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Correlation of Trehalulose Content with Physicochemical Parameters in Adulterated Kelulut Honey †

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Abstract: Kelulut (stingless bee) honey (KH) possesses a wide range of benefits for human consumption and can exhibit medical effects. Due to its high value, this premium honey is often adulterated with different types of cheaper sugars, causing low nutrients and potential food safety threats in the final product. This study aims to determine the correlation between trehalulose content of sugar-based adulterated KH from the stingless bee species *Heterotrigona itama* with the physicochemical properties. Adulterated samples were prepared using pure honey mixed with different concentrations of high fructose corn syrup (HFCS), i.e., 10%, 20%, 30%, 40%, and 50%. Water activity, colour, total soluble solids, pH, turbidity, and viscosity of KH were determined. In addition, the primary sugar composition (fructose, glucose, and trehalulose) was determined by high-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELSD). This study shows that the increasing percentage of HFCS addition in the KH samples significantly increases ($p < 0.05$) the total soluble solids, colour, pH, turbidity, viscosity, glucose and fructose content; meanwhile, the water activity and trehalulose were reduced significantly ($p < 0.05$). Pearson's correlation analysis demonstrated that trehalulose content in adulterated KH has strong positive correlation with water activity and strong negative correlation with glucose, fructose, total soluble solids, pH, and colour. In conclusion, the physicochemical properties of HFCS adulterated KH and authentic KH can be differentiated. This data is vital for the governing bodies to ensure that KH sold in the markets is free from HFCS adulteration.

Keywords: Sugar adulteration; Kelulut stingless bee honey; trehalulose; high fructose corn syrup; physicochemical properties; *Heterotrigona itama*

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1. Introduction

In Malaysia, stingless bees of Trigona spp. produce multiflora honey or well-known as Kelulut honey (KH), named after the bees producing it, commonly called Kelulut [1]. KH is more diluted than other honey and is famous for its unique flavour with sour-like taste and smell [2]. Research showed that honey from *Heterotrigona itama* has shown excellent antioxidant properties and possesses high mineral content [3]. Traditional medicine practitioners have used KH due to the beliefs on its medicinal usefulness and value [4]. According to current regulations, no other compounds or additives can be added to honey sold as authentic honey [5]. However, honey added with inexpensive sweeteners like maltose syrup and fructose syrup are the most typical forms of honey adulteration practiced by irresponsible sellers [6]. These adulteration process lowers its nutritional value and consequently gives adverse effects to human health [7].

Adulteration of authentic honey with sugar may also give changes to the physicochemical properties of KH. The recent discovery of trehalulose as a distinctive biologically active disaccharide in KH, specifically from species *Geniotrigona thoracica* and *Heterotrigona itama* is considered a potential marker to determine its authenticity [8]. Similarly, authentication of Manuka honey also uses specific marker compounds [9]. Adulteration of honey with products containing disaccharides can mimic the primary carbohydrate profile of honey [10]. This research was conducted to differentiate the physicochemical properties of authentic KH with HFCS adulterated KH stingless bee species, *Heterotrigona itama.*

2. Materials and Method

2.1. Procurement and Preparation of Sample

Raw KH *(Heterotrigona itama)* was harvested and purchased from a beekeeper in Bahoney Farm, Kampung Rinching Hilir, Kajang, Selangor, Malaysia. The sample was collected using sterilised, dried, and sealed Schott glass bottles and stored in a refrigerator with a temperature range between 0 °C to 5 °C. High fructose corn syrup was purchased from a local supermarket in Kuala Lumpur and stored at room temperature. To prepare adulterated samples, authentic KH was mixed with different concentrations of high fructose corn syrups, i.e., 0%, 10%, 20%, 30%, 40% and 50% (*w*/*w*).

2.2. Physicochemical Analysis

All physicochemical properties were analysed according to Official Methods of Analysis [11], Malaysia Standard, MS 2683: 2017: Kelulut (Stingless bee) honey—Specification [12] and harmonised methods of the International Honey Commission [13]. All measurements were carried out in triplicate (n = 2 × 3).

2.2.1. HPLC-ELSD Analysis

High-performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) was used to determine the composition of fructose, glucose and trehalulose in the samples according to Malaysian Standard, MS 2683: 2017 [11] with modification.

2.2.2. Water Activity (a_w), Total Soluble Solids, pH, Colour and Turbidity

A small aliquot of honey (1 g) sample was used to determine the water activity by using an Aqualab water activity meter (Decagon, Pullman, WA) at 20 °C.

The total soluble solid of the sample was analysed using a handheld manual refractometer (Master-3M, ATAGO, Japan) at 25 °C.

