



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF HIGH PRESSURE PROCESSING ON PHYSICOCHEMICAL,
ANTIOXIDANT AND MICROBIOLOGICAL PROPERTIES OF FRESH AND
STORED MALAYSIAN HONEYS***

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ABSTRACT

The changes of physicochemical (pH, free acidity, water activity, diastase activity, hydroxymethylfurfural content, sugar analysis), antioxidant (total phenolic content, antioxidant activity with DPPH assay) and microbiological properties (total plate count, total yeast and mould count) of *Apis Mellifera*, *Trigona Itama* and *Trigona Thoracica* honeys after HPP treatment and throughout 6 months of storage. The honey was pressurized at 450 and 600 MPa with 7 minutes holding time at ambient temperature. Thermal processing of honey at 60 °C for 3 minutes was conducted as comparison with HPP. This study reported that *Trigona* spp. had higher acidity and water activity with lower diastase enzyme, sugar composition, antioxidant activity and total plate count as compare with *Apis Mellifera*. Good retention of HMF and diastase were shown in HPP-treated honey where no significant alternation ($P > 0.05$) in both HMF and diastase over storage. Antioxidant activity maintain ($P > 0.05$) while TPC declined significantly ($P < 0.05$) in HPP-treated honey during storage. HPP also demonstrated the ability to reduce total plate count after 6 months, especially total plate count of *Apis Mellifera* (reduced 860 to 680 CFU/g) in 600 MPa for 7 minutes. Hence, this study suggested that HPP at 600 MPa with 7 min processing time as evident by the retention of HMF formation, diastase enzyme and antioxidant properties while inhibiting the microorganism growth of Malaysian honey.

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LIST OF ABBREVIATIONS

AOAC – Association of Official Agricultural Chemists

AM – *Apis Mellifera*

ANOVA – Analysis of variance

BAM – Bacteriological Analytical Manual

CFU – Colony forming units

DN – Diastase number

DPPH – 2,2-diphenyl-1-picrylhydrazyl

ELSD – Evaporating light scattering detector

FDA – Food and Drug Administration

FAOSTAT – Food and Agriculture Organization of the United Nations, Statistics Division

FRAP – Ferric reducing ability of plasma

GAE – Gallic acid equivalent

HHP – High hydrostatic pressure

HMF – Hydroxymethylfurfural

HPLC – High performance liquid chromatography

HPP – High pressure processing

HTST – High temperature short time

IHC – International Harmonised Commission

meq – milliequivalents

MRPS – Maillard reaction products

SPLC – Spiral plate count

TI – *Trigona Itama*

TPC – Total phenolic content

TT – *Trigona Thoracica*

UHP – Ultra-high pressure

CHAPTER 1

INTRODUCTION

Honey is a primary and natural sweet substance produced from the gathered nectar of flowers or plants, combined with enzyme secreted by bees and concentrated in honeycomb cells. The global honey production exceeds 1.8 million tonnes in 2017 (FAOSTAT, 2018) and is increasing due to its health promoting and disease preventing effects besides of its nutritional (Alvarez-Suarez et al., 2010; Zulkhairi Amin et al., 2018). Freshly harvested honey undergoes fermentation and results in deterioration in honey quality in terms of physical and nutritional changes when stored for a period of time as it ferments (Fauzi & Farid, 2017). Conventionally, thermal treatment of honey has been used to stop the fermentation besides inactivating spoilage microorganism, reduce moisture content to retard microbial growth and delay granulation for crystallisation (Wang et al., 2004). However, commercial thermal processing of honey leads to a detrimental effect on its quality in terms of change of antioxidant activity (Chaikham & Prangthip, 2015; Kowalski, 2013; Wang et al., 2004), formation of hydroxymethylfurfural (HMF) (Escriche et al., 2009; Önür et al., 2017), reduction of diastase enzyme (DN) activity (Eshete & Eshetie, 2019; Slim et al., 2017),

off-flavour and changes in colour (Kędzierska-Matysek et al., 2016). Diastase number (DN) and HMF content are the common parameters to evaluate the quality of honey in honey industry. These two parameters are used to detect the thermal-treated intensity, storage duration of honey and adulteration of honey with sugars (Shapla et al., 2018). HMF is a cyclic aldehyde produced by sugar degradation through Maillard reaction during heat treatment or long storage period of honey (Feather, Harris, & Nichols, 1972). Extravagant amount of HMF indicates excess heating of honey, loss of honey freshness and darkening of colour (Subramanian, Hebbar, & Rastogi, 2007). HMF and its derivatives have been proved to be potent with organotoxic, carcinogenic and genotoxic effects on human health (Shapla et al., 2018). Diastase is a honey enzyme with high resistance of heat, hence, low DN is an evidence of aging or overheating of honey (De-Melo et al., 2017). The unfavourable effects of heat on honey due to its thermolabile components has encouraged non-thermal treatment processes for honey in search of a better preservation of its quality and nutrition.

High pressure processing (HPP) is a non-thermal treatment that has the potential to eliminate microorganisms, produce high quality product in preserving nutritional, physical and functional properties of food (Considine et al., 2008). HPP applies hydrostatic pressure in the regime of 50 to 1000 MPa isostatically into food (Abdul Ghani & Farid, 2007; Considine et al., 2008). HPP affects noncovalent bonds macromolecules such as enzymes and microbial cells, but give relatively restricted effects to the small molecules such as vitamins, phenols and antioxidants (Grainger et al., 2014; Wang et al., 2016). Hence HPP is excellent for extending shelf life of food while maintaining its nutritional characteristic and original freshness when compared with thermal treatment. Conversely, the effect of thermal treatment is associated by activation energy which can destroy nutrition of food (Oey et al., 2008). Al-Habsi &

Niranjan (2012) claimed that thermal treatments from 50 to 70 °C for 15 to 20 minutes had resulted a loss of antimicrobial activity in Manuka honey, but enhancement of antimicrobial effect was found with pressurized processing at 100 to 800 MPa for 15 to 120 minutes. Razali et al. (2019) also evidenced that heat treatment at 60 and 90 °C for 10 and 30 minutes illustrated detrimental effects on antioxidant activity of *Kelulut* honey, while HPP of 200 and 600 MPa for 5 and 10 minutes showed better retention of antioxidant activity.

The use of HPP on honey processing is relatively new with the first study published on Manuka honey in 2013 by Fauzi and coworkers. They have evaluated on the improvement of total phenolic content (TPC) (Akhmazillah, Farid, & Silva, 2013), antioxidant activity and colour (Fauzi, Farid, & Silva, 2014) of HPP treated and preserved Manuka honey. Both researches have proven the enhancement of high nutritional quality of high pressure processed honey in the pressure regime of 600 MPa for 10 minutes as the most appropriate treatment. Al-Habsi & Niranjan (2012) compared the effects of HPP from 100 to 800 MPa for 15 to 120 minutes and thermal treatment of 60 and 90 °C for 10 and 30 minutes on diastase number (DN) and hydroxymethylfurfural (HMF) of Manuka honey. The finding showed that DN and HMF of HPP-treated honey remained stable, but in thermal treatments, DN values decreased and HMF contents increased with temperature and time. Chaikham and Prangthip (2015) evidenced the significant increment of total phenols, flavonoids, antioxidant activities by DPPH and FRAP assays of longan-flower honeys with pressurization at 500 MPa for 20 minutes. They found higher residual phenols, flavonoids, antioxidant capacity in HPP-treated honey as compared with untreated honeys during 4 months of storage. Fauzi and Farid (2017) claimed that the effect of improved antioxidant properties of HPP-treated Manuka honey at 600 MPa for 10

minutes was not temporary as the increase of antioxidant activity and TPC was observed over 1 year's storage. Recent investigation by Razali et al. (2019) found unnoticeable change of DN in *Kelulut* honey treated at 600 MPa for 10 minutes and 3% increment of antioxidant activity by HPP-treated compared to thermal treated honey. With the few studies on the effects of HPP treatment on honey quality generalised by the HMF, DN, TPC, antioxidant activity measurements, effects on these properties changes during storage after HPP treatment is still limited.

1.1 Objective

The objective of this study was to investigate the changes of physicochemical, sugar, antioxidant and microbiological properties of Malaysian honey after HPP treatment and during storage of 6 months. The three types of honey used are from two types of bees, the honey bee *i.e. Apis Mellifera* and the stingless bees, *i.e. the Trigona Itama* and *Trigona Thoracica*. Honey bee belongs to the genus of *Apis* whereas stingless bee can be classified into two genera of *Melipona* and *Trigona* (Michener, 2007). Stingless bee honey has a distinct taste, more dilute in texture and undergoes slow crystallization compared to honeybee honey (Fabíola C. Biluca et al., 2014).

1.2 Scope of Work

In this study, the focus is given on high pressure processing (HPP) of honey, the application of emerging non-thermal technology which has the potential for high-quality and safe food products. Honey samples were pressurized at 450 or 600 MPa for 7 minutes. In thermal processing, honey samples were heated for 3 minutes at 60 °C. Treated honey samples were stored for storage studies for half year where

physicochemical, HMF, DN, predominant sugar, antioxidant analyses were measured at monthly interval from month 0 to 6 while microbiological properties of total plate count and total yeast and mould at every 6 months for 0 and 6th month.

1.3 Thesis Structure

This thesis is separate into five chapters including introduction, literature review, methodology, results and discussion and lastly the conclusion and recommendation for future works. Chapter 1 discussed an overview of honey and high pressure processing, and the objective of the project. Chapter 2 summarizes the studies of effects of high pressure processing in honey properties. The honey composition, types of honey, physicochemical, antioxidant and microbiological properties of honey were summarized in this chapter. The materials and equipment used were detailed up in chapter 3 as well as the sample preparation and experimental works. Following is chapter 4 which includes the result and discussion of the effect of honey properties towards high pressure processing and thermal process, and the changes of HPP-treated honey for 6 months of storage. Lastly, chapter 5 concludes the thesis and recommended the possible future works to investigate the application of HPP of honey.

CHAPTER 2

LITERATURE REVIEW

2.1 Honey

Honey is primary nature original sweet substance that produced from the nectar of flowers or plants, gathered and combined with enzyme secreted by bees, then concentrated in the honeycomb cells. The aroma, flavour, colour and consistency of honey are depended from the flowers that the bees have been foraging. The factors of flavour and aroma of honey are subjective, honey in darker colour tends to have strong taste, and light-coloured honey has milder taste (National Honey Board, 2011).

According to Food and Agriculture Organization of the United Nations, Statistics Division (FAOSTAT, 2018), global honey production exceeds 1.8 million tonnes in 2017 and it is increasing. The demand of high quality honey attracts the attention of public as honey is one of the major types of functional foods having satisfied the criteria of health promoting and disease preventing effects apart from being nutritional. It posses with antimicrobial, anti-inflammatory, antioxidant and antitumor effects properties (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). The antimicrobial activity of the honey occurs due to hydrogen peroxide, high osmolarity, acidity and sugar content, hence, honey can serve as natural preservative (Western,

2000). Furthermore, consuming honey can prevent diseases due to the presence of antioxidant properties and phytochemicals such as phenolic acids, flavonoids, carotenoids and amino acids (Akhmazillah, Farid, & Silva, 2012).

2.2 Honey Bee

There are approximately 20,000 species of bee in the world which have been identified until today. Honey bees represent only a small fraction throughout 20,000 species, however, it is one of the most popular bees. Honey bees vary in their traits across the species around the world that lead to differences in attributes such as temperament, colour, and productivity. Genetic is the basis from the characteristic difference of honey bee.

Honey bees belong to family *Apidae* and fall in genus *Apis*. *Apis mellifera* is the most widespread species of honey bees in the world, it also known as *Apis mellifera* honey bee. *Apis mellifera* is known as Western honey bee. It is widely believed that *Apis mellifera* honey bee first evolved 40 million years ago in African tropics and subtropics, then migrated to colder climates into Europe and Asia (Culliney, 1983; Barnsley Beekeepers Association, n.d.). The main distribution of this species include Africa, Europe and western Asia, and now they can be found in all around the world, including east Asia, Australia and America (Sammataro & Avitabile, 1998) (Figure 1). Besides that, Eastern *Apis* honey bee species are found in a vary range from northern India, across southeast Asia to Malaysia, Indonesia, Borneo, Philippines and Japan (Figure 1). The Eastern *Apis* species include *Apis cerana*, *Apis dorsata*, *Apis florea*, *Apis andreniformis*, *Apis koschevnikovi* and *Apis nigrocincta* (Barnsley Beekeepers

Association, n.d.). The distribution of the three major groups of eusocial bees – honey bees, stingless bees and bumblebees are illustrated in Figure 1 (Kwong et al., 2017).

