentration of coconut water (GAE in mg/ml) =

$$\frac{Standard\ phenolic\ concentration\ x\ volume\ madeup}{vol.of\ phenolic\ extract\ taken\ for\ estimation\ x\ vol.of\ sample\ taken} \text{ …….(4)}$$

2.4.3 Antioxidant activity

The antioxidant activity of the extract was measured in terms of its DPPH radical scavenging ability. It represents the ability of the food product to resist oxidation. The advantage of the DPPH method is that free radicals are allowed to react with the whole sample and the relatively long time given in the method allows the free radical to react slowly even with weak antioxidants (Kedare and Singh, 2011) [18]. Methanolic extract of coconut water was used for the analysis of DPPH free radical scavenging activity and it was prepared as described for total phenol content. The DPPH assay was carried out according to the procedure of Goupy *et al*. (1999) [15] with slight modifications as described by Wijngaard and Brunton (2010) [29]. The change in color of the DPPH solution from purple to yellow, resulting from the addition of different quantities of methanolic extract of coconut water or gallic acid (GA) standard (20 to 200 µL) was measured at 517 nm after allowing the solution to stand in the dark for 30 min. The decrease in absorbance of DPPH after 30 min was calculated and expressed as mg of GA equivalents antioxidant capacity (GAEAC) per 100 mL of the sample using the formula given in Eq. (5)

$$\text{GAEAC} = \frac{\Delta Abs_{Sample}}{\Delta Abs_{GA}} \times C_{GA} \times \left(\frac{V}{W}\right) \times 100 \text{ … (5)}$$

Where,

ΔAbs_{sample} is the change of absorbance after addition of coconut water extract

C_{GA} is the concentration of GA standard solution (0.02 mg/mL);

ΔAbs_{GA} is the change of absorbance obtained from a calibration curve when the same volume GA standard solution as that of coconut water extract was added;

V is the final makeup volume of extract and

W is the volume of the sample used for extraction

2.5 Enzyme Activity Measurement

2.5.1 Assay of polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) was determined using Pyrocatechol solution as phenol substrate proposed by Tan *et al*. (2014) with slight modifications. Briefly, 5.5 mL of 0.2 M Sodium phosphate buffer of pH 6 and 1.5 mL of 0.2 M pyrocatechol were added into a test tube. The test tube was then immersed in a controlled temperature water bath at 25 ºC for 2 min for thermal stabilization. Then add 2 mL of coconut water mix properly and measure the change in absorbance at 420 nm using UV-1700 UV Visible spectrophotometer with respect to the blank solution consist of 7.5mL buffer and 1.5 mL 0.2 M pyrocatechol.

2.5.2 Assay of peroxidase (POD)

Peroxidase (POD) was Determined according to the method proposed by Augusto *et al*. (2015) [2] with slight modifications. 5% (w/v) pyrogallol solutions used as phenol substrate. In each assay 0.32 mL of 5% pyrogallol solution, 2.36 mL buffer and 0.16 mL coconut water were mixed in a cuvette. Then 0.16 mL of 0.5% H_2O_2 added to this mixture (reaction will start after adding H_2O_2). The change in absorbance was measured at 420 nm with respect to the blank solution contained 0.32 mL 5% pyrogallol, 2.52 mL buffer and 0.16 mL 0.5% H_2O_2.

2.5.3 Protein concentration

For the estimation of protein concentration in the crude enzyme extract Bradford's Method was followed (Sadasivam and Manickam, 2011) [24]. Bradford's reagent was prepared by dissolving 100 mg of Coomassie brilliant blue-G250 in 50 mL 95% ethanol and 100 mL concentrated orthophosphoric acid. Make up the volume to 200 mL with distilled water. It can be diluted 4 times before use. Take 0.1 mL of enzyme extract and add 5 mL of Bradford's reagent. Record the absorbance values in a UV-Visible spectrophotometer against the blank (without sample extract) at 595 nm.

2.5.4 Enzyme Activity

For both the enzymes, the absorbance was measured at every 10 sec interval for 15 min then the slope of the absorbance curve drawn against time will give the enzyme activity of coconut water. The enzyme activity was expressed in $U.mL^{-1}$ min^{-1} (µg of protein)$^{-1}$. The relative activity (A_{rel}) can be calculated by using Eq. (6)

$$Y = \frac{Ai\ x\ Po}{Pi\ x\ Ao} \quad \ldots (6)$$

Where, A_o and A_i represent the slope of OD vs time curve in the untreated sample and sample, respectively; Pi represents the relative absorbance differences with respect to blank got from Bradford analysis for enzyme concentration in the extract in sample and Po represents the same as previous but for the untreated sample. The slope was taken for every measurement in which correlation coefficient (R^2) is greater than 0.95 and it was done in Microsoft Excel 2013 software along with a precision up to four decimal places.

