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Effect of Fast Freezing with Liquid Nitrogen on Quality of *Kelulut* (stingless bee) Honey

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Abstract: *Kelulut* honey is a highly saturated sugar solution produced by the enzymatic conversion of nectar or plant secretion. *Kelulut* honey contained around 80 to 85 % carbohydrates, 15 to 17 % water, 0.3 % proteins and 0.2 % ashes. *Kelulut* honey undergo rapid alcoholic fermentation after harvested due to its high moisture content and the presence of osmophilic yeast. This problem causes deterioration effect of honey products. Hence, liquid nitrogen was used in this study to preserve the quality of *kelulut* honey in term of protein content, enzyme, and antioxidant compound. However, the effectiveness of liquid nitrogen in preserving *kelulut* honey was questionable. Therefore, this study aims to partially characterize the protein content, diastase activity and antioxidant properties of *kelulut* honey after freezing with liquid nitrogen. For identification of protein content, Fourier-transform infrared spectrometer (FTIR) was used to detect amide groups present in the frozen *kelulut* honey. Apart from that, DPPH assay was conducted to determine the radical scavenging activity while diastase assay was adopted to identify diastase activity in frozen *kelulut* honey using T60 UV-vis spectrophotometer. This study found that protein content, diastase activity and antioxidant properties of frozen *kelulut* honey were 4.6 %, 3.364 Schade units and 85.12 % respectively while raw *kelulut* honey were 4 %, 2.884 Schade units and 84.18 % respectively. This study revealed that protein content, diastase activity and antioxidant properties of frozen *kelulut* honey were higher than raw *kelulut* honey. Based on the results obtained, it can be concluded that liquid nitrogen can be used as freezing method for preserving the quality of *kelulut* honey.

Keywords: *Kelulut* Honey, Stingless Bee, Liquid Nitrogen, Protein, Enzyme, Antioxidant

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1. Introduction

The conversion of nectars by bee's enzyme is producing honey with highly saturated sugar solution [14]. Bee honeys are categorized into two which are stingless bee honey and sting bee honey. *Kelulut* honey is a common name for stingless bee honey in Malaysia. In Malaysia, honey and other bee products are mainly produced by *Apis dorsota*, *Apis cerana*, *Apis mellifera* and stingless bee which came from Trigona genus [10]. In term of nutritional composition, carbohydrates, water, proteins, and ashes have been discovered by previous researcher with 80 to 8 %, 15 to 17 %, 0.3 % and 0.2 % respectively [5]. Apart from that, honey has its own potential benefit and biological characteristics such as antibacterial, anti-inflammatory and antioxidant. Active compounds such as phenolic compounds, flavonoids and some enzymes that are found in honeys are contributing beneficial effect to human health.

Due to overwhelming of honey demand in market, numerous honey products are introduced to fulfill consumer's desire. Preservation of honey is required to prevent any problems such as storage that may reduce the quality of honey or shorten the shelf life. Moreover, because of high water content which is about more than 30% and the presence of osmophilic yeast, *kelulut* honey experiences rapid alcoholic fermentation after harvesting [4], [17]. Therefore, freezing with liquid nitrogen has been introduced in this study for preservation of honey. Nevertheless, quality factor such as degradation of nutritional compound in honey is another problem need to be considered. Thus, the protein content, diastase activity and antioxidant properties of frozen *kelulut* honey were determined in this study.

Several analyses were conducted to analyze frozen *kelulut* honey in term of protein content, enzyme activity and antioxidant properties. For protein content, the protein was identified using Fourier-transform infrared spectrometer (FTIR) to identify specific functional groups that build the protein which is amide group [21]. For diastase activity, diastase assay was conducted to demonstrate the presence or absence of enzyme and to evaluate the quantity of enzyme in the sample by assessing the absorbance using Ultraviolet-visible spectrophotometer (UV-Vis) [3]. For antioxidant properties, antioxidant activity was determined using 1,1-diphenyl-2-picryhydrazyl (DPPH) method. The function of DPPH assay is to discover free radical scavenging activity in frozen *kelulut* honey [11].

The goal of this research is to compare protein content in frozen and raw *kelulut* honey by FTIR. Apart from that, this research is also targeted to study the antioxidant properties and diastase activity in frozen *kelulut* honey. This research information will give a worth knowledge in term of nutritional composition, awareness, health information, research study to manufacturer, researcher and the community about the protein content, diastase activity and antioxidant properties in frozen *kelulut* honey by liquid nitrogen.