Each sample with a concentration of 10% (w/v) in distilled water was analysed with a pH meter at a temperature of 25 °C [1].

The colour of the honey was analysed using a chromameter (Konica Minolta Chroma Meter, model CR-400) by measuring the values of *L**, *a**, and *b**.

The turbidity of the samples was measured with a portable turbidimeter (Orbeco-Hellige Model 966), and the reading was stated in Nephelometric Turbidity Unit (NTU).

2.2.3. Viscosity

The viscosity of the samples was determined by using a rheometer (AR G2 rheometer, UK) using a parallel plate with a diameter of 40 mm for every sample. The gap between the plates was set to 0.5 mm. All analyses were conducted at 25 °C with the range of shear rate set from 0.1 to 100 s^{-1}. The apparent viscosity was determined as a function of shear rate.

2.3. Statistical Analysis

All the experiments were performed in triplicates except for sugar analysis in duplicates. The data were analysed using the computational software of Minitab 19 (Minitab Ltd., Coventry, UK) and Microsoft Excel (USA). One-way analysis of variance (ANOVA), Tukey Pairwise Comparison and Pearson's Correlation Analysis were performed. The results of the analysis were presented as mean ± SD (standard deviation) of triplicates.

3. Results and Discussion

All physicochemical properties of adulterated KH with HFCS at different percentages were determined. Sugar analysis was carried out in duplicate (n = 2 × 2), and the rest analyses were carried out in triplicate (n = 2 × 3). The results of the analysis are presented in Table 1.

Table 1. Physicochemical properties of Kelulut honey adulterated with HFCS at different percentage.

Sample		HFCS	0%	10%	20%	30%	40%	50%	p-Value
Fructose		55.0 [14,15]	14.15 ±1.23[d]	16.39 ±0.58[cd]	20.44 ±0.57[bc]	21.28 ±0.69[b]	23.56 ±1.42[b]	31.97 ±1.98[a]	0.00
Glucose		45.0 [14,15]	14.01 ±0.95[d]	15.21 ±0.49[cd]	17.76 ±0.38[bc]	18.36 ±0.32[bc]	19.48 ±1.09[b]	25.11 ±1.41[a]	0.00
Trehalulose		NA	24.11 ±3.39[a]	20.63 ±1.02[ab]	19.36 ±0.48[abc]	14.08 ±1.28[bc]	13.25 ±3.27[bc]	12.41 ±1.25[c]	0.002
Water activity		0.738 [16]	0.761 ±0.007[a]	0.740 ±0.004[b]	0.730 ±0.011[bc]	0.723 ±0.004[c]	0.697 ±0.003[d]	0.686 ±0.001[d]	0.00
Total soluble solid		75.6–77.0 [15,17]	69.067 ±0.115[d]	69.667 ±0.231[c]	70.00 ±0.00[c]	70.867 ±0.231[b]	71.733 ±0.115[a]	72.133 ±0.058[a]	0.00
pH		4.65–4.80 [17]	3.190 ±0.00[d]	3.223 ±0.006[cd]	3.243 ±0.006[bc]	3.257 ±0.006[abc]	3.290 ±0.044[ab]	3.297 ±0.006[a]	0.00
Color	***L***	63.46–65.26 [17]	31.513 ±0.218[d]	32.127 ±0.263[d]	33.70 ±1.89[cd]	35.717 ±0.142[bc]	37.827 ±0.392[b]	40.123 ±0.040[a]	0.00
	a	6.20–7.42 [17]	12.410 ±0.616[d]	12.723 ±0.320[d]	14.053 ±0.760[c]	15.513 ±0.130[b]	16.520 ±0.240[ab]	16.920 ±0.339[a]	0.00
	b	26.87–29.47 [17]	7.590 ±0.370[e]	8.300 ±0.208[e]	9.903 ±0.620[d]	14.280 ±0.104[c]	16.103 ±0.323[b]	19.407 ±0.425[a]	0.00
Turbidity		NA	15.967 ±0.416[d]	11.533 ±0.306[f]	13.867 ±0.379[e]	26.433 ±1.305[c]	38.533 ±0.643[b]	92.77 ±0.208[a]	0.00

Note: Means that do not share a letter are significantly different.

