ANALYSIS OF COMPOSITIONAL LEVEL IN HONEY

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A project report submitted in partial fulfilment of the requirements for the award of Bachelor of Engineering (Honours) Chemical Engineering

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DECLARATION

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

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APPROVAL FOR SUBMISSION

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ABSTRACT

Honey is a sweet and natural sugar solution extracted by honey bee from floral nectar. It consists mainly of carbohydrates such as fructose, glucose and sucrose. Minor components in honey are vitamins, proteins, water and minerals. The physiochemical properties strongly correlated to botanical and geographical origin, processing condition, environment condition surrounds hive as well as storage condition and period. This study is undertaken to ascertain the physiochemical parameters such as sugar content, antioxidants and elements concentration of seven honey samples which six were from Malaysia and one from Taiwan. For sugar content analysis, total reducing sugars and individual sugar level (fructose, glucose and sucrose) were determined. Based on reducing sugar content, only three honeys (multi-floral honey E, dark and light raw unprocessed honey F and G) satisfied the minimum amount. Apart from that, in the analysis of individual sugar content, all honeys satisfied the allowable limit as adherence to Codex Alimentarius Commission. Meanwhile, fructose-to-glucose ratio was used to evaluate the quality of honey through the rate of crystallisation. Local multi-floral honey E has the fastest rate of crystallisation indicating its relatively low quality. Antioxidant properties of honey are crucial for the medical importance of honey. Concentration of antioxidants such as phenolic compound, lycopene and β-carotene in honey were determined. Least amount of antioxidants was found in multi-floral honey A while the highest concentration of antioxidants was found in dark raw unprocessed honey F. In the elemental analysis, all the honey samples were found to satisfy the tolerable limit of Zn, Ni, Cu and Cr. The pH values of honey are low to inhibit the growth of microorganism and honey with higher element content has higher electrical conductivity. The last part of this study is identification and characterization of pollen to track the floral source of each honey. Pollen types of two honeys (A and B) were unable to be identified. Overall, dark raw unprocessed honey topped the ranking while multi-floral honeys were relatively inferior in terms of quality. In a nutshell, no serious contamination was found in the tested honey but only with slight difference in terms of quality.

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LIST OF SYMBOLS / ABBREVIATIONS

A	Absorbance
A_{453}	Absorbance at 453nm
A_{505}	Absorbance at 505nm
A_{663}	Absorbance at 663nm
C	Gram invert sugar per 100g honey
T	Transmittance
W_1	Weight of honey according to first procedure
W_2	Weight of honey according to second procedure
Y_1	Volume of diluted honey solution consumed in first and second titration in mL.
Y_2	Volume of diluted honey solution consumed in first and second titration in mL.
F/G	Fructose to glucose
ppm	Parts per million

ANSA	3-amino-5-nitrosalicylic acid
DNSA	3,5-Dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELSD	Evaporative light scattering detector
EVM	Expert group on vitamins and minerals
FID	Flame ionization detector
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GC	Gas chromatography
GLC	Gas-liquid chromatography
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
MS	Mass spectrometry
PMTDI	Provisional maximum tolerable daily intake
RI	Refractive index

RID	Refractive index detector
TPC	Total phenolic content
UV/Vis	Ultraviolet–visible spectroscopy

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CHAPTER 1

INTRODUCTION

1.1 Physiochemical Parameters of Honey

Honey is always being kept in airtight containers because it is hygroscopic. A hygroscopic material likes water and able to absorb water at room temperature from the surrounding environment. Once water is absorbed, the temperature, colour and viscosity of honey will be affected (Helmenstine, 2019). The density of honey also relies on the water amount. The lowest amount of water content is 14% with density of 1.4404 kg/L and the ceiling of water content is 21% with density of 1.3550 kg/L. Honey usually acts like Newtonian fluids. At constant temperature, the viscosity of a Newtonian fluid stays unchanged regardless the amount of shear stress is applied to the fluid (Peters, 2015). However, viscosity of honey varies at different temperature. Figure 1.1 shows the viscosity of honey at different temperature.

	Temperature (°C)	Viscosity (Poise)
Honey 1[a]	13.7	600.0
	20.6	189.6
	29.0	68.4
	39.4	21.4
	48.1	10.7
	71.1	2.6
Honey 2[b]	11.7	729.6
	20.2	184.8
	30.7	55.2
	40.9	19.2
	50.7	9.5

[a] Melilot honey (*Melilotus officinalis*; 16.1% moisture).
[b] Sage honey (*Salvia officinalis*; moisture content 18.6%).

Figure 1.1: Honey's Viscosity at Various Temperatures (Belitz, Grosch and Schieberle, 2009).

Honey composition depends widely on the floral origin, species of bee, seasons, processing and environmental factors (Boukraa, 2013). Honey

primarily composed of carbohydrates, water, proteins, vitamins and many other minor components. It is a complex but a nutritious food as it contains at least 181 substances. Table 1.1 and Figure 1.2 show the compositional data for most of the honey.

Table 1.1: Chemical Composition of Honey (Belitz, Grosch and Schieberle 2009).

Constituent	Average Value	Variation Range
Nitrogen	0.06	0.05-0.08
Minerals (ash)	0.22	0.20-0.24
Free acids [a]	22	6.8-47.2
Lactones [a]	7.1	0-18.8
Total acids [a]	29.1	8.7-59.5
Moisture	17.2	13.4-22.9
Fructose	38.2	27.3-44.3
Glucose	31.3	22.0-40.8
Saccharose	2.4	1.7-3.0
Maltose	7.3	2.7-16.0
Higher sugars	1.5	0.1-8.5
Others	3.1	0-13.2
pH value	3.9	3.4-6.1
Diastase value	20.8	2.1-61.2

[a]mequivalent/kg.

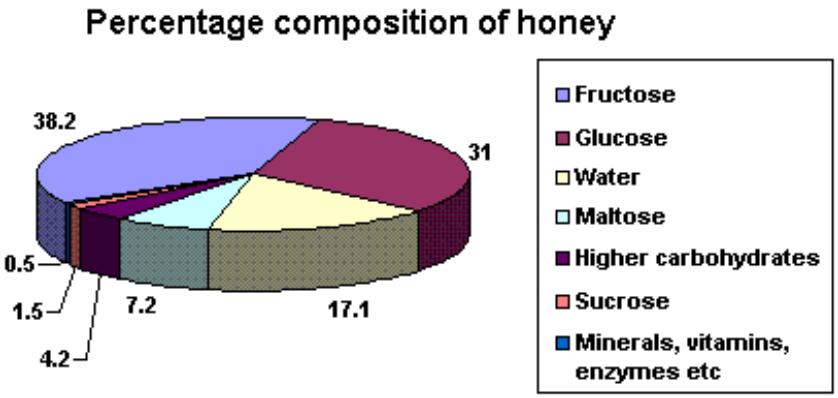


Figure 1.2: Chemical Composition of Honey (Loveridge, 2001).

Based on the Figure 1.2, the main constituents of honey are simple sugars (monosaccharide) like glucose, fructose and complex sugars

(disaccharide) such as sucrose. Among them, glucose and fructose are the major sugars in honey. Figure 1.3 showed the chemical structure of monosaccharide and disaccharide in honey.

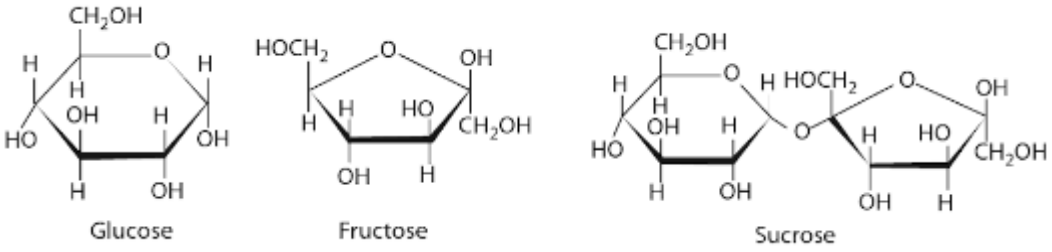


Figure 1.3: Chemical Structures of Simple and Complex Sugars in Honey (Boukraa, 2013).

Although honey does not contain other monosaccharide, there are almost 20 di- and oligosaccharides were found in honey and they were being shown in Table 1.2. The content of carbohydrates in honey depends mainly on the source of plants or floral sources.

Table 1.2: Di- and Oligosaccharides Found in Honey (Belitz, Grosch and Schieberle, 2009).

Common name	Systematic name
Glucose	
Fructose	
Saccharose	α-D-glucopyranosyl-β-D-fructo-furanoside
Maltose	O-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose
Isomaltose	O-α-D-glucopyranosyl-(1 → 6)-D-glucopyranose
Maltulose	O-α-D-glucopyranosyl-(1 → 4)-D-fructose
Nigerose	O-α-D-glucopyranosyl-(1 → 3)- D-glucopyranose
Turanose	O-α-D-glucopyranosyl-(1 → 3)-D-fructose
Kojibiose	O-α-D-glucopyranosyl-(1 → 2)-D-glucopyranose
Laminaribiose	O-β-D-glucopyranosyl-(1 → 3)-D-glucopyranose
α,β-Trehalose	α-D-glucopyranosyl-β-D-glucopyranoside
Gentiobiose	O-β-D-glucopyranosyl-(1 → 6)-D-glucopyranose
Melezitose	O-β-D-glucopyranosyl-(1 → 3)-O-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside
3-α-Isomaltosylglucose	O-α-D-glucopyranosyl-(1 → 6)-O-α-D-glucopyranosyl-(1 → 3)-D-glucopyranose
Maltotriose	O-α-D-glucopyranosyl-(1 → 4)-O-α-D-glucopyranosyl-(1 → 4)-D-gluco-pyranose
1-Kestose	O-α-D-glucopyranosyl-(1 → 2)-β-D-α-fructofuranosyl-(1 → 2)-β-D-fructofuranoside
Panose	O-α-D-glucopyranosyl-(1 → 6)-O-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose
Isomaltotriose	O-α-D-glucopyranosyl-(1 → 6)-O-α-D-glucopyranosyl-(1 → 6)-D-glucopyranose
Erlose	O-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranosyl-β-D-fructofuranoside
Theanderose	O-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-β-D-fructofuranoside
Centose	O-α-D-glucopyranosyl-(1 → 4)-O-α-D-glucopyranosyl-(1 → 2)-D-glucopyranose
Isopanose	O-α-D-glucopyranosyl-(1 → 4)-O-α-D-glucopyranosyl-(1 → 6)-D-gluco-pyranose
Isomaltotetraose	O-α-D-glucopyranosyl-(1 → 6)-[O-α-D-glucopyranosyl-(1 → 6)]$_2$-D-gluco-pyranose
Isomaltopentaose	O-α-D-glucopyranosyl-(1 → 6)-[O-α-D-glucopyranosyl-(1 → 6)]$_3$-D-gluco-pyranose

The moisture content of honey is beneath 20%. If the water content goes beyond the limit, the honey is vulnerable to fermentation caused by yeasts and spoiled the honey. For amino acids and proteins, they are less than 0.7% in honey. The primary amino acid in honey is proline ($> 200 mg/kg$) which measures the ripeness of honey. Meanwhile, the main protein in honey is enzyme. The predominant enzymes in honey are catalase, saccharase, diastase, glucose oxidase and acid phosphatise (Belitz, Grosch and Schieberle, 2009). Saccharase and diastase are important in judging the quality and freshness of honey (Bogdanov, 2011).

Since honey is collected by bees from flora, it is rich in phenolic compounds because they are plant derived secondary metabolites. To point out the antioxidant activities in honey, the dependable factor is the total phenolic content (Kek et al., 2014). Different uni-floral honeys have different total phenolic content. Darker honeys are found to have more phenolic compounds but with a lesser amount of flavonoids than honey with lighter colour.

There are a lot of mineral elements present in honey ranging from 0.02 to 1.03g/100g and it was an indicator for the quality of honey in the early days. Electrical conductivity has replaced the mineral content as a quality criterion of honey. The amount of trace elements in honey highly depends on the botanical sources of honey. The main mineral element in honey is potassium. Honey can be contaminated with heavy metals and increases the concentration of mercury, arsenic, cadmium and lead. The presence of different trace elements in honey allows researchers to differentiate between various uni-floral honeys (Bogdanov, 2011). The elements present in honey were shown in Figure 1.4.

Element	mg/100 g	Element	mg/100 g
Aluminium (Al)	0.01 - 2.4	Lead (Pb)*	0.001 - 0.03
Arsen (As)	0.014 - 0.026	Lithium (Li)	0.225 - 1.56
Barium (Ba)	0.01 - 0.08	Molybdenum (Mo)	0 - 0.004
Boron (B)	0.05 - 0.3	Nickel (Ni)	0 - 0.051
Bromine (Br)	0.4 - 1.3	Rubidium (Rb)	0.040 - 3.5
Cadmium (Cd)*	0 - 0.001	Silicium (Si)	0.05 - 24
Chlorine (Cl)	0.4 - 56	Strontium (Sr)	0.04 - 0.35
Cobalt (Co)	0.1 - 0.35	Sulfur (S)	0.7 - 26
Floride (F)	0.4 - 1.34	Vanadium (V)	0 - 0.013
Iodine (I)	10 - 100	Zirkonium (Zr)	0.05 - 0.08

*- elements regarded as toxic, can be partially of anthropological origin

Figure 1.4: Trace Elements in Honey (Bogdanov, 2011).

1.2 Antioxidant Properties of Honey

Oxygen is vital to living organisms' life and scientists found that it will bring illness and aging problems to human when it is metabolized. When this vital element is metabolized, it is known as "free radicals" and it will travel through the cell and cause damaging in cell. Here, antioxidant is introduced by neutralizing the free radicals. Therefore, the level of antioxidants in human body needs to increase through diet in order to combat the activities of free radicals (Khalil, Sulaiman and Boukraa, 2010).

Honey appears to work as an antioxidant which is rich in phenolic acids, carotenoids and flavonoids (Mungai, Nawiri and Nyambaka, 2017). Honey acts as a natural product that binds the free radicals of oxygen together to put off them from damaging the structure of DNA within the organism. Basically, honey with darker colour has higher level of antioxidants (Jaganathan and Mandal, 2009). The activity of antioxidants is expressed as radical scavenging acticity (%RSA).

Carotenoids are pigments produced by plants which serve as foragers of singlet oxygen. Such process is a chemical reaction between reactive oxygen and carotenoid where the singlet oxygen is inactivated in a permanent way. Carotenoids such as β-carotene, lycopene, lutein, β-cryptoxanthin and α-carotene are associated with many health effects. For example, β-carotene can help in reduction threat of cardiovascular disease and lycopene has protective effect against some cancers (Sartori and Silva, 2014). Flavonoids are natural chemical compounds and have two phenolic groups (OH) as minimum. They are classified based on their degree of oxidation and flavonex, flavanols and flavonols are the most plentiful flavonoids in honey (Cianciosi et al., 2018).

1.3 History of Honey

Honeys have their own history extending back over 10 000 years ago but the accurate of origin remains unknown. For humans in the earliest centuries who first encountered honey and tasted it, it seemed like a gods' gift as fruit was the sweetest thing they had ever tasted. Almost every society had a myth figuring the enduring sweetness and taste of honey. The earliest discover by archaeologists was in Egypt where the first honey comb was found buried in

tombs at the pharaohs. Even though the honey comb has been buried, it was still eatable.

The reason with sayings of usage of honey by humans at least 10 000 years ago is because there is evidence found in the early 1900's. There was a cave painting found in a city of Spain, Valencia. It is in the Cave of the Spider which is called Cueve de la Arana in Spanish. This is one of the first proofs of honey collecting that illustrates a honey hunter robbing a wild bee colony (Heathmont Honey, 2019). Back in thousands of years ago, human has to find a wild hive to gather honey until a clergyman in the mid-nineteenth century named Lorenzo Langstroth created the "collateral hive" that allowed the domestic bee-keeping to be possible (Danovich, 2013).

Humans use honey for many thousands of years ago for wide variety of purposes. Chinese was the first to start bee-keeping and this causes them to be ahead the remaining of the world in collecting, consuming and maintaining the quality of honey. Ancient Egyptian and people in Middle-Eastern used honey for "Mummification" which its purpose is to embalm the dead. In Roman, people used honey as valuable items to pay taxes, as sweetener and became cure for certain diseases. According to a Roman surgeon, Pedanius Dioscorides, he gave honey to his army to cure wounds, stomach diseases and coughing. Besides, "eating honey prolongs life", said Aristotle. Hippocrates also mentioned that "I eat honey and use it in the treatment of many diseases because honey offers good food and good health" (Crane, 1983). The Kings and Queens of England in the 10^{th} century had also fermented honey wine, Mead (Heathmont Honey, 2019).

1.4 Problem Statement

Honey adulteration is always a temptation due to the limited supply and relatively high price of honey. Corn syrups, molasses, acid-inverted syrups and hydrol are some examples of possible adulterants. It is vital to differentiate impure products from synthetic honeys that sold to provide 'honey flavour' (Mistry, 1987). Adulterated honey will has less medical usage and lose their original nutritional benefits. By comparing to pure honey, impure honey varies with respect to its physicochemical properties like pH value and electrical conductivity. Hence, it is necessary to determine pH and

conductivity of honey. Pure honey shall have low pH to inhibit the growth of microorganism. Pure honey shall also have low electrical conductivity to indicate the low concentration of heavy metal in it.

