EFFECT OF PROCESSING TREATMENT ON ANTIOXIDANT, PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF HONEY (*TRIGONA* spp.)

By

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ABSTRACT

EFFECT OF PROCESSING TREATMENT ON ANTIOXIDANT, PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF HONEY (*TRIGONA* spp.)

VIVIAN NGOI

Although honey is already known to possess nutritious health benefits, still many researchers are interested to investigate the ways to process honey without affecting its beneficial properties. Hence, the main objective of the project was to study the effect of processing temperature and duration on the antioxidant, physicochemical and enzymatic properties of Malaysian stingless bee honey namely Trigona honey. Five honey samples which were originated from *Trigona apicalis* and *Trigona itama* that have been processed at 41°C and 80°C for 100 minutes, 8 hours and 15 hours respectively were obtained and subjected to different assays such as ABTS, DPPH, FRAP, AEAC to determine the changes in antioxidant capacity of honey. The results of stated assays showed that antioxidant capacity of honey samples increased significantly along with greater processing temperature and duration. Besides, total phenolic and flavonoid contents were also measured before proceeding to physicochemical analyses (color characteristic, color intensity, electrical conductivity, free water activity, proline content, total reducing sugar and sucrose content) in order to determine the effect of processing on each aspect of honey properties. From the results, the levels of phenolics and flavonoids

were found to increase significantly when the processing temperature and duration increased. All the honey samples were classified as dark color and the color became darker when processing temperature and duration increased. Moreover, the electrical conductivity and free water activity increased when the processing temperature was getting higher but decreased when the processing duration was prolonged. Increment in proline content during higher temperature and longer duration was also observed. Lastly, the level of diastase enzymes was analysed to indicate the honey freshness. However, the diastase level decreased significantly as temperature and duration increased due to heat treatment. In brief, higher processing temperature and longer processing duration were found to enhance the antioxidant capacities and affect the physicochemical properties of honey.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for other degree at UTAR or other institutions.

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VIVIAN NGOI

APPROVAL SHEET

This project report entitled "**EFFECT OF PROCESSING TREATMENT ON ANTIOXIDANT, PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF HONEY (*TRIGONA* spp.)**" was prepared by VIVIAN NGOI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **VIVIAN NGOI** (ID No: **12ADB02692**) has completed this final year project entitled "EFFECT OF PROCESSING TREATMENT ON ANTIOXIDANT, PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF HONEY (*TRIGONA* spp.)" under the supervision of Mr. Ng Wen Jie from the Department of Biomedical Science, Faculty of Science, and Dr. Ee Kah Yaw from the Department of Agriculture and Food Science, Faculty of Science.

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LIST OF ABBREVIATIONS

ΔE^*_{ab}	Color difference
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AEAC	Ascorbic Acid Equivalent Antioxidant Content
$AlCl_3$	Aluminium chloride
$CaCl_2.2H_2O$	Sodium maleate buffer plus calcium chloride
CEQ	Catechin Equivalent
DN	Diastase Number
DNSA	3,5-Dinitrosalicylic acid
DPPH	Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium
Fe^{2+}	Iron (II) ion
Fe^{3+}	Iron (III) ion
$FeCl_3.6H_2O$	Ferric chloride hexahydrate
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
FRAP	Ferric reducing-antioxidant power
GAE	Gallic Acid Equivalent
HMF	Hydroxylmethyfurfural
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
$NaNO_2$	Sodium nitrite
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TPTZ	2,4,6-Tri(2-pyridinyl)-1,3,5-triazine

CHAPTER 1

INTRODUCTION

Honey is a natural sweet substance that is produced by honeybees from the nectar of blossomed flowers either unifloral (nectar from the same flowers) or multifloral (nectar of various types of flowers). It is a supersaturated sugar solution which is rich in proteins, minerals, vitamins, organic acids and polyphenols (Saba, Suzana, Yasmin-Anum, 2013).

Honey has been used as a traditional natural therapeutic agent to boost up the immune system and combat against diseases. It contains high nutritional values and prophylactic medical values (Adetuyi, Ibrahim and Ogundahunsi, 2009). However, each type of honey gives different color, flavor and composition according to climate, environment, handling and storage processes (Syaliza, Maisarah and Norhilmiah, 2009).

Recently, stingless bee honey has grabbed the attention of researchers due to its higher nutritional values as compared to ordinary honey. It is also called as "Mother Medicine" and is popular among traditional practitioners and researchers. Hence, researchers began to explore the chemical composition of stingless bee honey and its biological effects. According to Rintos (2014), a Borneo Post reporter, stated that having stingless bee honey regularly can promote anti-ageing, enhance immune system and libido, fight against bacteria

and treat sore throat, coughs, colds and bronchial catarrh. It is also used as antiseptic and therapeutic agent to sooth pain, promote healing, relieve cough and also effective in curing burns, carbuncle, boils and diabetic wounds.

