Effect of thermal treatment on the biochemical composition of tropical honey samples

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Abstract

Honey is usually subjected to filtration and heating for bottling before commercialization. However, there is no standard procedure available for thermal treatment on honey. Honey is thermally heated at various temperature and duration based on individual experience to prolong the shelf life of honey in the market. The heating methods might decrease the biochemical components such as nutrients, enzymatic activities and vitamins to certain extent. In addition to water reduction, thermal treatment on sugar rich honey usually accompanied by the formation of 5-hydroxymethylfurfural (HMF). In the present study, the biochemical components in three commonly consumed honey in Malaysia, namely tualang, gelam and acacia honey were investigated before and after thermal treatment at 90°C for 30 min. The short period of heating time was found to degrade nutrients, enzymatic activities and water soluble vitamins in honey. The degradation of protein and enzyme via proteolytic digestion had attributed to the increase of free amino acids in honey. Based on the multivariate analysis, the most thermally affected biochemical components are crude fat, panthotenic acid (Vitamin B5) and diastase activity which explain for 86.4% of the total variance. The kinetic studies on the HMF formation revealed that the honey samples followed zero order kinetic model for the first 60 min of heating at 90°C. The findings indicate that the temperature and duration of heating during honey processing is essential to be investigated according to the honey origin. The initial biochemical composition of honey would affect the kinetic profile of HMF formation.

Introduction

Raw honey after harvesting is usually strained and filtered to remove suspended materials including pollen and bee wax prior to heating. Honey is thermally treated before packaging for several reasons. Heating not only eases the processing of bottling by reducing the viscosity of honey (Anklam, 1998), but also reduces the water content in honey to prevent fermentation (Subramanian et al., 2007), dissolves the sugar crystal nuclei to retard granulation (Turhan et al., 2008; Escriche et al., 2009), homogenizes honey colour for the preference of consumers (Abu-Jdayil et al., 2002) and destroys the sugar tolerant osmophilic yeasts to prolong the shelf life of honey (Guo et al., 2011). Heating is the utmost important processing step in honey production because it directly affects the quality of honey.

Even though heating is of great important, no guideline is available till to date for the use of heating temperature and time for a particular type of honey. It is believed that the optimum heating conditions are mainly relied on the geographical and botanical origins of honey. Different origins of honey vary in their biochemical composition. A wide range of heating temperatures ranging from 30 to 140°C for a few seconds up to several hours had been practiced by honey producers worldwide, with the aim to reduce the water content in honey below 20% for shelf life prolongation (Wakhle et al., 1996; Turhan et al., 2008; Guo et al., 2011).

While measuring the water content in honey, studies were also focused on the hydroxymethylfurfural (HMF) content and its enzymatic activity before and after thermal treatment. This is because both HMF content and enzymatic activity are the indicators for honey freshness (Subramanian et al., 2007). The HMF content will increase, whereas the diastase activity of honey will decrease for over-heated, aged and poorly stored honey samples. However, the extent of thermal effects on these biochemical components including nutrients and vitamins is relatively limited in the literatures, particularly for honey samples from tropical country like Malaysia.

The objective of this study was to investigate the effects of thermal treatment on the biochemical components of honey samples such as tualang, gelam and acacia honey in Malaysia. In the present study, the two recommended heating temperatures such as 63°C (National Honey Board, 2013) and 90°C (Kowalski...
et al., 2012) were applied to reduce the water content of honey samples. Besides water reduction, the alteration of the other biochemical components such as nutritional content, various enzyme activities, HMF content, free amino acids and water soluble vitamins in honey were also measured after thermal treatment. The kinetic profile of the HMF formation resulted from the thermal treatment were also investigated in this study.

Materials and Methods

Honey samples and chemicals

Honey samples such as tualang (T), gelam (G), acacia (A) honey were harvested between the month of April and May 2011 from suppliers. Tualang and gelam honey samples were purchased from Federal Agriculture Marketing Authority (FAMA), Kedah, whereas acacia honey sample was supplied by MB An-Nur Apiary, Johor. They were stored in amber glass bottles at 20°C before analysis. The experimental works were completed within two months after sample receipt. Thiamine (vitamin B1, ≥99%), Riboflavin (vitamin B2, ≥98%), Nicotinic acid (vitamin B3, ≥99.5%), pantothenic acid (vitamin B5), folic acid (vitamin B9, ≥97%), cyanocobalamin (vitamin B12, ≥98.5%), ascorbic acid (vitamin C, ≥99%) and 5-hydroxymethylfurfural were sourced from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade of methanol (MeOH), acetonitrile (CH$_3$CN), acetic acid (100%) and formic acid (98-100%) were obtained from Merck (Darmstadt, Germany).