3.1. Physicochemical Properties

Main sugar compositions in adulterated KH obtained from HPLC-ELSD analysis is provided in Table 1. For each sugar, linear regression was conducted producing linear equations as follows; $y = 499301x - 532357$ ($R^2 = 0.9977$), $y = 518226x - 815764$ ($R^2 = 0.9995$) and $y = 288093x - 145393$ ($R^2 = 0.9918$) for fructose, glucose and trehalulose respectively. The R^2 values obtained for the standard curve of each sugar are greater than 0.99 ($R^2 > 0.99$), which indicates a linear relationship between chromatographic linear response areas with the concentrations of all compounds. The fructose and glucose content increased while the trehalulose content reduced significantly ($p \leq 0.05$) as the level of HFCS added increased. The fructose, glucose, and trehalulose content are ranged between 14.15 to 31.97 g/100 g, 14.01 to 25.11 g/100 g, and 12.41 to 24.11 g/100 g, respectively. Recently, trehalulose was discovered as the main sugar composition in stingless bee honey for the first time through research conducted on honey from different species of stingless bees from Malaysia, Australia, and Brazil [8]. Trehalulose is a natural structural sucrose isomer that releases monosaccharides into the bloodstream slowly and gradually comparing to sucrose [18]. The addition of HFCS increases the amount of fructose and the glucose content, which can be explained by the composition of HFCS, which contains monosaccharides glucose and fructose [19] primarily.

The range of water activity in the samples is between 0.686 to 0.761 as presented in Table 1. The water activity of KH decreased as the percentage of HFCS added increased ($p \leq 0.05$). Water activity refers to the "ratio of the vapour pressure of water in a system to the vapour pressure of pure water at the same temperature" [20]. Generally, KH have higher water activity compared to *Apis* spp. honey [21]. Previous research has observed changes in water activity associated with the adulteration of HFCS at different levels [22]. Another research conducted to evaluate adulteration of fructose, and hydrolysed inulin syrup in honey samples showed that as the level of the adulteration agent increased, the water activity increased proportionally [23].

Table 1 also shows that the total soluble solids in the samples increase significantly with the increasing amount of HFCS adulterated in the samples ($p \leq 0.05$). Total soluble solid is one of the ways to estimate sugar content and increasing the value of total soluble solid can be associated with increasing sweetness level [24]. Organic compounds like acids and minerals are components can also influence the total soluble solid in honey [25].

The pH value of the samples increased significantly with increasing HFCS (Table 1). pH is one of the significant parameters in honey for extraction method and the condition of product storage which influence the texture, stability, and shelf life [26]. A study on the impact of blossom honey adulteration with HFCS at different concentrations from 0% to 100% showed a significant increase in the pH values of the honey [27]. The same trend was observed in research on Pakistani honey, in which the pH values of adulterated honey with sugar syrup are greater than pure honey [28].

The colour of the adulterated KH was determined by measuring L^*, a^*, and b^* *values.* L^* values indicate the brightness level, a^* indicates red to green meanwhile b^* indicates yellow to blue. The increasing amount of HFCS added into the KH significantly increased the L^*, a^*, and b^* values ($p \leq 0.05$) as presented in Table 1. The highest L^* value is obtained from 50% HFCS, whereas the lowest is 0 % HFCS with L^* values of 40.123 and 32.127, respectively. As for the* values, 50% HFCS shows the highest value of 16.920 and the lowest value of 12.410 of 0% HFCS added into KH. The same trend was observed on b^* values, ranging between 7.590 (0% HFCS) and 19.407 (50% HFCS). L^*, a^*, and b^* values for KH from *Heterotrigona Itama* are 24.90, 1.90, and 2.52, respectively [21]. Colour is one of the indicators for the quality deterioration indicator of honey during storage and is affected by moisture content and storage temperature [29]. Previous research reported that colour analysis shows authentic honey contains more red components (more reddish), whereas adulterated honey is usually luminous [30].

Turbidity (presented in NTU: Nephelometric Turbidity Units) for each sample with different amounts of HFCS adulteration ranged between 11.533 to 92.770. Table 1 shows that the increasing value of HFCS added in the samples significantly increases the value of NTU as indicated by p-Value ($p \leq 0.05$). 50% HFCS adulteration has the highest turbidity (92.77) compared to other levels of adulteration. Turbidity is a parameter that can indicate the crystallisation of honey [31]. The crystallization process in honey can influence customers' preferences by lowering their acceptability [32]. The increasing percentage of glucose can explain the increasing turbidity values due to the strong effect of glucose on the crystallisation of honey [33].

