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Optimization of Double Boiling Condition for Kelulut Honey Processing Using Response Surface Methodology

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This study was conducted to investigate the combined effects of temperature and time of double boiling treatment on the quality of Kelulut honey using Response Surface Methodology (RSM). Three-level factorial design employed nine runs with duplicates under different combination of temperature (30 – 60 °C) and time (10 – 60 min) was developed. Quality of Kelulut honey in this experiment was analyzed under six parameters which are physicochemical properties (pH, moisture content, total solids, and total soluble solids), and nutritional properties (brown pigment and antioxidant activity). Statistical analysis revealed that treatment variables give coefficient of determination, R2 in the range of 46.21 % to 83.16 % that indicates an adequate to strong correlation between the experimental and predicted response values. As treatment temperature increases, total solids and total soluble solids increase while moisture content decreases. In addition, treatment conditions give significant effect on brown pigment along with highest R^2 value (83.17 %). The optimal setting of the experimental factors has been determined by desirability approach at temperature of 60 °C with treatment time of 35 minutes. This study is currently a novel report on optimization of combined temperature and time of double boiling treatment which provides a new insight on Kelulut honey processing.

1. Introduction

The Trigona spp., known as ‘Kelulut’ is the stingless bee species found in Malaysia. In the recent years, Kelulut honey has been slowly introduced to the public in Malaysia as a nutritional super food that has a lot of health benefits. However, the biggest concern in production of Kelulut honey is maintaining the quality, especially physicochemical and nutritional properties when it is being treated. In conventional honey processing, the use of heat is found to be essential for fast handling and to dissolve large sugar granules at a temperature of more than 50 °C or even up to 77 °C (Subramanian et al., 2007). Recent studies have stated that the quality of the honey such as nutritional value, texture and taste is proved to be compromised when thermal treatment was applied (Fauzi and Farid, 2015). The quality of honey which are physicochemical properties (pH, moisture content, total solids, total soluble solids (TSS)), brown pigment and antioxidant activity were investigated. Physicochemical analysis of honey is crucial in the global trade since honey is used in an ever-increasing array of food products (Belay et al., 2015). pH is important during the extraction process as it affects the texture of honey, its stability as well as the shelf life (Rebiai et al., 2015). Equally important is moisture content as high moisture content may lead to undesirable honey fermentation (Khalil et al., 2012). The moisture content is correlated with total solids and total soluble solids hence strongly associated with quality of honey. Whereas, the formation of brown pigment that result in darkening of honey is associated with loss in quality hence affecting consumer’s acceptance. Adding to nutritional properties, antioxidant is one of the prominent characteristics of honey (Fauzi and Akhmazillah, 2014). Therefore, due to numerous crucial properties that can be detrimental by direct heat application, an alternative indirect thermal treatment that is double boiling treatment will be investigated in this research. Local beekeepers have informed that the use of double boiling treatment on Kelulut honey has been practiced widely. However, the understanding of the combined effect of temperature and time of double boiling treatment on Kelulut honey is very limited.
The Response Surface Methodology (RSM) is a faster and more efficient method for optimization of processes that provides information at less cost and in a shorter time (Myers and Montgomery, 1995). To the

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best of knowledge, there is no study has been conducted on optimization of combined factors of temperature and time treatment of double boiling on physicochemical and nutritional properties of Kelulut honey. Thus, the objective of this study is to investigate the combined effects of double boiling treatment condition (temperature and time) to achieve optimal results on the quality of Kelulut honey.

2. Experimental works

2.1 Sample preparation

The raw Kelulut honey samples of coconut (Cocos nucifera) were collected from a local beekeeper in Kampung Bintang, Batu Pahat, Johor. The samples were collected fresh from raisin pot, packed and sealed in a glass bottle and stored at 4 °C. The untreated Kelulut honey possesed a pH of 3.475 ± 0.021 and total soluble solids of 68.3 ± 0.141°Brix.