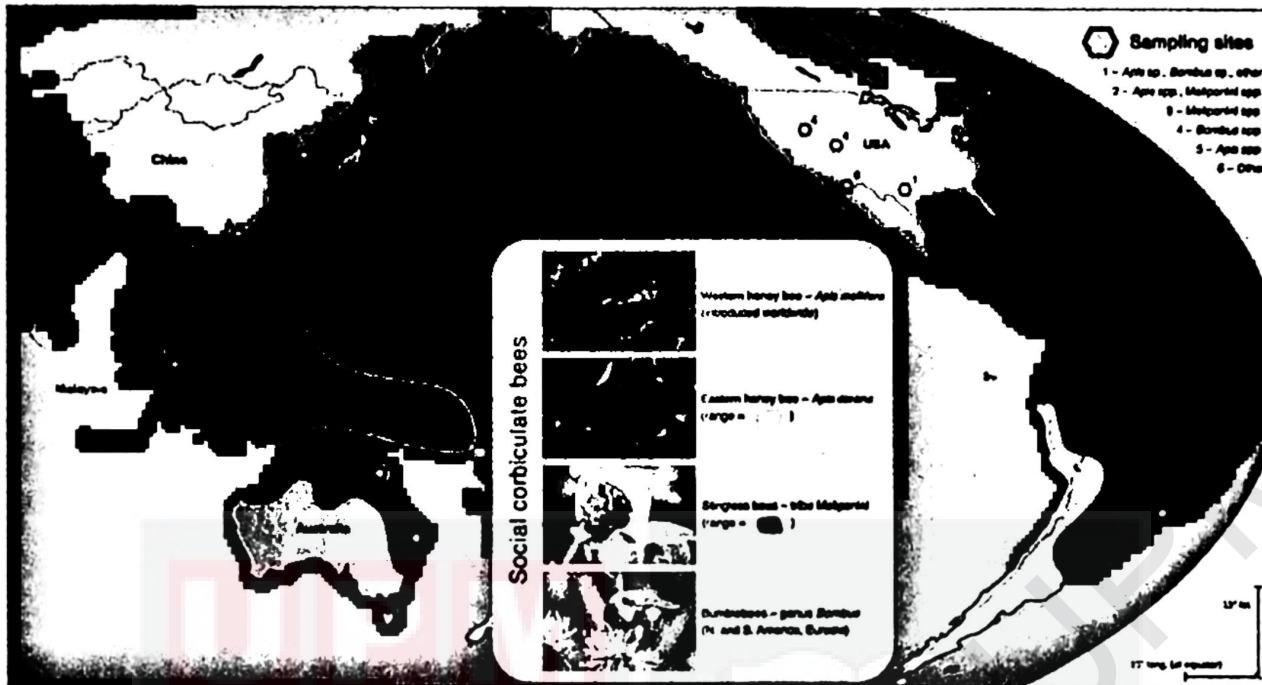


Figure 1: Distribution of eusocial bees. (Kwong et al., 2017)

Generally, honey bees are about 15 mm of body length and oval-shaped. Honey bees are brown with black bands and orange yellow rings on abdomen (Figure 2). They have hairy and reddish brown thorax and less hair on abdomen. They also have a pollen basket on their hind legs to harvest pollen and carry back to the hive. The light and dark stripes serve a purpose for the survival of the honey bee, it acts as a warning for the predators of the honey bee ability to sting (Barnsley Beekeepers Association, n.d.). Honey bees require an abundant supply of flowering plants in their habitat such as open wooded areas, gardens and meadows as their food source. They usually nest in cavities such as hollow trees and tree branches.



Figure 2: European honey bee, *Apis Mellifera*

2.3 Stingless Bee

There are approximately 500 species within stingless bee genus all around the world. Stingless bees are from the tribe of *Meliponini* in the family of *Apidae*. Stingless bees have populated tropical earth for over 65 million years, which is longer than *Apis* honey bee (Roubik, 2006). The stingless bees can be found in tropical and subtropical parts of the world such as Central and South America, Africa, Asia, and northern Australia (Boorn et al., 2010) (Figure 1). Stingless bees can be classified into 5 genera, namely, *Trigona*, *Melipona*, *Meliponula*, *Dectylurina* and *Lestrimelitta*, however, only *Melipona* and *Trigona* are the stingless bee species that can produce honey (Ngoi, 2016). In Malaysia, the *Trigona* species are vary found in Malaysia, it is also known as *Kelulut*. The most popular stingless bee species in Malaysia are *Trigona itama* (83.2%), followed by *Trigona thoracica* (11.2%) according to the study from Kelly et al., (2014).

Stingless bees are diminutive bee species with approximately 4 mm long. Stingless bees are black in colour and they have hairy thorax and legs as shown in figure 3. According to Malaysian Agricultural Research and Development Institute (MARDI), stingless bees have the ability to pollinate small-sized flowers due to their body figure which other big honey bee could not achieved (Abd Jalil, Kasmuri, & Hadi, 2017). Unlike other eusocial bees, stingless bees have atrophied sting which they are unable to sting but they will defend by biting if their nest is disturbed. Therefore, they can form very huge colonies made formidable by way of numerous defenders (Roubik, 2006).



Figure 3: Stingless honey bee, *Trigona Itama*

In Malaysia, *Trigona* spp. honey is known as *Kelulut* honey which has been claimed to exhibit outstanding antioxidant activity and qualitatively antibacterial capacity to reduce inflammation and inflection (Ewnetu, Lemma, & Birhane, 2013; Zainol et al., 2013). Stingless bee honey is unique depends on its originates from the rich vegetation in its native environments. Based on the finding from Biluca et al. (2014), they proved that stingless bee honey has a distinctive sweetness mixed with sour taste and aroma, it is more dilute in texture and undergoes slow crystallisation. As compared with *Apis Mellifera* honey that has been well-known internationally of its benefits, the antioxidant properties of stingless bee honey have gained massive attention and interest for many studies in recently (Zulhairi Amin et al., 2018). However, the distribution of stingless bee honey in world market is still comparatively lower than honey bee honey. This is due to insufficient knowledge of its composition which has led to lack of institutional quality standard, a lower shelf life and less industrial supply of stingless bee honey (Guerrini et al., 2009; Nordin et al., 2018).

2.4 Production of Honey

Honey bees collect nectar from plants and flowers. Nectar is an aqueous solution of sugars that contents 80 to 95 percent water and 5 to 20 percent sucrose

(Wykes, 1953). Nectar is sucked out of the flowers using long, tube like tongues called proboscis and stored in stomach of bees. Honey bees transport the nectar back to the hive, an enzyme called invertase that produced from hypo pharyngeal gland of bees, break down disaccharide sucrose in nectar into monosaccharide fructose and glucose (Kek et al., 2017). Then, bees create heat and air flow in the hive by fluttering their wings to evaporate water out of the nectar in order to concentrate it. Once water has been dehydrated out of the mixture with approximately 17.8% of water content, honey is produced and stored it in beeswax combs to maintain the low moisture content and move on to the next empty comb (Ngoi, 2016).

The hive of honey bees is in a series of combs that consists of two layers of hexagonal-shape cells made of wax in the nests. This is also known as comb honey. Meanwhile, the stingless bee honey is stored in clusters of small and horizontal resin pots made of cerumen near the extremities of their nests, this honey usually called as pot honey (Patricia Vit, Medina, & Enríquez, 2004).

2.5 Composition of Honey

Honey contains approximately 181 substances, mostly consists of approximately 80% of sugars and 17% of water, the remaining 3% is made up of minerals, proteins, enzymes, acids and vitamin (Alvarez-Suarez et al., 2010; Zulkhairi Amin et al., 2018). Carbohydrates are the main constituents in honey with composing about 95% of its dry weight. It is a highly complex mixture of sugars, most of which are in the immediately digestible form in the small intestine of human (Alvarez-Suarez et al., 2010). Other than that, honey also comprised with wide range of minor constituents which known as phytochemicals that have antioxidant properties. These

include polyphenols, phenolic acids, flavonoids, certain enzymes (glucose oxidase, catalase) and amino acids (Stefan Bogdanov et al., 2008). Table 1 displays the comparison of composition and physicochemical characteristics of honey bee honey, *Apis Mellifera* and stingless bee honey, *Trigona* spp.

Table 1: General physicochemical characteristics of European bee honey and stingless bee honey. (Zulkhairi Amin et al., 2018)

Physicochemical properties	European bee Honeys Manuka honey, <i>Apis mellifera</i>	Stingless bee honeys <i>Trigona</i> spp.
Appearance	Light dark brown	Amber brown
Moisture content (%)	18.70	25.00–31.00
pH	3.20–4.20	3.15–4.66
Total reducing sugars (%)	75.80	54.90–87.00
Glucose (%)	35.90	8.10–31.00
Fructose (%)	40.00	31.11–40.20
Sucrose (%)	2.80	0.31–1.26
Maltose (%)	1.20	ND
Calcium (%)	1.15	0.017
Potassium (%)	1.00	0.07
Sodium (%)	0.0008	0.012
Magnesium (%)	1.00	0.004
Specific gravity	1.39	ND
Electrical conductivity (mS/cm)	0.53	0.49–8.77
Hydroxymethylfurfural HMF (mg/kg)	400.00	8.80–69.00
Ash content (g/100 g)	0.03	0.01–0.12

ND: not detected.

Nonetheless, the composition of honey is majorly dependent on the plant source and botanical origin, besides, it also varies according to the external factors such as environmental factors, seasonal and handling processing during harvesting and storage (Kek et al., 2017). According to Nordin et al. (2018), stingless bee honey has proved to have higher moisture content, lower sugar composition, lower enzyme activity and higher acidity as compared with *Apis Mellifera* honey. Several studies claimed the composition of stingless bee honey revealed a significant difference with honey bee honey, the harmonized international standard set by Codex Alimentarius Commission is unsuitable to use to evaluate the quality of stingless bee honey (Biluca et al., 2014; Oddo et al., 2008; Souza et al., 2006).

2.6 Physicochemical Properties of Honey

Physicochemical properties are important indicators to analysis the quality of honey. The physicochemical parameters are pH, free acidity, water activity, diastase enzyme activity, hydroxymethylfurfural (HMF) content and sugar composition.

2.6.1 pH

pH of honey is usually low as honey is naturally acidic, which it is low enough to be inhibitory to the growth of microorganism (De-Melo et al., 2017). Generally, the pH of *Apis Mellifera* honey is in a range of 3.20 to 4.20, whereas pH of *Trigona* spp. honey is 3.16 to 4.66 (Zulkhairi Amin et al., 2018). There is no standard limit of pH value set by International Harmonised Commission (IHC). According to Malaysian Standard, the acceptable pH value of stingless bee honey is range in 2.50 to 3.80 (Nordin et al., 2018).

2.6.2 Free acidity

Free acidity of honey is contributes to its characteristic flavour stability against microbial attack and enhancement of chemical reactions (Al-Ghamdi et al., 2017; Cavia et al., 2007). Honey acidity is correlated to organic acids, internal esters and inorganic ions. According to IHC standard, the maximum acceptance of free acidity of honey is not more than 50 meq/kg. Free acidity increases with time and during fermentation due to the conversion of sugars into organic acids by honey yeasts (Cavia et al., 2007). Free acidity of stingless bee honey is also higher than *Apis Mellifera* due to the higher moisture content of stingless bee honey (Souza et al., 2006). Nordin et al.

(2018) summarised that the free acidity of stingless bee honey has been reported in the range from 5.9 meq/kg to 592 meq/kg.

2.6.3 Water activity

Water activity is criterion that determines microorganism spoilage instead of moisture content. Water activity (a_w) is the amount of water available to microorganism growth. Water activity of honey is mainly depending upon the concentration of glucose and fructose in the water of honey to lesser extent on some disaccharides sugar (Zamora & Chirife, 2006). Microorganism requires certain minimum water activity below which it cannot grow and minimum water activity required for osmophilic yeast found in honey is about 0.61 to 0.62 (Bogdanov, 2011). In *Apis Mellifera* honey, water activity ranges from 0.55 to 0.75 (Bogdanov, 2009), while water activity ranges from 0.76 to 0.87 for *Trigona* spp. honey (Shamsudin et al., 2019). Kek et al. (2017) reported that the water activity of *Apis* spp. honeys varied from 0.60 to 0.67, whereas *Kelulut* honey had highest water activity of 0.76. This indicated that *Kelulut* honey had high tendency of fermentation due to high water activity (Vit, Pedro, & Roubik, 2013).

2.6.4 Diastase activity

Diastase activity is a common parameter to evaluate the honey's freshness and quality in honey industry. Diastase activity is used to detect the thermal-treated intensity and storage duration of honey (Shapla et al., 2018). Diastase is part of a small fraction of proteins in honey and also known as alpha-amylase enzymes (da Silva et al., 2016). Diastase is also a honey enzyme with high resistance of heat, therefore, low

diastase number (DN) is an evidence of aging or overheating of honey (De-Melo et al., 2017). According to International Honey Commission (IHC), the set standard of DN of honey is not less than 8 diastase units, while for honeys with natural low enzymes should more than 3 diastase units (Bogdanov et al., 2009). Based on the study of Nordin et al. (2018), the diastase value of stingless bee honey were in the range of 0.15 to 32.28 DN.

2.6.5 Hydroxymethylfurfural (HMF) content

Hydroxymethylfurfural (HMF) content is widely recognised as a parameter of honey freshness. HMF is usually absent or very low amount in fresh honey and the concentration increases over time or through heating process (Shapla et al., 2018). HMF is the production of sugar degradation through Maillard browning reaction, hence the presence of high reducing sugars content can further the formation of HMF (Shapla et al., 2018). Extravagant amount of HMF indicates aging of honey, excess heating of honey, loss of honey freshness and/or adulteration of honey with sugars (Subramanian, Hebbar, & Rastogi, 2007). Therefore, the set standard of HMF content limit is 80 mg/kg for Codex Alimentarius and 40 mg/kg for European quality standard (Bogdanov et al., 2009).

2.6.6 Predominant Sugars Content

Honey sugar consists of 75% of monosaccharides and 10% of disaccharides and small amounts of other sugars (da Silva et al., 2016). Honey sugar is the most important physicochemical properties that contribute to sweetness, nutritional values, and antimicrobial factor (Doner, 1977; Jeddar et al., 1985). The monosaccharides

fructose and glucose are the major portion of sugar in honey. The summation of fructose and glucose (F + G) is used as a parameter to evaluate the quality of honey. Good quality honey should have the summation of fructose and glucose with a minimum of 60g/100g stated by International Honey Commission (IHC). According to Vit et al., (2004), the standard of (F + G) for stingless bee is lowered to 50 g/100g. Based on the findings of Kek et al. (2017), they suggested certain genera of stingless bee honey has possibility of low fructose and glucose content.