Thermal treatment on honey samples

Honey samples were thermally treated by two conventional methods, namely at 63 and 90°C for 60 min. The former method is a standard pasteurization procedure that widely applied in food industries (National Honey Board, 2013), while the latter method is based on the maximum temperature allowed to prevent deterioration of honey quality (Kowalski et al., 2012). The honey samples (10 g) were placed in test tubes and heated in a water bath without stirring. At an interval of 15 min, sample was drawn and cooled to room temperature for moisture content analysis based on the standard method from the Association of Official Analytical Chemists (AOAC) Official Method 969.38, 2000 using a handheld honey refractometer (Atago, Tokyo, Japan). Each experiment was carried out in triplicate. Since the thermal treatment at 90°C for 30 min could reduce the moisture content of honey samples to less than 20% (w/w), this method was used in the subsequent analysis.

Nutritional analysis

The honey samples with and without heat treatment were analyzed for their nutritional composition such as fat (AOAC method 14.019, 1984), protein (AOAC method 991.20, 2005) and ash (AOAC method 923.03, 1998). Dietary fiber was assumed zero in honey. Total sugar and energy were estimated based on Eq. (1) and (2), respectively (Charrondiere et al., 2004). All experiments were carried out in triplicate.

\[
\text{Total carbohydrate} (\%) = 100 - (\text{water} + \text{ash} + \text{protein} + \text{fat} + \text{dietary fiber})
\]

\[
\text{Energy} (\text{kcal/g}) = 9 \times (\text{fat}) + 4 \times (\text{protein}) + 4 \times (\text{carbohydrate})
\]

Enzyme assay

Glucose oxidase activity by peroxide test

The glucose oxidase activity of honey samples was screened for peroxide accumulation using Merckoquant test strip (no.10011) from Merck, Germany as described by Kerkvliet (1996). Results are expressed in milligram of hydrogen peroxide accumulation in a liter of sample solution for an hour at 20°C.

Diastase activity by Phadebas

The diastase activity (DN) of honey samples was determined based on the method described in the International Honey Commission (IHC, 2009) by using Phadebas tablets (Pharmacia Diagnostics, Sweden). Results are the mean value of triplicate absorbance measurement at 620 nm spectrophotometrically ($A_{620}$). One DN is expressed as a diastase unit per gram of honey as presented in Eq. (3).

\[
\text{DN} (\text{units/g}) = 35.2 \times A_{620} - 0.46
\]

Invertase activity by EIVT-100

The invertase activity of honey samples was determined by using the EnzyChrom™ Invertase Assay Kit (EIVT-100, BioAssay Systems, USA). Enzyme Mix consisted of invertase and sucrase was used to hydrolyze sucrose into fructose and glucose at 30°C for 20 min. The absorbance was spectrophotometrically measured at 570 nm. The slope of the glucose calibration curve (0-100 µM) was used for the calculation of invertase activity as shown in Eq. (4). One unit of invertase activity is equal to the formation of 1 µmole glucose per minute.
Invertase activity = \frac{R_{\text{sample}} - R_{\text{control}}}{t} (U/L) \quad (4)

Where,
R_{\text{sample}}: \text{Absorbance of sample}
R_{\text{control}}: \text{Absorbance of buffer solution}
t: \text{Incubation time (20 minutes)}

**Determination of 5-hydroxymethylfurfural in honey**

The concentration of 5-hydroxymethylfurfural (5-HMF) before and after thermal treatment was determined by using a high performance liquid chromatography system (Waters 2690 HPLC, Milford, MA) integrated with a photodiode array detector (Waters 996 PDA, Milford, MA). The sample preparation was based on the method described by Bogdanov (2002) with minor modification. Heat-treated honey sample (2.5 g) was dissolved in 100 mL of water and filtered (0.45 µm) before injection. A C18 Synergy column (Synergy 4U fusion-RP, 150 x 4.60 mm, 4 µm) from Phenomenex (Torrance, CA) was used for the separation. The mobile phase, 10%(v/v) methanol with 1% acetic acid was flowed (1 mL/min) at isocratic condition. The injection volume was 20 µL. A range of standard 5-HMF solution (0-0.1 mg/mL) was used for calibration. The chromatogram was monitored at 285 nm. The results were expressed in milligram per kilogram of honey (mg/kg).