Adulterated honey may have extremely high level of sucrose. Pure honey contains sucrose with concentration of only 5% or below (Codex Alimentarius Commission, 1981). Ratio of fructose-to-glucose reflects the rate of crystallisation. Low fructose-to-glucose ratio increases the rate of crystallisation and also reflects the addition of corn syrup (Singhal, Kulkarni and Rege, 1997). Therefore, concentration of the individual sugar contents like sucrose, glucose and fructose is important to justify the quality of a honey.

Concentration of heavy metal in honey should be as low as possible to indicate the absence of contamination of honey. Honey collected from location nearer to industrial areas tends to have higher concentration of heavy metals. However, pure honey should contain essential elements required for human body with tolerable limit (Mejias, E. and Garrido, T., 2017). Elemental analysis is required to perform on all honey samples. Apart from that, pure honey contains high amount of antioxidants to help in slowing down aging, wound healing, avoiding cardiovascular diseases and other medical purposes (Hagr et al., 2017). Honey with low amount of antioxidants is said to have poor quality. Determination of antioxidants is one of the important parameters to determine the quality of honey.

To identify the botanical origin of honey, pollen is often used as the indicator. Pollen serves as the fingerprints to determine the specific plant species. Analysis of pollen can be used to distinguish adulterated honey from pure honey as well because pollen can be found in pure honey only (Rosdi et al., 2016).

In recent years, the study of physicochemical properties of honey is getting focused because they are important for the determination of honey quality. However, only a few reports have been conducted in Malaysia regarding the quality of honey which includes only certain specific parameters (Kek et al., 2014; Yaacob et al., 2018; Rosdi et al., 2016). In short, the principal part of this research is to determine physical and chemical parameters of honey which can be used in evaluating and justifying the quality of each honey.

1.5 Aim and Objectives

The main focus of this research is to study the physical properties and chemical composition of different honey samples in Malaysia and Taiwan. In essence, well-defined objectives are established as below aim to achieve the desired goal.

1. To evaluate pH value and electrical conductivity of honey samples.

2. To determine sugar content in honey through UV/Vis Spectroscopy and Fehling test.

3. To perform heavy metal analysis in honey through inductively coupled plasma ICP-OES.

4. To interpret antioxidant properties of honey through UV-VIS Spectroscopy.

5. To identify and characterize the type of pollen present in honey.

6. To justify the quality of each honey sample by comparing the values obtained to the standard.

CHAPTER 2

LITERATURE REVIEW

2.1 Adulteration of Honey

Honey is always an aim for adulteration due to its limited supply and soaring price. There are many adulterants available nowadays and the common one includes corn syrups, glucose syrup, molasses, acid-inverted syrups and high-fructose insulin syrup in order to enhance the sweetness of honey.

In the past, HMF content is one of the main parameters used to detect the presence of adulterants, mainly acid-inverted syrup. However, sugar and proline content as well as electrical conductivity were later being suggested to replace HMF method because the level of HMF in honey varies when the honey is subjected to heat or originated from tropical environment. Meanwhile, proline and electrical conductivity is reported to be a wise choice for the determination of adulterant, sucrose syrup (Soares et al., 2017). On the other hand, corn syrup adulterated to honey can be distinguished when the laevulose: dextrose ratio is lowered. However, this task becomes more challenging when the adulterant is high fructose corn syrup because it resembles honey more strictly (Singhal, Kulkarni and Rege, 1997).

In many countries, the official method to analyse honey adulteration is stable carbon isotope ratio analysis (SCIRA). However, this method shows high accuracy in detecting adulteration when honeybees are overfeeding with corn or sugar cane but low accuracy for sugar syrups. Alternative chromatographic techniques are suggested to analyse sugars in honey such as GC and anion exchange chromatography coupled to pulse amperometric detector (HPAEC-PAD). The drawback of chromatography is that oligosaccharides cannot be detected. Several sugar syrups contain huge amount of oligosaccharides as they are acquired from enzymatic hydrolysis of starch (Soares et al., 2017).

Currently, spectroscopic approaches are the most comprehensive as compared to other analytical techniques. They include the use of Raman spectroscopy, NMR and infrared (IR). They are fast, low cost and not complex. For example, FTIR-ATR success to detect wide variety of

adulterants in honey including those well-known and common one: invert sugar, corn syrup, and sucrose syrup (Soares et al., 2017).

2.2 Factors Affecting Honey Composition

Production of honey starts from foraging of honey bees. Foraging activities are divided into nectar, water, pollen and resin foraging. Honey bees will collect and suck up from floral sources and store in their honey sac. This is where the production of honey starts as honey bees will enrich the nectar and floral pollen with their own substances. They deposit collected nectar in honeycombs for storage and ripening once they return to the hive (Belitz, Grosch and Schieberle, 2009). Honey composition is strictly affected by the process from the production stage in honey sac of bees till storage by consumers.

Chemical changes of honey begin at flower source and hive. Since nectar composition varies depending on the species of plant and condition of environment, this will affect the honey composition directly as the component honey bees suck up from flower is nectar. Nectar is an aqueous solution or secretion from nectarines in leaves, stems or flowers of plant depending on the species. Nectar contains fructose, glucose, sucrose and traces amount of proteins, acids, salts and oils (Petruzzello, 2019). The amount of sugar in nectar varies from 3% to 80% and thus, the sugar content in honey will vary as well. The foraging behaviour of different bees will also affect the composition of honey. They are the key transitional pace between nectar and honey. There are two groups of hunter bees which are scout bees and reticent bees. Scout bees responsible to look for finest food sources and pass the information to the latter bees. Factors affecting foraging activity of honey bees are classified into in-colony and out-colony factors. Meanwhile, in-colony factors are referring to colonies headed by queen or without queen. Higher foraging activity was found in queen headed. Out-colony factors are referring to the availability of appropriate plant species and environmental factors like temperature. Some forager bees have preference over some plant species (Abou-Shaara, 2014). After the forager bees disgorge nectar, they will give it to house bees. Once the house bees drink nectar, they will regurgitate and re-consume nectar multiple times for 15 to 20 minutes. In this process, secretions containing

enzyme mix with nectar to convert sucrose into simple sugar which are glucose and fructose. Ripening of honey includes conversion of complex sugar to monosaccharide and the evaporation of water to less than 20%. The rate of evaporation of water from honeycomb is enhanced by the fanning of bees using their wings (Alwazeer, Yildiz and Yalinkiliç, 2018).

Honey can have different composition and properties depend on the location where hives are located including the surrounding environment. One can observe differences between honeys from different country. This is because the pollen or nectar's compositions are different which depends on soil characteristic, moisture, sunlight and other factors. Even for the same flower species, the honey composition may vary from country to country (Kaškonienė and Venskutonis, 2010). According to El Sohaimy, Masry and Shehata (2015), honeys from different origin were collected from Egypt, Yemeni, Saudi and Kashmir to determine the characteristic between them. Based on the research, physical properties of these honeys are different. They show different colour ranging from light amber to amber where Yemeni and Egyptian honey are lighter in colour and darker for Saudi and Kashmiri honey. Honey extracted from different areas with varies environmental condition like air quality, temperature and humidity have found to contain different chemical composition. For example, honey from areas with poor air quality such as heavy industrial areas possesses high heavy metal content. Mercury levels in bees and honey are effective in reflecting the mercury loads in the environment (Singhal, Kulkarni and Rege, 1997). Honey is often used to indicate the environmental condition of the area where they are extracted. Honey can tell environmental data for around $7km^2$ from its sources (Czipa, Borbély and Kovács, 2008).

Composition of honey is commonly affected during the industrial processing of honey. In order to prevent crystallisation of honey, to facilitate packaging by reducing viscosity and prolong the lifetime of honey, producers tend to treat the honey with high/ uncontrolled heat (Alwazeer, Yildiz and Yalinkiliç, 2018). Two common processes are normally done by producers during the processing of honey: pasteurization and flash-heating. It is a general knowledge that producers apply pasteurization to kill microorganisms where rapid heating and cooling are involved. In the making of honey, pasteurization

is used to kill osmophilic yeasts. Meanwhile, flash-heating is applied to delay the crystallisation of honey. Although these processes have their pros and functions but they do affect the quality of honey by reduce the enzymatic activity of honey. Enzymes are sensitive to heat and they are important in converting nectar into honey. HMF concentration in honey is an indicator to evaluate freshness of honey and occurrence of overheating during processing. As a result of long-term heating, the content of HMF in honey will increase. However, the HMF amount of honey from tropical temperatures will be higher. Therefore, exceptions are present in standard established to determine the quality of honey (Soares et al., 2017).

Generally, honey can be stored for long period of time under suitable conditions. Upon storage, honey will become darker, aroma intensity decreases and HMF content increases at different pH, storage temperature and time (Belitz, Grosch and Schieberle, 2009). Honey should be stored in environment with temperature ranging from 10 to 16 degree C. The effect of storage temperature on honey was shown in Table 2.1. Honey storage containers shall resist the acidity of honey. These materials include glass, plastic and stainless steel.

Table 2.1: Influence of Storage Temperature on Honey Parameters (Bogdanov, 2008).

Storage temperature ℃	Time for the formation of 40 mg HMF/kg	Half life of Amylase activity	Half life of Invertase activity
10	10-20 years	35 years	26 years
20	2-4 years	4 years	2 years
30	0.5-1 years	200 days	83 days
40	1-2 months	31 days	9,6 days
50	5-10 days	5,4 days	1,3 days
60	1-2 days	1 day	4,7 hours
70	6-20 hours	5,3 hours	47 minutes

Besides, crystallisation of honey tends to occur over a time period because the content of water in glucose reduced. Upon crystallization, physiochemical and biological properties of honey will change. The appearance of crystalline phase will affect the impressions to consumers as well (Nurul Zaizuliana et al.,

2017). As time goes by, microorganisms will grow in honey and start to consume some sugars. Uptake of sugars allows microorganisms to produce metabolites like organic acids and modify the composition of honey (Alwazeer, Yildiz and Yalinkiliç, 2018).

In short, factors which influence the composition of honey ranging from the source of honey till the end of use of honey. Apart from the factors mentioned in earlier part, there are other minor factors that will change the quality and composition of honey such as higher yield prior to maturity, too much usage of veterinary drugs and overfeeding with sucrose.

2.3 Crystallisation of Honey

Honey consists of crystal phase and syrup phase naturally. The presence of crystal phase depends on the ratio of sugars and moisture content (Singhal, Kulkarni and Rege, 1997). Over a period of time, honey tends to crystallize due to the loss of moisture content.

There are several factors which will lead to the crystallisation of honey: sugar content, temperature and water content. According to Nurul Zaizuliana and Anis Mastura, 2017, the higher content of glucose, the higher the rate of crystallisation. Nectar honey and honeydew honey with glucose content higher than 28% and 10% respectively crystallise fast (Bogdanov, 2008). The optimal storage temperature to increase the crystallisation of honey ranges between 10 and 18℃. At low temperatures of deep-freezer, honey remains as liquid for longer period of time. Meanwhile, precipitation of honey at any temperature relies on another 2 factors. They are the saturation solubility and diffusivity of glucose where honey viscosity is considered. For instance, fructose is more soluble in water than glucose. Therefore, it slows down the rate of honey precipitation (Nurul Zaizuliana et al., 2017). When the water content of honey lies between 15 and 18%, honey crystallises optimally. The ratio of glucose or dextrose content to water is correlated to honey crystallisation. Dextrose is a simple sugar made up of corn and is the same with glucose chemically (Nall and Gotter, 2016). The correlation of dextrose to water ratio and crystallisation is shown in Figure 2.2.

Table 2.2: Honey Crystallisation Grading (Singhal, Kulkarni and Rege, 1997).

Crystallization grading (C)	Dextrose/ water	Description
0	1.58	No crystals
1	1.76	A few scattered crystals
2	1.79	Crystal layers 1/16–1/8 inch
3	1.86	A few clumps of crystals
4	1.83	Crystal layer 1/8–1/4 inch
5	1.99	Crystal layer 1/4 inch
6	1.98	Crystal layer 1/2 inch
7	2.06	Crystal layer 3/4 inch
8	2.16	Complete soft granulation
9	2.24	Complete hard granulation

Different honey have different crystallisation behaviour and is primarily depends on sugar level and storage conditions. Granulated honey can be liquefied by several heat treatments which will affect the composition of honey as mentioned before.

2.4 Acidity and pH of Honey

The sourness of honey comes from the content of acids in honey such as amino acid, citric acid, gluconic acid, formic acid, lactic acid and other organic acids, which is caused by different sources of nectar. The pH value of honey is naturally low in order to restrain the growth and presence of bacteria and spoil-ready organisms (Prica et al., 2014). Moreover, the acidity of honey allows honey to be compatible with wide variety of food products. In certain circumstances, the natural acidity of honey may rise especially when honey depreciates due to fermentation. As honey grows, the acidity will increase as well as when it is removed from propolis combs (Yadata, 2014).

In order to determine the acidity of honey, one can perform titration using 0.1M of NaOH solution or direct measurement by pH meter. By using titration method, the acidity of honey is expressed as in equation 2.1.

$$acidity\left(\frac{meq}{kg}\right) = \frac{mL\ of\ base * 10}{kg\ of\ honey} \qquad (2.1)$$

The acidity range of honey is from 8.68 to 59.49 meq/ kg with an average value of 29.12 meq/ kg. Among all the organic acids available in honey, gluconic acid is the major organic acid. It is produced by the achievement of glucose-oxidase enzyme. The organic acids in honey play their role as flavour and aroma-enhancing agent (Prica et al., 2014). Other than organic acids, amino acids are there in honey as well. Generally, honey contains about 18 free amino acids but with small amount (0.05 – 0.1%). Amino acids have little nutritional significance due to its small amount (National Honey Board, 2002).

The pH of honey ranges between 3.4 and 6.1 according to National Honey Board with an average pH of 3.9 (National Honey Board, 2002). There are some properties of honey are affected by pH of honey. They are shelf-life and stability, texture of honey and formation of HMF. As compared to the titration method, pH determination method has lower analytical complexity and this causes it to become a preferable and interesting quantification parameter (Salazar et al., 2017).

2.5 Electrical Conductivity of Honey

The determination of honey conductivity is widely employed in routine quality control of honey. It is also a good parameter to differentiate the botanical source of honey and honey purity. In the past, measurement of electrical conductivity of honey is only focused on honeydew honey. However in 2015, national legislation is harmonized with that of EU and causing the conductivity measurement expanded to other types of honey. Since then, conductivity is a good criterion to differentiate both honeydew and blossom honey. According to Serbian Official Regulation of honey, the conductivity of 0.8mS/cm is the maximum value for blossom honey but minimum value for honeydew honey (Vranić et al., 2017).

Honeydew honey is a unique kind of honey. It is different from blossom honey as it produced by bees using honeydew which is not collected from plants. Unlike nectar, insects like aphids absorb sap from plants for survival and the secretion they produced is called honeydew. Bees will find and collect the honeydew that is sticks to parts of plants (Gustorotondo, 2019).

The crystallisation rate of honeydew honey is lower than blossom honey and the colour is also darker.

The components like organic acids and minerals have the ability to dissociate into ions in aqueous solution. The free- moving ions contribute to the conductivity of honey. Usually, darker honey has greater conductivity than honey with brighter colour (Živkov-Baloš et al., 2018). Besides, there is a linear connection between conductivity and ash content. With higher ash content in honey, the electrical conductivity is higher as well (El Sohaimy, Masry and Shehata, 2015). The ash content is an environmental and geographical origin indicator. However, there are several other factors that will affect the conductivity of honey other than ash content such as storage time, sources of floral and amount of proteins. Therefore, high conductivity may not correspond to high ash content in some circumstances (Živkov-Baloš et al., 2018). The EC of honey is usually defined as 20 w/v % in solution at around 20℃. The 20% refers to anhydrous or the dry honey matter (Yadata, 2014).

2.6 Carbohydrates Analysis of Honey

In nature, carbohydrates are the most abundant category of organic compound which are made up of carbon, hydrogen and oxygen. They can be categorised into several clusters based on their chemical structure: mono- and disaccharides, oligosaccharides and polysaccharides. Both the mono- and disaccharides belong to simple carbohydrates (Herrero et al., 2011). As mentioned in earlier part, honey is made up of abundant amount carbohydrates. Monosaccharide in honey refers to fructose and glucose. There are extra 25 oligosaccharides found in honey such as sucrose, maltose and palatinose.

To analyse carbohydrates present in honey, there are more than one method available from previous researches including HPLC, GLC and thin-layer chromatography. Gas-liquid chromatography is also recognized as gas chromatography (GC). Before the usage of HPLC, GLC is widely used to detect carbohydrates in honey as the detection limit for oligosaccharides can reach the order of 40 ppb with the help of flame ionization detector (FID).

However, nothing is perfect in the world. GLC possesses some drawbacks and this is the reason why HPLC started to receive attention. One

of the negative sides of GLC is the component to be detect must be in a single tautomeric form. Tautomers are isomers which the carbon skeleton of the compound remains unchanged but the position of protons and electrons will change (Department of Chemistry, 2019). In addition, carbohydrates need to be derivatized before the analysis because it is not volatile. Due to these drawbacks, HPLC is getting popular as it requires small amount of sample and carbohydrates can be isolated for further study. By using HPLC, carbohydrates need not to be derivatized. The analysis of carbohydrates can be done by refractive index detector (RID), electrochemical detector, evaporate light-scattering detector (ELSD) or mass spectrometry (MS) (Swallow and Low, 1990). In HPLC, RID is the considered as the only universal detector and the principles is based on RI difference between mobile phase and sample (Kazakevich, 2019). However, the detection of minor oligosaccharides is difficult in HPLC because the amount is low and the structure is similar. The core oligosaccharides in honey are among glucose-glucose or glucose-fructose related. To determine the trace concentration of oligosaccharides, methodology of charcoal/Celite chromatography followed by anion-exchange HPLC analysis together with pulsed amperometric detection system is recommended (Swallow and Low, 1990).