Apart from the monosaccharides, disaccharides such as sucrose and maltose are also commonly found in honey. Sucrose and maltose can be hydrolyzed enzymatically to monosaccharides. Hydrolyzation of disaccharides is carried out by invertase enzyme to convert sucrose or maltose into fructose and glucose (da Silva et al., 2016). Sucrose content is a criterion to determine the honey's purity and maturity. High sucrose content may indicate early harvest of honey due to incomplete conversion of sucrose content to monosaccharides; adulteration of honey, such as artificial high fructose syrup, cane sugar or refined beet sugar (da Silva et al., 2016; Escuredo et al., 2014). The standard limit of sucrose should not more than 5 g/100g based on International Harmonised Honey (Bogdanov et al., 2009). For Malaysian Standards, the maximum sucrose threshold of 7.5 g/100g was set to regulate honey adulteration (Nordin et al., 2018).

2.7 Antioxidant Properties of Honey

Honey is rich in antioxidant compounds such as phenolic acids, flavonoids, and amino acids (Abd Jalil et al., 2017; De-Melo et al., 2017). Antioxidant is free-radical scavengers that reduce or neutralize the formation of free radicals. Antioxidant

in honey has proved to slow down food spoilage as oxidative reactions occurs to prevent oxidation (McKibben & Engeseth, 2002). The presence of high phenolic content in honey indicates that honey possess of high radical scavenging activity, as they are highly correlated (De-Melo et al., 2017). The measurement of total phenolic content (TPC) is a fast and simple method to assist complex phenolic compounds in honey (Chua et al., 2013), while antioxidant activity is usually determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a stable radical to estimate honey's antioxidant capacity (Molyneux, 2004). Several researchers have studied regarding of antioxidant activity of honey with time showed that honey's antioxidant activity decreased over time. Šarić et al. (2012) indicated significant decrement in TPC and antioxidant activity of acacia and multi-floral honey after one year of storage. The similar finding from Chaikham and Prangthip (2015) also described that significant curtailment of antioxidant activity and TPC obtain during 4 months storage.

2.8 Microbiological Properties of Honey

One common problem that caused deterioration in honey quality is due to fermentation (Ghazali, Ming, & Hashim, 1994). Fermentation is the production of ethyl alcohol and carbon dioxide by yeast as it grows and feeds on sugar. *Saccharomyces cerevisiaewas* found as the dominant yeast fermenting honey, as it is osmophilic or sugar tolerant yeast that can withstand high sugar content and low water content (16 to 21 %) conditions (Snowdon & Cliver, 1996). *Clostridium botulinum* is associated with honey and is known to cause a disease called botulism (Küplülü et al., 2006). Although the majority of yeasts and moulds are obligate aerobes which require free oxygen for growth, their acid and alkaline requirement for growth is quite broad,

ranging from pH 2 to above pH 9 (Rawat, 2015). Water activity values needed to achieve below 0.60 in order to inhibit the growth of osmophilic yeasts that can cause fermentation of honey (Bogdanov, 2011). According to Snowden and Cliver (1996), the total plate count of honey samples can vary from zero to tens of thousands per gram without apparent reason. Yet, high total plate count honey is not likely to be marketable. According to Sereia et al. (2017), the maximum limit of yeast and mould of honey is 100 CFU/g.

2.9 Thermal Treatment of Honey Processing

Thermal processing is the most common technology for food industry to inactivate microorganism, eliminate the growth of microbial, reduce moisture content of food in order to ensure safety of the products. In honey industry, the major issue of honey is the rapid honey's quality deterioration due to fermentation (Ghazali et al., 1994). Hence, heat is heat processed before storage to reduce moisture content and viscosity, inhibit microorganism growth and to retard honey fermentation process (Subramanian et al., 2007).

Thermal treatment of honey has been conducted in a wide range of heating temperatures range from 30 to 140 °C for various holding time from a few seconds up to several hours (Sereia et al., 2017). In the commercial honey processing plant, the processes of honey are usually started with collecting of raw honey, preheating honey for purification (approximately 32 to 40 °C), straining honey from combs and filtering to clarify honey (Subramanian et al., 2007). Next, liquefaction (approximately 55 °C) and pasteurization (operated up to 70 °C) processes for 25 to 30 minutes are the two stages of thermal treatment of honey to ensure honey remains in liquid phase and to

kill the microorganisms in honey (Subramanian et al., 2007; E. A. Tosi et al., 2004). Lastly, the processed honey is cooled and packed to be stored.

Studies have shown that heating honey at 63 to 68 °C for 7.5 to 35 minutes can completely destroy yeast cells in honey (Wakhle & Phadke, 1995). Tosi et al. (2004) proved that heating honey by using high temperature-short time (HSTS) at 80 °C for 60 seconds in transient and 30 seconds in isothermal stage can inhibit yeast and fungi completely. In general, the different combination of time and temperature of thermal technique is necessary to eliminate all types of microorganisms.

2.9.1 Effect of Thermal Treatment on Honey Properties

The main aim of thermal process of honey is destroying microorganisms, facilitating packaging and delaying crystallization of honey. However, uncontrolled thermal processing of honey may cause product quality deterioration and caramelisation due to significant losses and changes occur in colour, texture, flavour, enzymatic activity and nutritive value (Slim et al., 2017). This is due to the unstable and thermolabile components in honey when it is heated at 60 °C and above (Eshete & Eshete, 2019). The quality of honey is determined based on its physicochemical, antioxidant and microbiological properties.

There are many researches that reported the heating treatment influences the quality parameters of HMF content and diastase enzyme activity, which results in increasing or decreasing the parameters respectively. Wang et al. (2004) demonstrated that HMF content of processed (55 °C for 12-16 hours) Clover honey and Buckwheat honey increased (57% and 23% respectively) higher than raw honeys. Tosi et al. (2004) also reported that HMF content of natural honey increased significantly with heating

at 100-160 °C for 60 seconds. Escriche et al. (2009) proved that HMF concentration increases with increasing temperature and treatment time. According to the finding from Tosi et al. (2008), diastase activity of natural honey reduced with increased in temperature (60-100 °C for 120-1200 seconds) and became zero at 100 °C of thermal treatment. Recent study from Kędzińska-Matysek et al. (2016) reported that heating in mild temperature (50-80 °C for 15 minutes) did not significantly degrade the quality of honey to standard set by IHC, but enhanced HMF formation and reduced diastase activity of *Brassica napus* honey.

Several studies of the effect of heat treatment on antioxidant properties of honey are leading to contradictory results. Some papers described antioxidant properties of honey decreased with heating, meanwhile, some researchers studied that it increased with temperature. Wang et al. (2004) investigated that the total phenolic profiles of clover honey decreased and antioxidant activity of buckwheat honey reduced after thermal process at 55 °C for 12-16 hours. The antioxidant constituents of longan-flower honey had no significant reduce for thermal treatments at 50 and 70 °C for 1-5 minutes, while the sample heated at 100 °C contained the lowest levels of phenols and antioxidant capacity (Chaikham & Prangthip, 2015). On the other hand, Turkmen et al. (2006) found out that antioxidant activity of sunflower, cotton and canola honey increased with increasing heating time at 50 °C to 70 °C. Šarić et al. (2013) reported that the obtained results showed uneven changes of antioxidant activity and total phenolic content among individual samples after 95 °C for 5 minutes. The antioxidant changes of honey are due to the production of Maillard reaction products (MRPS) during heat treatment (Šarić et al., 2013).

2.10 High Pressure Processing

High Pressure Processing is a non-thermal food processing technology applied when the food is subjected to high hydrostatic pressure at 100 MPa and above (Considine et al., 2008). HPP is also known as ultra-high pressure (UHP) or high hydrostatic pressure (HHP). This technology is a method of food preservation to cause destruction of micro-organisms at room or low temperature, enzymes and even spore forming bacteria can be inactivated by pressure and thermal combinations and cause changes in conformation of biopolymers leading to changes in functionality and phase changes (Knorr, 1996).

In line with the consumer demand for fresh and minimally-processed food. HPP becomes the alternative technology to maintain the quality of honey, improves product safety and allow longer shelf life. The absence of significant heating makes HPP not to break covalent bonds, therefore it eliminates undesirable effects (colour change, nutrient destruction, texture defects and off-flavour) that are produced by thermal treatment (Wang et al., 2016).

HPP process is semi-continuous and has a treatment phase that stops product throughput in the treatment cycle (Figure 4). The typical industrial high pressure system consists of a high pressure process vessel, a pressure generation means, a temperature control device and a material handling system (Elamin et al., 2015). The process of HPP starts with chamber load-up which the products are placed in the chamber, hermetically sealed and flooded with water fully. A high-pressure pump forces water into the chamber in order to produce an isostatic and instantaneous increase in pressure. Once the pressure reached the set point, it is held for the desired

holding time. At the end of the process, the vessel is depressurized rapidly before the chamber is opened, then the HPP-treated products are done (Martin, 2016).

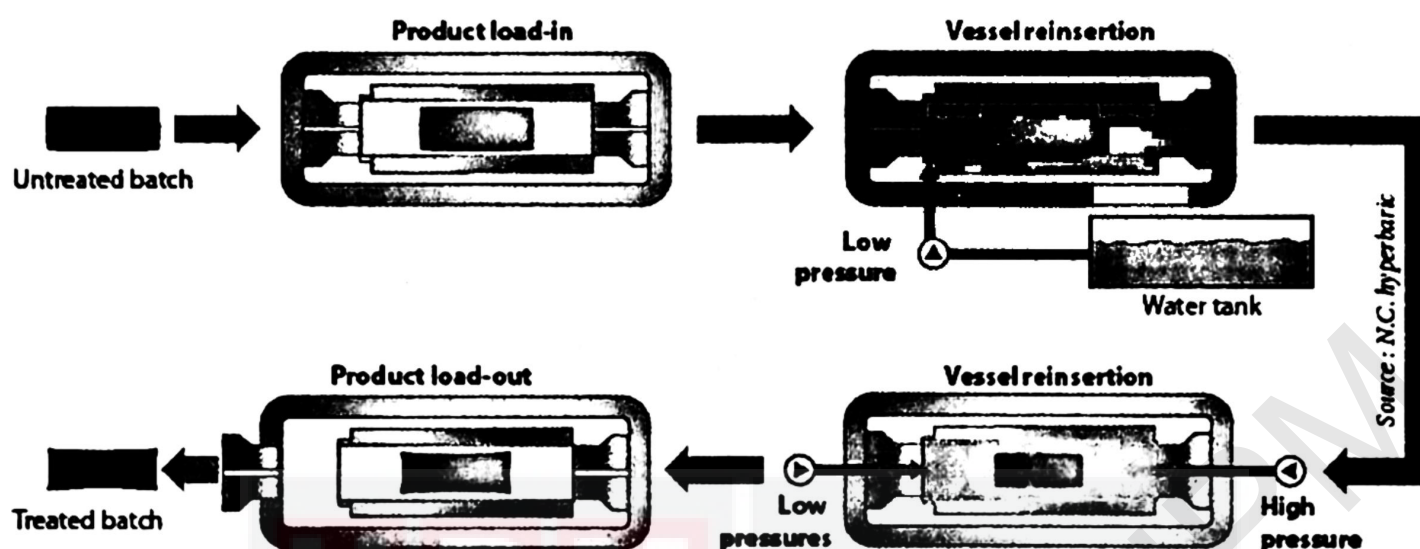


Figure 4: Schematic diagram of high pressure process

2.10.1 Principle of High Pressure Processing

The general principles of HPP that affects the behaviour of foods are based on Le Chatelier and isostatic principle.

Le Chatelier principle is the effect of pressure on the basis of absolute reaction rate theory. It states that if a system at equilibrium experience a change in concentration, temperature, volume or partial pressure, the equilibrium shifts to counteract the imposed change and a new equilibrium is established (Le Chatelier, 1884). HPP enhances reactions that are associated with volume compression. Overall volume changes favour the disruption of hydrophobic bonds and dissociation of ionic interactions. Hence, the HPP effects on low-molecular mass compounds such as vitamins, phenols and flavones are expected to be limited (Oey et al., 2008).

Isostatic principle is also known as Pascal's principle. The pressure is transmitted in a uniform manner in all directions, then the material returns to its initial shape during the decompression. The products are compressed independently of the

product size and geometry because transmission of pressure to the core is not mass or time dependent thus the process is minimized (Yordanov & Angelova, 2010).

2.10.2 Effect of High Pressure Processing on Honey Properties

High pressure processing has been known for more than hundred years to destroy microorganisms and preserve food quality. However, the HPP technology of honey started to be widely studied in recent. The quality of honey is evaluated based on physicochemical, antioxidant properties and microbiological properties.

The physicochemical parameters of honey are pH, moisture content, diastase activity (DN) and HMF formation. According to the finding from Al-Habsi & Niranjana (2012) that compared the effects of HPP and thermal treatment on DN and HMF content Manuka honey. They proved that DN and HMF of HPP-treated honey remained stable, however, DN decreased and HMF content increased with temperature and time in thermal process. Similar finding from Önür et al. (2017) also investigated a significant reduction on the formation of HMF in Turkish honey during HPP treatment for 220-330MPa for 23 minutes. Fauzi and Farid (2017) investigated that no alternation of pH and moisture content in untreated and HPP-treated Manuka honey after 1 year storage. Recent study from Razali et al. (2019) also found unnoticeable change of DN in Kelulut honey treated at 600 MPa for 10 minutes. All these researches indicate that HPP treatment has high ability to maintain diastase enzyme and slow down the formation of HMF content in honey.

Total phenolic content (TPC) and antioxidant activity are common parameters used to identify the antioxidant properties of honey. Researches proved that HPP treatment can improve TPC and antioxidant activity of Manuka honey with pressurized

at 600 MPa for 10 minutes (Akhmazillah et al., 2013; Fauzi et al., 2014). Similar findings from Chaikham and Prangthip (2015) evidenced the total phenols, flavonoids, antioxidant activities by DPPH and FRAP assays of longan-flower honeys increased significantly with pressurization at 500 MPa for 20 minutes. They also found higher residual phenols, flavonoids, antioxidant capacity in HPP-treated honey as compared with untreated honeys during 4 months of storage. Razali, Mohd Fauzi, et al. (2019) also investigated HPP (600 MPa for 10 minutes) has been shown to maintain the fresh-like quality and enhanced TPC of Kelulut honey.

