DETERMINATION OF THE MARKER COMPOUNDS IN ADULTERATED AND NON-ADULTERATED STINGLESS BEE HONEY USING GC-MS

BY

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CERTIFICATE

This is to certify that dissertation entitled “Determination of the marker compounds in adulterated and non-adulterated stingless bee honey using GC-MS” is bonafide record of the research work done by Ms. Mouylin Chem during the period from October 2016 to July 2017 under my supervision.

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

%	Percentage
µg/mL	Microgram per millilitre
µL	Micro litre
BSTFA	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
CS	Corn syrup
FAO	Food and agriculture organization
GCMS	Gas chromatography-mass spectrometry
HFCS	High fructose corn syrup
IHC	International honey commission
IS	Inverted sugar
mg	Milligram
mg/ Kg	Milligram per kilogram
mL/min	Millilitre per minute
N/A	Not applicable
^{O}C/ min	Degree celsius per minute
RSD	Related standard deviation
SD	Standard derivation
WHO	World health organization

ABSTRAK

Madu sejak dahulu lagi seringkali dicampur dengan alasan mengaut keuntungan ekonomi. Secara amnya, madu dicampurkan dengan menambah bahan asing atau digunakan untuk memberi makan lebah. Sehingga kini, kaedah pengesanan pencampuran madu adalah terhad kerana komposisi kompleks sebatian madu yang dihasilkan oleh lebah yang bergantung kepada bunga tumbuhan serta persekitaran geografi. Dalam kajian ini, tumpuan kepada pembangunan kaedah yang mudah dengan menggunakan GC-MS untuk mengesan pemalsuan di dalam madu lebah kelulut. Dalam usaha untuk mengesan perbezaan diantara madu yang dipalsukan dan asli, kajian ini telah menggunakan sampel madu lebah kelulut asli untuk mengesan dan menyaring sebatian mudah meruap yang terdapat didalam madu. Tiga sebatian seperti asid propionik, gliserol dan myo-inositol didapati terdapat didalam madu asli yang di analisis dan digunakan sebagai sebatian penanda. Sebatian-sebatian kimia ini dapat membezakan madu asli, tiruan serta produk gula. Di samping itu, kajian mengenai sebatian-sebatian penanda ini juga menunjukkan bahawa penyimpanan boleh memberi kesan terhadap kandungan sebatian penanda. Dalam tempoh 5 bulan penyimpanan, gliserol dan myo-inositol didapati terdegradasi. Walau bagaimanapun, asid propionik tidak terdegradasi. Kesimpulannya, kaedah mudah menggunakan GC-MS untuk menganalisis asid propionik, gliserol dan myo-inositol dapat membezakan madu lebah kelulut yang asli ataupun palsu dalam tempoh 5 bulan selepas menuai daripada sarang.

ABSTRACT

Honey has been adulterated since long time due to economical reason. Generally, honey is adulterated by adding the foreign substances to pure honey or used for feeding the bee. The method of detection for honey adulteration is limited due to complex composition of honey compounds, which depend on the bee's flowers preference and geographical region. This study had focused on the development of a simple method by using GC-MS to detect adulteration in stingless bee honey. In order to detect the difference between adulteration and non-adulteration stingless bee honey, this study had used pure stingless bee honey sample to screen honey volatile compounds. Three compounds such as propionic acid, glycerol and myo-inositol were found and used as marker compounds. These 3 marker compounds were able to differentiate the pure stingless bee honey, adulterated honey and sugar products. In addition, study on these marker compounds also showed that storage time can have an effect on the marker compounds content of honey. Within 5 months of storage, glycerol and myo-inositol were significantly degraded. However, propionic acid was not degraded. In conclusion, a simple method of using GC-MS to analyse propionic acid, glycerol and myo-inositol was able to differentiate adulterated and non-adulterated stingless bee honey within 5 months period after harvesting from the nest.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The natural honey is well known as the valuable products due to its medicinal properties (Buba *et al.,* 2012). Codex Alimentarous or 'food code' is a collection of standards, guidelines and codes of practice by FAO and WHO joint commission defined honey as natural sweet substance product which is produced by honey bees from the nectar of plants or from secretions of living part of plants or excretions of plant sucking insects on the living part of plants (Standard and Honey, 2001). The honey bees consume many of plants and transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature. Honey is a viscous solution containing sugar, mostly fructose and glucose 80-85%, water 15- 17%, ash 0.2% and also other substances which are organic acid, enzymes, vitamins and solid particles (Bogdanov *et al.*, 1999). Biological properties and therapeutic activities of honey are well known in wound healing. It contributes in wound healing processes due to antimicrobial, anti-inflammatory and antioxidant activities, boosting effect on immune system, debridement action and stimulating role in wound regeneration (Oryan, Alemzadeh and Moshiri, 2016). While, antioxidants compound such as phenolic compounds (p-hydroxibenzoic acid, chrysin, pinobanksin, pinocembrin) vitamin C, and catalase also present in honey which prolong cell or tissues oxidative damage in human body. Moreover, phenolic compounds in honey have similar antimicrobial capacity of inhibition in both dark and clear honey extraction (Estevinho *et al.*, 2008).

Stingless bee honey is prized bee product that has been traditional consuming for many years, due to advantages of medical properties. Stingless bee honey was excluded in the international standards for honey because of the limited of information to the product (Bogdanov *et al.*, 1999). The aim of the International Honey

Commission (IHC) is the creation of quality standards of bee products, sting-less bee honey was considered, along with pollen, beeswax, propolis and royal jelly. Stingless bee honey is a type of honey which consist of huge amount of bioactive substances include antioxidant substances (phenolic acids, flavonoids and enzymes glucose oxidase catalase) (Aljadi and Kamaruddin, 2004; Silva *et al.*, 2013). Due to advantages of stingless bee honey in term of medication, it would help in antioxidant activities. Nevertheless, compounds which present in stingless bee honey is changing because of botanical resources, origin, and type of plants (Silva *et al.*, 2013). Stingless bees consist of many compounds such as antiseptic, antimicrobial, anticancer, anti-inflammatory, and wound-healing properties and may give defence for and help cell function in erythrocytes (Alvarez-Suarez *et la*., 2013).

