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Preservation of Kombucha Tea—Effect of Temperature on Tea Components and Free Radical Scavenging Properties

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Kombucha tea is sugared black tea fermented with a consortium of acetic acid bacteria and yeasts (tea fungus) for 14 days. The tea tastes slightly sweet and acidic. The formation of tea fungal biofilms during storage is a big problem when kombucha tea is being stored and commercialized. Various thermal treatments have been tried for long-term storage of kombucha tea. The present study revealed the influence of heat on the biochemical constituents and the free radical scavenging properties of kombucha tea. Heat treatment at 60, 65, and 68 °C for 1 min controlled biofilm formation in kombucha tea without changing its clarity, taste, and flavor. However, tea polyphenols and black tea quality parameters showed varying stability during the storage period. A decrease in free radical scavenging properties was also found during the storage period. Because the biological activities of kombucha tea depended on the biochemical constituents, it was concluded that heat treatment was not a suitable method for kombucha tea preservation.

KEYWORDS: Kombucha tea; tea fungus; preservation; shelf life

INTRODUCTION

As the growth of the beverage industry enables the massive production of tea products, the market for canned tea products has expanded rapidly during the past few years. Ready-to-drink black and green teas are now increasingly consumed in the world, especially in Japan and China, because of their health benefits (*1, 2*). Kombucha tea is sugared black tea fermented for about 14 days with a consortium of acetic acid bacteria and yeasts, named as "tea fungus". The name tea fungus is a misnomer since there is no fungus involved in the fermentation (*3*). Like green tea and black tea, kombucha black tea can also be bottled for commercialization. The findings of various health benefits of kombucha tea have led to a general consumer's appreciation for its functional properties. Thus, kombucha tea is consumed not only to satisfy consumers' fine taste buds but also to impart health benefits. Tea fungus is an excellent example of a biofilm that consists of bacteria and yeasts. Several bacterial and yeast species are reported to be present in the tea fungal consortium (*4*). After fermentation, the kombucha tea is filtered through a cheese cloth and is consumed as a health drink. When kombucha tea is stored at ≥20 °C, the biofilm continues to form due to the presence of microorganisms in it. The ability of tea fungal microbes to form a biofilm is a big problem when the kombucha tea is stored and commercialized. Hence, it is essential to kill or remove the microbes in kombucha tea after fermentation, thus preventing biofilm formation during the storage period.

Kombucha microorganisms can be killed by physical and chemical treatments. However, it has been reported that yeast has developed resistance against the chemical preservatives used to control their growth. Heat treatment is an effective method to inactivate spoilage yeasts. However, exposure of yeast cells to heat without causing death can result in metabolic and structural debilitation. Resistance of yeasts to heat inactivation and injury can be influenced by a large number of factors, including inherent differences among strains and species and the composition of the medium in which yeasts are grown before and during heat treatment (*5*). Until now, there has been no specific study available in the literature regarding the processing of kombucha tea for preservation. In the present study, various

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Table 1. Effect of Temperature on Kombucha Tea Quality

temperature[a]	color	clarity	mat formation	taste and flavor	bacteria (CFU $\times$ 10^3 mL^{-1})	yeast (CFU $\times$ 10^6 mL^{-1})
control	normal	turbid	mat formed	no change	15 ± 1.5	7 ± 1.8
50 °C	normal	turbid	mat formed	no change	10 ± 2.5	6 ± 1.2
60 °C	normal	clear	nil	no change	nil	nil
65 °C	normal	clear	nil	no change	nil	nil
68 °C	normal	clear	nil	no change	nil	nil
70 °C	becoming dark	clear	nil	no change	nil	nil
75 °C	becoming dark	clear	nil	changed	nil	nil
80 °C	becoming dark	clear	nil	changed	nil	nil
85 °C	becoming dark	clear	nil	changed	nil	nil
90 °C	becoming dark	clear	nil	changed	nil	nil

[a] All treatments were given for 1 min; CFU, colony-forming units. Values are means $\pm$ SDs; $n = 3$.

heat treatments were tried for long-term storage of kombucha tea. Changes in color, taste, flavor, clarity, tea fungal mat formation, epicatechin isomers [(−)-epicatechin (EC), (−)-epicatechin-3- gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG)], gallic acid, caffeine, black tea quality parameters [theaflavins (TFs), thearubigins (TRs), highly polymerized substances (HPS), and total liquor color (TLC)], total phenolic compounds, and free radical scavenging properties were studied during the storage period with an interval of 30 days.