2.7 Determination of Antioxidant Properties of Honey

Antioxidants or sometimes known as “free-radical scavengers” are natural or human-made substances that have the ability to slow down or prevent cells damage caused by free radicals in body. Antioxidant sources can be from diet intake or produced by body naturally. As body digests food intake and reacts to environmental changes, free radicals are created by cells. However, if the radicals produced cannot be removed, the consequence is the presence of oxidative stress (Ware, 2018). Antioxidants will bind these waste substances to prevent cellular damage (Khalil, Sulaiman and Boukraa, 2010).

In honey, there are various compounds found to have antioxidant properties. They are phenol compounds, catalase and glucose oxydase enzymes, carotenoids and flavonoids (Khalil, Sulaiman and Boukraa, 2010). The total content of phenolic in honey has strong relationship with the antioxidant action (Kek et al., 2014). The concentration of these compounds

varies with environmental and geographical factors. The main factor that affects their concentration is the botanical source of honey because these compounds are passed to honey through plants' nectar which is collected by bees. Due to this reason, phenolic compounds also appear to be valuable for honey classification and determination of botanical or geographical origin (Soares et al., 2017).

Until today, there are many modes proposed for determining the antioxidant activity in honey. Among them, the few common methods are determination of total phenolic content, 2.2-diphenyl-1-picrylhydrazyl (DPPH) analysis, the ferric-reducing/antioxidant power (FRAP) assay and with the application of HPLC (Ferreira et al., 2009). For the investigation of total phenolic content, Folin and Ciocalteu's phenol reagent is used due to its high sensitivity and the presence of poly-phenol entitles together with other electron-donating antioxidants. Using this method, the result is expressed as mg GAE/kg honey because gallic acid is used as the reference (Kek et al., 2014). For DPPH analysis, DPPH assay is used and the free-radical foraging activity of its radical is investigated. The activity is determined by measuring the absorbance of honey and DPPH radical mixture. The higher activity the mixture possesses the higher antioxidant ability the honey has. In addition, the working principle of FRAP assay is similar to DPPH analysis as their results are based on absorbance. FRAP assay can measure the presence of reductants or antioxidants directly. To be more specific, phenolic compound can be extracted out and detected by using HPLC. Solid-phase extraction (SPE) is developed to extract and identify the identity of different phenolic compounds including various phenolic acids and flavonoids (Moniruzzaman et al., 2014).

Other than phenol content, carotenoids can be determined with the help of UV-VIS spectrophotometer as well. In this analysis, contents of lycopene and β-carotene were determined with specific equations (Ferreira et al., 2009).

2.8 Elemental Analysis of Honey

Heavy metals are elements occur naturally all over the earth's crust. However, some of the elements may induce toxicity even at low exposure such as arsenic. Besides, environmental pollution and human exposure which has risen in recent years result from activities involving production and the use of heavy

metals. Environmental pollution can occur from leaching of heavy metals, metal corrosion and deposition of metal ions while human exposure results from the utilization of heavy metals in industrial, agricultural, domestic and other purposes. Apart from the bad side, some metals are essential nutrients such as iron, copper, magnesium, zinc and nickel without going beyond the tolerance limits (Tchounwou et al., 2012).

Heavy metals appear as trace elements in honey due to its low concentration (0.02-1.03%) (Kiliç Altun et al., 2017). The type and concentration of elements present in honey depends largely on the type and origin of floral materials. The sources of metals may be coming from external sources like heavy metal pollution and improper processing of honey. In country with serious heavy metal pollution like Iran, the quality of products manufactured including honey is strongly affected. The pollution of heavy metals due to mining, smelting and metal-based treatment threats human being and animals through food chain (Aghamirlou et al., 2015). The heavy metal concentration of honey is often used as quality pointer (Kiliç Altun et al., 2017). Figure 2.1 outlines how heavy metals will transfer to and remain in honey.

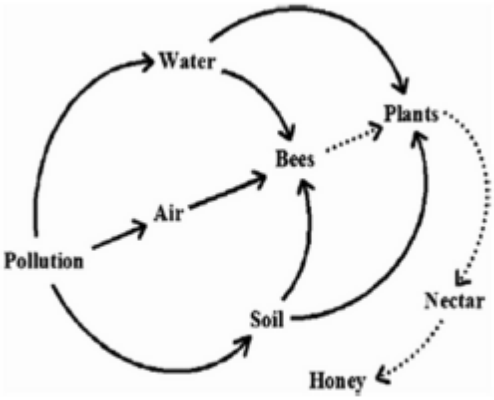


Figure 2.1: Natural and Anthropogenic Basis of Heavy Metals in Honey (Aghamirlou et al., 2015).

Determination of the identity of trace elements present in honey can be done by inductively coupled plasma ICP method. The analysis can be done by either ICP mass spectrometry (ICP-MS) or ICP optic emission spectro-photometry (ICP-OES). Both of the methods require sample pre-treatment

using microwave acid digestion. The operating conditions for ICP-OES and ICP-MS are shown in Table 2.3 and 2.4 respectively.

Table 2.3: ICP-OES Operating Conditions (Aghamirlou et al., 2015).

Spectrometer	Agilent 7500ce with ORS
Nebulizer	Micromist
Interface	Interface
RF generator (W)	1550
Argon flow rate (L min^{-1})	0.85
Nebulizer pump (rps)	0.1
Scanning condition	Number of replicate 5, dwelling time 1s
Scanning mode	Pulse
Reduction gas flow (L min^{-1}):	
H_2	3.5
He	4
Internal standard	^{45}Sc, ^{89}Y, ^{159}Tb

Table 2.4: ICP-MS Operating Conditions (Kilic Altun et al., 2017).

RF power (W)	1500
Plasma gas flow rate (L min^{-1})	15
Auxiliary gas flow rate (L min^{-1})	1
Carrier gas flow rate (L min^{-1})	1.1
Spray chamber T (°C)	2
Sample depth (mm)	8.6
Sample introduction flow rate (mL min^{-1})	1
Nebulizer pump (rps)	0.1
Extract lens (V)	1.5
Number of replicates	3

The inferior detection limit of ICP-OES is ppb, parts per billion while ICP-MS is ppt, parts per trillion (Thermo Fisher Scintific, 2019). If lower detection limit is not required, ICP-OES is good enough as the overall cost is cheaper.

2.9 Honey Pollen

Pollen is a vital part of diet for bee larvae. Bee larvae are legless white grub that never leaves their wax cell (Bee Health, 2019). Pollen is present in honey

because when bees collect nectar from floral, they do collect pollen as well. Pollens are fine grains produced by flora's male reproductive organs, anther.

The present of pollen is a useful indicator and representation of the honey's botanical origin. Like fingerprints, pollen is often used to identify certain species of plants unique to their group. With the combination of pollen shape, size and surface structure, it is sufficient enough to characterize the species of plants but only to the level of family (Kiew and Muid, 1991). This is called as melissopalynogical studies which involves qualitative and quantitative analysis of pollen in honey. Qualitative analysis allows one to identify the types of pollen while quantitative analysis is performed to determine the main sources of nectar and pollen. Analysis of pollen can also evaluate the quality of honey and differentiate pure honey from adulterated or contaminated honey (Rosdi et al., 2016).

There are three layers on the pollen wall: sexine, nexine and intine. Most of the pollen grains can be recognized by surface structures in the outermost layer, sexine. These surface structures are useful tool for the identification on genus on taxonomy rank or sometimes species. The shape of pollen grains is normally spherical or elliptical. However, there are some unique and distinct shapes like two air bladders, triangular, square or polygonal. These unique shapes are caused by the pores in the pollen grain. In some cases, the shapes are different in different viewing angle. By combining the shape, size and surface structure, one can identify the family where the plant belongs (Rosdi et al., 2016).

2.10 Medical Importance of Honey

Honey is recognized by its clinical significance in treating several diseases including cancer. Figure 2.2 shows the properties of honey such as antioxidant, antibacterial and anti-inflammatory which contributes to the medical use of honey.

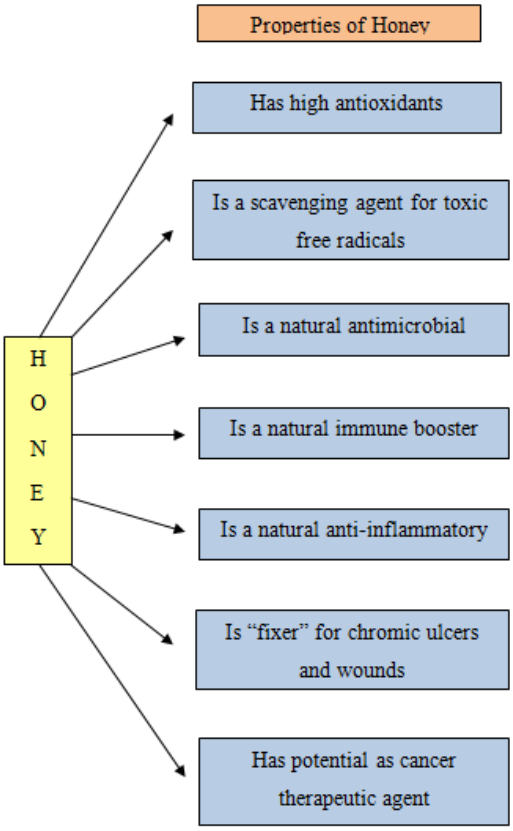


Figure 2.2: Properties of Honey that Reflect Its Medical Importance (Mohamed and Hamad Alfarisi, 2017).

Since ancient times, honey is widely used to treat wounds. The antibacterial and antioxidant properties of honey allow it to heal and prevent infection to wound. Due to its viscosity and moisture content, honey creates a protective barrier and allows the wound to stay in moist condition. Within the first two days of wound healing, wounds are prior to inflammation due to disordered network of cytokines. Cytokines are proteins secreted by immune system cells (Sino Biological, 2019). The anti-inflammatory effects of honey exert help to prevent wound inflammation and increase the rate of wound healing (Alvarez-Suarez et al., 2014). Although current therapeutic products used in medical for wound healing are hydro-fibre silver and aquacel plain, natural products with antimicrobial property like honey is getting more attention. This is because present therapeutic products have contributed to the problem of bacterial resistance. Honey is one of the alternatives in wound healing. For instance, Tualang honey has high efficiency in treating full-thickness burn wounds. It reduces wound size and restricts from partial-

thickness wounds from growing. In addition, Tualang honey even helps in treating diabetes patients who are suffering from diabetic foot (Mohamed and Hamad Alfarisi, 2017).

The antioxidants in honey such as Caffeic acid, Quarcetin and Apigenin evolved in treating cancer and cardiovascular diseases. According to Ahn et al. (2009), Caffeic acid and quarcetin have the ability to inhibit tube formation and proliferation of endothelial cells. Although caffeic acid itself is considered carcinogenic, it was found that colon tumors in rats are suppressed if caffeic acid is applied together with other antioxidants. Furthermore, quarcetin and apigenin have anti-proliferation effects on glioma, breast cancer cells and liver, breast, breast cancer cells respectively. In animal test, it was found that caffeic acid reduces blood pressure and heart rate while quarcetin helps to restore dysfunction of endothelial (Khalil, Sulaiman and Boukraa, 2010). In previous study, tualang honey can enhance the antioxidant levels in cardiac muscle and decrease peroxidation of lipid (Mohamed and Hamad Alfarisi, 2017).

Other clinical importance of honey includes learning and memory improvement, reproductive benefits, anti-diabetic activity, restoring osteoporosis and hepatoprotective effect. Clinically, honey can increase concentration of sperm and improve motility and morphology of males suffering from oligospermia. In the study of Tualang honey, ovarism toxicity of prepubertal animal model induced by Bisphenol-A is reduced significantly by consuming Tualang honey. Since antioxidants are present in honey, oxidative stress of brain can be reduced which will help in improving learning and memory. Honey also has the ability to improve morphology and cholinergic system of brain. Figure 2.3 shows the responsibilities of Tualang honey found in learning and memory improvement.

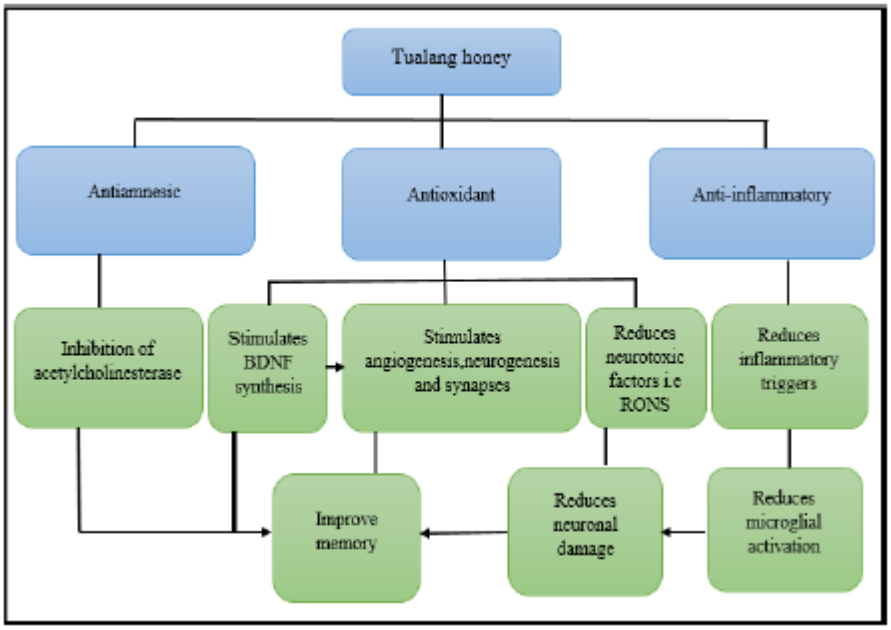


Figure 2.3: Tualang Honey's Role in Learning and Memory (Mohamed and Hamad Alfarisi, 2017).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Introduction of Different Honey Samples

There are seven honey samples used in current study and each of them is shown in Figure 3.1. Honey samples A to E are processed honey purchased from market while honey F and G are raw unprocessed honey. The geographical origin and type of each honey was tabulated in Table 3.1.

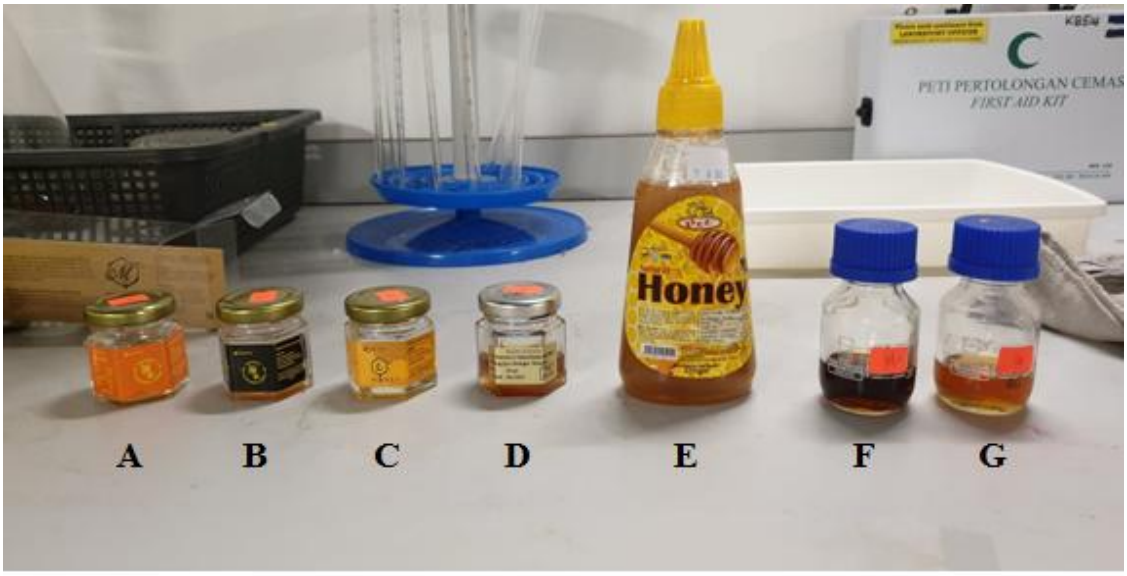


Figure 3.1: Seven Honey Samples Involved Throughout the Analysis.

Table 3.1: Type and Geographical Origin of All Honey Samples.

Honey Sample	Type of Honey	Geographical Origin
A	Multi-floral honey	Taiwan
B	*Apis Cerena* honey	Bentong, Pahang
C	Stingless bee honey	Bentong, Pahang
D	Tualang honey	Maran, Pahang
E	Multi-floral honey	Malaysia
F	Dark raw honey	Selayang, Selangor
G	Light raw honey	Selayang, Selangor

3.2 Analysis of pH

The steps involved in pH determination of honey were very simple. A 10% (w/v) solution of honey as solute was prepared in ultrapure water (El Sohaimy, Masry and Shehata, 2015). W/V is one of the simple ways used to express concentration of solution. W refers to the weight of solute to be dissolved and V is the volume of entire solution in millilitres. After the sample solutions were being prepared, a pH meter was employed to determine their pH values.

3.3 Electrical Conductivity

Similarly, the sample solution for electrical conductivity was prepared in milli-Q water with certain concentration. 20% (w/v) honey solutions were prepared for each honey sample.

A conductivity meter's working principle is based on the conductance when a sensor is dipped in a solution. The sensor detects the size of resulting signal which has linear relation with conductivity. The unit of measurement currently is "microSiemen/cm". The conductivity of ultrapure water is less than 10 μS/cm and the value increases as the addition of electrolytes like acids or bases (Omega Engineering Inc., 2019a).