Adulteration is a fake adjustment of foods carried out by adding inactive or hazardous material or substances of less quality, or subtracting those components which confer food properties and value (Li *et al.*, 2012). Adulteration happens mostly because of less expensive substances being added. Because of high price due to natural food, honey has been adulterated with inexpensive products since long time ago. Honey is mostly composed of carbohydrates (around 80% of honey), therefore, honey adulterations mainly involve the addition of inexpensive sugar products (Cotte *et al.*, 2003; Cordella *et al*, 2005). Adulterants in honey have been demonstrated the present of molasses, caramels, sugar syrups from corn, sugar cane and sugar beet, inverted sugar (IS) by acids and enzymes, as well as corn syrups (CS) or high fructose corn syrups (HFCS) obtained by isomerization from corn syrup (White and Doner, 1980; Ruoff and Bogdanov, 2004).

Using gas chromatography (GC) and mass spectrometry (MS) is very effective combination for chemical analysis. GC-MS has been using for drug testing and environmental contaminant identification. GC analysis separates all of the components in a sample and provides a representative spectral output (Hajšlová and Čajka, 2007). The resolution of GC capillary columns gives chromatographic profiles

with contains qualitative and quantitative information useful for characterization purpose. GC-MS is a beneficial technique for honey analysis since it combines high separation efficiency and sensitivity besides providing qualitative and quantitative data for these compounds (Cuevas-Glory *et al.*, 2007). Since aromatic compounds are present in honey at very low concentrations as complex mixtures of volatile components of different functionality and relatively low molecular weight, GC-MS is a suitable technique for honey analysis as it combines high separation efficiency and sensitivity besides providing qualitative data for these compounds (Cuevas-Glory *et al.*, 2007). GC-MS is required derivatization of polar function group prior to analysis due to low thermal stability and insufficient volatility of carbohydrates (Ronald Bentley *et al.*, 1963)

1.2 Problem Statement

Recently there is highly interest in producing high quality of stingless bee honey. Because of its nutritional and medicinal price, stingless bee honey continues to be a popular food. One of the important quality parameters in honey is it should not contain any adulterant. However, the analyse of honey adulterations can be a difficult task, especially due to high variability in honey compounds which depends on its botanical or geographical origin (Anklam, 1998; Prodolliet and Hischenhuber, 1998). The adulterated honey with syrups were also having a similar chemical compositions compare to natural honey (Cordella *et al.*, 2005). Honey can easily be adulterated with various cheaper sweeteners, such as refined cane sugar beet sugar, high fructose corn syrup and maltose syrup, resulting in higher commercial profits (Li *et al.*, 2012).

1.3 Objective

The main objective of this study is to differentiate between pure and adulterated stingless bee honey using selected marker compounds in the GC-MS

analysis. In addition, this study is including three specific objectives such as:

i. To determine the volatile compounds in stingless bee honey as a marker for pure stingless bee honey using GC-MS analysis.
ii. To compare the selected markers compounds in stingless bee honey and adulterated honey by using peak area of GC-MS.
iii. To measure selected marker compounds degradation in pure honey at different time of storages.

1.4 Scope of Study

The scope of this study is focused on how to differentiate between pure stingless bee honey with adulterated honey. The samples of stingless honey are harvested from Syamille Agrofarm in Kuala Kangsar, Perak. For sugars which used to adulterate in adulterated honey are bought from market nearby Advance Medical and Dental Institute (AMDI), and adulterated honey samples were manually mixed in laboratory. This study was done to determine the compounds present in pure stingless bee honey by using GC-MS. The marker compounds which were consistently present in the pure honey will be used to differentiate the adulterated and non-adulterated honey. In addition, the level of each compound in pure stingless bee honey was measured at different time of storage, in order to see the degradation of compounds within time.

1.5 Significant of Study

This study will be able to differentiate the pure and adulterated stingless bee honey using a relatively easy, fast and simple method, which is GC-MS. The compounds were measured in GC-MS, will be selected as biomarker to differentiate adulterated and non-adulterated stingless bee honey and for future use to determine the quality of pure stingless bee honey.

CHAPTER 2

LITERATURE REVIEW

2.1 Honey

Honey is a strong modulator of wound healing based on its biological and therapeutic activities. The wound healing processes is due to its anti-microbial, anti-inflammatory and anti-oxidant activities, debridement action and stimulating role in wound regeneration (Oryan *et al.*, 2016). Honey contains high amount of different sugar concentration with some amounts of nutrients include mineral, proteins, vitamins, organic acid, flavonoids, phenolic acids, and other phytochemicals which contributes to the antioxidant activity of honey (Chaikham *el al*., 2016). In addition, present of phenolic acids and flavonoids play an important roles in preventing from depletion of intracellular antioxidant enzymes and prevent red blood cells against hemolysis and lipid peroxidation (Alvarez-Suarez *et al.*, 2012). Honey was used since ancient time due to nutrient and also medicine for therapeutic purpose, moreover many compounds in honey prevent bacterial, fungal (Dias *et al.*, 2008; Estevinho *et al.*, 2008).