2.2 Double boiling treatment

Double boiling treatment was conducted using a double boiler that was set up conventionally by placing a 250 mL round bottom flask on top of a 500 mL beaker (Sigma-Aldrich, USA) that fit tightly as shown in Figure 1.

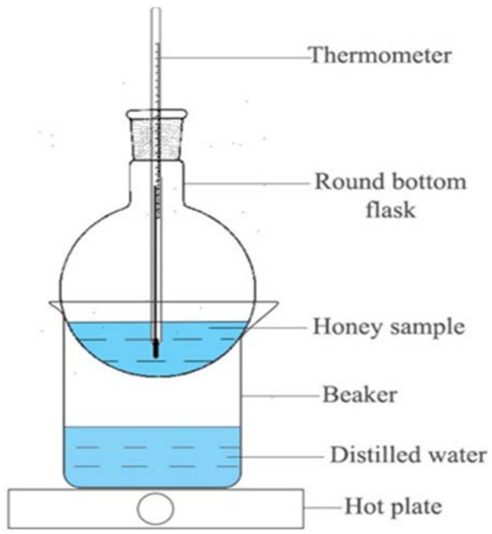

Figure 1: Schematic diagram of double boiler used in this study

Distilled water inside the beaker was let simmered on the hot plate before round bottom flask filled with Kelulut honey was placed on top of the beaker. The beaker was capped with aluminium foil to make sure there is no gap between the flask and the beaker. The simmering distilled water was made sure to not touch the bottom of round bottom flask to avoid direct heating to occur. Accordingly, the steam released by simmering water was completely trapped hence softly heat the samples. The honey samples were heated at different temperatures (30 – 60 °C) for different treatment time (10 – 60 min). The actual temperatures were obtained by measuring the thermometer readings (which was located in the center of honey samples inside the round bottom flask) during the treatments. Additionally, the hold times for the samples to reach targeted temperature were between 3 to 8 minutes. Once the treatment was completed, the sample was cooled in iced water to 20 °C.

2.3 Physicochemical properties analysis

The pH of honey samples was determined by diluting honey to 10 % (w/v) solution with milli Q water and measured using a pH meter (Eutech Instruments PH 700, USA) (Stefan, 2009). Refractive index of samples was measured using a RX-5000a digital refractometer (Atago®, Tokyo) at 20 °C and used to determine moisture content and soluble solids. Moisture content corresponding to the refractive index was calculated using Wedmore's table from International Honey Commission (Stefan, 2009) and total soluble solids (in °Brix) was calculated using sucrose conversion table (United State Department of Agriculture, 1981). Additionally, percentage of total solids was determined from measured moisture content. All measurements were carried out in duplicates.

2.4 Determination of brown pigment

Brown pigment was determined according to the (Turkmen et al., 2006). One gram of honey was dissolved in 5 mL distilled water and mixed using vortex-mixer. The solution was then centrifuged for 10 min at 10,000xg (Hermle Z 323 K, Germany). Once the supernatant was filtered through Whatman No. 1, it was precisely diluted to 4 °Brix with distilled water using a digital refractometer (Atago®, Tokyo). Lastly, brown pigment was determined by measuring absorbance at wavelength 420 nm using a spectrophotometer (Labomed Inc., USA). The experiment was carried out in duplicates.

2.5 Determination of antioxidant activity

The antioxidant activity was determined based on the ability of antioxidants to block the 2,2-diphenyl-1-picrylhydrazyl radical in the honey samples based on the method of (Rauter et al., 2012). Antioxidant activity was expressed as percentage inhibition of DPPH radical and determined by the following equation (Fauzi and Akhmazillah, 2014):

$$AA\ (\%)=\frac{Abs_{control}-Abs_{sample}}{Abs_{control}}\times 100 \quad (1)$$

where AA (%) is the antioxidant activity in percentage, $Abs_{control}$ is the absorbance of control while Abs_{sample} is the absorbance reading of the sample.