Akhmazillah et al. (2012) proved that HPP has the ability to destroy and inactivate microorganisms of honey. The results showed that the high pressure more than 350 MPa and temperature higher than 40 °C is needed to completely destroy microorganisms in Manuka honey. Therefore, HPP has potential to produce high quality foods that are microbiologically safe with 'fresh-like' characteristics and improved functionalities.

CHAPTER 3

METHODOLOGY

3.1 Honey Sampling and Processing

8 kg of raw honey was collected for each of the honey, i.e. *Apis mellifera*, *Trigona itama* and *Trigona thoracica* directly from a local bee farm in Johor, Malaysia from the same batch of harvest. 200 g of honey was filled into 250 ml transparent plastic bottles suitable for HPP. The bottles were sealed and labelled before HPP.

High pressure processing (HPP) was conducted using a commercial unit, Hiperbaric 55 (Hiperbaric, Miami, USA) at Kara F&B Production Sdn. Bhd. in Semenyih, Selangor. The HPP vessel has 55 litre volume with 200 mm diameter, a pressure intensifier pump, an automated loading and unloading conveyor and a control system linked with software. Honey samples were subjected to HPP at 450 or 600 MPa for 7 minutes. For comparison with thermal processing, honey samples in 250 ml beakers were heated for 3 minutes at 60 °C in a thermostatic water bath (BS-21, JEIO TECH, Korea) filled with distilled water following the Codex Standard of Honey (Codex Alimentarius Commission, 1999). After heating, honey samples were immediately placed in ice-cooled water to ambient temperature before bottling.

Treated honey samples were stored for storage studies for half year where physicochemical, HMF, DN, predominant sugar, antioxidant analyses were measured at monthly interval from month 0-6 while microbiological properties of total plate count and total yeast and mould at every 6 months for 0 and 6th month. 7 bottles of honey samples were prepared for each honey type for the HPP at 450 MPa and 600 MPa. Similar was prepared for unprocessed as control. For thermal treatment, only 1 bottle of honey sample was prepared for the 0-month analysis for each sample. Figure 5 illustrates the overview of research framework of this study.

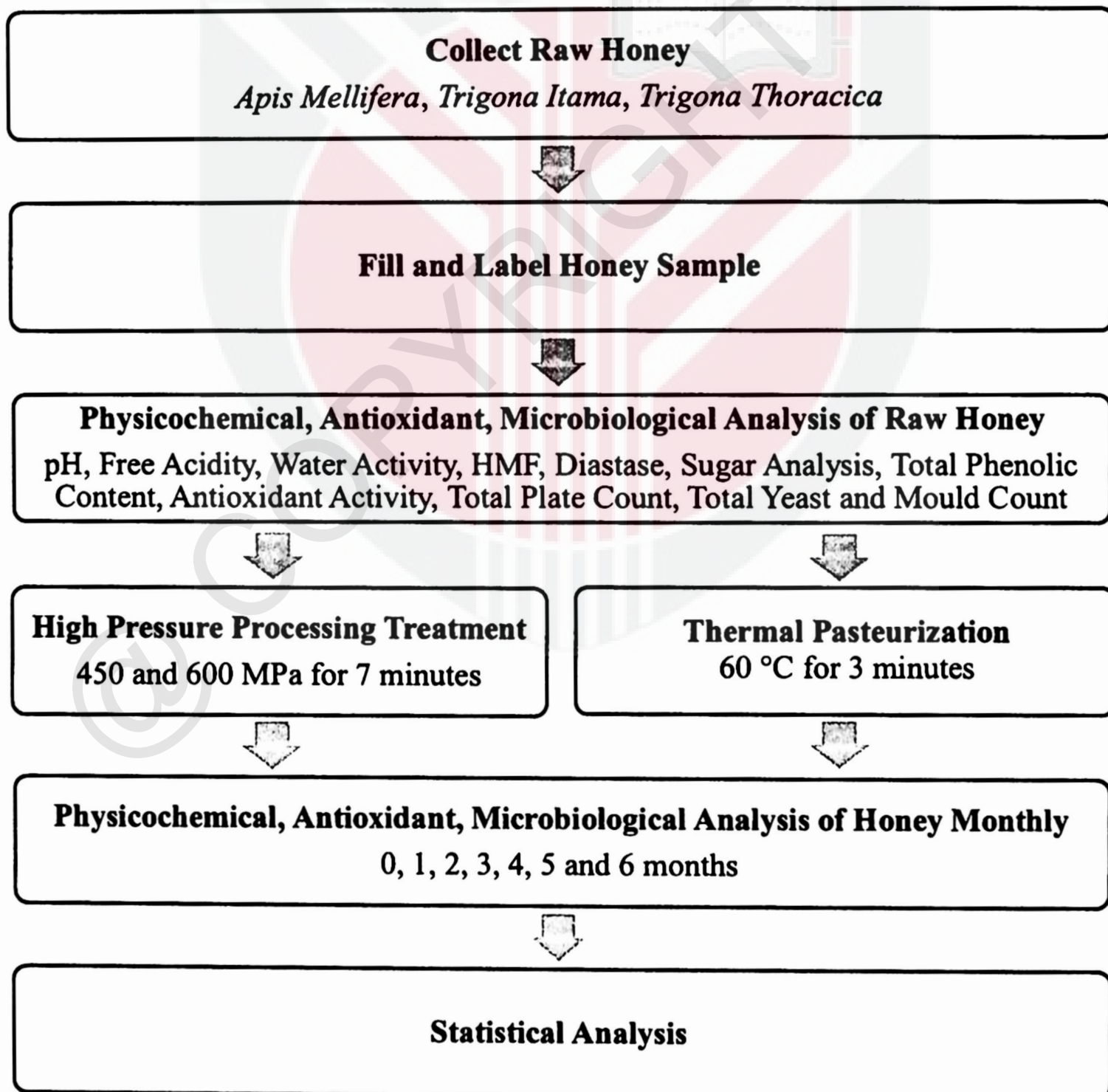


Figure 5: Overview of Research Framework

3.2 Physicochemical Analysis

3.2.1 pH and Free Acidity

The pH and free acidity was determined based on AOAC official method 962.19 (AOAC, 1990). 10 g of honey sample was dissolved in 75 ml of distilled water in a 250 ml beaker. After calibrating the pH meter (Milwaukee Mi805, USA) with a standard buffer solution with pH of 4 and 7, the pH electrode was immersed in the solution and the pH was recorded. The sample solution was titrated with 0.1M sodium hydroxide, NaOH to pH 8.30. Free acidity of honey was determined as 10 times the volume (ml) of 0.1 M sodium hydroxide, NaOH used in titration and expressed as milliequivalent acid per kg of honey (meq/kg). Each determination was done in duplicates.

3.2.2 Water Activity

Water activity of honey sample was determined by using water activity meter (AquaLab Pre, Decagon Device Inc., USA) in duplicates. Each determination was done in duplicates.

3.2.3 Hydroxymethylfurfural (HMF) Content

Hydroxymethylfurfural (HMF) content of honey sample was determined using White method as described in Harmonised Methods of International Honey Commission (Bogdanov et al., 2009). 5g of honey was weighed in a 50 ml centrifugal tube. The sample was dissolved in 25 ml of water. Carrez solution I was prepared by dissolving 7.5 g of potassium hexacyanoferrate (II), $K_4Fe(CN)_6 \cdot 3H_2O$ in water and

filled up to 50 ml. Carrez solution II was prepared by dissolving 30 g of zinc acetate, $Zn(CH_3COO)_2 \cdot 2H_2O$ and filled up to 50 ml. 0.5 ml of Carrez solution I, 0.5 ml of Carrez solution II and 0.5 ml of ethanol were added and mixed into the sample solution. Water was added and made up to the mark to 50 ml before filtering through filter paper (Grade 1 Qualitative, Whatman). The first 10 ml of the filtrate was rejected and 5 ml of filtered honey solution was pipetted into two 15 ml centrifugal tubes each. 5 ml of water was added into one of the centrifugal tubes, mixed well and known as sample solution. 5 ml of 0.2% sodium bisulphite solution was added to the second test tube, mixed well and known as the reference solution. The absorbance of the sample solution against the reference solution were determined at 284 nm and 336 nm using UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA) in duplicates.

The HMF content was calculated using equation 3.1:

$$\text{HMF in mg/kg} = (A_{284} - A_{336}) \times 149.7 \times 5 \times D/W \quad (3.1)$$

where A_{284} and A_{336} are the absorbance at 284 nm and 336 nm respectively, D is dilution factor, W is the weight of honey sample in gram.

3.2.4 Diastase Activity

Diastase activity (DN) of honey sample was determined using Phadebes method as described in Harmonised Methods of International Honey Commission (Bogdanov et al., 2009). 0.5 g honey was weighed into 15 ml centrifugal tube. Acetate buffer was prepared by dissolving 13.6 g of sodium acetate trihydrate in water, the pH of the solution was adjusted to 5.2 with glacial acetic acid and diluted to 1L with water. The honey solution was dissolved in acetate buffer and filled to 50 ml mark. A blank solution was prepared by pipetting 5 ml acetate buffer into another tube. 5 ml of the

honey solution was transferred into 15 ml centrifugal tube and placed in water bath at 40 °C for 5 minutes. Both solutions were added with a Phadebas tablet and mixed using a vortex mixer. The solutions were placed in water bath at 40 °C, 130 rpm for 30 minutes. 0.5 M sodium hydroxide, NaOH was added into the solutions and mixed using vortex mixer. The solutions were filtered immediately through filter papers (Grade 1 Qualitative, Whatman). The absorbance of filtrate was measured at 620nm using UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA). Distilled water was used as reference. The absorbance of the blank was subtracted from that of the sample solution (ΔA_{620}). Each determination was done in duplicates.

The diastase activity was calculated using equation 3.2:

$$DN = 28.2 \times \Delta A_{620} + 2.64 \quad (3.2)$$

where 28.2 and 2.64 are the slope and the intercept of the best straight line obtained by linear regression of ΔA_{620} on DN respectively.

3.2.5 Sugar Analysis

Sugar contents of honey samples were determined using a high performance liquid chromatography (HPLC) with an evaporating light scattering detector (ELSD) as described in AOAC Official Method 977.20 (AOAC, 2000). 2.5 g of honey was weighed into a 50 ml centrifugal tube and was dissolved in 20 ml water. 12.5 ml of methanol was pipetted into the honey solution and filled to the mark with water. The solution was poured through a membrane filter and collected in sample vials. The chromatographic separation was performed using a Prevail carbohydrate ES column (5 μm particle size, 250 \times 4.6 mm inner diameter) with an isocratic elution mobile phase of acetonitrile-water (75:25, v/v). The injection volume of the samples was 10

μl at flow rate of 1.3 ml/min. Honey sugars were identified and quantified by comparing the retention times and the peak area of honey sugars with those of the standard sugars. Summation of fructose and glucose (F + G) and ratio of fructose to glucose (F/G) were calculated. Determinations were done in duplicates.

The mass percentage of the sugars, W in g/100g was calculated as equation 3.3:

$$W = A_1 \times V_1 \times m_1 \times 100 / A_2 \times V_2 \times m_0 \quad (3.3)$$

where A_1 and A_2 are the peak areas of the given sugar compound in the sample solution and standard solution respectively, V_1 and V_2 are the total volume of the sample solution and standard solution respectively in ml, m_1 is mass amount of sugar in gram in the total volume of standard, and m_0 is sample weight in gram.

3.3 Antioxidant Analysis

3.3.1 Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined following the spectrophotometric Folin-Ciocalteu method as described by Singleton, Orthofer, & Lamuela-Raventós (1999) with minor modification. Each 1 g of honey sample was diluted to 20 ml with distilled water. 1 ml of honey solution was then mixed with 5 ml of 0.2 N Folin-Ciocalteu reagents. A control was prepared using 1 ml distilled water instead of the honey solution. The mixture was incubation in the dark for 5 minutes. 4 ml of 75% w/v aqueous sodium carbonate solution was added and the mixture was incubated in the dark at room temperature for 2 hours. The absorbance of the reaction mixture was measured at 765 nm against a distilled water blank using the UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA). Gallic acid

was used to produce the standard calibration curve with concentration from 5 to 100 µg/ml. The total phenolic content was expressed in mg of gallic acid equivalent (GAE) per kg of honey. Determinations were done in duplicates.

3.3.2 Antioxidant Activity (DPPH assay)

Radical scavenging activity was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a stable radical following Chaikham & Prangthip (2015) and Molyneux (2004) with some modifications. 0.5 g of honey was added with 10ml of methanol. 2ml of supernatant were mixed with 2ml of 0.1mM DPPH radical methanol solution. Methanol was used as blank and a control was prepared using 2ml methanol instead of the honey solution. The mixture was mixed well and incubated in the dark for 30 min at room temperature. Absorbance was immediately measured at 517nm using an UV-VIS spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, USA). Determinations were done in duplicates.

The radical scavenging activity was calculated using equation 3.4:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_s / A_c)] \times 100\% \quad (3.4)$$

where A_s and A_c are the absorbance of sample and control, respectively.

3.4 Microbiological Analysis

3.4.1 Total Plate Count

The total plate count is intended to indicate the level of microorganism in a product. Total plate count of honey was determined by using FDA, Bacteriological Analytical Manual (BAM) Chapter 3 Method. Spiral plate count (SPLC) method is an

official analysis developed by AOAC and APHA. The spiral plate count method conforms to AOAC Official Methods of Analysis 977.27 (BAM, 1998b). A mechanical plater inoculated a rotating agar plate with liquid sample. The sample volume dispensed was decreased as the dispensing stylus moved from the centre to the edge of the rotating plate. The microbial concentration was determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determined microbial densities between 500 and 500,000 microorganism/ml. Additional dilutions may be made for suspected high microbial concentrations.

