THE DEVELOPMENT OF KIT SYSTEM FOR HONEY QUALITY QUICK CHECK FROM DIFFERENT SPECIES OF STINGLESS BEE HONEY IN MALAYSIA

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ABSTRAK

Lebah kelulut adalah di dalam keluarga Apidae yang mempunyai keupayaan untuk menghasilkan pelbagai jenis produk yang berharga bagi manusia seperti madu, roti lebah, dan propolis. Permintaan madu yang semakin meningkat di pasaran telah menyebabkan berlakunya madu dicampur dengan bahan asing serta telah meningkatkan pengeluaran madu tiruan. Terdapat banyak ujian makmal untuk analisa madu tiruan namun kebanyakan ujian adalah rumit dan melibatkan kos yang tinggi . Oleh itu, perlu ada pembangunan dan ujian berterusan kepada kaedah alternatif yang membolehkan madu tiruan dikesan dengan lebih cepat dan mudah. Kajian ini telah membangunkan biosensor amperometrik berasaskan kertas menggunakan elektrod karbon yang dicetak dan diubah suai menggunakan Prussian Blue (PB). Untuk kakjian ini, kertas selulosa digunakan sebagai matriks immobilisasi untuk glukosa oksidase (GOx), invertase (INV) dan hidrogen peroksidase (HRP) dimana ia telah berjaya diimobilisasi ke dalam matriks gentian kertas melalui penjerapan fizikal. Biosensor berasaskan kertas ini hanya mengunakan sejumlah kecil (8 µL) sampel bagi kedua-dua analisis glukosa dan sukrosa. Biosensor glukosa mempunyai julat kalibrasi linear antara 0.5 mM hingga 4.5 mM (R2 = 0.9925) dan had pengesanan 0.15 mM. Untuk biosensor sukrosa, julat kalibrasi adalah dari 0.1 mM hingga 1.1 mM (R2 = 0.9897) dan had pengesanan 0.1 mM. Kajian terhadap bahan yang berpotensi untuk menggangu tindak balas biosensor juga telah disiasat. Di samping itu, analisis enam sampel madu menggunakan biosensor ini juga dikaji. Hasil yang diperoleh menggunakan biosensor glukosa disokong dengan baik dengan kaedah kromatografi cecair prestasi tinggi (HPLC) namun untuk biosensor sukrosa, lebih banyak kajian perlu dilakukan untuk meningkatkan hasil yang diperolehi kerana ia mempunyai lebih daripada 70% perbezaan dengan rujukan. Dari kajian ini, dapat disimpulkan bahawa, prototaip sensor untuk menentukan pemalsuan gula dalam madu lebah telah berjaya dihasilkan.

Kata kunci: Madu kelulut, skrin cetak elektrod, biosensor berasaskan kertas, glukosa, sukrosa, amperometrik

ABSTRACT

Stingless bee is a large group of bees in family Apidae that have the ability to produce different types of products that valuable to humankind which are honey, bee bread, and propolis. The growing demand for honey in the market has led to the occurrence of the tampering honey with foreign substances and increases the production of artificial honey. There are lots of laboratory tests for honey adulteration however most of tests are tedious, high cost and complicated. Therefore, there should be a continuously development and testing of alternative methods that would allow faster and easier detection of honey adulteration. This research attempt to develop a simple inexpensive paper-based amperometric biosensor based on Prussian Blue (PB)-modified screen-printed carbon electrodes (SPCEs). To do so, cellulose filter paper was used as immobilization matrix for glucose oxidase (GOx), invertase (INV) and hydrogen peroxidase (HRP) as it was successfully embedded within the fibre matrix of paper via physical adsorption. The paper-based biosensor allowed a small amount (8 µL) of sample solution for both glucose sucrose analysis. The glucose biosensor had a linear calibration range between 0.5 mM to 4.5 mM ($R^{2=}$ 0.9925) and a detection limit of 0.15 mM. For sucrose biosensor the calibration ranges from 0.1 mM to 1.1 mM (R^2=0.9897) and detection limit of 0.1 mM. Interference study of selected potential interfering compounds on the biosensor response was investigated. In addition, its performance was demonstrated in the analysis of six honey samples. The results obtained using glucose biosensor corroborated well with high performance liquid chromatographic (HPLC) method however for sucrose biosensor, more study should be done to improve the result obtained as it has more than 70% differences with reference. From this research, it can be concluded that, the prototype sensor to determine sugar adulteration in stingless bee honey was successfully developed.

Keywords: stingless bee honey, Screen-printed electrode, Paper-based biosensor Glucose, Sucrose, Amperometry

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

%	percentage
°C	Celcius
mm	millimetre
g	gram
mL	millilitre
M	moles of solute per litre of solution
mL/min	millilitre per minute
µL	microlitre
nm	nanometre
mEq/kg	milliEquivalents per kilogram
mS/cm	millisiemens per centimetre
g/100g	gram per 100 gram
mg/kg	milligram per kilogram
h	hour
M	molarity
CFU/g	Colony Forming Unit per gram
CFU/L	Colony Forming per liter
IN	Invertase number
DN	Diastase number
µA	Current
V	Potential
U/mL	Activity unit enzyme per liter

LIST OF ABBREVIATION

HMF	Hydroxymetylfurfural
POC	Point of care
PADs	Paper based analytical device
µPADs	Microfluidic paper based analytical device
SPCEs	Screen Carbon Printed Electrode
PB	Prussian blue
INV	Invertase
GOX	Glucose oxidase
HRP	Hydrogen peroxidase

CHAPTER 1 INTRODUCTION

1.1 Background

Stingless bee is a small, all black creature that can be found in most tropical areas of the world, such as Australia, Africa, Southeast Asia, and tropical America. There are approximately more than 500 species of stingless bees was found worldwide with the number of stingless bee species in Malaysia are varies between 17 to 32 species (Kelly et al., 2014). However, *Geniotrigona Thoracica* and *Heterotrigona Itama* are two main species widely used by bee farmers in Malaysia. Stingless bees are very special insects as it can produce three different products which are honey, propolis and bee bread.

According to Malaysia Agriculture Research and Development Institute (MARDI), stingless bee honey is twice as nutritious as ordinary honey. This is due to their small size (2 to 14mm) which allows them to suck the nectar from the flowers to the deepest space (Norowi et al., 2010). Honey is a natural sweetener that is widely used for various applications where it contains approximately 200 distinct chemical compositions (Ramanauskiene et al., 2012). The composition inside honey includes fructose and glucose (80–85%); water (15–17%); ash (0.2%); proteins and amino acids (0.1–0.4%) and trace amounts of enzymes, vitamins and other sub-stances, such as phenolic compounds (Rao et al., 2016). However, honey composition varies depending on the plants types as well as the nectar which the bee consumes (Rao et al., 2016).

The increasing demand of honey in the market nowadays leads to the phenomenon of the dishonest act of honey adulteration and production of synthetic

honey. Honey adulteration refers to the immoral act of producers by adding sugar syrups into natural product (Islam et al., 2014). Adulterants such as water, sucrose, inverted sugar, hydroxymethyl cellulose, dextrin and starch a have been regularly detected by regular physicochemical analysis (Serrano et al., 2004).

Over the years, a lot of laboratory techniques and tests for honey adulteration have been developed to identify the purity of honey samples. Methods such as high-performance liquid chromatography, isotope mass spectrometry, capillary electrophoresis and rapid detection technique such as spectral technology and sensory technology are commonly used to detect adulteration in honey (Gan et al, 2016). However, not all of the tests are suitable for consumers as the cost to test the honey samples in the laboratory are expensive, complicated and time consuming (Morales et al., 2008; Tu et al., 2011; Luo et al., 2012). Therefore, there should be a continuously development and testing of alternative methods that would allow faster and easier detection of honey adulteration (Yilmaz et al, 2014).

The portable on-site testing device or known as Point Of Care (POC) devices are those technologies that are able to provide the alternative methods for detection of honey adulteration. The POC technologies has sparks the interest in both clinically and industrial as it can provide rapid data output, easy to use, portable and inexpensive (Bee et al., 2018). POC devices can be developed using various platforms such as glass, ceramic, plastic and finally paper which has become the most attractive and promising platforms because of its advantages (Bee et al., 2018). By using paper as the platform for POC devices, the devices are now known as paper based analytical devices (PAD) or also called paper-based sensor.

The idea of PADs is to carry out test on small piece of paper where the most common paper used are filter papers, graphite paper and chromatograpy paper (Fu & Wang, 2018; Songjaroen et al., 2011). Material of paper were chosen according to the fabrication method and the sensor application where the most used material is Whatman brand chromatography paper because of its exceptional wicking ability (Singh et al., 2018). With the advanced development of PADs, variety of detection techniques are widely applied to rapidly quantified the fabricated PADs, however, among of these methods, the electrochemical detection (Potentiometry, coulometry, polarography and amperometry) is more relevant with the paper-based analytical device due to the potential for miniaturization and portability, low fabrication cost and higher sensitivity (Dungchai et al., 2010; Kong et al., 2014; Bee et al., 2018).

Enzymes have been associated with electrochemical PADs (paper based biosensor) because of the potential to be applied in a various clinical diagnostics (Gianini et al., 2017). The most common enzyme analysis on paper based biosensor are the peroxidase coupled enzymatic system which enabling detection of various analytes including glucose, lactate, uric acid, cholesterol and sarcosine (Gianini et al., 2017). The immobilization techniques of enzymes are crucial as it also demonstrating the paper-based biosensor performance. The choice of paper materials should be good enough to provide strong support for enzyme immobilization through simple adsorption procedure (Lawrence et al., 2014).

Interestingly, in this research, the screen-printed carbon electrode (SPCE) was integrated together with the paper substrate as immobilization matrix for the enzymes. The enzymes (Glucose Oxidase, Horseradish Peroxidase, and Invertase) were dropped onto paper disc, allowing the physical adsorption of enzyme within the porous paper

matrix before the paper disc were placed on top of a SPCE. The SPCE is well known for electrochemical detection in biosensor and often chosen due to their simplicity, ability for mass production, modest cost, portability and ease of chemical alteration (Rao et al., 2006; Parkash et al.,2014; Bian et al., 2017). However, here, instead of using bare SPCE, the screen printed carbon electrode (SPCE) was first modified by using Prussian Blue (PB) in order to prevent disruption from interference species (Lee et al., 2016). Prussian blue (PB, Fe4[Fe(CN)6]3) act as "artificial peroxidase" in electrochemical sensors and by modifying the SPCE with Prussian Blue (PB), the selectivity towards oxidase enzyme substrates can be improved (Haghighi et al., 2004; Ricci & Palleschi, 2005; Lee et al., 2016).

This paper-based biosensor was developed using simple fabrication technique and does not required any pre-processing steps. Therefore, the sensor is convenient for POC test application as the analytes were able to be measured not only in the laboratory but also for outdoors application. In addition, this paper based biosensor also were cost effective especially in reagent consumption as only 8 µL of analytes (glucose and sucrose) was used to detect the glucose and sucrose adulteration in stingless bee honey (Sekar et al., 2014).

1.2 Problem Statement

Recent years have seen growing interest among the consumers into food that helps maintaining their health with honey being one of them. Honey is used for nutritional and medicinal purposes where it has become an important commodity in the market (Buba et al., 2013). With the rapid increase of consumer demand, the honey supply in market is slowly decreasing. The limited availability and increasing price of pure honey has led to the problem of honey adulteration and production of synthetic honey in the market. Thus, it has increase the awareness among the honey consumers about the quality and purity of the commercial honey in the market.

Since the available method of adulterant detection such as high-performance liquid chromatography and spectrophotometry is expensive, complex and suffers from low sensitivity, the rapid detection techniques such as spectral technology, sensor technology and chemical kits became popular in testing of species, habitat, grades, freshness, nutrient quality, and drug residues in agricultural products (Gan et al., 2106). These methods are widely used since they are time-saving, more convenient and accurate than traditional methods.

Although many substantial works on rapid adulteration detection methods has been reported on both honey and stingless bee honey, the development of paper based amperometry biosensor for stingless bee honey adulteration detection in Malaysia has not yet been explored. Therefore, this research aims to develop the prototype devices that is effective, simple, and affordable for detection of glucose and sucrose adulteration in stingless bee honey.

1.3 Objectives

The objectives of this research are:

1. To characterize and compare the harvested stingless bee honey (*Heterotrigona Itama* and *Geniotrigona Thoracica*) and stingless bee honey available in local market based on the physicochemical, enzymatic and microbiological properties.
2. To develop the prototype detection device for measurement of stingless bee honey adulteration by focusing on point of care (POC) technology.
3. To investigate the performance of developed prototype devices for detection of glucose and sucrose adulteration in stingless bee honey.

1.4 Scopes of study

The scope of this study is focusing on how to differentiate between pure stingless bee honey with adulterated honey. Two pure stingless bee honeys from *Heterotrigona Itama* and *Geniotrigona Thoracica* species were harvested from Universiti Malaysia Pahang stingless bee farm, Kuantan and Aqif Kelulut Farm, Pekan, while other four commercial honey samples were bought from Agrobaazar and online medium, Shopee.

For objective 1, the characteristic of harvested honey with commercial honey is compared based on the physicochemical, enzymatic and microbiological properties where all analyses were assessed according to International Honey Commission's harmonized methods, Malaysian Kelulut Standard as well as reported methods in past studies. For physicochemical properties, the pH, free acidity, moisture content, electrical conductivity, ash content, sugar profile and hydroxymethylfurfural (HMF) values were determined. The enzymatic activity such as diastase activity and invertase activity of honey also were analysed. Finally, the bacteria growth (*Bacillus sp*., and total coliform), the standard plate count, the yeast and mould count as well as the antibacterial activity (Agar Disc Diffusion) in honey sample were evaluated.

For objective 2, the screen-printed electrode surface was first modified with Prussian blue (PB). After modification, the modified PB- SPCE undergoes cyclic voltammetry analysis to study the electrochemical behaviour of the modified electrodes. Then, the enzymes (Glucose Oxidase, Invertase and Peroxidase) were immobilized onto the paper disc (diameter ca. 10 mm) and were allowed to dry before placing them on top of the modified creen-printed carbon electrode (SPCE). Then, again the electrochemical behaviour was performed using cyclic voltammetry.

Objective 3 started with determining the best condition of several parameters including the effect of buffer pH, effect of applied potential and effect of enzymes concentration using One Factor At Time (OFAT). By using the determined optimum conditions, the calibration graph of the sensor was obtained. Then, the developed paper-based biosensor

was evaluated on its performance by conducting the selectivity study, reproducibility, and stability study. Finally, to conform the applicability of the paper-based biosensor, the validation study using real samples (honey samples) were analyzed. The study involved in detecting the presence of glucose and sucrose adulterant in stingless bee honey sample where the results were compared with the results obtained from High Performance Liquid Chromatography (HPLC).

1.5 Significant of study

The increasing adulterated and synthetic honey in the market is the main intention for this research. The results on physicochemical, enzymatic and microbiological of harvested stingless bee honey, commercial honey and adulterate honey are useful to evaluate and differentiate the quality of these samples. Besides that, the information obtained from this research are handy as it can add some important values to improve the current legislation of Malaysian stingless bee honey.

Development of prototype honey sensor is significant as it can be developed to be portable, simple, rapid and cost effective compared to the conventional method in laboratory which is tedious and time consuming. The developed prototype is able to differentiate the difference between adulterated and non-adulterated stingless bee honey based on its glucose and sucrose content without using any complicated instrumentation. This research is important as it may provide relevant data to develop full scale and advance devices in future where it can be used to determine the quality of stingless bee honey on the spot.

1.6 Thesis Layout

The structure of the remainder thesis is summarized as followed:

In Chapter 2, the literatures can be divided into two main parts where the first part is focusing on the nature of stingless bee, stingless bee honey and the characteristic of good quality honey as well as the various honey analysis such as physicochemical, enzymatic and microbiological analyses. The second part of this chapter is focusing on

the detail description of biosensor including the materials, fabrication methods and detection technologies that is used to develop the prototype device. Moreover, previous studies on amperometric biosensor applications in various samples is also discussed in this chapter.

Chapter 3 presented the methods and procedures performed to characterize stingless bee honey which includes the three main analysis of honey; physicochemical, enzymatic and microbiological analysis. In order to develop the prototype sensor, the modification, immobilization technique and detection method is applied. The parameters tested include effect of pH, effect of scan rate, effect of enzymes concentration, selectivity study, reproducibility, and stability study. Lastly, the developed sensor is tested for its performance to detect glucose and sucrose adulterant in all samples of stingless bee honey.

Chapter 4 presents the research findings where firstly, the characterization of honey based on the physicochemical, enzymatic and microbiological analyses is discussed. The figures and graphs are also provided for a better understanding. The performance of developed sensor also is discussed in this chapter where it demonstrated the overall outcome for this research.

Chapter 5 is the conclusion for the whole thesis which concludes the findings of current works and provides the recommendation required for future works.

CHAPTER 2 LITERATURE REVIEW

2.1 Stingless Bees

Stingless bees or simply meliponines belong to the family *Apidae* where over 400 species have been described by the scientist (Aidoo et al., 2011). They are variable in size, ranging from 2mm to 14 mm and generally found in tropical region all over the world (Norowi et al., 2010). Stingless bees are eusocial insects with lack of functional stings (Amano et al., 2000). Most species do not harm human as they are safe to handle and can be managed at ease (Rahman et al., 2015). However, they may sting if disturbed. Stingless bees are really special for their ability to produce three different product that is honey, propolis and bee bread. In addition, stingless bees also value as effective pollinator for both wild and cultivated crops (Mathiasson et al., 2015).

Figure 2.1 *Heterotrigona Itama* (Adapted from Discover Life)

In Malaysia, the number of stingless bee species varies between 17 to 32 species depending on the study areas (Norowi, 2010; Salim, *et al.*, 2012; Schwarz, 1939). However, according to

MARDI, the most commercialized stingless bee species in Malaysia are *Heterotrigona Itama* dan *Geniotrigona Thoracica* where the two species contributed 94.4% of the total stingless bee species in the farm (Kelly et al., 2014). Stingless beekeeping is known as meliponiculture (Cortopassi-Laurino et al., 2006). This activity involved transferring wild colonies nest in the log hive into wooden boxes with a hole in centre and place on the top of tree trunks (Kelly et al., 2014; Kek et al., 2017).

Figure 2.2. *Geniotrigona Thoracica* (Adapted from Discover Life)

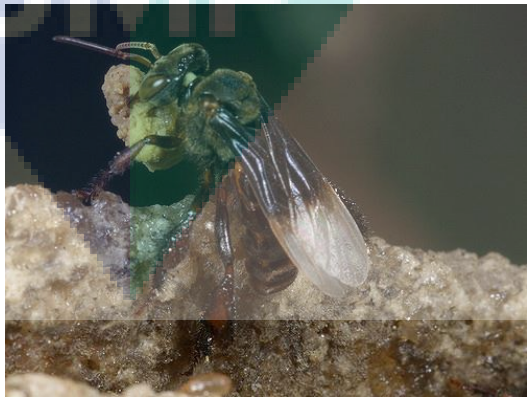

Figure 2.3.*: Tetrigona Binghami* (Adapted from Discover Life)

2.2 Stingless Bees Honey

Honey is valuable products produce by stingless bee honey. It is a viscous solution containing approximately 200 compounds, such as vitamins, enzymes, amino acids and minerals, with the major content being water and sugars (Rao et al., 2016). Stingless bee honey is differing in terms of its color, taste and viscosity (Almeida-Muradian et al., 2014; Guerrini et al., 2009). Besides that, stingless bee honey is more fluid in texture and undergoes slow crystallization (Biluca et al, 2014). Stingless bees honey is not very popular to consume due to its sour and bitter taste (Chanchao et al., 2006). However, since there are an increasing number traditional practitioners and researchers suggesting of using it, the honey has increased in popularity.

Not only honey is nutritional, but it also inhibits the growth of pathogens and due to its antimicrobial activities, honey is effective to treat bacterial infection (Chanchao et al., 2006). Stingless bees honey is rich in antioxidants and is known for its medicinal value. The variety of phenolic acids shows that the honey has a lot of nutrients (Roowi et al., 2012). The stingless bee honey can enhance immune system, fighting bacteria, slowing the growth of cancer cell, relieving sore throat, coughs and colds. Honey also can be used as restorative after an illness and said to sooth pain, act as antiseptic, hasten healing, effective in curing burns, carbuncle, boils and diabetic wounds (Mail, 2014). Due to limited honey production, the price of stingless bee honey can reach up to RM 120 per bottle compare to normal honey bees.

Figure 2.4: Harvested stingless bee honey

2.3 Pure and Adulterate Honey

Pure honey is commonly referring as organic and natural honey. It is made naturally by bees that were fed only organically grown flowers. According to the international honey standard, pure honey should be free from any food ingredient, including food additives, or any other additions when marketed as honey or used in any product intended for human consumption (Alimentarius, 2001). The honey should be maintained to its natural composition, hygienic and pure to preserve its nutritive and healing properties (Wu et al., 2017). Honey contains high nutritional value and has a unique flavour characteristic thus making it one of popular natural sweetener in the market. However, the price of honey in the market is quite expensive making it a target of adulteration for economic profit. In order to decrease the cost of honey, producers tend to adulterate honey with less expensive substances (Sivakeseva & Irudayaraj, 2002).

Honey adulteration refers to the immoral act of producers by adding sugar syrups into natural product (Islam et al., 2014). The most common honey adulteration methods are by addition of

sucrose syrups that are produced from sugar beet, high-fructose corn syrup (HFCS), maltose syrup or by adding industrial sugar (glucose and fructose) syrups obtained from starch by heat, enzyme or acid treatment, or by feeding the bee with sugar during the main nectar period (Wu et al., 2017). Honey adulteration is relatively easy to make but it is difficult to detect since the adulterated honey may have similar physical properties to natural honey (Mehryar & Esmaiili, 2011; Siddiqui et al., 2017). Therefore, continuous study should be done in order to develop newer, simpler and more sensitive detector for honey adulteration.

2.4 Honey Quality Attributes

The major concern of honey quality is to ensure that honey is authentic with respect to the legislative requirements such as in codex alimentarius standard, the EU Honey Directive and other legislations (Siddiqui et al., 2017). There are two major aspects of honey authenticity: (i) the mode production of honey (to identify the potential fraud and adulteration) (ii) the geographical and botanical origin of the honey (Bogdanov & Martin, 2002). The mode of production of honey is subjected to the harvesting of honey hives and honey processing. After harvesting, the honey is treated with either centrifugation or filtration in order to remove any unwanted debris (Bogdanov & Martin, 2002). Heat treatment such as pasteurisation (to kill osmophilic yeast) and liquefaction should not change the essential composition of honey or impair its quality (Danilcenko et al., 2011). Besides that, honey quality may be characterized by physical and chemical parameters such as sugar content (fructose, glucose, and sucrose), moisture, amino acid profile, hydroxymethylfurfural content (HMF) and enzyme activity (Danilcenko et al., 2011).

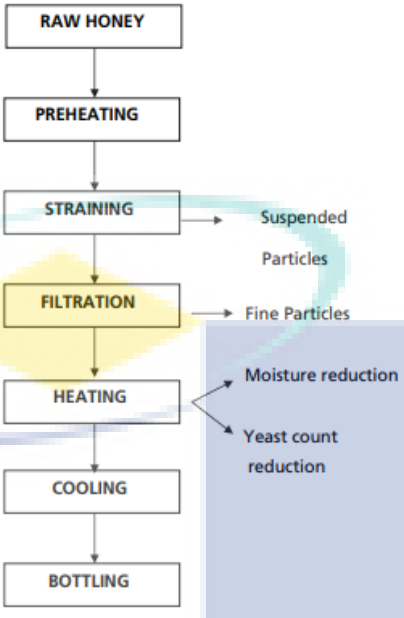

Figure 2.5. Conventional method of processing honey (Subramanian et al., 2007)

Honey is mixture of different sources as the bee's forage different plants. Thus, the authenticity with respect to geographical and botanical origin of honey is important to guarantee the quality of honey (Bogdanov & Martin, 2002). Pollen analysis is one of the most important analyses in determining the geographical and botanical origin of honey. If the geographical differences are less pronounced, the determination of pollen spectrum may not result a confident authenticity proof (Anklam, 1998).

2.5 Stingless Bee's Honey Analysis

For this research, the characterization is focusing on physiochemical, enzymatic and microbiological analysis.

2.5.1 Physicochemical Analysis

The main criteria for physicochemical analysis are moisture content, electrical conductivity, ash content, sugar profiles, free acidity, diastase activity and hydromethylfurfural (HMF) content

(Gomes et al., 2010). Other criteria such as pH, colour, and water activity also can be analysed. Although in most research mentioned diastase activity as part of physicochemical properties, for this research, diastase activity is included in enzymatic analysis since enzyme is involved in the analysis.

2.5.1.1 pH

According to past studies, most honey has low pH where the minimum pH value is ranging between pH 3.5 to 5.5 (Gangwar, 2016). However, the pH values are varies depending on their geographical origin, floral sources as well as the bee's species (De Sousa et al., 2016). The low pH suppresses the growth of many species of bacteria (Buba et al., 2013) as most bacteria and moulds are stable in neutral and mildly alkaline conditions (Gangwar, 2016). The low pH demonstrates by the stingless bee honey can also indicate the freshness or a possible adulteration of the honey as the pH of adulterate honey is higher (more than pH 5.5) compared to pure honey because it is related to the extraction, storage factor and temperature of honey (Gomes et al., 2010).

2.5.1.2 Free Acidity

The acidity in honey is quite low, but it influences the taste, stability against microorganisms, improvement of chemical reactions, antibacterial and antioxidant activities of honey (Cavia et al., 2007). Free acidity is caused by the presence of organic acid with gluconic acid is the main acid in honey (Moo-Hoochin et al., 2015). Gluconic acid is a product of glucose oxidation by the enzyme glucose oxidase (Buba et al., 2013). Low acidity indicates the freshness of honey though the value may increase with time (Yadata et al., 2014). Meanwhile the high acidity values can be indicative of fermentation of sugars into organic acid (Shobham et al., 2017).

2.5.1.3 Moisture Content

Based on the past studies, stingless bee honey has higher moisture content compare to apis mellifera honey (Chuttong et al., 2016). This may due to the humidity of tropical forest in which it is difficult to extract nectar with low water content (Biluca et al., 2016). Besides being exposed to the fermentation process, higher moisture content can also indicate the honey is being adulterated (Gangwar, 2016). The floral origin may cause the variation in moisture content of honey although other factors such as climatic conditions, soil, collection period and processing aspects also affect this parameter (De Sousa et al., 2016). Honey is hygroscopic where it tends to absorb moisture from the air (Malika et al., 2005). The low moisture content of honey inhibits the growth of bacteria and yeast. This is due to the high sugar concentration ties up the water molecules leaving the bacteria with insufficient water to growth (Adenekan et al., 2010).

2.5.1.4 Electrical Conductivity and Ash Content

Both electrical conductivity (EC) and ash content are related to the minerals content of the honey (Malika et al., 2005) where electrical conductivity measures all ionisable organic and inorganic substances and ash directly measures the inorganic residue after carbonization (Andualem, 2014). Though both parameters are quite similar to each other, electrical conductivity is often been used in routine honey control which is important in determining the botanical origin of honey (Malika et al., 2005). Generally, stingless bees honey has higher conductivity ranging from 0.32 to 3.10 $mscm^{-1}$ (Chuttong et al., 2016). The differences in electrical conductivity of the diverse honey not only correspond to the differing geographical and floral sources but also the amount of organic acids, proteins and storage time (Malika et al., 2005; De Sousa et al., 2016).

Colour of the honey also influence the electrical conductivity values as dark honey gives higher conductivity due to higher levels of microelements than light honeys (Alqarni et al., 2014).

According to legislation for honey, the maximum limit allowed for ash content is 0.6% (Alimentarius, 2001). However, based on the study of stingless bee's honey in Thailand, the ash is ranging from 0.040 to 3.10%, which is more than the limit allowed (Chuttong et al., 2016). The higher ash content in stingless bee's honey may due to the chemical composition of differing nectar during honey production.

2.5.1.5 Hydromethylfurfural (HMF)

Hydroxymethylfurfural or HMF is one of the indicators used to evaluate the quality of honey which literally absence in fresh honey and yet the values are increasing during processing, preparation, aging and storage of honey (Zappala et al., 2005; Gomes et al., 2010). High values of HMF suggest that the honey is either overheating or stored in poor condition (Boussaid et al., 2014). In addition, pH, climate, botanical sources and difference geographical region also control the HMF values. Thus, the tropical climate honey will eventually provide HMF if being exposed to high temperature for quite some time (Biluca et al., 2016). Besides that, HMF also depends to the types of sugar present in honey as HMF is formed during acid- catalysed dehydration of hexoses; simple sugars mainly fructose (Boussaid et al., 2014; Zappala et al., 2005).

2.5.1.6 Sugar Profile

Generally, honey is a supersaturated sugar solution with fructose (38%) and glucose (31%) being the two main sugar presents in honey. Additionally, due to the presence of invertase enzyme, sucrose found in honey is rather small approximately 1% w/w (Anklam., 1998; Kaskoniene et al., 2010; Gangwar., 2016). If the sucrose concentration is high, it may due to the diversity of floral sources, early harvest of honey as the sucrose not fully transform into fructose and glucose, overfeeding the bees with sugars, syrups or artificial honey and lastly, the honey is adulterated by

addition of commercial sugar (Anklam., 1998; Suntiparapop et al., 2012; De Sousa et al., 2016) .The ratios of fructose to glucose (F/G) are the other significant factor in determining honey quality where fructose content should be higher than glucose content (El Sohaimy et al., 2015). The F/G ratios implying the ability of honey to crystallize because when fructose is higher than glucose, the honey is fluid (Boussaid et al., 2014). The F/G ratios not only depend on the source of the nectar but it also depends on the variation of bee species and climate of different regions (Biluca et al., 2014). Furthermore, the F/G ratios influence the taste of honey as the fructose is sweeter than glucose (De Sousa et al., 2016).

2.5.2 Enzymatic Analysis

One of the interesting components in honey is enzymes as the activity of enzymes is important in biological value of honey (Vorvola & Čelechovská, 2002). The predominant enzymes in honey are diastase (α-amylase), invertase (α- glucosidase), glucose oxidase, catalase and acid phosphatase (Dinkov & Vashin, 2001). Enzymes have always been an important subject in research involving honey as it can be used to differentiate between pure and adulterated honey. However, this research mainly focused on diastase and invertase activity as both enzymes are largely used to measure honey freshness.

2.5.2.1 Diastase Activity

Enzyme α-amylase or its common name diastase is an enzyme that responsible in converting starch into simpler compound (Assia & Ali, 2015). Almost all honey contains diastase where the activity can be measured and expressed as Diastase Number (DN) (White & Doner, 1980). DN is defined as the amount of enzyme that will convert 0.01 starch in one hour at 40ºC (Bhargava & Mothilal, 2014). Diastase values are control not only by geographical and botanical origin but also by pH values, nectar flow and foraging patterns of the bees (Assia & Ali, 2015; da Silva et al., 2016). Similar to HMF, diastase can be used as indicative of freshness in honey (Pasias et al., 2017). Lower diastase value may mean that the honey is aging or has been heated since the enzymes are very sensitive towards heat (above 35ºC) (Iftikhar et al., 2014).

2.5.2.2 Invertase Activity

Invertase is the enzymes that hydrolysed sucrose to fructose and glucose where the activity can be expressed as Invertase Number (IN) (Assia & Ali, 2015). One unit of IN indicates the amount of sucrose per gram being hydrolysed in one hour (Bhargava & Mothilal, 2014). Even though invertase work is complete when honey is ripened, the enzymes still remained in honey and can be deactivating by exposing the honey to heat (White and Doner, 1980). Both diastase and invertase activities are steadily deteriorate on prolonged storage and heating of honey (Buba et al., 2013) and can be considered as freshness index along with HMF. However, invertase is considered as better freshness indicator than diastase because it is more susceptible towards prolonged storage and heat (Oddo et al., 1999).

2.5.3 Microbiological Analysis

Microorganisms may influence the quality or safety of honey. The microorganisms that may be found in honey are mostly yeasts and spore-forming bacteria, but there are no bacteria causing disease had been detected in honey (Adenekan et al., 2010). Microbial contamination commonly occurs in honey are from primary sources where it is difficult to control. The sources include pollen and nectar sources, the digestive tracts of honey bees, dust, air and earth (Snowdon & Cliver, 1996). Besides that, organisms are also found from the secondary sources which are from honey post-harvest including air, food handlers, cross-contamination, equipment and buildings (Snowdon & Cliver, 1996). However, these sources of contamination are easier to control by good manufacturing practices (Gomes et al., 2010).

Nevertheless, honey exhibit powerful antibacterial properties against the growth or persistence of many organisms (Agbagwa et al., 2014). Thus, honey is expected to contain low

numbers and a limited variety of microbes. The antibacterial effect has sparks the interest of using honey as a natural medicine mainly in diseases healing and surgical infections (Molan, 1996 ; Malika et al., 2005). The antibacterial activity of honey is likely to depend on geographical origin, floral sources, harvesting, processing and storage conditions along with water activity, pH, accumulation of hydrogen peroxide, and different phytochemicals compounds for example methylglyoxal (Baltrusaitytė et al., 2007; Andualem, 2014).

The analyses that was conducted under microbiological properties are Standard Plate Count (provide general information on microbial growth), Detection of *Bacillus Spp*, Total Coliforms (indicator of sanitary quality), Yeast and Mould Fungi and finally the Antibacterial Activity Analysis which includes Agar Diffusion Assay.

2.6 Paper based analytical devices (PADs)

In recent years, lot of techniques have been developed in order to test the purity of honey sample. These techniques mostly are complex and expensive. Therefore, there is a need to develop more efficient, reliable and high in sensitivity sensing and detection technologies. Point of care (POC) technologies can be the best solutions to the rapid detection of honey purity as it emphasized on the onsite diagnosis that allowed test results to be obtained directly to users (Liana et al., 2012). Though POC technologies are often practiced to clinical diagnostic applications, the demand for this technologies in industrial research are increasing.