MATERIALS AND METHODS

Starter Culture. A starter culture or tea fungal mat of *Medusomyces gisevii* was obtained from the tribal people of Kolli hills, Tamil Nadu, India, and was maintained in sugared black tea. Bacterial components were isolated on nutrient agar medium containing cycloheximide (0.5% peptone, 0.3% beef extract, 0.2% yeast extract, 0.5% sodium chloride, 2% agar, and 0.0004% cycloheximide, pH 7.0), and yeasts were isolated on glucose-yeast extract agar containing oxytetracycline (0.5% glucose, 0.2% yeast extract, 2% agar, and 0.0004% oxytetracycline, pH 5.0). Samples were plated on the respective medium and were incubated at 30 °C for bacteria and at 25 °C for yeasts. The bacterial components were identified as *Acetobacter aceti* MTCC 2945, and the yeast components were identified as *Zygosaccharomyces bailii* MTCC 8177 and *Brettanomyces claussenii* MTCC 7801 at the Institute of Microbial Technology (IMTECH) (Chandigarh, India). Identified cultures were deposited in Microbial Type Culture Collection, IMTECH.

Preparation of Kombucha Tea. Brooke Bond Red Label Tea (Hindustan Lever Limited, Mumbai, India) was used for the preparation of kombucha tea. Tea was added to boiling water (1.2%) and allowed to infuse for about 5 min after which the infusions were filtered through a sterile sieve. Sucrose (10%) was dissolved in hot tea, and the preparation was left to cool. The cooled tea (200 mL) was poured into 500 mL glass jars that had been previously sterilized at 121 °C for 20 min and inoculated with 3% (w/v) freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) previously fermented liquid tea broth aseptically. The jar was covered with a clean cloth and fastened tightly. The fermentation was carried out in the dark at 24 ± 3 °C for 14 days (*4, 6*).

Heat Treatment. The fermented kombucha tea was added to 100 mL airtight pet bottles in 50 mL portions. A set of bottles were subjected to various heat treatments (50−90 °C) in a temperature-controlled water bath for 1 min. To measure the internal temperature of the kombucha tea during heat treatment, an opened pet bottle containing kombucha tea was used. Heat-treated kombucha tea was cooled rapidly by placing it in ice water for 5 min. The bottles were kept at room temperature (24 ± 3 °C) for 3 days and observed for color, clarity, mat formation, and changes in taste and flavor. On the basis of the results, kombucha teas heated to 60, 65, and 68 °C were selected for the shelf life study.

Shelf Life Study. Bottles containing kombucha tea heated to 60, 65, and 68 °C were incubated in an environmental chamber maintained at 28 °C and 65% relative humidity for 90 days. Sampling was performed every 30 days by taking one pet bottle from each treatment at a time to avoid contamination. Kombucha tea without any treatment served as a control. Tea polyphenols content (EGCG, EGC, ECG, EC, catechins, and gallic acid), caffeine, black tea quality parameters (TF, TR, HPS, and TLC), and free radical scavenging activities of control and heat-treated kombucha tea broth were analyzed.

Analysis of Tea Polyphenols, Caffeine, and Black Tea Quality Parameters. All of the analyses were carried out in Parry Agro Industries Ltd., R&D Centre (Murugalli Bazaar, Valparai, Coimbatore (Dist), Tamil Nadu, India), which is a NABL (National Accreditation Board for Testing and Calibration Laboratories) accredited laboratory for both chemical and microbiological testing as per IS/ISO/IEC 17025: 2005. Tea catechins (EC, ECG, EGC, EGCG, catechins, and gallic acid) and caffeine in kombucha tea were measured by the method followed by Jayabalan et al. (*3*). Five milliliters of kombucha tea was extracted with 25 mL of methanol. The contents were filtered through a membrane filter (0.45 μM) into high-performance liquid chromatography (HPLC) vials. A 10 μL sample of filtrate was injected into a Shimadzu (Kyoto, Japan) HPLC system equipped with a diode array detector (SPD-M10Avp). A Phenomenex Luna C-18(2) column (4.6 mm i.d. $\times$ 25 cm, 5 μm) was used for the analysis. The mobile phase was a mixture of 0.1% orthophosphoric acid (A) and acetonitrile (B). The gradient used was as follows: 0−12 min, 15% B; 12−22 min, 25% B; and 22−30 min, 15% B. The flow rate and column temperature were maintained as 1.0 mL min^{-1} and 35 °C, respectively. Tea polyphenols were detected at 280 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound. The concentrations of tea polyphenols were quantified from standard curves. Black tea quality parameters (TF, TR, HPS, and TLC) were determined according to the method of Takeo and Oosawa (*7*) as modified by Ramaswamy (*8*) and Thanaraj and Seshadri (*9*).

Total Phenolic Compounds. The total phenolic compounds present in tea samples were quantified by the method described by Singleton, Orthofer, and Lamuela-Raventos (*10*). The fermented tea broths (0.1 mL) were transferred to 100 mL Erlenmeyer flasks, and the final volumes were adjusted to 46 mL by addition of distilled water. Folin−Ciocalteau reactive solution, 1 mL, was added to each flask, and the flask was incubated at room temperature (27 ± 2 °C) for 3 min. Then, 3 mL of 2% sodium carbonate solution was added, and the mixture was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm. Gallic acid was used as the standard for plotting the calibration curve. The phenolic compound content was expressed as gallic acid equivalent (GAE, μg).