2.6 Modelling

Non-linear regression (Quadratic function) modelling

A second order polynomial equation was used to fit the experimental data. The propesed polynomial equation to predict the response is

$$Y_R = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 \ \ldots \ (7)$$

Where Y_R is response varibles Y_1=PPo; Y_2=PPD; Y_3=TPC; Y_4=LAA and Y_5=AA; X_1=time, X_2=temperature; β_0, β_1 are the linear regression coefficents; β_{11}, β_{22} are the coefficients of qudratic effects and β_{12} is coefficient of interaction. Response surface analysis of experimental data was carired out using Design Expert software. The regression analysis and analysis of variance (ANOVA) were conducted by fitting the experimental data with the eqaution to determine the regreession coefficents and stastical significance of the model. The significance of the model was decided based on the p-value. The performance of the model was assessed by the stastical parameters R^2, CV, and MSE. The three dimenssional surface graphs were generated inorder to visualize the relationship between dependent and independet varbiels.

3. Results and Discussion

3.1 Compositions of raw tender coconut water

The nutritional properties and enzyme activity of TCW were analyzed before treatment. The compositions of TCW varied from fruit to fruit depending upon variety and maturity of fruit (Jackson *et al*, 2004 and Tan *et al*, 2014) [17, 26]. Although there was an important initial difference exist in physicochemical properties of TCW between different verities of fruit. But for comparison, these parameters kept as constant for the whole experiment. The compositions of fresh TCW were measured and presented in Table 1.

Table 1: Enzyme activity and biochemical characterization of fresh TCW.

Parameters	Value
Ascorbic acid	2.7 ± 0.25
Total phenolic content (mg of GAE/ L)	63.1 ± 0.4
Antioxidant activity (mg of GAEAC/ L)	8.1 ± 0.5
PPO ($U.mL^{-1}$. Min^{-1} $^oBrix^{-1}$)	0.59 ± 0.015
POD ($U.mL^{-1}$. Min^{-1} $^oBrix^{-1}$)	0.06 ± 0.024

Note: Values reported as mean ± standard deviation (N = 12).

3.2 Effect of temperature and time

3.2.1 PPO

PPO and PDO are the two most important enzymes that present in the fresh coconut water. Generally, Polyphenol oxidase (PPO) is copper containing oxidoreductases that catalyze the hydroxylation and oxidation of phenolic compounds in the presence of molecular oxygen. The individual and interaction effect of temperature and time on PPO inactivation is illustrated in Fig 1. The Individual and interaction effect of temperature and time are highly significant. The PPO inactivation increased with increase in temperature and time at a given time and temperature respectively. The individual effect has a positive relation with the PPO but the interaction effect has negative relation (Table 2). A quadratic model has been developed to predict the PPO inactivation at any combination of time and temperature is shown in the table. The coefficients of factors and statistical results obtained from the analysis are tabulated in Table. The R^2 and MSE of the model was 0.991 and 2.084 respectively. The same trend was reported by Falguera *et al.* (2011) [12] conducted on apple juice. The reason for such type of changes mainly attributed to an increase in temperature may affect the biosynthesis process which results in protein degradation in TCW.

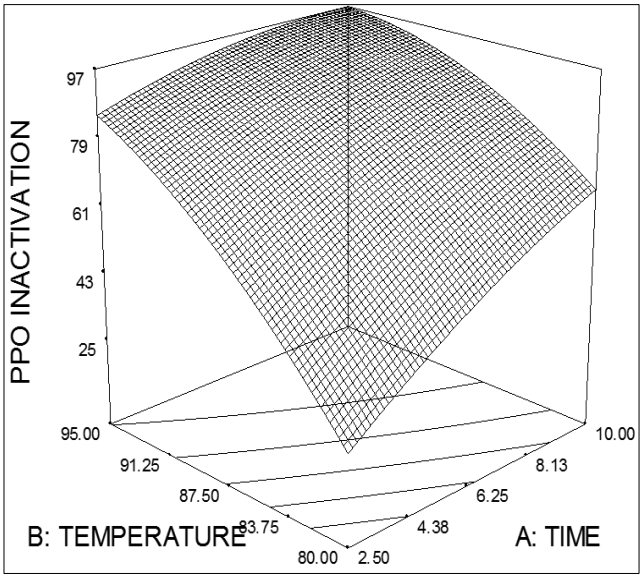

Fig 1: Effect of treatment temperature and time on PPO of TCW.