3.4 Determination of Sugar Content

Total of 2 methods were employed to determine the sugar level of honey samples. The first method was Fehling Test. It was used to calculate the percentage of reducing sugar by substitute results obtained into 2 equations accordingly. In the first part of Fehling Test, 1 g of honey was dissolved in distilled water to the amount of 100 mL followed by boiling the Fehling A and B reagents with amount of 5 mL each in 150 mL of distilled water. While maintaining the temperature of the solution, titration was performed. Diluted honey solution was used as the titrant and was added into the Fehling mixtures until blue colour almost disappeared. At this stage, the mixture remained as murky-blue colour with red precipitate. Two drops of methylene blue were then added to the mixture after the most of the blue colour was gone. The titration was continued until the blue colour disappeared completely with only red precipitate and yellowish clear solution left. The solutions were yellowish

in colour due to the colour of honey itself. After the first part was done, equation 3.1 was used to calculate percentage of total reducing sugar:

$$C = 25 \times 1000/W_1Y_1 \quad (3.1)$$

Then, the second part of Fehling test started with addition of 1mL less the amount of honey solution consumed in first titration into the Fehling solutions. Again, the mixture was boiled for 1 to 2 minutes and the titration was performed in the similar way with the first part of this test. Equation 3.2 was applied in the second part:

$$C = 25 \times 1000/W_2Y_2 \quad (3.2)$$

Where,

C = Gram invert sugar per 100g of honey

W_1 & W_2 = Weight of honey according to first and second procedure

Y_1 & Y_2 = Volume of diluted honey solution consumed in first and second titration in mL.

The second method was performed by using UV/Vis Spectroscopy. It was applied as an alternative for HPLC due to its simplicity and the absence of column as well as detector for HPLC. For the determination of sugar content in honey, 3 standard solutions are needed: sucrose, fructose and glucose. They were prepared with concentration of 5000 ppm respectively in distilled water and diluted to 1000, 2000, 3000 and 4000 ppm as standards. Diluted honey solutions were prepared by dissolving 0.1 g of honey in 50 mL, 12 mL and 10 mL for sucrose, glucose and fructose analysis respectively. By taking sucrose as example, 2 mL of each standard solution and samples were pipetted into different test tubes. Same amount of deionised (DI) water was used as blank. Then, 2 mL of 6 M hydrochloric acid (HCl) solution was added to each test tube and placed in boiling water for 10 minutes. Next, 8 mL of 2.5 M sodium hydroxide (NaOH) solution and 2 mL of 3,5-Dinitrosalicylic acid (DNSA) solution were introduced before the tubes were covered by parafilm and shook

to mix. The mixtures were then placed in boiling water for another 5 minutes followed by 10 minutes in ice water. The Absorbance of standards, blank and samples were measured at 580 nm and the concentrations were obtained from the standard calibration curve. For glucose and fructose, the steps of adding HCl solution and 10 minutes staying in boiling water were skipped because they can react readily with DNSA reagent (Perkin Elmer, 2015). The absorbance of glucose and fructose were measured at 540nm and 490nm respectively.

3.5 Analysis of Antioxidants & UV-Vis Spectroscopy

For the analysis of total phenolic content, Folin and Ciocalteu's phenol reagent was utilized. The method used was spectrophotometric Folin-Ciocalteu method with slight modification. Before the analysis of samples, gallic acid standard solutions were used to create calibration curve. The concentrations involved were 50, 100, 150, 200 and 250 mg/L. 0.5 mL of each standard was pipetted and mixed with 2.5 mL of Folin and Ciocalteu's reagent as well as 2 mL of 0.7 M sodium carbonate (Na_2CO_3) solution. The mixtures were allowed to rest for 7 minutes before incubation in dark for 2 hours. For sample preparation, 1 g of each honey sample was mixed with 1mL of Folin and Ciocalteu's reagent and then 1 mL of sodium carbonate (Na_2CO_3) was added to the mixture after 3 minutes respectively. The mixtures were diluted to 10 mL with distilled water and incubated for 2 hours. After that, UV-VIS spectrophotometer was used to measure the absorbance at 760 nm. The total phenolic content is expressed as mg gallic acid equivalent (GAEs) /kg honey (Moniruzzaman et al., 2014).

The absorbance of a compound at certain wavelength was obtained from the transmittance (T) when the UV-Vis light passed through the sample. It could be computed from the following formula:

$$A = -\log T \tag{3.3}$$

UV-Vis spectroscopy or spectrophotometry is one of the widely used characterization techniques as it can detect nearly all molecules. For

qualitative analysis, it helps to determine or confirm identity of a compound by comparing with the absorbance spectrum. For quantitative purpose, one can obtain the concentration of the analyte by relating absorbance to Beer-Lambert Law (JoVE Science Education Database, 2019).

Other than total phenolic content, concentration of another antioxidant could be obtained using UV-Vis techniques, which is carotenoid. For this antioxidant, the absorbance of honey sample was measured at 453, 505 and 663nm. To prepare the sample, 100mg of honey was shook with 10mL of acetone-hexane mixture vigorously. The ratio of acetone to hexane was 4: 6. The mixture was shoke for 1 minute and filtered through filter paper. Two carotenoids are determined: β-carotene and lycopene by using equation 3.4 and 3.5 respectively.

$$\beta - carotene \left(\frac{mg}{100mL}\right) = 0.216A_{663} - 0.304A_{505} + 0.452A_{453} \quad (3.4)$$

$$lycopene \left(\frac{mg}{100mL}\right) = -0.0458A_{663} + 0.372A_{505} - 0.0806A_{453} \quad (3.5)$$

The final results found were expressed as mg carotenoid per kg of honey (Ferreira et al., 2009).

3.6 Elemental Analysis & ICP-OES

To analyze mineral content of honey, inductively couples plasma – optic emission spectrophotometry (ICP-OES) and microwave system were applied. Before analysis started, pre-cleaning of the microwave vessels was performed. 9mL of 65% HNO_3 and 1mL of 37% HCl were introduced into all microwave vessels. Microwave was heated with the following program : up to 170℃ for 5 minutes, then 200℃ for 15 minutes and cooled to 50℃ for 10 minutes. Sample analysis was carried out on the next day. For sample preparation, 500 mg of honey was mixed with 3mL of concentration nitric acid (HNO_3) (65 v/v %) and 0.5mL of HCl with concentration of 37 v/v% in digestion vessels . Before closing the vessels , the mixture was stirred well to mix . The microwave was heated up to 150℃ for 8 minutes, then to 210℃ for 20 minutes and cooled down to 50℃ for 10 minutes. A blank was digested in the same way as well.

Post-cleaning was also performed in the similar way as pre-cleaning to the microwave vessels. ICP-OES was applied to analyse the concentrations of elements in the digested samples (Aghamirlou et al., 2015). Standard solutions of Copper (Cu), zinc (Zn), chromium (Cr) and nickel (Ni*)* with concentration of 1000 ppm were prepared in deionised water. Then, they were diluted to the concentration shown in Table 3.2 to plot calibration graphs (Aljohar et al., 2018).

Table 3.2: Concentrations of each Element for Calibration.

Elements	**Concentration (ppm)**
Zn	10, 20, 30, 40 and 50
Cu	1, 2, 3, 4 and 5
Cr	0.2, 0.4, 0.6, 0.8 and 1.0
Ni	0.5, 1.0, 1.5, 2.0 and 2.5

ICP-OES is a method to identify the composition of an element using plasma energy. The element gets excited when plasma energy is given to it. The elevated temperature and electron density of plasma enable it to excite the atoms. Plasma can be produced from ionised argon gas. To ionise the gas, high frequency electric current is supplied to the torch coil. Next, when the excited atoms fell back to low energy state, rays are emitted and those corresponded to the wavelength of photon will be measured. The location of photo rays is utilized to verify the type of element present. They ray intensity can then be used to obtained the concentration of element (Hitachi, 2019).

3.7 Pollen Identification and Characterization

The principle involved in microscopy pollen analysis was concentrating the microscopic elements by centrifugation. In current study, pollen analysis was done without acetolysis. 10 g of honey was weighed and dissolved in 20 mL of distilled water. The solution was centrifuged at 2500 rpm for 10 minutes and the supernatant was extracted. To remove sugars in honey more completely, the supernatant was diluted to 10 mL with distilled water and centrifuged again for 5 minutes. Lastly, the sediment was extracted out and

spread on glass slides for analysis. Pollen of each honey was observed and evaluated at 40x magnifications (Louveaux et al., 1978). The images of pollen were characterized accordingly to determine the floral origin of each sample.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Sugar Content

Two methods were employed to determine and evaluate sugar levels in honey. Fehling test was used to measure the total content of reducing sugar in the honey samples. Determination of the individual sugar content including glucose, sucrose and fructose were done by utilizing UV/Vis Spectrophotometer.

4.1.1 Overview of Sugar Content Analysis

Table 4.1 and Figure 4.1 showed the overall results of sugar content in tested honey samples (A-G) with the involvement of Fehling Test and UV/Vis Spectrophotometer.

Table 4.1: Sugar Content in Examined Samples Obtained from Fehling Test and UV/Vis Spectrophotometer.

Honey Samples	Total reducing sugars %	Sucrose %	Fructose %	Glucose %	Fructose/ Glucose ratio
A	21.45	4.909	37.33	36.99	1.009
B	27.95	4.966	37.04	36.51	1.014
C	29.70	4.842	36.59	35.67	1.026
D	32.53	4.955	36.16	36.14	1.001
E	95.00	4.839	35.66	35.82	0.996
F	87.79	4.736	34.97	34.60	1.011
G	69.91	4.667	34.73	33.53	1.036

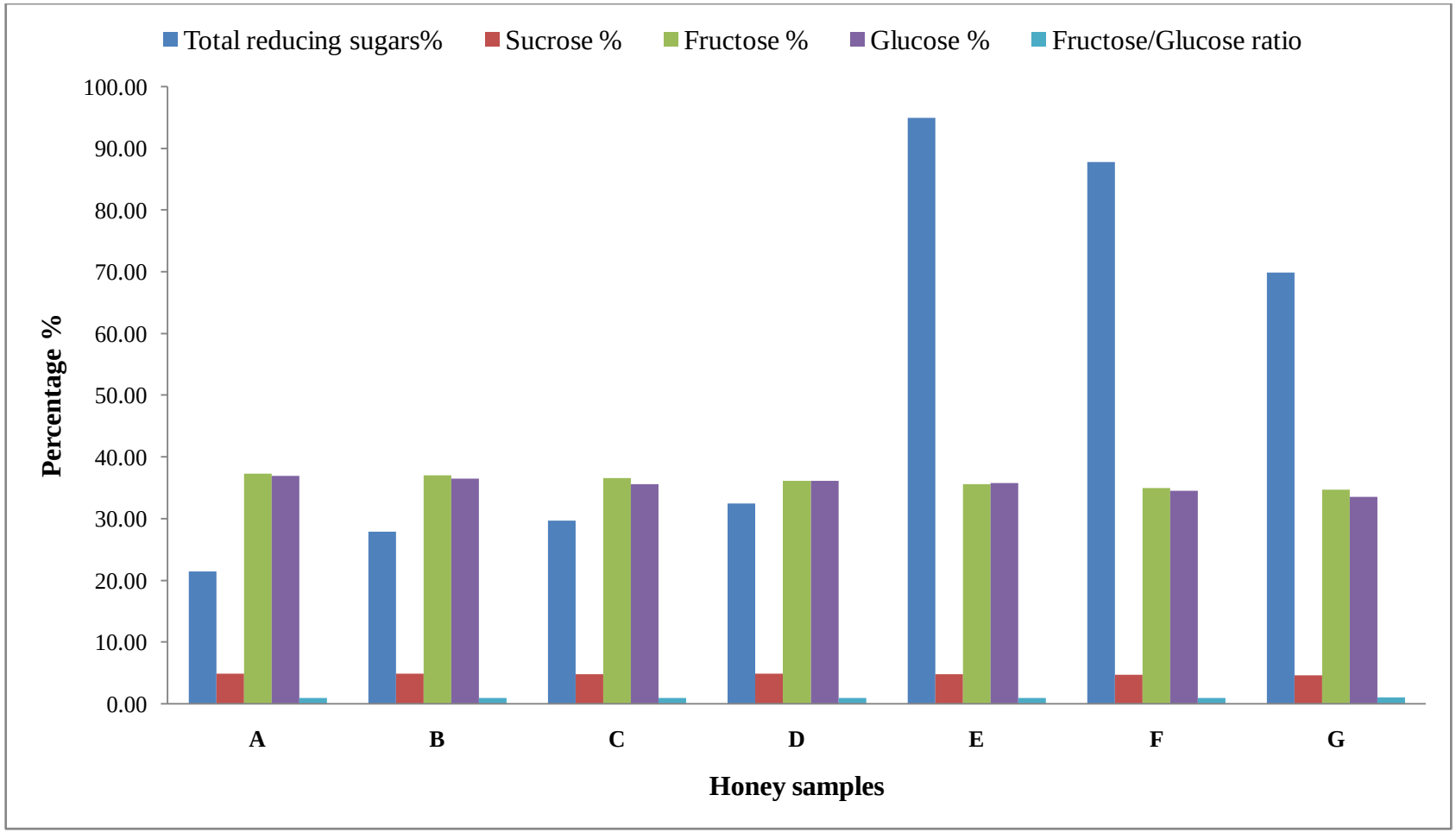


Figure 4.1: Overall Results of Sugar Content Analysis in Honey A to G.

4.1.2 Fehling Test

Since 1800's, Fehling test has been widely employed to determine the level of glucose and other reducing sugars. The working principle of this test is the reduction of Cu^{2+}ions to Cu^{+} ions by aldehyde-containing sugars whereby Cu^{2+} ions are contributed by Fehling A reagent containing $CuSO_4 \cdot 5H_2O$. Here, Fehling B which contains potassium tartrate is needed because Cu^{2+} ions tend to form insoluble precipitate with hydroxide ions. Thus, tartrate ions are added to avoid the precipitation. The redox reaction between Cu^{2+} ions and aldehyde-containing sugars is illustrated in equation 4.1.

$$RCHO(aq) + 2Cu^{2+}(aq) + 5OH^{-} \rightarrow RCOO^{-}(aq) + Cu_2O(s) + 3H_2O(l) \qquad (4.1)$$

Once the deep blue Cu^{2+} tartrate complex disappeared and rust red precipitate, Cu_2O appeared, the equivalence point of the reaction is indicated. However, as the Cu^{2+}ions decrease, the blue colour intensity is reduced and led to difficulty in determining the end point of the titration. To overcome this trouble, methylene blue is added. It can be reduced by aldehyde-containing sugars while removing the blue colour completely. The end point of this test is the complete disappearance of colour (Lamp, 2016). The amount of total reducing sugars in honey samples depends on the amount of diluted samples used to reach the end point of titration. Appendix A showed the condition of honey sample that reached the end point of the titration with clear yellowish solution and rust red precipitation ofCu_2O.

According to Codex Alimentarius Standard, the amount of total reducing sugars in pure honey should be greater than 60% (Codex Alimentarius Commission, 1981). Results obtained from the 7 honey samples collected in Malaysia showed that the percentage of total reducing sugars ranges from 21% to 95%. As illustrated in Figure 4.2, only three honey samples (E, F and G) contained more than 60% of reducing sugars which means that only three honeys among seven honey samples met the minimum requirement for total reducing sugars in pure honey. This was because honey is naturally made up of mainly glucose and fructose which belong to reducing sugars. The remaining honeys with total reducing sugars of lesser than 60% showed that they might be adulterated by adulterants in which reduced the

content of reducing sugars present naturally in honey. Figure 4.3 illustrated the percentage of honey samples that had and did not have the minimum requirement of reducing sugars in honey. However, determination of total reducing sugars might not distinguish adulterated honey from pure honey accurately. This was because there are few factors time that could affect the content of reducing sugars such as collection time of honey, plants from which the honey was derived and storage (Aljohar et al., 2018). Another limitation of analyzing reducing sugars in honey was the presence of most significant non-reducing sugar, sucrose (Centre for Food Safety, 2017). Therefore, determination of total reducing sugars in honey might not be the most appropriate technique to verify adulteration of honey. Appendix B tabulated the exact data for total reducing sugars for all honey samples.

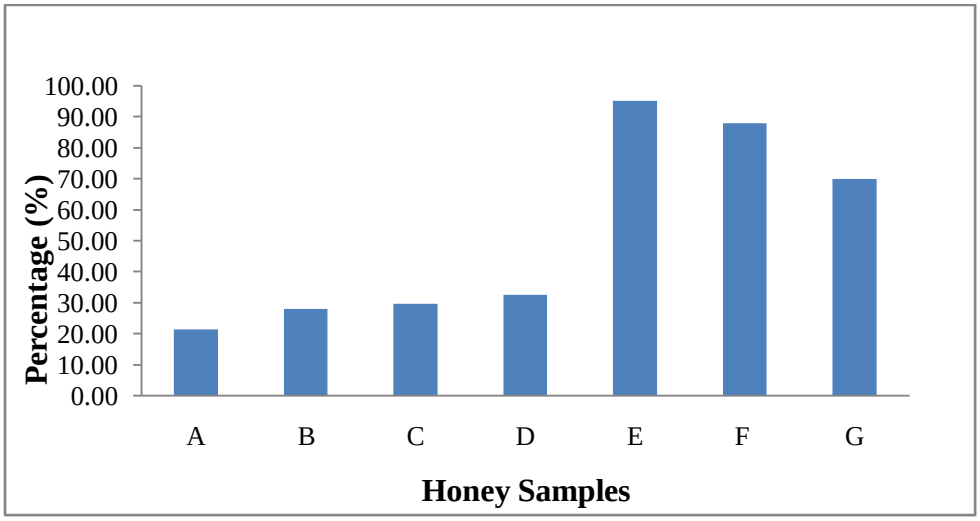


Figure 4.2: Total Reducing Sugars % of All Seven Honey Samples.