2.2 Stingless Bee Honey

Stingless bee honey has been celebrated as a therapeutic honey and used traditionally since ancient time in many ancient culture such as Mayan (Bogdanov, 1997). Although the studies on stingless bee is lacking, it is still regards as medicinal honey, which possess healing properties. Stingless bee nests were found in living trees and the colonies are characteristically long-lived but have low productiveness of honey (Eltz and Bru, 2003). In Malaysia, there are currently 32 species which have been inventoried with few more newer species of stingless bee have been found recently (Herbert F. Schwarz, 1939; Norowi and Fahimie, 2010). Twelve species out of 32 species were commonly found such as Trigona *itama*, T. *thoracica*, T. *apicalis*,

T. *terminata*, T. *respani*, T. *melanocephala*, T. *valdezi*, T. *collina*, T. *atripes*, T. *canifrons*, T. *iridepennis* and T. *rufibasalia* (Norowi and Fahimie, 2010). However, only two stingless bee species, which are *Trigona (Geniotrigona) thoracica*, and Trigona (Heterotrigona) *itama* preferred by bee farmers. T. *itama* contributed to 83.2% and T. *thoracica* 11.2% of the total domesticated stingless bee species. Other species, which are less common domesticated are Trigona (Lepidotrigona) *terminata* 4%; Hypotrigona (Lisotrigana) *scintillans* 1% and Trigona (Tetragonula) *laeviceps* 3% (Kelly *et al.*, 2014). The bee keepers usually kept the stingless bees in wooden hives or in the original log with hive-box on top of the log for easier harvesting the honey. Honey, pollen and propolis are the main products of stingless bee, although the production per hive is much less compared with honeybee. Stingless bee honey was produced by local beekeeper is predictable to grow rapidly due to high demand by the public due to its health benefits. As awareness increases, the price of the commercial stingless bee honey is also increase, in which may lead to adulteration of the honey product. Physicochemical analysis from Australia and Brazil showed that the colour of stingless bee honey was darker than apis mellifera honey (76mm Pfund) (Oddo *et al.*, 2008). The physicochemical parameters were shown in Table 2.1.

Table 2.1: Physicochemical parameters of stingless bee honey.

Physicochemical compounds	Amount
Moisture content (g/100g honey)	25.30 - 27.50
Water activity content	0.73 - 0.75
pH	3.20 - 3.45
Ash content (%)	0.33 - 0.56
Total acidity (mEq/kg)	98.20 - 161.70
Flavonoids (mg EQ/100g)	8.12 - 12.77
Polyphenol (mg EGA/100g)	48.53 - 63.43
Nitrogen content (mg/100g)	109.70 - 693.00
HMF (mg/kg)	0.40 - 2.10
Mn (mg/kg)	2.49 ± 0.06
Fe (mg/kg)	1.97 ± 0.27
Cu (mg/kg)	2.18 ± 0.01
Zn (mg/kg)	6.23 ± 0.01
Co (µg/kg)	18.12 ± 0.26
Ni (µg/kg)	28.51 ± 1.72
Pb (µg/kg)	22.10 ± 0.48
Cd (µg/kg)	3.19 ± 0.15
Nonaromatic organic acids (g/kg) • D-gluconic acid • Citric acid • Malic acid	 7.70 - 11.80 0.11 - 0.36 0.04 - 0.20
Total sugar contents (g/100g) • Fructose • Glucose • Maltose • Sucrose	60.50 - 63.30 21.80 - 27.40 14.30 - 22.70 15.30 - 22.80 0.90 - 2.20
Total antioxidant activity (TTA) (µM Trolox equivalents)	175.23 - 303.40
Radical scavenging activity (RSA) (% ascorbic acid equivalent)	32.50 - 67.67

Stingless bee honey consists very reach amount of flavour or fragrance qualities (Jerković *et al.*, 2006). Mostly, honey with light-coloured has less flavour comparable to honey with darker-coloured (Castro-Vázquez *et al.*, 2003). Stingless bee honey, when compared to Apis mellifera honey indicates the higher values of

moisture, water activity, electrical conductivity, free acidity and low of enzymes activity, and a distinct sugar spectrum (Assegid Garedew, Erik Schmolz, 2003; Paxton *et al.*, 2007; Oddo *et al.*, 2008; Vit, 2008; Boorn *et al.*, 2010). Stingless bee honey showed strong antimicrobial activity, high antioxidant and an excellence wound healing activity.

2.2.1 Antimicrobial Activity

Stingless bee honey was used traditionally since long time ago to treat and prevent different illnesses and to heal the wound. Higher concentration of honey able to prevent the growth of gram positive and negative bacteria. Honey can be use along with dressing agent during treatment of wound (Assegid Garedew, Erik Schmolz, 2003). Stingless bee honey was shown to be an effective treatment for Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes, Pseudomonas aeruginosa and Candida albicans (Boorn *et al.*, 2010; Jenkins and Cooper, 2012; Maddocks *et al.*, 2012). The effect of antimicrobial of stingless bee honey is related to the effect of compound such as hydrogen peroxide, phenolic and flavonoids and lower pH (Boukraa, 2008).

2.2.2 Anti-Oxidation Activity

Antioxidant activities measurement in stingless bee honey was higher than other types of honey (Oddo *et al.*, 2008). Natural antioxidant activity in honey is produced by phenolic compounds. The phenolic compound present in stingless bee honey are caffeic acids, caffeic acid phenethyl ester, acacetin, kaempferol, galangin, chrysin, acacetin, pinocembrin, pinobaksin and apigenin; which important for maintaining health; and improved some chronic diseases (Tomfis Barberfin *et al*., 1993; Cushnie and Lamb, 2005; Lianda R *et al*, 2012). Due to honey consists of sugars, antioxidant compounds which are amino acids, proteins, carotenes, phenolic compounds, flavonoids, ascorbic acid; and other organic acid, honey are a potential

diet for human in term of antioxidant activity (Erejuwa *et al*., 2012). Polyphenol compounds in honey was showed to have high correlation with antioxidant capacity (Alzahrani *et al.*, 2012).