2. Materials and Methods

2.1 Materials

Kelulut honey from *Heterotrigona Itama* species were collected from Parit Botak, Batu Pahat, Johor. Distilled water, starch solution, sodium chloride, NaCl, acetate buffer, iodine stock, methanol, 1- diphenyl-2-picryl hydrazyl or DPPH, Zinc Selenium (ZnSe) and fresh milk.

2.2 Freezing of *kelulut* honey with liquid nitrogen

Freezing of *kelulut* honey was carried out by submerging in liquid nitrogen. The method was adopted from Deng *et al.*, (2004) [7] with some modification. Firstly, 1.5 mL of *kelulut* honey was inserted into 2 mL Polymerase Chain Reaction (PCR) tube. Next, PCR tube containing *kelulut* honey was fully submerged in the liquid nitrogen for 5 minutes.

2.3 Protein identification by using Fourier-transform Infrared Spectrometer (FTIR)

FTIR was used for measuring protein content in frozen *kelulut* honey and raw *kelulut* honey. The procedure was referred to Sjahfirdi *et al.*, (2012) [21]. For the sample preparation, about 0.5 µl of each sample was placed on Zinc Selenium (ZnSe) window using a pipette. Next, the window was placed on the holder and scanned at 400 - 4000 cm^{-1} wavelength with resolution of 4 cm^{-1}. The scanning results were the percentage absorbance of specific wavenumber for every amide functional group in each sample. Each amide has specific marker group as shown in Table 1 below. The identification of functional group was done by comparing wavenumber of amide functional groups of the samples with the existing standard. Relative protein level was calculated by comparing absorbance of specific functional groups with the absorbance of fatty acid functional groups in every sample while protein level in percentage was obtained by comparing absorbance of sample with the absorbance of fatty acid functional groups in fresh milk. Carbonyl group was detected at 1746 cm^{-1} as fatty acid functional group.

Table 1: Amide functional groups

Amide Types	Functional Groups	Wavenumber (cm^{-1})
Amide A	N-H	3300
Amide B	N-H	3100
Amide I	C=O	1600-1690
Amide II	C-N, N-H	1480-1575
Amide III	C-N, N-H	1229-1301
Amide IV	O-C-N	625-767
Amide V	C=O	640-800
Amide VI	N-H	537-606
Amide VII	Skeletal torsion	200

2.4 Diastase activity via UV-vis Spectrophotometer

Diastase assay was carried out to determine the diastase activity of raw *kelulut* honey and frozen *kelulut* honey. This method was referred from Omar *et al.*, (2019) [18] with minor modification. UV-Vis Spectrophotometer (T60 PG Instruments, USA) was used to evaluate the diastase activity. Firstly, 10 g honey was mixed with 15 mL of water and 5 mL acetate buffer (pH 5.2). Then, the solution was mixed with 3 mL of sodium chloride (NaCl) and distilled water in a 50 mL volumetric flask. Two flasks containing 10 mL of honey solution and 10mL starch solution were prepared separately and placed in a water bath (40 ^{o}C). After 15 minutes, 5 mL of starch solution was transferred into a flask containing the honey solution. After 5 minutes, 0.2 g of the honey solution was mixed directly with a 5 mL of diluted iodine solution. Then, the sample mixture was immediately measured against distilled water (blank) at 620 nm wavelength. Diastase number was calculated by using 1, as follow:

$$\text{Diastase number} = 28.2 \times \Delta A_{620} + 2.66 \quad Eq.1$$

2.5 Antioxidant properties by DPPH assay

Antioxidant activity or free radical scavenging activity of frozen *kelulut* honey and raw *kelulut* honey were determined by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) method. This method referred from Ferreira *et al.*, (2009) [8]. For the sample preparation, 0.5 mL of 0.2 g/mL *kelulut* honey extract with methanol (0.2 g honey sample in 1 mL methanol) was mixed with 2.7 ml methanolic DPPH radical solution (0.024 mg DPPH in 1 mL methanol). Next, the mixture was vigorously shaken and incubated for 60 minutes in dark condition before the absorbance was measured at 517 nm by using UV-Vis Spectrophotometer (T60 PG Instruments, USA). The radical-scavenging analysis (RSA) was using Equation 2:

$$\% \text{ RSA} = \frac{(A_{DPPH} - A_S)}{A_{DPPH}} \times 100 \quad Eq. 2$$

where,

A_S = The absorbance of the sample with DPPH solution.

A_{DPPH} = The absorbance of the DPPH solution.

3. Results and Discussion

This section consists of three parts which are identification of protein content by determining the absorbance of specific amide groups via FTIR, characterization of diastase activities by diastase assay and determination of antioxidant properties via DPPH assay.