Studies have shown that honey possesses antioxidant properties and the major components responsible for such activity are divided into enzymatic and non-enzymatic categories. The components which are significantly expressed in every honey include phenolic acids, flavonoids, ascorbic acids, catalase, peroxidase and carotenoids (Khalil, et al., 2011). Honey also contains a variety of phytochemicals such as organic acids (gluconic acid and acetic acid), vitamins (ascorbic acid, niacin and pyridoxine), and enzymes (diastase, invertase, glucose oxidase and catalase) that serve as dietary antioxidants (Gheldof and Engeseth 2002). Besides, the enzymes originated from bees or floral sources also play an important role in the formation of honey from nectar. The content of enzymes in honey can be used to classify a good quality of unadulterated honey. As stated by Bogdanov, et al. (2000), a fresh honey contains low amount of hydroxymethylfurfural (HMF) with natural levels of enzymes. Diastase and invertase are normally used as the parameter to determine the freshness of a honey (Dustman, 1993; Bogdanov, et al., 2000).

The quality of honey is primarily determined by its sensorial, chemical, physical and microbiological characteristics (Alvarez-Suarez, et al., 2010). Since the quality of honey varies according to geographical and seasonal conditions as well as floral sources, each honey exhibits various sensory and physicochemical properties. However, the major criteria of interest that define

the physicochemical quality of honey in this project are proline content, electrical conductivity, free water activity, color characteristics, color intensity, total reducing sugar and sucrose content.

Nowadays, concerns aroused on whether heat makes honey toxic and do the properties of honey change upon heating. Therefore, the major aim of present study was to investigate on the antioxidant, physicochemical and enzymatic properties of stingless bee honey (*Trigona* spp.) upon different heat treatments.

Thus, the objectives of this study were:

1. To determine the antioxidant, phytochemical, physicochemical and enzymatic properties of honey.
2. To screen the compositions and properties of stingless bee honey originated from *Trigona apicalis* and *Trigona itama*.
3. To investigate the changes on the compositions and properties of stingless bee honey under different processing temperature and duration.

CHAPTER 2

LITERATURE REVIEW

2.1 Honey

Honey is a collection of nectar that is processed by honey bees such as bumblebees, stingless bees or other hymenopteran insects. The color and flavor of honeys vary depending on the nectar sources or the blossoms encountered by the honey bees. The range of honey color differs from colorless to dark brown; and flavor differs from mild to strong, depending on the location of honey bees buzzed. Generally, dark-colored honey is stronger in taste and light-colored honey is milder (National Honey Board, 2010).

Besides, honey is well known for its antioxidant properties due to the presence of phenolic acids, flavonoids, catalases, peroxides, carotenoids and nonperoxidal components. This antioxidant properties enable honey to prevent some chronic diseases such as coronary heart disease, strokes, chronic respiratory disease and even cancer (Jennifer and Michael, 2007). Moreover, honey also exhibits antimicrobial activity due to low water content, low pH and presence of hydrogen peroxide. Such high osmolality in honey can inhibit the growth of bacteria and promote wound healing due to low free water activity (Office of Complementary Medicines, 1998). High sugar content in honey also causes the withdrawal of water from bacteria through osmosis process.

However, honey can sometimes contaminated by *Clostridium botulinum*, which is dangerous to infants as the endospores can transform into toxin-producing bacteria in their immature intestinal tract, leading to illness and even death. Therefore, infants and people with weakened immune system should not eat honey to avoid the risk of bacterial or fungal infection (Kowsalya, 2012).

2.1.1 Production of Honey

Nectar is a sugary liquid that is extracted from flowers. Honey bees convert this nectar into honey through a process called ‘regurgitation and evaporation”. When the honey bees return to hive, they pass the nectar to other bees by regurgitating the liquid into other bees’ mouths (Palermo, 2013). Honey bees use their "honey stomachs" which contain digestive enzymes (e.g. diastase, invertase, glucose oxidase, etc) and gastric acid to digest the nectar repeatedly until it is partially digested (Suarez, et al., 1996). Gastric acid hydrolyses the sucrose from nectar into glucose and fructose which provides sweet taste of honey. The product is then stored in honeycombs. Then, honey bees keep fluttering their wings to evaporate the water from the honey in order to increase the sugar concentration. Lastly, the bees will seal the cap with wax to prevent fermentation (Binkley, 2014).

2.1.2 Composition of Honey

The composition of honey is highly dependent on the geographical and botanical origin, as well as the handling process during harvesting and storage (Gheldof and Engeseth, 2002). However, type of flora sources is the major factor in determining the composition of each honey. Honey is primarily made up of carbohydrates (82.3%), namely glucose (31%) and fructose (38%) and also water (17-20%) (Office of Complementary Medicines, 1998; Alvarez-Suarez et al. 2010). It also contains disaccharides (8%) such as sucrose, maltose, kojibiose, turanose, isomaltose, and maltulose and trisaccharides such as melezitose and raffinose (National Honey Board, 2010). However, most of these sugars are not found in nectar as they are only formed by the bees during the ripening and storage of the honey (El-Soud, 2012). According to White (1975), there are other important substances such as amino acids (>200 ppm), proteins, enzymes and minerals (<0.02%) which are the minor constituents of honey. Honey also contains organic acid such as gluconic acid which is formed by glucose oxidase during ripening of honey (Office of Complementary Medicines, 1998).