2.2.3 Wound Healing

Stingless bee honey was used for wound healing due to complex composition of its sugar (glucose and fructose), proteins, vitamins, minerals, peroxide compound (glucose oxidase for the production of hydrogen peroxide and D-gluconic acid), non-peroxide compounds (flavonoids, polyphenols), high acidity and high water content (Oddo *et al.*, 2008; Guerrini *et al.*, 2009; Suntiparapop *et al*., 2012). In wound healing process, honey plays a role as antiseptics and antibiotics to avoid wound infection and also improve the rate of wound healing. The main mechanism of honey in wound healing process is strongly related to its antioxidant, which prevent the detrimental effects on wound caused by oxidative stress (Khalil *et al.*, 2011), antibacterial, and anti-inflammatory properties (Dobanovački *et al.*, 2012).

2.3 Adulteration of Honey

The nutritional characteristics and its medicinal properties of honey are important to support human health, in which honey is highly sought after and the demand is increased. The stingless bee honey is always being considered as medicinal honey and the price of this honey is high as compared to honey from honeybee. Because of this economical reason, stingless bee honey is often adulterated. There are two types of honey adulteration, which is indirect and direct adulteration. Indirect honey adulteration is commonly used by beekeepers by feeding the bee with different type of sugar and the detection of indirect adulteration is more difficult compare to direct adulterated. Direct adulteration is made by adding the foreign substances to pure honeys (Zábrodská and Vorlová, 2014). One of the important adulterant is HFCS. It is generally used by the beekeepers to feed the honey

bee in order to increase brood production in the spring. HFCS is also used by the beekeepers to feed the bees if there is insufficient of nectar and pollen (Barker and Lehner, 1978). HFCS is also added to the pure honey at certain ratio to increase the honey volume for adulteration. Others adulterant that are commonly used by the unscrupulous sellers for profit gain are brown sugar, corn syrup, white sugar and starch-based sugar syrups (Tosun, 2014).

2.4 Gas Chromatography Mass Spectrometry

One of the method to detect honey adulteration is GC-MS, and the use of GC-MS in detection of honey adulteration was showed previously by detecting the sugar composition of HFCS used for bee-feeding (Ruiz Matute *et al.*, 2010). GC-MS has a high separation efficiency and sensitivity and able to quantitative measures the volatile compounds found in the sample of interest (Cuevas-Glory *et al.*, 2007) such as carbohydrate (mono-saccharides, di-saccharides, and tri-saccharides) in honey (Sanz *et al.,* 2004a). Honey which was produced by bee using HFCS as their feed was detected as frucosyl-fructose in GC-MS (Zábrodská and Vorlová, 2014). The other previous study had used GC-MS to detect adulterations of honey with high fructose inulin syrup (HFIS). Carbohydrate composition of HFIS was detected such as fructose, sucrose, dianhydrides of fructose (DFAs), inulobiose, kestoses and inulotriose. Inulotriose was used as fingerprint to differentiate between adulteration and pure honey since inulotriose was not detected in pure honey (Ruiz Matute *et al.*, 2010). In this study, a method was developed for detection of adulterated and non-adulterated in stingless bee honey by screening the compounds present in the pure stingless bee honey using GC-MS. This study hypothesized that the marker compounds present at certain concentration in stingless bee honey may distinguish between the pure and adulterated honey.

CHAPTER 3

METHODOLOGY

3.1 Honey

The stingless bee honey samples were collected from Syamile Agrofarm, Kuala Kangsar, Perak. Commercial sugar and syrup were purchased from local supermarket at Bertam, Kepala Batas, Penang (see Table 3.1). All collected samples were kept in 50 mL Falcon tube and stored at 4^{o}C until future use.

Table 3.1: Samples used in the experiment were, collected and obtained from the stingless bee farm and local supermarket. Adulterated honey samples were prepared by adding certain proportion of sugar to pure honey.

Type of samples	The Number of Samples
Stingless bee honey	8 samples derived from 3 species; – 2 samples from *Trigona itama* (T. *itama*) – 3 samples from *Trigona thoracica* (T. *thoracica*) 3 samples from *Trigona apicalis* (T. *apicalis*)
Sugar	5 samples – 1 type of brown sugar – 2 types of high fructose corn sugar syrup 2 types of white sugar.
Adulterated honey	4 samples, which were manually mixed at laboratory in different concentration
Commercial honey	2 types of commercial honey.

3.2 Reagents

Samples were derivatived prior to GC-MS analysis. Pyridine and Bis (trimethylsily) trifluoroacetamide (BSTFA) were used as derivatization agents. Pyridine was purchased from Sigma Aldrich and BSTFA was bought from Supelco (US).

3.3 Optimization of Sample Amount

The optimization of sample concentrations used for GC-MS analysis in this study was adopted from method by (Graikou *et al.*, 2011). Different samples amount of pure stingless bee honey (T. *thoracica* species) (1 mg to 5 mg) were weight, derivatives with fixed ratio of pyridine and of BSTFA. The mixture was then heated in water bath for 20 min at 80 ^{o}C prior to GC-MS analysis. Samples were mixed with pyridine and BSTFA concentrations at different amount for the optimization procedure (Table 3.2).

Table 3.2: Different concentrations of samples, pyridine and BSTFA used to determine the optimum sample concentrations for GC-MS analysis.

Weight of samples (mg)	Concentration of derivatives compound
1	10 µL Pyridine , 15 µL BSTFA
2	20 µL Pyridine, 30 µL BSTFA
3	30 µL Pyridine, 45 µL BSTFA
4	40 µL Pyridine, 60 µL BSTFA
5	50 µL Pyridine, 75 µL BSTFA

3.4 Optimization of Pyridine and BSTFA Concentration

Once the effective or optimum sample concentration was known, it was further tested with different pyridine and BSTFA concentrations to determine the best concentrations for GC-MS analysis. Table 3.3 showed the different concentration of pyridine and BSTFA used to determine the best combination for GC-MS analysis.