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Scavenging Activity. The scavenging activity on DPPH was assessed by the method of Blois (*11*) with slight modification. To 100 μL of fermented tea broths, 1 mL of 0.1 mM DPPH in ethanol solution and 450 μL of 50 mM Tris-HCl buffer (pH 7.4) were added and incubated at room temperature (27 ± 2 °C) for 30 min. The reduction of DPPH free radicals was measured by reading the absorbance at 517 nm. Tubes without tea solutions served as the control. The activity was given as % DPPH radical scavenging calculated as per the following equation:

DPPH radical scavenging activity (%) = [(control absorbance − sample absorbance)/control absorbance] × 100 (1)

Reducing Power. The reducing powers of tea samples were estimated by the method proposed by Yildirim, Mavi, and Kara (*12*). To 20 μL of fermented tea broths, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide were added, and the reaction mixture was incubated at 50 °C for 30 min. The reaction was stopped by the addition of 2.5 mL of 10% trichloroacetic acid and centrifuged for 10 min at 3000 rpm. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicated increased reducing power.

Inhibitory Activity on Hydroxyl Radical-Mediated Linoleic Acid Oxidation. The inhibitory activity of tea broths on hydroxyl radical mediated linoleic acid oxidation was measured through ammonium thiocyanate assay (*13*). A 100 μL amount of fermented tea broth, 200 μL of diluted linoleic acid (25 mg mL^{-1} in 99% ethanol), and 400 μL of 50 mM phosphate buffer (pH 7.4) were incubated at 40 °C for 15 min. An aliquot of the reaction mixture (100 μL) was mixed with a reaction solution containing 3 mL of 70% ethanol, 100 μL of ammonium thiocyanate (300 mg mL^{-1} distilled water), and 100 μL of ferrous chloride (2.45 mg mL^{-1}, 3.5% hydrochloric acid) and incubated at room temperature (27 ± 2 °C) for 3 min. The absorbance was measured at 500 nm. A linoleic acid emulsion without tea solution served as the control. Inhibition of linoleic acid oxidation was calculated by the formula:

inhibition on hydroxyl radical-mediated linoleic acid oxidation (%) = [(control absorbance − sample absorbance)/control absorbance] × 100 (2)

Scavenging Activity onto Superoxide Anions. The scavenging ability on superoxide radical ($O_2^{\bullet -}$) was assessed by the method described by Lee et al. (*13*) with a slight modification. To 100 μL of fermented tea broths, 1.7 mL of 0.94 mmol EDTA containing 0.05 mmol of xanthine and 0.025 mmol of 2-(4-iodophenyl-3- (4-nitrophenol)-5-phenyltetrazolium chloride (INT) were added. The reaction mixture was incubated at room temperature (27 ± 2 °C) for 3 min, and then, 250 μL of xanthine oxidase (80 U L^{-1}) was added and again incubated for 20 min at room temperature. The absorbance was measured at 505 nm with control tubes having all of the reaction compounds except the tea solutions. The scavenging of superoxide anions was calculated by the formula:

superoxide radical scavenging activity (%) = [(control absorbance − sample absorbance)/control absorbance] × 100 (3)

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was determined by Klein, Cohen, and Cederbaum (*14*). Fermented tea broths (100 μL) were taken in test tubes and evaporated to dryness. To these tubes, 1 mL of iron−EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA (0.018%), and 1 mL of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added, and the reaction was initiated by the addition of 0.5 mL of 0.22% ascorbic acid. The tubes were capped tightly and heated on a water bath at 80−90 °C for 15 min. The reaction was terminated by the addition of 1 mL of ice-cold trichloroacetic acid (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid, and 2 mL of acetyl acetone was mixed and made up to 1 L with distilled water) was added to the tubes, and the tubes were kept at room temperature (27 ± 2 °C) for 15 min for color development. The intensity of the yellow color formed was measured spectrophotometrically (Shimadzu, Kyoto, Japan) at 412 nm against reagent blank. The percentage hydroxyl radical scavenging activity is calculated by the formula

hydroxyl radical scavenging activity (%) = [1 − (absorbance of sample/absorbance of blank)] × 100 (4)