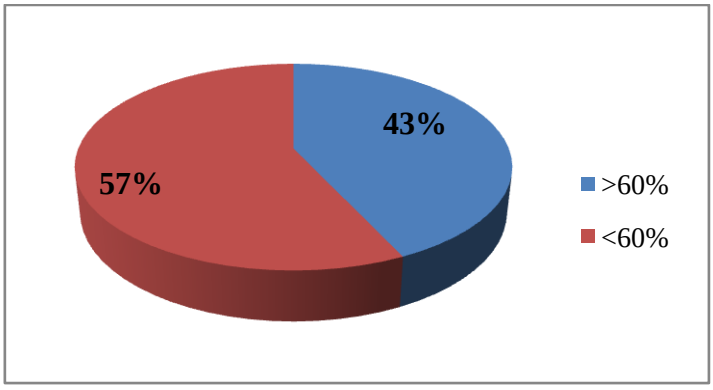


Figure 4.3: Percentage of Honey Samples with <60% And >60% of Total Reducing Sugars Among All Samples.

4.1.3 UV/Vis Spectroscopy Analysis

To compensate the limitation of Fehling test, alternative quality measurements including level of glucose, sucrose and fructose and also fructose/glucose ratio were performed using UV/Vis spectrophotometer. Glucose and fructose were the two most significant monosaccharides in honey while sucrose was the main disaccharide. Therefore, estimation of the individual sugar content for all three sugars is essential and was done by using colorimetric method. This method was based on the colour changes of 3, 5-dinitrosalicylic acid (DNSA reagent) when it was reduced by the oxidation of reducing sugars (glucose and fructose) and was previously described by Rajbhar et al. (2015). DNSA is more sensitive and less complicated than Benedict's reagent (National Centre for Biotechnology Education, 2018). After the reduction reaction, DNSA will be converted into 3-amino-5-nitrosalicylic acid (ANSA) and the colour change is from yellow to orange. The reduction of DNSA to ANSA was shown in Figure 4.4. The presence of ANSA changed the absorbance of the honey samples. The absorbance determined is directly proportional to the concentration of reducing sugars (Rajbhar et al., 2015).

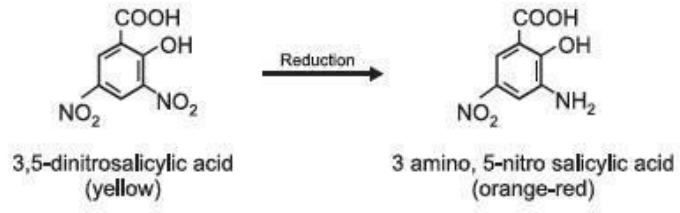


Figure 4.4: Reduction of DNSA to ANSA and the Colour Change.

Sucrose, the non-reducing sugar will not react with DNSA. So, it was broken down into simple sugars in advance by boiling the samples with hydrochloric acid (Perkin Elmer, 2015).

Glucose, fructose and sucrose showed different colour intensities. Therefore, calibrations were done in advance with concentration of 0, 1000, 2000, 3000, 4000 and 5000 mg/L. The calibration curves for sucrose, fructose and glucose were illustrated in Figure 4.5, 4.6 and 4.7 respectively. The absorbance was detected at their representative wavelength (nm) as shown in Table 4.2. Detail data for each calibration curve was tabulated in Appendix C.

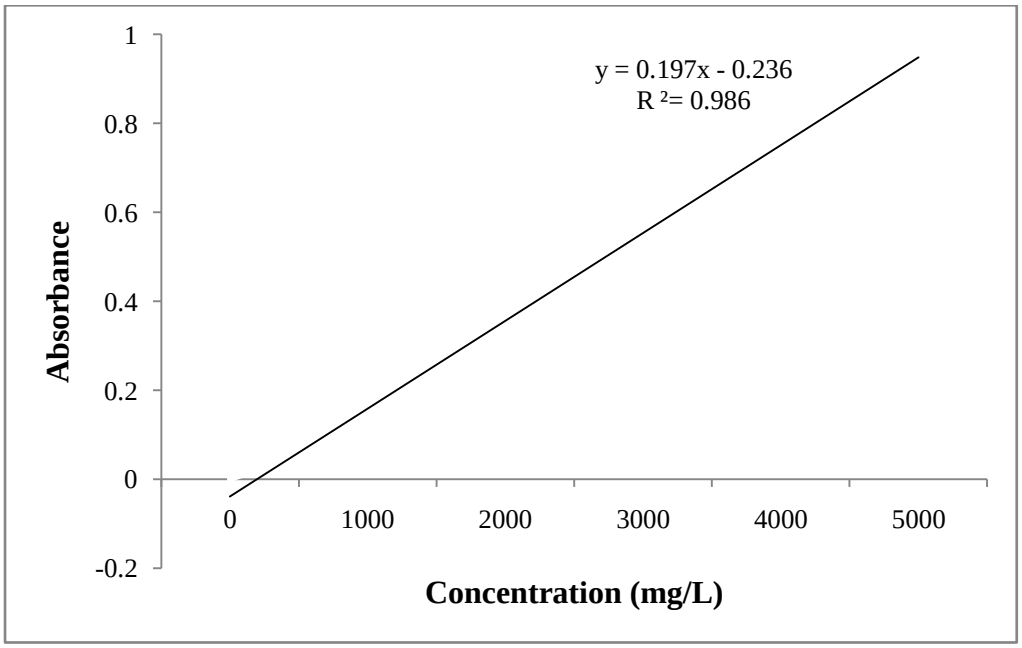


Figure 4.5: Sucrose Calibration Curve.

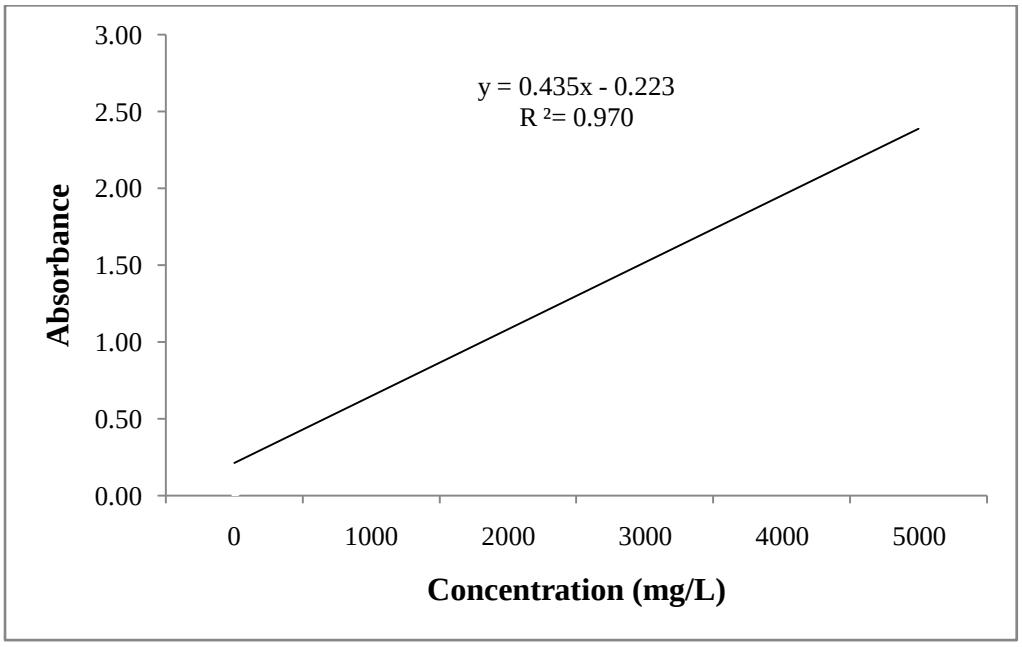


Figure 4.6: Fructose Calibration Curve.

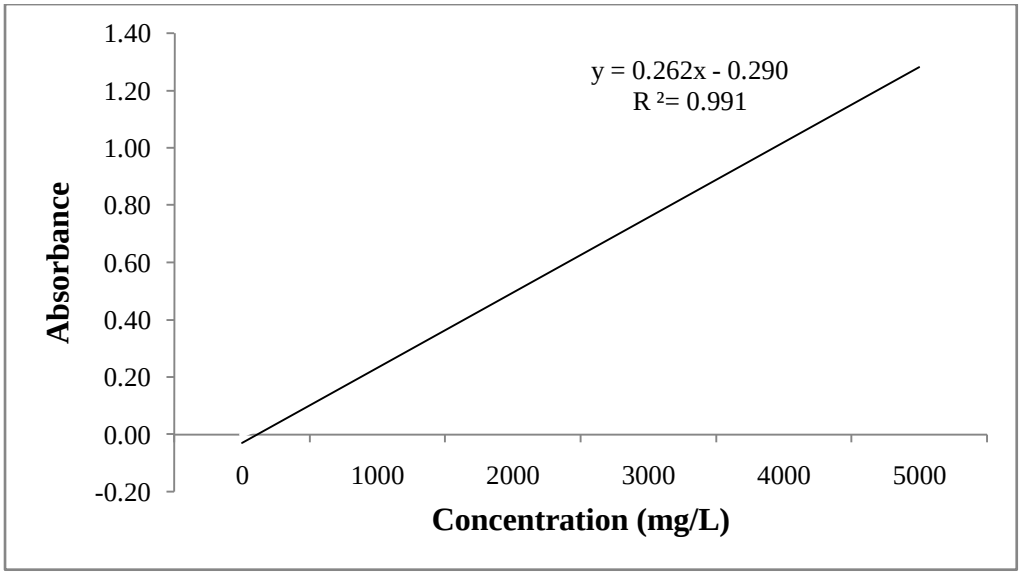


Figure 4.7: Glucose Calibration Curve.

Table 4.2: Wavelength (nm) for Glucose, Fructose and Sucrose.

Sugars	Wavelength (nm)	Sources
Glucose	540	(National Centre for Biotechnology Education, 2018)
Fructose	490	(Chow and Landhäusser, 2004)
Sucrose	580	(Perkin Elmer, 2015)

According to Belitz, Grosch and Schieberle (2009) honey contains approximately 38% of fructose and 31% of glucose and sucrose with concentration of not more than 5% based on Codex Alimentarius Commission (1981). The total concentration of glucose and fructose shall not be less than 60% (Codex Alimentarius Commission, 1981).

The analysis results of the individual sugar content were shown in Figure 4.8. Detail results of sugar analysis for each sample were presented in table form Appendix D.

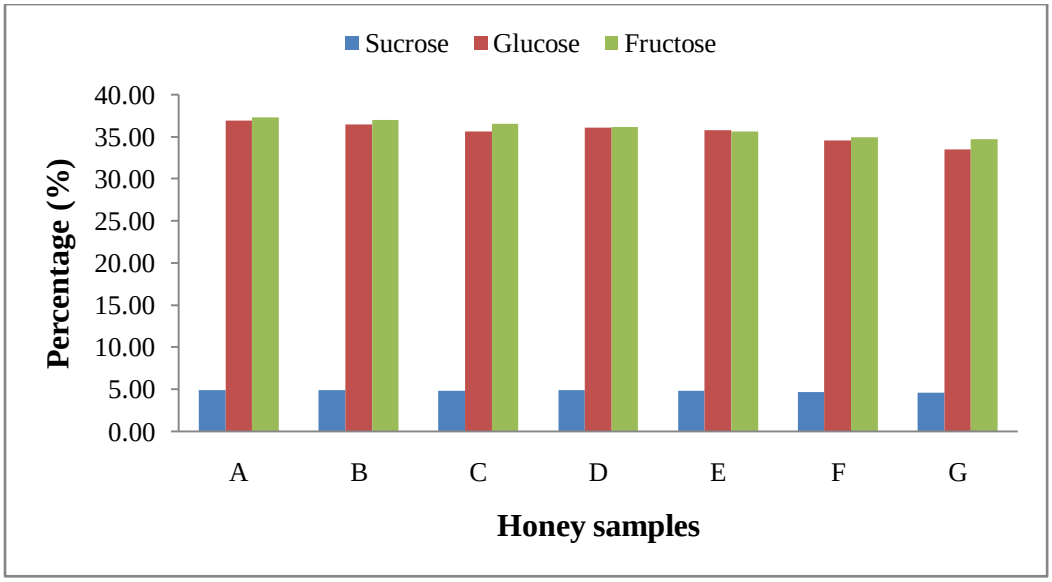


Figure 4.8: Concentration of Glucose, Fructose and Sucrose in Honey Samples.

Fructose levels in honey A to G were in the range of 34 to 38%. Results obtained were similar to that of Belitz, Grosch and Schieberle (2009). Fructose in honey provides the sweetness taste of honey. Even so, it has the lowest glycemic index, GI (19) as compared to glucose (100) and sucrose (60). GI is a ranking tool used to categorize the concentration and the rate of a carbohydrate increases blood glucose level. Carbohydrates with low GI raise blood glucose level slower and in a small portion (Diabetes Canada, 2013). Studies conducted by Atayoğlu et al. (2016) on healthy and diabetic people found that foods with low GI will reduce the blood glucose level and raise the secretion of insulin. Carbohydrates with indices smaller than 55 is categorised in the group of low GI. It was also found that fructose reduces blood glucose level in diabetic animal models (Erejuwa et al., 2012).

The concentration of glucose in examined honey samples were in the range of 33 to 37%. Even though the values were slightly higher than the amount as mentioned earlier, they were still in the specification range written in Codex Alimentarius Commission (1981) which was between 22 to 40.7%. The concentration of glucose in honey relied on the source of nectar. With the help of glucose, absorption of fructose and its hepatic action were enhanced (Bobiş et al., 2018).

Among the examined honey samples, sucrose concentrations were all below 5% with fluctuations around 4%. Highest concentration of sucrose

found in honey B and D might be due to the processing in industry. Adulterants might be added into these honeys during the processing and it is common by adding sucrose syrup. Adulterated honey or over-heated honey has sucrose content of more than 8%. Over-heating would denature the enzyme and led to high sucrose content remained (Aljohar et al., 2018). Determination of sucrose content was one of the criterions to assess the quality of honey. Pure honey has low sucrose content due to the enzymatic activity of invertase enzymes in honey. The enzyme breaks down sucrose into glucose and fructose. Based on the results obtained, the purity of all honey samples are said to be in good category.

Apart from analysis of individual sugar content, the most important parameter of quality evaluation is the fructose to glucose ratio (F/G). The results shown in Table 4.3 showed that the F/G ratios are in the range of 0.99 to 1.04 which matched with that in Codex standard (Codex Alimentarius Commission, 1981). According to the standard, the F/G ratio shall be greater than 0.95.

Table 4.3: Fructose/ Glucose Ratio for Honey Samples (A to G).

Sample	Fructose concentration (%)	Glucose concentration (%)	F/G ratio
A	37.33	36.99	1.0091
B	37.04	36.51	1.0145
C	36.59	35.67	1.0258
D	36.16	36.14	1.0006
E	35.66	35.82	0.9957
F	34.97	34.60	1.0106
G	34.73	33.53	1.0358

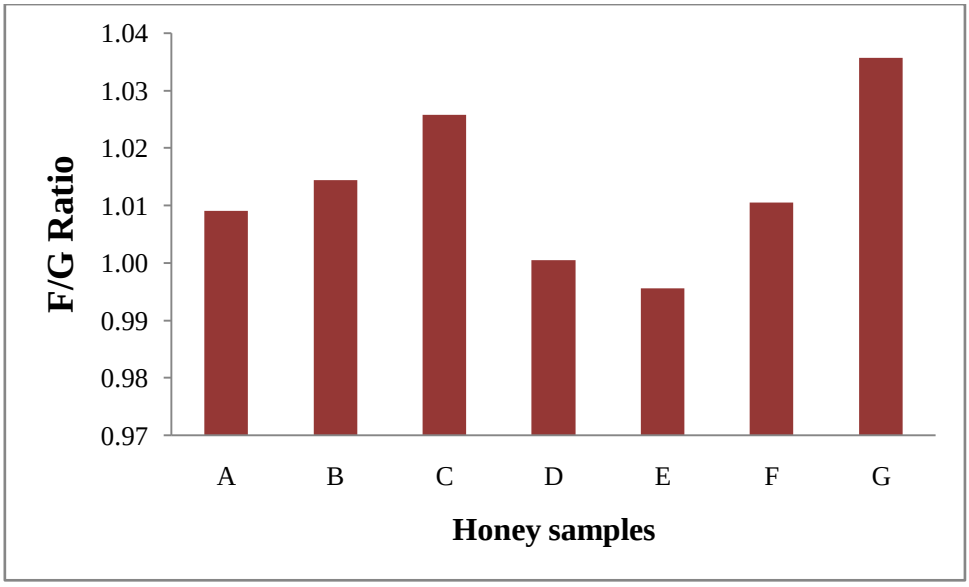


Figure 4.9: Fructose to Glucose Ratio of All Honey Samples.

Crystallisation of honey was affected by the fructose-to-glucose ratio. When the fructose content was lower but glucose content was higher in honey, crystallisation might be observed. By referring to the results, multi-floral honey E had the lowest F/G ratio and crystallisation had occurred during storage. This again confirmed that having low F/G ratio could increase the tendency of honey crystallisation. Lower fructose to glucose ratio might also reflect adulteration of honey by the addition of corn syrup or dextrose syrup (Singhal, Kulkarni and Rege, 1997).