Table 3.3: The different concentrations of pyridine and BSTFA used to determine the best concentration for sample derivatization. The '4' mg represent the weight of the sample after optimization.

Weight of samples	Concentration of derivatives compound
4 mg	30 µL Pyridine , 60 µL BSTFA
	40 µL Pyridine, 40 µL BSTFA
	40 µL Pyridine, 60 µL BSTFA
	40 µL Pyridine, 80 µL BSTFA
	50 µL Pyridine, 60 µL BSTFA

3.5 The Process of Adulteration

Adulteration of honeys were prepared in the laboratory. Adulterated honeys were made by manually mixed of pure stingless bee honey with corn syrup at different concentration, to mimic the adulterated stingless bee honey as shown in Table 3.4.

Table 3.4: The adulteration of stingless bee with corn sugar syrup.

Product used for adulteration	Concentration of product used for adulteration (%)	Concentration of pure stingless bee honey (%)
Lite corn syrup	75	25
	50	50
Biker corn syrup	75	25
	50	50

3.6 GC-MS Analysis

GC-MS was used for the analysis of both type of samples (honey and sugar). GC-MS was preferred method for analysis of volatile compounds. The used of GC-MS requires samples derivatization to improve detection of targeted analysts in the samples. The derivatized samples were injected into GC-MS in a portion of 2 µL at a split ratio 1:20. GC-MS analysis was performed by Agilent Technologies 7890A GC-Agilent 5975C Series MSD system equipped with split injector. A non-polar capillary column Agilent HP5-MS (30 m × 0.25 mm × 0.25 µm film thickness) was used. Helium

was used as the carrier gas at a flow rate of 1 mL/min. The injector was set at 200 ^{o}C, and oven temperature was ramped from 100 to 300 ^{o}C at rate of 5 ^{o}C/min. The identification of compounds was based on the retention indices (RI), retention time (RT), and comparison of mass spectra with those from NIST GC-MS library. Peak area value of each marker compound was used to calculate the abundances of the compounds.

3.7 Quantification of Marker Compounds in Stingless Bee Honey

The marker compounds were quantified by the abundance of peak area value. A total of 8 samples of pure honey from colonies of stingless bee which includes T. *itama*, T. *thoracica*, and T. *apicalis* were used to determine the peak area value range of marker compounds.

3.8 Statistical Analysis

All data were calculated as mean $\pm$ standard deviation (SD), except for the range value of marker compounds. Student t-test was used to analyse the significant difference between the two parameters.

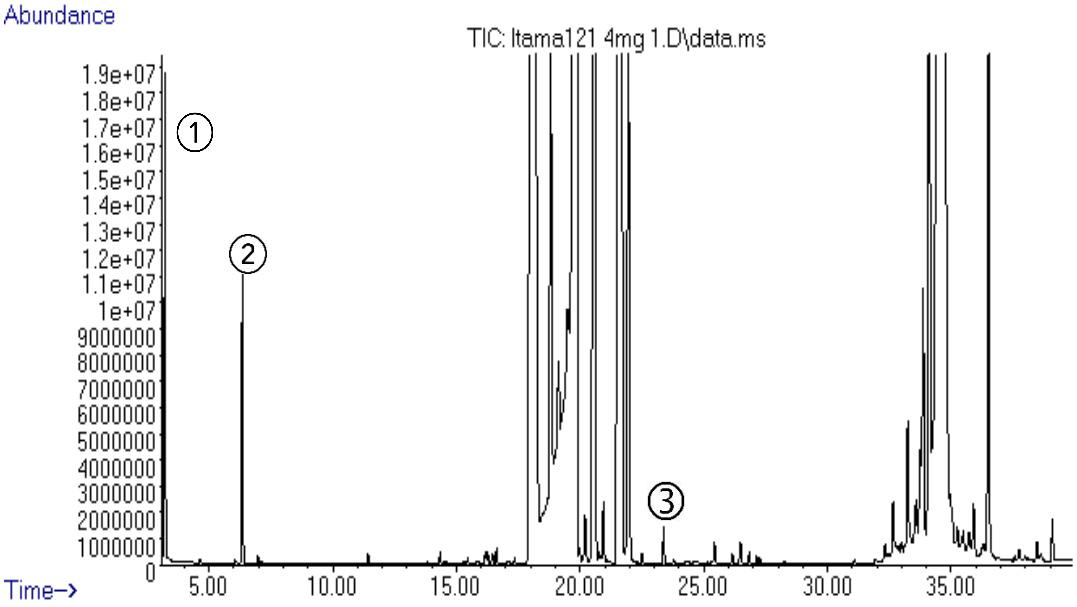
CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Screening of Marker Compounds in Pure Stingless Bee Honey

In general, all pure stingless bee honey samples were analysed using an established GC-MS method to identify the marker compounds as shown in Fig 4.1. From the GC-MS analysis of three species of stingless bee honey samples, three types of volatile compounds were identified at retention time 3.2 min (propionic acid), 6.3 min (glycerol) and 23.3 min (myo-inositol) (Table 4.1). The analysis time of GC-MS was set 40 min. The presences of the three marker compounds were consistent in all samples tested. Therefore, they were selected as the marker compounds for stingless bee honey in this study.