Antilipid Peroxidation. The lipid peroxidation was measured by the method of Halliwell and Gutteridge (*15*). Institute Ethical Committee (IRT Perundurai Medical College and Research Center, Perundurai, Erode District, Tamil Nadu, India) clearance was obtained prior to our study to use rat liver in antilipid peroxidation study (588/02/A/CPCSEA; CPCSEA, 2003). Liver homogenate was prepared by grinding fresh normal albino rat liver using phosphate buffer saline, pH 7.4 (10% w/v). The homogenate was centrifuged at 3000 rpm for 15 min, and the clear supernatant was used for analysis. The fermented tea broths (100 μL) were taken in test tubes and evaporated to dryness. To this, 1 mL of 0.15 M potassium chloride and 0.5 mL of rat liver homogenate were added. Peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride. The tubes were incubated at 37 °C for 30 min. The reaction was stopped by adding 2 mL of ice-cold hydrochloric acid (0.25 N) containing 15% trichloroacetic acid, 0.38% thiobarbituric acid, and 0.5% butylated hydroxyl toluene. The reaction mixture was heated at 80 °C for 1 h. The samples were cooled and centrifuged, and the absorbance of the supernatant was measured at 532 nm. A similar experiment was performed in the absence of tea solutions to determine the amount of lipid peroxidation obtained in the presence of inducing agents, which served as control. The percentage of antilipid peroxidation was calculated by the formula:

antilipid peroxidation activity (%) = [(control absorbance − sample absorbance)/control absorbance × 100 (5)

RESULTS AND DISCUSSION

In the present study, kombucha black tea was heated to various temperatures (**Table 1**) for 1 min and was observed for mat formation and changes in clarity, color, taste, and flavor. Tea fungal mat formation and growth of bacterial (10×10^3 CFU mL^{-1}) and yeast colonies (6×10^6 CFU mL^{-1}) were observed in kombucha black tea heated up to 50 °C after 3 days of storage. On rising temperature, no mat formation was observed up to 90 °C. However, the color of the kombucha tea turned dark brown when it was heated above 70 °C for 1 min. No color changes and microbial colonies were observed in kombucha black tea heated to 60, 65, and 68 °C. So, these three temperatures were selected for the shelf life study. Analysis of heat-treated kombucha tea during the storage period (90 days) revealed nonlinear degradation of the tea components and a linear decrease in free radical scavenging properties.

Effect on Tea Components. In the untreated kombucha tea (control), a linear decrease in quality parameters was observed during storage up to 90 days. TF was decreased up to 60% and TRs up to 87%, while HPS and TLC decreased up to 63%. EGCG and catechins were found to be decreased during the initial storage periods (up to 30 days) and thereafter increased. Similarly, EGC and caffeine contents decreased up to 60 days and then increased. ECG and EC showed a linear decrease during the storage period. Gallic acid was observed to be increased up to 60 days and later decreased (**Figures 1** and **2**). In kombucha tea heated to 60 °C for 1 min, TR, HPS, ECGC, and ECG contents declined linearly during the storage period, and TF was low on the 30th and 90th days and high on the 60th day. TLC and gallic acid contents were increased up to 60 days and thereafter decreased. EGC and catechins were found at low levels on the 30th day, and then, there was an increase. The EC content was high on the 30th and 90th days and low on the 60th day. Caffeine was observed to be decreased up to 60 days and increased afterward (**Figures 1** and **2**).

In kombucha tea, heated at 65 °C for 1 min, there was an initial decrease (up to the 30th day) in TF, TR, HPS, TLC, EGC, and EC contents, and then, an increase was observed. EGCG, ECG, and catechins contents linearly decreased during the storage period. The caffeine content was low up to the 60th day storage and then increased. The gallic acid content was