Paper based analytical devices (PADs) are one of the potential POC devices that have the ability to be built as user friendly, portable, cost saving and producing rapid results (Liana et al., 2012; Kong et al., 2014). Paper based analytical devices (PADs) is currently undergo rapid development especially in medical point-of-care diagnostics as it can be used as low-cost substitutes due to abundant of the paper resources (Bee et al., 2018). The most widely used PADs are called Paper-based microfluidic analytical devices (μPADs) which involved the distinct design of hydrophilic/hydrophobic zones on paper substrates using current printing and cutting techniques such as wax printing, screen-printing, photolithography, and many more (Almeida et al., 2018; Fu & Wang, 2018). Though μPADs is able to wick fluids better due to separation of hydrophilic/hydrophobic zones, for this research we will be focusing on the simplest and cheapest method for PADs fabrication which only involved manually cutting the paper substrate in order to avoid using any complicated fabrication equipment's.

2.6.1 Paper substrate

As the most abundant biopolymer on the Earth, cellulose is mostly used to produce paper for industrial use (Liu et al., 2016). That is why paper is the most suitable for PADs materials due to the cost saving and its availability. Paper has been chosen as the substrate for analytical purposes due to several advantages (Altundemir et al., 2017; Almeida et al., 2018): its porosity, fast wicking rate, fondness to different analyte types, lightweight and easy to transport and it is also disposable and biodegradable. The choice of paper materials is important as it will help to enhance the sensitivity of the PADs. The most favourable paper substrate of choice has been chromatography filter paper. Besides that, filter paper, blotting paper, art paper and office paper of different grades also can be chosen as PADs substrates (Bee et al., 2018).

For this research, Whatman grade 1 chromatographic filter were specifically chosen as paper substrate for the developed PADs. Besides it is the most used paper substrates in literature, the Whatman filter paper offers lots of advantages such as smooth surface, reproducibility, uniformity, suitable for immobilization of enzymes, proteins and DNA due to its high degree of non-specific binding towards biomolecules and high alpha-cellulose content which helps increasing its wicking ability (Silveira et al., 2016; Bee et al., 2018).

2.6.2 Detection methods

Various detection techniques have been employed in paper-based sensors such as colorimetry, chemiluminescence, electrochemiluminescence and electrochemical to detect the presence of target analytes (Singh et al., 2018). These techniques have been used due to highly sensitive results compared to traditional techniques such as the ELISA.

2.6.2.1 Colorimetric techniques

Colorimetric assays include methods utilized to detect the presence and concentration of an analyte by evaluating the color formation or color change via i) direct imaging using a single-lens reflex (SLR) camera, mobile devices, or low-cost desktop scanners in combination with software such as MATLAB for quantification (Ellerbee et al., 2009) or ii) traditional spectrophotometers by measuring the absorbance of the sample at specific wavelengths. This detection technique is the most commonly used one as it provides accurate results at a lower cost (Singh et al., 2018). Figure 2. shows the overview of colorimetric detection.

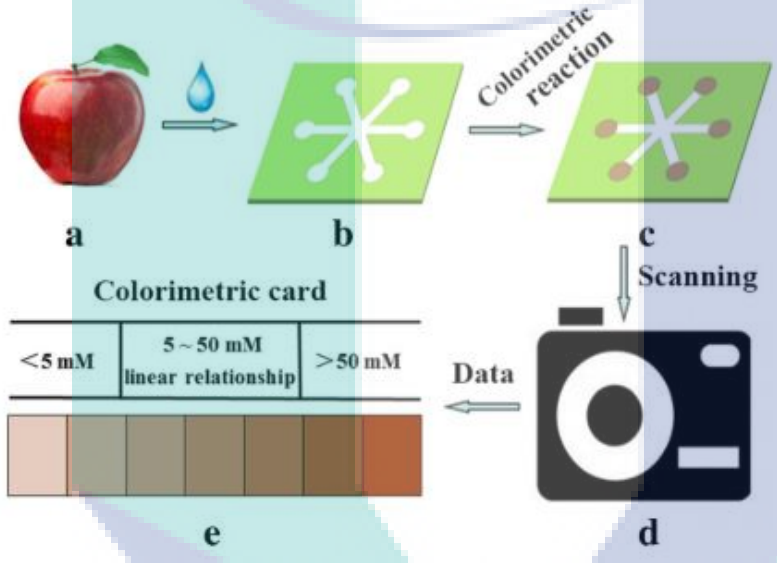

Figure 2.6 Overview of colorimetric detection

2.6.2.2 Luminescence Detection

Luminescence detection, which includes fluorescence, chemiluminescence (CL) and electrogenerated chemiluminescence (ECL), has been used in conjunction with PADs. A fluorescence measurement is inherently more sensitive than a photometric measurement because the analyte signal is a function of both the intensity of the incident excitation radiation and the analyte concentration (Almeida et al., 2018). A reagent that can produce luminescence is oxidized at the working electrode (usually screen-printed carbon) with triethylamine (TEA) or hydrogen peroxide obtained by reduction of the sample. This reaction produces photons resulting in a luminescent color change. An image evaluation software (e.g., Python, NIH Image J) can be employed to study the pixel intensity for each color, indicating the concentrations of the analyte (Singh et al., 2018).

2.6.1 Electrochemical detection

Electrochemical detection techniques are very compatible with paper-based analytical devices to extent that they are often referred to separately as "paper-based electrochemical devices" (PEDs)(Almeida et al., 2018). Electrochemical detection offers simplicity, low cost, portability, high selectivity, good sensitivity, low electrical power consumption, and minimal instrumentation (Fu & Wang, 2018). PEDs generally comprise of three electrode systems (working, counter and reference electrodes), which are used to perform stripping and cyclic voltammetry, amperometry and coulometry. The electrodes are usually screen- or inkjet-printed onto the paper using conductive carbon or metal inks or drawn using graphite pencils (Almeida et al., 2018). The electrochemical reading on paper substrate is highly comparable to results produced by the more conventional benchtop techniques, such as UV-Vis spectroscopy, liquid chromatography and inductively coupled plasma–mass spectrometry, in terms of sensitivity and specificity (Bee et al., 2018). Figure 2.7 are the examples of paper based analytical devices used for electrochemical detection.

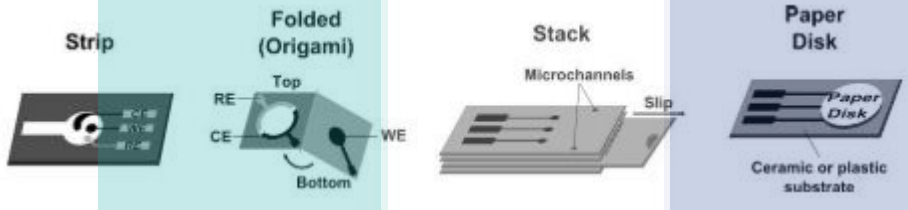

Figure 2.7 Configurations of paper based analytical devices used for electrochemical detection.

2.7 Paper - Based Biosensor

In this work, the detection method that were used is the electrochemical detection which includes the amperometric evaluation and cyclic voltammetry measurement. The electrochemical PADs (PEDs) or paper-based biosensors was integrated with printed electrodes (Screen printed carbon electrodes) for signal transduction and multiple enzymes were immobilized into paper substrate to identify the target analytes which is the glucose and sucrose.

2.7.1 Biosensor

For this section, the working scheme of biosensor were explained in order to get the overview needed to develop this prototype device. Biosensor concept is mostly applied in medicine, agriculture, food safety, homeland security, bioprocessing, environmental and industrial monitoring (Luong et al. 2008). Biosensor offer few advantages over conventional techniques using

spectrophotometry and high-performance liquid chromatograph including 1) targeted specificity to carry out selective measurements in complex media; (2) fast measurement through the use of high sample throughput in automated analyzers; and (3) continuous measurement (Mulchandani & Bassi, 1995)

The aim of biosensor is to produce a digital electronic signal which corresponds to the concentration of specific chemical or sets of chemical (Turner et al., 1987). There are three main elements in biosensor; bioreceptor, a transducer and a signal processing system (Morrison et al. 2007). Biological recognition element or bioreceptor is consists of an immobilized biocomponent that is able to detect the specific target analyte (Kahn & Plaxco 2010). This biocomponents are mainly composed of nucleic acids, enzymes, cells and antibodies (Touhami, 2014). Transducer is a convertor which converts the biochemical signal into an electric signal. The electrical signal is then amplified by the signal processing system and sent to a microelectronics and data processor where finally a measurable signal such as digital display, print-out or optical changes is produced (Perumal & Hashim, 2014).

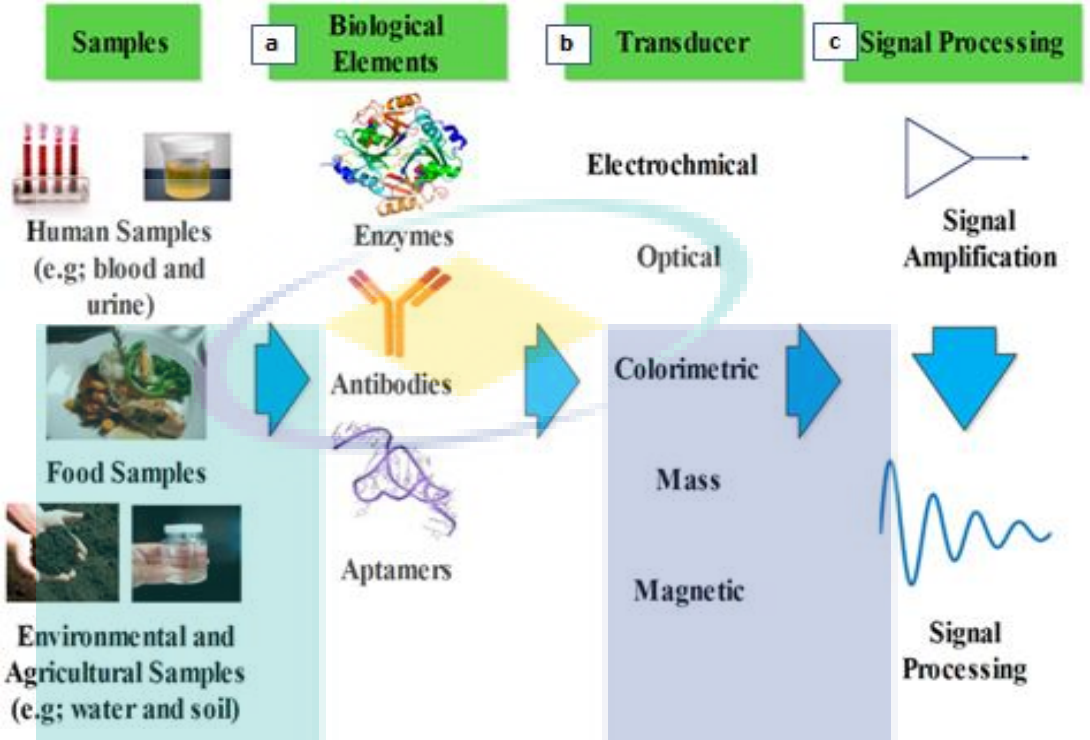

Figure 2.8. The schematic diagram of a typical biosensor. a) Biological element that specifically bind to the analyte; b) Transducer element convert biochemical signal into electronic signal c) The signal is amplified by a detector circuit using appropriate reference and sent for processing (Touhami, 2014)

The paper electrochemical biosensors trend is an added value to the diagnostic assays since it combines paper as a solid support, to the high sensitivity and selectivity of bio-electrochemical detection. The working electrode is the key component, because it is the place where the recognition event is transduced. Typically, the electrode surface is coated with an enzyme extract that catalyzes an electron transfer reaction in the presence of its substrate, the analyte. Depending on the species that interacts with the working electrode, namely a reaction's product/co-substrate, a redox mediator (mediated electrochemistry) or the enzyme itself (direct electron transfer), one can have first, second or third generation biosensors, respectively (Silveira et al., 2016).

For this research, electrochemical detection was applied to obtain the signal from target analytes. The amperometric detection and cyclic voltammetry measurement were involved in this paper-based biosensor development.

2.8.1 Amperometric Biosensor

Amperometric continuously measure current resulting from the oxidation or reduction of an electroactive species in a biochemical reaction. Clark oxygen electrodes perhaps represent the basis for the simplest forms of amperometric biosensors, where a current is produced in proportion to the oxygen concentration. Table 2.1 shows the summary of amperometric detection in food quality evaluation.

Table 2-1 Amperometric biosensor application for food quality evaluation

Target analyte	Samples	Recognition enzyme	Electrodes & sensing materials	Transduction system	Detection limit	Reference
Fructose	Honey	Fructose dehydrogenase	Ferrocyanide-SPCE	Amperometric	0.05 mM	Biscay et al., 2012
Fructose	Fruit juice	Fructose dehydrogenase	Graphite-nanoparticle SPCE	Amperometric	8 µM	Nicholas et al.,2017
Glucose	Honey, drinks for baby, glucose drink	Glucose oxidase and horseradish peroxidase	Ferrocyanide-SPCE	Amperometric	0.025 mM	Biscay et al., 2011
Glucose	Glucose beverages	Glucose Oxidase	Prussian Blue -SPCE	Amperometric	0.01 mM	Sekar et al., 2014
Glucose	Honey	Glucose oxidase	Palladium-MWCNT SPCE	Amperometric	0.04 mM	Guzsvány et al., 2017
Sucrose	Fruit juices	Invertase and Fructose dehydrogenase	Osmium-polymer - SWCNTP electrode	Amperometric	2 µM	Antiochia et al., 2014

2.8.2 Cyclic voltammetry

Voltammetry is one of the amperometric technique as it also measured the current of target analytes by varying a potential (Grieshaber et al., 2008). In a cyclic voltammetry measurement, the voltage is the working electrode potential is changed linearly versus time. Cyclic voltammetry experiment ends when it reaches a set potential value. When cyclic voltammetry reaches the set

potential, potential ramp of the working electrode is inverted back. **Figure 2.9** showing the overview of cyclic voltammetry graph.

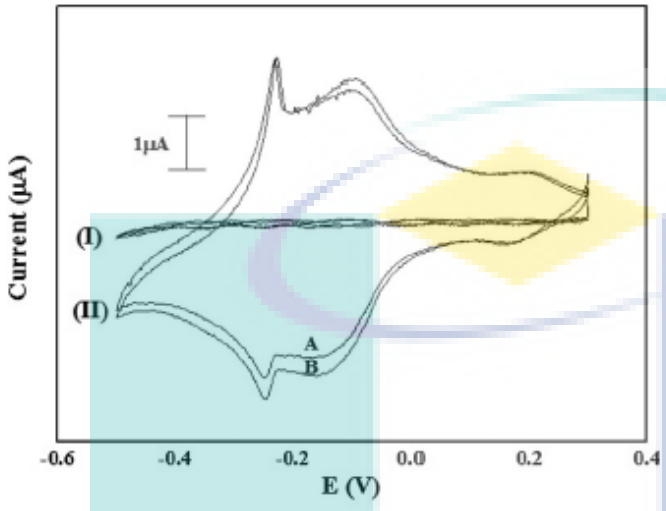

Figure 2.9 Overview of cyclic voltammetry diagram (Sekar et al., 2014)

2.8.2 Screen printed electrode (SPEs)

Due to their sensitivity and simplicity, electrodes are often used as signal transducers in biosensors (Abdulbari & Basheer, 2017). For amperometric electrodes to work, two-electrode (working/counter) system or a three-electrode (working/counter/reference) system can be designed (Prodromidis & Karayannis, 2002). For this research, the three - electrode system were employed and the electrodes were fabricated using thick film technology. Thick-film technology are widely used to fabricate disposable screen printed electrode where various paste with different composition were pressed onto selective transducer layers such as baking plastic and glass (Hu, 1998). Usually, the paste is a mixture of materials with the ability to transfer electrons, such as gold, silver, and carbon (Abdulbari & Basheer, 2017). The screen- printed electrode were purchased at local manufacturer, Rapid labs and **Table 2.2** described the specification of screen-printed electrodes that were used throughout this research.

Table 2.2 Screen-printed electrode specification

Specifications	Description
Dimensions	Length: 47 mm Width: 18mm
Thickness	0.35 mm
Electrode Material	Carbon for WE & CE, Silver for RE
Dimension of WE	5mmØ equivalent. to 19.638 mm^2
Backing Material	PET (Poly-Ethylene- Terepthalate)

Note: WE: Working electrode; CE: Counter electrode; RE: Reference electrode

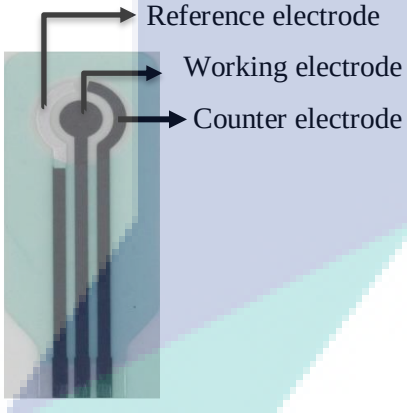

Figure 2.10 Screen printed carbon electrode

2.8.2.1 Prussian blue (PB) modification

There are many efforts have been made to improve the selectivity of carbon based electrochemical sensors, mainly by modification with redox mediators (Ricci et al., 2003). One of the most used mediators are Prussian-blue (PB) due to its competency in improving the selectivity towards oxidase enzyme substrates (Ricci et al., 2003). The immobilization of oxidase enzymes on the modified PB-SPCE makes it possible to detect the enzyme substrate (Ricci et al., 2003). PB is a ferri/ferrocyanide complex, which can act as an electrocatalyst for hydrogen peroxide reduction and was also referred as "artificial peroxidase" due to its capability catalyzing the reduction of hydrogen peroxidases at low potentials (−50 mV). (Haghighi et al., 2004). In addition to the ability

of PB to detect hydrogen peroxide at low potential, PB also is easy to deposit onto SPCE at lower cost.

2.8.3 Enzymes

The enzymes that were used in this research are glucose oxidase (GOX), invertase (INV) and hydrogen peroxidase (HRP). GOX enzymes are for glucose detection and for sucrose detection multienzymes were required that is GOX, INV and HRP. In order for paper-based biosensor to work, the techniques of immobilization enzyme are the most important as both stability and the biological function of the enzyme mainly depend of the immobilization (Biscay et al., 2011). The enzymes were physically attached to a water-insoluble matrix, supporting the material or carrier where among of the support materials used are inorganic, organic and organic synthetic carriers. Immobilization method can be divided into physical and chemical methods.

2.8.3.1 Physical Methods

a. Adsorption

Enzymes are adsorbed on the surfaces of support carriers by H-bondings, van der Waals forces, electrostatic or hydrophobic interaction. This method is easy to be implemented where just by mixing them for a certain incubation time and relatively low cost. No additional coupling agents and modification steps are required. The interaction between enzymes and support carriers are weak and reversible in physical adsorption.

b. Entrapment

In this method, enzymes are occluded in polymeric network. Enzymes are immobilized by gel/fibre entrapping or microencapsulation. Substrates and products are allowed to pass through while enzymes are retained in the networks. This will decrease the leaching of enzymes, improve the stability, and allow the generation of enzymatic reactions. Covalent bond between enzymes and support matrices do not exist but then the enzymes conformation is maintained ensuring the high

catalytic activities. The demerit of this technique is where the high diffusion barriers prevent the macromolecular substrates from passing through the networks.

2.8.3.2 Chemical Methods

a. Covalent Attachment

Covalent bonds are formed through chemical reactions between support materials and enzymes' side chain amino acids. Covalent bonds are stable to prevent the leakage of enzymes from the support matrices this improving the stability of the immobilized enzymes. Despite that, the enzyme active sites might be inactivated due to chemical reactions between enzyme molecules and support matrices where will decrease the catalytic activities.

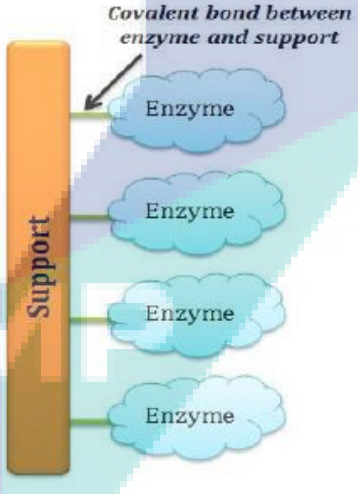

Figure 2. 11: Illustration of covalent attachment

b. Cross-linking

Enzymes are cross-linked to the support matrices using bifunctional reagents. In this method, enzymes are immobilized firmly to improve the reusability and stability with the help of covalent bonds. However, enzymes may lose their catalytic activities during the cross-linking process.

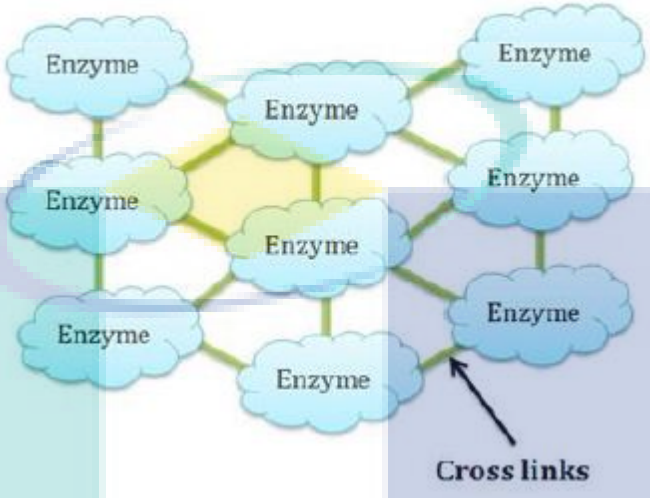

Figure 2. 12: Illustration of cross-linking

For this research, the chosen immobilization techniques are the physical adsorption since the enzymes were only dropped into the paper disc and were allowed to dry before directly applied to the SPCE. This method is easy and cost saving as no further modification or additional chemicals are required.

2.7.4 Target analytes

The target analytes for this research are the glucose and sucrose due to the presence of both sugar in stingless bee honey. Besides that, both analytes especially the sucrose has become a target for adulteration activity where the producer most likely to add these sugars into pure honey.

2.7.4.1 Glucose

Due to the occurrence in the literature, glucose is chosen as one of the target analytes for this paper- based biosensor. Glucose biosensor are widely generally applied to many industries such as in dairy, wine, beer, and sugar industry. The majority of glucose amperometric biosensors are based on enzymes glucose oxidase that consume oxygen and produce hydrogen peroxide (oxidase enzymes)(Monosik et al., 2012). The general equation is as followed:

$$\mathrm{D-Glucose} + \mathrm{O2} \xrightarrow[Glucose\ Oxidase]{} \mathrm{D-gluconic\ acid} + \mathrm{H2O2}$$

2.7.4.2 Sucrose

One of famous and cheap and easily available commercial honey adulterant in Malaysia is cane sugar. Cane sugar is extracted from perennial grass where it is known for its high sucrose content (Se et al., 2018). That is why sucrose are chosen as one of the target analytes for this research because in pure honey, sucrose content should be less than 5% or otherwise the honey maybe is tainted with the addition of commercial sugar (Anklam, 1998).

Sucrose determination requires a multienzyme system in which sucrose is first hydrolyzed by the enzyme invertase:

$$\mathrm{D-Sucrose} + \mathrm{H2O} \xrightarrow[Invertase]{} \mathrm{D-fructose} + \alpha\mathrm{-D-glucose} \qquad (2)$$

Invertase usually will work together with GOx to detect sucrose but there is a need to convert α-D-glucose to its β-isomer, for which GOx is more specific (Monosik, et al., 2012). For this purpose enzyme mutarotase often used however due to unavailability of the enzyme, we changed it to peroxidase enzyme according to method by Jokerst et al., 2012.

2.8 Summary

In summary, this research is focusing on the development of paper-based biosensor using electrochemical detection (chronoamperometry) as sensing method, screen printed electrode as the transducers, enzyme glucose oxidase, invertase and peroxidase as the biorecognition elements and finally glucose and sucrose as target analytes. Many substantial works on biosensor to detect adulterants in different types of honey has been reported. However, to date, there is no work on paper-based biosensor integrating with screen printed carbon electrode has been developed to quantify sugar adulterants in Malaysian stingless bee honey. The research is relatively new and not

yet explored by researchers in Malaysia. Thus, this work attempted to develop the paper based amperometric prototype sensor that are able to differentiate between pure and adulterated honey. Besides that, the developed paper-based biosensor aimed to be as simple as it could as we want it to be cost effective, able to replicate it easily without using any complicated materials and instrumentation.

CHAPTER 3

METHODOLOGY

3.1 Overview

As indicated in the title above, this chapter is focusing on the methods used to achieve the required objectives for this research. Figure 3.1 describing the overall methodology for this research starting with the collection of raw material which is the stingless bee honey. The samples preparation, utilized materials and all of experimental procedures is described in the following sections. Besides that, the details for all equipment used in this research were also included in this chapter. For characterization of honey samples, mostly the methods have followed the Malaysian Kelulut Standard and International Honey Commission. As for the development of paper-based biosensor, the procedures were done following the methods from past publications where some modification is applied to suit the objectives of this research.

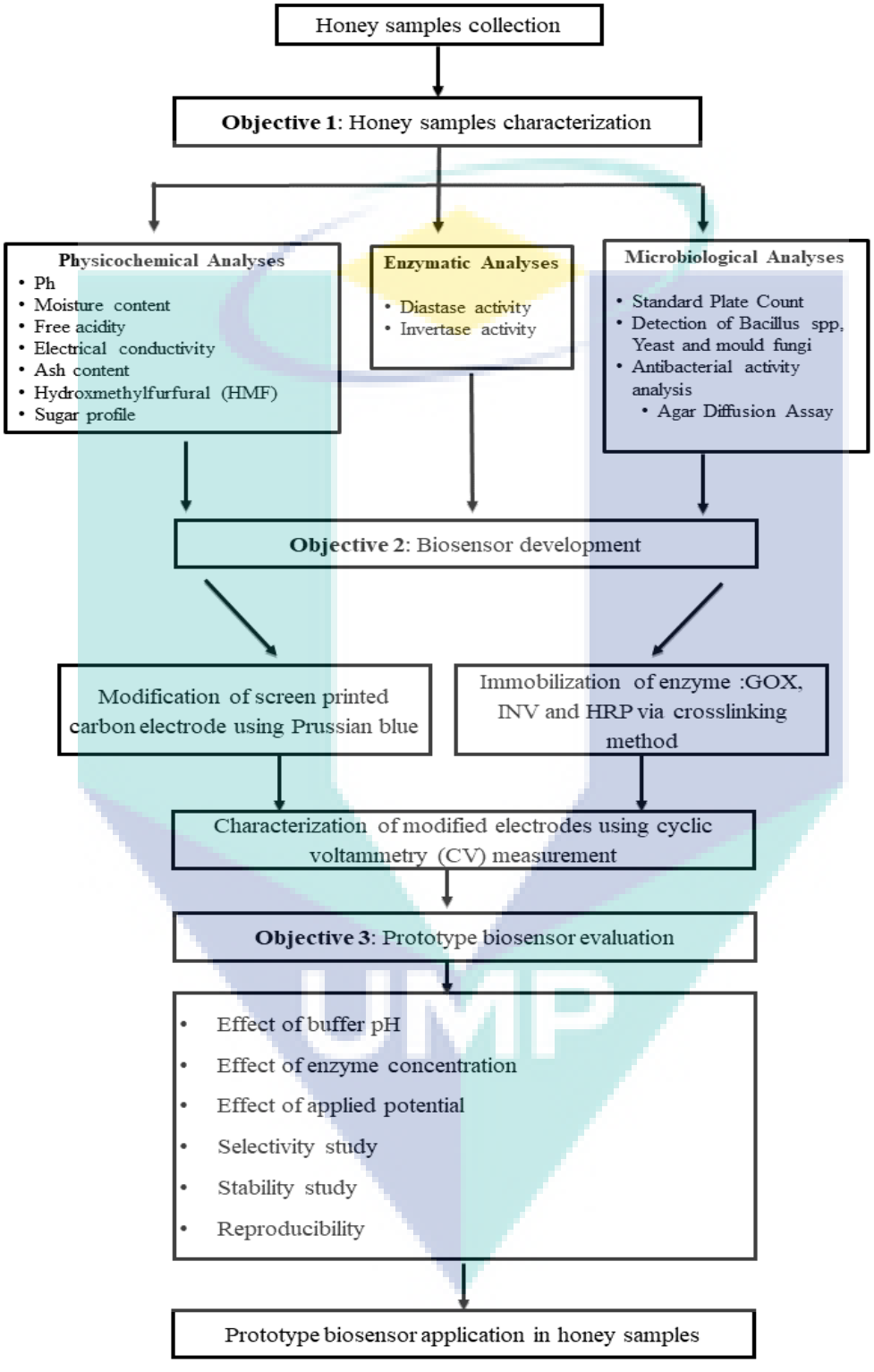

Figure 3. 1 Overall methodology applied in this research

3.2 Samples Collection

In this study, six samples of stingless bee's honey were collected from different regions in Malaysia (Table 3.1). Two samples, *Heterotrigona Itama* and *Geniotrigona Thoracica* were harvested from Universiti Malaysia Pahang stingless bee farm, Kuantan and Aqif Kelulut Farm, Pekan while other four samples of commercial stingless bee honey were randomly obtained from local market around Malaysia. All honey samples were stored at 4°C and left at room temperature 25°C for 4–5 h before the analysis.

Table 3.1 Sampling Location, description and time collection of six sample of stingless bee honey from Malaysia

Sample Code	Description	Sampling Location	Time of collection
H1 (*Heterotrigona Itama*)	Harvested	Taman Pertanian Sultan Haji Ahmad Shah, Kuantan, Pahang	March, 2017
H2 (*Geniotrigona Thoracica*)	Harvested	Pekan, Pahang	March, 2017
H3	Commercial	Selangor	October, 2017
H4	Commercial	Kedah	October, 2017
H5	Commercial	Kuala Lumpur	November, 2017
H6	Commercial	Pahang	September, 2017

3.3 Materials, Chemicals and reagents

All of the chemicals and reagents used were of analytical grade. Sugar standard (Fructose, Glucose, Sucrose), acetonitrile, potassium hydrogen phosphate (KH_2PO_4), di-potassium phosphate ($K_2HPO_4.2H_2O$), sodium chloride (NaCl), hydrochloric acid (HCl), ethanol, sodium bisulfate, acetate buffer, sodium hydroxide (NaOH), Bovine serum albumin (BSA) (V fraction), enzymes Invertase (INV) from baker's yeast (*S.cerevisiae*), Grade VII, >300 units/mg solid, Glucose Oxidase (GOx) from *Aspergillus niger*, Type VII, lyophilized powder, ≥100,000 units/g solid (without

added oxygen), 10KU were purchased from Sigma-Aldrich (USA), and enzyme Horseradish peroxidase (HRP), 145.7 U/mg are from Aladdin chemicals (China). P-Nitrophenyl-α-D-glucopyranoside (pNPG) as well as Carrez solution I and II was purchased from Merck (Germany) while Tris- (hydroxymethyl) aminomethane and chloramphenicol was purchased from Nacalai Tesque (Japan). Iodine, Iron(III) Chloride, Potassium Ferrocyanide and Glutaraldehyde (GA), 50% in H_2O were obtained from R&M (Malaysia). Plate count agar (PCA), violet red bile glucose agar (VRGB), Sabouroud Dextrose agar (SDA) and Mueller Hinton agar were purchased from Oxoid, UK.

Whatman Filter paper Grade 1 (90mm Ø, Pore size: 11 µm) were obtained from Tay Scientific (Malaysia), Instrument 3-Channel Screen Printed Electrode (O-ring) 7.20 100 720: Working Electrode (Carbon), Reference Electrode (Ag/AgCl) and Counter Electrode (Carbon) and the connector to the potentiostat were purchased from Rapid Genesis Sdn Bhd (Malaysia).

3.4 Physicochemical Analysis

3.4.1 pH Determination

The pH of honey samples was measured using the pH meter by diluting 10 g of honey with 75 mL of distilled water. The pH electrode was immersed into the solution after it was mixed using magnetic stirrer. The reading is recorded in two decimal places.

Figure 3.2 SevenCompact pH meter (Mettler Toledo, USA)

3.4.2 Free Acidity

Free acidity is determined by the titrimetric method. 10 g of honey were dissolved in 75 mL of distilled water, and this solution was titrated with NaOH 0.1 M solution until the pH reached 8.5, with the result expressed in mmol/l. The pH is measured by pH meter (Biluca et al., 2016).

3.4.3 Moisture content

The official method for moisture content determination is the drying oven however in order to achieve a faster result, moisture content was determined using handheld refractometer (RHB 90ATC, China) where it measured the refractive index of the honey.

Figure 3.3 Hand-held refractometer

3.4.4 Electrical Conductivity and Ash Content

Electrical conductivity of the honey samples was measured by Hi 8733 conductivity meters (Hanna Instruments, USA) and the results were expressed in milliSiemens per centimetre (mS/cm). For this analysis, 20 g honey was diluted in 100 mL distilled water (20% w/w). The ash content were analysed by burning the samples at 550 ºC in the Carbolite CWF 1200 muffle furnace (Carbolite Gero Limited, UK) until constant mass was obtained (Malika et al., 2005). The honey ash content was determined by placing the crucible in an oven for 1 h. After cooling, the crucible was weighed. 5 g of the honey sample was placed into the crucible and burnt in a 500 ºC furnace for 2 hours. The sample was then reweighed and ash percentage was calculated.

Figure 3.4 Carbolite CWF 1200 muffle furnace (Carbolite Gero Limited, UK)

3.4.5 Hydroxymethylfurfural (HMF) determination

HMF was determined by using the White spectrophotometric method which was described in the International Honey Commission's harmonized methods.5 g of honey sample was weighed and completely dissolved in 25 mL of distilled water before transferred into 50 mL volumetric flask. 0.5 mL carrez solution I (Merck, Germany) was added into the volumetric flask and mixed well by vortex. The mixed solution was then added with 0.5 mL Carrez solution II (Merck, Germany) and mixed before adding distilled water up to 50 mL mark. A drop of ethanol was added to suppress the foam that formed during mixing. The mixture was filtered through filter paper, and the first 10 mL of the filtrate was discarded. Aliquots of 5.0 mL was placed in two test tubes where in Tube 1(sample solution): 5.0 mL of distilled water was added and in Tube 2 (reference solution): 5.0 mL of 0.2% sodium bisulphate solution (Sigma- Aldrich, USA) was added. The absorbance of the solutions at 284 and 336 nm were determined using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, USA).