2.2 Stingless Bees

Stingless bees belong to family *Apidae*, which is further divided into subfamily Meliponinae. The process of keeping stingless bees is known as "meliponiculture" (Kelly, et al., 2014). Stingless bees are active all the time except during cold weather. They are highly sociable, with one queen lives together with thousands of workers (Chuttong, et al., 2015). They normally

inhabit in tropical and subtropical parts of the world such as Central and South America, Africa, Asia and northern Australia as highlighted in Figure 2.1 (Boorn, et al., 2010).

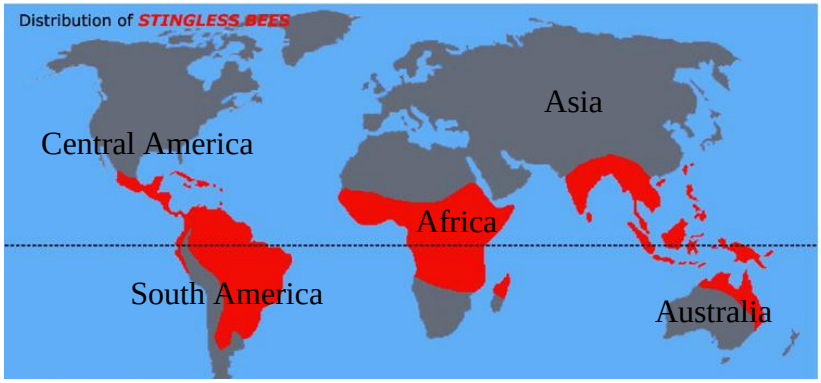

Figure 2.1: Distribution of stingless bees around the world (Adopted from Sakagami, 1982).

There are around 500 species of stingless bees around the world and they are classified into five genera: *Trigona*, *Melipona*, *Meliponula*, *Dectylurina* and *Lestrimelitta*. However, the only types of stingless bees that can produce honey are *Trigona* and *Melipona*. According to Kelly, et al. in 2014, the most common stingless bee species found in Malaysia are *Trigona itama* (83.2%) and *Trigona thoracica* (11.2%). However, the diversity of stingless bees throughout Peninsular Malaysia is poorly documented (Salim, et al., 2012). According to Liow, Sodhi and Elmquist in 2001, the study showed that stingless bees in Peninsular Malaysia were ubiquitous in rainforest especially primary and secondary forests than in more disturbed sites.

Generally, as displayed in Figure 2.2, stingless bees are smaller in size with approximately 4 mm of body length. As indicated by the name, they have atrophied sting which makes them incompetent for defence. As highly eusocial

insects, a stingless bee colony can contain hundreds to thousands of bees. They usually live in hollow trunks, tree branches, underground cavities or rock crevices (Pyper, 2001). One uniqueness of stingless bees is that they store the honey in resin pots instead of honey combs and they produce less honey as compared to other honey production. Nonetheless, according to the Malaysian Agricultural Research and Development Institute (Mardi) stated in Borneo Post newspaper on 31st August 2014, stingless bee honey is twice as nutritious as ordinary honey. It contains higher amount of potassium, magnesium, iron and zinc (Rintos, 2014).

Figure 2.2: Side view of a stingless bee (Adopted from Cockerell, 1918).

As shown in Figure 2.3, the nest structure of stingless bees is different from honey bees. Honey bees usually make vertical hanging wax combs while stingless bees build horizontal brood combs (Bradbear, 2009). The brood chamber has a protective wall made of wax and propolis, named "involucrum". There are pots for honey and pollen outside the involucrum and inside the involucrum, brood cells and food pots are separated. The size of food pots is larger than brood cells and is sealed once filled. The brood cells are arranged compactly in clusters (Gajanan, et al., 2005). The nest is then enclosed with

batumen, which is made of a mixture of resin, wax, mud, oil, paint, and sometimes, animal faeces. The batumen is very strong and thick that provides protection for colony against water and enemies (Sommeijer, 1999).