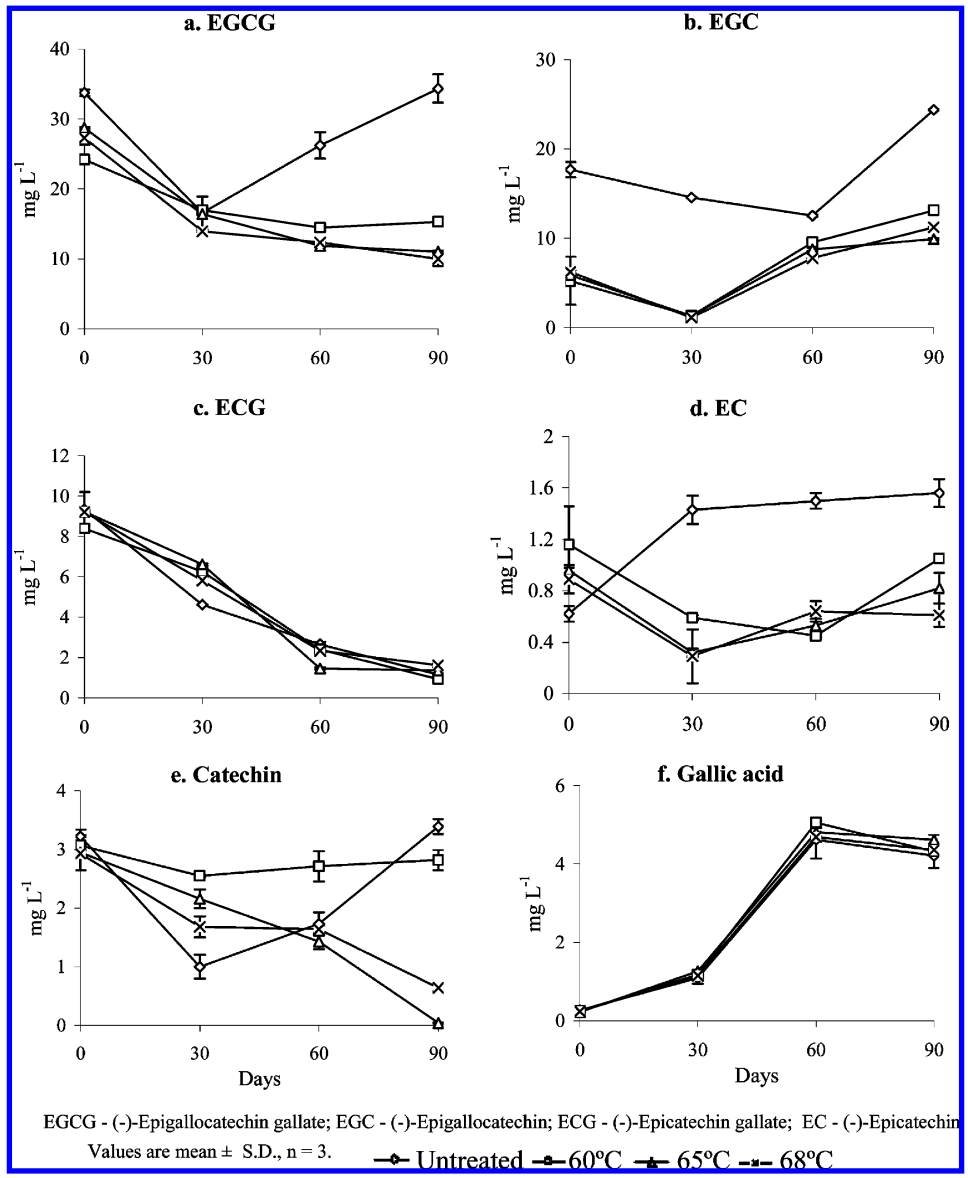

Figure 1. Effect of heat treatment on the stability of polyphenols in kombucha tea.

found to be increased on the 30th and 60th days and decreased on the 90th day (**Figures 1** and **2**). Heat treatment at 68 °C for 1 min reduced the TF, EGCG, EGC, and catechin contents during storage, and a linear increase was observed in HPS and TLC. TR, caffeine, and gallic acid contents were reduced up to the 60th day of storage and then increased. The EGC content was decreased up to the 30th day and then increased. The EC content was observed to be low on the 30th and 90th days and high on the 60th day (**Figures 1** and **2**).

Over the past few years, there has been an increasing interest in natural antioxidants and their role in human health and nutrition. The facts that the oxidation process plays an important role in several degenerative diseases and can contribute significantly to the risk of human aging and cancer has focused the interest on this subject (*16*). It is well-known that many food antioxidants can be significantly lost as a consequence of sterilization, pasteurization, dehydration, and during prolonged storage. Processing and storage are not always responsible for depletion in the antioxidant properties of foods. In some cases, these factors can induce the formation of compounds with novel antioxidant properties, which can maintain or enhance the overall antioxidant potential of foods. Recent studies carried out on various red wines containing polyphenols showed that prolonged air exposure caused a progressive increase in the chain-breaking activity (*17*). These changes were attributed to the partial oxidation of the wine polyphenols to form macromolecular compounds, which still maintain a remarkable radical scavenging activity. In the literature, little information is available on the changes in individual phenolic constituents during storage. For orange juices, mostly experimental, the influence of storage time and temperature on the content of ferulic and *p*-coumaric acids has been investigated (*18*). The present study showed the changes in contents of tea polyphenols in kombucha tea to temperature and storage over a prolonged period. It was found that the biochemical constituents were unstable not only in heat-treated kombucha tea but also in untreated kombucha tea during the storage period. This clearly showed the influence of time on the stability of tea polyphenols in kombucha tea.

The results of the present study were in agreement with the results of Kim et al. (*19*). They demonstrated that with an increase of temperature from 85 to 120 °C, the green tea liquor became darker and less green and attained a deeper yellow color. During heating, epigallocatechin gallate, epigallocatechin, epicatechin, and epicatechin gallate partially epimerized, and the concentration of total catechins decreased. Some of the volatile flavor compounds (pentanol, *cis*-3-hexenol, linalool oxide I,