3.4.2 Total Yeast and Mould Count

Total yeast and mould count of honey was determined by using FDA BAM Chapter 18 Method (BAM, 1998). Spread-plate method was used for plating and incubation the samples. 0.1 ml of each dilution was aseptically pipetted on pre-poured, solidified DRBC agar plates and inoculum was spread with a sterile, bend glass rod. Each dilution was plated in triplicate. The plates were incubated in dark at 25 °C. The plates were count after 5 days of incubation. If there was no growth at 5 days, the plates were re-incubate for another 48 hours. Plates containing 10-150 colonies were counted. If mainly yeasts were present, plates with 150 colonies were usually countable. However, if substantial amounts of mould were present, depending on the type of mould, the upper countable limit may have to be lowered at the discretion of the analyst. The results were reported in colony forming units (CFU/g) based on average count of triplicate set.

3.5 Statistical Analysis

Results were given as mean \pm standard deviation of duplicated samples. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at confidence level of 0.05 ($P < 0.05$) among the mean values by using Tukey's test. All statistical analyses were performed with Minitab statistical software (Version 18, Minitab Inc., USA).



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physicochemical Properties of Honey Samples

Table 2 shows the pH value, free acidity, water activity, hydroxymethylfurfural (HMF) content and diastase activity (DN) of samples immediately after treatment. Unprocessed honey can be regarded as fresh or raw honey as control. Both HPP and thermal treatment of stingless bee honeys, the *Trigona Thoracica* and *Trigona Itama* changed very slightly with significant difference ($P>0.05$). For *Apis Mellifera* from the honey bee, both HPP and thermal treatments have lowered its pH. Malaysian honey is generally acidic in nature and its pH is usually low which helps to inhibit growth of microorganisms (Adenekan et al., 2012; De-Melo et al., 2017). Both stingless bee honeys, the *Trigona Thoracica* and *Trigona Itama* have lower pH values when compared with *Apis Mellifera*. The lower acidic value is the cause of sourness in stingless bee honey (Fuad et al., 2018). The pH of *Apis Mellifera* honey was found in the range of 3.20-4.20 (Zulhairi Amin et al., 2018). The pH values for *Trigona* spp. honey has been reported in the range between 2.79-2.95 by Fatima et al. (2017) and 2.40-3.40 by (Lani et al., 2017). The acceptable pH value of stingless bee honey is range in 2.50 to 3.80 following the Malaysian Standard (Nordin et al., 2018). A low

pH of honey suggests honey acidity which is important for its characteristic flavour and stability against microbial attack (Al-Ghamdi et al., 2017), thus reflected in the lower free acidity values in stingless bee honey. Ananias et al. (2013) reported that moisture content and acidity levels of honey were positively correlated. The high moisture content in stingless bee honey has frequently proved its the higher free acidity than the *Apis* spp (Souza et al., 2006). Free acidity of honey is the presence on the production of organic acids, internal esters and inorganic ions (De-Melo et al., 2017). HPP treatment has generally reduced free acidity of the stingless bee honey while heat treatment has given both effects, increase for *Thorocica* but decrease for *Itama*. There were no changes in free acidity of *Apis Mellifera* by both HPP and thermal treatments. Recently, no previous study in the changes of free acidity of honey on HPP treatment was found.

Both *Trigona* spp. have higher water activity than the *Apis Mellifera* honey. This is due to higher moisture in *Trigona* spp. honey compared to *Apis Mellifera* honey (Souza et al., 2006). In *Apis Mellifera* honey, water activity ranges from 0.55 to 0.75 (Stefan Bogdanov, 2009) while water activity ranges from 0.76 to 0.87 for *Trigona* spp. (Shamsudin et al., 2019). As expected, thermal treatment will reduce water activity of honey which is seen in the decrease in both *Trigona* spp. (significantly) and *Apis* spp. (non-significantly) honeys. HPP treatment did not give changes in water activities of all honey. Currently, limited research is available regarding on the change of water activity of honey on HPP treatment. Water activity is the amount of water available for microorganisms to grow, which act as a criterion for the determine of bacterial spoilage. Rockland and Beuchat (1987) proved that the amount of free water is response to the growth of yeast instead of moisture content. Water activity less than 0.60 can restrain osmophilic yeast growth and honey fermentation (Stefan Bogdanov,

2009; De-Melo et al., 2017). The high water activity of *Trigona* spp. is the reason of the higher tendency of honey fermentation compared with *Apis Mellifera* honey.

No record of HMF content and diastase number (DN) were found in *Trigona* spp. honeys. The HMF indicates that the stingless bee honeys are freshly harvested while the zero DN implies it has very low or no diastase enzyme. This agrees with other studies where very low DN values of 0.4 ± 0.5 was found in *Trigona Carbonaria* honey (Oddo et al., 2008), 1.62 ± 0.23 for *Kelulut* honey (Razali et al., 2019) compared to *Apis* honey. Both HPP and thermal treatment did not give any effect to the HMF and DN of both *Trigona* and *Apis* honeys despite *Apis* honey has recorded positive values of HMF and DN. Al-Habsi & Niranjan (2012) showed that HMF and diastase number of Manuka honey was also unaffected in their HPP treatment. Razali et al. (2019) also reported that DN of *Kelulut* honey had no significant change with pressurized at 600 MPa for 10 minutes. These honey samples displayed HMF content within the limit set by International Honey Commission (IHC)'s Codex Alimentarius for European honey quality standard of HMF content 40 mg/kg and with a higher limit of 80 mg/kg for tropical honey (Bogdanov et al., 2009). The set standard of DN of not less than 8 diastase units, and 3 diastase units for honeys with natural low enzymes (Bogdanov et al., 2009).

Table 2: Physicochemical properties of fresh, HPP and thermal treated honey

Treatment	pH	Free Acidity (meq/kg)	Water Activity	HMF Content (mg/kg)	Diastase Activity (DN)
<i>Trigona Thoracica</i>					
Unprocessed	2.76 ± 0.02^a	425.00 ± 7.07^c	0.861 ± 0.002^a	0.00 ± 0.00^a	0.00 ± 0.00^a
HPP at 450 MPa	2.82 ± 0.02^a	$436.00 \pm 5.66^{b,c}$	0.865 ± 0.000^a	0.00 ± 0.00^a	0.00 ± 0.00^a
HPP at 600 MPa	2.80 ± 0.00^a	$453.00 \pm 1.41^{a,b}$	0.860 ± 0.001^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Thermal at 60 °C	2.82 ± 0.03^a	461.00 ± 1.41^a	0.855 ± 0.000^b	0.00 ± 0.00^a	0.00 ± 0.00^a
<i>Trigona Itama</i>					
Unprocessed	3.05 ± 0.02^a	165.00 ± 4.24^a	$0.804 \pm 0.003^{a,b}$	0.00 ± 0.00^a	0.00 ± 0.00^a
HPP at 450 MPa	3.06 ± 0.02^a	$145.00 \pm 4.24^{b,c}$	0.813 ± 0.001^a	0.00 ± 0.00^a	0.00 ± 0.00^a

HPP at 600 MPa	3.05 ± 0.00 ^a	156.00 ± 0.00 ^{a,b}	0.806 ± 0.002 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
Thermal at 60 °C	3.10 ± 0.04 ^a	143.00 ± 1.41 ^c	0.796 ± 0.002 ^b	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
<i>Apis Mellifera</i>					
Unprocessed	3.47 ± 0.01 ^a	54.00 ± 2.83 ^a	0.656 ± 0.027 ^a	16.39 ± 2.43 ^a	13.95 ± 5.45 ^a
HPP at 450 MPa	3.41 ± 0.01 ^b	53.00 ± 1.41 ^a	0.723 ± 0.011 ^a	17.14 ± 0.11 ^a	15.70 ± 1.25 ^a
HPP at 600 MPa	3.42 ± 0.00 ^b	53.00 ± 1.41 ^a	0.688 ± 0.001 ^a	15.87 ± 0.42 ^a	19.29 ± 0.10 ^a
Thermal at 60 °C	3.42 ± 0.00 ^b	55.00 ± 1.41 ^a	0.660 ± 0.026 ^a	16.39 ± 1.16 ^a	18.95 ± 0.87 ^a

Mean ± standard deviation values in the same column with different superscript letters are significantly different ($P < 0.05$) in each type of honey.

4.2 Predominant Sugar of Honey Samples

Honey is also defined as a supersaturated sugar solution as its main constituents are sugars forming carbohydrates in honey (Alvarez-Suarez et al., 2010). The reducing sugars fructose and glucose are often reported as the major proportion of honey sugar. Table 3 shows that the highest fructose and glucose content was detected in *Apis Mellifera* honey at 68.15 g/100g and 65.20 g/100g respectively, whereas *Trigona Thoracica* had the lowest fructose content of 23.43 g/100g and *Trigona Itama* had the lowest glucose content of 27.85 g/100g. According to International Honey Commission (IHC), the set standard of the summation of fructose and glucose (F + G) should not be less than 60 g/100g for a good quality European honey (Bogdanov et al., 2009), and the (F + G) standard has been lowered to 50 g/100g for stingless bee honey (Vit et al., 2004). The (F + G) values of all honeys had reached the limit. Studies have reported various genera of stingless bee honey having low fructose and glucose (F + G) content include the *Homotrigona Fimbriata* (22 g/100g) and *Lepidotrigona Doipaensis* (38.50 g/100g) from Thailand (Chuttong et al., 2016), *Heterotrigona Itama* (24.99 g/100g) from Malaysia (Kek et al., 2017), *Geniotrigona Thoracica* (29.64 g/100g) and *Heterotrigona Itama* (40.5 g/100g) from East Malaysia, and *Melipona Bicolor* (60.14 g/100g) from Brazil (Biluca et al., 2016).

The ratio of fructose to glucose (F/G) is honey flavour indicator. Fructose tends to give a sweeter taste than other sugars whereas glucose content is dependent from its nectar source collected by bees (Chua & Adnan, 2014). *Apis Mellifera* honey was the sweetest honey as it had the highest F/G ratio of 1.05. *Trigona Thoracica* honey had the lowest F/G ratio of 0.51 and this stingless bee honey is most sourish. The *Apis Mellifera* also had the highest sucrose content of about 15 g/100g. No sucrose content was found in *Trigona Thoracica* honey and relatively low range of 1.04-1.71 g/100g in *Trigona Itama* honey. Sucrose content is a criterion to determine the purity of honey (Aljohar et al., 2018) as measured by the incomplete sucrose conversion into fructose and glucose by invertase enzymes which is also known as honey immaturity (Chua & Adnan, 2014). High sucrose level in honey can be resulted by adulteration with high inverted sugar syrup, beetroot sugar or cane sugar (Chua & Adnan, 2014), thus Codex Alimentarius Committee has set the standard limit of sucrose to be not more than 5 g/100g (Bogdanov et al., 2009). For Malaysian Standards, the maximum sucrose threshold is 7.5 g/100g (Nordin et al., 2018). This *Apis Mellifera* honey did not comply with the standard set by IHC and Malaysian Standards. In this study, HPP treatment of honey has increased the sucrose content significantly while fructose, glucose and maltose contents insignificantly. Leyva-Daniel et al. (2017) found that no significant changes in fructose and glucose of HPP-treated multifloral honey from Mexican were observed. As no research has been conducted on the effect of HPP treatment on sucrose and maltose of honey, further investigation is needed to support this observation.

Table 3: Predominant sugars of fresh, HPP and thermal treated honey

Treatment	Fructose (g/100 g)	Glucose (g/100 g)	Sucrose (g/100 g)	Maltose (g/100 g)	F + G (g/100 g)	F/G
<i>Trigona Thoracica</i>						
Unprocessed	23.43 ± 0.48 ^a	44.38 ± 0.65 ^a	0.00 ± 0.00 ^a	21.87 ± 0.55 ^a	67.81 ± 1.14 ^a	0.53 ± 0.00 ^a
HPP at 450 MPa	25.27 ± 1.21 ^a	48.11 ± 2.50 ^a	0.00 ± 0.00 ^a	23.80 ± 1.66 ^a	73.39 ± 3.71 ^a	0.53 ± 0.00 ^a
HPP at 600 MPa	26.95 ± 1.31 ^a	48.17 ± 2.63 ^a	0.00 ± 0.00 ^a	24.01 ± 1.28 ^a	75.12 ± 1.32 ^a	0.56 ± 0.06 ^a
Thermal at 60 °C	26.69 ± 3.16 ^a	51.88 ± 5.27 ^a	0.00 ± 0.00 ^a	26.04 ± 1.62 ^a	78.57 ± 8.43 ^a	0.51 ± 0.01 ^a

<i>Trigona Itama</i>						
Unprocessed	25.18 ± 1.35 ^a	27.85 ± 1.52 ^a	1.04 ± 0.37 ^a	39.24 ± 3.98 ^a	53.02 ± 2.88 ^a	0.90 ± 0.00 ^a
HPP at 450 MPa	25.40 ± 2.71 ^a	29.31 ± 0.03 ^a	1.18 ± 0.02 ^a	44.23 ± 0.97 ^a	54.71 ± 2.75 ^a	0.87 ± 0.09 ^a
HPP at 600 MPa	33.44 ± 2.65 ^a	35.57 ± 2.73 ^a	1.50 ± 0.15 ^a	52.12 ± 4.98 ^a	69.01 ± 5.38 ^a	0.94 ± 0.00 ^a
Thermal at 60 °C	31.34 ± 6.56 ^a	34.16 ± 7.18 ^a	1.71 ± 0.28 ^a	47.78 ± 9.40 ^a	65.50 ± 13.74 ^a	0.92 ± 0.00 ^a
<i>Apis Mellifera</i>						
Unprocessed	65.72 ± 0.30 ^a	63.59 ± 0.48 ^a	15.58 ± 0.02 ^{ab}	5.04 ± 0.01 ^a	129.31 ± 0.17 ^a	1.03 ± 0.01 ^a
HPP at 450 MPa	63.13 ± 0.39 ^a	60.32 ± 1.80 ^a	14.66 ± 0.03 ^b	4.53 ± 0.28 ^a	123.45 ± 2.19 ^a	1.05 ± 0.02 ^a
HPP at 600 MPa	68.15 ± 2.41 ^a	65.20 ± 3.52 ^a	15.98 ± 0.53 ^a	5.21 ± 0.59 ^a	133.35 ± 5.93 ^a	1.05 ± 0.02 ^a
Thermal at 60 °C	63.08 ± 6.72 ^a	63.22 ± 2.04 ^a	15.78 ± 0.23 ^{ab}	4.80 ± 0.14 ^a	126.30 ± 8.76 ^a	1.00 ± 0.07 ^a

Mean ± standard deviation values in the same column with different superscript letters are significantly different ($P < 0.05$) in each type of honey. F + G summation of fructose and glucose; F/G ratio of fructose and glucose

4.3 Antioxidant Properties of Honey Samples

Table 4 shows that the *Apis Mellifera* honey in this study had higher antioxidant activity and TPC compared to both *Trigona* spp. It is contrarily with research by Abu Bakar et al., (2017) who found that *Trigona* spp. honey had higher antioxidant activity and TPC of 435.69-516.07 mg GAE/kg and 52.33-97.30%, respectively when compared with *Apis Mellifera* honey (243.01 mg GAE/kg and 83.74%). The variation of phenolic compounds composition of honey is dependent on the floral source, geographic origin, environment factors and harvesting methods (Khalil et al., 2011). Nevertheless, TPC in honey samples correlated with its antioxidant activity. Chua et al. (2013) claimed that antioxidant capacity of honey was correlated with its biochemical constituents of total phenol, total flavonoid content and total water-soluble vitamins.