(a)



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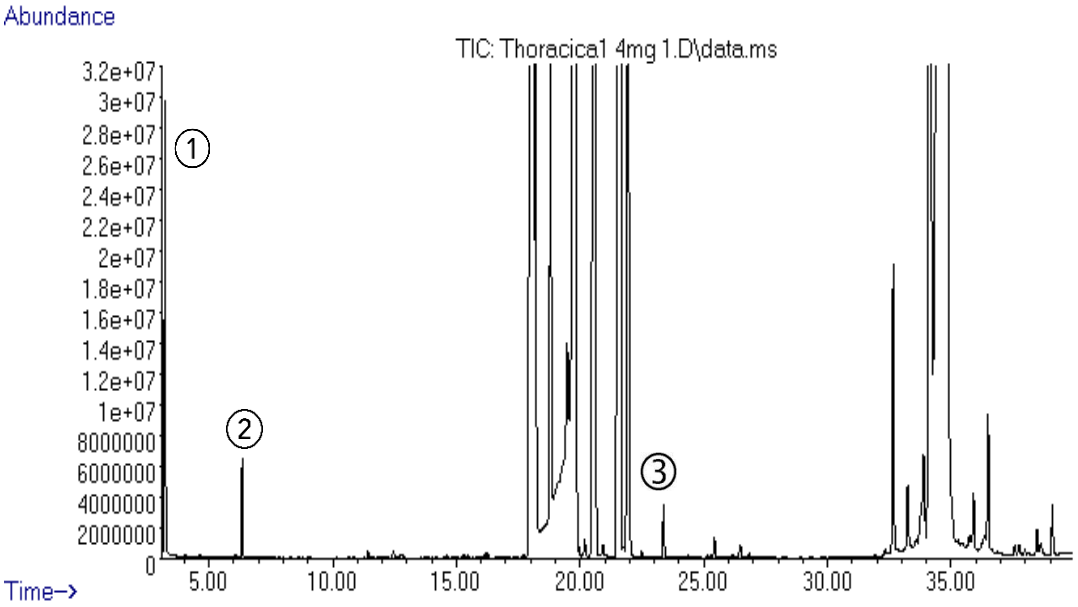

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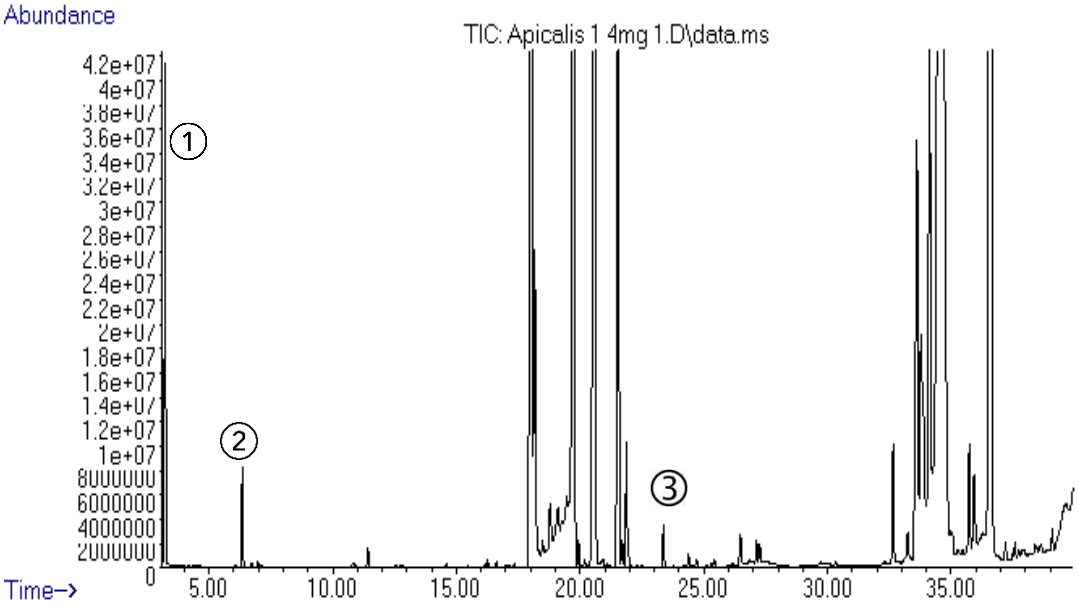

Figure 4.1: GC-MS chromatogram of volatile compounds in (a) T. *itama*, (b) T. *thoracica*, and (c) T. *apicalis*. Number 1, 2 and 3 representing propionic acid, glycerol and myo-inositol.

Table 4.1: Volatile compounds used as marker compounds of stingless bee honeys with retention time.

Marker compounds of stingless bee honey	Retention time (min)
Propionic acid	3.2
Glycerol	6.3
Myo-inositol	23.3

Propionic acid in stingless bee honey was detected at retention time 3.2 min, whereas the retention time for glycerol was at 6.3 min and myo-inositol was at 23.3 min. Based on the data, it had demonstrated that propionic acid, glycerol and myo-inositol can be used as marker compounds to differentiate between stingless bee honeys, adulterations, and other sugar products, due to the presence of these marker compounds in every pure stingless bee honey samples.

Naturally present of organic acids in honey play very important role in honey's antimicrobial activity (Bogdanov, 1997). However, the presence of organic acids was less than 0.5 % of out of other compounds in honey but, they are really important in term of contributions to organoleptic, physical, and chemical properties of honey (Mato *et al.*, 2006). Organic acids can be used to differentiate honey in differences location and botanical origin as it presents in honey are common. A study by Mohandes and Sawsan, (2011) indicated the presence of organic acid such as formic acid, malonic acid, tartaric acid, shikimic acid, maleic acid, citric acid, succininc acid, and propionic acid in Egyptian honey by (Mohandes and Sawsan, 2011). While a study by Nafea *et al*, (2013) revealed the presents of oxalic, formic, malonic, tartaric, shikimic, maleic, citrice, succinic, propionic, butyric, isobutyric and benzoic acid among 8 types of Saudian honey (Nafea *et al.,* 2013). Our study also showed similar findings with Mohandes and Sawsan, (2011) and Nafea *et al.*, (2013), which detected the presence of propionic acid in our stingless bee honey samples. According to Lee *et al*, (2010) propionic acid is organic acid which is a strong flavour compound and its present in food is related to fermentation food. Propionic acid is also commonly found