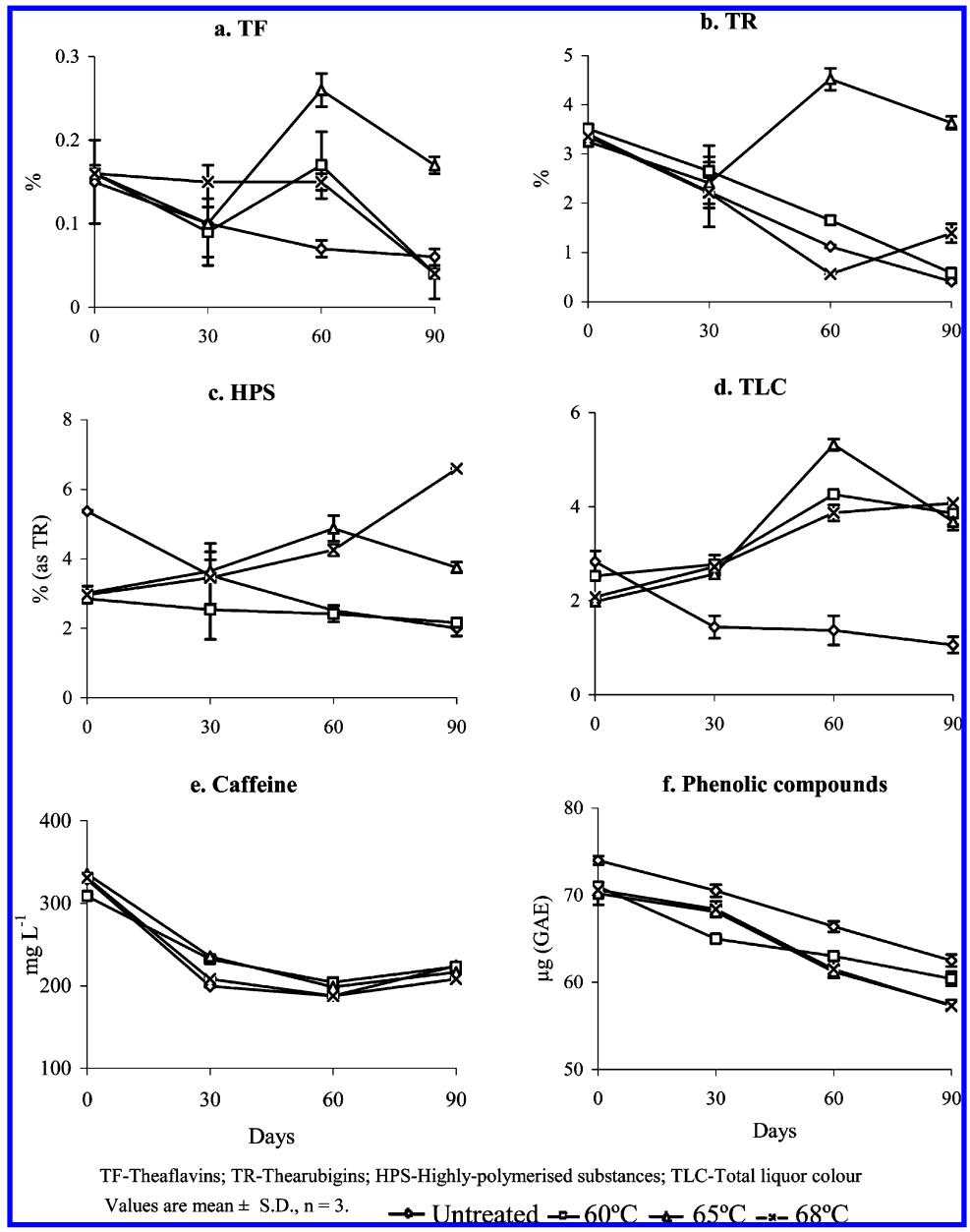

Figure 2. Effect of heat treatment on the stability of black tea quality parameters and caffeine and phenolic compounds contents in kombucha tea.

linalool oxide II, and β-ionone) were found to be decreased in their study. Kenji and Hideki (*20*) showed that 3-methyl butanol, methanol, β- damascenone, dimethyl trisulfide, and 2-methoxy-4-vinylphenol were decreased after heat processing of the black tea sample. Fallico et al. (*18*) showed that the content of ferulic and *p*-courmaric acids in freshly squeezed blood orange juices stored for 4 months at 25 °C increased from 1.0 to 3.2 and 0 to 5.2 mg/L, respectively. Klimczak and Malecka (*16*) also showed the same kind of results with ferulic acid. Klimczak et al. (*21*) stated that the contents of hydroxycinnamic acids in orange juices were decreased up to 38% after 6 months of storage. They also showed that there was a significant decrease in the total polyphenols during 4 months of storage at 38 °C, and after the storage period, the total phenolic content was significantly increased. Larrauri et al. (*22*) observed a significant reduction in both total and extractable polyphenols (18.6 and 32.6%) and condensed tannins (11.1 and 16.6%) as well as a decrease of 28 and 50% in the antioxidant activity, respectively, when red grape pomace peels were dried at 100 and 140 °C. Zafrilla et al. (*23*) described that the anthocyanin content was decreased up to 88% in conventional wine and 91% in ecological wine during 7 months of storage in the dark. Kabasakalis et al. (*24*) reported that the content of vitamin C in different juices decreases during storage, depending on storage conditions, such as temperature, oxygen, and light access. Klimczak et al. (*21*) have also described that an increase of temperature by each 10 °C caused a distinct decrease in the concentration of vitamin C, and the contents were decreased up to 81% after 6 months of storage at 38 °C. A study on the phenolics in the hawthorn furits and the drink conducted by Chang et al. (*25*) showed that the epicatechin isomer was degraded up to 50% after 6 months of storage at 23 °C.