4.2 Elemental Analysis (ICP-OES)

Honey composition including element concentration is strongly reliant on the origin, geographical and botanical factors, anthropogenic factors and natural factors (Vinceviča-Gaile, 2010). According to Mejías and Garrido (2017), hives near to contamination sources like volcanoes, mining sites, factories or railways will affect the presence of metals in honey. If the botanic sources of honey were exposed to unwanted residues like metals, honey will definitely inherit their characteristics and biological properties. Besides, the concentration of elements is closely affected by the type of raw floral materials such as pollen and nectar (Aljohar et al., 2018). Elements are considered as minor constituents in honey due to their low concentration. Based on one of the previous research studies, elements in honey are classified into two groups: macro-elements and micro-elements. Macro-group consists of Ca, Zn, Cu, Mg, Mn, P, S, Fe, K while micro-group includes Al, B, Ba, Cd, Cr, Hg, Ni, Pb, Se, Ti, U (Vinceviča-Gaile, 2010).

Elements in honey will possess risk to human health through diet intakes, depending on their concentration. Although various heavy metals (Fe, Ni, Zn and Cu) are important for growth of plants and metabolism of plants, their toxicity increases as well at higher concentration. Macro- and micro-elements such as chromium, copper, nickel and zinc are considered as toxic elements at elevated concentrations (Mejías and Garrido., 2017). Due to the human health risk possessed by these elements, determination of element concentrations in honey samples is necessary for quality evaluation.

4.2.1 Pre-treatment of Elemental Analysis (Microwave Digestion)

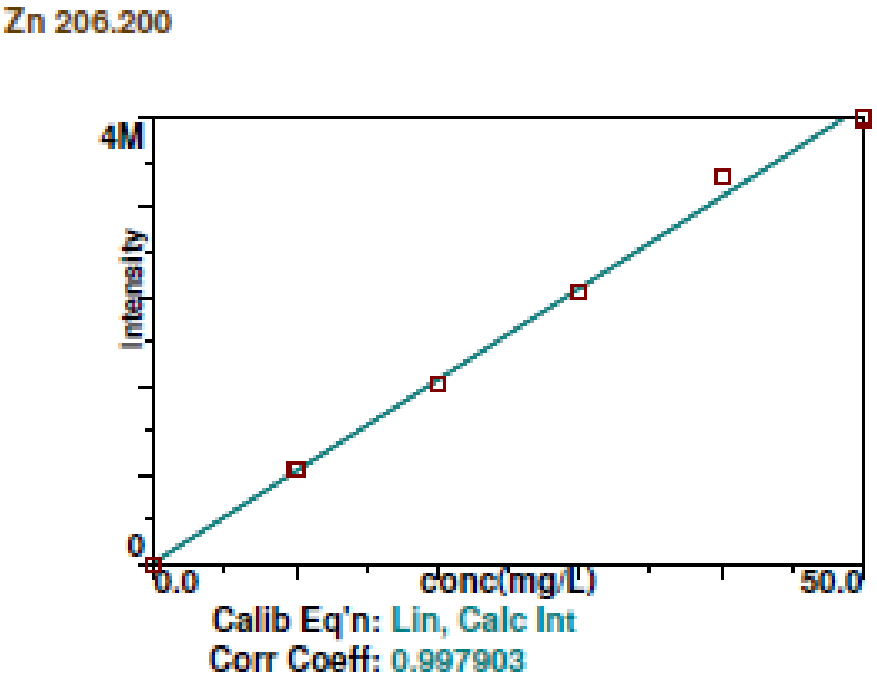
Microwave digestion was performed prior to elemental analysis to diminish the consequences of the organic matrix (Moniruzzaman et al., 2014). This pre-treatment is applied on all examined honey samples. The purpose was to breakdown the samples completely for the ease of elemental analysis (Labcompare, 2009). It was also known to be the most common technique used to dissolve heavy metals prior to elemental analysis with the presence of organic molecules. The working principle of microwave digester was by increasing the pressure and temperature through microwave irradiation in the closed vessel. Strong acids were used to provide low pH environment to the

samples which will help to speed up the solubility of heavy metals and thermal decomposition (Kingston and Jassie, 1988). The advantages of treating samples with microwave digestion prior to elemental analysis are improves QA/QC, decreases preparation time, improve cleanliness of preparation environment and lessen factor of skill level (J.Mangum, 2009).

4.2.2 Calibration of Examined Elements

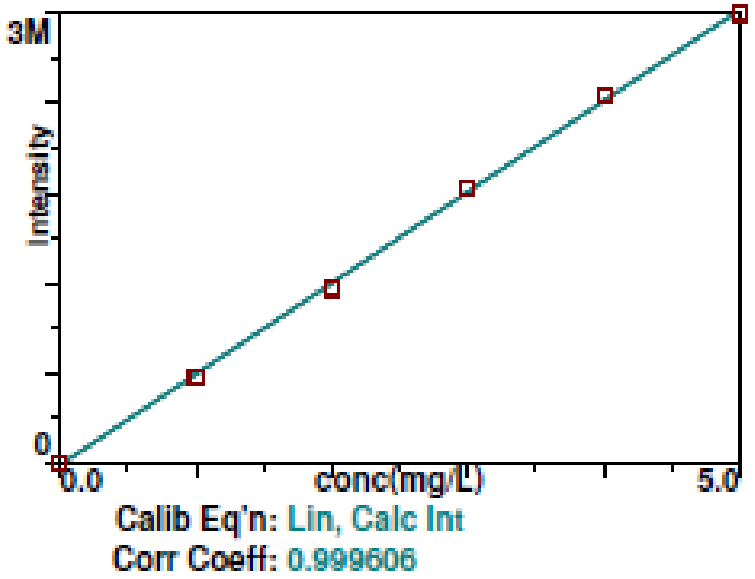
Digested honey samples were transferred to ICP-OES in contemplation of quantitative determination, mainly concentration of zinc, copper, nickel and chromium. Analysis was focused on these four elements because zinc and copper are macro-elements in honey while nickel and chromium belong to micro-elements. Standard solutions of each element were prepared in the concentration of 1000ppm and subjected to dilution prior to analysis, intrinsically facilitated the linear relationship between light intensity and concentration as shown in Figure 4.10 (a) to (d).

(a)



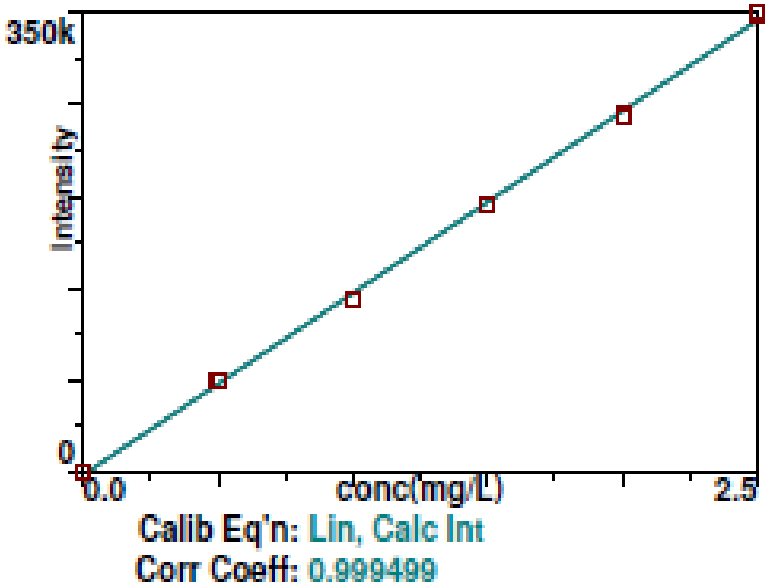
(b)

Cu 327.393



(c)

Ni 231.604



(d)

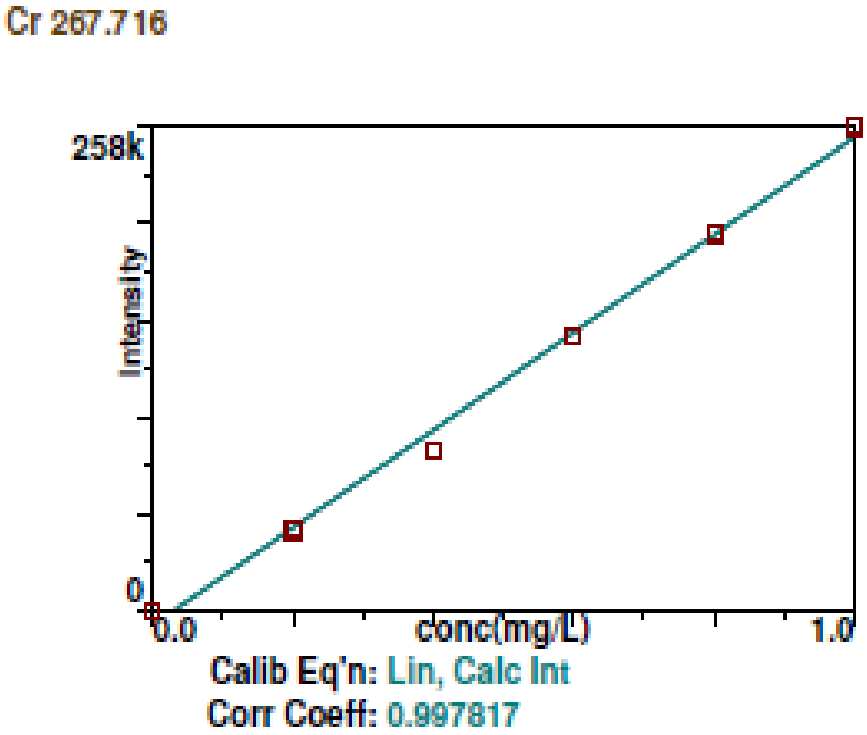


Figure 4.10: ICP-OES Calibration Curve for elements analysed, (a) Zinc, (b) Copper, (c) Nickel and (d) Chromium.

Theoretically, Beer's Law claimed that analytes' concentration is depend on the attenuation of light by absorption through dissolved constituents in produced solvent. Be in agreement with Beer's Law, certain extent of concentration will affect the linearity of intensity-calibration curve. Analyte with high concentration will excite molar absorptivity due to intermolecular interactions and lead to deviation from linearity. Thus, low concentration of solvent is likely to postulate linear relationship with coefficient close to 1 (Hou et al., 2006).

4.2.3 Overview of Elemental Analysis

Appendix E tabulated the experimental data obtained from ICP-OES for each honey sample with their respective Zinc, Copper, Nickel and Chromium concentrations. Likewise, graphical results of each sample were illustrated in Figure 4.11.

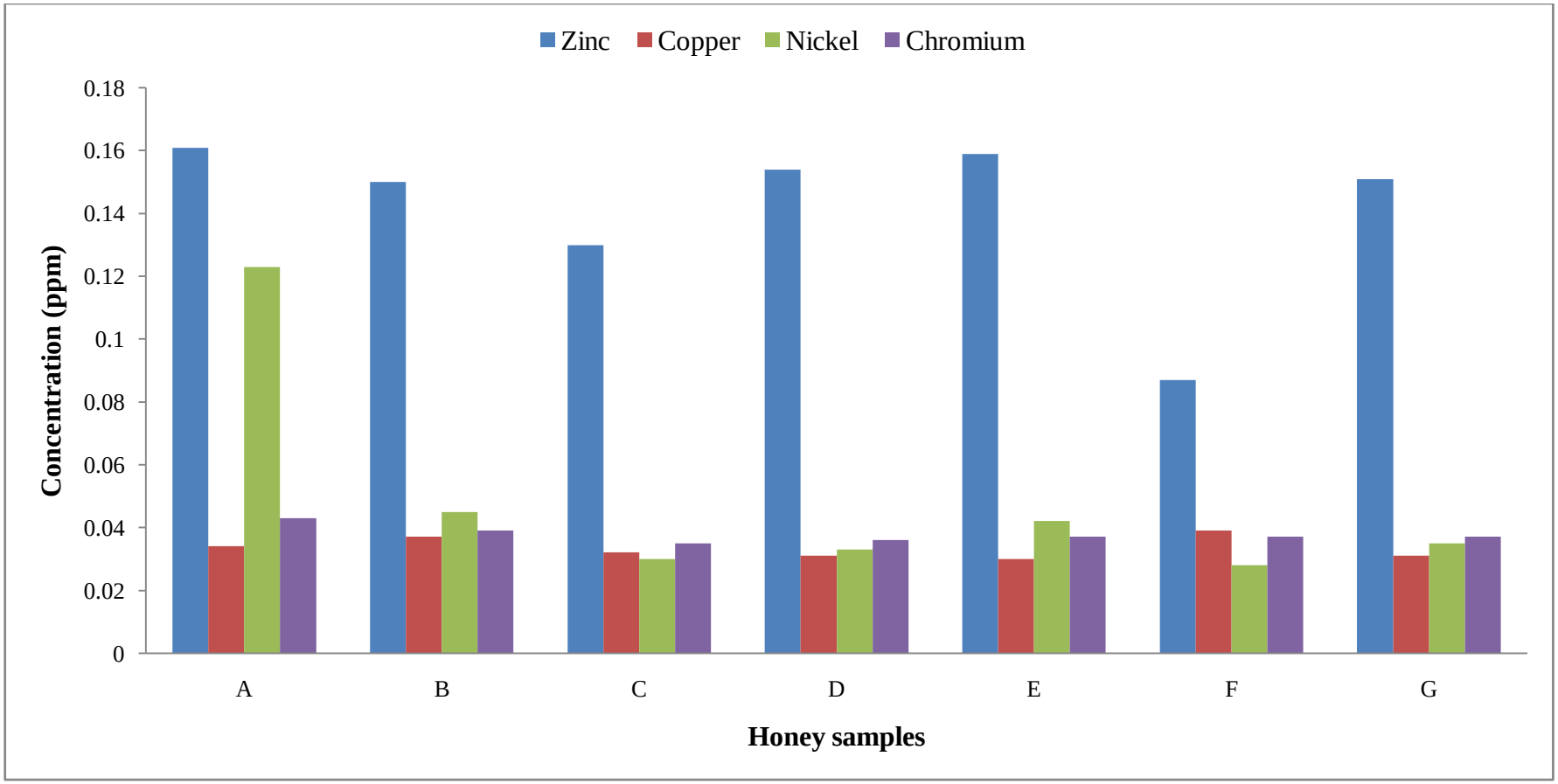


Figure 4.11: Concentration of Zinc, Copper, Nickel and Chromium across Honey Samples.

4.2.4 Concentration of Zinc in Examined Honey Samples

Zinc is necessary for humans, plants and animals for different biological functions. It also has a significant position in more than 300 enzymes in human body (Altundag et al., 2016). Zinc considered as an antioxidant which helps in treating bad skin conditions like acne and healing wounds (Moniruzzaman et al., 2014). It is known to be the second most abundant element in organisms after iron (Kılıç Altun et al., 2017). Even so, zinc may possess toxicity to organisms at high concentration. Excess zinc will produce reactive oxygen species which will then replace other metals in proteins. For human, the daily intake of zinc through diet is approximated at 12 to 15 mg/day (Aghamirlou et al., 2015). Acute effects like gastrointestinal irradiation and vomiting are likely to happen if the daily intake of zinc exceeds allowable limit. For adults, the average daily intake of zinc has been estimated to be ceiling at 20mg/day (Codex Alimentarius Commission, 2011).

The concentration of zinc in the examined honeys ranged between 0.08 to 0.17 ppm. Among all honeys investigated, multi-floral honey A from Taiwan was the richest in zinc concentration (0.161 ppm), followed by honey E (0.159 ppm), D (0.154 ppm) and B (0.150 ppm) from rainforest Malaysia. Dark raw unprocessed honey F (0.087 ppm) had the least zinc concentration among all studied honeys. The concentration of zinc in tested honey samples fell within the provisional maximum tolerable daily intake (PMTDI), which was 0.3 to 1.0 ppm (Codex Alimentarius Commission, 2011). So, all honey samples were safe to be consumed in terms of zinc. Figure 4.12 illustrated the concentration of zinc in each honey tested.

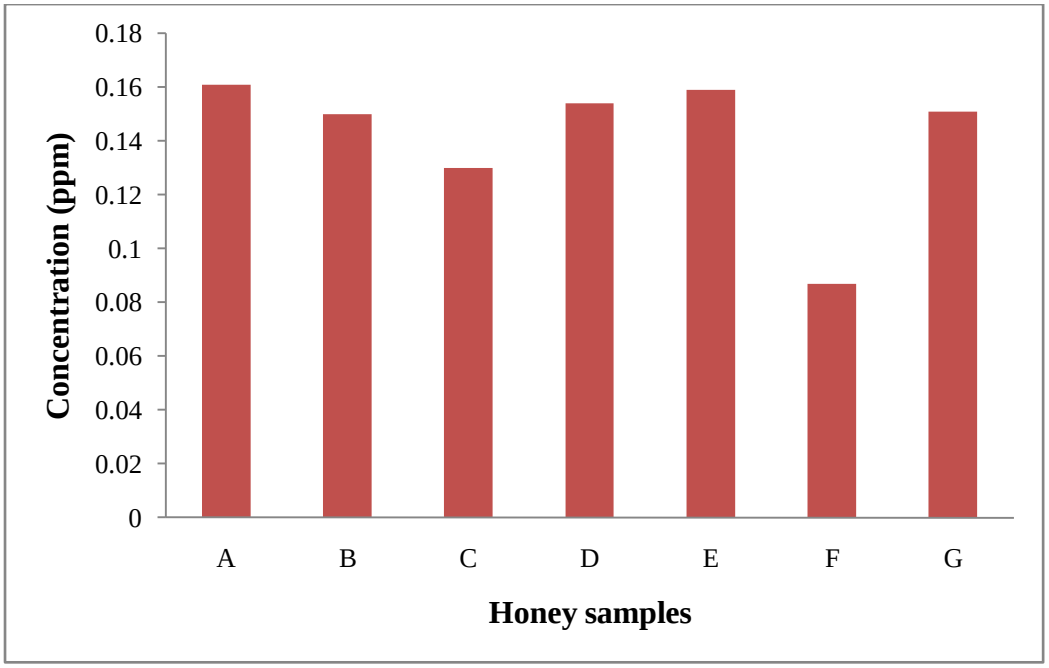


Figure 4.12: Zinc Concentration in Examined Samples.