Figure 3.5 GENESYS 10S UV-Vis spectrophotometer

3.4.6 Sugar profile

The determination of sugar profiles (fructose, glucose, sucrose) was performed using 1260 Infinity II LC System (Agilent Technologies, USA) where the method is following the method of Malaysian Kelulut Standard. First, Firstly, the standards solution and sample solution were prepared by adding 25 mL acetonitrile into different 100 mL volumetric flask. The amount of each standard and sample substances as in **Table 3.2** were dissolved in 40 mL of ultrapure water (UPW) before being transferred quantitatively into the flasks. UPW were added until reached the mark of the flasks. The solution was filtered through 0.45µm membrane filter and stored in sample vials before being eluted through Zorbax column (Zorbax NH_2 150 x 4.6 mm, Agilent Technologies, USA) and detected by refractive Index detector (RID). The conditions of HPLC is as shown in **Table 3.3.** The retention times obtained from the standards were compared to obtained HPLC sample peaks. Duplicate

injections were performed where the average peak areas are used for the quantification (de Sousa et al., 2016).

Table 3.2 Amount of standard and samples for HPLC analysis

Types of substances	Amount (g)
Fructose	2.0
Glucose	1.50
Sucrose	0.25
Honey	5

Table 3.3 HPLC conditions for sugar analysis of honey

Conditions	Description
Flow rate	1.3 mL/min
Mobile phase	Acetonitrile:Water (75:25,v/v)
Column and detector temperature	35 ˚C
Sample volume	2 µL

3.5 Enzymatic Analysis

3.5.1 Diastase Activity

The diastase activity was determined by following the method of International Honey Commission. A 5.0 g of honey samples were dissolved in 15 mL distilled water and then mixed with 2.5 mL of acetate buffer (1.59M, pH 5.3). The solution was then mixed with 1.5 mL of 0.5 M

sodium chloride solution in 25 mL volumetric flask before 10 mL of this solution is taken and combined with 5 mL of 2% starch solution in a test tube. Then, the test tube was kept in BS -21 shaking water bath (Lab Companion, Jaio Tech Inc, South Korea) at 40ºC. After 5 minutes, 1 mL of the solution was mixed with 10 mL of 0.0007M diluted iodine solution. The absorbance was recorded using a spectrophotometer at 660 nm until the reading reached less than 0.235 absorbance. The diastase activity was expressed in Diastase Number (DN). DN was the amount of enzyme that hydrolysed/ converts 1% starch solution/ 0.01g of starch for 1 h at 40ºC. Diastase Number (DN) was calculated using formula shown below (Buba et al, 2013) :

Diastase Number (DN) was calculated according to Equation 3.1:

$$Diastase\ Number\ (DN) = \frac{60}{tx} \times \frac{0.10}{0.01} \times \frac{1.0}{2.0} = \frac{300}{tx} \qquad \text{(Equation 3.1)}$$

Where, tx = reaction time in minute

3.5.2 Invertase Activity

The invertase activity was determined by following the method of International Honey commission. A 5.0 g of honey with buffer solution was transferred into a 25 mL flask and fill to the mark. This solution can be kept in the refrigerator for 1 day. Next, 5.0 mL of substrate solution;p-Nitrophenyl-α-D-glucopyranoside (pNPG) was placed in a test tube and transferred into the water bath at 40 °C for 5 minutes before adding the 0.50 mL honey solution (starting time). The contents were briefly mixed and incubated at 40 °C. After 20 minutes, 0.50 mL of the reaction-terminating solution was added into the solution and mix (sample solution). For the blank, 5.0 mL of substrate solution was incubated at 40 °C at the same time. After 5 minutes, 0.50 mL of reaction-terminating solution was added into substrate solution; mixed and finally 0.50 mL of honey solution was added. Separate blank was made for each honey samples. The samples were determined using

spectrophotometer at 400 nm. The value is indicated as IN (Invertase Number). The IN indicates the amount of sucrose per gram hydrolysed in one hour by the enzymes contained in 100 g of honey under lab conditions.

The readings were taken after about 15 minutes and in any case within one hour. The absorbance of the blank was subtracted from that of the sample solution (=ΔA400). Invertase Number (IN) was calculated using formula shown below (Bogdanov, 2009) :

$$IN = 21.64 \times \Delta A400 \quad \text{(Equation 3.2)}$$

Where,

ΔA – the value of absorption

21.64 – the slope of the linear regression of IN (y-axis) on ΔA400 (x-axis)

Buffer solution is made by dissolving 11.66 g of potassium hydrogen phosphate KH_2PO_4 and 2.56 g of disodium hydrogen phosphate $Na_2HPO_4.2H_2O$ in water and dilute to 1 L. Reaction-terminating solution (3 M, pH = 9.5) made by dissolving 363.42 g of tris- (hydroxymethyl) aminomethane in water and dilute to 1L. Adjust to a pH value of 9.5 with 3 M hydrochloric acid.

3.6 Microbiological Analysis

Ten grams of each honey samples were mixed with 90 mL of saline water (8.5 g/L) to prepare the initial dilution. This solution is used as the mother dilution for further serial dilutions, 10^{-1}, 10^{-2}, 10^{-3}. All colonies growth (standard plate count, detection of *Bacillus badius*, total coliform, yeast

and mold fungi) were observed and counted in CFU/g. The experiment was conducted using (ECSO Class II Bio Safety Cabinet) as shown in **Figure 3.6** below.

Figure 3.6 ECSO Class II Bio Safety Cabinet

3.7 Preparation of test organisms

Micro-organisms were obtained from the Central Laboratory, University Malaysia Pahang. Two strains of the gram-positive bacteria and gram-negative bacteria: *Escherichia coli* and *Bacillus badius* were used in this research.The isolates were identified based on standard microbiological techniques, and sub-cultured in nutrient agar slopes at 37 °C for 24 h. Colonies of fresh cultures of the different microorganisms from overnight growth were picked with sterile inoculating loop and suspended in 3-4 mL nutrient broth contained in sterile test tubes and incubated for 8 hours at 37 °C using (Memmert incubator) as shown in **Figure 3.7**. This was diluted with distilled water to set inoculum density used in this study.

Figure 3.7 Memmert biological incubator

3.8 Standard Plate Count

Twelve grams of standard plate count agar (PCA) was prepared and mixed with 600 ml distilled water. 100 µL of each dilution was plated onto each plate. The honey samples that are diluted in saline water were plated on standard plate count agar (PCA) and incubated at 30 °C for 48 h. The calculation of colonies as shown in Equation 3.3 (Buba et al, 2013):

$$\text{CFU/g} = \frac{\text{no.of colonies x dilution factor}}{\text{a gram of sample}} \qquad \text{(Equation 3.3)}$$

3.9 Detection of *Bacillus badius*, Total coliform and Yeast and mould fungi

3.9.1 Detection of Bacillus badius

Bacillus badius was detected by plating the aerobic spore-forming bacteria on plate count agar (PCA) and incubated at 30 °C for 48 h using Memmert incubator. A 12 g of standard plate count agar (PCA) was prepared and mixed with 600 mL distilled water. 100 µl of each dilution was plated onto each plate. Before plating the bacteria, the initial dilution was heated at 80 ºC for 10 minutes and cooled immediately in iced water. The calculation of colonies was used Equation 3.3.

3.9.2 Detection of Total Coliform

Total coliform was counted on violet red bile glucose agar (VRGB). The plate was incubated at 35 ºC for 24 h to 48 h using Memmert incubator. A 12 g of violet red bile glucose agar (VRGB) was prepared and mixed with 600 mL distilled water. 100 µL of each dilution was plated onto each plate. The calculation of colonies was used Equation 3.3.

3.9.3 Detection of Yeast and Mould Fungi

Yeast and Moulds were enumerated on Sabouroud Dextrose agar (SDA) supplemented by 100 mg/L chloramphenicol (antibiotic). 12 g of Sabouroud Dextrose agar (SDA) was prepared and mixed with 600 mL distilled water. 100 µL of each dilution was plated onto each plate. The plates were incubated at 25°C for 3-5 days using Memmert incubator. If there is no growth at day 5, need to re-incubate for another 48 h (total 7 days). The calculation of colonies was used Equation 3.3.

3.10 Antibacterial activity analysis

The bacteria that is used in the analysis are gram positive bacteria (*Bacillus badius*) and gram-negative bacteria (*Esherichia coli*). The inoculants are prepared by sub culturing and incubating the test cultures for 24 h. The colonies willsuspended in 0.85% saline and the cell suspension is adjusted to concentration of c. 10^8 CFU mL^{-1} for bacteria.

3.10.1 Agar disc diffusion assay

Fresh culture suspension of the test microorganisms (100 µL) was spread on Mueller Hinton agar plates. The concentration of cultures was $1x10^7$ CFU/mL. For screening, 5 mm sterile diameter filter paper disc was impregnated with 10 µL of honey equivalent to 0.1 mg of honey. The plates were placed at 4 °C for 2 h before being incubated using (Memmert incubator) in Figure 3.5 under

optimum conditions for 24 h. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The zone diameters of inhibition (ZDI) was measured in millimeter, including the diameter of the disc. The controls were set up with equivalent quantities of water as a control.

3.11 Construction of prototype paper-based biosensor

The prototype paper-based biosensor was developed starting with the modification of screen printed electrode, immobilization of enzymes, development of glucose and sucrose calibration curve, testing the behaviour of the paper-based biosensor with cyclic voltammetry, performance evaluation of developed paper-based biosensor with several parameters and finally the paper-based biosensor were tested on honey samples. **Figure 3.8** shows the flow of paper-based biosensor development for this research.

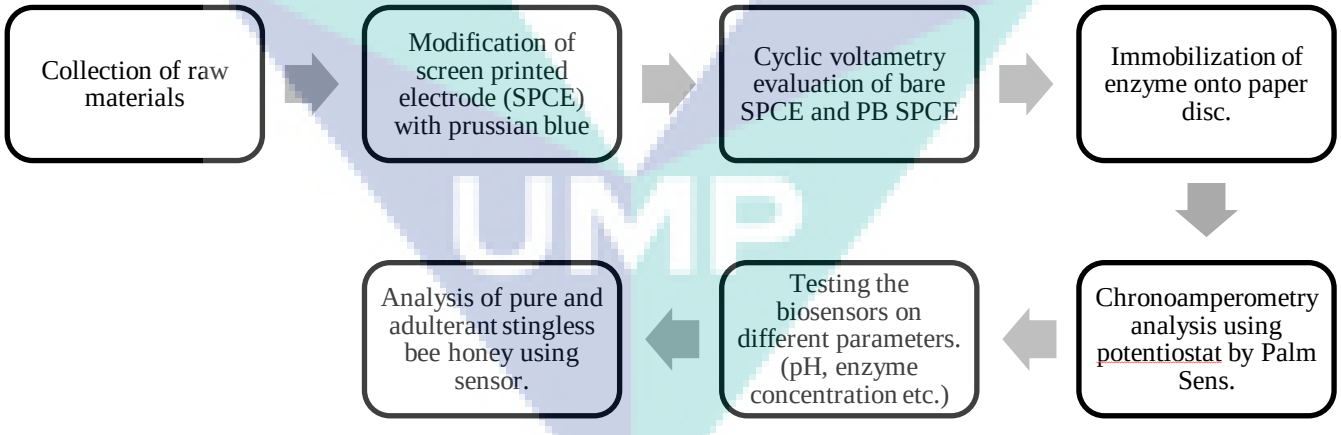

Figure 3.8 Flowchart for paper-based biosensor development

3.12 Preparation of phosphate buffer and Hydrochloric acid

Most of experiments for biosensor construction were conducted using 0.1 M phosphate buffer solution (PBS), pH 7.0 (Biscay et al., 2011). First, 1.0 M of K_2HPO_4 (87.09g were diluted in 500 mL distilled water) and 1.0 M of KH_2PO_4 (68.04g were diluted in 500 mL) were prepared. Then, the combined 1 M stock of solutions were diluted again to 1 L with distilled water. In order to prepare pH 7.0, 61.5 ml K_2HPO_4 and 38.5 ml KH_2P0_4 were mixed and pH of the new buffer solution were measured. Below are the recipes to prepare different pH of phosphate buffer.

Table 3.4 The amount of the volume of chemical needed to achieve the targeted pH value

Phosphate Buffer pH	Volume of KH_2PO_4, (mL)	Volume of K_2HPO_4, (mL)
6.0	13.2	86.8
6.5	28	72
7.0	61.5	38.5
7.5	85	15
8.0	94.0	6.0

For 10 mM hydrochloric acid (HCl), 0.17 mL of 37% concentrated HCl were diluted in 200 mL of distilled water.

3.13 Modification electrode by Prussian blue (PB)

Prussian blue modification of SPEs was done according to method by (Piermarini et al., 2011; Ricci et al., 2003) by dropping 30 µL of mixed solution containing 10 µL of 0.1 M $K_3Fe(CN)_6$ (1.65 g were diluted in 10mM 50 mL HCL) with and 10 µL of 0.1 M FeCl3 (0.811 g were diluted in 10 mM 50 ml HCL onto electrode surface. The electrode was dried off for approximately 10 min and rinsed with 10 mM HCL before left in the oven at 60 °C for 90 min oven to secure a stable and active layer of PB. The PB-modified electrodes were then stored dry at room temperature (25-27°C) in the dark.

3.14 Enzyme Immobilization on Paper Disc

The method of enzymes immobilization on paper disc were done by following method from past literature with some modification (Sekar et al., 2014; Kuek Lawrence et al., 2014; Sekar et al., 2015). Whatman Grade 1 filter paper was cut into round disks with ca. 10 mm. Then, 8 µL of enzyme GOX solution (50 U/ml in of 0.1 M of phosphate buffer, pH 7.0) was carefully dropped to paper disc surface and allowed to dry at room temperature. These GOX paper disks were used for glucose detection. The same procedure was applied to sucrose detection where 8 µL mixture of enzyme INV and GOX were also dropped on the paper discs. If paper discs are to be used in the following days or weeks, it must be kept in dark and at 4 ºC.

3.15 Paper- based biosensor preparation

After the drying steps, the paper discs were placed on the top of PB-SPCE fully covered the working, counter and reference electrodes. As shown in **Figure 3.9,** 12 µL of 0.1 M of phosphate buffer, pH 7.0 and 8 µL of 1 mM glucose or 1 mM sucrose solutions was dropped on the paper disc before each electrochemical measurement was performed. All paper disc and PB-SPCE were disposed after single used. **Figure 3.10** are the setup for biosensor measurement for this research.

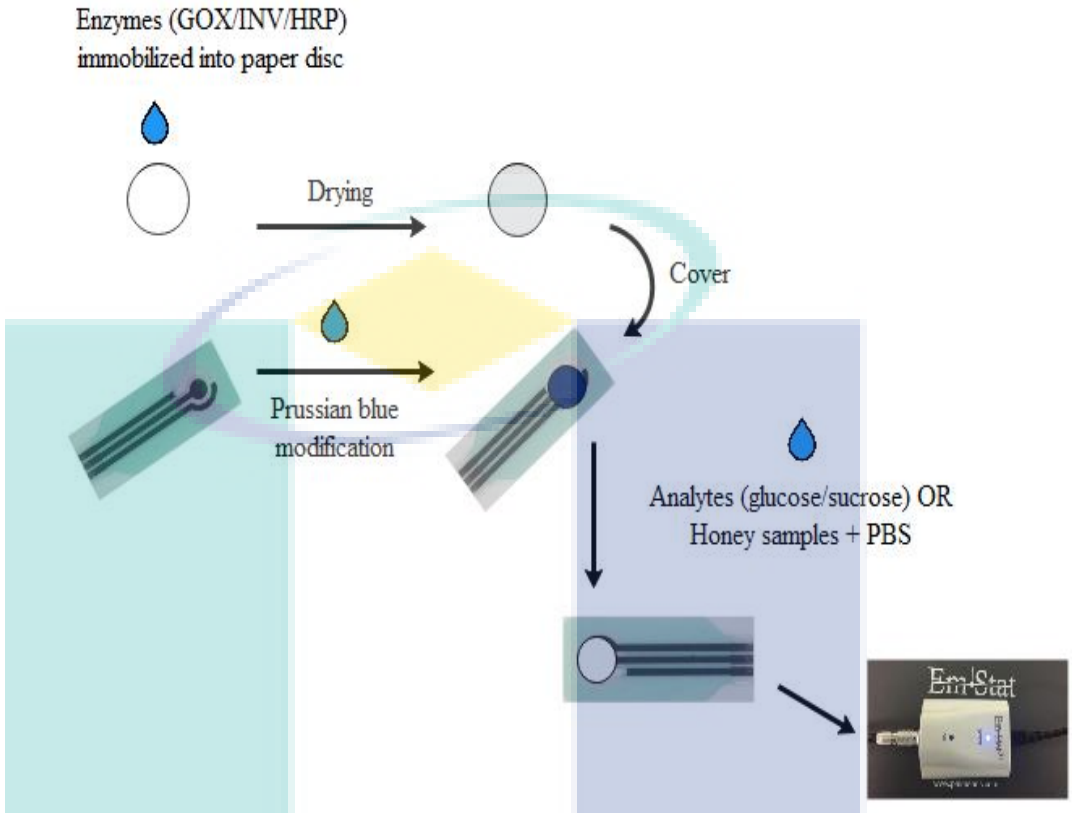

Figure 3.9 Schematic illustration of paper-based biosensor fabrication process

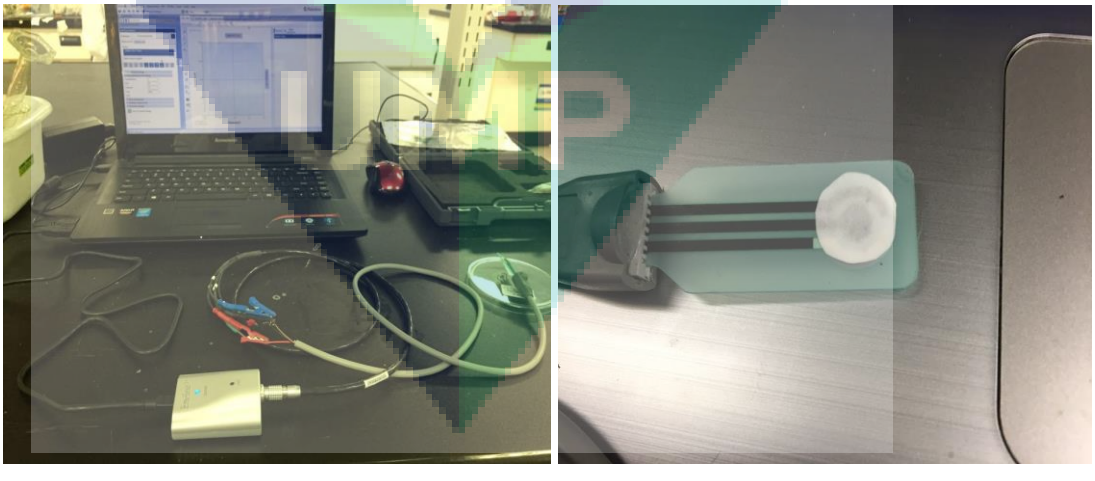

Figure 3.10 Setup for paper-based biosensor

3.16 Measurements

Cyclic voltammetry was monitored from an initial potential of -0.2 V to a vertex potential of +0.3 V where the scan rate was at 40 mV s^{-1}. The functionality of PB layer on SPCE was tested by cyclic voltammetry. The detection of glucose and sucrose by PB-SPCE was recorded chronoamperometrically at fixed potential of +0.8 V versus Ag/AgCl for 100 s. The electrochemical response was recorded as current (µA). The Cyclic voltammetry (CV) measurements and chronoamperometry measurements were carried out using a Palmsens Emstat3 (Palmsens, Netherlands) potentiostat **(Figure 3.11)** controlled by PSTrace 5.5 software.

Figure 3.11 Palmsens Emstat3 portable potentiostat

3.17 Application in honey samples

Stingless bee honey samples and the market samples were prepared by dissolving 1 g of honey in 50mL of Millipore milli-Q water and diluted to 100 times in 0.1M phosphate buffer of pH 7.5 (Biscay et al., 2011). For analysis, 8 µL of the samples was dropped on paper disc and tested chronoamperometrically at +0.8 V potential. The obtained values from biosensor were compared with HPLC method.

CHAPTER 4

RESULT & DISCUSSION

4.1 Introduction

As a starter, the characterization of six honey samples which comprises of three different analyses that is physicochemical, enzymatic and microbiological analyses were examined. Physicochemical analyses cover the evaluation of honey samples pH, moisture content, free acidity, electrical conductivity, ash content, hydroxymethylfurfural (HMF) and sugar profile while enzymatic analyses discuss on the diastase activity and invertase activity of honey samples. Then, the microbiological properties explained on the standard plate count, detection of bacillus, total coliform and yeast and mould and the antibacterial activity of all honey samples. After the development of the paper-based biosensor, the best conditions from each parameter which includes the effect of drop volume, effect of enzyme concentration, effect of applied potential and effect of enzymes concentration were discussed. Additionally, by using the optimum conditions, the calibration graph of the paper- based glucose and sucrose biosensor were recorded. The sensor response on selectivity, reproducibility, and stability was also conducted before the applicability of the paper-based biosensor on the detection of glucose and sucrose adulteration on stingless bee honey samples were applied.

4.2 Physicochemical Analyses

Physicochemical of six honey samples using various analyses such as pH, free acidity, electrical conductivity, ash content, moisture content, hydroxymethylfufural (HMF), and sugar analysis was determined and discussed in following section.

4.2.1 pH determination

The most consistent parameter which displays the least variability, measured in this study is pH. Based on **Table 4.1** and **Figure 4.1**, all stingless bee honey samples were acidic with pH values ranging from 2.51 to 3.26. Comparable data from South American stated that pH value for stingless bee honey is generally between 2.9 to 5.3 (Biluca et al, 2016). There is no significant difference in pH value of H1 and H2, however, when compared with the other commercial honey, the result is significantly different. The variation in pH value is due to the enzyme reaction in honey. Enzyme functions and catalyzed reactions will determine pH level, honey composition, and antibacterial properties (Fuad, 2017).

Theoretically, pH value is depending on their geographical origin, floral sources and bee's species (Sousa et al, 2015). Since the fresh harvested honey is a culture at the same place, the floral sources

do not affect the differentiation of the pH. However, it is influenced by bee species. H2 has lower pH because it comes from species *Trigona Thoracica* that have sour taste compared to H1 from species *Heterotrigona Itama*. In comparison to the commercial honey, lower pH may cause by the different harvested area that has different air relative humid rate. High humid area usually leads to low pH due to the high-water content of the honey. The different composition of nectar that used to produce the honey also influences the variation in pH of the commercial honey. Moreover, the low pH value can also indicate the freshness or possible honey adulteration.

Commonly, the honey that has undergo adulteration will have higher pH value which is more than 5.5 because it is related to the extraction process, storage factor and temperature of honey (Gomes et al.,2010). Due to acidic properties of stingless bee honey, it will prevent the development of microorganisms that require neutral or basic pH values, significantly limiting the spectrum of potentially contaminating microorganisms.

Table 4.1 pH reading for six stingless bee honey samples

Sample	pH reading
H1	3.26± 0.11
H2	3.20± 0.08
H3	2.52± 0.03
H4	2.79± 0.01
H5	2.51 ± 0.01
H6	2.57 ± 0.01

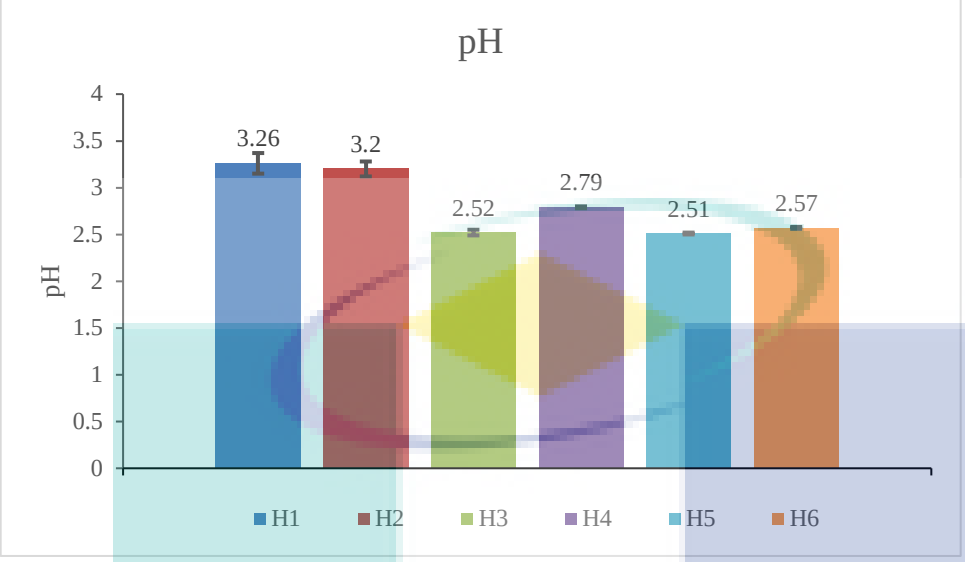

Figure 4.1 pH reading for six samples of stingless bee honey from Malaysia

4.2.2 Free Acidity

The mean value for free acidity found in six samples of stingless bee honey in the range of 121.1 to 318 mEq/kg. Based on **Table 4.2** and **Figure 4.2**, H5 has the highest free acidity with the value 318.7 mEq/kg. According to Malaysia kelulut standard, there is no fixed limit of free acidity for stingless bee honey, however, all the samples values exceeded the value that was reported by International Honey Commission which is not more than 50 mEq/kg. This result may explain the sour taste that the stingless bee exhibit. H1 and H2, which is the harvested honey have the lower free acidity value compare to the commercial honey. This is influenced by maturation state of the honey. The honey might be harvested after it just reached early mature state that leads to the small amount of organic acids present in the honey.

Meanwhile, high free acidity content in the commercial may be due to a different type of floral sources used to produce the honey. Since the commercial honey is located at a different location, there is a variation of floral sources provided in the area. The floral sources in the area may have a high organic compound that will lead to the high free acidity in honey. Other factor

that influenced the result is maturation state of the honey. The honey might be harvested after it have reached very mature state that lead to the increasing of organic acids in the honey. The maturation of honey will increase due to fermentation. The fermentation of sugar into organic acids clarified the high acidity in the honey whereas the low acidity indicates the freshness of the honey though the value may increase with time (Yadata, 2014). The variation of acidity values also influenced by flower sources and the bee species since it conforms to the balance of organic acids present in honey.

Table 4.2 Free Acidity value for six samples of stingless bee honey from Malaysia

Sample	Free acidity (mEq/kg)
H1	146.4 ± 7.46
H2	121.1 ± 1.24
H3	257.8 ± 2.39
H4	318.7 ± 7.76
H5	250.7 ± 3.19
H6	241.7 ± 3.46

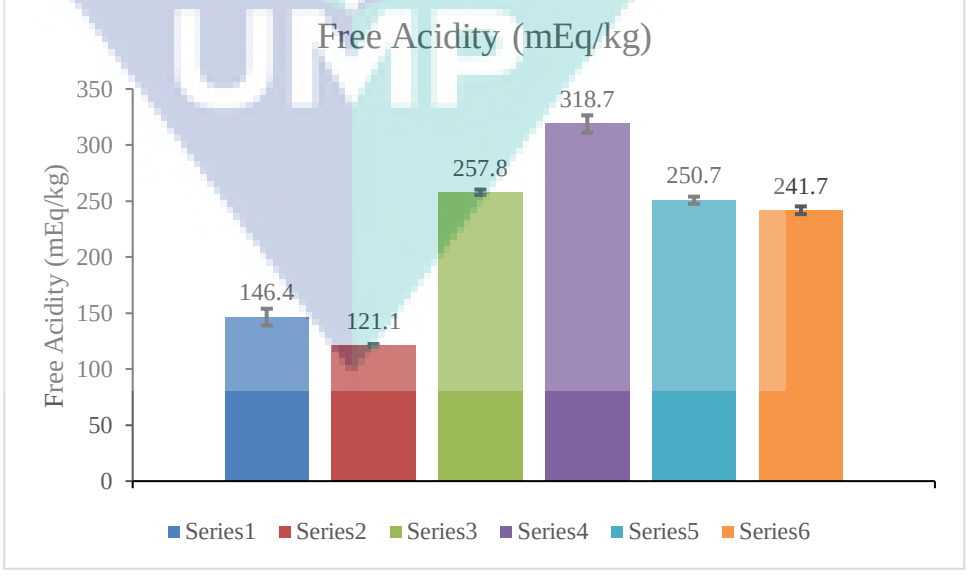

Figure 4.2: Free Acidity value of six samples of stingless bee honey from Malaysia

4.2.3 Moisture Content

By referring to **Table 4.3** and **Figure 4.3**, six honey samples showed that the percentage of moisture content fluctuates from 19.4 to 30.9 %. The average moisture content is calculated as 25.7 ± 4.01 %. In comparison with the data of stingless bee honey from Brazil, the average moisture content is Thailand is 31 ± 5.4 %. The variation of moisture content may due to the humidity of tropical forest, floral sources, collection period as well as processing aspects. The highest moisture content is recorded by H2 whereas the lowest moisture content is H5. This may due H2 production area has high air relative humidity and near to water sources while H4 might undergo a process of extraction that will remove the water content in honey. The rest of honey samples show the normal moisture content for a stingless bee.

The main factor that influences the high moisture content in the samples is because of the warm and humid environment in Malaysia. According to Bijlsma et al (2006), stingless bee honey has higher moisture content compare to normal honey bee because stingless bee collects small amounts of honey and use it rather than ripening and storing it. The moisture content of stingless bee should not be more than 20 % in order to decrease the probability of fermentation to occur. Moisture content within the reference standards able to enhance the shelf life of the product, since it provides an un-favorable condition for microorganism growth and fermentation. The high moisture content of honey will increase the probability of fermentation to occur. Besides that, fermentation process that occurs in honey also indicates that the honey has undergone adulteration.

Table 4.3 Moisture content of six samples of stingless bee honey in Malaysia

Sample	Moisture content (%)

H1	25.4 ± 0.09
H2	30.9 ± 0.06
H3	23.4 ± 0.53
H4	28.4 ± 0.51
H5	19.4 ± 0.21
H6	26.7 ± 0.61

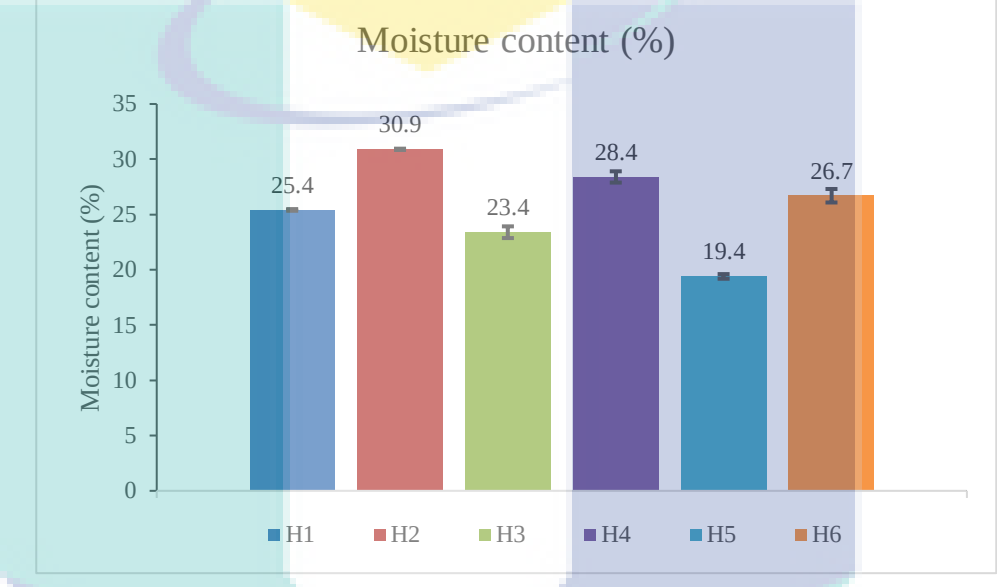

Figure 4.3 Moisture content of six samples of stingless bee honey in Malaysia

4.2.4 Electrical conductivity

Based on **Table 4.4** and **Figure 4.4**, the reading of electrical conductivity is ranging from 0.33 to 0.69 mS/cm. The average conductivity of six samples of stingless bee honey is 0.55 ± 0.15 mS/cm. This is comparable to the report of (Biluca et al, 2016) who reported that the electrical conductivity of stingless bee from Brazil is ranging between 0.15 to 1.34 mS/cm. From the six samples, H2 showed the lowest reading of electrical conductivity with 0.33 ± 0.01 mS/cm which may be influenced by the intake of floral sources. Nectar composition in the floral sources intake might have low mineral content. Meanwhile, the highest reading of electrical conductivity is H5 with 0.69 ± 0.06 mS/cm which may indicate the honey is rich with mineral content. Electrical

conductivity is closely related to the concentration of minerals, organic acids as well as it shows great variability depending on the floral source of honey (Nascimento et al, 2015) Besides that, the color of honey also influence the electrical conductivity reading. The darker the color intensity, the higher the conductivity reading due to high levels of microelement (Alqarni et al, 2014).