Effect on Free Radical Scavenging Properties. Free radical scavenging properties and total phenolic contents of untreated and heat-treated kombucha tea were linearly decreased during the storage period (**Figures 2** and **3**). Klimczak et al. (*21*) explained that the DPPH radical scavenging activity of orange juices was increased during the first 2 months of storage at 18 and 28 °C, but the activity was decreased by 13% when stored in 38 °C. They have also showed that after 6 months of storage of orange juices at 38 °C, the antioxidant activity was decreased by 84%. Arena et al. (*26*) showed the increase in the antioxidant

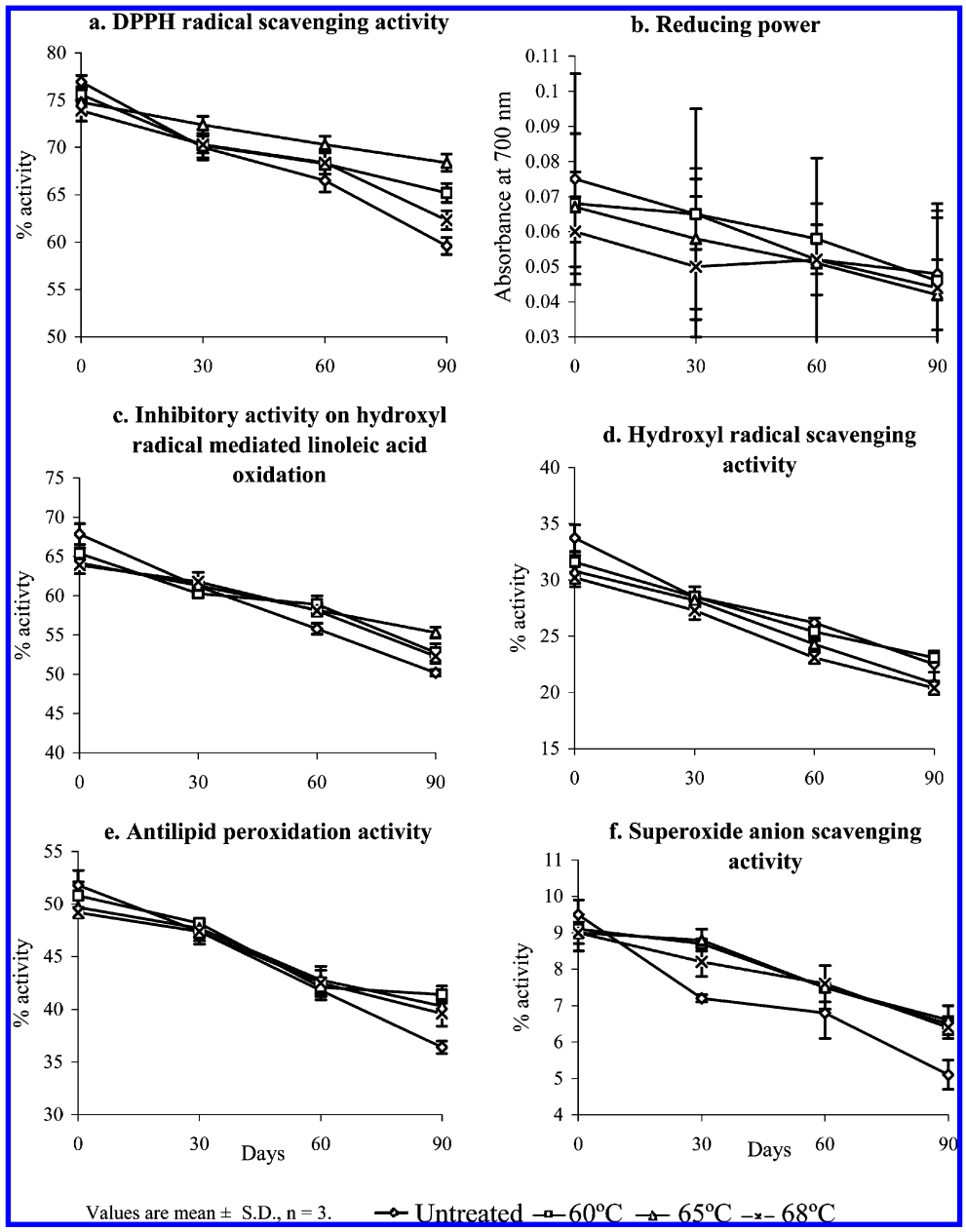

Figure 3. Effect of heat treatment on free radical scavenging activities of kombucha tea.