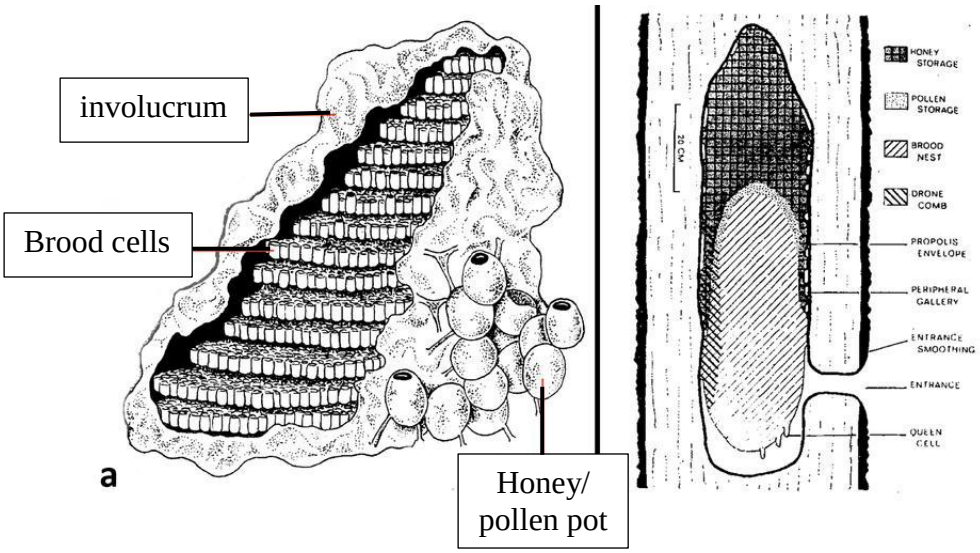

Figure 2.3: Comparison of stingless bee and honeybee nests (Adopted from Cockerell, 1918).

2.3 Trigona Honey

In Malaysia, Trigona honey is commonly known as "Kelulut" and is believed to have high medicinal value (Tualang Honey Malaysia, 2014). It is produced by specific stingless bees from genus *Trigona*. The nest of Trigona bees is mostly found in between the roots or in a tree stump, near the land surface which unlike normal bees nest that is found on a tree (Khasiat Madu, n.d.). Since Trigona bees are smaller than normal honey bees, they can collect the flower nectar from the deepest region of blossoms. As a result, Trigona honey contains higher nutritional values (Tualang Honey, n.d.).

Besides, Trigona bees can produce propolis through the mixing of saliva with food substances such as pollen, bark, shoots of trees and flowers. Propolis is good for health as it contains amino acids, glucose, vitamins A, B, C, D and E, bioflavonoids and minerals (Rintos, 2014).

Stingless bee honey has distinct characteristics as compared to honey bee honey in term of color, taste, viscosity, water and sugar content. According to Biluca, et al. (2014), they proved that stingless bee honey had distinct taste and aroma, more fluid in texture and undergone slow crystallization. Generally, Trigona honey is darker in color and has slightly sour taste (Garedew, Schmolz and Lamprecht, 2003). The taste originated from plant resin where the bees build their hives and honey pots. The taste varies from one another depending on the flowers and trees they visited. However, the quantity of honey harvested is lesser as compared to other honey varieties. Honey bees can produce approximately 75 kg of honey per hive while stingless bees can only produce less than 1 kg of honey per hive (Pyper, 2001). Even though so, it still carries good benefits as it can enhance metabolism, maintain beauty and improve various diseases and internal injuries (Foong, 2012).

From the research done by Oddo and colleagues in 2008, Trigona honey showed to have lower values of pH, reducing sugar and enzymatic activities (diastase and invertase) whereas higher values of moisture, water activity, electrical conductivity and free acidity. Hence, Trigona honey is said to be more susceptible to fermentation if it is not stored properly (Garedew, Schmolz and Lamprecht, 2003). According to Boorn, et al. in 2010, Trigona honey

exhibited broad spectrum antibacterial activity but limited antifungal activity. However, it is still highly appreciated by Aboriginal people in northern Australia due to social traditional and rituals (Isaacs, 2000). It has also been used as traditional medicine in Central and South America, and Africa and is believed to have therapeutic effect similarly to current medicine honey such as Manuka honey from New Zealand (Cooper, Wigley and Burton, 2000; Cortopassi-Laurino, et al., 2006; Adams, et al., 2008). In Ethiopia, Trigona honey is also used for traditional treatment, for example respiratory ailments, surface infections and other diseases. It was suggested that stingless bee honey might be more effective than honey bee honey to treat infectious disease (Andualem, 2014). However, according to Codex Alimentarius Commission (2001), stingless bee honey is not included in international standards for honey due to limited knowledge.

2.4 Antioxidant Properties of Honey

Reactive oxygen species (ROS) are very reactive substances which are produced during enzymatic reactions in cells (Hu and Brindle, 2005). ROS are normally existed in low level during normal physiological conditions to maintain normal cellular functions. However, when ROS are produced excessively, it causes cellular oxidative stress and consequently leads to chronic diseases such as brain damage, ischemic heart disease, atherogenesis and cancer (Migliore and Coppede, 2009). It is also believed that these free radicals are the culprits which contribute to aging by causing cellular and

molecular damage, such as DNA damage, mitochondrial collapsing and oxidation of protein, lipid and carbohydrate (Watanabe, et al., 2010). Hence, antioxidants function to combat against these free radicals that are found in the body by neutralizing them and protect the cells from damage.