Table 4.4 Electrical Conductivity reading of six samples of stingless bee honey from Malaysia

Sample	Electrical conductivity (mS/cm)
H1	0.56 ± 0.01
H2	0.33 ± 0.01
H3	0.68 ± 0.01
H4	0.40 ± 0.03
H5	0.69 ± 0.06
H6	0.65 ± 0.04

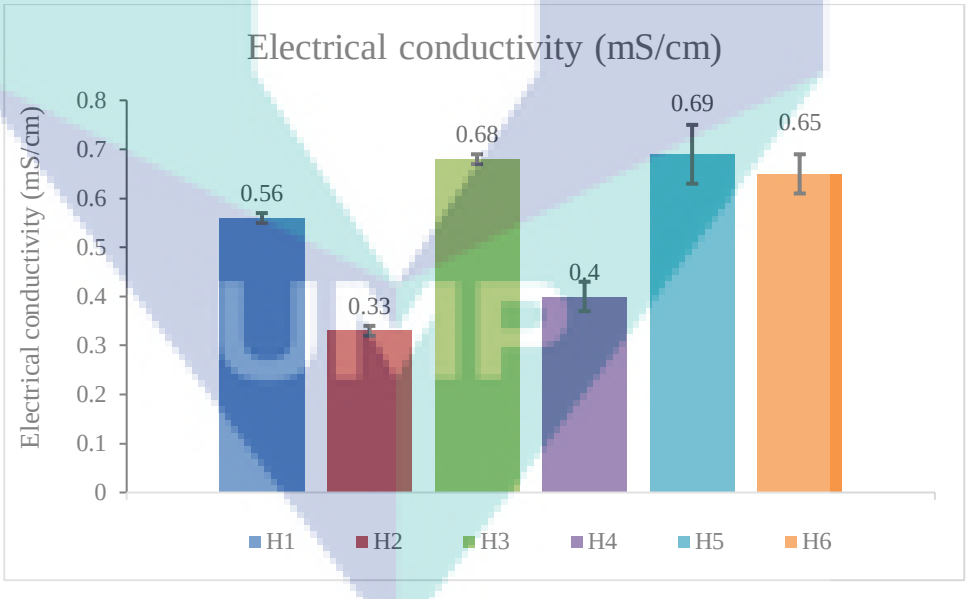

Figure 4. 1: Electrical Conductivity reading of six samples of stingless bee honey from Malaysia

4.2.5 Ash Content

By referring to the **Table 4.5** and **Figure 4.5**, only H2 and H6 were observed to be within the range of Malaysia kelulut standard which is lower than 1.0 g/100g. While, H1, H3, H4, and H5 have higher ash content ranging from 2.75 to 4.31 g/100g. The variation of ash content in honey may be due to the material collected by bees during the foraging on the flora, different botanical origin as well as the characteristic of nectar in some floral. High ash contents in H1 and H4 are influenced by the chemical composition of the dominant nectar of each floral source or also known as monofloral honey. Monofloral honey usually has the highest representation in the honey and dominates the flavor profile and appearance (Lixandru, 2017). The wide distribution of values detected in the six samples may cause by an irregular pattern of the harvested process and different in meliponiculture techniques used as well as different nectar composition of the flora.

Besides that, the ash content related to the color where the darker the honey color, the higher the honey content. This is due to amounts of mineral in the honey, while the mineral content depends on the soil type.

Table 4. 5: Ash content of six samples of stingless bee honey from Malaysia

Sample	Ash content (g/100g)
H1	3.11 ± 0.23
H2	0.72 ± 0.11
H3	2.75 ± 1.12
H4	3.86 ± 1.02
H5	4.31 ± 1.53
H6	0.52 ± 0.16

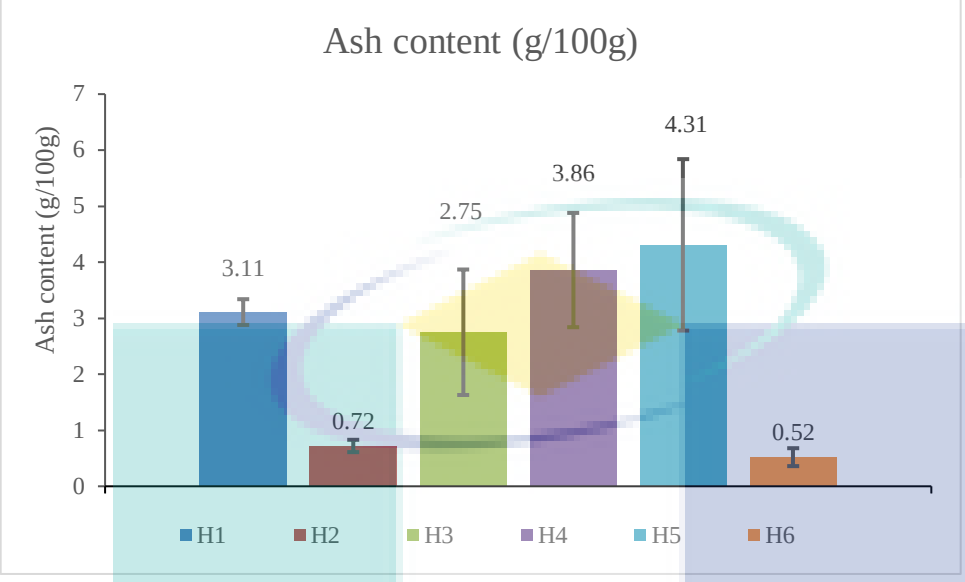

Figure 4. 2: Ash content of six samples of stingless bee honey from Malaysia

4.2.6 Hydroxymethylfurfural (HMF)

HMF content is a parameter to evaluate the freshness and overheating of the honey. HMF is a cyclic aldehyde (C6H603) formed by decomposition of fructose in the presence of acid (Nascimento et all, 2015). **Table 4.6** and **Figure 4.6** below shows the HMF content of six samples of stingless bee honey is ranging from 35.4 to 461.7 mg/kg. The result shows that H4 has the lowest HMF values, 35.4 ± 0.71 mg/kg but still exceeded the limit set by Malaysian kelulut standard which is less than 30 mg/kg. H3, H5, and H6 exceeded the HMF limit in both Malaysia kelulut and international honey commission standard which is more than 400 mg/kg.

From the result, this indicates that the three commercialized honey are non- fresh honey due to it has undergone the heating process. Usually, honey undergoes heat treatment to remove the water content. The other reason that can relate to the high HMF value of all commercial honey is due to honey adulteration, where the honey was added with other foreign substances such as syrup corn starch and fructose-rich syrup. According to Codex Alimentarius (2011), honey from tropical ambient temperature shall not be more than 80 mg/kg. Hence, H1, H2, and H4 can be considered as a good quality of stingless honey. The high HMF content may be caused from the poor storage condition or the sample is old. HMF value in honey will increase when honey is exposed to high temperature, inadequate storage condition and aging (Zappala & Verzela, 2005)

Table 4. 1: HMF reading of six samples of stingless bee honey from Malaysia

Sample	HMF (mg/kg)
H1	74.3 ± 6.88
H2	85.9 ± 8.40
H3	157.2 ± 0.81
H4	35.4 ± 0.71
H5	461.7 ± 5.69
H6	456.6 ± 2.72

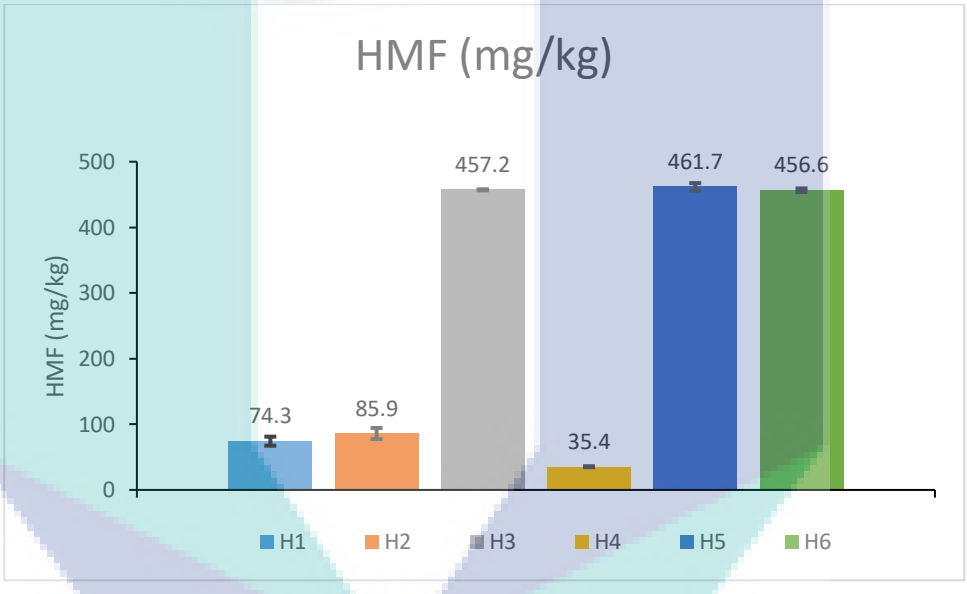

Figure 4. 3: HMF reading of six samples of stingless bee honey from Malaysia

4.2.7 Sugar profile

Table 4.7 shows the results of carbohydrates, which indicates substantial variation among themselves. The summation of fructose and glucose of the six samples are ranging from 27.2 to 69.08 g/100g. The summation results met the requirement by Malaysian kelulut standard which is no more than 90.0 g/100 g. However, some samples slightly exceeded the codex standard of sugar profile for honey (< 60 g/100 g). Based on the result, the content of fructose and sucrose are lower compared to others which followed the result trend by (Chuttong et al ., 2016). Chuttong et al. (2016)

have suggested that possibility of low fructose and glucose content for certain species of stingless bee honey. The proportion of sugar content in honey is depending on floral sources, seasonal climate and geographical origin (Biluca et al, 2016).

H5 has high sucrose content of 7.47 ± 1.04 g/100g, but it is still within the maximum limit stated by Malaysian kelulut standard which is no more than 8.0 g/100 g. The high sucrose content could be attributed to floral sources, overfeeding with sugar syrup, sucrose not being fully transformed into glucose and fructose, or adulteration (Suntiparapop et al, 2015). Addition of cheaper sweeter, corn syrup, other foreign substance into the honey significantly will increase the content of sucrose in the honey. According to Siok et al, (2017), the data from Colombia reported that the content of fructose and glucose in stingless bee honey should be lower than 36 g/100g and 30 g/100g respectively.

The ratio of fructose to glucose (F/G) could be an indicator of honey flavor due to the fact that fructose is sweeter than glucose and sucrose (de Sousa et al., 2016; Kek et al., 2017). Commercial honey, C11 has the highest F/G ratio of 1.63 thus, the honey is sweeter compare to others. Besides that, F/G ratio implying the honey ability to granulate because when the amount of fructose is greater than glucose, the honey is in fluid state (Boussaid et al., 2013). When the F/G ratio is below 1.0, the crystallization of honey is quicker, however when this ratio is greater than 1.0, the honey stays in liquid forms for a long time (El Sohaimy, Masry, & Shehata, 2015; Radia et al., 2015). F/G ratios not only depend on the source of the nectar but it also depends on the variation of bee species and climate of different regions (Biluca et al., 2014). Low ratio of F/G will speed up the process of crystallization. This is because, as the glucose is less water soluble than fructose, thus it tends to come out from honey solution in order to form a crystal.

Table 4. 2: Sugars content in six samples of stingless bee honey from Malaysia

Sample	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)	F + G (g/100g)	F/G (g/100g)
H1	17.5 ± 1.3	16 ± 0.8	1.10±0.03	33.5 ± 2.27	1.10 ± 0.10
H2	15.03 ± 1.22	12.17 ± 0.47	0.93±0.01	27.2 ± 0.69	1.24 ± 0.09
H3	42.81 ± 0.72	26.27 ± 0.63	3.34±0.08	69.08 ± 1.35	1.63 ± 0.01
H4	32.52 ± 0.59	30.09 ± 0.70	0.80±0.15	62.62 ± 0.67	1.08 ± 0.04
H5	33.51 ± 0.54	31.94 ± 0.07	7.29±1.04	65.45 ± 0.61	1.05 ± 0.01
H6	22.83 ± 0.42	25.47 ± 0.50	2.81±0.06	48.3 ± 0.95	0.89 ± 0.01

4.3 Enzymatic analyses

For this research, only diastase and invertase activity for all six samples were reported and discussed. These enzymes activities play important roles in honey quality evaluation.

4.3.1 Diastase Activity

Table 4.8 and **Figure 4.7** below shows the diastase activities of commercial honey ranged from 2.71 to 6.11 DN where harvested honey of known species, *Heterotrigona Itama* (H1) and *Geniotrigona Thoracica* (H2) had some small differences between them, 5.85 and 5.95 DN respectively. These results clearly showed that the amount of diastase number of samples bought from the market (except H5) was lower compared to harvested honey. However, all the samples were below the range of international honey standard, 8 DN or 3 DN (low enzyme content).

Table 4. 8 Diastase activity (DN) of various honey samples ± standard deviation

Samples	H1	H2	H3	H4	H5	H6
Diastase Number (DN)	5.85±0.08	5.96±0.25	3.56±0.09	3.12±0.41	6.11±1.57	2.71±0.01

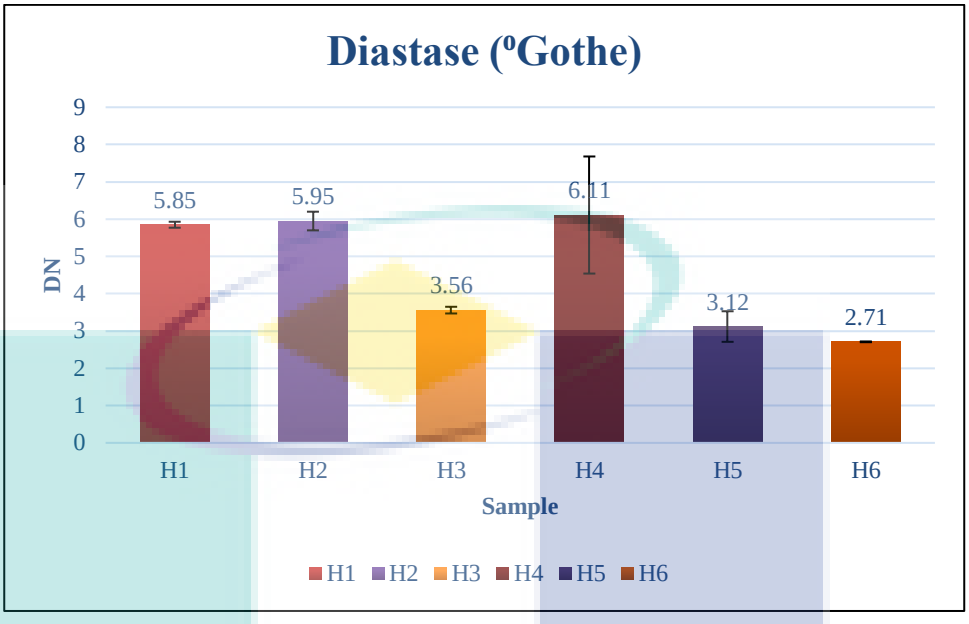

Figure 4. 7 Diastase activity (DN) of various honey samples

Diastase is one of the enzymes mostly secreted from bee salivary found in honey but not the floral source and it measures the combined activities of both α- and β-amylases in honey (Kek, 2017). It is also considered as a quality factor in the international honey standards of the Codex Alimentarius Commission (1969, 1989). There is also no fixed limit for diastase number in Malaysian stingless bee standard but according to Codex Alimentarius Commission (2001), Diastase Number have a minimum value of 8 DN. Souza et al (2006) recorded the diastase number for Trigona honey ranged from 0.9 to 23.0 DN.

The variability in enzyme activity found in the different honey types is probably due to a series of factors, such as: nectar collection period, abundance of nectar flow and its sugar content, age of the bees and pollen consumption (L Persano Oddo et al, 1990). A very low diastase activity allegedly indicates that the honey has been subjected to unfavorable temperature conditions since diastase activity of natural honey is rapidly reduced when honey is heated or stored at unfavorable

temperatures. According to Schade et al (1958), heating honey to a very high temperature is believed to destroy certain dietetic values of honey.

4.3.2 Invertase Activity

Table 4.9 and **Figure 4.8** below shows the results of invertase activity for all honey samples. The invertase activity of harvested honey ranged widely from 0.265 to 0.529 IN. On the other hand, the invertase activity at commercial honey the range was from 1.059 to 3.760 IN.

Table 4.9 Invertase activity (*IN*) of various honey samples ± standard deviation

Samples	Invertase (IN)
H1	0.265 ± 0.183
H2	0.529 ± 0.091
H3	1.748 ±0.159
H4	3.760±0.242
H5	1.059 ±0.091
H6	2.119 ±0.091

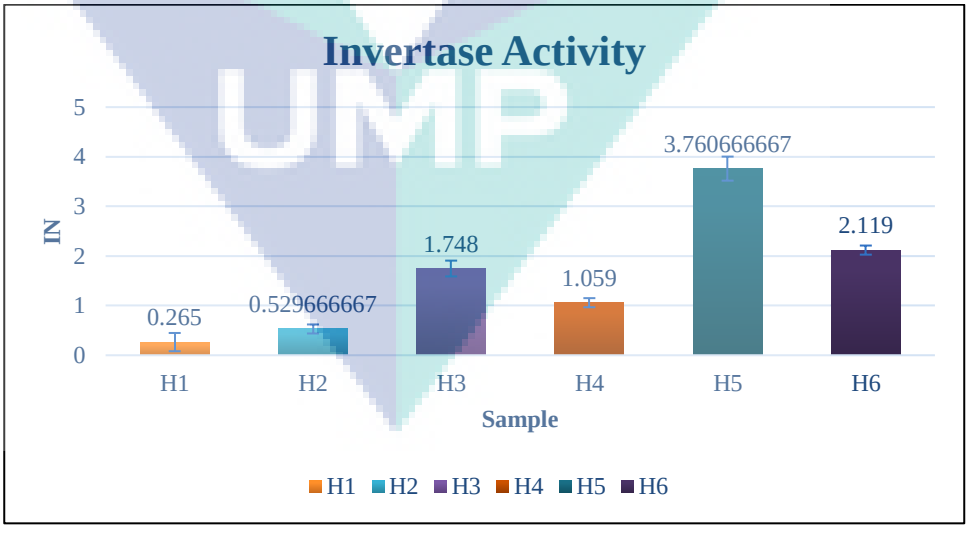

Figure 4. 8 Graph of invertase activity of various honey samples

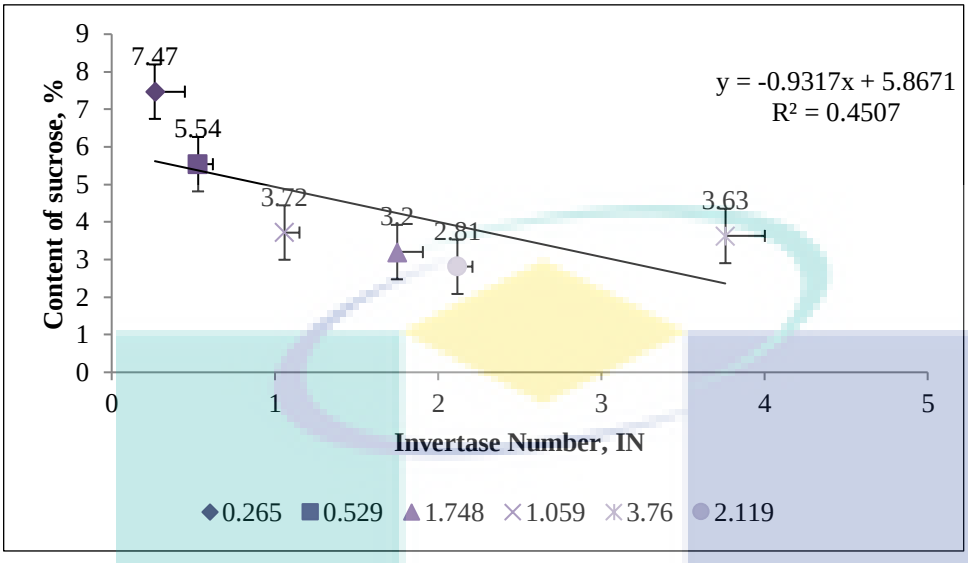

Figure 4.9 Influence of invertase activity on the content of sucrose in honey

From previous studies, the invertase value of stingless bee honey samples in Brazil ranged from 19.80 to 90.10 (Souza et al, 2006) and Jayalekshmi (2015) reported the invertase activity of stingless bee honey was 65.63 – 98.00 IN which had a huge difference with results of honey in Malaysia. According to Fredijs Dimins et al (2008) in conformity to EU recommendations, it was proposed that fresh and unheated honey should have an invertase number (IN) higher than 10, but for honey with low enzymatic activity IN higher than 4 is recommended. The fact that the honey samples were refrigerated, low enzyme content of honey samples < 4 IN from Figure 4.8 above had to be regarded as a natural feature of these honey, rather than an index of scarce freshness or lowered in quality.

Vorlov & Piidal (2002) also stated that the commercial honey could not be strictly classified in the most cases because they have been during processing mixed with more honey sorts with the aim to produce a honey with better taste feature. The amount of the invertase in honey depends on many factors such as the condition of a bee colony, the age of the bees, food, temperature and

intensity or type of honey flow. Moreover, the invertase activities decrease depending on the temperature and time of processing as proved by Dinkov at al (2001) where the invertase number of its honey samples dropped drastically from 11 IN to 0.67 IN after processing at 70 °C. White, Landis, & Doner (1980) demonstrated that invertase was destroyed more quickly than amylase when honey is heated, so invertase activity could be a better indicator of honey quality than diastase activity.

As we know that enzyme invertase hydrolyzes sucrose glucose and fructose. The correlation between activity enzyme invertase and the content of sucrose in honey can be observed from Figure 4.3 where there was some dependence between the content of sucrose in honey and invertase activity. We can see that the more the content of sucrose in honey, then less is activity invertase in honey. The amount of sucrose increase probably because of the factor of storage period and the harvested honey was not matured enough in which sucrose was not thoroughly transformed into glucose and fructose by enzyme invertase (Nascimento et al, 2015). However, when the invertase activity of a sample is measured in this study, it is hard to interpret the result in terms of freshness: since the initial value is unknown, there is no correct starting point from which to evaluate possible overheating or aging effects. Therefore, it seems hard to draw any conclusion about the use of this parameter for detecting honey freshness.

4.4 Microbiological Analyses

Microbiological properties of honey including the standard plate count (SPC), total coliforms, detection of Bacillus sp., fungi (yeasts and molds) and antibacterial activity were evaluated in this section.

4.4.1 Standard Plate Count

The microbial counts in the different samples of honey are reported in **Table 4.10**. below.

Table 4.10 Standard Plate count of various honey samples

Sample	**Microbial count (10^{-1}) CFU/g**
H1	3.6×10^2
	4.5×10^2
	1.5×10^2
H2	3.0×10^2
	8.0×10^2
	4.4×10^2
H3	3.6×10^2
	4.5×10^2
	1.5×10^2
H4	2.6×10^2
	2.7×10^2
	9.7×10^2
H5	1.5×10^2
	2.2×10^2
	3.5×10^2
H6	1.0×10^2
	1.0×10^2
	1.0×10^2

*CFU/g = colony forming unit per gram

Standard plate counts (SPC) in the harvested samples, H1 and H2 ranged from 1.5×10^2 to 8.0×10^2 CFU/g while in commercial samples, H3 to H6 ranged from 1.0×10^2 to 9.7×10^2. The standard plate count (SPC) were found in low numbers in most samples of honey with a minimum count of 100 CFU/g and a maximum 970 CFU/g. Other than that, based on the results obtained, the colonies at every dilution factor were successfully grown but the most obviously at dilution factor 10^{-1}.

The result on standard plate count as shown on Table 4.4 above is lower than that reported by Omafuvbe & Akanbi (2009) which studied about commercial Nigerian honey but falls within the range reported by (Kornkanok Suntiparapop et al, 2012). This variation in bacterial counts may be due to the type of sample, freshness of the honey, the time of harvest and the analytical techniques used. The standard plate count provides very general information and is useful as a point of comparison to other data and as a general indicator of the microbial quality of honey. Honey with high standard plate counts (10 000/g) could be acceptable if other microbial criteria example like indicating the presence of yeast or freedom from fecal contamination were satisfied.

4.4.2 Detection of *Bacillus badius* , Total coliforms, Yeast and mold fungi

Table 4.11 below shows the results of some microbiological parameters of honey samples. Total coliforms were not detected in all six samples whereas yeast and mold were detected on both harvested honey, H1 and H2 and only on one commercial honey, H3. In addition, Bacillus count was detected on all the commercial honey.

Table 4.11 Detection of Bacillus badius, Total coliforms, Yeast and mold fungi of various honey samples

Sample	Microbial count (x 10^2 CFU/g)			
	Total coliform	**Yeast**	**Mould**	***Bacillus badius***
H1	ND	ND	ND	ND
H2	ND	ND	ND	ND
H3	ND	D	D	D
H4	ND	D	D	D
H5	ND	D	D	D
H6	ND	ND	ND	D

D = Detectable; ND=Not Detectable

Coliforms and yeasts are indicative of sanitary or commercial quality concern. According to Adenekan et al (2012), bacteria are not able to multiply in honey. Their high number could indicate contamination during processing, handling, and storage because during the honey production, processing and storage there are chances of pollens, bee digestive tract residues, dust, and polluted air contact with honey which could lead to microbial contamination This should be controlled by good manufactory practices.

Bacillus sp is a type of environment pathogenic bacteria. The famous type of Bacillus sp is *Bacillus cereus* and *Bacillus badius* which is a group of ubiquitous facultative anaerobic spore-forming gram-positive rods bacteria commonly found in soil, vegetables, and in many raw and processed foods. The previous study has revealed the most Bacillus species had been reported

common in honey are B. cereus, B. megaterium, B. coagulans, and B. pumilus (Andualem, 2014). The presence of *Bacillus* spp. would be expected in honey, since a symbiotic relationship between this microorganism with insects, including honeybees, solitary bees, and stingless bees had been reported (Pucciarelli et al, 2014). Since, there was no detection of total coliform, yeast, and mold and also spore-forming bacteria, *Bacillus badius* on harvested honey samples, H1 and H2, this may be explained by the evidence that honey is well preserved against bacteria so that these organisms would not survive unfavorable conditions. This result agreed with the report that Moroccan honey was very low in bacterial and coliform counts (Adenekan et al, 2012).

4.5 Antibacterial Activity Analysis

The results of antibacterial activity of the six honey samples with different dilutions used in this study are shown in Table 4.12. In this study, we used *Bacillus badius* as gram-positive bacteria and *E. coli* as gram-negative bacteria as showed in Figure 4.10 and Figure 4.11 respectively. From the table 4.12, also can observe that the harvested honey, H1, and H2 had a greater inhibitory effect on both gram-negative and gram-positive bacteria when tested using undiluted honey. The results of commercial honey samples also showed they had an inhibitory effect only on gram-positive bacteria when tested using undiluted honey (except H4) but all the commercial honey samples were not active against gram-negative bacteria, *E. coli.*

4.5

Table 4. 12 Antibacterial Activity of various honey samples on Gram-positive and Gram-negative bacteria

Sample	Dilution	*Bacillus badius* (mm)	E. coli (mm)
H1	Undilute	15	11
	10^{-1}	-	-
	10^{-2}	-	-
H2	Undilute	24	11
	10^{-1}	-	-
	10^{-2}	-	-
H3	Undilute	20	-
	10^{-1}	-	-
	10^{-2}	-	-
H4	Undilute	-	-
	10^{-1}	-	-
	10^{-2}	-	-
H5	Undilute	12	-
	10^{-1}	-	-
	10^{-2}	-	-
H6	Undilute	10	-
	10^{-1}	-	-
	10^{-2}	-	-

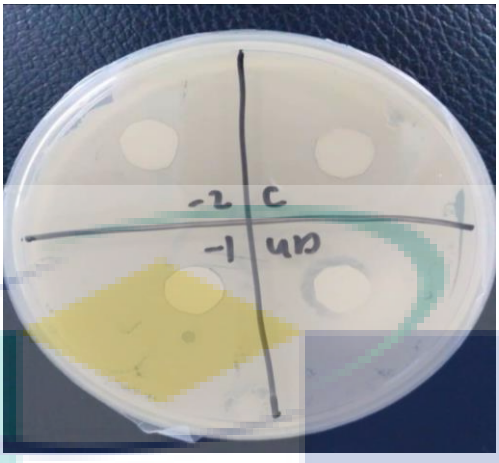

Figure 4.10 The inhibition zone by using different dilution factor on *E. coli*

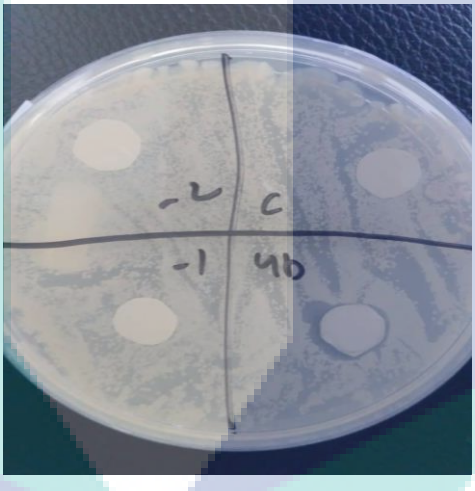

Figure 4. 11 The inhibition zone by using different dilution factor on *Bacillus badius*

The disc diameter of harvested samples was ranged from 11 to 24 mm where commercial honey, H3, H5, and H6 ranged from 10 to 20 mm. There were no zone diameters of inhibition (ZDI) was measured on H4 sample for both bacteria tested. The result indicated that different concentrations of this compound in different kinds of honey result in their varying antimicrobial effects (Moussa et al, 2012). All honey samples had a broad spectrum antibacterial activity because stingless bee honey can act against a wide range of bacteria that able to cause disease (Yaacob at al, 2018). According to Moussa et al (2012), the antibacterial activity was classified as: no sensitive,

for diameters lower than 8 mm; sensitive, for diameters from 8 to 14 mm; very sensitive, for diameters from 15 to 19 mm; extremely sensitive, for diameters higher than 20 mm.

As we know, *Escherichia coli* (E. coli) bacteria normally live in the intestines of people and animals. Most E. coli are harmless and an important part of a healthy human intestinal tract. However, some E. coli are pathogenic, meaning they can cause illness, either diarrhea or illness outside the intestinal tract. *Bacillus badius* also can be found in the human intestine. From the studies, stingless bee honey can reduce the risk of infection to humans by this pathogen. This might be one of the basic reason that the society widely uses honey for the treatment of the wound. Therefore, harvested honey samples the highest potential to become an antibacterial agent to treat disease compared to commercial honey samples because they could inhibited the growth of bacteria on plate.

4.6 Electrochemical characterization using Cyclic Voltammetry (CV)

The electrochemical behaviour of the bare SPCE, PB modified SPCE, PB-SPCE with immobilized enzymes and analytes additions on paper disc was investigated by cyclic voltammetry and is intepreted in **Figure 4.12** . The CV performed from +0.30 V to −0.2 V at a scan rate of 40 mV s−1. As shown, the bare SPCE does not display any redox peaks. However after the modification of SPCE by PB, there are some increament in reduction current whereas only slight increase in oxidation current was observed. This conformed that PB was a good electrocatalyst for the reduction of hydrogen peroxide (H_20_2) where the same observation has been reported in the literature (Chandra Sekar et al., 2014).

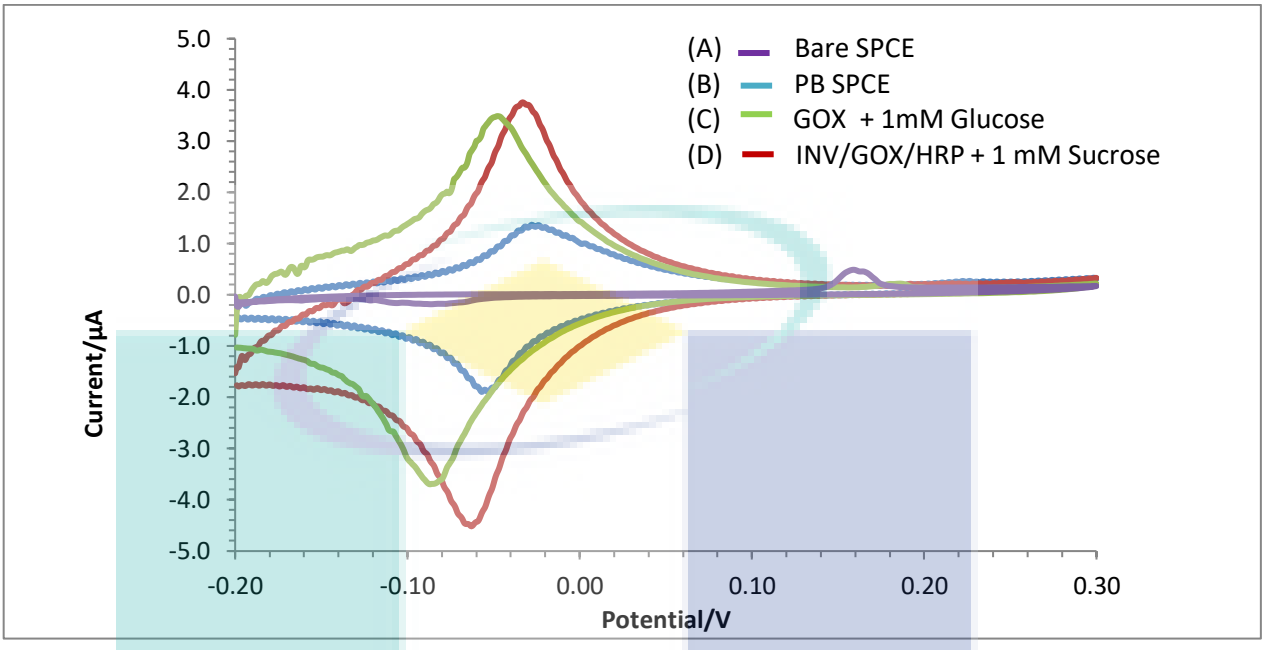

Figure 4.12 Cyclic voltammograms of the (A) Bare SPCE (B) PB-SPCE (C) GOx immobilized on paper disk + addition of 1 mM glucose (D) INV/GOX/HRP immobilized on paper disk + addition of 1mM sucrose in 0.1 M PBS (pH = 7.0) at a scan rate of 40 mV s−

After the addition of glucose and sucrose on the enzymes paper disc, the reduction current were increased significantly where the reduction current for glucose and sucrose biosensor only differed by 1µA. Sucrose paper- based biosensor shows greater current due to the presence of various enzymes on the paper disc. In addition, the oxidation peaks did not significantly increase as much as in comparison to the reduction peaks. This also can be concluded that only a small sample volume of 8 µL was sufficient in producing excellent signals. Based on previous research, these scans are characteristic of the enzymatic reaction of GOx on glucose and H_2O_2 reduction was catalyzed by PB (Chi & Dong, 1995). Moreover, the enhancement of reduction current in the scans clearly demonstrates that the close proximity of PB and the GOx disc allowed for its efficient electrocatalytic role for the H_2O_2 reduction reaction

4.7 Operating Conditions of paper-based biosensor

In order for paper biosensor to response well is by selecting the best operating conditions which includes the study on effect of applied potential, effect of buffer pH and effect of enzyme concentration where the response were recorded using amperometric detection.