**Determination of water soluble vitamins in honey**

Water soluble vitamins in honey samples were determined by using the method reported by Ciulu et al. (2011) with minor modification. Four water soluble vitamins (WSVs) such as B2 (riboflavin), B3 (niacinamide), B5 (panthotenic acid) and vitamin C (ascorbic acid) was quantified by using an hyphenated system (UPLC-MS/MS) consisted of ultra-high performance liquid chromatography (Waters Acquity UPLC; Milford, MA) integrated with a triple quadrupole and linear-ion trap mass spectrometer (AB SCIEX 4000 QTRAP; Foster City, CA).

A C18 reversed phase Acquity column (4.6 x 100 mm, 1.7 µm) was used for the separation. The mobile phase was a binary solvent system consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile). The UPLC gradient was: 0–5 min, 10% B; 5–15 min, 10–90% B; 15–20 min, 90% B; 20–25 min, 90–10% B; 25–30 min, 10% B for final washing and equilibration of the column for the next run. The strong wash and weak wash solution were 10 and 50% of acetonitrile, respectively. The flow rate was 0.15 mL/min and the injection volume was 5 µL.

The mass spectra were acquired using multiple reactions monitoring (MRM) in positive ionization mode. Low energy collision-induced dissociation (CID) was used to confirm the characteristic ion of metabolites in MRM mode. The capillary and voltage of ion source were maintained at 400°C and 4.5 kV, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulization, 40 psi; for drying solvent, 40 psi; curtain gas, 10 psi; collision gas, high; declustering potential, 40 V, and collision exit energy, 10 V. The scan rate was 1000 amu/s.

A serial of standard solution consisted of a mixture of WSVs (0-1 ppm) was prepared by diluting the stock solution. The stock solution was freshly prepared by dissolving 2.5 mg of each vitamin into buffer solution consisted of 1 ml of ammonium acetate (2M) and 12.5 ml of phosphate buffer (1M, pH 5.5). The stock solution was then topped up to the mark with water in a 25 mL of volumetric flask. Honey sample (2 g) was weighed and dissolved in 2 ml of water. A 0.2 ml of ammonium acetate (2M) and 2.5 ml of phosphate buffer (1M, pH 5.5) were added into the honey solution and then topped up to 5ml with water. Honey sample was filtered (0.22 µm) before injection to UPLC-MS/MS.

**Determination of free amino acids in honey**

The similar analytical system, UPLC-MS/MS was used to determine the composition of free amino acids in honey samples under similar operating conditions otherwise stated. The sample preparation was carried out based on the method described by Nimbalakar et al. (2012) with minor modification. A mixture of amino acid standard (AA-S-18, Sigma-Aldrich, Missouri) was used to build the calibration curve (0.2-1.0 mg/L). Honey sample (1 g) was weighed and dissolved in 5 ml of 20% (v/v) methanol with 0.1% (v/v) formic acid. The honey solution was vortexed for 10 minutes and followed by centrifugation at 10,000 rpm for 20 minutes. The supernatant was filtered through 0.2 µm nylon membrane filter before injection. The mobile phases consisted of water with 0.1% (v/v) formic acid (Solvent A) and 50% methanol with 0.1% (v/v) formic acid (Solvent B) at a flow rate of 0.15 mL/min. The gradient was; 0-1 min, 2% B; 1-10 min, 2-80% B; 10-12 min, 80% B; 12-13 min, 80-2% B and 13-18 min, 2% B for equilibration.

**Results and Discussion**

Thermal treatment was applied to the honey samples at two different temperatures; 63 and 90°C at pH 4.5 under the stipulated assay conditions.
Table 1. Moisture content of honey samples before and after treatment at 63 and 90°C

<table>
<thead>
<tr>
<th>Honey</th>
<th>Moisture (%) at 63°C (±SD)</th>
<th>Moisture (%) at 90°C (±SD)</th>
<th>Equation (90°C)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tualang</td>
<td>28.17 ± 0.09</td>
<td>24.20 ± 0.20</td>
<td>y = 0.112x + 27.56</td>
<td>0.981</td>
</tr>
<tr>
<td>Gelam</td>
<td>28.49 ± 0.21</td>
<td>20.03 ± 0.20</td>
<td>y = 0.141x + 25.36</td>
<td>0.972</td>
</tr>
<tr>
<td>Acacia</td>
<td>21.36 ± 1.83</td>
<td>19.30 ± 0.10</td>
<td>y = 0.127x + 25.22</td>
<td>0.917</td>
</tr>
</tbody>
</table>