3.2.2 POD

The POD inactivation positively correlated with the temperature and time ($p<0.001$) but the interaction effect is non-significant. The surface plot for individual and interaction effect of temperature and time on POD inactivation is illustrated in Fig 2. The POD inactivation increased with an increase in temperature at a given time and similar results are obtained with time at constant temperature (Falguera *et al.*, 2011) [12]. The rate of inactivation of POD at a given temperature and time can be estimated from the quadratic model. The coefficients and statistical results of this model are shown in Table 2. The R^2 and MSE of the model are 0.916 and 5.7583 respectively. The complete inactivation of POD achieved after thermal processing at 95 ^oC for 5 min. Though the experiment was continued up to 10 min because at this stage the PPO didn't inactive completely. It indicates that PPO is more heat resistant than POD in coconut water. The same trend was reported by Falguera *et al.* (2011) [12] conducted on apple juice.

Table 2: Coefficients and standard error of quadratic model for different quality attributes of coconut water.

Factor	PPO	POD	TPC	LAA	AA
Intercept	Coefficients (Standard Error)				
	78.41 (0.62)**	80.56 (1.69) **	6.25 (0.04)**	7.91 (0.04)**	0.71 (0.01)**
X_1	13.09 (0.41)**	6.94 (1.12) **	-0.27 (0.03)**	1.05 (0.03) **	-0.02 (0.01) **
X_2	22.77 (0.41) **	21.94 (1.12) **	-0.41 (0.03)**	4.69 (0.03) **	-0.04 (0.01) **
X_{12}	-7.29 (0.55) **	-8.01 (1.5) **	-0.22 (0.03) **	0.36 (0.04) **	0.01 (0.01)
X_1^2	-3.43 (0.68) **	-2.11 (1.88)	0.1 (0.04) *	-0.13 (0.05) *	0.01 (0.01)
X_2^2	-6.8 (0.68) **	-2.3 (1.88)	-0.14 (0.04) *	0.45 (0.05) **	-0.01 (0.01)*
Statistical results					
R^2	0.991	0.916	0.934	0.999	0.973
MSE	2.0837	5.7583	0.1097	0.1261	0.0051
CV (%)	2.87	7.37	1.76	1.56	0.73

X_1-*time*; X_2-*temperature*; PPO-*polyphenol oxidase*; POD-*peroxidase*; TPC-*total phenolic content*; LAA-*loss of ascorbic acid*; AA-*antioxidant activity*; MSE-*mean square error*.

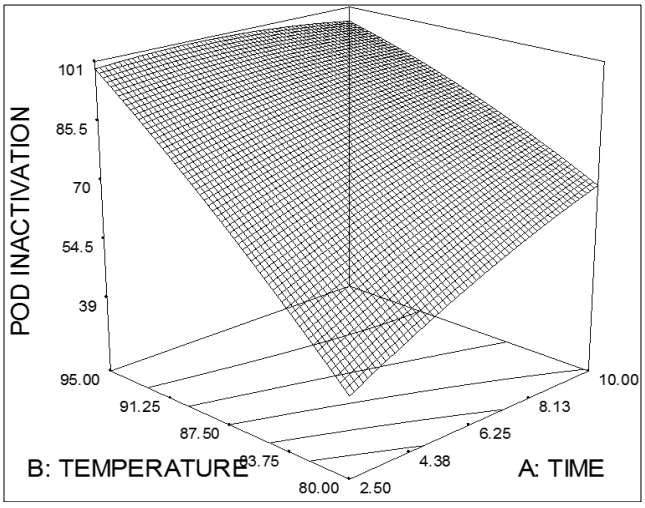

Fig 2: Effect of treatment temperature and time on POD of TCW

3.2.3 Total Phenolic Content (TPC)

Phenolic compounds are beneficial compounds mainly found in fruits and vegetables. They have been implicated in the reduction of degenerative diseases in human beings primarily because of their antioxidant potential. The TPC of thermal treated coconut water at different temperatures and time combinations is illustrated in Fig 3. From this figure, it is evident that the TPC decreases with increase in time and temperature at a given temperature and time respectively. The reason for such type of changes attributed to the increase in temperature may destruct the phenolic compounds those are initially present in fresh coconut water. The estimated coefficient of respective factors and statistical results obtained from the statistical analysis are shown in Table 2. Form this it is observed that time, temperature and their interaction effect on TPC are negatively correlated with TPC. The R^2 and MSE of the fitted quadratic model are 0.934 and 0.1096 respectively.