3.1 Identification of Protein Content via FTIR

FTIR was used in this study to identify amide groups which are the specific functional groups that build amino acids or structural units to make up protein in the sample. Thus, the identification of protein and its level in the frozen *kelulut* honey and raw *kelulut* honey were conducted.

According to the result shown in Table 2, protein level of frozen *kelulut* honey was slightly higher (4.6 %) than raw *kelulut* honey (4 %). The protein level in the *kelulut* honey was slightly maintained after freezing with liquid nitrogen. Although, the small differences of protein level between frozen *kelulut* honey and raw *kelulut* honey had been identified with 0.6 differences. This is because during freezing at -195 °C with liquid nitrogen, the *kelulut* honey sample in the tube was completely frozen *kelulut* honey became a glassy amorphous solid that is non-crystalline. Then, when it reached -20 °C and below, *kelulut* honey underwent crystallization due to high content of sugar and all water particles had already completely frozen before reaching -20 °C. At the same time, the rapid freezing with extreme temperature with liquid nitrogen was believed to preserve the protein stability and structure from denatured. According to Deng *et al.*, (2004) [7], freezing cause damage of proteins through concentration of protein and pH changes that promote the denaturation and aggregation but extremely rapid freezing with liquid nitrogen able to minimize the problem.

Table 2: Relative protein level and protein level of samples

Sample	Relative Protein Level	Protein Level (%)
Fresh Milk (Positive Control)	6.0044	6
Frozen *Kelulut* Honey	4.6352	4.6
Raw *Kelulut* Honey	4.4062	4

Freezing as preservation method contributes to retaining high amount of free amino acid as compared to other preservation methods such as curing or canning [15]. This is because low temperature can effectively reduce the deterioration of amino acid component and some amino acids decreased at a lower rate under the lower storage temperature due to relatively lower enzyme activity and respiration rate while the degradation of amino acid at room temperature cannot be avoided and it degrades faster than at low temperature [23]. The lower the temperature, the higher the protein level from the *kelulut* honey as well as the lower the risk of protein degradation and denaturation. Therefore, frozen *kelulut* honey had the highest protein level with 4.6% as compared to raw *kelulut* honey with 4% which was placed at room temperature. The application of liquid nitrogen as preservation of *kelulut* honey could

be considered by researchers. Although, the value of protein level between raw *kelulut* honey and frozen *kelulut* honey are very small difference, further analysis such as physicochemical properties, colour and rheological behaviour of honey could be carried out to justify the advantages of using liquid nitrogen as quick freezing compared to other methods. For example, gradient thawing can retain more than 95% of polyphenols and other nutritional compounds (including pectin, soluble sugar, and vitamin C) in thawed blueberries compared with fresh blueberries, acclaimed by Cheng *et al.*, (2015) [6].

3.2 Characterization of Diastase Activity by Diastase Assay

Diastase activity in *kelulut* honeys (frozen and raw) was conducted based on Kuc *et. al.*, (2017) [13] where starch was used as substrate. According to Tosi *et al.*, (2008) [22], diastase activity is an important quality parameter to identify whether honey has been extensively heated during processing. Thus, it is necessary to preserve the quality of diastase activity in *kelulut* honey by freezing with liquid nitrogen as preservation method. In this study, diastase activities in the frozen and raw *kelulut* honey were characterized and compared to determine the effectiveness of liquid nitrogen to preserve diastase stability.

According to the result as shown in Table 3 and Figure 1, frozen *kelulut* honey had the highest diastase number (DN) of 3.364 Schade units as compared to raw *kelulut* honey with 2.884 Schade units. It means that diastase activities in the frozen *kelulut* honey higher than raw *kelulut* honey. After freezing, the diastase activities were completely inactive due to extremely low temperature and below its optimal temperature (30 °C to 50 °C) [9]. Enzyme is a type of protein that made up of amino acids that fold up into very specific shape. Enzymes were not losing their shape during freezing in liquid nitrogen and the regions around their active sites were frozen too. It prevents the enzyme from reacting. According to Deng *et al.*, (2004) [7], extreme cold temperature prevents the enzyme from losing their shape and structure due to denaturation and degradation at high temperature.