**Different superscript in the same row showed significant difference (p < 0.05)**

Table 2. Nutritional components, enzyme activities and water soluble vitamins in honey samples before and after thermal treatment (90°C for 30 min)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Honey</th>
<th>Raw</th>
<th>Gelam</th>
<th>Acacia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/g)</td>
<td>6.78 (±0.92)</td>
<td>6.84 (±0.41)</td>
<td>7.86 (±0.23)</td>
<td>7.95 (±0.19)</td>
</tr>
<tr>
<td>Ash (±SD)</td>
<td>0.23 (±0.06)</td>
<td>0.22 (±0.08)</td>
<td>0.30 (±0.06)</td>
<td>0.35 (±0.09)</td>
</tr>
<tr>
<td>Total Carbohydrate (±SD)</td>
<td>72.31 (±6.25)</td>
<td>71.56 (±5.75)</td>
<td>75.56 (±5.15)</td>
<td>82.16 (±0.75)</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>286.64 (±9.78)</td>
<td>256.12 (±4.98)</td>
<td>206.84 (±2.75)</td>
<td>310.86 (±0.18)</td>
</tr>
<tr>
<td>Glucose oxidase (±SD)</td>
<td>5.34 (±0.25)</td>
<td>5.39 (±0.25)</td>
<td>5.47 (±0.25)</td>
<td>5.51 (±0.25)</td>
</tr>
<tr>
<td>Invertase (±SD)</td>
<td>3.03 (±0.13)</td>
<td>3.04 (±0.13)</td>
<td>3.06 (±0.13)</td>
<td>3.08 (±0.13)</td>
</tr>
<tr>
<td>Riboflavin (µg/g)</td>
<td>2.57 (±0.53)</td>
<td>2.59 (±0.53)</td>
<td>2.61 (±0.53)</td>
<td>2.63 (±0.53)</td>
</tr>
<tr>
<td>Nicotinic acid (±SD)</td>
<td>0.14 (±0.03)</td>
<td>0.15 (±0.03)</td>
<td>0.16 (±0.03)</td>
<td>0.17 (±0.03)</td>
</tr>
<tr>
<td>Pantothenic acid (±SD)</td>
<td>0.26 (±0.05)</td>
<td>0.28 (±0.05)</td>
<td>0.30 (±0.05)</td>
<td>0.32 (±0.05)</td>
</tr>
<tr>
<td>Ascorbic acid (±SD)</td>
<td>0.24 (±0.04)</td>
<td>0.25 (±0.04)</td>
<td>0.26 (±0.04)</td>
<td>0.27 (±0.04)</td>
</tr>
</tbody>
</table>

**Different superscript in the same row showed significant difference (p < 0.05)**

Table 3. Zero-order kinetic equations and their correlation coefficient for HMF formation

<table>
<thead>
<tr>
<th>Honey</th>
<th>Initial HMF (mg/g)</th>
<th>Equation</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tualang</td>
<td>0.92 (±0.14)</td>
<td>y = 0.44x + 39.64</td>
<td>0.98</td>
</tr>
<tr>
<td>Gelam</td>
<td>1.36 (±0.24)</td>
<td>y = 0.31x + 27.56</td>
<td>0.96</td>
</tr>
<tr>
<td>Acacia</td>
<td>0.32 (±0.08)</td>
<td>y = 0.18x + 19.30</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**Different superscript in the same row showed significant difference (p < 0.05)**

SD: Standard deviation
d: not detected

Figure 1. Schematic diagram of HMF formation in honey.

(a) is a process of acid catalyzed dehydration

oxidase was detected using Merckquant test strip (Table 2). This kind of test strip might be not sensitive enough to detect the low concentration of glucose oxidase in honey samples. Both diastase and invertase activities are commonly used in Europe as an indicator for honey freshness (Manzanares et al., 2011). This is because the enzyme activities decrease in heated or aged honey. A slight decrease in the enzyme activities was observed because of the denaturation of proteinaceous enzyme (Ozcan et al., 2006) and decrease of water content (Gomes et al., 2010) resulted from heating. It is believed that certain level of water is required for the exhibition of enzyme activity. Protein denaturation involves a conformational change from the native and folded state to the denatured and unfolded state accompanied by an endothermic heat effect (Al-Malah et al., 1995).