Changes in the total phenolic content (TPC) and antioxidant activity of honey was not significant ($P > 0.05$) in all honey samples for all treatments. It is similar to Razali et al.'s (2019) study who found that HPP treatment at 200-600 MPa for 5-10 minutes did not change the antioxidant activity of *Kelulut* honey while thermal treatment of 10 and 30 minutes at 60 °C has resulted slightly reduced. Several studies

of HPP treatment on honey TPC and antioxidant activity have reported contradictory results. Akhmazillah et al. (2013) and Razali et al. (2019) both have reported enhancement of TPC of Manuka honey at 600 MPa for 5-15minutes, and *Kelulut* honey at 600 MPa for 10 minutes, respectively. Improvement of antioxidant activity was reported in Manuka honey at 200-600 MPa for 10 minutes (Fauzi et al., 2014) and longan-flower honey at 500 MPa for 5-20 minutes (Chaikham & Prangthip, 2015). The contradictory results may due to difference in HPP pressure and holding time used where more comprehensive researches are needed to explain the mechanisms of pressure and holding time on antioxidant properties of honey. HPP treatment at 600 MPa for 7 minutes in this study suggested that it can maintain and make slight enhancement of the antioxidant properties of Malaysian honey.

Table 4: Antioxidant properties of fresh and HPP and thermal treated honey

Treatment	Total phenolic content (mg GAE/kg)	Antioxidant activity (%)
<i>Trigona Thoracica</i>		
Unprocessed	533.7 ± 32.2 ^a	6.59 ± 1.93 ^a
HPP at 450 MPa	548.6 ± 13.6 ^a	5.02 ± 3.25 ^a
HPP at 600 MPa	504.8 ± 30.9 ^a	3.85 ± 2.55 ^a
Thermal at 60 °C	523.2 ± 7.43 ^a	5.43 ± 4.46 ^a
<i>Trigona Itama</i>		
Unprocessed	667.6 ± 16.1 ^a	36.84 ± 2.93 ^a
HPP at 450 MPa	649.2 ± 32.2 ^a	33.88 ± 7.41 ^a
HPP at 600 MPa	596.7 ± 17.3 ^a	37.59 ± 0.20 ^a
Thermal at 60 °C	665.8 ± 21.0 ^a	41.11 ± 5.18 ^a
<i>Apis Mellifera</i>		
Unprocessed	978.3 ± 9.9 ^a	58.41 ± 6.75 ^a
HPP at 450 MPa	868.9 ± 58.2 ^a	54.89 ± 7.88 ^a
HPP at 600 MPa	969.5 ± 34.7 ^a	66.29 ± 9.21 ^a
Thermal at 60 °C	905.7 ± 21.0 ^a	64.34 ± 5.17 ^a

Mean ± standard deviation values in the same column with different superscript letters are significantly different (P < 0.05) in each type of honey

4.4 Microbiological Properties of Honey Samples

Table 5 shows the total plate count and total yeast and mould count in honey samples. While the total yeasts and moulds count in all the honey samples were consistently less than 10 CFU/g, the total plate count was in high variability. *Trigona Thoracica* had exceptional high total plate count of 17000 CFU/g. Snowdon & Cliver (1996) mentioned that total plate count of honey samples can be varied from zero to tens of thousands per gram for no apparent reason. Based on the findings from Tysset & Rousseau (1981), they found that the total plate count was in the range of 3 to 9500 CFU/g and total yeasts and moulds count was in the range of 0 to 2500 CFU/g. HPP treatment has only reduced the total plate count of *Trigona Thoracica* honey but not the *Trigona Itama* and *Apis Mellifera* honeys. Although HPP treatment and thermal processes is expected to can bring significant influence on microbial survival and growth in terms of considerable microbial inactivation and microbial growth reduction (Considine et al., 2008), these treatments however did not show in this study. Akhmazillah et al. (2012) reported that pressure above 350 MPa and temperature more than 40 °C is needed to inactivate microbial in Manuka honey. It is therefore anticipated that higher temperature may be required to inactivate microbial cells (Considine et al., 2008). The total plate count and total yeast and mould count in HPP and thermal-treated honey samples in this study are still in the acceptable limit and safe for consumption with MERCOSUL approval that the maximum yeast and mould of honey is 100 CFU/g. Sereia et al. (2017).

Table 5: Microbiological properties of fresh and HPP and thermal treated honey

Treatment	Total plate count (CFU/g)	Total yeast and mould count (CFU/g)
<i>Trigona Thoracica</i>		
Unprocessed	17000	<10
HPP at 450 MPa	30	<10

HPP at 600 MPa	20	<10
Thermal at 60 °C	30	<10
<i>Trigona Itama</i>		
Unprocessed	20	<10
HPP at 450 MPa	370	<10
HPP at 600 MPa	220	<10
Thermal at 60 °C	150	<10
<i>Apis Mellifera</i>		
Unprocessed	440	<10
HPP at 450 MPa	520	<10
HPP at 600 MPa	860	<10
Thermal at 60 °C	640	<10

4.5 Changes of Physicochemical Properties During Storage

Figure 6(a) shows that the pH of honey decreased significantly ($P < 0.05$) over 6 months' storage. *Trigona Itama* honey had more significant reduction of pH in comparison with other honeys. Both *Trigona Itama* and *Trigona Thoracica* had similar pH value after 6 months of storage. Lani et al. (2017) also reported that unprocessed *Heterotrigona itama* and *Geniotrigona thoracica* honey showed drastic decrease in the storage of 6 weeks. Similar results were found by Bath and Singh (2000) who reported that pH raw *Helianthus annuus* and *Eucalyptus lanceolatus* honeys decreased significantly with storage. pH greatly influences the flavour characteristic, stability and the shelf-life of honey (Cavia et al., 2007). The decrease of pH is due to the increase of free acidity which also may indicate fermentation of honey where honey sugar transforms into organic acids by yeasts (Nordin et al., 2018) as shown in Figure 6(b). The higher increase in free acids content of *Trigona Thoracica* honey is likely due to higher water activity and higher moisture content (Figure 7) (Jiménez et al., 1994). From the overall pH decrease, the HPP-treated honeys have maintained better in terms of slightly higher pH and less obvious increase of free acidity when compared with unprocessed throughout 6-month storage. This is similar for the Fauzi & Farid

(2017) however found no significant change in unprocessed and HPP-treated Manuka honey after 1 year of storage.

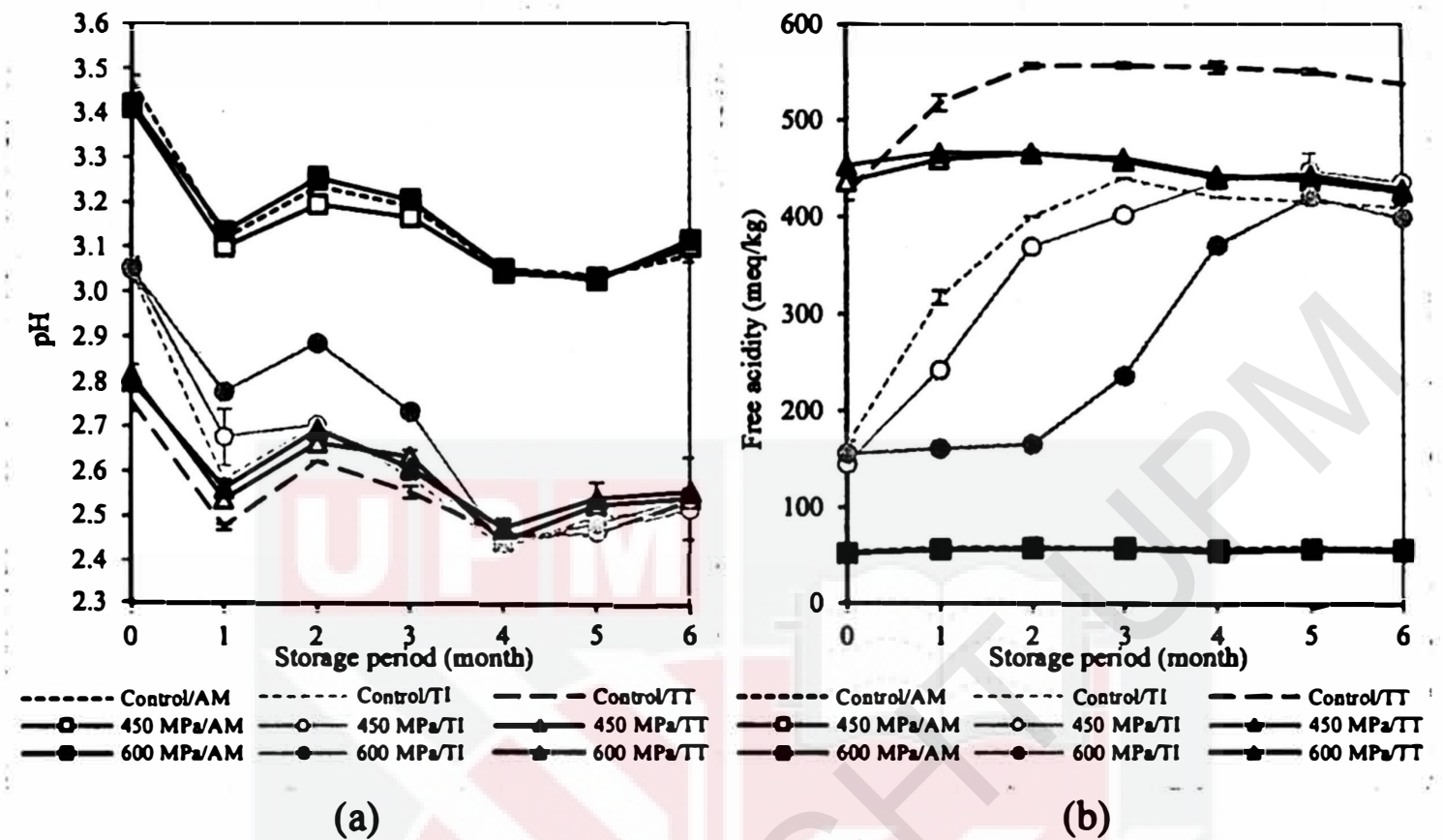


Figure 6: Physicochemical changes of HPP-treated honey stored for 6 months (a) pH, (b) free acidity (TT = *Trigona Thoracica*, TI = *Trigona Itama*, AM = *Apis Mellifera*)

Figure 7 shows the water activity of both *Trigona* spp. honeys increased significantly ($P < 0.05$) while decrease was detected for *Apis Mellifera* during storage. Water activity of honey is mostly determined by the molar concentration of main sugars, especially the monosaccharides fructose and glucose (Zamora & Chirife, 2006). The increase of water activity is due to the formation of glucose and fructose concentration (Figure 9(a) and 9(b)) in the water of honey which lowers the liquid concentration of honey (Khadra et al., 2018; Shafiq et al., 2014). In the results, water activity of stingless bee honey was influenced by HPP treatment in 600 MPa effectively, which leads to slower increment of water activity in storage.