Table 3: Diastase number of each sample

Sample	Diastase Number (DN)				
	1st trial	2nd trial	3rd trial	Mean	Std
Frozen *Kelulut* Honey	3.401	3.430	3.260	3.364	0.074
Raw *Kelulut* Honey	2.950	2.894	2.809	2.884	0.058

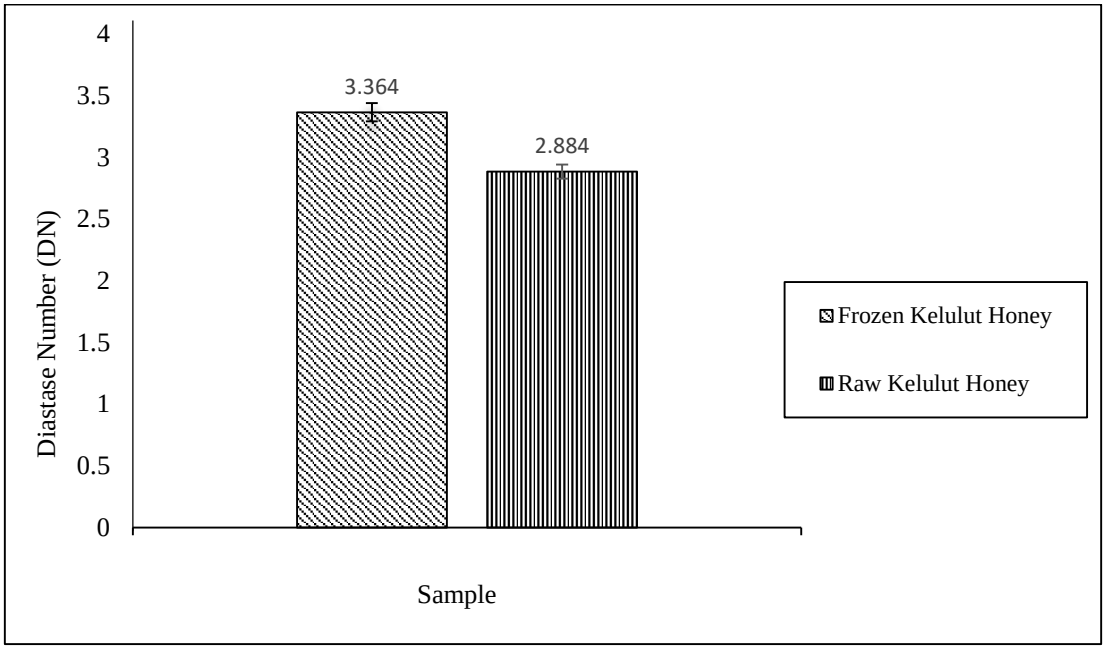

Figure 1: Diastase number of samples

As the temperature was increased during thawing of the frozen *kelulut* honey sample, the enzymes and substrate were gaining back their kinetic energy and move quickly. Then, it increases the frequency of collisions and the formation of enzyme-substrate complex. At room temperature, enzymes, or diastase in the raw *kelulut* honey were undertaking their role to speed up the rate of biochemical reaction by breaking down substrates without being disturbed by the heating or cooling process. The biochemical reaction will achieve equilibrium at certain time after all the substrates have been degraded. Thus, activity of enzyme will decrease. However, enzymes in frozen *kelulut* honey were inactive and no biochemical reaction occur. The biochemical reaction between enzymes and substrates only happens after thawing the frozen *kelulut* honey. During the thawing process, enzymes were activated. Enzyme activity and rate reaction were increasing due to the temperature increase [22]. Therefore, frozen *kelulut* honey had higher number of diastase than raw *kelulut* honey based on the statement above by Tosi *et. al.*, (2008) [22].

3.3 Determination of Antioxidant Properties by DPPH Assay

Antioxidant activity of frozen *kelulut* honey sample was carried out using DPPH assay based on a method established by Ferreira *et al.*, (2009) [8] with minor modification. DPPH method or assay is one of the assays that usually used to determine the antioxidant properties of honey. DPPH assay contributes to identifying the percentage of radical scavenging that was trapped by the potential antioxidant compound in frozen *kelulut* honey sample. According to Kek *et al.*, (2018) [11], the scavenging activity of frozen *kelulut* honey was regulated by deep purple coloured DPPH radical's reduction after receiving a hydrogen donated by free radical scavenging antioxidant from *kelulut* honey sample. In this study, ascorbic acid was used as positive control.

Average of absorbance reading of methanol solution (blank) at 517 nm was 0.771. Based on the result shown in Table 4 and Figure 3, radical scavenging activity or antioxidant activity of frozen *kelulut* honey (85.12 %) was slightly higher than raw *kelulut* honey (84.18 %). This might be due to the extremely rapid freezing with liquid nitrogen preserved antioxidant compounds in the frozen *kelulut* honey sample. While raw *kelulut* honey sample which was stored at room temperature may reduce the antioxidant compound due to unstable environment condition. According to Lohachoompol *et al.*,

(2004) [16], flavonoids can be well preserved during freezing and no significant reduction in the levels of the flavonoids even after three months of freezing. Flavonoids as well as phenolic acids are naturally and abundantly found in honey which act as natural antioxidants and exhibit a wide range of biological effects [19].