4.2.5 Concentration of Copper in Examined Honey Samples

Copper is another element with numerous biochemical functions in living organisms. Other than storing in liver, it helps in absorption of iron in the intestine which will combine with haemoglobin and then transport from tissues to plasma. With the presence of copper, immune system of organisms can be strengthening as well. For plants, copper helps in growth and can be found in protein structures (Altundag et al., 2016). However, over-digestion of copper can bring adverse effects to health. Excess copper will put off the performance of some enzymes. Adverse effects caused by copper are much influenced by interaction with other minerals in food. Excess concentration of copper can cause nausea, haemoglobinuria, coma or even death. The fatal oral human intake of copper is 200mg/kg, concluded by WHO (1974). For infants (<6 months), the daily intake is recommended at 0.5 to 0.7 mg while for adults, the intake can be tolerated up to 3 mg (Codex Alimentarius Commission, 2011). A person is recommended to intake 1-1.5 mg/day of copper to maintain routine activities (Altundag et al., 2016).

Based on the findings, the concentrations of copper ranged between 0.03 and 0.04 ppm. According to Codex Alimentarius Commission (2011), the PMTDI for copper in food was 0.05 to 0.5 ppm. The results obtained were

below the tolerable daily intake. PMTDI is the maximum permissible of certain substance exposes to human in food. Therefore, all honey samples were harmless but deficiency of copper in daily food intake might cause anaemia, lymphoma and neutropenia (Mohamed, Haris and Brima, 2019). On the contrary with zinc concentration, dark raw unprocessed honey F had the highest concentration of copper (0.039ppm). Although honey F was the richest in copper, the concentration was still considered very low. The condition of soils for the plants where the honey was extracted from may affect the content of copper in honey. Figure 4.13 showed the content of copper in honey investigated.

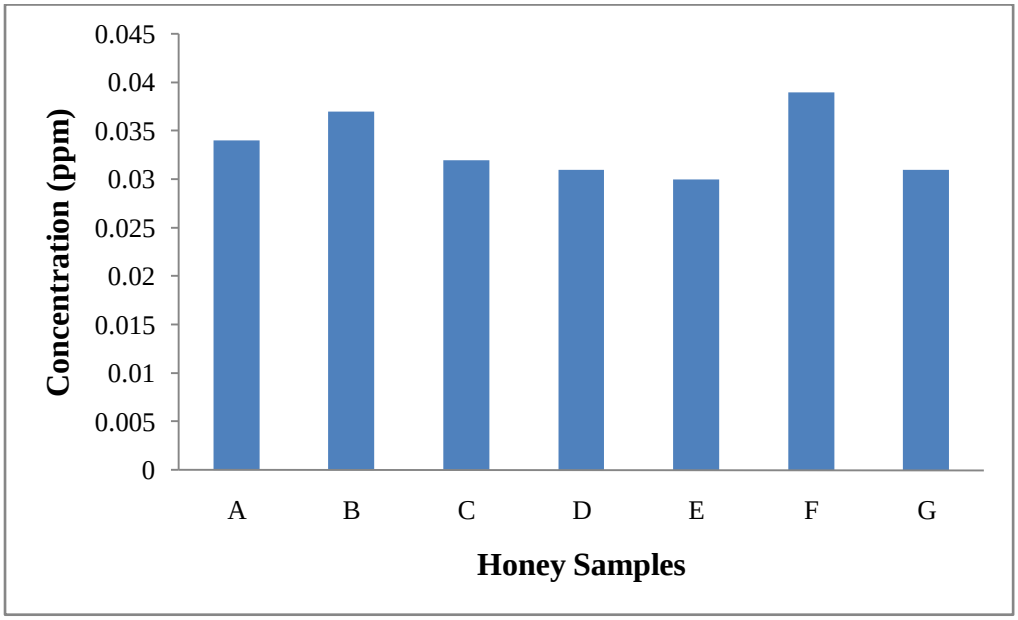


Figure 4.13: Copper Concentration in Examined Samples.

4.2.6 Concentration of Nickel in Examined Honey Samples

Unlike zinc and copper, nickel is the micro-nutrient present in honey. Nickel is available in the water, soil and air. It will distribute through soil profile and affect the concentration in honey. Nickel is important for living organisms at low concentration but there is no scientific evidence to support the case of nickel deficiency. The most noteworthy route of exposure for nickel is through inhalation where lung and respiratory tract are the target organs. For oral intake, ingestion of excess nickel can cause gastric irritation. Daily dietary

intake of nickel has been estimated at 200 to 300 µg/ day (World Health Organization, 2000).

In this study, the concentration of Nickel was found to be the lowest 0.028 ppm and the highest 0.123 ppm in the honey samples. Multi-floral honey A from Taiwan had the highest content of nickel (0.123ppm), followed by *Apis Cerena* honey B and raw unprocessed honey G from Malaysia. Honey A led the trend much higher than the other honey samples. Similarly to zinc concentration, dark raw unprocessed honey F had the least nickel (0.028ppm). According to World Health Organization (2000), nickel content shall be less than 0.5 ppm. All analysed honeys fell within the standard range. Figure 4.14 showed the concentration of nickel in examined honeys.

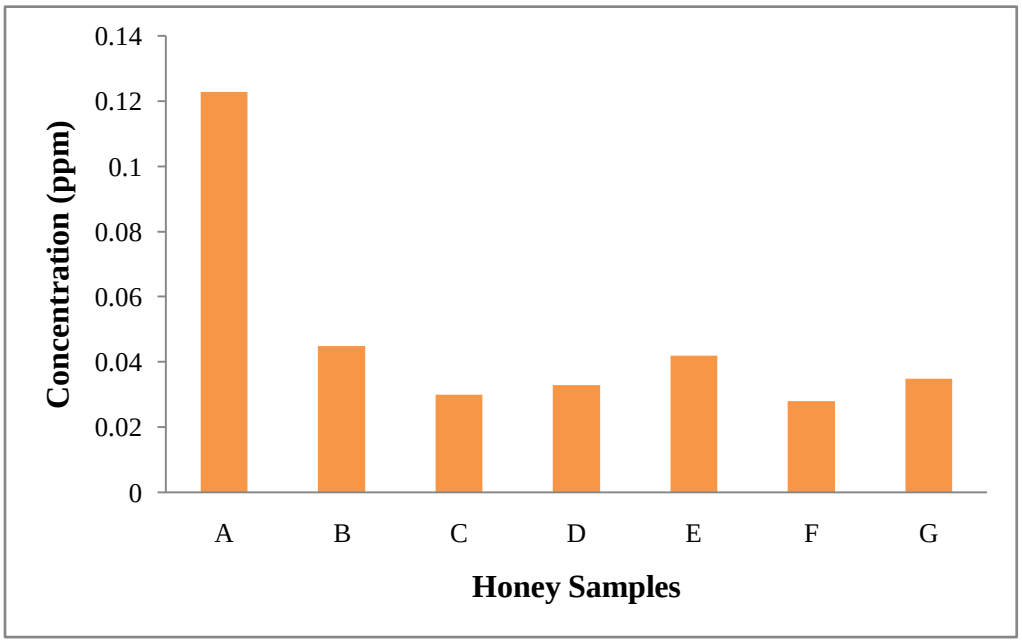


Figure 4.14: Concentration of Nickel in Examined Samples.

4.2.7 Concentration of Chromium in Examined Honey Samples

In the Environmental Protection Agency, chromium is listed as one of the 14 most harmful heavy metals. Chromium is commonly present as trivalent form including in foodstuffs. It is one of the trace elements that are vital to living organisms. Chromium (III) helps to convert glucose into energy, maintain blood glucose and pressure levels as well as body mass by suppressing appetite. It was found to be an important element in treating type 2 diabetes mellitus (Swaroop et al., 2019). Unlike chromium (VI), chromium (III) does

not possess any adverse effect to human health. In a study of workers exposed to insoluble chromite ore with both chromium (III) and (VI), only 10% of them had ulcer formation and 6% has hypertrophic gastritis. The route of exposure for this study was through inhalation where the workers breathed using mouth and ingested the insoluble chromate dust (Wilbur et al., 2012). The daily intake of chromium (III) for adults is recommended as 50 to 200 µg/ day (World Health Organization, 2000).

Based on the results obtained, chromium concentration is honey ranged between 0.035 ppm to 0.045 ppm. Again, the multi-floral honey A from Taiwan had the highest concentration of chromium, followed by *Apis Cerena* honey B (0.039 ppm). Stingless bee honey C had the least amount of chromium. According to EVM, the Expert Group on Vitamins and Minerals, the chromium content in food shall be less than 0.15ppm (Aghamirlou et al., 2015). All studied honey samples were considered to be in an acceptable range. Figure 4.15 denoted the concentration of chromium in analyzed honeys.

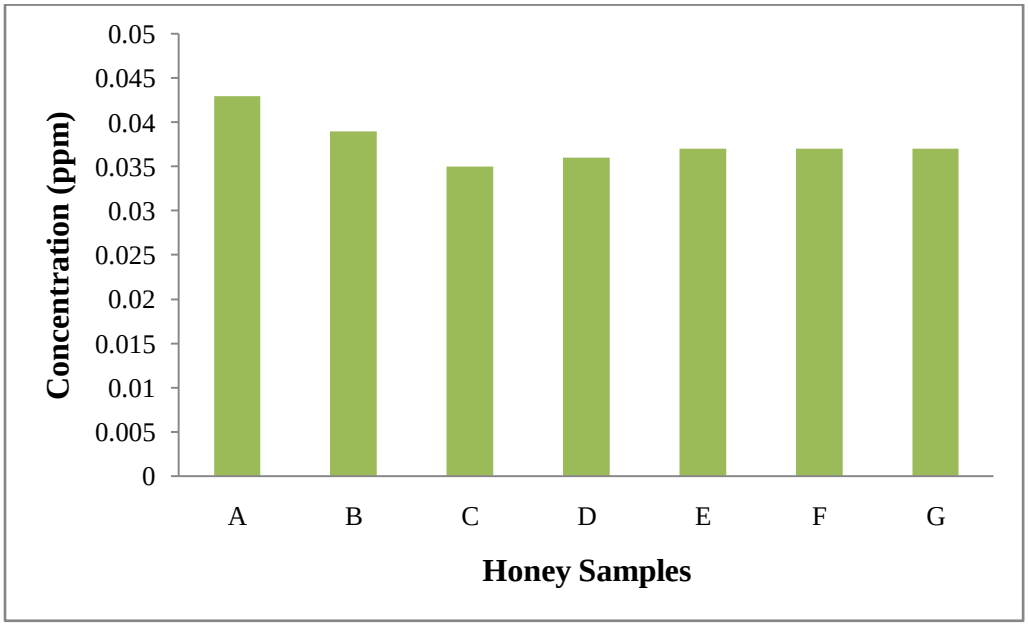


Figure 4.15: Chromium Concentration in Examined Samples.

4.3 Determination of Antioxidants in Honey

Many determination and quantification methods have been used to evaluate antioxidant activities of honey. Antioxidants present in honey can be classified into enzymatic and non-enzymatic substances. Phenolic compounds and carotenoid belong to non-enzymatic antioxidants (Ferreira et al., 2009). Antioxidants play important role in preventing and curing oxidative stress-related diseases such as cancer, cardiovascular diseases and liver diseases (Hagr et al., 2017).

4.3.1 Overview of Antioxidants in Honey

Results obtained for total phenolic content and carotenoids in investigated honey were tabulated in detail in Appendix F and G. Graphical results of antioxidants were illustrated in Figure 4.16.

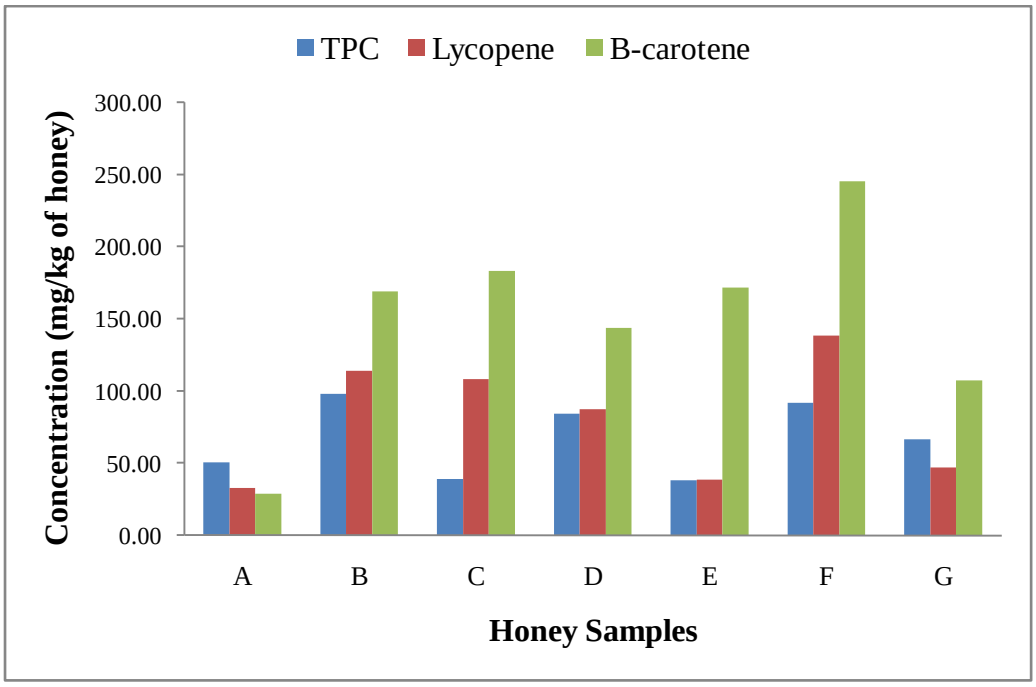


Figure 4.16: Concentration of Antioxidants in Examined Honey.

4.3.2 Total Phenolic Content in Honey

Honeys possess antioxidant properties due to the antioxidants present in it such as phenolic compounds. Phenolic compound is available in honey because it is extracted from the plants by bees. Since total phenolic content is correlated to the antioxidant properties of honey, it can be used as a

dependable parameter to specify antioxidant activities in honey. Determination of total phenolic content is said to be the simplest and quickest technique to assess total phenol in complex matrix like honey. For the quantification of total phenolic content in honey, Folin-Ciocalteu spectrophotometric assay is widely used since 2005 (Chis and Purcarea, 2016). It is widely utilized in UV/Vis spectroscopy due to low-cost, fast and ease of performance. Phenolic compound reacts with Folin-Ciocalteu reagent to produce a blue complex and this reaction is known as colorimetric reaction. Folin-Ciocalteu method depends on the movement of electrons in alkaline medium from phenolic compound to form the blue complex. The blue complex formed is made up of phosphotungstic-phosphomolybdenum complex. The concentration of phenolic compounds and the alkaline solution affects the absorbance of samples. Due to the possibility of decomposition of Folin-Ciocalteu in alkaline solutions, lithium salts are included in the reagent to prevent turbidity that will affect the absorbance of samples (Blainski et al., 2013). Gallic acid was employed as the reference standard and thus, results were expressed as in gallic acid equivalents (GAE). The calibration curve of gallic acid with concentration of 0, 50, 100, 150, 200 and 250 mg/L were shown in Figure 4.17.

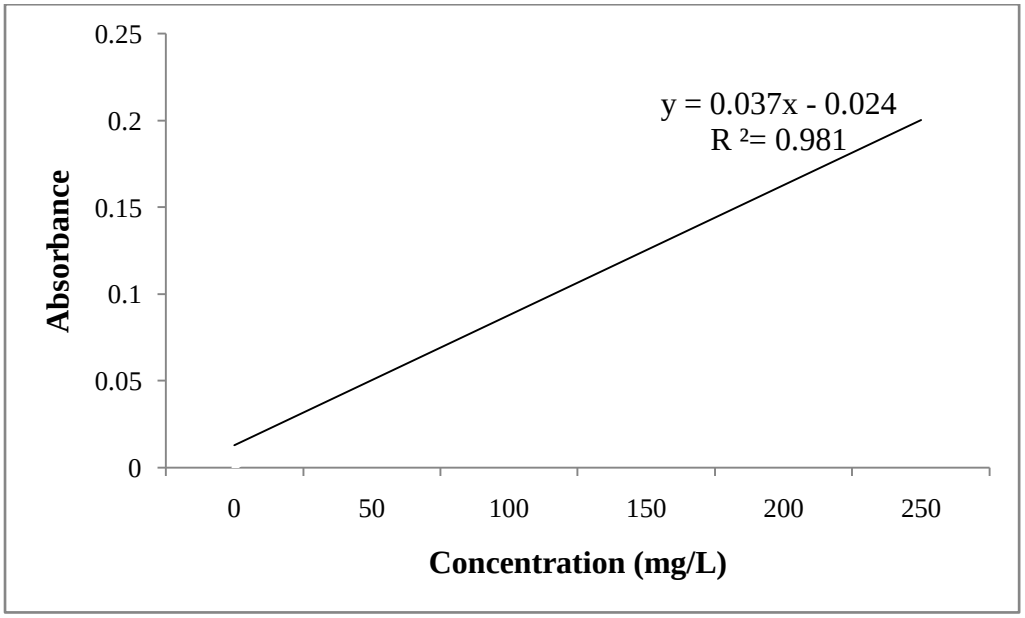


Figure 4.17: Calibration Curve of Gallic Acid as the Reference Standard.