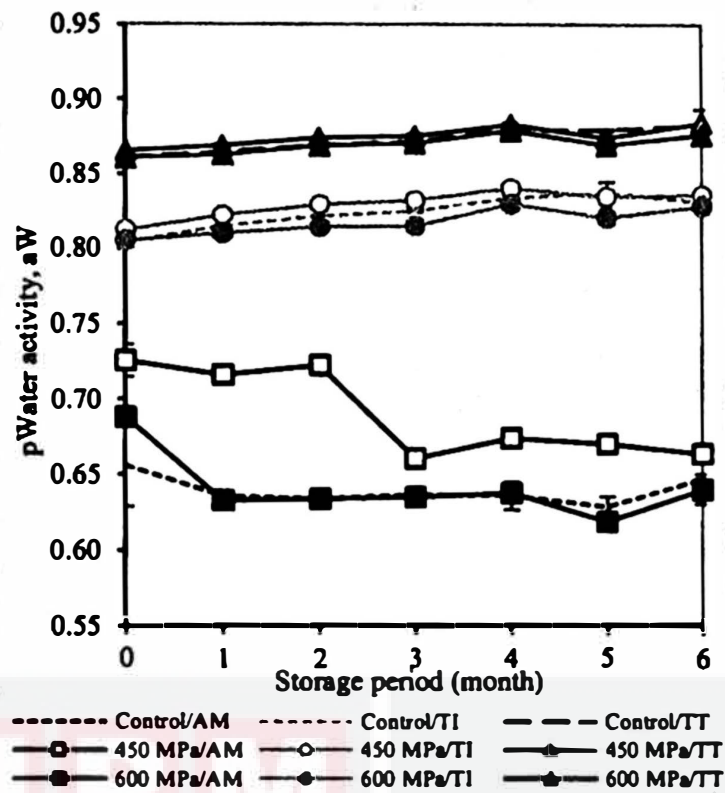


Figure 7: Change of water activity of HPP-treated honey stored for 6 months (TT = *Trigona Thoracica*, TI = *Trigona Itama*, AM = *Apis Mellifera*)

The diastase number (DN) values of all honeys during the 6 months' storage fall within the range of International Honey Commission standard with *Trigona Thoracica* and *Trigona Itama* honey recording zero (Figure 8 (a)). The changes of DN of *Apis Mellifera* honey was insignificantly with regards to storage time and treatments which implied that DN was unaffected by exposing to high pressure. The HMF content of both *Trigona* spp. honeys no significant changes ($P > 0.05$) but the *Apis Mellifera* honey has decreased significantly ($P < 0.05$) in the first month and raised insignificantly raised ($P > 0.05$) the following months. Islam et al. (2012) reported that the HMF content of raw Bangladeshi honeys remained low for 1.5 years of storage, which the finding was similar with the result from *Trigona* spp. honeys. However, the reduction of HMF in *Apis Mellifera* honey is unknown. This implied that HPP may have the tendency to degrade and impeded the formation of HMF, which requires further investigation.

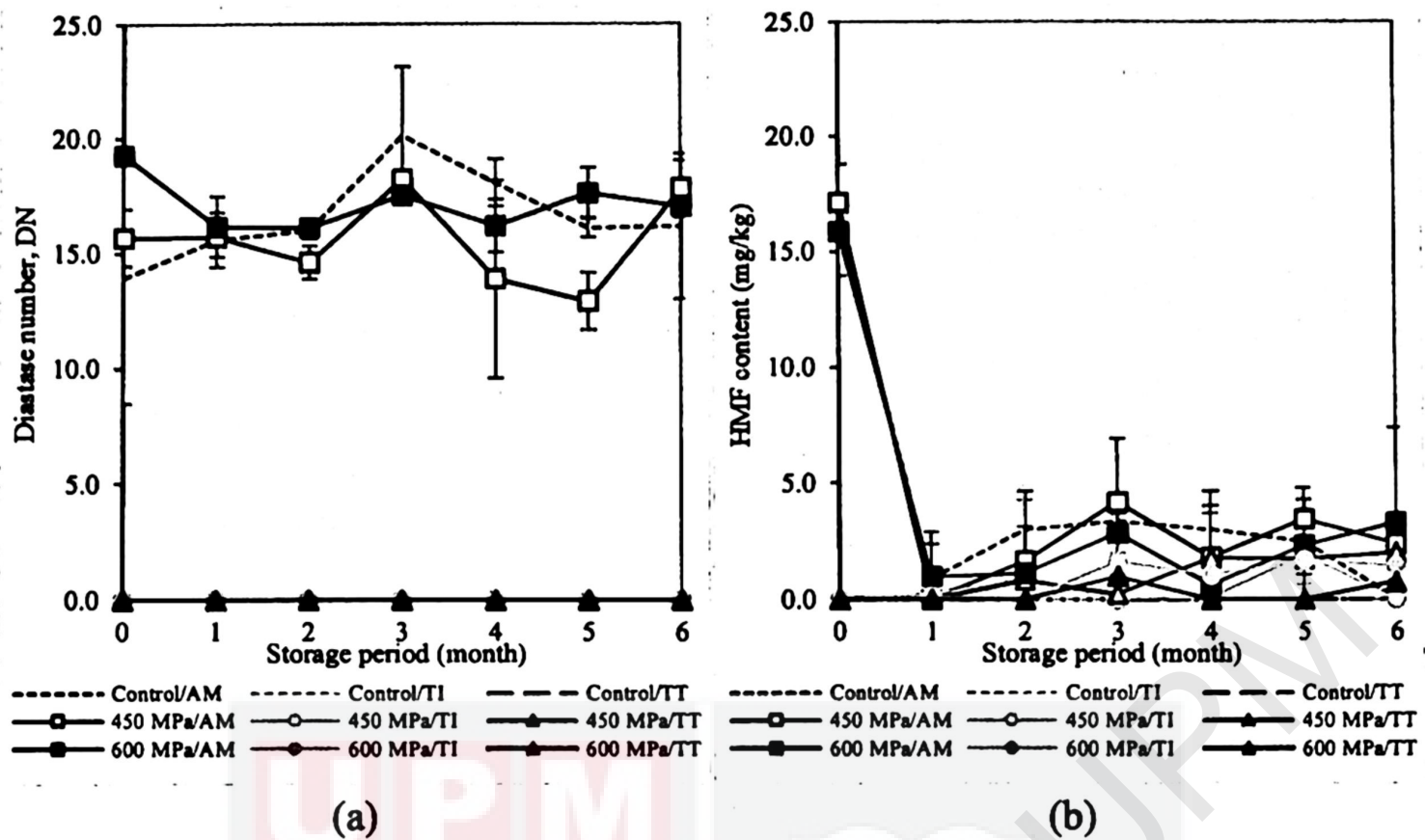
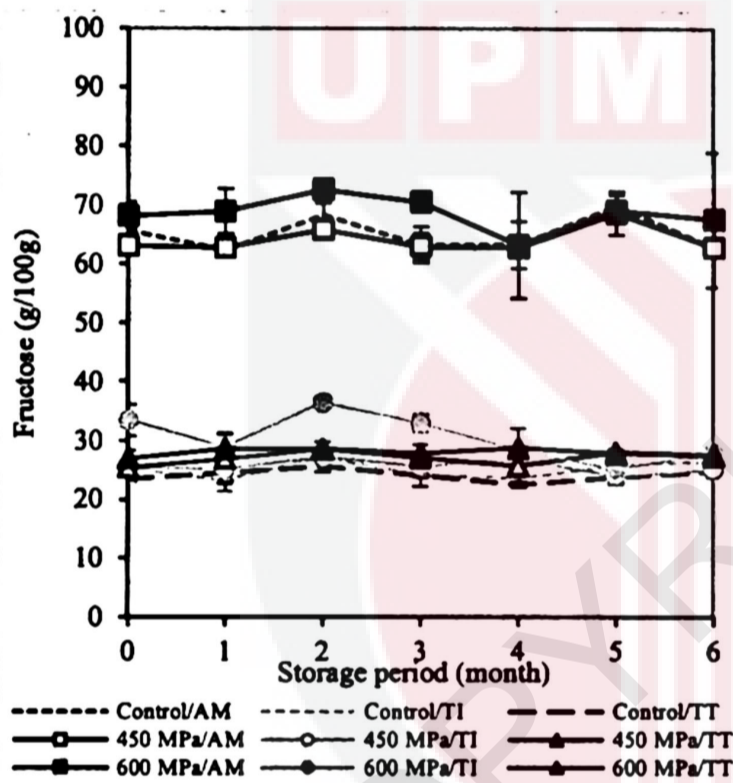


Figure 8: Physicochemical changes of HPP-treated honey stored for 6 months (a) diastase number, (b) HMF content (TT = *Trigona Thoracica*, TI = *Trigona Itama*, AM = *Apis Mellifera*)

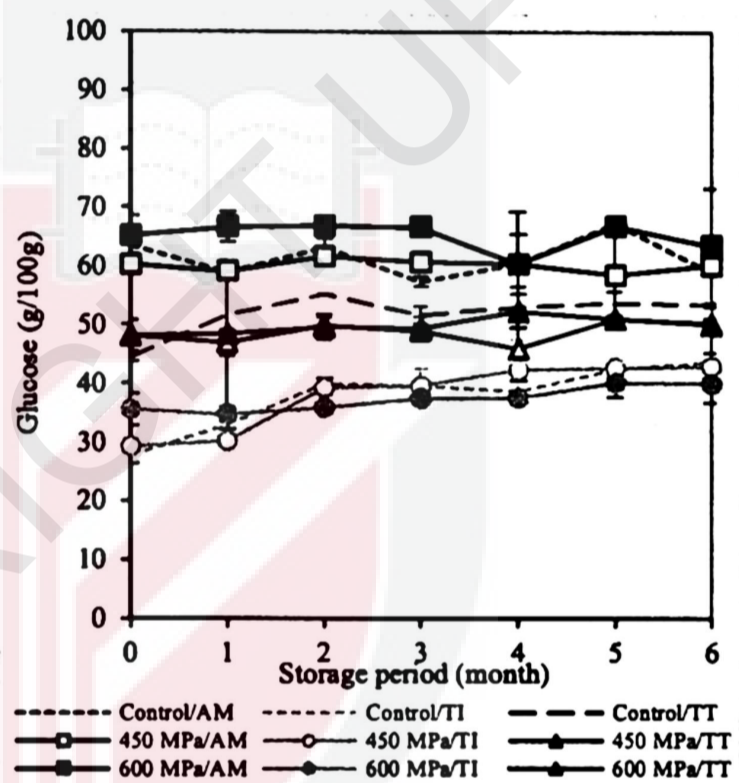
4.6 Changes of Predominant Sugar During Storage

Figure 9 shows that the fructose of all honeys increased slightly ($P > 0.05$) over storage. Glucose of unprocessed and HPP treated *Trigona Thoracica* at 450 MPa showed slight increase ($P < 0.05$) during the first 3 months and remained stable in the following months. Sucrose of *Trigona Itama* honey reduced to 0.00 g/100g after 1st month ($P < 0.05$) while significant reduction ($P < 0.05$) was observed for *Apis Mellifera* honey except for 600 MPa treatment. Maltose of *Trigona Itama* honey showed significant increase ($P < 0.05$) in unprocessed and 450 MPa treatment. The degradation of sucrose is caused by invertase enzyme that convert sucrose into glucose and fructose (Lichtenberg-Kraag, 2012). This is often linked to explain the increment of fructose and glucose of honey during storage. In the findings from Lichtenberg-Kraag (2012) in various multifloral honeys with 6 months storage, Lichtenberg proposed that the presence of invertase enzyme is interrelated with the degradation of sucrose content,

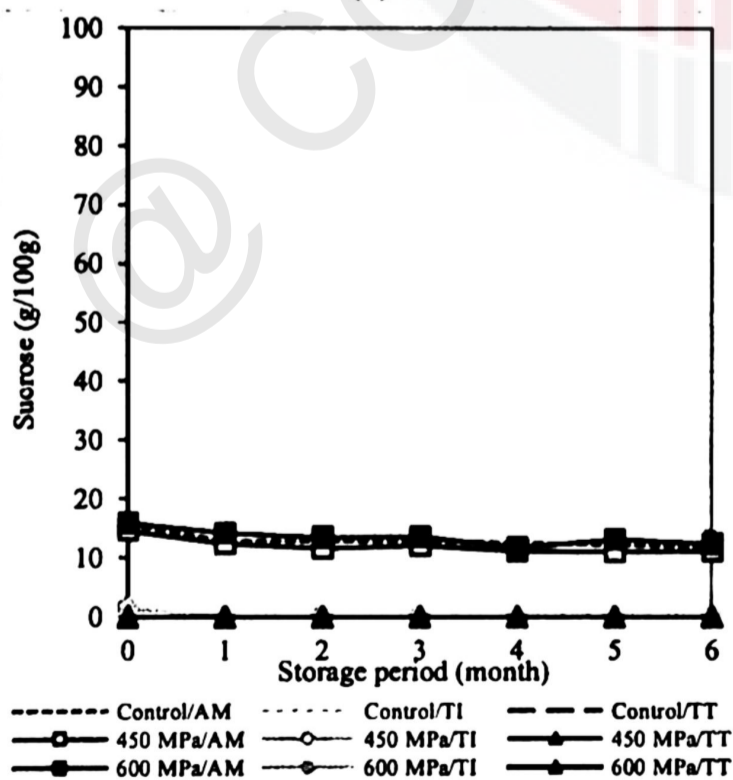
as the reduction of invertase enzyme will slow down the sucrose degradation. A similar research from Rybak-Chmielewska (2007) on unpasteurized honey stored at room temperature with half-year storage reported sucrose content decreased 79%, fructose and glucose concentration increased 7% and 8.8% respectively. No study has reported the change of sugar with HPP-treated honey during storage. No major change of sugar contents was observed in HPP treatment, this indicated that high pressure processing had the ability to maintain the sugar contents in honey.