With this, scientists began to explore the way to increase the antioxidant levels in body through dietary supplements and realize that honey could be one of the best options which supplies antioxidants that can uptake the free radicals in the body (Bashkir Bee Honey, 2009). Many studies have proved that honey could serve as a natural source of antioxidants to reduce the risk of heart disease, cancer, immune system deficiency, cataracts, different inflammatory processes and so on (National Honey Board, 2002a). Honey contains significant antioxidant compounds such as phenolic acids, flavonoids, vitamins and enzymes (Meda, et al., 2005). Among all, polyphenols attained the most interest in research due to its functional properties. Polyphenols can act as both radical scavenger and immune modulator as they contain high mobility of hydrogen in the molecular structure (Havsteen, 2002). According to Alzahrani, et al. in 2012, the study showed that there was a high correlation between polyphenols and antioxidant capacity, suggesting that phenolic compounds and flavonoids were the main antioxidants found in honey. Besides, phenolic acid, which is one of the major components among polyphenols in honey, could also affect the flavour and color of honey (Alvarez-Suarez, et al., 2010). Interestingly, it has been proved in previous research done by Jaganathan and Mandal (2009), showing that there was a correlation between color and

antioxidant capacity, with the darker honey providing higher level of antioxidants.

Previous researches had investigated extensively on the honey produced by *Apis* spp. but less on stingless bee honey. This has led to limited knowledge on the antioxidant and physicochemical information of Trigona honey (Boorn, et al., 2010). According to Kek, et al. (2014) in a research done on the comparison between *Apis* spp. and *Trigona* spp. showed that Trigona honey contained higher total phenolic content than Apis honey by 33%. It could suggest that Trigona honey has higher antioxidant capacity too as phenolic content is positively correlated with antioxidant capacity (Alzahrani, et al., 2012). Recently, Ibrahim, et al. (2016) has compared the phenolic content of propolis produced by two Malaysian stingless bees (*Heterotrigona itama* and *Geniotrigona thoracica*) and concluded that *H. itama* contained higher phenolic content and flavonoid content than *G. thoracica*, hence showing that *H. itama* possessed greater antioxidant capacity than *G. thoracica*.

There are several methods to measure the antioxidant properties of honey. The phenolic content of honey is primarily determined by using Folin-Ciocalteu method which measures the inhibition of low density lipoprotein oxidation mediated by cupric ions (Prakash, Rigelhof and Miller, n.d.). The flavonoid content is measured by using aluminium chloride method based on the formation of complexes between hydroxyl and carbonyl groups of flavones with aluminium ions (Al^{3+}) and flavonols (Popova, et al., 2004). Besides, the antioxidant properties of honey can also be accessed by measuring the free

radical scavenging activities against DPPH and ABTS. Moreover, ferric reducing antioxidant power (FRAP) assay enables direct evaluation of antioxidant level based on the ability of reducing ferric to ferrous couple (Moniruzzaman, et al., 2013a).

2.5 Physicochemical Properties of Honey

Apart from the determination of antioxidant properties, physicochemical parameters are also important indicators for the analysis of honey quality. These parameters include pH, color, acidity, hydroxymethyfurfural (HMF) content, electrical conductivity, water content, water activity and sugar composition (Boussaid, et al., 2014). Each honey exhibits different properties depend on the botanical origin, floral source, season, weather, storage method and treatment of honey by beekeepers (Kaskoniene and Venskutonis, 2010; El-Metwally, 2015).

HMF is normally not present in fresh food but is only generated during heat treatment. It serves as an indicator for excessive heat-treatment. According to Codex Alimentarius Commission (2000), fresh honey only contains low amount of HMF at approximately 15 mg/kg. The HMF content of honey from other countries at 40 mg/kg or 80 mg/ kg for honey imported from tropical regions while honey with low diastase enzymatic level (8–3 Schade Units) at 15 mg/kg (Codex Alimentarius Commission, 2000). Other than HMF content, fructose/ glucose ratio can also be used as another parameter for honey quality

analysis to indicate honey crystallization (White and Doner, 1980; Kaskoniene and Venskutonis, 2010; El-Sohaimy, Masty and Shehata, 2015). Besides, water content and water activity also play important roles for the stability of honey against fermentation and granulation. Low water content and activity can inhibit microbiological activity and prolong preservation period (Akhtar, et al., 2014; El-Metwally, 2015). There were various studies done by researchers from different countries such as Europe, Africa, South America, Australia and New Zealand on the influence of geographical origin on the physicochemical properties of honey and the results were found to be significant (Boussaid, et al., 2014).