2.6 Experimental design

Response Surface Methodology (RSM) was implemented by using STATGRAPHICHS CENTURION XVII software (Statpoint Technologies, Inc., Virginia, USA) to determine the combined effect of two experimental factors on six quality parameters of Kelulut honey.The independent treatment variables were temperature, X1 (30 – 60 °C) and time, X2 (10 – 60 min). A three-level factorial design with 2 factors (32 design) for optimization of process variables consisting of nine treatment combinations was employed. All experiments were conducted at duplicates. The range and levels of independent variables and code values were presented in Table 1. Experimental data was analyzed by multiple regressions using the least-squares method. A second-order polynomial equation was fitted to data which is given below:

$$Y= \beta_0+ (\beta_1X_1)+ (\beta_2X_2) + \beta_{11}X^2{}_1+\beta_{22}X2_2+\beta_{12}X_1X_2 \quad (2)$$

where Y is the response calculated by the model, β_0 is a constant, and β_i, β_{ii}, and β_{ij} are linear, squared and interaction coefficients, respectively. Significance was judged by determining the probability level that the F-statistic calculated from the data is less than 5 % by using ANOVA. The adequacy of the model was checked by R^2 and Fisher's test ratio (F-value) obtained from the analysis of variance (ANOVA). Optimization of double boiling temperature and time was conducted using desirability approach by multi-response analysis of the response surface design using STATGRAPHICS statistical software.

Table 1: Two-factor, three levels full factorial design used for RSM and experimental data of the investigated responses of double boiling treatment

Standard Order	Temperature, °C (X_1)	Time, min (X_2)	pH	Moisture Content (%)	Total Solids (%)	Total Soluble Solids	Brown Pigment	Antioxidant Activity (%)
1	30 (0)	35 (1)	3.39	29.46	70.54	69.10	0.07	61.03
2	60 (2)	10 (0)	3.43	29.02	70.98	69.60	0.09	35.94
3	30 (0)	10 (0)	3.45	30.00	70.00	68.60	0.07	60.59
4	60 (2)	35 (1)	3.46	27.86	72.14	70.75	0.08	65.44
5	45 (1)	35 (1)	3.40	28.48	71.52	70.15	0.09	28.07
6	60 (2)	60 (2)	3.43	29.30	70.70	69.30	0.08	34.46
7	45 (1)	60 (2)	3.42	28.32	71.68	70.30	0.08	56.32
8	45 (1)	10 (0)	3.39	29.08	70.92	69.50	0.08	28.74
9	30 (0)	60 (2)	3.43	29.38	70.62	69.25	0.08	61.03

3. Results and discussion

3.1 Statistical analysis of results obtained by experimental design

The physicochemical and nutritional properties of Kelulut honey was determined by the combined effect of two treatment variables that were temperature and time. The combination treatments of independent variables were followed as in Table 1. The effects of independent variables on each of the responses are presented in Table 2. Predicted and experimental values for each of the responses are presented in Table 3.

Table 2: Multifactor ANOVA on the effects of independent variables (X_1: temperature, X_2: time) on dependent variable responses

Source	pH		Moisture Content (%)		Total Solids (%)		TSS (°Brix)		Brown Pigment		Antioxidant Activity	
	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value
X_1	0.35	0.59	5.62	0.09	5.62	0.10	5.54	0.10	12.00	0.04	1.15	0.36
X_2	0.00	0.95	0.96	0.39	0.96	0.40	1.01	0.39	0.37	0.59	0.37	0.59
X_1*X_1	1.99	0.25	2.81	0.19	2.81	0.19	2.76	0.20	0.81	0.43	1.48	0.31
X_1*X_2	0.16	0.71	0.96	0.39	0.96	0.40	1.03	0.39	1.62	0.29	0.00	0.96
X_2*X_2	0.07	0.80	3.24	0.16	3.24	0.16	3.02	0.18	0.02	0.89	0.18	0.70

R_2 values: 0.4621 (pH), 0.8193 (moisture content), 0.8193 (total solids), 0.8165 (total soluble solids), 0.8317 (brown pigment) and 0.5146 (antioxidant activity).