4.7.1 Effect of applied potential

The effect of applied potential for both glucose and sucrose paper-based biosensor was illustrated in **Figure 4.13**. The signals produced in response to glucose and sucrose were measured in the potential range of + 0.2 V to + 1.0 V. Both biosensor started with small current from +0.10 V and increased gradually until reached maximum current at +0.8 V before the response drop

beyond +0.8 V to +1.0. The increased sensitivity with applied potential was attributed by upsurge force for the reduction of H_2O_2 (Sekar et al., 2014). Thus, in order to attained the greater sensitivity potential of +0.8 V was selected for the following experiments.

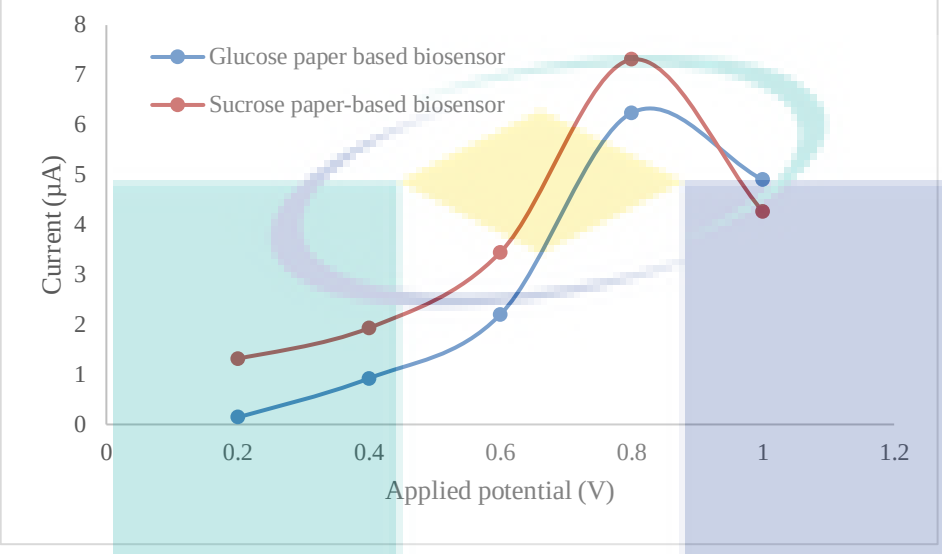

Figure 4.13 The effect of applied potential on the glucose and sucrose paper based biosensor amperometric response with 1mM glucose and 1 mM sucrose in 0.1 M PBS (pH = 7.0)

4.7.2 Effect of buffer pH

The effect of buffer pH is important to the sensitivity of glucose biosensors as it influences both the bioactivity of the GOx and electrochemical behaviour of PB. **Figure 4.14** showed the response of buffer pH which are ranging from pH 5.0 to pH 9.0. The analytical signal increases with pH until a pH value of 7. At pH values higher than 7.0, the catalytic activity might decrease due to irreversible denaturation of the enzyme. Those results are as expected because they correspond to the optimal pH range of the enzymes as well as pH of biological substances in food (Biscay et al., 2011; Polan, et al., 2011). For further studies a pH of 7 was chosen which corresponds to the pH commonly used in biological samples.

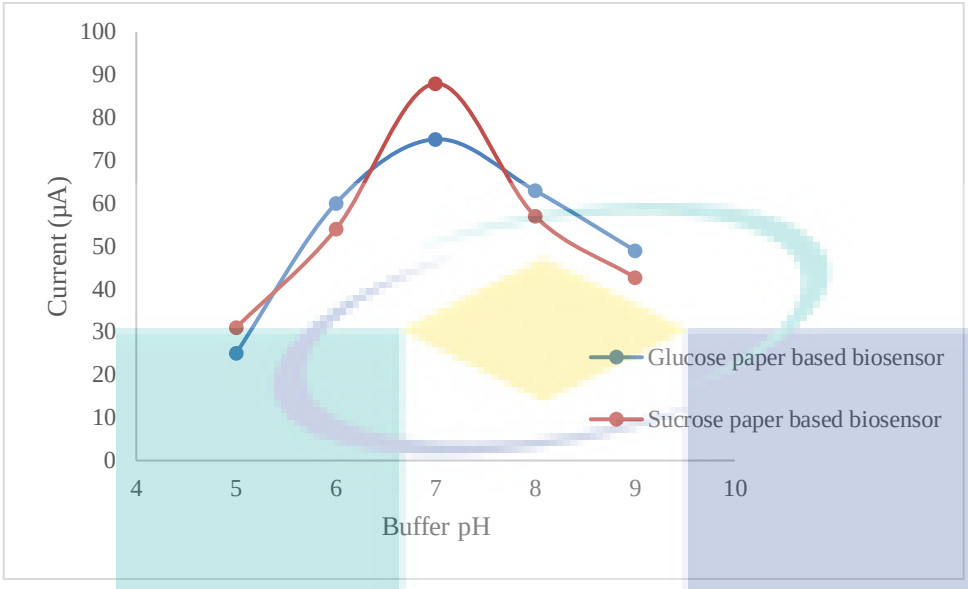

Figure 4.14 The effect of 0.1 M pH buffer on the glucose and sucrose paper based biosensor amperometric response with 1mM glucose and 1 mM sucrose.

4.7.3 Effect of enzyme concentration

The performance properties of the biosensors depend on the enzyme activity therefore, the effect of enzymes concentration was investigated. For glucose biosensor, different amounts of GOx (5, 15, 35 and 50 U/ mL) were studied in working buffer were shown in **Figure 4.15**. The highest amount was found to be 35 U/ mL of enzyme activity and higher enzyme amount beyond 35 U/ml resulted in a decrease in the PB-SPCE GOx response. The presence of a larger amount of enzyme causes diffusion problems for the oxygen as well as substrate transfer to the bioactive layer and therefore a lower current response was obtained (Demirkol , et al., 2017). Thus, for further experiments in glucose paper- based biosensor, concentration of 35 U/ mL was chosen.

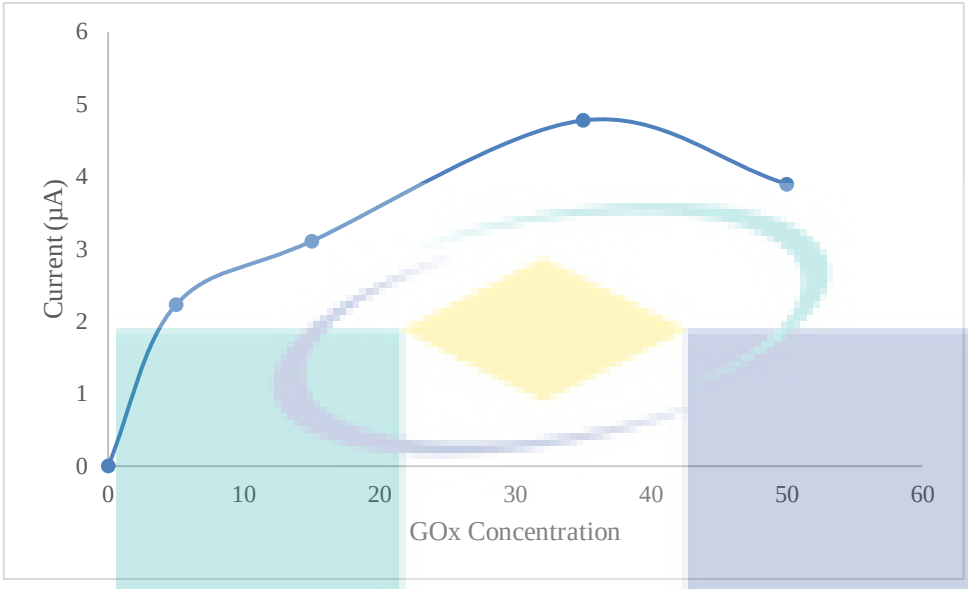

Figure 4.15 The effect GOx concentration on the glucose paper based biosensor amperometric response with 1mM glucose in 0.1 M PBS (pH = 7.0)

For sucrose paper based-biosensor, multienzymes was used in order to enhance the sensitivity of the biosensor.

The effect of three different enzymes INV, GOX and HRP concentration at various concentration (5, 10, 25, 30 and 50 U/ mL) were illustrated in **Figure 4.16.**

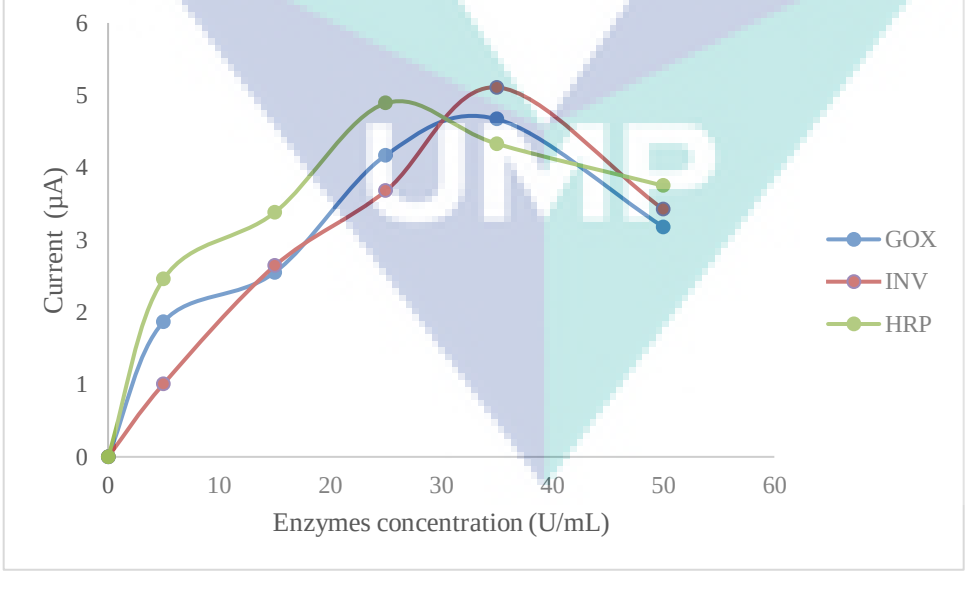

Figure 4.16 The effect of various enzyme concentration on the sucrose paper-based biosensor amperometric response with 1mM sucrose in 0.1 M PBS (pH = 7.0)

The higher enzyme concentration has resulted in a decrease in the biosensor response. For further experiments in sucrose paper-based biosensor, 35 U/ mL of INV, 35 U/ mL GOx and 25 U/ mL HRP was chosen. As shown in the result, MUT and GOx shows the lowest electrode response towards sucrose compare to INV enzymes. This indicate that the SPCEs response to sucrose increase when the concentration of INV increase.

4.8 Paper -based biosensor response characteristics

The evaluation of glucose and sucrose paper- based biosensor was determined by using several parameters such as development of glucose and sucrose calibration curve, selectivity against potential interference, reproducibility of the biosensor and storage ability. These parameters were studied using the best condition that has been done in previous section. The conditions are summarized in **Table 4.13**.

Table 4.13 Operating condition for both glucose and sucrose paper-based biosensor

Conditions	Glucose paper-based biosensor	Sucrose paper-based biosensor
Applied potential	+ 0.8 V	+ 0.8 V
Buffer pH	pH 7.0	pH 7.0
Enzyme concentration	35 U/ mL GOx	35 U/ mL of INV, 35 U/ mL GOx and 25 U/ mL of HRP

4.8.1 Calibration curve and reproducibility

By employing the best conditions from previous section, the calibration graph of both sensor was obtained as shown in **Figure 4.17** and **Figure 4.18**. The glucose biosensors have linear range between 0.5 mM to 4.5 mM. The linear regression equation was $y = 0.2993x + 2.2843$ where y signifies the current in µA and x is the glucose concentration in mM with R^2 value was found to be 0.9925. The relative standard deviation (RSD) for this biosensor is 3.74% thereby showing good reproducibility and limit of detection (LOD) is 0.15 mM.

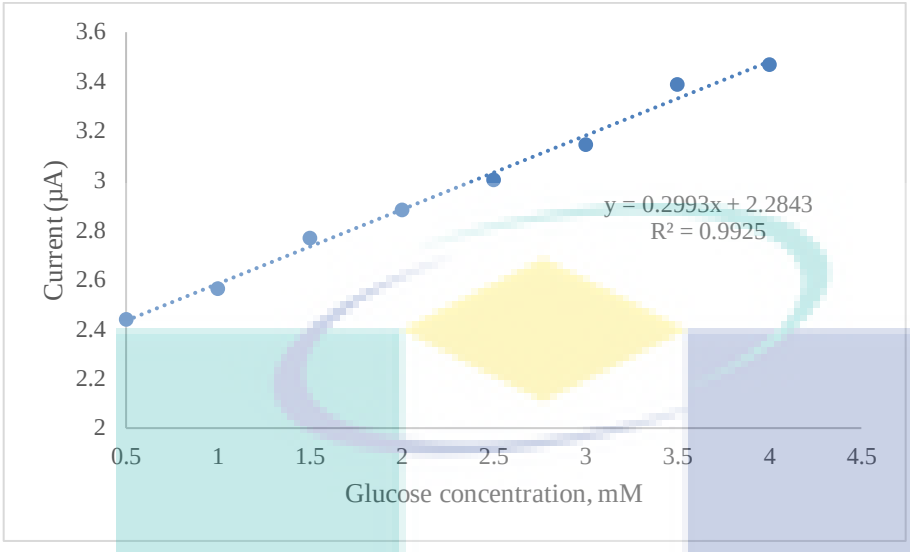

Figure 4.17 Calibration curve of the amperometric response of the glucose paper-based biosensor on PB-SPCE with various glucose concentrations at an applied potential of + 0.8 V in 0.1 M PBS (pH = 7.0).

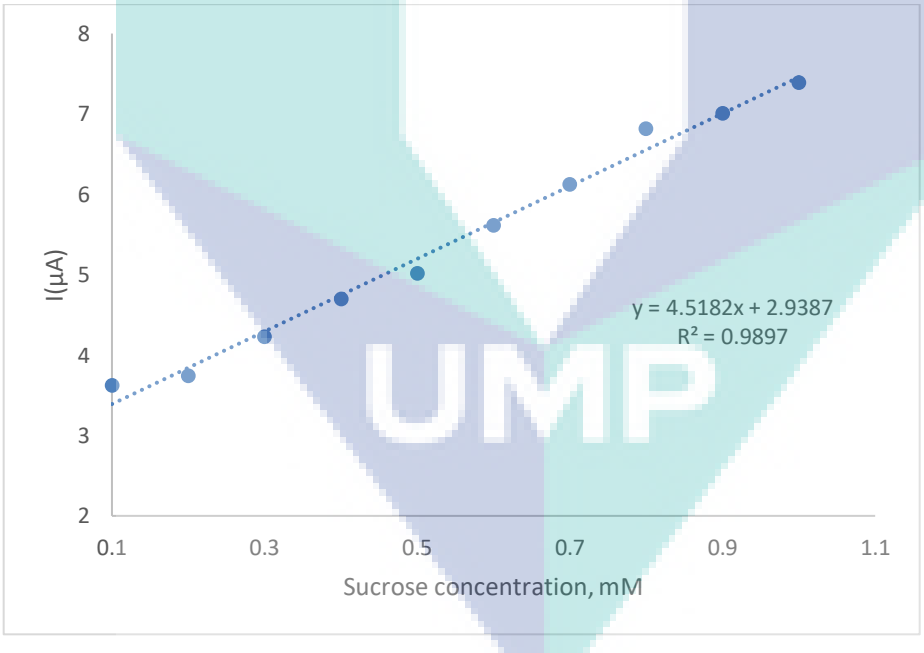

Figure 4.18 Calibration curve of the amperometric response of the sucrose paper-based biosensor on PB-SPCE with various glucose concentrations at an applied potential of + 0.8 V in 0.1 M PBS (pH = 7.0).

The sucrose biosensors exhibited a linear range between 0.1 mM to 1.1 mM. The linear regression equation was y = 4.5182x + 2.9387 with R^2 value was found to be 0.9897. In addition,

the RSD of the biosensor at 1 mM sucrose was 3.37% (n = 3) and and the LOD was at 0.1 mM. It reveals that the biosensor remains invertase activity and have higher affinity towards sucrose. Despite the simplicity of the present biosensor, it produced a lower LOD compared to the highly complex paper-based electrochemical (Cinti et al., 2017; Guzsvány et al., 2017)

4.8.2 Selectivity against interferences

The possible interference on the paper-based biosensor was investigated. The selection of interference was done according to the presence of sugars found in stingless bee honey, thus for glucose paper-based biosensor, the potential sugar interferences are sucrose and fructose. The current for each sugar interference at 1mM concentration were evaluated in the presence of 1 mM glucose (I^0) (Sekar et al., 2014; Sekar et al., 2015). Then, the current obtained is compared to the current of 1mM glucose alone (I) where the results are exhibited in **Table 4.14**. As can be seen, the fructose and sucrose do not cause any significance interference. This may due to the enzyme specificity as only enzyme GOX were presence in glucose paper- based biosensor.

Table 4.14. The selectivity study of interference substance for glucose paper-based biosensor

Interference substance	Current ratio [a] (I^0/I)
Fructose	0.8
Sucrose	1.0

[a] Current ratios (I^0/I) for mixtures of 1 mM interfering substance with comparison to the presence of 1 mM glucose alone (0.1 M PBS, pH 7.0, [GOX] = 35 U/mL)

For sucrose paper- based biosensor, the potential interference are the glucose and fructose. However, for sucrose biosensor, the glucose interfered significantly due to the presence of GOX enzyme in multienzyme system for sucrose detection. The result for potential interference substance in sucrose paper-based biosensor are shown in **Table 4.15.**

Table 4.15. The selectivity study of interference substance for sucrose paper-based biosensor

Interference substance	Current ratio[a] (I^0/I)
Glucose	0.6
Fructose	0.9

[a] Current ratios (I^0/I) for mixtures of 1 mM interfering substance with comparison to the presence of 1 mM sucrose alone (0.1 M PBS, pH 7.0, [INV = 35 U/mL, GOX = 35 U/mL, HRP= 25 U/mL])

4.8.3 Storage stability

Several glucose and sucrose sensors were prepared forehand and kept into the refrigerator at 4˚C as it is the optimum stored temperature for enzymes and light protected until their further use. The stability was observed out in different times 1 ,5, 15, 20 and 30 days duration. As shown in **Figure 4.19**, the stability of the sensor based on immobilized GOx was studied. As a result, the response shows a quite decrease by 24.6% after a week and on the 30th day, the activity had lost about 75.2%. Therefore, it is believed that the biosensor can stay long for 20 days for future use.

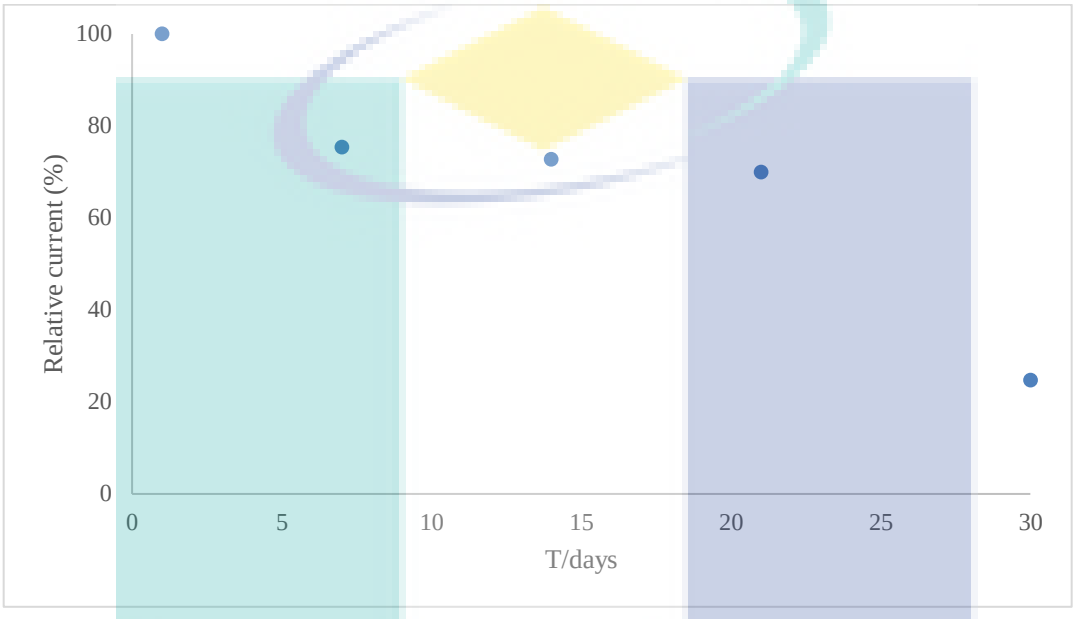

Figure 4.19 Stability of glucose paper-based biosensor for 30 days duration.

Figure 4.20 shows the study for sucrose paper-based biosensor at 1 day, 5 days, 15 days, 20 days and 30 days. As can be seen, the sensors are most stable at day 1. Then, it beginning to lose its stability until at 30th day where the sensor stability was significantly dropped. Compared to glucose biosensor, this sensor has lost 98.2 % from day 1 until day 30. The possible cause of biosensor losing it stability may due to inactivation of immobilized enzyme. Based on a case study written by Zhang for an article name Electrolysis, it stated that the stability of immobilized INV was the limiting factor for the stability of their sucrose sensor.

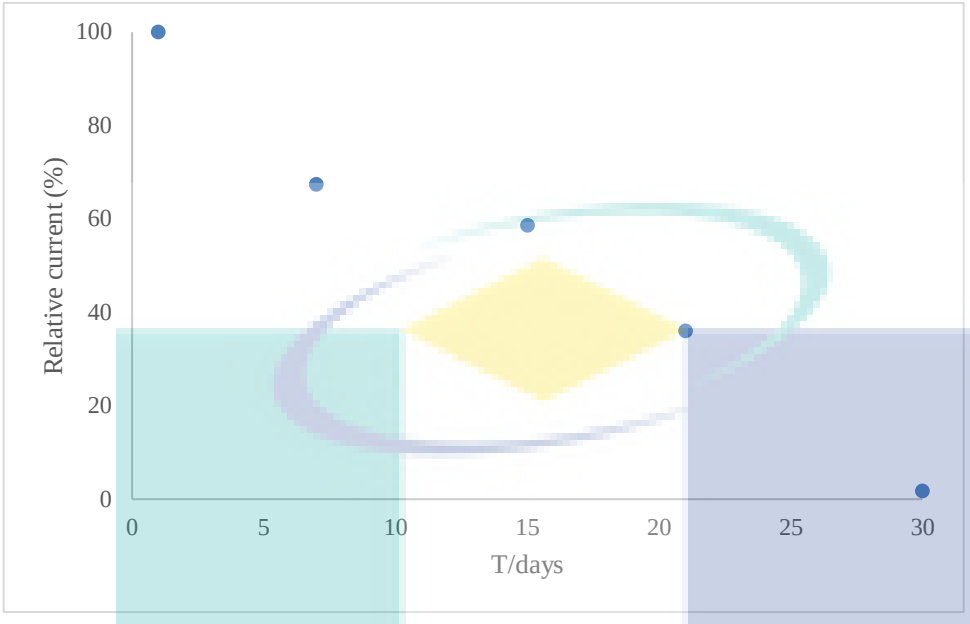

Figure 4.20 Stability of sucrose paper-based biosensor for 30 days duration.

4.9 Application to honey samples

To ascertain the applicability of the paper-based biosensor, all six stingless bee honey samples were measured by the both glucose and sucrose paper-based biosensor and the results were recorded in **Table 4.16** and **Table 4.17**. The results were compared with the results obtained from high performance liquid chromatography methodology in that have been done in **Section 4.2.7.**

For glucose measurement, most of the results obtained are lower compare to the prototype but this results are still comparable compared to the literature (Sekar et al., 2014; Lawrence et al., 2014). The percentage difference of the results and references was calculated by using equation 3.

$$\frac{Result - Reference}{Result + Reference/2} \times 100\ \% \qquad (3)$$

The difference of the glucose paper-based biosensor is ranging from 2.6 % to 17.9% which still considered as a good result. It shows that the glucose biosensor was successfully developed. However, for difference situation occurred for sucrose measurement as the results for developed sucrose paper-based biosensor shows larger percentage difference compared to the reference. Since there is no work on sucrose paper-based biosensor was developed to date, we not able to compare the obtained results with previous literature. The large differences of the results may have occurred due to the selection of enzymes HRP.

Table 4.16 The measurement of glucose with the proposed sensor in real samples; Data are given as average ± SD (n=3)

Samples	Reference (g/100g)	Glucose paper-based biosensor (g/100g)	% Difference
H1	16 ± 0.80	13.43 ± 0.17	17.5
H2	12.17 ± 0.47	10.16 ± 1.0	17.9
H3	26.27 ± 0.63	23.09 ± 0.08	12.5
H4	30.09 ± 0.70	27.44 ± 0.05	9.2
H5	31.94 ± 0.07	28.38 ± 0.43	10.3
H6	25.47 ± 0.50	24.62 ± 0.66	2.6

Table 4.17 The measurement of sucrose with the proposed sensor in real samples; Data are given as average ± SD (n=3)

Samples	Reference (g/100g)	Sucrose paper-based biosensor (g/100g)	% difference
H1	1.10 ± 0.03	0.56 ± 0.42	67.5
H2	0.93 ± 0.01	0.71 ± 0.05	26.3
H3	3.34 ± 0.08	2.21 ± 0.20	40.4
H4	0.80 ± 0.15	0.48 ± 0.88	50
H5	7.29 ± 1.04	4.36 ± 0.03	50.5
H6	2.81 ± 0.06	1.25 ± 0.27	76.8

CHAPTER 5

CONCLUSION

5.1 Overall Conclusion

A low cost, amperometric biosensor used for glucose and sucrose concentration determination was developed by using filter paper as a matrix for the enzyme (GOx/INV/HRP) immobilization via a simple adsorption step. The developed paper-based biosensor was used to quantify the sugar adulteration in stingless bee honey. The best conditions for the fabrication and operation of the biosensor were systematically studied. In addition, the characterization of all stingless bee honey samples was also determined. The characterization includes the physicochemical analyses, enzymatic analyses and microbiological analyses. The major findings for this research can be concluded as followed.

From four commercial samples, only H3 samples have similar characteristic when compare with harvested honey *H. Itama* (H1) and *G.Thoracica* (H2). This result may suggest that these samples are good quality honey. The rest of commercial honey (3 samples) shows high value of HMF (> 100 mg/kg) and higher sugar content which may indicate poor honey processing and possibility of adulteration.

The amount of the diastase and invertase in all six samples was quite low compared to Malaysia Kelulut Standard and it may depend on many factors such as the condition of a bee colony, the age of the bees, food, temperature and intensity or type of honey flow. There was some dependence between the content of sucrose in honey and invertase activity where the more the content of sucrose in honey, then the less activity of invertase.

The parameters standard plate count in the harvested honey samples, H1 and H2 comply with the requirements of the Malaysia Kelulut Standard. The presence of bacteria with the low count in some of the harvested samples in some of the microbiological analysis may be attributed mainly to contamination due to poor handling at harvest, packaging or storage.

The developed biosensor exhibits good linear range but sadly, both glucose and sucrose biosensor had lost more than 70% of their activities on the 30th day.

Based on the results of the experiments, it was confirmed that the proposed biosensor was sensitive and capable detecting addition amount of glucose in stingless bee honey. However, for sucrose determination, the percentage difference was highly significant, therefore more study should be done to improve this developed biosensor. Nevertheless,

this paper-based biosensor has promising prospects for applications in the detection of glucose and sucrose adulteration in stingless bee honey.

5.2 Future recommendation

1) More samples can be added for future research, at least 20-100 honey samples in order to obtained more information and to confirmed the quality of stingless bee honey

2) Modification for SPCE such as addition of polymers may be one of the practical solutions to enhance the biosensor stability.

3) In order to improve the sucrose biosensor, selection of enzymes for sucrose measurement should be investigated further. Other enzymes should be used since HRP produced quite poor result.

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Review

Classical and novel approaches to the analysis of honey and detection of adulterants

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ABSTRACT

Honey is an extract of floral and secretions from a variety of bees. Some honey manufactures adulterate pure honey with industrial sugar, chemicals, and water either directly or indirectly. Many methods have been developed to detect honey adulterants including physicochemical analysis, microscopy, chromatography, immunoassay, thixotropicity, DNA metabarcoding, sensors, and spectroscopy. However, the most promising methods for the development of a portable test kit for honey adulterant detection are ELISA, electronic tongue, and NIR. The most sensitive and accurate method is NIR. These methods have shown satisfactory results when used individually or combined. Further research is still required to trial different combinations of methods to improve accuracy and the ability to detecting a wide variety of adulterants simultaneously. There is a need to develop a portable honey adulterant detection method, such as NIR spectroscopy using a smartphone.

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1. Introduction

1.1. Definition of honey

Honey is a yellowish liquid that acts as Newtonian fluid (Abu-Jdayil, Ghzawi, Al-Malah, & Zaitoun, 2002). It consists of secretions of bees and extracts of plant nectar. Several species of bees visit plant nectar, collect the extract and store them as food. The classification of honey is thus based on the source of the nectar. Based on color there are two types of honey; light and dark. The dark honey is considered more nutritious such as richer in minerals (Anthony & Balasuriya, 2016; White, 1978). Honey can also be classified as honeybee (*Apis mellifera*) and stingless bee (meliponini) honey (da Silva et al., 2013). The honeybee is bigger in size and it sting while stingless honey bee does not sting and is smaller in size (Jalil, Kasmuri, & Hadi, 2017). Honeybee honey is sweet in taste while stingless bee honey is a mixture of sweet and sour taste (Aziz, Giribabu, Rao, & Salleh, 2017). Stingless bee business is a potential and fast growing in Malaysia. In 2014 Malaysian researchers found five species of stingless bee; *Hypotrigona scintillans*, *Trigona laevicepts*, *Trigona thoracica*, *Trigona Terminata* and *Trigona itama*. Among this *Trigona itama* is the most widely used by farmers (meliponiculture) (Kelly, Farisya, Kumara, & Marcela, 2014).

1.2. Composition of honey

Honey is nutritious and has medicinal value. Sugars, amino acids, organic acids, and biologically active compounds in honey make it nutritious and medicinally beneficial (Ahmed, Prabhu, Raghavan, & Ngadi, 2007). In the honey, main constituents are carbohydrate (70–80% w/w) and water (10–20% w/w). Other varieties of minor components such as free amino acids, proteins, phenolic compounds, minerals, vitamins and organic acids are also recorded in the honey (Ouchemoukh, Louaileche, & Schweitzer, 2007b). Amino acid content in honey is about 1% among which proline is dominant (50–80%) (Hermosín, Chicón, & Cabezudo, 2003). Carbohydrate content in honey by dry weight is recorded as 95% w/w and include mainly glucose and fructose (65–80% w/w), and saccharose/sucrose (disaccharides such as glucose and fructose bonded by glycosidic bonds) (de la Fuente, Sanz, Martínez-Castro, & Sanz, 2006). Propolis is one of the natural honey products that are waxy and resinous (Jalil et al., 2017). Physicochemical analysis revealed that propolis is rich in carbohydrates (49%) and crude fibre (44%). It also consists of 23% moisture, 21% crude fat, 4% ash and 3% crude protein (Ibrahim et al., 2016).

1.2.1. Honey composition standard

Codex Alimentarius (CODEX STAN 12-1981) standardized the composition of honey. Honey should have a moisture content not be more than 20%, sugar content not less than 60 g/100 g, sucrose not more than 5 g/100 g, free acidity not more than 50 milliequivalents acid/100 g, diastase activity not less than 8 Schade units, hydroxymethylfurfural (HMF) content not more than 40 mg/kg, electrical conductivity not more than 0.8 mS/cm and water-insoluble content not more than 0.1 g/100 g (Codex Alimentarius, 2001, p. 8).

1.3. Honey quality

Honey quality is decided based on physicochemical parameters; water, sugar, HMF, acidity, ash (mineral content), density, electrical conductivity, invertase activity and diastase level (Bogdanov & Gallmann, 2008; Bogdanov, 1999; Olugbenga & Obasanmi, 2014; Pasias, Kiriakou, & Proestos, 2017). The honey with high water content, low density, and high electrical conductivity easily ferments and degrades the quality resulting in a reduced shelf life. Water content indicates the honey density, extraction method and is also related to the maturity of the honey. The increase of water content decreases the honey density (Ouchemoukh et al., 2007b).

Sucrose content of the authentic honey is less than 5% (Ouchemoukh, Louaileche, & Schweitzer, 2007a). Therefore, honey that contains more than 5% sucrose maybe unripe; sucrose is not converted completely into glucose and fructose by invertase enzyme (Ouchemoukh et al., 2007a).

Invertase activity, diastase, and HMF are quality indicators that indicate freshness and overheating of honey (Bogdanov et al., 1999; Pasias et al., 2017). Lower diastase content may also indicate that the honey contains naturally low amylase content (Ouchemoukh et al., 2007a). HMF also indicates the purity of honey; a higher HMF value indicates that the honey has been overheated, aged or stored under poor for too long. For instance, honey samples stored for more than 12–24 months contained 128–1131 mg/kg of HMF which is greater than the recommended standard (80 mg/kg). Honey should be consumed within one year of storage (Khalil, Sulaiman, & Gan, 2010).