Figure 1 shows the impact of high fructose corn syrup adulteration on KH at a different percentage at a constant temperature of 25 °C. For each sample, the viscosity increases with increasing shear rate. This result can be associated with decreasing water content contributed by the adulterants added as viscosity is influenced by the flow behaviour [34]. The viscosity of HFCS (55% fructose) at 25 °C is between 0.212 Pa.s to 0.700 Pa.s [35,15]. The viscosity of the syrup is varied and affected by the composition of fructose and glucose and comparable at the same solid content. A high concentration of fructose results in lower viscosity, whereas a high glucose concentration, will increase the viscosity. Different shear rates significantly affect the viscosity of the sample with the *p*-value of 0.00 ($p \leq 0.05$).

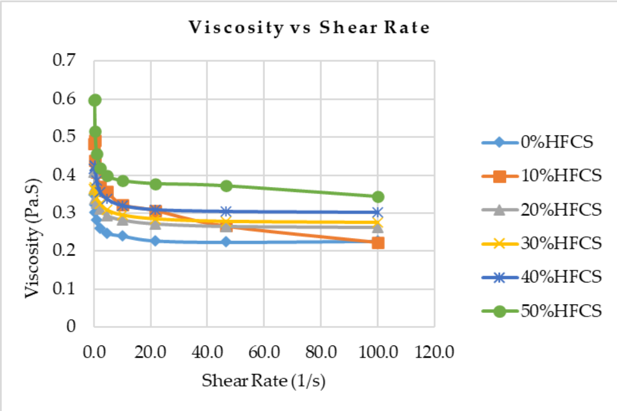

Figure 1. Effect of HFCS adulteration on the viscosity of adulterated KH at a different shear rate.

This result supports the correlation between shear stress and shear rate data, as shown in Figure 2. The graph indicates that the shear stress increased linearly with the shear rate, which indicates that the honey samples are having Newtonian flow behaviour [36–38]. The result from two-way ANOVA shows a significant interaction between the effects of shear rate and sample (formulation) on shear stress of the samples ($p \leq 0.05$). The *p*-value of shear rate is 0.00 showing that different shear rate has a significant effect on the shear stress of the samples. The shear stress of the samples is also associated with different levels of HFCS added with *p*-value = 0.00. Another study suggested that the dynamic viscosity of adulterated honeydew honey is affected by the type of adulterating agent used. The results from the study proved that the dynamic viscosity declined with the addition of fructose but increased with glucose and hydrolysed inulin syrup [36].

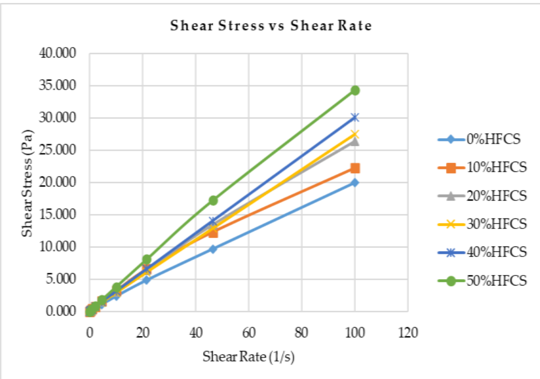

Figure 2. Correlation between shear stress with a shear rate on adulterated KH.

3.2. Pearson's Correlation Analysis between Trehalulose and Physicochemical Parameters of Adulterated KH at Different Concentration

The Pearson's correlation analysis (Table 2) revealed that trehalulose content has strong negative correlation with glucose, fructose, total soluble solids, pH and color (L*, a* and b* values), with *p*-value < 0.05 which indicates that the correlation coefficients are significant. The correlation between trehalulose and these variables is negative indicating that these variables decrease as trehalulose increase. Meanwhile, trehalulose is significantly positively correlated with water activity value (*p*-value < 0.05) in which the water activity increases as trehalulose increase. However, the result shows weak correlation between trehalulose and turbidity.

Table 2. Pearson's correlation analysis between trehalulose and physicochemical parameters of adulterated KH at different concentration.

Trehalulose	Correlation	*p*-Value
Glucose	−0.727	0.007
Fructose	−0.749	0.005
Water activity	0.806	0.002
Total soluble solids (brix)	−0.919	0.000
pH	−0.875	0.000
Turbidity	−0.640	0.025
L*	−0.855	0.000
a*	−0.922	0.000
b*	−0.884	0.000

4. Conclusions

In this study, physicochemical properties were used as the criterion to investigate the effect of adulteration of KH with different amount of HFCS. Significant differences were observed after KH honey were adulterated. Based on Pearson's correlation analysis, trehalulose concentration can be used as a marker for adulterated KH. It is recommended to develop effective, inexpensive, and time-saving methods to detect adulterated KH honey using these parameters in the future. This study focuses only on direct adulteration, so further investigation of indirect adulteration on KH should also be pursued to ensure honey quality in the market.

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