Table 3 Predicted (Pred.) and experimental (Exp.) values of pH, moisture content, total solids, total soluble solids, brown pigment and antioxidant activity of double-boiled Kelulut honey. Values are means of duplicates.

Exp no.	pH		Moisture content (%)		Total Solids (%)		Total Soluble Solids (°Brix)		Brown Pigment		Antioxidant Activity (%)	
	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
1	3.42	3.42	28.32	28.81	71.68	71.19	70.30	69.81	0.08	0.08	56.32	52.38
2	3.43	3.41	29.38	29.25	70.62	70.75	69.25	69.36	0.08	0.07	61.03	60.18
3	3.39	3.42	29.08	29.17	70.92	70.83	69.50	69.43	0.08	0.08	28.75	43.54
4	3.46	3.43	27.86	28.55	72.14	71.45	70.75	70.07	0.08	0.09	65.44	40.16
5	3.45	3.41	30.00	29.62	70.00	70.38	68.60	68.98	0.07	0.07	60.59	51.34
6	3.43	3.43	29.30	28.36	70.70	71.64	69.30	70.26	0.08	0.08	34.46	44.58
7	3.39	3.41	29.46	29.43	70.54	70.57	69.10	69.17	0.07	0.07	61.03	55.76
8	3.43	3.43	29.02	28.73	70.98	71.27	69.60	69.88	0.09	0.09	35.94	35.74
9	3.40	3.42	28.48	28.99	71.52	71.01	70.15	69.62	0.09	0.08	28.07	47.96

3.2 Effects of treatment variables on physicochemical properties of Kelulut honey

The R^2 value for the variation in pH was adequate at 0.4621. Additionally, the p-values for all interactions of factors are more than 0.05, hence temperature and time of double boiling treatments did not significantly affect the pH values. The pH values obtained were in the range of 3.39 to 3.46 in which the values are low enough to inhibit the growth of most bacteria as they grow optimally at pH between 7.2 and 7.4 (Vandamme et al., 2013). For moisture content, there were no significant influences at any linear and quadratic factors. However, from the data obtained, F-value of X_1 was at the highest while F-value for X_2 was at the lowest proving that treatment time scarcely affects moisture content of Kelulut honey. The R^2 value for moisture content was satisfactory at 0.8193 indicating a strong relationship between factors and response. Generally, floral source has been linked to moisture content of honey, although climatic conditions, soil, collection period and processing aspects may also strongly affect this parameter (de Sousa et al., 2016). Therefore, the high moisture content of Kelulut honey may be resulted from stingless bees feeding on multifloral sources. The results for moisture content were similar for total solids as both are correspond to each other. As for TSS, there are no significant effects of linear factors, nor quadratic factors. However, the fit of model was convinced with a satisfactory R^2 of 0.8165. The TSS maximized as the temperature increased. Maximum TSS was obtained at highest temperature (60 °C) with time treatment of 35 min. The range of TSS obtained is slightly lower than Manuka honey (79.67 ± 0.50 °Brix) (Akhmazillah Fauzi and Mehdi Farid, 2016). It has been reported that a high negative correlation existed between TSS and moisture content in honey (Anupama et al., 2003). Therefore, the lower TSS of Kelulut honey may due to its higher moisture content.