activity after 2 months of storage in orange juices reconstituted from concentrate. According to Piga et al. (*27*), storage of mandarin juices for 15 days at 4 °C resulted in an increase in the DPPH radical scavenging activity. In contrast to Piga et al. (*27*), Del Caro et al. (*28*) described a slight decrease in the TEAC (trolox equivalent antioxidant capacity) value obtained by the DPPH method for orange juice stored in the same conditions. Manzocco et al. (*29*) observed that pasteurization at 105 °C for 20 min and then storage at 25 °C of green and black tea extracts caused an unexpected increase in the chain-breaking activity of tea extracts. Radical scavenging properties appeared to be enhanced as a consequence of the thermal treatment and the storage that followed. The oxygen scavenging properties were gradually decreased during pasteurization and storage. The reduction in the oxygen scavenging ability of tea extracts was correlated with a progressive increase in the redox potential value for the black tea extracts. These results indicate that gain in radical scavenging activity is associated with a corresponding decrease in the reducing power of the tea extracts. It is known that processing and storage can promote a progressive polymerization of phenolic compounds to form brown-colored macromolecular products. In some cases, the oxidation of polyphenols leads to the formation of stable intermediates, which can still exhibit strong antioxidant activity. Modification in chain-breaking activity and oxygen uptake detected in tea extracts could be ascribable to the progressive oxidation of polyphenols, which leads to the formation of macromolecular compounds with stronger or decreased radical scavenging power. A possible explanation could be that the enzymatic and chemical oxidations of polyphenols follow different pathways, which lead to the formation of compounds having markedly contrasting radical scavenging capacities. A further hypothesis could be that although both reactions follow the same pathway, chemical oxidation proceeds much slower than the enzymatic one and the compounds formed during pasteurization and storage could have an intermediate oxidation status when compared to those formed by enzymatic oxidation (*29*). In the present study, free radical scavenging properties of untreated kombucha tea also linearly decreased during the storage period. It clearly indicates the influence of time on free radical scavenging properties of

kombucha tea. Tea flavanoids are very reactive species, which can easily undergo enzymatic and chemical reactions, which may be responsible for changes in antioxidant properties of kombucha tea (*29*) during storage. It can be concluded that chemical modification of flavanoids/tea polyphenols may take place during the fermentation and storage period.

The effect of pH on the stability of plant phenolic compounds has been extensively investigated in previous studies (*30*). These studies have consistently showed that the phenolic compounds such as chlorogenic acid, caffeic acid, gallic acid, flavanoids, and green tea catechins were pH-sensitive: The lower the pH, the greater was the stability. For instance, the tea catechins are extremely unstable in alkaline solution (pH > 8) and degrade almost completely in a few minutes, whereas they are very stable in acidic solution (pH < 4) (*30*). The pH of kombucha tea is below 4 due to the significant amounts of various organic acids (*4, 6*). In the present study, temperature was found to markedly influence the stability of studied compounds, even if they were in acidic conditions. Hence, the tea polyphenols that do not degrade due to acidic pH may degrade due to thermal processing and enzymes liberated by microorganisms in the kombucha tea during the storage period. Biotransformation of tea polyphenols present in kombucha tea to some other simpler molecules may be the possible explanation for the changes observed in contents of tea polyphenols in heat-treated kombucha black tea. On the basis of the cited references, it can be concluded that temperature and storage time have a greater effect on the content of tea polyphenols and its antioxidant activity. The protective effect of kombucha tea is mainly due to the activity of polyphenols, the compounds produced during the fermentation period, and the synergistic action among different potential compounds found in kombucha tea. Because the content of polyphenols and antioxidant activity of heat-processed kombucha was decreased, heat treatment may not be an appropriate method to destroy bacteria and yeasts in kombucha tea for commercialization. Nevertheless, besides polyphenols, there are many other bioactive compounds such as organic acids, vitamins, and biotransformed compounds as well as their synergistic effects, which may also be related to the protective effect of kombucha tea. The consumption of kombucha tea should provide an excellent means of increasing antioxidants in the diet.

The present study explained the influence of heat on biochemical constituents and free radical scavenging properties of kombucha tea. Heat treatment at 60, 65, and 68 °C for 1 min controlled biofilm formation in kombucha tea without changing its flavor characteristics. However, biochemical constituents of tea showed varying stability during the storage period. Free radical scavenging properties were also found to be decreased during the storage period. Because the biological activities of kombucha tea depended on the biochemical constituents, it can be concluded that heat treatment is not a suitable method for kombucha tea preservation.

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