2.5.1 Color

According to USDA-approved color standards, color is the first characteristic that is taken for honey classification and the honey color comes from its botanical origins. The color is ranged from light yellow, amber, dark amber or even black (Diez, Andres and Terrab, 2004). The. Analysis of color characteristic and intensity enables the determination of antioxidant potential and presence of pigment compounds such as phenolics, flavonoids and carotenoids (Moniruzzaman, et al, 2013b). According to Estevinho, et al (2008), darker honey exhibited higher amount of antioxidants. However, several factors can cause changes in the color of honey, for example, exposure to light and high temperature will cause the honey to become darker in color (White and Doner, 1980).

2.5.2 Electrical Conductivity

Electrical conductivity reflects the mineral content of honey (Nascimento, et al., 2015). It is one of the parameters to determine the physical characteristics of honey as it measures the ash and acid content in the honey (Serrano, et al., 2004). However, the electrical conductivity of honey varies according to botanical origins and geographical regions (Bogdanov, et al., 1997). It is important to note that electrical conductivity is different from ash whereby ash measures the only inorganic residues after carbonization while electrical conductivity measures all the ionizable organic and inorganic substances in honey (Andualem, 2014). However, the amount of ash showed positive correlation with electrical conductivity. High level of ash and acid content in honey is associated with high electrical conductivity (Sancho, et al., 1991).

2.5.3 Free Water Activity

Water content is an important parameter in determining a quality of food, including honey. It affects not only the microbial growth but also chemical and physical stabilities (Abramovic, et al., 2008). Moisture or water content alone is not reliable for microbial responses or any chemical reactions as it only analyses the total amount of water present. The main concern of determining the susceptibility of microbial growth is the presence of ‘free’ water in the food product which provides opportunities for binding of microbes. The availability of ‘free’ water in a sample is known as water activity (Olaitan, Adeleke and Ola,

2007). Hence, water activity (a_w) is a preferred method that is used to define the quality of a product (Decagon, 2006).

Free water can be affected by the production process or by packing and storage. Presence of high 'free' water decreases the quality and shelf life of a food product as microorganisms can grow optimally in this environment (Novasina, 2010). Therefore, it is commonly used to examine the shelf life of the food products. There are two methods in determining the water activity, including refractometer or hygrometer. The operation of measurement is very simple by just putting the sample into a close chamber until the equilibrium is achieved to give the free water activity value (US Food and Drug Administration, 2015). As an overall, water content is the quantitative measurement of water present while water activity is the qualitative measurement of product such as stability and shelf-life (Decagon, 2006).

2.5.4 Proline

Honey usually contains approximately 11-21 free amino acids (Dimins, et al., 2006). According to Wu, et al. in 2003, some amino acids possess antioxidant properties. The major amino acid that composites honey is proline which is produced by the bees in their salivary glands during the conversion of nectar to honey. It also serves as a sign of honey ripeness and sugar adulteration (Bogdanov, 1999). Refering to International Honey Commission, the minimum proline value for a genuine honey is around 180 mg/kg (Bogdanov, et al., 1997). However, the amount of proline could vary between different honey,

depends on the types of flower that bees visited during collection of nectar (Bosi and Battaglini, 1978). Values fall below 180 mg/kg could suggest that the honey is probably adulterated or non-ripen (Almeida, et al, 2013). The proline content of a honey is usually measured by using ninhydrin. When proline reacts with ninhydrin, it forms color complex which is then measured spectrometrically at 520 nm (Dimins, et al., 2006).

2.5.5 Diastase Level

Honey contains various enzymes either originated from flower nectar or produced by the bees. The common enzymes that are found in honey include diastase, invertase, catalase, glucose oxidase, peroxidases, inulase and phosphatase. These enzymes are vital as they involve in the conversion of nectar to honey (Vorlova and Celechovska, 2002). Diastase is introduced by the bees into honey. It functions to convert starch into dextrin, oligo-, di- and monosaccharides like maltose. Diastase is very sensitive to temperature and therefore it is usually used as an indicator for processing and honey freshness (Bogdanov, et al., 2000). The level of diastase is normally affected by floral origin. According to Codex Alimentarius Commission standard for honey (1994), general diastase activity of honey should be more than 8 Diastase Number while for honeys with natural low enzymes should more than 3 Diastase Number. However, inappropriate storage condition and high temperature can reduce the amount of diastase in honey (Ng, Chin and Khoo, 2014).

2.6 Heat Treatment on Honey

Heat treatment is normally involved in food processing. To our knowledge, natural nutrients tend to lost after heating as most of the bioactive compounds are unstable at higher temperature. Undeniably, such treatment can cause more or less changes in the nutritional value, chemical composition as well as antioxidant activity in food (Saric, et al., 2013). Therefore, it is important to study the consequences of food processing in order to obtain a correct and reliable interpretation of results (Nicoli, Anese and Parpinel, 1999).