3.3 Effects of treatment variables on nutritional properties of Kelulut honey

There was a significant influence of treatment temperature on brown pigment but not for X_2 and all quadratic factors. A satisfactory R^2 value was obtained proposing a strong relationship between factors and the response. Additionally, the brown pigment was highest as the time increased but at lowest temperature (60 °C, 10 min) while the lowest brown pigment was obtained at lowest temperature with same time treatment (30 °C, 10 min). This indicates that temperature strongly affected the brown pigment of Kelulut honey but not treatment time. From the results, there were no significant effects of linear and quadratic factors observed. However, R^2 value of 0.5146 showed an adequate strength on relationship of treatment temperature and time to antioxidant activity of Kelulut honey after double boiling. The highest antioxidant activity was observed when the treatment temperature was set at 60 °C for 35 min. The big range (28.07 ± 0.06 to 65.44 ± 3.12) gained proved that processing of honey strongly affected the antioxidant activity of Kelulut honey. Antioxidant activity has strong correlation with physical properties (pH, color, electrical conductivity and TSS) of honey, hence resulting a higher antioxidant activity range from recorded by (Hussein et al., 2011) (22.40 to 41.30 %).

3.4 Optimization

Optimum conditions of double boiling treatment on enhancing the physicochemical and nutritional properties of Kelulut honey were slightly different for each parameter. Few combinations of double boiling conditions resulting in best results for moisture content, total solids, TSS and antioxidant activity while still achieving good pH and brown pigment. As a result, optimum double boiling treatment condition counting all parameters in was depicted in Figure 2. Desirability approach from multi-response analysis of response surface design was used to optimize double boiling temperature and time. Multiple response optimization indicates that physicochemical and nutritional properties of double-boiled Kelulut honey may be optimized by treatment temperature at 60 °C for 34.69 min. This is a promising finding as the treatment time is short and the temperature is lower than boiling temperature. The response predicted value when optimum double boiling condition was applied were 3.43, 28.34 %, 71.66 %, 70.27 °Brix for pH, moisture content, total solids and TSS respectively. Additionally, for nutritional properties, the predicted value for brown pigment was 0.084 while antioxidant activity was 48.78 %. A validation experiment was conducted by applying double boiling treatment on Kelulut honey at the optimized condition. The results for physicochemical properties obtained were 3.38, 29.66 %, 70.34 %, and 68.95 °Brix for pH, moisture content, total solids and TSS respectively. Brown pigment of treated Kelulut honey was obtained at 0.102 and an antioxidant activity of 38.06 %. The validation hence indicated a satisfying result and closely to the predicted values.

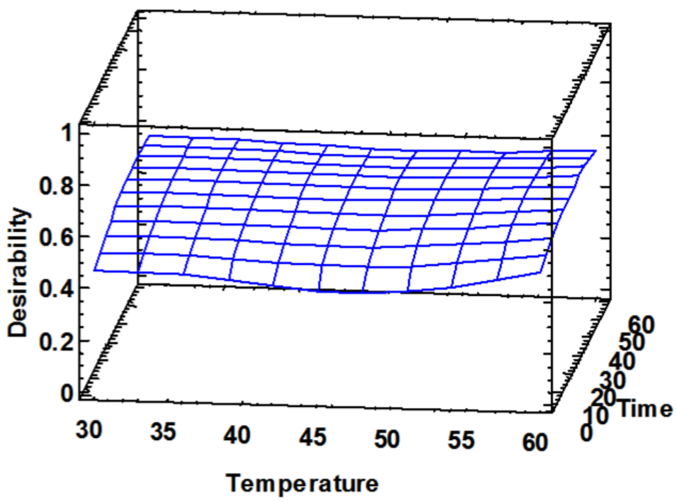

Figure 2: Response surface plot showing optimized effect of double boiling temperature (°C) and time (min) to maximize both physicochemical and nutritional properties of Kelulut honey

4. Conclusions

The effects of double boiling treatment temperature and time on physicochemical (pH, moisture content, total solids, TSS) and nutritional (brown pigment and antioxidant activity) properties were determined by response surface methodology. Moisture content was found the lowest at 27.86 % with the highest total solids, TSS and antioxidant activity at 72.14 %, 70.75 °Brix and 65.44 % respectively when the double boiling treatment was conducted at 60 °C for 35 min.

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