In line with the honey colour, the initial HMF content was the highest in Gelam honey sample, followed by Acacia and Tualang honey samples. The HMF content appeared to be proportionally increased with the increase of heating time up to 60 minutes. The formation of HMF upon heating followed the zero-order kinetic model. It was found that the kinetic constant of Gelam honey sample was the highest, which was about four and six times higher than Acacia and Tualang honey samples, respectively (Table 3). From the table, the higher initial HMF content, the faster rate of HMF formation is. HMF was produced as a result of the action honey acidity on hexoses, and further accelerated at high temperature during processing (Coco et al., 1996). This acid-catalyzed dehydration was reported to be faster from fructose than from glucose. Fructose is likely to enolize rapidly as presented in Figure 1 (Lee and Nagy, 1990). The results indicated that different honey showed different kinetic profile of HMF formation. As reported by Abu-Jdyil et al. (2002), dark-colored honey tend to be affected by heat faster than light-colored honey. A higher number of honey samples are required to establish the kinetic model of HMF formation for local honey.

Although honey is not a vitamin rich food, the variance of vitamin concentration indicates the...
extent of its freshness resulted from either treatment or storage. The effect of thermal treatment on water soluble vitamins such as vitamin B2, B3, B5, and C were investigated because vitamins are sensitive to heat (Table 2). All honey samples had almost similar vitamin C concentration, approximately 20 mg/kg, but no vitamin B3 was detected. The results showed that the concentration of vitamin C was only reduced for about 11-14% after heated at 90°C for 30 min. A short period of heating time (30 min) did not degrade vitamin C significantly even at high temperature, 90°C. This observation might be contributed by high sugar content of honey as a stabilizer to prevent degradation of vitamin. However, this explanation was not applicable to vitamin B2 and B5 because they were degraded more than 50%, except riboflavin in acacia honey.

Interestingly, certain free amino acids were increased after thermal treatment. Phenylalanine, proline, tyrosine and valine were the most abundant amino acids in all honey samples, covering for about 66-82% of the total free amino acid. The finding was in line with the results of Conte et al. (1998) who reported that phenylalanine and proline were the major amino acids in floral honey. Proline has been identified as the most important biochemical component in honey. It is mainly from honey bees because proline is also present in sugar-fed colonies (Ohe von der et al., 1991). A higher value of proline is typical for honeydew honey as reported by Baroni et al. (2009). The concentration of free amino acids which were increased after thermal treatment was found to be higher in floral honey such as tualang and gelam honey. The increase most probably was due to the degradation of protein in honey. The degradation of protein was approximately contributed to 0.01-0.04% of the increase in total free amino acid. On the other hand, the decrease in protein content was noticed in proximate analysis, which was about 35% of reduction for both tualang and gelam honey, but only 10% of reduction in acacia honey.

An unsupervised multivariate data analysis approach such as principle component analysis was used to analyze the biochemical components statistically. The results showed that the first three biochemical components that most significantly reduced after thermal treatment were crude fat, panthotenic acid and diastase as presented in Figure 2(a). These three biochemical components are located at the positive region of the score plot. However, free amino acids are located at the negative region of the plot. This was because the concentration of most of the free amino acids was increased after thermal treatment resulted from the degradation of protein. Tyrosine and valine are the most affected free amino acids after thermal treatment as they are located at the highest score of the negative region. On the other hand, the loading plot indicates that there is relatively small in the difference of biochemical composition for honey samples in the first principle component (PC1). The difference appears to be greater at the second principle component (PC2) to distinguish between floral honey (tualang and gelam) and honeydew honey (acacia) as presented in Figure 2(b).

**Conclusion**

As a conclusion, thermal treatment would degrade biochemical components in honey to certain extent depending upon its initial biochemical composition. This study found that crude fat (66.7-100.0 %), panthotenic acid (18.0-93.6%) and diastase (43.1-66.7%) were the most affected biochemical components by thermal treatment at 90°C for 30 min. The thermal treatment had degraded certain percentage of nutritional content, water soluble vitamins, and enzymatic activities and simultaneously increased in HMF content (12.8-38.4%). Both HMF and diastase activity are the international parameters used to control the limit for thermal treatment to honey.

**Reference**