Table 4: Radical scavenging activity of samples

Sample	Radical Scavenging Activity (%)				
	1st trial	2nd trial	3rd trial	Mean	Std
Ascorbic Acid (Positive Control)	94.94	94.94	94.94	94.94	0.000
Frozen Honey	84.95	85.60	84.82	85.12	0.341
Raw Honey	84.44	83.79	84.31	84.18	0.281

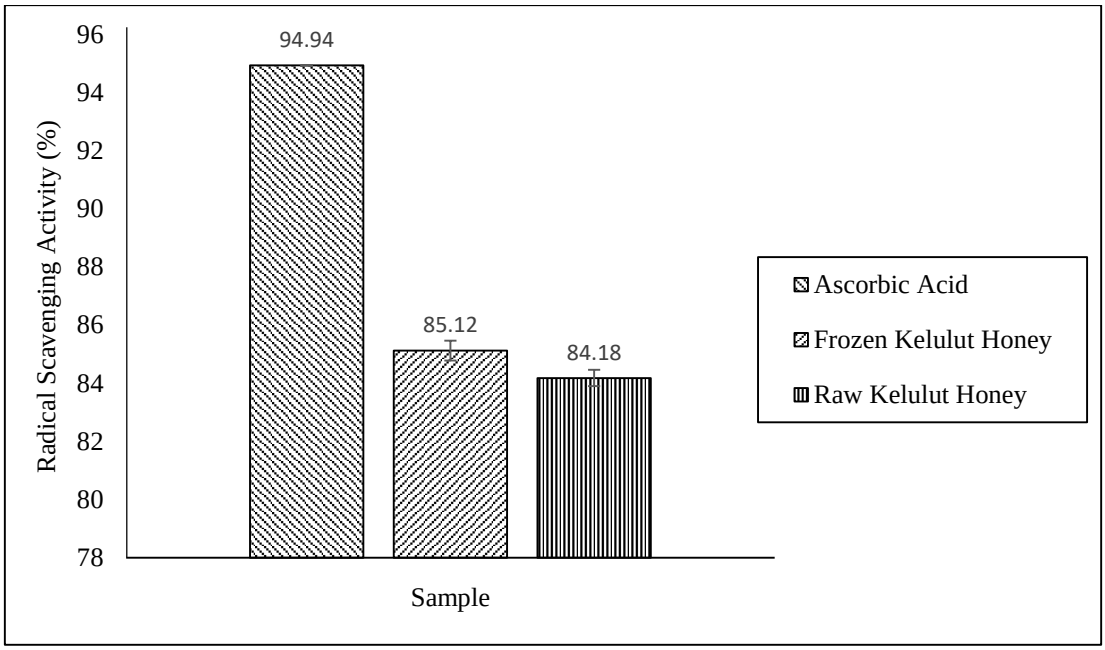

Figure 2: Radical scavenging activity of samples

Temperature is one of the most crucial factors affecting antioxidant properties. High heating temperature promotes an acceleration of the initiation reactions that causes reduction in the activity of the existing antioxidants [20]. Thus, many researchers were trying to preserve the antioxidant compound due to its benefits to human body. Based on study reported by Abu Bakar *et al.*, (2017) [2], *kelulut* honey usually exhibited high radical scavenging activity due to high flavonoid and phenolic content. Antioxidant compound is one of the essential components that act as wound healing. According to Abdul Jalil *et al.*, (2017) [1], the major therapeutic action of honey in improving the wound healing process comes through its antioxidant activity since it can prevent the detrimental effects on the wounded site caused by oxidative stress. *kelulut* honey can be applied for the wound treatment due to the presence of high antioxidant content [12]. Apart from that, antioxidant characteristics of raw honeys from Malaysia was used as markers for identifying its entomological source of bee species [11]. This is because as reported by Abu Bakar *et al.*, (2017) [2] that antioxidant properties of the honey influenced by various factors like botanical origins, floral sources, season, environment, the presence of polyphenols and treatments. Therefore, rapid freezing with liquid nitrogen can be used as preservation or cryopreservation of antioxidant compound in *kelulut* honey.

4. Conclusion

This study found that freezing with liquid nitrogen able to preserve the quality of *kelulut* honey due to protein level, diastase activity and antioxidant activity of frozen *kelulut* honey with liquid nitrogen were slightly higher than raw *kelulut* honey. From this study, rapid freezing with liquid nitrogen can be considered as good preservation method for preserving nutritional composition such as protein content, enzyme, and antioxidant compound.

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