in shellfish and Korean medicinal plants (Lee *et al.*, 2010). Propionic acid can be converted to glucose, carbohydrates, amino acids, and lipids (EPA, 1991). Propionic acid is commonly used in food, perfume and plastic industries. It uses in bakery products as preservative, propionic acid functions as anti-microbial and recognized to be safe usage as food additives. Propionic acid was showed to inhibit the mold in animal feeds at dose over 200µg/mL (Ibáñez, 2003; Kirbaşlar *et al.*, 2006; Al Azzamet *et al.*, 2010).

Generally, glycerol is used as excipient and formulation aid in pharmaceuticals, chemical synthesis, cosmetics, industrial fluids as antifreezes, lubricants and hydraulic fluids (Oecd, 2002). The glycerol contents in honey in the form of polyol glycerol (ethanol), which is present as a minor constituent in honey (Laub, E.,& Marx, 1987). It is produced by the activity of microorganisms exist in the nectar and honey dew where bees collected from plants and flowers. Study by Jose F. Huidobro *et al*, (1996) had showed that an average of glycerol content in honey bee was 172 mg/kg, whereby the maximum value was 601 mg/kg. The majority of honey had glycerol content less than 100 mg/kg (Jose F. Huidobro *et al.*, 1996). Glycerol is used as a reactant or as an additive. It is non-toxic and safe. Due to its cheaper price and safe, glycerol is commonly used as polyols in confectionery, food, oral care, pharmaceutical, cosmetic, paint, automotive, tobacco, leather and industrial applications (Wang *et al.*, 2001). Polyols are the sweeteners with sugar-free, normally it can reduce calories, provide a pleasant sweetness and have ability to prevent moisture and enhance processing. Glycerol contain about 27 calories per teaspoonful and has the same sweetness as 60% of sucrose (da Silva *et al.,* 2009). Moreover, glycerol is used as hygroscopic to reduce water loss and prolongs shelf life. Glycerol is also used in medical and pharmaceutical preparations in order to increase the smoothness, providing lubrication and also preserving moisture (Pagliaro *et al*., 2008). The biological effects of glycerol are contributed due to its chemical structure (Fluhr *et al.,* 2008). Glycerol acts as moisturizing agents by preventing water loss and

increase skin barrier properties. It prevents the phase transformation of the stratum corneum (SC) lipid from liquid to solid crystalline structure, which resulting in increased skin barrier properties and preventing water losses (Claudia *et al.*, 1990). Glycerol has keratolytic effect by desmosome degradation (Rawlings *et al.*, 1995). It helps to protect the skin against irritation and penetration of compound through SC by plasticizes the SC and decrease the tissue scattering. It also help to stabilizes skin collagen and significant help in healing process (Fluhr *et al.,* 2006; Fluhr *et al.,* 2008).

Myo-inositol is known as vitamin B8 is a part of vitamin B complex family. It is found in the cell membrane phospholipids, plasma lipoproteins and (as phosphate form), with potential chemoprotective properties. Human can get it from animal and plants sources as its free form as inositol consisting of phospholipid (phosphoinositides) or as phytic acid (Holub, 1986). Derivatives of myo-inositol and its phosphate necessary as a secondary messenger molecules for cellular activities (such as for cell growth, apoptosis, cell migration, cell differentiation, stress response, seed germination, gene expression, hormone function, protein trafficking) (Shen, 2003). Myo-inositol is known as valuable nutrient for every animal species whereby in plants, the other forms of myo-inositol are cylitols and methyl inositols (Prezoto and Gobbi, 2003). The study by Sanz *et al.,* (2004) revealed that myo-inositol was found in 28 examined honey samples, with the range of 0.14 to 2.78 mg/g (Sanz *et al*., 2004b). Clemants and Darnell, (1980) had studied on the amount of myo-inositol content in food and showed that milk consists of low myo-inositol content whereas cereal seed and vegetable showed higher amount of myo-inositol. Fresh vegetables were observed to have higher amount compared to frozen, canned and salt-free products. In addition, Cantaloupe and the citrus fruits were found to consist of extremely amount of myo-inositol (Clements and Darnell, 1980). Myo-inositol was documented useful in the treatment of polycystic ovary syndrome (PCOS), acting by inducing ovulation induction and oocyte maturation (Ciotta *et al.*, 2011). Recommended dosage of myo-inositol is 1 to 4 g per day, in which it is considered

safe and effective to increase of the hormonal and metabolic disturbances of PCOS (Unfer *et al.*, 2012).

4.2 Optimization of Marker Compounds.

4.2.1 Optimization of Sample Amount

In order to get the best result in the analysis process, the optimum weight of the sample for GC-MS analysis was determined. The samples of pure stingless bee honey (T. *thoracica* species) was used for optimization of sample amount. Five different concentrations of the sample were mixed with derivative compounds include samples of 1 mg mixed with 10 µL pyridine and 15 µL BSTFA, 2 mg mixed with 20 µL pyridine and 30 µL BSTFA, 3 mg mixed with 30 µL pyridine and 15 µL BSTFA, 4mg mixed with 40 µL pyridine and 60 µL BSTFA, and 5 mg mixed with 50 µL pyridine and 75 µL BSTFA. The mixed of honey sample and derivative agent was then subjected to GC-MS analysis. Percentage relative standard deviation (%RSD) values were calculated from the data obtained. Figure 4.2 demonstrates the %RSD value of sample weights.