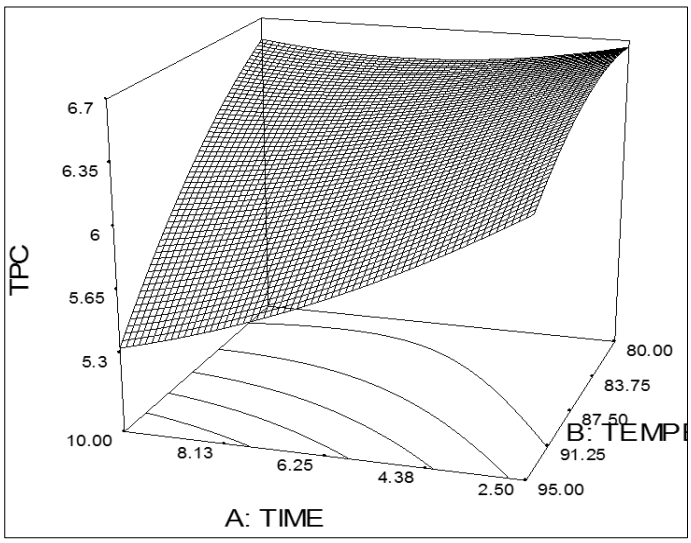

Fig 3: Effect of treatment temperature and time on total phenolic content of TCW

3.2.4 Ascorbic acid content

Ascorbic acid is a heat-sensitive bioactive compound that plays a vital role in human health and can act as an antioxidant. The change in ascorbic acid with respect to time and temperature is shown in Fig 4. The increase in temperature and time has a positive effect on loss of ascorbic acid and the interaction effect too. The ascorbic acid sensitive to extreme temperatures. The individual and interaction effect of temperature and time are highly significant (p<0.001). Heating effects the degradation of ascorbic acid in anaerobic pathway due to its heat-sensitive characteristic in the presence of oxygen. In addition to this, the depletion of ascorbic acid may be due to the formation of free hydroxyl radicals by photochemical reaction, related to oxidative processes. The similar results were reported by Goh *et al*. (2012) [14]. The loss of ascorbic acid is increased with temperature in thermal treatment.

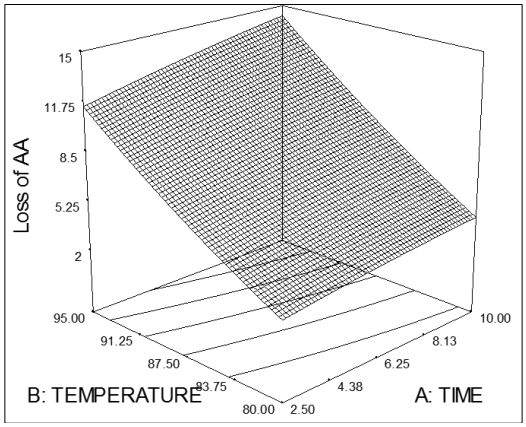

Fig 4: Effect of treatment temperature and time on loss of ascorbic acid of TCW

3.2.5 Antioxidant capacity

The antioxidant activity of coconut water at different temperatures and time combinations is shown in Fig 5. The antioxidant activity has a negative correlation with the temperature and time (p<0.001) i.e AA decreases with increase in temperature as well as time at a given time and temperature respectively. But the interaction effect is positively correlated but non-significant. The reason for such type of changes attributed to the increase in temperature may destruct the phenolic compounds initial present in coconut water. A quadratic model was developed to express the relationship among the temperature, time and AA of coconut water. The coefficients and statistical results of the analysis are illustrated in Table 2.

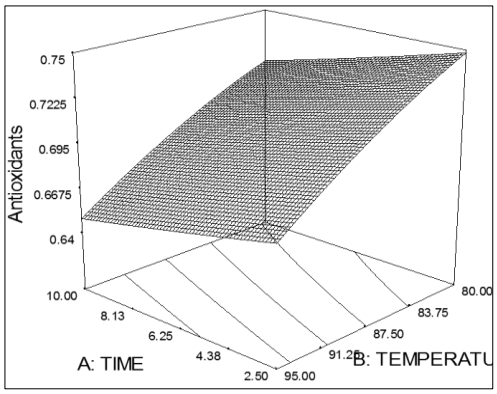

Fig 5: Effect of treatment temperature and time on antioxidant activity of TCW

4. Conclusion

Effect of thermal treatment on Enzymes (PPO and POD) and nutritional properties (*viz.* Ascorbic acid, Total phenolic content and Antioxidant activity) of tender coconut water (*Cocos nucifera*) were studied during this research work. The process conditions for thermal treatment were temperature (80, 85, 90, 95 ^{o}C) and treatment time (2.5, 5, 7.5, 10 min). The results obtained from this study showed that the thermal treatment conditions had a significant effect on ascorbic acid, total phenols, antioxidant activity, PPO and POD. Further, inactivation kinetics parameters (*viz.* D value and Z value) were calculated for PPO and POD at different temperatures. The complete inactivation of POD achieved after thermal processing at 95 ^{o}C for 5 min. Though the experiment was continued up to 10 min because at this stage the PPO didn't inactive completely. These results evident that the PPO was more heat resistant than POD in thermal treatment. Further, the results were compared with enzyme activity and nutritional properties of tender coconut water after UV-C treatment. From the results, the study concluded that, although the thermal treatment was better processing option pertaining to enzyme inactivation, but ultraviolet treatment was found superior based on retention of nutritional attributes.

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