Nonetheless, honey is also subjected to heat treatment during processing to reduce viscosity for facilitating the process of bottling, decrease the water content to prevent fermentation, dissolve the sugar crystal nuclei to slow down granulation, homogenize color of honey for favourable of customers and eliminate microorganisms to prolong shelf life of honey (Anklam, 1998; Abu-Jdayil, et al., 2002; Subramanian, Umesh-Hebbar and Rastogi, 2007; Irfan, 2008; Turhan et al., 2008; Guo et al., 2011). However, if the thermal treatment is not applied properly, then it could adversely damage the quality of honey (Mihaly-Cozmuta, et al., 2011). There were two ways of heat treatment proposed by Fallico, et al. (2004), which included air ventilation at 45-50°C for 4-7 days or in hot water. Other than that, there are also other alternative thermal processing methods developed to replace the conventional heating process. According to Subramanian, Umesh-Hebbar and Rastogi (2007), the alternatives included microwave heating, infrared heating, ultrasound processing, and membrane processing. Among these methods, microwave heating is the most rapid method in reducing microorganisms with lower

thermal damage (Subramanian, Umesh-Hebbar and Rastogi, 2007). Inevitably, microwaves still can alter the quality of food including honey by denaturing the proteins and reducing the activity of enzymes (Hendrickson, 2011). However, according to Chua, et al. (2014) stated that until now, there is still no guideline available on the application of heating temperature and time for a particular type of honey. The knowledge of thermal effects on the biochemical components such as vitamins and nutrients in honey samples, especially from tropical country such as Malaysia is also limited (Chua, et al., 2014).

2.6.1 The Effect of Heat Treatment on Antioxidant Properties

There are various statements regarding the effect of heat treatment on antioxidant properties of food. Some reports showed that thermal process did not cause loss of natural antioxidants in food (Hong, Barrett and Mitchell, 2004, Amin and Lee, 2005; Oszmianski, et al., 2007). Meanwhile, some reports stated that heat treatment increased the antioxidant activity in food (Dewanto, et al., 2002; Turkmen, Sari and Velioglu, 2005; Durmaz and Alpaslan, 2007).

However, Wang, Gheldof and Engeseth (2004) demonstrated that heat treatment did not cause significant effect on the antioxidant activity of honey samples. On the other hand, a group of Turkish scientists yet found out that the antioxidant activity of honey could increase upon thermal process (Turkmen, et al., 2006). According to Saric, et al. in 2013 stated that heat treatment could sometimes increase the antioxidant activity of food yet sometimes do not cause

any changes. All these antioxidant changes could be explained by the production of Maillard reaction products (MRPs) during heat treatment (Saric, et al, 2013). It is also supported by Manzocco, et al. (2001) stating that the loss of natural antioxidants during heating could be compensated by the formation of non-nutrient antioxidants like MRPs.

Besides, according to Lachman, et al (2010), the main components which contribute to antioxidant activity in honey are phenolic compounds, which originated from the pollen of flowering plants and trees. The amount of phenolics and flavonoids were found to increase proportionally with the heating temperature (Jahan, et al, 2015). Soon later in 2011, Brudzynski and Miotto subsequently found that melanoidins are the one responsible for radical scavenging capacity of honey. However, the melanoidins isolated from different foods showed different antioxidant capacity (Turkmen, Sari and Velioglu, 2005). They also suggested MRPs and phenolic content might possess the same chemical entity to exert antioxidant activity.

2.6.2 The Effect of Heat Treatment on Physicochemical Properties

According to EU Standards, the quality of honey can be characterized by various chemical and physicochemical parameters such as color, moisture, proline content, electrical conductivity and sugar content (Dimins, et al., 2006). The color of honey can be affected by heat and storage time. High temperature and storage duration can cause the honey becomes darker (National Honey

Board, 2002b). According to the research done by Turkmen, et al. in 2006, they demonstrated that increased treatment temperature would increase brown pigment formation which led to darker coloration. Their results also suggested that there was a correlation between antioxidant activity and browning of honey samples (Turkmen, et al., 2006).

According to Czipa (2012), there was no changes observed in pH, moisture and sugar content of honey samples upon heating, but proline content and electrical conductivity reduced significantly. The results revealed that the higher the temperature, the faster the changes of these parameters. Besides, the viscosity of the honey was found to decrease as the temperature increased. It suggested that higher temperature will reduce the average intermolecular forces and increase the kinetic energy of molecules, causing the molecules to become more mobile (Patil and Muskan, 2009).

However, a study done on the effects of treatment temperature and duration on honey showed interesting results. It was found that light-colored honey which had been heat-processed experienced changes in viscosity only at higher temperatures as compared to fresh untreated samples while dark-colored, heat-processed honey experienced viscosity changes at all levels of heating temperatures. The authors concluded their studies by suggesting that increase heating temperature will increase the viscosity of heat-processed honey (Abu-Jdayil, et al., 2002).

2.6.3 The Effect of Heat Treatment on Diastase and HMF activity

Heat treatment is a common processing method before placing the honey to the market. Hence, diastase activity and hydroxymethylfurfural (HMF) ultimately serve as the parameters to access the honey freshness and overheating of honey (Rotarescu and Vidican, 2010). Naturally, HMF is absent or present in very low amount in food as it is only produced during Maillard reactions (Mihaly-Cozmuta, et al., 2011).