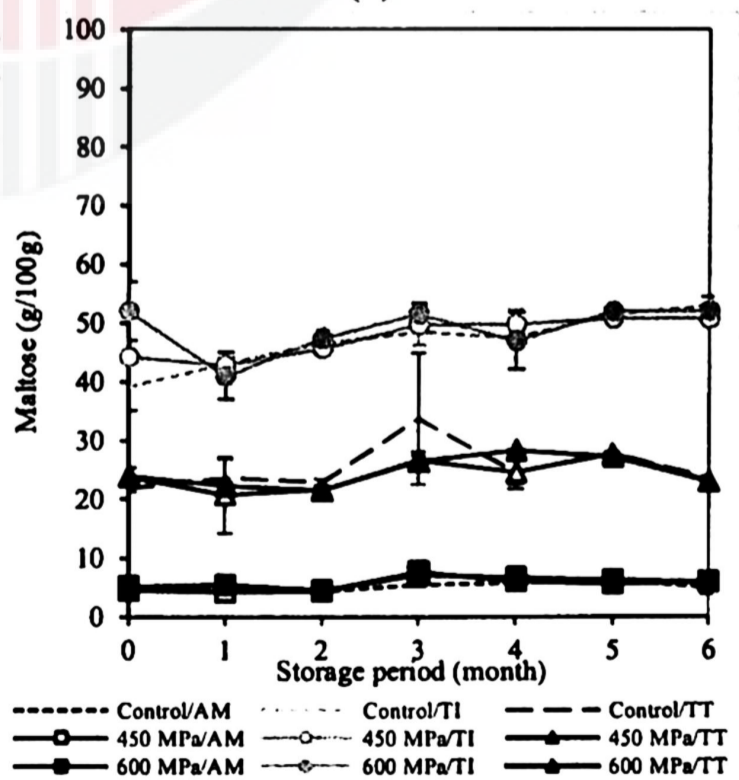
(a)



(b)



(c)



(d)

Figure 9: Changes of predominant sugar of HPP-treated honey stored for 6 months (a) fructose, (b) glucose, (c) sucrose, (d) maltose (TT = *Trigona Thoracica*, TI = *Trigona Itama*, AM = *Apis Mellifera*)

4.7 Changes of Antioxidant Properties During Storage

Figure 10 shows changes of total phenolic content (TPC) and antioxidant activity during storage. The TPC in all honey samples significantly reduced significantly ($P < 0.05$) with the increase of storage time. The TPC of HPP-treated samples however showed higher values than unprocessed sample, indicating HPP treatment could be a good preservation method for antioxidant properties of honey. The similar findings were also reported for unprocessed longan-flower honey with pressure treatment at 500 MPa for 20 minutes that exhibited significant curtailment of TPC and antioxidant activity during 4 months of storage, where HPP treated honey had higher antioxidant capacity than unprocessed honey (Chaikham & Prangthip, 2015). Šarić et al. (2012) also reported the significant decrement in TPC and antioxidant activity of untreated acacia and multi-floral honey after one year of storage.

Antioxidant activity of all honey samples had no notable changed ($P > 0.05$) in all processes throughout 6 months of storage. Gheldof and Engeseth (2002) reported no significant change of antioxidant capacity in bulk and clover honey for around 2 years storage. Contrarily to the previous findings above, Fauzi and Farid (2017) demonstrated that antioxidant activity of unprocessed and HPP-treated Manuka honey increased significantly over 1-year storage, HPP-treated honey at 600 MPa for 10 minutes exhibited higher antioxidant activity and TPC than unprocessed honey. The different findings show the effect of antioxidant properties can vary based on types of honey. This is due to the alternation of chemical composition of honey which depends on its botanical source, environment and seasonal changes (De-Melo et al., 2017; Kek

et al., 2018). The effect of intensity of pressure and holding time applied onto honey samples can also be the reason that leads to contradictory results from different findings. These research findings nonetheless showed that antioxidant properties of honey were preserved better with high pressurization.

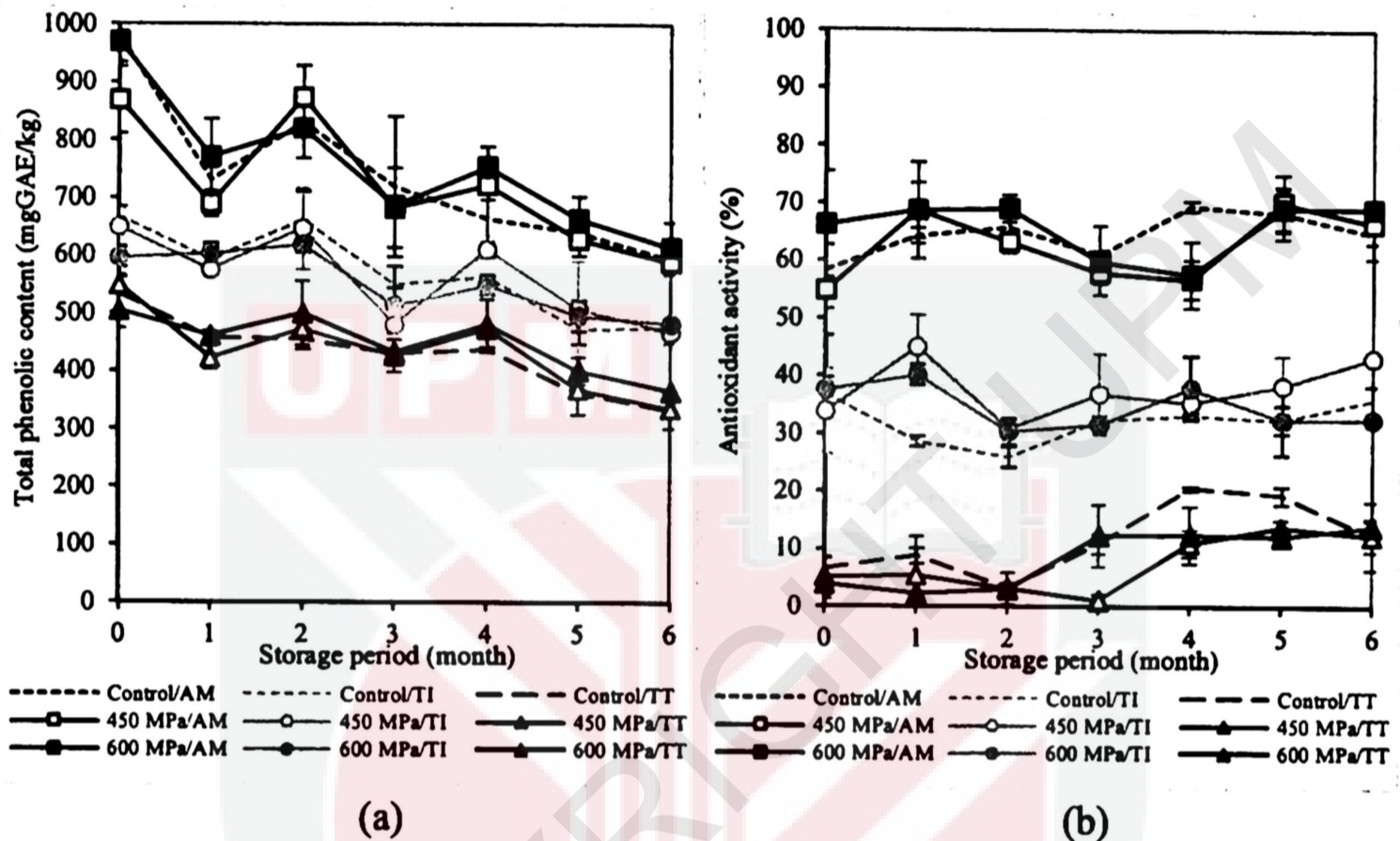


Figure 10: Antioxidant properties changes of HPP-treated honey stored for 6 months (a) total phenolic content, (b) antioxidant activity (TT = *Trigona Thoracica*, TI = *Trigona Itama*, AM = *Apis Mellifera*)

4.8 Changes of Microbiological Properties During Storage

Table 6 shows the change in total plate count and total yeast and mould count of honey after 6 months of storage. Total yeast and mould count of all honey samples remained in less than 10 CFU/g for all treatments after 6 months. The total plate count of *Apis Mellifera* honey decreased from 860 to 680 CFU/g for the 600 MPa. As expected, a higher pressure gives higher impact to microbial inactivation and reduction of total plate count in honey. Total plate count of *Trigona* spp. honeys showed significant reduction to less than 10 CFU/g after 6 months. Tuksitha et al. (2018) found that *Heterotrigona itama* had the ability to inhibit the growth of gram-positive

microorganisms, while *Geniotrigona thoracica* can inactivate both gram-positive and gram-negative microorganisms. *Trigona* spp. honeys have been postulated to have high antibacterial activity that can inhibit the growth of microbial in unprocessed honey naturally.

Table 6: Changes of microbiological properties of honey samples for 6 months of storage.

Treatment	Total plate count (CFU/g)		Total yeast and mould count (CFU/g)	
	Initial	6 months	Initial	6 months
<i>Trigona Thoracica</i>				
Unprocessed	17000	<10	<10	<10
HPP at 450 MPa	30	<10	<10	<10
HPP at 600 MPa	20	<10	<10	<10
<i>Trigona Itama</i>				
Unprocessed	20	<10	<10	<10
HPP at 450 MPa	370	<10	<10	<10
HPP at 600 MPa	220	<10	<10	<10
<i>Apis Mellifera</i>				
Unprocessed	440	720	<10	<10
HPP at 450 MPa	520	660	<10	<10
HPP at 600 MPa	860	680	<10	<10

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

HPP and thermal treatment have resulted slight change in pH of *Trigona* spp. honey and decrease of pH for *Apis Mellifera* honey. Both HPP and thermal treatments have resulted free acidity increase in *Thoracica*, decrease for *Itama* and no changes in free acidity of *Apis Mellifera*. HPP treatment did not affect water activities of all honey, but thermal treatment reduced water activity of all honey. Both HPP and thermal treated honey showed no significant effect on diastase number and HMF content. The HPP-treated honey had minimal increment of sucrose and no significant alteration was found in fructose, glucose and maltose. Both HPP and thermal process did not give any effect to TPC and antioxidant activity of *Trigona Thoracica*, *Trigona Itama*, and *Apis Mellifera*. Over a storage time of 6 months, pH decreased significantly and free acidity of honey increased over time in all type of honey. Water activity of *Trigona* spp. honey showed significantly increment, whereas a significant declination of water activity of *Apis Mellifera* during the storage. Diastase number did not show significant changes, and insignificant minimal formation of HMF was found during storage in

HPP treatment. HPP-treated honeys showed slightly degrade in sucrose content and increased in fructose and sucrose. TPC of honey reduced significantly and antioxidant activity (DPPH assay) had no notable change throughout storage. In terms of microbiological properties, HPP showed effective microbial inactivation as highly reduce of total plate count in all honeys after 6 months. HPP treatment of 600 MPa for 7 minutes is suggested as the most effective treatment towards all honey in this study. This HPP treatment demonstrated better retention in physicochemical properties, sugar contents and antioxidant capacity as compared with unprocessed honey during storage.

5.1 Recommendation

The application of emerging non-thermal technology has potential for high-quality and safe food products. A higher pressure and longer holding time give higher impact to microbial inactivation as expected. Therefore, further analysis can be carried out by investigating high pressure effects on antioxidant and microbiological properties of honey for using longer pressurizing time.

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APPENDICES

Table 7: Number of samples of unprocessed, HPP and thermal treatment

Month	Unprocessed	450 MPa	600 MPa	Thermal	Total
Initial	3	3	3	3	
1 month	3	3	3		
2 month	3	3	3		
3 month	3	3	3		
4 month	3	3	3		
5 month	3	3	3		
6 month	3	3	3		
Total	21	21	21	3	66

Calculation of HMF Content

Sample- Unprocessed *Trigona Thoricica* honey, 0 month

$$A_{284} = -0.011$$

$$A_{336} = 0.008$$

$$\text{Dilution, } D = 1$$

$$\text{Weight, } W = 5 \text{ kg}$$

$$\begin{aligned} \text{HMF in mg/kg} &= (A_{284} - A_{336}) \times 149.7 \times 5 \times D/W \\ &= (-0.001 - 0.008) \times 149.7 \times 5 \times 1/5 \\ &= -2.8 \text{ mg/kg} \\ &= 0 \text{ mg/kg} \end{aligned}$$

Calculation of Antioxidant Activity

Sample- Unprocessed *Trigona Thoricica* honey, 0 month

$$A_s = 0.417, A_c = 0.440$$

$$\begin{aligned} \text{DPPH radical scavenging activity (\%)} &= [1 - (A_s / A_c)] \times 100\% \\ &= [1 - (0.417 / 0.440)] \times 100\% \\ &= 5.23 \% \end{aligned}$$

Calculation of Sugar Content

Sample- Unprocessed *Trigona Thoricica* honey, 0 month

$$A_{1, \text{fructose}} = 3593962$$

$$,A_{2, \text{fructose}} = 12094381$$

$$A_{1, \text{glucose}} = 5779477$$

$$,A_{2, \text{glucose}} = 7733039$$

$$A_{1, \text{sucrose}} = 0$$

$$,A_{2, \text{sucrose}} = 1521101$$

$$A_{1, \text{maltose}} = 3055039$$

$$,A_{2, \text{maltose}} = 823428$$

$$V_1 \text{ and } V_2 = 0.01$$

$$m_0 = 2.5 \text{ g}$$

$$m_{1, \text{fructose}} = 2 \text{ g}$$

$$,m_{1, \text{glucose}} = 1.5 \text{ g}$$

$$m_{1, \text{sucrose}} = 0.25 \text{ g}$$

$$,m_{1, \text{maltose}} = 0.15 \text{ g}$$

$$W = A_1 \times V_1 \times m_1 \times 100 / A_2 \times V_2 \times m_0$$

$$\begin{aligned} \text{Fructose} &= (3593962 \times 0.01 \times 2 \times 100) / (12094381 \times 0.01 \times 2.5) \\ &= 23.77 \text{ g/100g} \end{aligned}$$

$$\begin{aligned} \text{Glucose} &= (5779477 \times 0.01 \times 1.5 \times 100) / (7733039 \times 0.01 \times 2.5) \\ &= 44.84 \text{ g/100g} \end{aligned}$$

$$\begin{aligned} \text{Maltose} &= (3055039 \times 0.01 \times 0.15 \times 100) / (823428 \times 0.01 \times 2.5) \\ &= 22.26 \text{ g/100g} \end{aligned}$$

$$\text{Sum of Fructose and Glucose, } F + G = 23.77 + 44.84$$

$$= 68.62 \text{ g/100g}$$

$$\text{Ratio of Fructose to Glucose, } F/G = 23.77/44.84$$

$$= 0.53$$