Electrical conductivity (EC) increases as the mineral and acid content of the honey increases. Honey mineral contents were found significantly correlated ($P < 0.05$) to EC. Yemeni and Egyptian honey had 4.18 and 1.98 ms/cm EC, respectively. Saudi and Kashmiri honey had 0.53 and 0.67 ms/cm, respectively. Therefore, Saudi and Kashmiri honey is within the standard limit (not more than 0.8 mS/cm) while Egyptian and Yemeni honey exceeds the limit (>0.8 mS/cm) (El Sohaimy, Masry, & Shehata, 2015). The acidity of the honey is due to organic acids such as gluconic acid, esters, lactones and inorganic ions of chloride and phosphate. Besides that, the extraction season varies the pH of a honey. The honey with pH below 3.5 is susceptible to spoilage (Bogdanov et al., 1999; El Sohaimy et al., 2015). El Sohaimy et al. (2015) found that the honey samples they tested were fresh as the acidic values (pH 4.1–4.6) comply with standard limits (pH 3.4–6.1) (Codex Alimentarius, 2001, p. 8). When the acidic value exceeds the standard limit it indicates fermentation of honey sugar into organic acids. The acidity controls the microbial spoilage and maintains the honey flavor (Bogdanov & Gallmann, 2008).

1.4. Storage stability

During storage, honey is fairly stable. However, honey adulterated with water will deteriorate faster. Besides that honey adulterated with chemicals lower the medicinal value as well as may harm the consumers (Anthony & Balasuriya, 2016). Jiménez, Mateo, Huerta, and Mateo (1994) investigated storage stability of honey for 2 years at 4–7 °C and 28 °C. The changes in pH, colour, sugar composition, water content, yeast and mould counts of honey were

analyzed. Over two years of storage the color of the honey darkened, sugar content changed but the total yeast level increased significantly ($P < 0.05$). However, there was no microbial growth and pH was found stable. Maltulose and turanose increased during storage while glucose, fructose, sucrose kojibiose, maltose, trisaccharides and isomaltose decreased. The yeast identified were *Schizosaccharomyces*, *Zygosaccharomyces*, and *Saccharomyces*. The moulds isolated were from the genera of Aspergillus, Fusarium, Alternaria, and Penicillium (Jiménez et al., 1994).

1.5. Production of honey

In the world, 1.5 billion kg honey is produced per year from 2005 to 2010. Worldwide twenty countries produce honey of which China is the largest producer (436000 Mt) followed by Turkey (88162 Mt). India is the 7th largest honey producer (6100 Mt) while Central African Republic is the least producer (1600 Mt) (FAOSTAT, 2016). Honey production is declining due to high labor costs and low profits from the honey business. Therefore, to overcome this decline pure honey is adulterated with chemicals and water (Anthony & Balasuriya, 2016). According to Codex Alimentarius, the honey intended for human consumption should not have any food ingredient other than honey thus must be free from food additives, organic and inorganic matters that are foreign to its original constituents (European Commission, 2001). Therefore, for honey to remain complied with international food standards honey adulteration need to be identified and enforced. For identification of honey that has been adulterated various methods need to be explored and developed. Thus the aim of this review is to explore the possibility of developing a portable test kit, which would detect adulterant of honey on the spot, for the consumers or regulatory authorities to check before buying or prior approval of honey to be sold in the market. Therefore, honey adulteration methods and potential honey adulterant detection methods are briefly described for exploring into a kit development possibility.

2. Adulteration of honey

Adulteration alters the quality and safety of honey. For instance, honey adulterated with chemicals lower the medicinal value as well as may harm the consumers (Anthony & Balasuriya, 2016). Honey adulterants are mainly starch syrup, inverted syrup, starch or inverted syrup fed to bees and low-quality honey added to high-priced honey. Adulteration methods (See Fig. 1) of honey can be direct or indirect (Zábrodská & Vorlová, 2015). Direct adulteration is the direct addition of a substance into honey. Indirect methods are when the honeybee is fed with honey, chemicals and industrial sugars (Fig. 1) and thus detection of indirect adulteration is a challenge (Zábrodská & Vorlová, 2015) compared to direct contamination.

Honey is adulterated directly; adding industrial sugar or honey into ready-made honey (Fig. 1). Main adulterants of honey are sugar such as the addition of high fructose corn syrups (HFCS), high fructose inulin syrups (HFIS), invert syrups (IS) and corn syrups (CS). Syrup or invert sugar constituents are same as the natural constituents in the honey thus these adulterants are not easily detected; a challenge for the scientists to discover a new method of distinguishing the differences of pure and adulterated honey (Mehryar & Esmaiili, 2011).

Most honey is produced from plants such as rice, wheat and beet (C3), and as well as maize and sugar cane (C4). Honey adulterated by plant sources are categorized as C3 and C4 as per their carbon metabolism. Plants that are categorized as C3 fix carbon dioxide via Calvin (C3 cycle) which has a low 13C/12C ratio to that of C4 plants fixing carbon dioxide using the Hatch-Slack (C4) cycle (Zábrodská & Vorlová, 2015).

3. Adulterant detection methods

Traditionally, honey adulterants are detected by physicochemical methods. Adulteration of honey by crystallized cane sugar, invert sugar syrup, and cane sugar syrup can be detected with chemical determinations including HMF, glucose, sucrose, fructose, and diastase (Codex Alimentarius, 1989; White, 1979). Geographically the honey can be categorized by physicochemical parameters such as HMF, fructose, sucrose, glucose, electrical conductivity, free acidity, moisture and color (Siddiqui, Musharraf, Choudhary, & Rahman, 2017). Also, the botanical origin of the honey can be identified by electrical conductivity (Bogdanov et al., 1999). Besides, uni-floral honey has been characterized by electrical conductivity, water content, color, fructose, and sucrose (Bogdanov et al., 1999; Mateo & Bosch-Reig, 1998).

As the honey adulteration detection is complex more advanced methods of adulterant detection have been developed constantly. For example, oligosaccharides of the honey were adsorbed and fractionated by activated charcoal to prepare the samples for analysis. Then, high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) was used to detect high fructose corn syrups (HFCS) and corn syrups (CS) adulterants in the sample which identified adulterants down to 5% (Morales, Corzo, & Sanz, 2008).

Methods used until 2014 for detection of adulterants from honey were summarized by Yilmaz et al. (2014) as electrochemical analysis, enzymatic methods, thin layer chromatography (TLC), carbon isotopy, flow injection analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC), anion-exchange liquid chromatography (LC), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), mid-infrared, near-infrared (NIR) transfectance spectroscopy, gas chromatography-mass spectrometry (GC-MS), high performance (HP) anion exchange chromatography with pulsed amperometric detection method (HPAEC- PAD), high performance thin layer chromatography (HPTLC), isotope ratio mass spectrometry coupled with an elemental analyzer, and low field nuclear magnetic resonance (Yilmaz et al., 2014). For interested readers could refer Yilmaz et al. (2014) paper for the details of the mentioned methods. Methods used to detect honey adulterants also include microscope combined with real-time PCR (Kast & Roetschi, 2017; Siddiqui et al., 2017), three-dimensional fluorescence spectroscopy (3DFS) coupled with multivariate calibration (Chen et al., 2014), electronic honey quality analyzer (Anthony & Balasuriya, 2016), fiber optic displacement sensor (FODS) (Bidin et al., 2016), electronic tongue (Gan et al., 2016), and nuclear magnetic resonance (NMR) (Siddiqui et al., 2017). However, none of the methods to date could be used to identify all the adulterants in the honey simultaneously. Instead of going into details about all the honey adulteration detection methods, this work focused on methods that have the potential for developing a portable method for honey adulterant detection.

Wu et al. (2017) thoroughly reviewed sugar based adulterant detection methods including SCIRA, GC, HPAEC, HPLC, IR-based analysis, NMR, Raman spectroscopy and Q-TOF-MS that differentiate C3 plant honey adulterants, HFCS, C3 and C4 starch and rice syrups. However, the authors did not address the potential of these methods to develop a portable detection method that could be used on-site. The main difference between this present review and that of Wu et al. is that the authors focused on sugar-based honey adulterants and detection methods while the present review focuses on potential portable honey adulterant detection methods using classical and advanced adulterant detection methods. This review also includes recent studies that have been published after

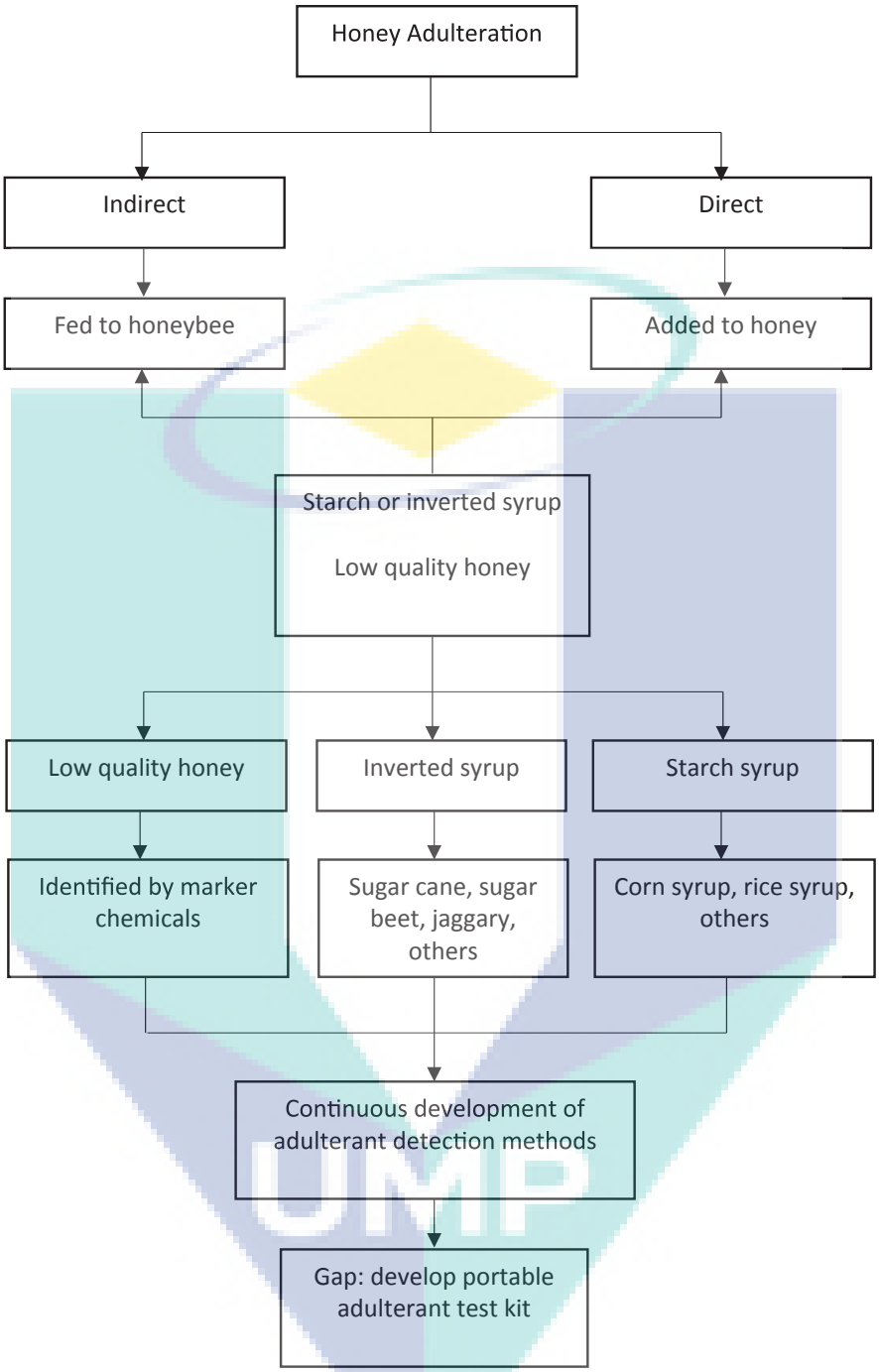

Fig. 1. Honey adulteration and detection research gap.

the publication of Wu et al. (2017). Fig. 1 illustrates the types of honey adulteration and the continuous development of honey adulterant detection methods and the need for to focus on portable honey adulterant detectors or kits.

4. Potential for the development of honey adulterant detection kits

Since available methods of adulterant detection in honey are complex and not portable to use for on the spot inspections a kit needs to be developed for a similar purpose which with one drop of honey may change the color and qualitatively detect if the honey is adulterated or not. For exploring the best fit method for honey adulterant analysis it is important to know the details of the pure honey compositions as stated in section 1.2, such as moisture content, sugar content and others. When the honey has adulterated some changes to this composition will occur which could be used as indicators for developing methods for adulterants detection in honey. Honey adulterant detection methods are briefly discussed in section 4 to explore for a potential development of a rapid detection or portable honey adulterant detector. Table 1 summarizes the various honey adulterant detection methods and possibilities of portable test kit development.

Table 1
Summary of potential adulterant detection methods as portable kits.

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
NIR transflectance spectroscopy (sample scanned: 1100 −2498 nm)	Beet invert syrup (BI) high fructose corn syrup (HFCS)	83	Irish honey and adulterant solution adjusted to 70° Brix with distilled water 18 honey samples adulterated; 8 with BI (7,10,14,21, 30,50, 70% w/w; n = 56), 10 with HFCS (10, 30, 50, 70% w/w; n = 40)	Raw spectra pre-treated: multiplicative scatter and second derivative spectra analyzed: unusual spectra detected using PCA model adulterants identified and quantified using SIMCA adulterants predicted using PLSR	Correlation coefficient of determination; BI = 0.79, HFCS = 0.72	Most accurate prediction; BI- with a multiplicative scatter correction pre-treatment, HFCS- second derivative calculated from pre-treatment	BI and HFCS: 20% w/w	After miniaturizing the equipment possible to develop a portable test kit	(J. D. Kelly, Petisco, & Downey, 2006)
FT-NIR spectrometer (sample scanned between 10,000 and 4000 cm^{-1})	HFCS, Maltose syrup (MS)	102	Natural and adulterated honey adjusted to 60 °Brix with distilled water honey adulterated with HFCS (n = 180) and MS (n = 180) at 10%, 20% and 40% w/w	PLS-LDA and CARS-PLS-LDA used to analyze data PLSR model predicted and quantified adulterated honey	Actual and predicted adulterant level at coefficient of determination of data sets ranged: 0.902 −0.992 and 0.901−0.981, respectively	Accurate for adulterant detection; PLS-LDA- 88.5% CARS-PLS-LDA 92%	NIR combined with CARS-PLS-LDA classified MS detection better compared to HFCS. The prediction for MS adulteration was satisfactory and non-satisfactory for HFCS	After miniaturizing the equipment possible to develop a portable test kit	(Li et al., 2017)
NIR Transflectance spectroscopy (1100 −2500 nm)	High fructose corn syrup (HFCS; 40% fructose, 33% glucose)	4	artifisanal Robinia honey adulterated with HFCS (0 −40%; n = 40)	PLSR model developed based on pre-treated spectra Leave-one-honey out cross-validation used to quantify adulterated honey	$RMSE_{CV}$ = 1.48 Coefficient of determination (R^2_{CV} = 0.987	1300-1800 nm spectral intervals identified adulterants and water	NIR combined with aquaphotomic is satisfactory to use for adulterant detection	After miniaturizing the equipment possible to develop a portable test kit	(Bázár et al., 2016)
spectrometer NIR spectroscopy (XDS™ Optiprobe analyzer reflection type) with Chemometrics	Jaggery syrup	4	honey samples mixed with jiggery syrup at various ratios and total 160 spectra collected using the XDSTM Optiprobe	PLSR was used to build a calibration model	Calibration error = 0.00751	coefficient of determination (R^2) = 0.9924	The honey adulteration was predicted satisfactorily	After miniaturizing the equipment possible to develop a portable test kit	(Kumaravelu & Gopal, 2015)
NIR filter-based technique (NIR transflectance method at different wave length)	jaggery syrup	56	Indian honey adulterated at different ratio with jiggery syrup	PCA used to compress data PSLR model developed	adulterants predicted at standard error of calibration of 4.55	R^2 = 0.81	determined adulterants successfully	After miniaturizing the equipment possible to develop a portable test kit	(Mishra, Kamboj, Kaur, & Kapur, 2010)
NIR transflectance spectroscopy (10,000-4000 cm^{-1})	Fructose:glucose mixtures	68 authentic	Natural and adulterated honey samples were set to 70 Brix	Spectra pretreated using SNV and	WT better in variable selection compared to PCA. Best model was LS-SVM. The recognition ratio of 95.2%	LSSVM model is better in generalizing than others; SVM, BP-ANN, KNN, and LDA	WT-LS-SVM model suitable for the adulterant detection	After miniaturizing the equipment possible to develop a portable test kit	(Zhu et al., 2010)
		67 adulterated Total 135 spectra	adulterated honey samples prepared in distilled water at different level (7, 14, 21, and 28% w/w)	WT Spectra data compressed using PCA and WT Five classical modeling used to detect adulterants: LS-SVM, SVM), BP-ANN, LDA and KNN	and the area under the receiver operating characteristic curves (AUC) of 0.952 by WT-LSSVM model	good accuracy (95.1%) and better generalization using WT-LS-SVM			
sensors (Electronic Tongue, ET; a-Astree ET	Rice syrup	35 pure honey total 259 samples (105 pure and	Pure honey adulterated with syrup (5%, 10%, 20%, 40%)	Adulterants determined using PLSDA and LS-SVM model-raw data pretreated; for sensor- SNV	ET-PLSDA model; The total discriminant accuracy of calibration = 98.43% prediction = 100%	Total accuracy for calibration and prediction sets: above 96% in NIR, MIR and ET by PLSDA model.	ET is more suitable for detecting honey adulteration	Further work needed as few studies on ET use to detect adulterants and compared to sensors spectra are more accurate	(Gan et al., 2016)
with seven potentiometric chemical sensors- with an Ag/AgCl standard electrode) and spectra (NIR- a FTNIR system; Mid Infrared spectrum, MIR- FT-IR equipped with an Attenuated Total Reflection (ATR)	Corn syrup	154 adulterated honey)	For ET: 120s for each evaluation and data recorded every 1s. Mean value recorded between 110 and 120 s. NIR: samples scanned at 10000-4000 cm^{-1} MIR: samples scanned at 4000-650 cm^{-1}	smoothing, auto-scale and derivatives Sensor and spectra (NIR, MIR) results optimized using SVMDA and iPLS data dimensions reduced using PCA	NIR: LS-SVM model total accuracy = 95.1%.		Spectra is more accurate than sensors Sensor-sample needed pretreatment but sensor better than traditional methods. ET, NIR and MIR successfully detected adulterants in honey.		

Honey protein: ELISA kit developed using polyclonal anti-apalbumin1 antibody for immunochemical quantification of apa1 in honey	Marker: apalbumin1 (apa1), the major royal jelly (RJ) protein Adulterant: corn syrup, high-fructose corn syrup	N = 46	Honey samples vortexed at room temperature with water (1:1, w/v) for 5 min Diluted samples filtered through a 0.8 μm membrane filter to obtain Pollen-free honeys	honey and bee pollen contain RJ proteins of molecular mass from 3 to 90 kDa Western-blot analysis using polyclonal anti-apa1 antibodies	apa1 used as a protein standard for ELISA analysis apa1: 55 kDa protein with N-terminal amino acid sequence N-I-L-R-G-E =	Results is effected based on the substrate type	The limit of detection for apa1 was 2 ng mL^{-1} concentration of apa1 in honey below 50 μg g^{-1} would be indicative of the presence of industrial glucose syrups in honey or dilution of floral honey with the honey obtained by feeding the honeybee colony with sucrose syrup	more research on other adulterants need to be tested. Possible for the development of portable kit	(Bilikova, Kristof Krakova, Yamaguchi, & Yamaguchi, 2015)
Honey enzyme: detection of foreign amylase addition based on the comparison of diastase determination using the Schade and Phadebas procedures,	Marker: activity of diastase (α-, β-, γ-amylase)	N = 15	The model samples of honeys with addition of foreign amylase (Aspergillus oryzae) were analysed	hydrolytic activity is expressed in g of starch/100 g honey when hydrolysed for 1 h at 40C	Diastase number quantify the general enzyme activity in honey, expressed as amylase activity on standardised substrate.	Results for same honey differed in both methods and thus reliability is poor	Diastase level varied in the samples and variation existed in both Schade and Phadebas method although in general there is a correlation between the results.	Potential method to make portable but further research required to improve the detection accuracy	(Voldřich, Rajchl, Čížková, & Cuhra, 2009)
Diastatic activity was determined spectrophotometrically by Schade and Phadebas method	Adulterants: sucrose, hydrolysed starch; HFCS- increase of adulterant decrease diastase number-addition of foreign amylases (eg bakery mould amylases) compensate this decrease and mask it			Adulterant detection is based on the substrate specificity of enzymes	Amylase activity differ in substrate specificity selection of substrate is the principal factor that affect the result	The results also differed within the results of Schade test- variability of substrate also observed			
DNA metabarcoding to detect indirect adulterants, and identify botanical and entomological origin of honey	Markers: gene regions; ITS2, rbcLa, COI Adulterants: indirect adulterant-low quality honey fed to bees	7	Honey prepared for DNA extraction DNA extraction PCR amplification Sequencing	Plant and insect sources identified in 5 samples Two samples only identified botanical or insect sources Two samples misrepresented	fail to identify when honey is rich in polyphenolic compound	honey crystallization effect the analysis	Satisfactory to identify plant and insect sources Detail study need to be done on only focusing to identify and quantify honey adulterants	Sample preparation tedious. Various field of research need to be joined to provide portable method for adulterant detection in honey- not easy	(Prosser & Hebert, 2017)
Thixotropicity: rheological analysis; rheometer, HPLC-RID	Fructose and saccharose syrups	Not stated	saccharose or fructose syrups mixed with water and adjusted to 75 °Brix at 60 °C adulterated honey prepared by mixing syrup with natural honey (0, 10, 20, 30, 40 and 50%, w/w). Then samples stirred and centrifuged at 2500 rpm for 3 min	Steady, dynamic and creep tests conducted using a stress or strain controlled rheometer equipped with a peltier system. sugar composition of adulterants analysed using HPLC-RID	Change in viscosity, flow and creep behavior of natural honey was clear adulterated honey showed decreased viscosity, storage, loss modulus values and obvious deformation	significant correlations were observed between sugar composition and rheology parameters Suggesting that these parameters could be prominent indicators for presence of saccharose or fructose syrups	Use of steady, dynamic and creep analysis satisfactorily detect adulterants	The equipments that test the rheological parameters need to be made portable and possibly made as one equipment testing all the parameters simultaneously to use this method. Currently not easy to apply	(Yilmaz et al., 2014)

4.1. Spectroscopy

Infrared (IR) spectroscopy can detect many adulterants in food and is regarded superior to other methods. Sample preparation is nil or minimal and sample size needed for the analysis is small. Moreover, the method is considered low-cost, fast, non-destructive and easy to use (Wu et al., 2017). Thus the equipment has potential to be portable to carry to the field for on-site analysis of adulterants from honey. Raman spectroscopic analysis is also a potential method to use on-site as the equipment can be made portable and is similar to IR spectroscopy in terms of low-cost, simple and rapid, requiring minimal sample preparation and is non-destructive. One advantage over IR is that the samples do not receive any interference by fluorescence (Wu et al., 2017).

The idea of IR spectroscopy to be made portable and miniaturized was recently designed and prototype released by a mobile company. On 6th January 2017, a UK online newspaper (dailymail.co.uk) published that Changhong released a breakthrough design of a smartphone (H2) that can detect chemical composition of a product. The phone possesses SCiO's material sensing technology that is a tiny NIR spectrometer built into the phone that emits a light and records the reflection where latter has a spectrum based on the product. These spectra are sent to the cloud for analysis and the detail of the materials is given to the owner of the phone. This technology can detect molecular properties of food and body metrics. The authenticity of the food can also be detected. Viagra and an identical imitation pill were used to demonstrate the ability of the smartphone to distinguish fake Viagra (Macdonald, 2017). Similar technology can be used to identify the adulteration of honey for on-site inspections. Since, as previously described, NIR is the method that can detect most varieties of adulterants of honey, this could be a potential solution to design a test similar to the smartphone system described.

4.2. Electronic tongue

Food for mankind relies on perception through our senses that help judge the quality and acceptability of the product. Biomimetics involves mimicking human senses to design such things as an electronic tongue and is an emerging technology that will advance science. Nanotechnology is used to minimize the size of these instruments (Twomey, de Eulate, Alderman, & Arrigan, 2009). The performance of these sensors is enhanced with computers and its software using calibration techniques (Ghasemi-Varnamkhasti, Mohtasebi, & Siadat, 2010; Lenau, 2009). The electronic tongue mimics the gustatory systems of the mankind. The effectiveness of the sensor depends on the absorption and catalysis of the materials into ions.

A taste sensor is a low selective sensor which identifies components in a solution mixture. The identification is through pattern recognition and multivariate calibration by computer software for data processing. The sense of taste contributes to 'umami', sweet, bitter, sour and salty tastes which are the basic tastes identified in different areas of the human tongue with specific receptors on the tongue, papillae. Once the food enters the mouth the information from the olfactory receptors are combined to judge the taste of the food. Sensing principles applied in the electronic tongue include voltammetry and potentiometry which are electrochemical methods. The electronic tongue takes the fingerprint of the food and then chemometrics tools attached to it are used to process the data. Methods to prepare a taste sensing system include the use of materials that have electrochemical sensing properties and semiconductors. For example, radical lanthanide bisphthalocyanines are intrinsic semiconductors that can be used to improve the sensitivity of taste sensors and electrical measurements (Ghasemi-Varnamkhasti et al., 2010).

The electronic tongue detects and identifies the complex material in the liquid, even if the different components are very similar by pattern-recognition and by multivariate calibration technique and qualitatively and quantitatively identify the target materials (Vlasov, Legin, & Rudnitskaya, 2002). These sensors are in the early stages of its technology but their applications in the food analysis are already established.

In recent years the electronic tongue has been used to analyze various beverages, water, and food components after modification of the sensor to the target analysis (Deisingh, Stone, & Thompson, 2004). These applications include analysis of sensory attributes of beer (Rudnitskaya et al., 2009), analysis of palatability, sourness and bitterness of nutritive drinks (Kataoka, Miyanaga, Tsuji, & Uchida, 2004), analysis of tomato taste (Beullens et al., 2008), salt prediction from minced meat (Labrador et al., 2010), umami taste flavor of food (Yang et al., 2013) and identification of honey (Wei, Wang, & Liao, 2009). Although many food analyses have been carried out using an electronic tongue, few studies have applied this to the analysis of honey. For instance, electronic tongue was used to analyze geographical and floral origins of honey (Wei et al., 2009), physiochemical characteristics and botanical origin of honey (Escriche, Kadar, Domenech, & Gil-Sánchez, 2012; Major et al., 2011), and adulterants of honey (Gan et al., 2016).

Electronic tongue, α-Astree ET, with seven potentiometric chemical sensors and an Ag/AgCl standard electrode was used to analyze honey effectively for its geographical and floral origins (Wei et al., 2009). Another electronic sensor was used in 2011 to analyze honey. This commercial electronic tongue (αAstree, Alpha M.O.S) was employed to identify physicochemical characteristics and botanical origin of honey; chestnut, acacia, and honeydew. The equipment was equipped with seven potentiometric sensors that contained an Ag/AgCl reference electrode. The physicochemical analysis (acidity, water content, invert sugar, total sugar, and electrical conductivity) was quantified using Artificial Neural Network (ANN) modeling and the reference value for these parameters was obtained from the traditional methods. The botanical classification was obtained from Principal Component Analysis (PCA), Canonical Correlation Analysis (CCA) and ANN modeling. ANN modeling was found to be the best (100% accurate). The authors concluded that the electronic tongue could be a potential tool to characterize honey (Major et al., 2011). In 2012 a potentiometric electronic tongue with metals and metallic compounds was developed to analyze honey. The sensor successfully identified the botanical origin and physiochemical parameters of honey. The data obtained was modeled using PCA and ANN. The authors suggested developing a new system of the electronic tongue for the honey sector (Escriche et al., 2012).

In 2016 adulterants of honey were tested using an electronic tongue. Gan et al. (2016) analyzed honey samples using sensors (electronic nose and tongue) and spectra and compared and concluded that the most effective method to analyze honey to be an electronic tongue. Adulterant and pure honey are divided into 3 groups and the adulterant honey is easily distinguished from pure honey. The electronic tongue (ET) was also found to be more sensitive to minerals, mono and disaccharides, amino acids, and phenols in the honey and the gustatory difference was easily observed by pure and adulterated honey using the ET. The adulterant was more accurately identified when the ET-Partial Least Squares Discriminant Analysis (ET-PLS-DA) model and ET-PCA models were combined. However, many more research studies are required as few studies have to date have focused on honey adulterant analysis using electronic tongues (Gan et al., 2016).

More research on the taste sensor systems needs to be explored as they are in the early stages of development. Scientists are now

trying to advance and expand the technology of the electronic tongue (Twomey et al., 2009). Since honey may be adulterated with multiple adulterants a multisensory system such as electronic tongue is suitable for honey adulterants detection. This electronic tongue development could focus on wholly on honey and how to detect added adulterants. Therefore, an electronic tongue is to be developed for all the adulterants of honey and the equipment must be miniaturized and portable for on-the-spot inspection.

4.3. Immunoassays

Immunoassays are based on antibody and its antigen interaction and are an analytical technique having the concept of immunology. The antibody, a glycoprotein, is produced in the body when it is exposed to a foreign body substance, antigen. In a favorable environment, these antigens induce antibodies production. Immunoassay is used to detect foreign bodies (antigens) in a sample matrix and these antigens could be a protein or a smaller molecule. The antibody is used to locate and capture the antigens in the sample matrix. The antibodies can be used as probes. When the antibody reacts with its antigen the antigen-antibody complex is formed and measured to identify and quantify the amount of foreign body in the samples. In enzyme immunoassay, an enzyme label is used that can change the color of the sample matrix for easy detection and quantification (Hsieh & Ofori, 2017). Honey adulterant kit development based on honey proteins and enzymes is discussed in sections 4.3.1, 4.3.2 and 4.3.3.

4.3.1. Honey protein

Honey contains very low amounts of protein (0.1–0.5%). Honey protein originates from hypopharyngeal glands and salivary glands of bees and from the enzymatic reaction of pollen and saliva of the bee (Baroni, Chiabrando, Costa, & Wunderlin, 2002). Early researchers (1900s) reported that honey contains protease, albumin, peptone, and globulin. Specific protein found in most honey is royal jelly protein (Won, Lee, Ko, Kim, & Rhee, 2008; Šimúth, Bíliková, Kováčová, Kuzmová, & Schroder, 2004). For example, Korean and European honey contain glycoprotein as a major protein (MRJP1, identical to apalbumin-1), one of the royal jelly proteins (Won et al., 2008). New Zealand honey was found to contain proteins such as apalbumins, arabinogalactan protein (AGPs) and apisimin (Gannabathula et al., 2017). In 2013 a review was published regarding the extraction methods of honey protein using mass spectrometry (Chua, Lee, & Chan, 2013). Honey from different regions was investigated for the presence of royal jelly protein using Western-blot that used polyclonal antibodies. The protein identified was apalbumin-1 with the size of 55 kDa, the most dominant protein among royal jelly proteins (Šimúth et al., 2004).

Honey protein can also be isolated and identified using LC-MS/MS (Liquid chromatography-mass spectrometry/mass spectrometry) after separating them using electrophoresis; SDS-PAGE. For instance, electrophoresis is used to detect protein (19 protein bands) in Australian honey using silver stain containing methylamine, followed by SDS-PAGE (Marshall & Williams, 1987). Honeybee protein is used as chemical markers to identify the floral origin of honey as the protein is common regardless of the type of honey. A combination of SDS-PAGE and immunoblot assays with anti-pollen antibodies raised from pollen extracts were used to identify the floral origin of honey (Baroni et al., 2002).

Few tests have been used to identify adulterated and pure honey using the honey protein. Biological tests developed for identification of pure and adulterated honey include immunological methods such as the development of anti-bee serum and rabbit anti-serum (White, 1957). The major protein of honey, apalbumin-1 was proposed as a marker for immunochemical testing to detect adulterants in honey (Šimúth et al., 2004). In light of these suggestions, it is feasible to develop a test kit for honey adulteration test similar to pregnancy test kits. The principle of pregnancy test kits is the detection of human chorionic gonadotropin (hcG) with the use of antibodies. The hcG rises rapidly during early pregnancy and thus is easily detected in the urine of a pregnant woman (Gnoth & Johnson, 2014). However, the drawback is honey protein is present in low concentrations unlike hcG in pregnant women. Therefore, unless a better option using the protein as a marker is developed, a honey adulterant test kit based on lateral flow devices such as the pregnancy test kit, may not be practical.

4.3.2. Honey enzymes

Enzymes found in honey include glucose oxidase, amylase, α-glucosidase, β-glucosidase (Won et al., 2008) and proteases (Rossano et al., 2012). Proteases were first discovered from honey in 2012. Bidimensional zymography (2-DZ), a very sensitive method for enzyme identification as it detects the enzyme in the order of nanograms, was used to analyze proteases where proteases were isolated using isoelectric focusing (IEF) and by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Proteolytic enzymes affect the quality and nutritional value of honey as they aid in the degradation of honey proteins (Rossano et al., 2012). Using enzymes few scientists have developed biosensors to quantify compositions of honey such as fructose, phenols, and glucose.

Fructose in honey was quantified using an amperometric biosensor based on D-fructose dehydrogenase that is immobilized on the electrode surface. The biosensor was developed using a CNTP electrode that is modified using 3,4-dihydroxybenzaldehyde, an electropolymerized film. The probe was then optimized by optimizing pH, temperature, enzyme immobilization and a lifetime of the probe. The biosensor reading was proportional to D-fructose content and the detection limit was 1×10^{-6} mol/L. After analyzing the fructose content of honey the biosensor was validated using a commercial enzymatic kit (Antiochia, Lavagnini, & Magno, 2004).