From the experiment data established by Mihaly-Cozmuta, et al. (2011), the number of diastase decreased and HMF content increased when the heating temperature and duration increased. According to Tosi, et al (2008), reduction of diastase by heat was due to structural changes in enzyme molecules. Heat provides kinetic energy to enzymes and leads to irreversible denaturation. Therefore, when the heating temperature increases, then enzymes will gain more energy and become denatured. The paper concluded that diastase activity was more sensitive to prolongation of heating time than increasing temperature. On the other hand, increment in HMF could be explained by the increase concentration of fructose which surmounted the energy barrier and activated the Maillard reaction to form HMF compounds.

However, recent study showed that diastase number was not only affected by heating treatment but also storage time while HMF was affected significantly by both heating and storage time (Hasan, 2013). Therefore, it is important to ensure the proper storage of fresh honey to minimize fermentation, granulation and heat damage (White and Doner, 1980).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Honey Samples

Pure honey samples were obtained from a bee farm located in Bahau, Negeri Sembilan. The stingless bee honey samples were originated from *Trigona* spp., specifically *T. itama* and *T. apicalis*. Each honey sample was processed and subjected to different temperature with different processing time as displayed in Table 3.1.

Table 3.1: Types of stingless bee honey samples.

Honey sample	Heating temperature	Heating duration
Trigona apicalis	41°C	15 hours
	80°C	100 minutes
Trigona itama	41°C	8 hours
	41°C	15 hours
	80°C	100 minutes

CHAPTER 4

RESULTS

4.1 Antioxidant Assays

4.1.1 DPPH and ABTS Radical Scavenging Activities

From Table 4.1, both DPPH and ABTS radical scavenging percentage values were slightly higher in honey samples processed at higher temperature. Relatively, honey samples originated from *T. apicalis* showed greater radical scavenging activities than *T. itama*. Among all the honey samples, *Trigona apicalis* honey processed at 80°C for 100 minutes showed the highest radical scavenging activities. Besides, the radical scavenging activities also increased when the processing duration was prolonged.

CHAPTER 5

DISCUSSION

5.1 Antioxidant Properties

The antioxidant properties of honey depend greatly on the geographical origin, climatic changes, processing and storage of honey. However, the major factor that influences the antioxidant capacity is the botanical origin (Al-Mamary, Al-Meeri and Al-Habori, 2002; Beretta, et al., 2005). DPPH assay is a fast and easy method to determine the antioxidant properties of honey by measuring the ability of antioxidants to scavenge the DPPH radical. It also tests the ability of antioxidants to act as hydrogen donor. The content of DPPH radicals reduced when there is the presence of antioxidants such as polyphenols (Lim and Tee, 2007). ABTS assay is commonly used together with DPPH assay to determine the radical scavenging activity (Miller, et al., 1993). However, there is a difference between two assays even though they produce similar result. DPPH radicals only react with lipophilic antioxidants whereas ABTS radicals react with both hydrophilic and lipophilic antioxidants (Prior, Wu and Schaich, 2005).

CHAPTER 6

CONCLUSION

In a nutshell, *Trigona apicalis* honey showed greater antioxidant capacity than *Trigona itama* honey with higher radical scavenging activities (DPPH and ABTS), FRAP and AEAC values. The antioxidant capacity of honey samples tested increased along with higher processing temperature and duration. Besides, the total phenolic compounds of *T. apicalis* honey were also greater than *T. itama* honey. It showed that the amount of phenolic compounds found in honey was responsible for the antioxidant activities. However, within the phenolic compounds, the amount of flavonoids was found higher in *T. itama* honey rather than *T. apicalis* honey. It indicated that the major class of phenolic compounds which contributed to the antioxidant activities in *T. itama* honey was flavonoids. Moreover, the results suggested that more phenolic compounds and flavonoids could be liberated by higher processing temperature and duration. For the color analysis, all the honey samples were classified as dark-colored honey. Furthermore, *T. apicalis* honey again exhibited higher electrical conductivity than *T. itama* honey, which showed that *T. apicalis* naturally contained more minerals than *T. itama* honey. However, higher processing temperature would increase the electrical conductivity of honey but longer processing duration would decrease it. The level of total reducing sugar, sucrose content and free water activity of all honey samples were similar to each other with no huge variation. However, processing temperature and

duration could cause significant changes on each aspect, for example higher processing temperature increased the sucrose level and free water activity while reduced the total reducing sugar level. Nonetheless, longer processing treatment increased sucrose content and decreased total reducing sugar and free water activity in honey samples. In addition, proline content increased and diastase level decreased significantly due to higher processing temperature with longer duration. It suggested the degradation of proteins and enzymes upon heat treatment which led to higher amount of proline and lower content of diastase. Nevertheless, more parameters should be added to the analysis for the confirmation of processing treatment on the honey quality.

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