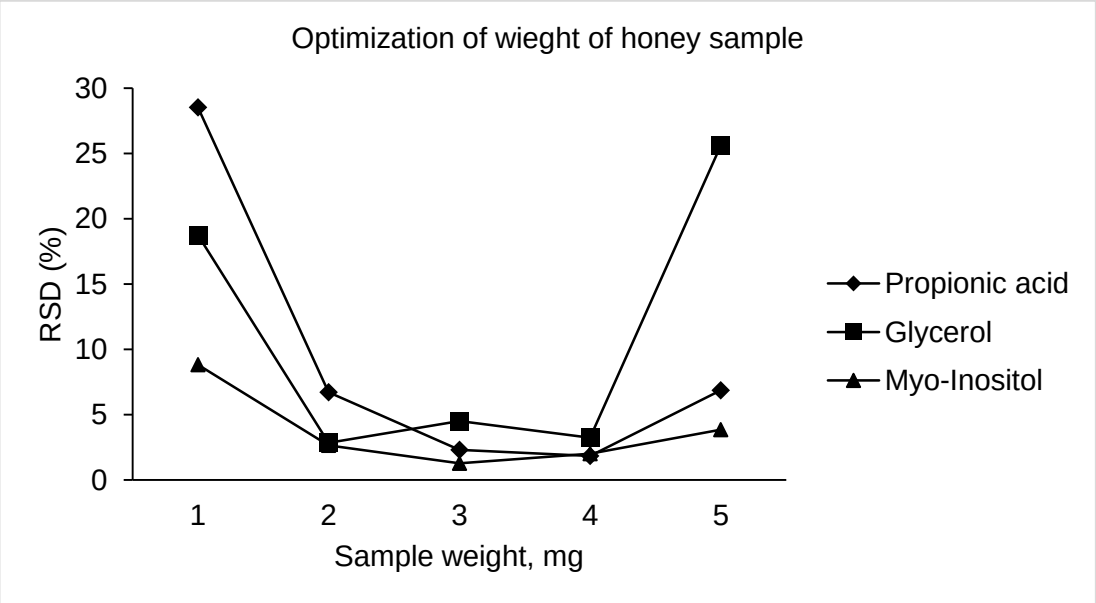

Figure 4.2: The RSD% of weight.

At the smallest weight of the sample, 1 mg showed a high %RSD value (propionic acid 28.54%, glycerol 18.76%, myo-inositol 8.83%). The lower %RSD value indicates lower data variability and uncertainty of variation. Five milligrams of sample also demonstrate the high %RSD value (propionic acid 6.87%, glycerol 25.58%, myo-inositol 3.86%). The lowest %RSD value was seen at 4 mg of sample (propionic acid 1.84%, glycerol 3.23%, myo-inositol 2.0%), followed by the %RSD value of 2 mg and 3 mg of samples. Therefore, 4 mg was chosen as an optimum sample amount for subsequent experiments.

The method of this study adapted from (Graikou *et al.*, 2011). Where in their study, the optimum sample weight used was 5 mg, derivatives with 50 µL pyridine and 75 µL BSTFA. Result showed that at 4 mg of sample, the RSD% value were less when compare to other concentrations. The RSD% value was used to represent the spread of the data where the lowest the RSD% value, the smaller of the data spread toward the mean. Our result also showed that although the derivatives agents concentrations were used in the same ratio, the sample weight still affecting the GC-MS analysis in term of consistency in every injection of samples. The lower sample weight such as 1 mg as well as 2 mg and 3 mg of honey samples demonstrated inconsistency as represent by higher RSD% values. Some injections using 1 to 3 mg of samples weight also didn't show any result of marker compounds peak in the chromatogram. This study had found out that 4 mg of sample weight was optimum to be used in the GC-MS analysis. It also reduces the amount of samples used in the study when compared to the sample's weight in Graikou *et al.,* (2011) study, which used 5 mg of sample.

4.2.2 Optimization of Pyridine and BSTFA Concentration

Apart from the optimization of the sample weight, the concentration of derivatization agents, pyridine and BSTFA were also studied to get the optimum concentration for complete derivatization. Sample (4 mg) was mixed with a different

volumes of pyridine and BSTFA, where pyridine at 30, 40 and 50 µL and BSTFA at 40, 60 and 80 µL were prepared as a part of the optimization parameter prior to GC-MS analysis. Results of %RSD for sample mixture with pyridine and BSTFA were shown in Table 4.2. By comparing the GC-MS chromatogram of different concentration, 40 µL pyridine and 60 µL BSTFA was the optimum condition for derivatization with the least %RSD when compared to other pyridine and BSTFA concentrations.

Pyridine and BSTFA were used as agents for derivatization for GC-MS analysis in this study. Pyridine was used as solvent and had the capacity to break off many natural substances in the environment (Health, 2010). While, BSTFA was used as derivatization compounds due to its volatility and polar in nature. It is able to performs as own solvent and the use of it can result of better derivatization compared with other types of derivation agent N,O-Bis(trimethylsilyle)acetaminde (BSA) in GC (Van Look *et al.,* 1995).

Table 4.2: The percentage relative standard deviation (%RSD) value of pyridine and BSTFA.

Percentage relative standard deviation percentage (%RSD)			
Concentration	Propionic acid	Glycerol	Myo-Inositol
4 mg 30 µl pyridine 60 µl BSTFA	1.88	2.89	5.70
4 mg 40 µl pyridine 40 µl BSTFA	N/A	85.76	N/A
4 mg 40 µl pyridine 60 µl BSTFA	2.43	0.12	1.14
4 mg 40 µl pyridine 80 µl BSTFA	5.68	4.94	1.56
4 mg 50 µl pyridine 60 µl BSTFA	3.67	2.31	N/A

Note: N/A (Not applicable)