A label-free potentiometric biosensor was developed to quantify total phenols in honey. The sensor was immobilized with tyrosinase via a covalent bond on a solid contact transducer surface. This transducer had two layers in which first layer itself had two layers; the first layer consisting of poly(vinyl)chloride carboxylated, potassium permanganate and graphite and to this layer 2nd layer was deposited using a mixture of graphite and poly (vinyl chloride) carboxylated. The second layer was immobilized with tyrosinase enzyme using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride. The biosensor detection limit was 7.3×10^{-7} M. The results obtained from the biosensor reading was validated using the Folin-Ciocalteu method (Draghi & Fernandes, 2017).

The screen-printed carbon electrode was used to quantify glucose in honey. Bulk and surface modified screen-printed carbon electrodes were prepared using multiwalled carbon nanotubes and palladium and the surface of the electrodes were immobilized with glucose oxidase. The electrodes were characterized in a 7.5 pH solution by hydrodynamic chronoamperometry and cyclic voltammetry. Gold nanoparticles were also added into the electrodes biolayer. The detection limit of the electrode was 0.07 mM glucose. The electrode (GOx/Pd-MWCNT-SPCE) was used to quantify glucose in multi-floral honey and the results obtained were validated using commercial equipment for glucose quantification (Guzsvány et al., 2017).

Although biosensors have been developing for honey composition detection, to our knowledge no biosensor was developed to quantify adulterants in honey. Honey enzymes may be used to develop a biosensor that could detect adulterants of honey. However, to determine the authenticity of honey, active enzymes in the honey would be needed. Honey enzymes do become inactive

during storage or when honey is heated (Šimúth et al., 2004) and therefore may not have the adequate enzyme to depend on for biosensor development. Another alternative approach could be to use an enzyme from another source to immobilize onto a biosensor that could detect honey adulterant. For example, fructose dehydrogenase was immobilized on carbon nanotubes to develop a nano-biosensor to detect fructose in honey with a detection limit of 1×10^{-6} mol/L (Verma, 2017; Zhao et al., 2007). Nanotubes may be immobilized with various enzymes that are able to detect adulterants simultaneously, this way one biosensor could be used to detect several adulterants.

4.3.3. Enzyme-linked immunosorbent assay

Enzyme immunoassay is named as enzyme-linked immunosorbent assay (ELISA) and is pioneered by Engvall and Perlmann (1971) in which reactants are bound to a 96-well plastic microtiter plate and is separated by unbound materials (Hsieh & Ofori, 2017). An ELISA test kit method was successfully used in the detection of streptomycin residues in honey (Cara, Dumitrel, Glevitzky, Mischie & Silaghi-Perju, 2013). Streptomycin, an aminoglycoside antibiotic, is used to protect bees from a variety of brood diseases during apiculture. MaxSignal® Streptomycin ELISA test kit has 10 mg/kg as a detection limit and was used to test streptomycin in honey samples. The ELISA plate wells were coated with a conjugate protein of streptomycin. The streptomycin is detected based on a competitive reaction; anti-streptomycin antibody is added into the reaction and this competes with free streptomycin and the conjugate streptomycin antibody and the unlinked antibodies get removed during washing. The ELISA test depends on the reaction between an antigen and an antibody (Cara et al., 2013). A similar ELISA test kit could be produced for the detection of adulterants in the honey. For this, specific adulterants would need to be targeted for the development of an appropriate kit. Using ELISA method, for each adulterant specific kit may be developed.

4.4. Microscopy

Microscopic analysis on honey could reveal some adulterants present in it in addition to the geographical and botanical origin of the honey.

4.4.1. Microscopic detection of pollen grains

Palynology (early branch called Melissopalynology) is the study of pollen grains and spores in geological deposits (Ohe et al., 2004). For survival honeybee require natural resources such as resin, nectar, water, and pollen (Seedley, 2001). Pollen contains protein, minerals, vitamins, and fats and is considered as a protein source of honey (Haydak, 1935). Honey contains pollen grains, and honeydew elements such as algae, fungal spores, and wax tubes. The pollen grain is from nectar which gets into honey as honeybee collect nectar. These pollen and honeydew elements are fingerprints to locate the geographical and botanical origin of honey (Ohe et al., 2004). The pollen can be identified under microscopic observation. Although pollen analysis is traditionally used for quality analysis of the honey it is tedious (Hermosín et al., 2003).

For pollen analysis, the honey samples are mixed, 10 g of honey in 20 ml warm water (40 °C), and centrifuged twice at 2000 rpm for 10 min. The sediment is dried, mounted on a slide with glycerine gelatine and stained with fuschin-alcohol solution. The slides are then observed under the microscope for the pollen identification (Kerkvliet, Shrestha, Tuladhar, & Manandhar, 1995; Louveaux, Maurizio, & Vorwohl, 1970).

For the details of how pollens are detected and quantified under the microscope refer to Louveaux et al. (1970). Pollen grains are not identified to genus or species level by this method but identified to in shape and morphological characteristics. The pollen grain is analyzed based on frequencies; 45% of pollen grain means very frequent, 16–45% is frequent, 3–15% is rare and less than 3% pollen grains found means sporadic. Predominant pollen means that the sample contains more than 45% of pollen grains, secondary pollen means 16–45%, 3–15% means important minor pollen and minor pollen is when pollen grain present in the sample is less than 3%. The pollen grains are expressed in percentages if equal or greater than 1200 pollen grains identified. Pollen grains of 1% are referred if greater or equal to 1200 pollens are identified in the sample materials. Pollen studies reveal the geographical origin of the honey but not the country of origin. The pollen spectrum of honey provides information about forest and floral agricultural conditions. The pollen also detects the botanical origin of honey. The frequencies of pollen types in honey can be used to identify the botanical origin (Louveaux et al., 1970).

Pollen studies can be used for identification of the geographical and botanical origin of honey. Floral origin of the honey is traditionally identified by pollen analysis (Hermosín et al., 2003). Honey samples from Algeria were identified by pollen quantity and pollen spectrum. The pollen grains present in samples were rich, greater than 45% (80,000 to 24, 832, 000) (Ouchemoukh et al., 2007a). In another study, pollen detection of honey samples from Austria, Canada, Germany, Pakistan, Saudi Arabia, America, and Australia were carried out and revealed that pollens were from 15 plant species. The pollen spectra identified nectarless and nectariferous sources bees visited. The pollen identifies climate, geographical location of the beehive and their vegetation. The composition of the pollen exposes the floral origin. For example, *Eucalyptus fibrosa* pollens were detected mostly from Saudi Arabian, Pakistani, Germans and Austrian honey (Bibi, Husain, & Malik, 2008).

In summary, pollen detection provides information about the geographical and botanical origin of the honey and it does not provide information about the adulterants of the honey. This method along with the physicochemical analysis of the honey can be used for identification of honey quality for trade purposes. The pollen detection is through microscopy which is tedious and in need of replacement with a more efficient method that could be made portable. There is potential to use pollen detection as an indicator of honey adulteration as honey from a particular region will have expected pollen profiles that are likely to be altered with adulteration.

4.4.2. Microscopic detection of adulterants

A microscopic (polarization microscope) procedure was used to detect cane sugar and acid-hydrolyzed can sugar syrup, honey adulterants fed to honey bees. Cane sugar contains particles from the cane stem such as epidermis cells, single rings of ring vessels, sugar cane starch, and sclereid (Kerkvliet et al., 1995). Supplementary tests used with the microscopic characterization of the honey can include HPLC to analyze glucose, sucrose, fructose, and HMF. Moreover, the honey pH, water content and electrical conductivity can also be measured. This combination of methods was used to identify 10 adulterated honey samples from Nepal and Philippines (Kerkvliet et al., 1995). Chemical analysis of these honey highlighted that samples were adulterated or heated.

Another combination of methods is the use of microscopy and real-time PCR assay to detect sugar, adulterants of honey using *Saccharomyces cerevisiae* (baker's yeast) as an indicator of the sugar adulterant (Kast & Roetschi, 2017; Siddiqui et al., 2017). *S. cerevisiae* (baker's yeast) is added into the sugar paste prepared to feed the honey bees. However, this yeast does not multiply in the honey and will only multiply in the presence of sugar adulterants in the honey. Within 10 days after the honey bees are fed with baker's yeast, if

the yeast has multiplied in adulterated honey, they can easily be detected by microscopy and real-time PCR assay.

In summary instead of the use of only microscopic analysis for adulterants, it is more accurate to combine it with other methods such as physicochemical analysis, HPLC, and PCR. Microscopic methods may be most useful in developing countries where alternative methods are impractical due to high cost.

4.5. DNA metabarcoding

Botanical and entomological origin of honey has been identified using DNA metabarcoding (Prosser & Hebert, 2017). Three gene regions were used to analyze pollen components; mitochondrial cytochrome c oxidase subunit (COI) to identify bee species and to classify entomological source of honey (Prosser & Hebert, 2017; Yao et al., 2010), nuclear ITS2 (for honey pollen signature identification to discriminate plant species) (Yao et al., 2010) and pollen-free plant material plastid gene (rbcLa) to recognize any plant DNA in honey (Prosser & Hebert, 2017). Nuclear ITS2 is present in all pollen grains while plastid markers are not always present (Bell, Burgess, Okamoto, Aranda, & Brosi, 2016). Prosser and Hebert (2017) discovered that the indirect adulteration of honey with low quality of honey can be detected by DNA metabarcoding without a study of pollen. This study revealed that flavored or dark-colored honey is not accurately identified by this method and this could be due to interference from secondary metabolites on PCR. A change in the buffer used during DNA extraction may solve the problem of PCR inhibition. The authors suggested the potential use of this method require various modifications in the future for the detection of adulterants in honey (Prosser & Hebert, 2017).

The current limitation to the DNA metabarcoding is that the genetic markers used for identification affect the taxonomic resolution of the assay (Prosser & Hebert, 2017). The other drawback of DNA metabarcoding is when honey is purified by filtration to remove impurities the pollen is also removed. The method does not work on creamed, flavored and darker honey. For darker and flavored honey, a new genetic marker needs to be developed. The accuracy and reliability of DNA metabarcoding depend on the effectiveness of the genetic markers. The genetic markers for problematic analysis could be developed through research on a wider analysis of honey authentication. Also for better identification, a combination of melissopalynology and DNA metabarcoding is recommended.

DNA metabarcoding could be a potential method that could identify all the adulterants in the honey, however, there are challenges in developing a portable kit; need more research and expertise in the different field that requires the kit to become reality.

4.5.1. Thixotropicity

Thixotropicity (stickiness or rheology) of honey could be explored for the possibility of developing a honey adulterant test kit. The following section provides details on this methodology. Honey adulterated with carbohydrates was identified based on the nitrogen content of the honey. For example, nitrogen content less than 10 mg/100 g honey in Venezuela honey was considered adulterated with carbohydrates (Anklam, 1998; Olivier, 1987). Another method to detect adulterated carbohydrates in honey is by rheological methods. For instance, adulterants such as fructose and saccharose syrups in honey can be detected using rheological methods (Yilmaz et al., 2014).

Yilmaz et al. (2014) adulterated natural honey with saccharose and fructose syrups at different levels (0–50% by weight) and then tested these honey by creep, dynamic and steady shear tests. For steady shear analysis, the samples were sheared between 0.1 and 100 s^{-1} at 25 °C. The viscosity was analyzed as a function of shear rate. The dynamic shear analysis was conducted in the strain range of 0.1–100% using the amplitude sweep test at 1 Hz and the linear viscoelastic region (LVR) was determined. The frequency of the sweep test was also investigated between 0.1 and 10 Hz at 25 °C using 1% strain, and this strain is obtained from amplitude sweep test. The storage modulus and viscous or loss modulus are viscoelastic parameters. Temperature sweep test at a 50s^{-1} shear rate and 1 Hz and between 5 and 50 °C was also conducted to test any variation between dynamic and steady shear parameters. Creep and recovery tests were conducted using constant stress at 0.1 Pa within the LVR. Within a given time at steady state, the viscoelastic material deformation was analyzed and then stress applied and released to examine for recoverable shear. This method was validated using parameters such as linearity, sensitivity, and repeatability. Statistical analysis was conducted to see the different adulterant levels on dynamic and steady shear parameters and bivariate correlations were conducted to check the relatedness between sugar composition and Pearson's test was carried out to analyze rheology parameters of the adulterated honey and principle component analysis (PCA) was used to categorize honey between sugar composition and rheological parameters (Yilmaz et al., 2014).

Rheological properties vary during the manufacturing processes such as mixing, filtering, heating, bottling and hydraulic transport. For example, the viscosity of the honey is one of the rheological parameters influenced by the quality, processing steps, and honey processing equipment design. Viscosity also depends on moisture content, colloids and crystals and other materials in the honey. The rheological parameters also depend on time, stress, shear rate and temperature which are also important factors during the manufacturing processes and its equipment design. Moisture content and the temperature of the honey influences the viscosity. The viscosity decreases with moisture increase up to 19% moisture with less effect with a further increase in moisture content. The viscosity decreases with increase in temperature up to 30 °C with less effect at higher temperatures. Viscosity also varies depending on the botanical origin of the honey (Yanniotis, Skaltsi, & Karaburnioti, 2006).

The physicochemical analysis reveals that the adulterated honey is brighter in color while the pure honey is more reddish. The pH of the adulterated honey decreases when the adulterant level increases while the water activity of the adulterated honey increases compared to that of the pure honey. The steady shear stress and viscosity values decrease with increase in the adulteration level (Yilmaz et al., 2014). The adulterants in the honey can be detected between the temperatures of 5–50 °C. Steady shear analysis revealed that the samples adulterated with 10% sucrose and fructose syrups could be detected between 5 and 20 °C. Dynamic shear properties revealed that the adulterated honey is not elastic but viscous in nature. Also adulterated honey decreased in resistance to deformation and the Newtonian model parameters that describe shear properties of the samples could be used to detect a 10–50% level of adulterated sugar content in honey. The creep-recovery analysis is suggested to be a potential approach to detect fructose and saccharose as the adulterated honey structure is easily deformed and this behavior can be picked up by creep-recovery analysis. The method is repeatable and the limit of detection of rheological parameters for adulteration ratio in honey is more than 4%. The study results revealed that the behavior of the natural honey such as its flow, creep and viscoelasticity was notable and pure. However, when the honey is adulterated with syrup, the viscosity loss and storage modulus values and deformation was prominent compared to natural honey. HPLC-RID was used to find the composition of the syrups. A significant correlation ($P < 0.05$)

was found between sugar composition and the rheology parameters (dynamic shear, creep and steady shear) of the honey when Pearson's correlation test was conducted (Yilmaz et al., 2014). The study concluded that these rheological parameters; creep and dynamic and steady analysis is a novel approach for detecting fructose and saccharose syrups as adulterants of honey (Yilmaz et al., 2014). The use of rheological parameters in combination with HPLC-RID is a promising method for the identification of fructose and saccharose syrup adulterated honey. However, the method is a challenge to make it portable for on-sight use as there is no portable rheological method.

Honey crystallization is based on storage time, temperature as well as the botanical origin (Smanalieva & Senge, 2009). Below 30 °C honey is known to crystallize (Venir, Spaziani, & Maltini, 2010). Although glucose and fructose content in the honey are approximately same, due to the lower solubility of glucose the latter crystallizes (Venir et al., 2010; Young, 1957). Parameters that affect honey crystallization are fructose and glucose concentration and water content (chemical composition) as well as mechanical processing and the storage temperature (Smanalieva & Senge, 2009). Crystallization affects the rheological properties of honey and the crystallization rate of the honey can be determined by the ratio of fructose and glucose, F/G. The glucose (α-D-glucose monohydrate) crystallizes below 50 °C (α-D-glucose anhydrous) while remains stable in the anhydrous forms between 50 and 80 °C and above 80 °C (β -D-glucose anhydrous form) (Venir et al., 2010; Young, 1957). The crystal size produced in the honey is determined by the F/G ratio and its storage condition. To remain stable, the F/G ratio must stay above 1.33 and below this value, it crystallizes (White, 1978). In natural honey the F/G ratio is 1–1.2 and the addition of adulterated glucose or fructose will change this ratio (Puscas, Hosu, & Cimpoiu, 2013). Rheological properties of honey are affected by temperature and the natural honey making process. The temperature and the F/G ratio is used to determine the size of the crystal formation in the honey (Smanalieva & Senge, 2009). Smanalieva and Senge (2009) tested 39 German honey to identify the botanical origin. Flowing behavior of honey depends on botanical origin and temperature (Smanalieva & Senge, 2009). Natural honey behavior is generally considered as Newtonian (Abu-Jdayil et al., 2002). However, all non-floral German honey demonstrated the non-Newtonian flowing behavior (Smanalieva & Senge, 2009) indicating they may be adulterated. However, for creamed honey in its natural form, the flow behavior is non-Newtownian (Karasu, Toker, Yilmaz, Karaman, & Dertli, 2015). Honey from *Eucalyptus spp* also exhibits non-Newtonian behavior (Trávníček & Přidal, 2017). Adulterated honey with fructose and saccharose is Newtonian (Yilmaz et al., 2014).

Adulterants in honey lower viscosity. For example, when saccharose and fructose syrup were added to natural honey, viscosity decreased and the decrease was enhanced as the concentration of the adulterants increased. Shear stress of the adulterated honey decreases as the adulterant content increases which results in the decrease of viscosity of the honey. These results highlight that the adulterants of honey, saccharose and fructose syrups, can be successfully detected by steady shear rheological analysis. The temperature range that honey adulteration can be detected using rheology is reported to be 5–20 °C. Honey is a viscous liquid that is non-elastic in nature and possesses liquid-like behavior. The resistance to deformation of the adulterated honey is low compared to the natural honey. Thus total resistance to deformation could be a good indicator for adulterant (10–50%) detection in the honey. The authors indicate that with the inclusion of adulterants in honey, the viscoelastic nature of the honey changes as the deformation is enlarged. The authors suggest that viscosity is also a good indicator to detect fructose and saccharose adulteration in honey. The researchers concluded that the adulteration between 0 and 50% level with saccharose or fructose was detected by a change of creep, flow and viscoelastic behavior of the pure honey (Yilmaz et al., 2014).

In summary, using thixotropicity honey adulterants such as carbohydrates; glucose, fructose and saccharose syrups, could be detected using viscoelastic and flow behavior, change of creep, shear stress, crystal formation, and nitrogen content. The storage time, temperature, and solubility are factors that effect in the detection of adulterants in honey. However, the sensitivity of the adulterant detection using viscoelastic behavior is questionable although it is good detecting the presence and absence of carbohydrate adulterants in honey. For quantification, more advanced detection methods are required. Further study is still needed to explore the thixotropicity of honey with other adulterants before selecting a method that could be considered feasible for a honey adulterant kit. Adulterants of fructose, saccharose syrup and glucose could be identified after developing a carbohydrate adulterant detection kit.

5. Conclusion

In conclusion, for honey adulterant detection there are many methods that have been developed. However, none of the methods are portable to use for on-site inspection. The methods used for honey adulterants include physiochemical analysis, microscopic methods, ELISA methods, rheological analysis, chromatographic methods, PCR, DNA-metabarcoding, sensors, and spectroscopic methods. The most promising methods among these for the development of a portable test kit are ELISA test kits, sensors such as an electronic tongue and NIR spectroscopy. These techniques may be very effective particularly if coupled with appropriate statistical analyses. These promising methods also need to be further researched for various honey adulterant detection and need to be miniaturized as portable honey adulterant detectors or kits, ideally compatible with smartphone technology. Convenience and growing applications through smart phones would suggest that in the future, some analytical tests could be conducted using this technology.

Abbreviations

DSC	Differential scanning calorimetry
FODS	Fiber optic displacement sensor
ET	Electronic tongue
HPLC	High-Performance Liquid Chromatography
IRMS	Isotope ratio mass spectrometry
NMR	Nuclear magnetic resonance
PCR	Polymerase Chain Reaction
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
NIR	Near Infrared Spectroscopy
3DFS	3-dimentional fluorescence spectroscop
HPAEC-PAD	High-performance anion-exchange chromatography-pulsed amperometric detection
TLC	Thin layer chromatography
GC	Gas chromatography
NIR	Mid-infrared NIR transflectance spectroscopy
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
FAO	Food and Agriculture Organization
HMF	Hydroxymethylfurfural
HFIS	High fructose inulin syrups
IS	Invert syrups
CS	Corn syrups

HPLC-IRMS	Liquid chromatography coupled to isotope ratio mass spectrometry
BP-ANN	Back propagation neural network
DFAs	Dianhydrides of fructose
BI	Beet Invert syrup
PCA	Principal component analysis
SIMCA	Soft independent modeling of class analogy
PLS/PLSR	Partial least squares regression
DPLS	Discriminant partial least squares
PLS-LDA	Partial least squares linear discriminant analysis
(CARS)-PLS-LDA	Competitive adaptive reweighted sampling
RMSECV	Error of cross-validation
WT	Wavelet transformation
SNV	Standard normal variate transformation
LS-SVM	Least square support vector machine
SVM	Support vector machine
BP-ANN	Back propagation artificial neural network
KNN	K-nearest neighbors
SVMDA	Support vector machine discriminant analysis
iPLS	Interval Partial Least Squares
HPTLC	High-performance thin-layer chromatography
HFCS	High fructose corn syrup
GS	Glucose syrup
SS	Saccharose syrup
DA	n-Decyl alcohol
OA	Oleic acid
DOP	Dioctyl phosphate (Bis[2-ethylhexyl]hydrogen phosphate
TOMA	Trioctyl methyl ammonium chloride
OAm	Oleyl amine
ANN	Artificial Neural Network
CCA	Canonical Correlation Analysis
ET-PLSDA	Electronic tongue-Partial Least Squares Discriminant Analysis
ET-PCA	Electronic tongue- Principal Component Analysis
2-DZ	Bidimensional zymography
IEF	Isoelectric focusing
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
hcG	Human chorionic gonadotropin

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Physicochemical and Microbiological Analysis of Stingless Bees Honey Collected from Local Market in Malaysia

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Abstract: *The growing demand for honey in the market has led to the occurrence of the tampering honey with foreign substances and increases the production of artificial honey. Due to this concern, this study works on the physicochemical and microbial characterization of stingless bee honey. The physicochemical analysis showed that the honey possessed pH (2.51–3.26), free acidity (121.1 to 318.7 meq/kg), moisture (19.4–30.9%), electrical conductivity (0.33–0.69 mS/cm), ash content (2.75–4.31 g/100g), Hydroxymethylfurfural (HMF) content (35.4 to 461.7 mg/kg) and diastase activity (2.71 to 6.11 DN). Also, sugar profile of honey showed that the honey contained fructose (15.03–32.52 g/100g), glucose (12.17–34.55 g/100g) and sucrose (0.01–7.29 g/100g). The harvested honey, H1, and H2 have the highest potential to become an antibacterial agent to treat disease compared to commercial honey samples because they were active against gram-negative bacteria. All analyzed samples were within the maximum limit of the quality criteria set by the Malaysian Kelulut Standard and Codex Alimentarius except for free acidity, HMF, and Diastase Number. All the data obtained is vital in order to create a specific statute for stingless bees honey in Malaysia that may help to protect the consumer from purchasing adulterated honey.*

Keywords: *honey; adulteration; physicochemical; microbiological; quality*

■ INTRODUCTION

Stingless bees are small, all black creatures that commonly reside in the tropical and subtropical regions of the world, such as Southeast Asia and tropical America. Stingless bees are very special insects as they can produce three different products; honey, propolis, and bee bread. In addition, stingless bees are also valued as an effective pollinator for both wild and cultivated crops [1]. Honey is a natural sweetener that is widely used for various applications where it contains approximately 200 distinct chemical compositions including 80–85% of carbohydrates such as fructose and glucose [2]. Besides that, water, proteins, and amino acids, ash, hint of enzymes, vitamins and phenolic compounds also constitute almost 15–20% in honey [2]. Nevertheless, the contents of honey differ depending on the types of plants as well as the nectar which the bee consumes [2].

The authenticity of honey is specified internationally by the Codex Alimentarius and European Legislation while locally; stingless bee honey is defined by Malaysian Standard Kelulut (Stingless Bee). The characterizations of honey, especially by physicochemical analyses, are well described by both standards [3]. Moisture content is the most popular criterion for stingless bees honey followed by free acidity, sugar profile, pH, HMF, ash content and electrical conductivity [4]. The moisture content was important as it influenced many other parameters in honey such as sugar content, hydroxymethylfurfural (HMF) and microbial properties. Based on the past studies, stingless bee honey has been reported to have a higher moisture content, higher electrical conductivity, lower enzyme activity, higher free acidity, and lower glucose and fructose content compared to *Apis mellifera* honey [4,32].

Microorganisms may influence the quality or safety of honey. Yeast and spore-forming bacteria are mostly found in honey, but to our concern, there are no bacteria causing disease had been identified in honey [5]. Microbial contaminations commonly occur in honey are from primary sources where it is difficult to control. The sources include pollen and nectar sources, the digestive system of honey bees, dirt, air and soil [6]. Good manufacturing practices may be the practical way to control the sources of contagion that are mostly found in secondary sources particularly from honey post-harvest including air, food operators, cross-contamination, equipment, and buildings [6-7].

Honey adulteration refers to the immoral act of producers by adding sugar syrups into natural product [8]. Commonly, adulterants such as water, sucrose, inverted sugar, hydroxymethyl cellulose, dextrin, and starch have been detected by routine analysis of physicochemical [9-10]. Adulteration activity of honey has increased the awareness among the honey consumers about the quality and purity of the commercial honey in the market. Thus, the study aimed to assess the quality of harvested honey and commercial stingless bee honey available in Malaysia market concerning the physicochemical properties and microbial profile.

■ EXPERIMENTAL SECTION

Materials

In this study, six samples of stingless bees honey were collected from different regions in Malaysia (Table 1). Two samples, H1 and H2, were harvested from Universiti Malaysia Pahang stingless bee farm and Aqif Kelulut Farm, Pekan. Four samples of commercial stingless bee honey were randomly obtained from the local market around Malaysia.

All of the chemicals and reagents used were of analytical grade. Sugar standard (fructose, glucose, sucrose), acetonitrile, ethanol, sodium bisulfate, acetate buffer and sodium hydroxide (NaOH), were purchased from Sigma-Aldrich (USA). Carrez solution I and II were purchased from Merck (Germany). Sodium chloride and iodine were obtained from R&M (Malaysia). Plate count agar (PCA), violet red bile glucose agar (VRGB), Sabouroud Dextrose agar (SDA) and Mueller Hinton agar were purchased from Oxoid, UK. Finally, chloramphenicol was obtained from Nacalai Tesque (Japan).

Two strains of the gram-positive bacteria and gram-negative bacteria: *Escherichia coli* and *Bacillus sp.* used in this study were obtained from the Central Laboratory, University Malaysia Pahang. The isolates were identified based on standard microbiological techniques, and sub-cultured in nutrient agar slopes at 37 °C for 24 h.

Instrumentation

Instrumentation used were SevenCompact pH meter (Mettler Toledo, USA), Hand-held refractometer (RHB 90ATC, China), Hi 8733 conductivity meter (Hanna Instruments, USA), Carbolite CWF 1200 muffle furnace (Carbolite Gero Limited, UK), GENESYS 10S UV-Vis spectrophotometer (ThermoFisher Scientific, USA), LP vortex mixer (ThermoFisher Scientific, USA), 1260 Infinity II LC System (Agilent Technologies, USA), BS -21 shaking water bath (Lab Companion, Jaio Tech, South Korea) and Incubator I (Memmert, Germany).

Procedure

Physicochemical analyses

Determination of pH and moisture content. The pH of the honey sample was measured by diluting 10 g of

Table 1. Sampling location, description and time collection of six samples of stingless bee honey from Malaysia

Sample Code	Description	Sampling Location	Time of collection
H1	Harvested honey	Pahang	March 2017
H2	Harvested honey	Pahang	March 2017
H3	Commercial honey	Selangor	October 2017
H4	Commercial honey	Kedah	October 2017
H5	Commercial honey	Kuala Lumpur	November 2017
H6	Commercial honey	Pahang	September 2017

of honey with 75 mL of distilled water while moisture content was determined by dropping approximately 1 to 2 mL of honey samples to the measuring surface of the handheld refractometer.

Determination of free acidity. The free acidity was measured by diluting 10 g of honey in 75 mL of distilled water before this solution was titrated with 0.1 M NaOH solution. pH readings were observed simultaneously during titration until the pH reached 8.5, and the results were expressed in mmol/L.

Determination of electrical conductivity and ash content. The electrical conductivity was measured by diluting 20 g of honey in 100 mL distilled water (20% w/w) where the results were expressed in milliSiemens per centimeter (mS/cm). The honey ash content was determined by placing the crucible in an oven for 1 h. After cooling, the crucible was weighed, and 5 g of the honey sample was added into the crucible before burnt in a 500 °C furnace for 2 h until constant mass was obtained. The sample was then reweighed, and ash percentage was calculated.

Determination of hydroxymethylfurfural (HMF). Determination of HMF was carried out by following the International Honey Commission's harmonized methods [11] where 5 g of honey sample was weighed and completely dissolved in 25 mL of distilled water. The volumetric flask containing a honey solution was added with 0.5 mL Carrez solution I and mixed well by vortex. The mixed solution was then added with 0.5 mL Carrez solution II and mixed before adding distilled water up to 50 mL mark. A drop of ethanol was added to suppress the foam that formed during mixing. The first 10 mL of the mixture was disposed of after being filtered through filter paper. 5.0 mL of the solution was placed in two test tubes where in Tube 1 (sample solution) was added with 5.0 mL of distilled water and in Tube 2 (reference solution) was added with 5.0 mL of 0.2% sodium bisulfate solution. The absorbance of the solutions was recorded at 284 and 336 nm, respectively.

Determination of sugar profile. The determination of sugar (fructose, glucose, sucrose) were performed following the method of Malaysian Kelulut Standard [12]. The sugars were eluted through Phenomenex column (PhenoSphere 5µ NH_2 80A, 250 × 4.6 mm, Phenomenex Inc, USA) and detected by Refractive Index detector (RID) operated at 40 °C. The mobile phase is acetonitrile: water (80:20, v/v) at a flow rate of 1.3 mL/min. The retention times obtained from the standards were compared to obtain HPLC sample peaks. The injections were performed in triplicate where the average peak area was used for evaluation.

Determination of diastase activity. The diastase activity of honey was determined by dissolving 5.0 g of honey in 15 mL distilled water and 2.5 mL of acetate buffer (1.59 M, pH 5.3). The samples solution was then mixed with 1.5 mL of 0.5 M NaCl solution before 10 mL of this solution was transferred in a test tube containing 5 mL of 2% starch solution. The test tube was then kept in a water bath at 40 °C for 5 min, and 1 mL of the solution was added with 10 mL of 0.0007 M diluted iodine solution. The absorbance was recorded at 660 nm in spectrophotometer until readings showed absorbance less than 0.235. The diastase activity was expressed in Gothe degrees. DN was the amount of enzyme that hydrolyzed/converts 1% starch solution/0.01g of starch for 1 h at 40 °C.

Microbiological analyses

Standard plate count. The honey samples that were diluted in saline water were plated on standard plate count agar and incubated at 30 °C for 48 h.

Detection of *Bacillus sp*. The initial dilution containing the aerobic spore-forming bacteria was heated in 10 minutes at 80 °C and immediately immersed in cold water afterward to cool down the temperature. *Bacillus sp.*
was detected by plating the dilutions on plate count agar (PCA) and incubated at 30 °C for 48 h.

Detection of total coliform. The samples were plated on violet red bile glucose agar (VRGB) and incubated at 35 °C for 48 h.

Yeast and Moulds count. Yeast and Moulds were quantified after plating the samples on Sabouroud Dextrose agar (SDA) supplemented by 100 mg/L chloramphenicol. The plates were incubated at 25 °C for 5 days. Microbial counts were expressed as colony-forming units per gram of honey sample (cfu/g).

Antibacterial analysis: Agar disc diffusion method

The agar diffusion method was determined by following the method by Moussa [13]. Fresh culture suspension of the test microorganisms (100 µL) was spread on Mueller Hinton agar. The concentration of cultures was 1×10^7 CFU/mL. For screening, 5 mm sterile diameter filter paper disc was infused with 10 µL of honey equivalent to 0.1 mg of honey. The plates were placed at 4 °C for 2 h before being incubated under optimum conditions for 24 h. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The zone diameters of inhibition (ZDI) was measured in millimeter, including the diameter of the disc. The controls were set up with equivalent quantities of water as a control.

Statistical analysis

All analyses were prepared in triplicate. The data obtained in the study were analyzed using analysis of variance (ANOVA) and followed by Tukey test (Minitab 18, Minitab Inc., USA) where the differences between mean values were significant at values of $p < 0.05$.

■ RESULTS AND DISCUSSION

Physicochemical Analyses

Table 2 reported the physicochemical properties of the different samples of honey obtained from a local market in Malaysia. According to the result, pH values ranged from 2.51 to 3.26 which met the criteria of pure honey. There was no significant difference in the pH values of H1 and H2 ($p > 0.05$). However, when compared with H3, H4, and H5, the results were significantly different. The pH values varied depending on their geographical origin, floral sources as well as the bee's species [14]. Honey from warm and humid countries usually has lower pH due to their high water content. The pH of adulterated honey is higher (more than pH 5.5) compared to pure honey due to the extraction, storage factor, and temperature of honey [7].

From the result, six selected honey samples showed that the percentage of moisture content fluctuated from 19.4 to 30.9% which is still in the range of Malaysian Kelulut standard. The variation may be due to the humidity of tropical forest, floral origin, soil, collection period and processing aspects [14-15]. H2 has the highest moisture content while H4 has the lowest moisture content. This may be due to the collection time of H2 which was during the rainy season while H4 may have undergone moisture removal. Besides increasing the probability of fermentation happening, higher moisture content (more than 35 %) can also indicate that the honey is adulterated [16-17].

Typically, a small amount of acid can be found in pure honey which is significant for a taste of honey [18]. From the result, H4 has the highest free acidity with a value of 318.7 meq/kg. There is no fixed limit of free acidity in Malaysian Kelulut standard, but all six samples

Table 2. Summary of physicochemical analyses of six sample of stingless bee honey from Malaysia (mean ± standard deviation, n = 3)

Sample	pH	Moisture (%)	F.A (meq/kg)	EC (mS/cm)	Ash (g/100g)	HMF (mg/kg)	Diastase Number (DN)	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)
H1	3.26±0.11^{a}	25.4±0.09^{d}	146.4±7.46^{b}	0.56±0.01^{b}	3.11±0.23^{a}	74.3±6.88^{b}	5.97±0.18^{a}	17.5±1.3^{a}	16.0±0.8^{b}	< 0.01
H2	3.20±0.08^{a}	30.9±0.06^{a}	121.1±1.24^{b}	0.33±0.01^{c}	0.72±0.11^{b}	85.9±8.40^{b}	5.85±0.08^{a}	15.03±1.22^{a}	12.17±0.47^{b}	< 0.01
H3	2.52±0.03^{c}	23.4±0.53^{e}	257.8±2.39^{a}	0.68±0.01^{a}	2.75±1.12^{a}	457.2±0.81^{a}	2.32±0.12^{b}	30.96±0.81^{b}	31.6±0.79^{a}	1.10±0.03^{a}
H4	2.79±0.01^{b}	28.4±0.51^{b}	318.7±77.6^{a}	0.40±0.03^{c}	3.86±1.02^{a}	35.4±0.71^{c}	6.30±1.16^{a}	32.52±0.59^{b}	30.09±0.70^{a}	< 0.01
H5	2.51±0.01^{c}	19.4±0.21^{f}	250.7±3.19^{a}	0.69±0.06^{a}	4.31±1.53^{a}	461.7±5.69^{a}	2.16±0.13^{b}	31.64±0.48^{b}	34.55±0.50^{a}	2.81±0.09^{a}
H6	2.57±0.01^{c}	26.7±0.61^{c}	241.7±3.46^{a}	0.65±0.04^{a}	0.52±0.16^{b}	456.6±2.72^{a}	2.79±0.01^{b}	22.83±0.42^{c}	25.47±0.50^{c}	7.29±0.18^{b}
Mean ± SD	2.81±0.34	25.7±4.01	222.7±74.5	0.55±0.15	2.54±1.58	261.8±216.1	4.23±2.0	25.08±7.70	24.98±9.02	3.73±3.20
Min Value	2.51	19.4	121.1	0.33	0.52	35.4	2.16	15.03	12.17	<0.01
Max Value	3.26	30.9	257.8	0.69	4.31	461.7	6.30	32.52	34.55	7.29

Note: $^{a-f}$=Means with a different superscript letter along the column are significantly different ($p < 0.05$); F.A: Free acidity; EC: Electrical conductivity; HMF: Hydroxymethylfurfural

acid values exceeded the international honey standard, which is no more than 50 meq/kg. This may explain the sour taste that the stingless bee exhibit. The lower value of acidity indicates the freshness of honey however the value may increase with time. This is due to the fermentation process where sugars converted into organic acids. [7,19]. Besides that, flower sources and bee species influence the variation of acidity value since it conforms to the balance of organic acids present in honey [15].

Electrical conductivity relies predominantly on the mineral content of honey [20]. From the six samples, H5 had the highest value of electrical conductivity with 0.69 ± 0.69 mS/cm which may indicate that the honey is rich in mineral content. The differences in electrical conductivity correspond to not only the different geographical and floral sources but also the number of organic acids, proteins and storage time [14,21]. The color of honey also influence the electrical conductivity values as dark honey gives higher conductivity due to higher levels of microelements than light honey [22].

From the result of ash, only H2 and H6 were observed to be within the range of Malaysian Kelulut standard (< 1.0 g/100g) while others reported being higher. The high value of ash may contribute by the floral source and nectar characteristic in some floral species [23]. There is a wide distribution of values detected in all six samples which may contribute by an irregular pattern of harvest processes and the different in meliponiculture techniques used by the producers [24]. Ash content and electrical conductivity are closely related to the mineral content in honey, but ash is differing as it directly measures the inorganic residue after carbonization [25].

HMF is one of the indicators used to assess the quality of honey which is absent in fresh honey [26]. Results showed that H4 has the lowest HMF values (35.4 ± 0.71 mg/kg) but still exceeded the limit set by the Malaysian Kelulut standard (< 30 mg/kg). The samples H3, H5, and H6, exceeded the HMF limit in both international standard and Kelulut standard (> 400 mg/kg) which suggest that the three honey samples have undergone a heating process or adulterated. Furthermore, high HMF content in honey also demonstrates the fabrication of honey with invert syrup since HMF can be formed by heating sugars in the presence of an acid to the inversion of sucrose [27-30]. Even though HMF of H1, H2, and H4 were quite high, the result may cause from poor storage condition, or the samples were old. The values of HMF could increase during processing, preparation, aging, and storage of honey [26].

In good quality honey, the fructose content should exceed the glucose content [16,31], except in three samples (H3, H5, and H6). Thus, H3, H5, and H6 probably had poor quality. Harvested honey samples, H1 and H2, had lower fructose and glucose content compared to other commercial samples but still comparable with the study of Thailand and Malaysian stingless bee honey [32-33]. Sucrose content should be within 8.0 g/100 g, and all analyzed samples were still within the Malaysian Kelulut standard limit. The high concentration of sucrose may due to the diversity of floral sources, early harvest of honey as the sucrose not fully transform into fructose and glucose, overfeeding the bees with sugars, syrups or artificial honey and lastly, the honey is adulterated by the addition of commercial sugar [14,34-35].

Denoted as Diastase Number (DN), diastase activity for six samples of honey ranged from 2.6 to 6.30 DN. There is no fixed limit for Diastase Number in Malaysian Kelulut standard, but according to international honey standard, Diastase Number should be no more than 3 for honey with low enzyme content. H3, H5, and H6 samples have low DN number (less than 3 DN) which may mean that these honey is aging or has been heated since enzymes are susceptible towards heat [36]. Diastase Number is not only controlled by geographical and botanical origin but also by pH values, nectar flow and foraging patterns of the bees [24-25].

Microbiological Analyses

The microbial counts of six stingless bee honey samples can be observed in Table 3. The standard plate counts (SPC) were found in every sample with a count of 1×10^2 cfu/g to 9.7×10^2 cfu/g. The total plate counts variation may be influenced by the honey characteristic, honey freshness, the harvest period and infection by pathogenic bacteria [6,25]. The most common types of

Table 3. Summary of the microbial profile of honey samples from Malaysia

Sample	Microbial count ($\times 10^2$ cfu/g)				
	SPC	*Bacillus sp.*	Total coliform	Yeast	Mold
H1	2.0×10^2 1.5×10^2 1.0×10^2	ND	D	ND	ND
H2	3.0×10^2 8.0×10^2 4.4×10^2	ND	ND	ND	ND
H3	3.6×10^2 4.5×10^2 1.5×10^2	D	D	D	D
H4	2.6×10^2 2.7×10^2 9.7×10^2	D	ND	D	D
H5	1.5×10^2 2.2×10^2 3.5×10^2	D	ND	D	D
H6	1.0×10^2 1.0×10^2 1.0×10^2	D	ND	ND	ND

SPC: Standard Plate Count; D = Detectable; ND=Not Detectable

Table 4. Summary of antibacterial activity of various honey samples on gram-positive and gram-negative bacteria

Sample	Dilution	*Bacillus sp.* (mm)	*E. coli* (mm)
H1	Undilute	15	11
	10^{-1}	-	-
	10^{-2}	-	-
H2	Undilute	24	11
	10^{-1}	-	-
	10^{-2}	-	-
H3	Undilute	20	-
	10^{-1}	-	-
	10^{-2}	-	-
H4	Undilute	-	-
	10^{-1}	-	-
	10^{-2}	-	-
H5	Undilute	12	-
	10^{-1}	-	-
	10^{-2}	-	-
H6	Undilute	10	-
	10^{-1}	-	-
	10^{-2}	-	-

Bacillus sp. in honey are *B. cereus, B. megaterium, B. coagulans, and B. pumilus* [25]. *Bacillus sp.* were absence in H1 and H2 which may indicate that the honey is well conserved against this bacteria. The symbiotic relationship

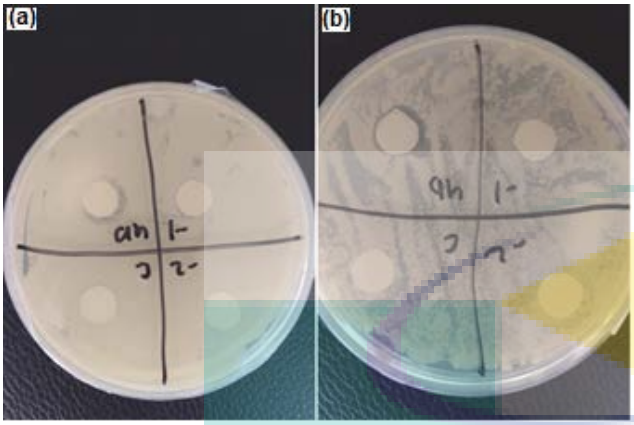

Fig 1. (a) The inhibition zone by using different dilution factor on *E. coli* (b) The inhibition zone by using different dilution factor on *Bacillus sp.*

between *Bacillus sp* and insects especially tropical honey bees and stingless bees may be the solid reason why these bacteria were present in the other four samples [37]. Total coliform indicates the sanitary quality of honey [21], and for this study, the coliform was present only in H1 and H3 samples. The low counts and limited variety of microbes are expected because of honey antibacterial properties against the growth or persistence of many organisms [31,38]. Yeast and mold were not detected for H1, H2, and H6 but, it was present in a high number (> 100) for the other three samples. The result for H3, H4, and H5 contradict the limit set by Malaysia Kelulut standard where the count should be less than 1×10^1 cfu/g.

Antibacterial Analysis: Agar Disc Diffusion

From Table 4, H1, and H2 had a greater inhibitory effect on both gram-negative (*E. coli*) and gram-positive bacteria (*Bacillus sp.*) when tested using undiluted honey. The results of commercial honey samples also showed they had an inhibitory effect only on gram-positive bacteria when tested using undiluted honey (except H4), but all the commercial honey samples were not active against gram-negative bacteria, *E. coli*. The disc diameter of harvested samples was ranged from 11 to 24 mm where commercial honey, H3, H5, and H6, ranged from 10 to 20 mm. There were no zone diameters of inhibition (ZDI) was measured on H4 sample for both bacteria tested. Among all honey samples, H1 and H2 are the only honey that have effects on gram-negative bacteria. Thus, the honey may have the potential as therapeutic or healing honey [13]. The antibacterial activity of honey depends on various factors such as geographical origin, botanical source, harvesting (season when honey was collected), processes and storage conditions as well as the presence of hydrogen peroxide, phenolic compounds, acidity, phytochemicals, pH of honey and higher osmotic pressure [13,25,39].

■ CONCLUSION

In this present work, six samples honey from the local market in Malaysia have been analyzed for their quality criteria concerning the physicochemical parameters and microbial profile. All analyzed samples are within the maximum limit of the quality criteria set by the Malaysian Kelulut Standard and Codex Alimentarius except for free acidity, HMF, and Diastase Number. Most samples have a high value of HMF (more than 400 mg/kg) and lower Diastase Number (lower than 3 DN) especially for H3, H5, and H6. These three samples may indicate poor honey processing and the possibility of adulteration. Even though H1 and H2 were purely harvested from the farm, the quality of both honey is quite poor which may be due to prolonged storage (more than six month) and the poor storage condition. There is no limit set by the Malaysian standard for free acidity, electrical conductivity, and Diastase Number. Hopefully, in future, these parameters can be added into the Malaysian Kelulut Standard to ensure the standard is as per with another international standard available. Honey is known for its antibacterial properties; however, microbial growth has been found in most samples, expressing poor hygienic procedures during harvesting, packaging or storage. Although this study was a preliminary work and only limited to six samples, more sample data are in preparation in order to be part of the process to create more specific legislation for stingless bees honey in Malaysia.

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Sugar profile and enzymatic analysis of stingless bee honey collected from local market in Malaysia

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Abstract. Commercial honey is widely available in the market, raising questions whether the honeys are good in quality or otherwise, Thus, this research was designed to compare the quality of harvested stingless bee honey and commercial honey available in the Malaysian market by measuring their sugar profile and enzyme activity. The analysis showed that the honey contained moisture between 16.6% - 32.1%, various sugar starting with fructose (15.03 – 48.44 g /100 g), glucose (12.16 – 40.09 g/100 g), sucrose (<0.01 – 7.29 g/100 g), Fructose + Glucose (F+G) (15.03- 80.25 g/100 g), Fructose/Glucose (F/G) (0.78 – 1.63), and G/W (0.47 – 1.89). Also, diastase activity and Invertase activity of the honey varied from 1.82 to 6.11 DN and 0.27 IN to 4.94 IN, respectively. Eight honey samples including harvested honey, *H. Itama* and *G.Thoracica* showing comparable results with past studies and within the limits of Malaysian Standard. However, all honey samples demonstrate lower enzyme activity suggesting that honey from stingless bee has low enzyme activity compare to *Apis mellifera* honey.

1. Introduction

Recent years have seen growing interest among the consumers into food that helps maintaining their health with honey being one of them. Honey is a golden sticky liquid food that is sweet and rich in nutrients due to their various components such as sugars (mostly fructose and glucose), enzymes, amino acid, proteins, organic acids and mineral [1,2]. Stingless bees honey is not very popular to consume due to its sour and bitter taste [3]. Furthermore, stingless bee honey colour are darker, more watery in texture and expected to have slower crystallization compare to *Apis mellifera* honey [4,5]. Enzymes have always been an important subject in research involving honey as it can be used to differentiate between pure and adulterated honey. The predominant honey enzymes are diastase (amylase), invertase, glucose oxidase, as well as catalase and phosphatase [6]. However, this research is focussing mainly on diastase and invertase activity as both enzymes are largely used to measure honey quality freshness. Almost 95–99% of honey's dry substance consist of sugars where fructose is the most dominant followed by glucose and sucrose [7]. There is only small amount of sucrose (1% w/w) are found in honey due to the presence of invertase enzyme[8–10]. Furthermore, the quality of honey also is relying on the sum of fructose and glucose, fructose/glucose ratio and glucose/water ratio [11]. Due to the increasing popularity of honey, the honey supplied are decreasing thus it has led to the

production of impure honey. In a year, stingless bee only can produce up to 4 kg honey per colony compared to *Apis meliffera* honey which can produce 5 to 9 kg honey per colony [12]. Therefore, to cater the additional demand and to gain more economical profits, the irresponsible producers tend to add cheap chemical and artificial syrup such as cane sugar into pure honey. The abundance adulterated honey in the market could negatively impact the consumer trust and most importantly their health thus in order to find the difference between pure and adulterate honey, this present study is intended to measure the sugar profile and the enzyme activity of several stingless bee honey samples in Malaysia. Simultaneously, the properties of harvested and commercial stingless bee honey were compared and the interaction between sugar profile and enzyme activity in stingless bee honey were also observed.

2. Materials and Methods

2.1 Sample Collection and Preparation

17 samples of stingless bee's honey were collected from May 2017 to March 2018. Two pure stingless bee honey, *Heterotrigona Itama and Geniotrigona Thoracica* were harvested from Universiti Malaysia Pahang stingless bee farm and Aqif Kelulut Farm, Pekan, while other 15 samples were randomly obtained from local market around Malaysia. The honey obtained was stored in the dark at ambient temperature until the experiment.

2.2 Chemicals and Reagents

All of the chemicals and reagents used were of analytical grade. Sugar standard (Fructose, Glucose, Sucrose), acetonitrile, potassium hydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium chloride (NaCl), and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (USA), and p-Nitrophenyl-α-D-glucopyranoside (pNPG) was purchased from Merck (Germany). Iodine was obtained from R&M (Malaysia) and tris- (hydroxymethyl) aminomethane was purchased from Nacalai Tesque (Japan).

2.3 Moisture content

Using the refractive index of the honey, the moisture content was measured by handheld refractometer (RHB 90ATC, China).

2.4 Sugar profile of Honey

Sugar profiles determination (fructose, glucose, sucrose) were performed using 1260 Infinity II LC System (Agilent Technologies, USA). The method is following the method of Malaysian Kelulut Standard [13]. The sugars were eluted through Phenomenex column (PhenoSphere 5µ NH_2 80A, 250 x 4.6 mm, Phenomenex Inc, USA) and detected by refractive Index detector (RID) operated at 35ºC. The mobile phase is acetonitrile: water (75:25, v/v) at a flow rate of 0.9 ml/min. The retention times obtained from the standards were compared to obtain HPLC sample peaks. The injections were performed in triplicate where the average peak area was used for evaluation.

2.5 Enzyme Activity

2.5.1 Diastase Activity

The diastase activity was determined by following the method of International Honey Commission [14]. A 5.0 g of honey samples were dissolved in 15 mL distilled water and then mixed with 2.5 mL of acetate buffer (1.59M, pH 5.3). The solution was then mixed with 1.5 mL of 0.5 M sodium chloride solution in 25 mL volumetric flask before 10 mL of this solution is taken and combined with 5 mL of 2% starch solution in a test tube. Then, the test tube was kept in BS -21 shaking water bath (Lab Companion, Jaio Tech Inc, South Korea) at 40ºC. After 5 minutes, 1 mL of the solution was mixed with 10 mL of 0.0007M diluted iodine solution. The absorbance was recorded using a spectrophotometer at 660 nm until the reading reached less than 0.235 absorbance. The diastase activity was expressed in Diastase Number (DN). DN was the amount of enzyme that hydrolysed/ converts 1% starch solution/ 0.01g of starch for 1 h at 40ºC.

2.5.2 Invertase Activity

The invertase activity was determined by following the method of International Honey Commission [14]. Substrate solution; p-Nitrophenyl-α-D-glucopyranoside (pNPG), was used in order for it to be dissolved into glucose and p-nitrophenol by enzyme invertase in honey [15]. The invertase activity of the samples was determined using UV- Vis spectrophotometer (ThermoFisher Scientific, USA) at 400 nm where the values obtained were expressed as IN (Invertase Number). The IN indicates the ability of enzymes to break down sucrose in 1 h [14].

2.6 Statistical Analysis

All analyses were prepared in triplicate where the differences between mean values were relevance at values of $p < 0.05$. The data obtained in the study were statistically analysed using analysis of variance (ANOVA) and followed by Tukey test (Minitab 18, Minitab Inc, USA).

3. Result and Discussion

3.1 Sugar profile of honey

The results of stingless bee sugar profile are presented in Table 1. The addition of fructose and glucose content of seventeen (17) honey samples were varied between 27.2 to 80.25 g/100 g with an average of 56.93 ± 16.5 g/100 g. The summation results met the requirement by Malaysian kelulut standard which is no more than 90.0 g/100 g. However, some samples slightly exceeded the codex standard of sugar profile for honey (< 60 g/100 g). For good quality honey, the glucose content should be lower than the fructose content [16] and this can be seen in most samples except for six commercial samples (C1, C4, C5, C10, C13 and C15). Thus, the six honeys probably were poor quality honey. Honey sample, C4, has high sucrose content of 7.29 ± 0.18 g/100 g, but it is still within the maximum limit stated by Malaysian kelulut standard which is no more than 8.0 g/100 g. High sucrose content could be contributed by various factors such as stingless bee species, floral sources, sucrose not completely converted into fructose and glucose and the trace of adulteration activity in honey [1,8,17].

3.2 Moisture Content

As shown in Table 1, moisture content varied from 16.6% to 32.1%, which is still in the range of Malaysian standard. For raw honey, the moisture should be no more than 35% and no more than 22% for processed honey. The results showed that, C11 had the lowest moisture content while C15 had the highest moisture content. Moisture is one of the significant characteristics in quality evaluation of honey as it control the maturation and preservation effects of honey, influences viscosity, weight, crystallization and finally the flavour of honey [18]. In addition, moisture content are able to prevent fermentation and granulation during storage [11]. Apart from being exposed to the fermentation process, higher moisture content can also indicate the honey was adulterated (> 35%) [10].

3.3 Ratio of Fructose/ Glucose (F/G) ratio and Glucose/ Water (G/W)

The F/G ratios influence the flavour of honey as the fructose is more sweet than glucose and sucrose [1,19]. Commercial honey, C11 has the highest F/G ratio of 1.90 (Table 1), thus the honey is sweeter compare to others. Besides that, F/G ratio implying the honey ability to granulate because when the amount of fructose is greater than glucose, the honey is in fluid state [20]. When the F/G ratio is below 1.0, the crystallization of honey is quicker, however when this ratio is greater than 1.0, the honey stays in liquid forms for a long time [11,21]. F/G ratios not only depend on the source of the nectar but it also depends on the variation of bee species and climate of different regions [4].Apart from F/G ratio, the G/W ratio also associated to honey crystallisation. Both ratios are useful to predict and control the chances of granulations in honey especially G/W ratio since the glucose contents and moisture content are crucial for honey granulation [11,16]. Honey with F/G more than 1.0 and G/W less than 1.0 are likely to crystallise slower compared to honey with low F/G (< 1.0) and high G/W (> 2.0) [19] . *G. Thoracica* has the lowest G/W value which is 0.46 (<1.0) thus, it may imply that the honey has the lowest ability to crystallize and will prolong as liquid for quite some time compared to other honey.

Table 1. Summary of sugar profile of various stingless bee honey samples from Malaysia (mean ± standard deviation, n = 3).

Samples	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)	F+G (g/100g)	F/G	Moisture content (%)	G/ W
H. Itama	17.5 ± 1.3^{j}	16 ± 0.8^{j}	< 0.01	33.5 ± 2.27^{i}	1.10 ± 0.10$^{c, d, e}$	30.3 ± 0.18^{b}	0.53 ± 0.03^{i}
H. Thora	15.03 ± 1.22^{i}	12.17 ± 0.47^{k}	< 0.01	27.2 ± 0.69^{j}	1.24 ± 0.09$^{b, c, d}$	26.4 ± 0.09^{d}	0.46 ± 0.02^{i}
C1	30.96 ± 0.81^{e}	31.6 ± 0.79^{d}	1.10 ± 0.03$^{e, f}$	62.56 ± 1.53^{e}	0.98 ± 0.02^{f}	19.43 ± 0.20^{g}	1.63 ± 0.06^{b}
C2	32.52 ± 0.59$^{d, e}$	30.09 ± 0.70$^{d, e}$	< 0.01	62.62 ± 0.67$^{d., e}$	1.08 ± 0.04$^{c, d, e, f}$	28.43 ± 0.51^{c}	1.06 ± 0.02^{h}
C3	33.51 ± 0.54^{d}	31.94 ± 0.07^{d}	0.05 ± 0.02^{h}	65.45 ± 0.61^{d}	1.05 ± 0.01$^{c, d, e, f}$	26.7 ± 0.61^{d}	1.19 ± 0.03$^{f, g, h}$
C4	22.83 ± 0.42^{g}	25.47 ± 0.50^{g}	7.29 ± 0.18^{a}	48.3 ± 0.95^{g}	0.89 ± 0.01$^{d, e, f}$	17.73 ± 0.65^{h}	1.44 ± 0.08$^{c, d, e}$
C5	31.64 ± 0.48$^{d, e}$	34.55 ± 0.50^{c}	2.81 ± 0.09^{c}	66.19 ± 0.95$^{d, e}$	0.92 ± 0.01$^{d, e, f}$	23.43 ± 0.25^{e}	1.47 ± 0.01$^{b, c, d}$
C6	31.67 ± 0.57$^{d, e}$	28.08 ± 0.95$^{e, f}$	0.45 ± 0.13$^{g, h}$	59.75 ± 1.45$^{d, e}$	1.13 ± 0.02$^{c, d, e}$	26.7 ± 0.61^{d}	1.05 ± 0.01^{h}
C7	24.13 ± 1.80^{g}	22.83 ± 1.04^{h}	3.34 ± 0.33^{b}	46.97 ± 1.77^{g}	1.06 ± 0.02$^{c, d, e, f}$	17.12 ± 0.76^{h}	1.33 ± 0.11$^{d, e, f}$
C8	20.6 ± 1.10^{h}	18.53 ± 0.50^{i}	3.55 ± 0.05^{b}	39.13 ± 0.61^{h}	1.11 ±0.05$^{c, d, e}$	16.7 ± 0.35^{h}	1.11 ± 0.03$^{g, h}$
C9	42.81 ± 0.72^{b}	26.27 ± 0.63$^{f, g}$	3.35 ± 0.05^{b}	69.08 ± 1.35^{b}	1.63 ± 0.01$^{a, b}$	24.0 ± 1.0^{e}	1.89 ± 0.09$^{g, h}$
C10	39.35 ± 0.60^{c}	40.9 ± 1.03^{a}	0.80 ± 0.07$^{f, g}$	80.25 ± 1.62^{c}	0.96 ± 0.01$^{c, d, e, f}$	21.67 ± 0.57^{f}	1.89 ± 0.09^{a}
C11	48.44 ± 0.42^{a}	25.47 ± 0.50^{g}	< 0.01	73.91 ± 1.01^{a}	1.90 ± 0.02^{a}	16.6 ± 0.53^{h}	1.54 ± 0.06$^{b, c}$
C12	17.58 ± 0.44^{i}	13.33 ± 0.67^{k}	0.03 ± 0.002^{h}	30.91 ± 0.87^{i}	1.32 ± 0.07$^{b, c}$	28.43 ± 0.51^{c}	0.47 ± 0.02^{i}
C13	38.91 ± 0.30^{c}	37.06 ± 0.97^{b}	< 0.01	75.96 ± 1.21^{c}	1.05 ± 0.02$^{c, d, e, f}$	26.73 ± 0.55^{d}	1.38 ± 0.01$^{c, d, e}$
C14	26.05 ± 0.60^{f}	28.9 ± 0.85^{e}	2.17 ± 0.15^{d}	54.95 ± 0.86^{f}	0.90 ± 0.04$^{d, e, f}$	26.33 ± 0.32^{d}	1.09 ± 0.02$^{g, h}$
C15	31.04 ± 1.0^{e}	40.03 ± 1.0^{a}	1.47 ± 0.28^{e}	71.07 ± 1.01^{e}	0.78 ± 0.04$^{e, f}$	32.01 ± 0.30^{a}	1.25 ± 0.04$^{e, f, g}$
Mean ± SD	29.68 ± 9.42	27.25 ± 8.67	2.20 ± 2.06	56.93 ± 16.5	1.12 ± 0.28	24.04 ± 4.98	1.22 ± 0.43

Note: $^{a - k}$ =Means with different superscript letter along the column are significantly difference ($p<0.05$); F+G: Summation of fructose and glucose; F/G: Ratio of fructose to glucose; G/W: Ratio of glucose to water (moisture content)

3.4 Diastase Activity

Almost all honey contains diastase where the activity can be measured and expressed as Diastase Number (DN) [22]. Diastase activity for seventeen (17) samples of stingless bee honey ranged from 1.82 to 6.11 with average value of 3.89 ± 1.56 (Table 2). According to Codex Alimetarius [23], Diastase Number should be no less than 8 DN and for honey with low enzyme content, more 3 DN is acceptable. There is no fixed limit for diastase number in Malaysian kelulut standard. From the results, we can conclude that stingless bee honey has low diastase number compared to *Apis mellifera* honey since all samples are less than the limits for international standards. Our results also comparable with the study from Thailand [23] where the average diastase reported ranging from 0.050–4.9 DN. Diastase are regarded as one of quality criteria by international standard [24] where the values is influenced by honey storage and heating [25]. Besides that, it also can be used as a mark for honey freshness and overheating [26]. Diastase values are not only affected by geographical and botanical origin but also by pH values, nectar flow (honey flow) and foraging behaviour of the bees [27,28].

3.5 Invertase Activity

Invertase is the enzyme that hydrolyses sucrose to fructose and glucose. The invertase activity can be represented by either invertase units (IU kg-1) or as invertase number (IN), where 1 IN is equal to 7.344732 IU kg^{-1} [29,30]. Table 2 shows the invertase activity result for all samples. The invertase activity ranges were between 0.27 IN to 4.94 IN with average value of 2.77 ± 1.22 IN. No limits were proposed by Malaysian kelulut standard but according to Bogdanov [25], it was suggested that honeys should have more than 10 IN while for low enzyme honey, the activity should be greater than 4 IN. The fact that harvested honey, *H. Itama* and *G.Thoracica* had almost none invertase activity may suggest that the stingless bee honey in Malaysia has low enzyme activity compare to *A. mellifera* honey. Due to the low enzyme content, this may be regarded as a natural feature of these honey, rather

than an index of scarce freshness or lowered in quality. Both diastase and invertase activities steadily deteriorate on prolonged storage and heating of honey [31]. Nevertheless, invertase is considered as better freshness indicator than diastase because it is more susceptible towards prolonged storage and heat [25].

Table 2. Summary of predominant enzyme activity of various stingless bee honey samples from Malaysia (mean ± standard deviation, n = 3).

Samples	Diastase Number (DN)	Invertase Number (IN)
H. Itama	5.85 ± 0.08^{a}	ND
H. Thoracica	5.87 ± 0.48^{a}	ND
C1	$3.11 \pm 0.41^{f, g}$	3.2 ± 0.18^{d}
C2	6.11 ± 1.11^{a}	ND
C3	$3.79 \pm 0.01^{d, e, f}$	4.29 ± 0.41^{b}
C4	2.11 ± 0.20^{h}	0.27 ± 0.01^{g}
C5	$2.79 \pm 0.01^{f, g, h}$	$2.19 \pm 0.07^{e, f}$
C6	$5.36 \pm 0.25^{a, b}$	4.25 ± 0.13^{b}
C7	$4.33 \pm 0.17^{c, d, e}$	2.13 ± 0.07^{f}
C8	$3.33 \pm 0.17^{e, f}$	0.55 ± 0.05^{g}
C9	$2.16 \pm 0.13^{g, h}$	1.82 ± 0.07^{g}
C10	1.82 ± 0.16^{a}	3.79 ± 0.03^{c}
C11	$4.58 \pm 0.02^{b, c, d}$	ND
C12	5.65 ± 0.37^{a}	4.94 ± 0.12^{a}
C13	$5.12 \pm 0.11^{a, b, c}$	ND
C14	$2.32 \pm 0.12^{g, h}$	2.58 ± 0.09^{e}
C15	1.93 ± 0.06^{h}	$2.24 \pm 0.06^{e, f}$
Mean ± SD	3.89 ± 1.56	2.68 ± 1.46

Note: $^{a\text{ - }h}$ =Means with different superscript letter along the column are significantly difference ($p<0.05$); ND= Not detected

3.6 Relationship between sucrose content and invertase activity

The correlation between the amount of sucrose in stingless bee honey and invertase activity was determined in Figure 1. From the regression analysis, there is some reliance between the amount of sucrose and invertase activity where the higher amount of sucrose resulting the lower invertase activity in honey. The amount of sucrose increased probably because of the factor of storage period and the honey was not matured enough due to sucrose was not wholly converted into fructose and glucose by enzyme invertase [18]. Five honey samples including *H. Itama* and *G.Thoracica* had low sucrose level and almost none invertase activity, thus it seems hard to conclude that these samples are lower in quality. The amount of the invertase in honey depends on many factors such as the condition of a bee colony, the age of the bees, food, temperature and intensity or type of honey flow [15].

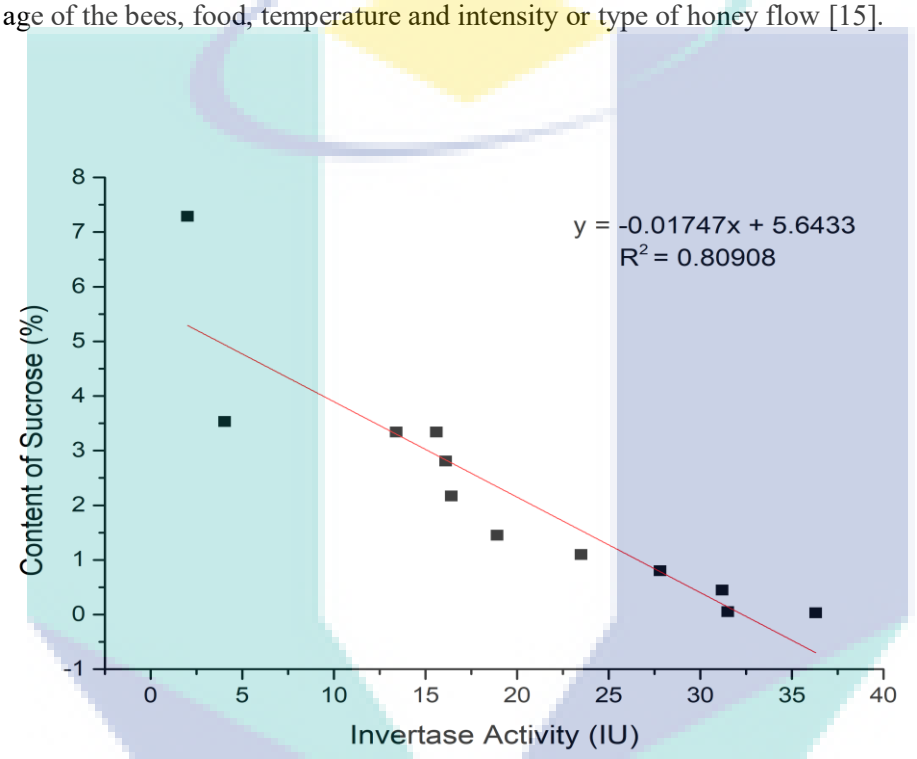

Figure 1. Influence of invertase activity on the content of sucrose in stingless bee honey samples from Malaysia

4. Conclusion

Seventeen samples of stingless bee honey collected from local market in Malaysia were analysed for their sugar profile and enzyme activity. From all commercial samples, six samples including C2, C3, C6, C11, C12 and C13 have similar characteristic when compare with harvested honey *H. Itama* and *G.Thoracica*. This result may suggest that these samples are good quality honey. The rest of commercial honey (9 samples) shows high value of glucose and sucrose content and lower enzyme activity which may indicate poor honey processing and possibility of adulteration. Since diastase and invertase result are lower than the international standard of *Apis mellifera* honey, we can conclude that enzyme activity in stingless bee honey are lower compare to *Apis mellifera* honey. This study has revealed that there is difference between good and adulterated honey however in future more samples can be added so that a decent data on sugar and enzyme content in stingless bee honey can be proposed and established which it may help the consumers from purchasing adulterated honey.

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