From the analysis of Figure 4.18, a significantly higher total phenolic content could be observed in 3 honeys: *Apis Cerena* honey B (97.89mg GAE/ kg honey), Tualang honey D (84.38mg GAE/ kg honey) and dark raw unprocessed honey F (91.84mg GAE/ kg honey) from Malaysia. Lowest concentration of total phenolic content, approximately 38 mg GAE/ kg honey was found in stingless bee honey C and local multi-flora honey E. Phenolic compounds is vital and responsible for therapeutic properties of honey as natural oxidants. Hence, honey B, D and F with higher total phenolic content had higher antioxidant activity by inhibiting oxidative reactions of free radicals and thus reducing DNA damage. High phenolic content in these three honeys also demonstrated greater anti-viral activity in inhibiting HIV replication, anti-inflammatory activity in wound healing and anti-ulcer activity in treating gastricis, gastric mucosa and duodenal ulcers (Jibril, Hilmi and Manivannan, 2019). Hence, honeys with lesser phenolic compound were concluded to have lesser therapeutic properties and medical importance. The difference in total phenolic content in tested honeys might be influenced by collection region, floral source and harvest technology. Although honey F and G came from the same floral source, the difference in total phenolic content might be affected by storage time, storage mode and collection season (Kek et al., 2014). Concentrations of total phenolic content of honey A to G were illustrated as in Figure 4.18.

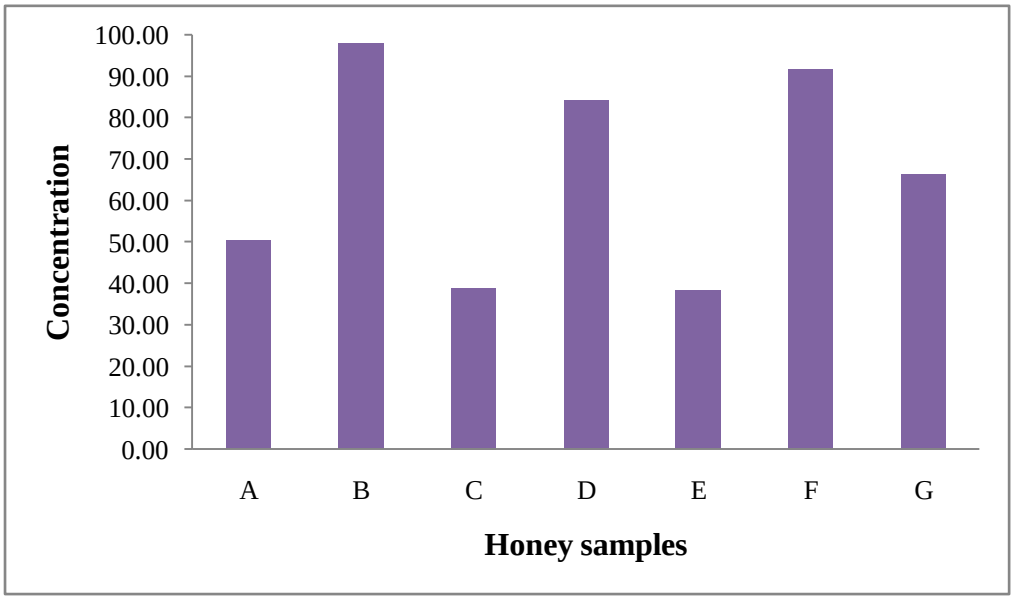


Figure 4.18: Total Phenolic Content per Gallic Acid Equivalent.

4.3.3 Concentration of Carotenoids in Honey

Carotenoid is one of the important antioxidants for living organisms but is unable to be synthesized by the organism itself. Therefore, it can be only obtained through dietary intake. Due to the hydrophobic nature of carotenoid, only organic solvents can extract it for analysis (Saini and Keum, 2018). There are various organic polar solvents can be used: acetone, dichloromethane and mixture of solvents such as hexane/acetone, acetone/methylic alcohol and dichloromethane/methylic alcohol). In this study, mixture of solvents (acetone/hexane) with ratio of 4:6 is used. Carotenoids are classified into two groups: hydrocarbon carotenoids (40 carbon atoms) and oxygenated derivatives of the hydrocarbon type. Among them, lycopene and β-carotene are the two significant hydrocarbon carotenoids and their concentrations were determined in this study (Butnariu, 2016).

Based on the findings, the concentration of lycopene in tested honeys ranged between 30 to 140 mg/ kg honey. The highest concentration was found in dark raw unprocessed honey F (138.3 mg/kg), followed by *Apis Cerena* honey B (113.9 mg/kg) and stingless bee honey C (108.1 mg/kg). Multi-floral honey A from Taiwan had the least lycopene. For β-carotene, the overall concentration ranged between 28 to 250 mg/kg honey. The concentration was higher than that of lycopene which was similar to the findings by Butnariu (2016) and Ferreira et al. (2009). Similar to lycopene, dark raw unprocessed honey F was the richest in β-carotene (245.6 mg/kg), followed by stingless bee honey C (183.3 mg/kg), local multi-floral honey E (171.1 mg/kg) and *Apis Cerena* honey B (169 mg/kg). Multi-floral honey A from Taiwan had the least content of β-carotene with only 28.76 mg/kg. From the results, dark raw unprocessed honey F had the highest concentration of carotenoid among seven honeys. Similar to phenolic compound, high concentration of carotenoids contributed to better antioxidant properties of honey which could protect human body from free radicals damage. Among the antioxidants, carotenoids specifically help in reducing the risk of heart disease, improving eye sight and contributing to stronger bones (Petre, 2018). The difference in concentration of carotenoids in each sample might be affected by weather, geographical factor and environmental factors (Boussaid et al., 2018). In short, dark raw

unprocessed honey F possessed the greatest antioxidant properties with highest content of phenolic compounds and carotenoids. Figure 4.19 illustrated the concentrations of both lycopene and β-carotene in each sample.

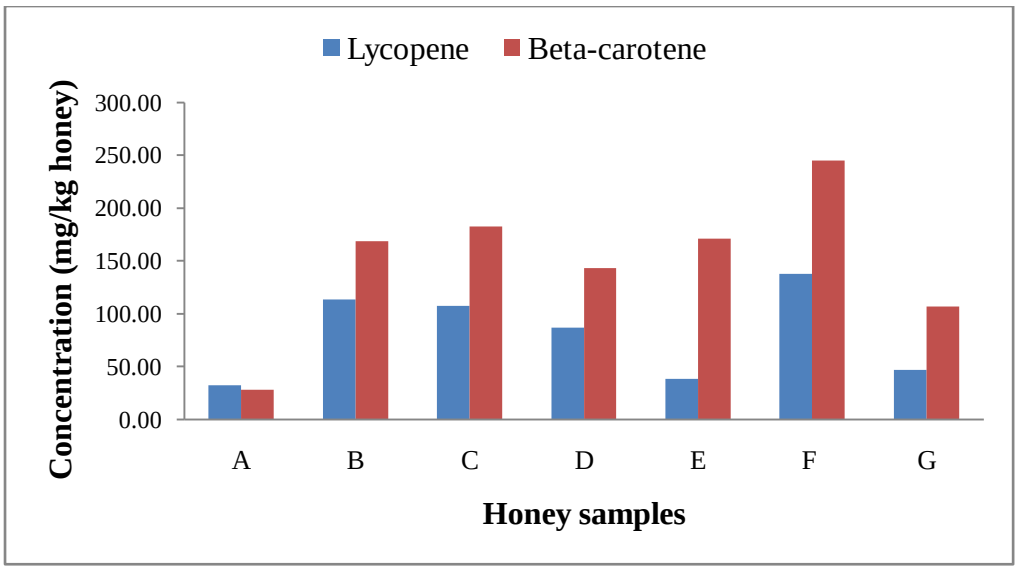


Figure 4.19: Carotenoids Content in Examined Samples.

4.4 Analysis of pH

Quality of honey closely related to the acidity of honey. Hence, the pH values were detected in contemplation of adulteration studies. Direct measurement of pH is more favourable than determine the acidity of honey due to its low complexity (Prica et al., 2014). Appendix H outlined the triplicate results of pH.

4.4.1 Variable pH of Honey

The low pH in honey is caused by the presence of organic acid arise from nectar or bees' secretions. Some of the acids were formed during storage and is strongly affected by storage environment and condition of processing. According to Živkov-Baloš et al. (2018), darker honey has higher acidity than lighter honey. Besides, acidity of honey tends to increase as it grows older and is also caused by fermentation process of sugars into organic acid. Determination of pH in honey is a quality indicator as well as the pH will change in adulterated honey. According to Yadata (2014), honey adulterated with inverted sugar has lower pH while higher pH for honey adulterated with

sugar syrup. The pH of honey causes variation in honey shelf-life, texture and stability (Salazar et al., 2017).

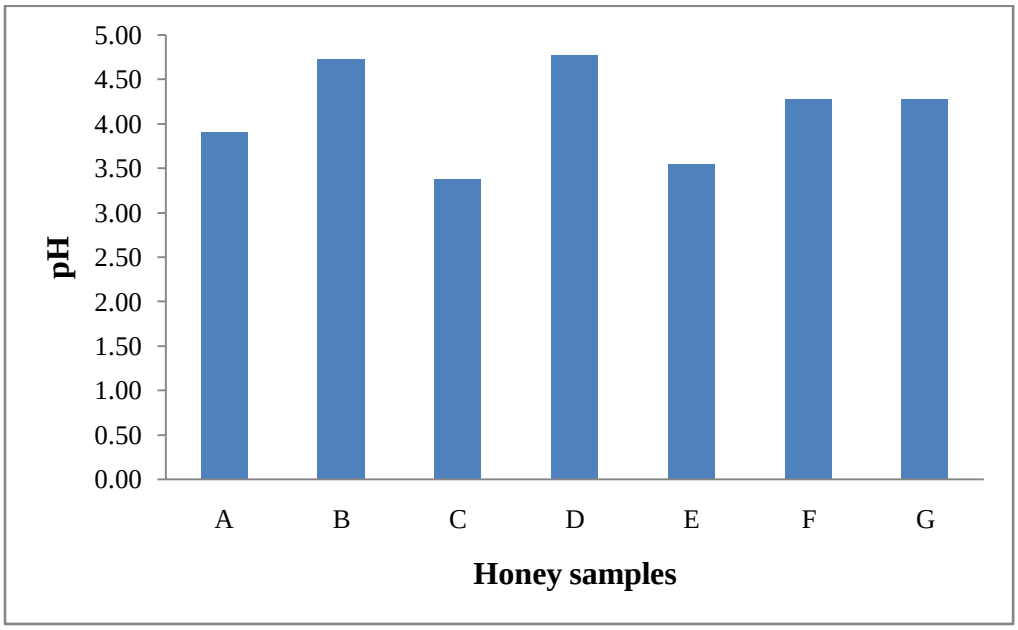


Figure 4.20: The pH Values of Examined Samples.

From the analysis of Figure 4.20, it could be noticed that stingless bee honey C was the most acidic with pH value of 3.38, followed by local multi-floral honey E (pH 3.55) and multi-floral honey A (pH 3.90). The lowest acidity was found in Tualang honey D (pH 4.78) and *Apis Cerena* honey B (pH 4.72). The pH values of all investigated honey samples except sample C fell within the standard limit of 3.40 to 6.10 (Codex Alimentarius Commission, 1981). Generally, low pH brought advantages in inhibiting microorganisms growth and proliferation (Boussaid et al., 2018). Furthermore, relatively low pH might indicate the improper way of storage, low purity, high concentration of minerals and fermentation of sugars (Aljohar et al., 2018).

4.5 Analysis of Electrical Conductivity

Electrical conductivity is another good indicator of quality and purity of honey. Hence, the conductivity was detected in contemplation of routine quality control. Appendix I outlined the triplicate results of electrical conductivity.

4.5.1 Variable Electrical Conductivity of Honey

Since honey contains minerals and organic acids, they dissociated into ions whenever they are in aqueous solution. Dissociated ions conduct electricity and resulted in conductivity results. Higher concentration of minerals, organic acids and protein resulted in higher electrical conductivity. Therefore, determination of conductivity can distinguish floral origins of honey. According to Živkov-Baloš et al. (2018), dark colour honey has higher conductivity than light colour honey. It was found that linear relationship is noticeable between the conductivity and ash content. Higher acid concentration and higher ash content led to higher conductivity (El Sohaimy et al., 2015).

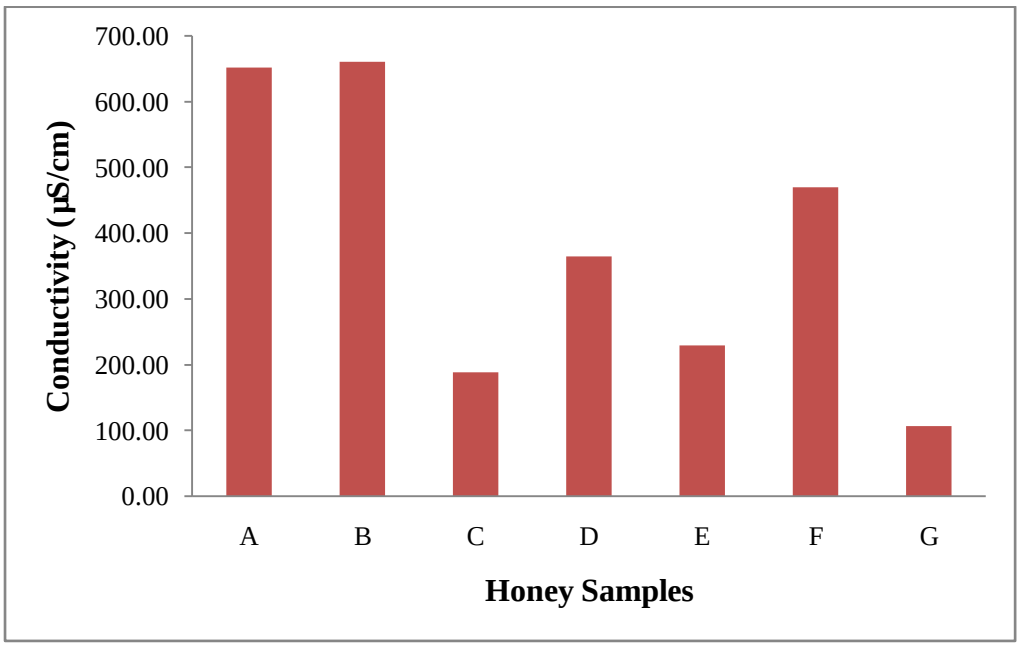


Figure 4.21: Electrical Conductivity on Examined Samples.

Based on Figure 4.21, bright raw unprocessed honey G and stingless bee honey C had the lowest conductivity values, 107.17 and 188.43 µS/ cm respectively. Honey G and C had the brightest colour among all honey samples. This was supported by Živkov-Baloš et al. (2018) who mentioned that bright colour honey has lower conductivity than dark colour honey. *Apis Cerena* honey B had the highest conductivity (661.33 µS/cm), followed by multi-floral honey A from Taiwan (652.00 µS/cm). The results fell within the standard limit of not more than 800 µS/cm (Codex Alimentarius Commission,

1981). Electrical conductivity was closely correlated to the concentration of minerals and organic acids. Honey G had the best quality based on electrical conductivity.

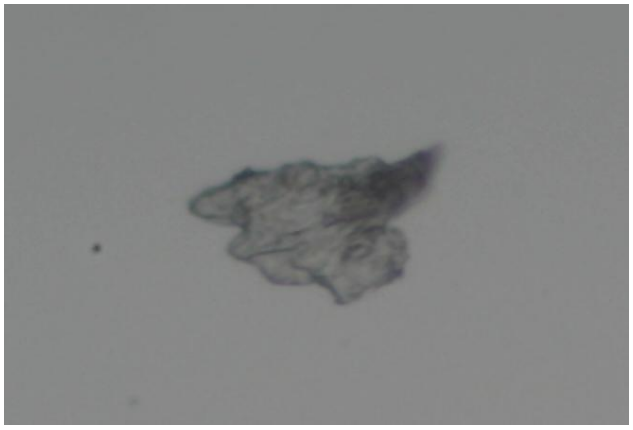
4.6 Analysis of Pollen

Pollen was collected by honey bees while they extract nectar from flowers and remained in honey. It is produced by the male reproductive organs of flora, anther and each of it has unique shape and size. Identification of honey is widely used to determine the botanical origin or geographical origin of honey. This analysis is an indicator of honey quality as it helps to determine contamination in honey (Rosdi et al., 2016). The wall of pollen is made up of three layers: sexine, nexine and intine. Most of the pollen grains could be identify by morphology in the sexine which is the outermost layer (Weber, 1998). Light microscopy is the least complex method and huge amount of samples can be analyzed. Therefore, it is popular for routine laboratory analysis (Sharma and Bhat, n.d.).

4.6.1 Identification & Characterization of Pollen

Identification of pollen was performed by comparing the microscopy image with published references of pollen and flora description. Figure 4.22 showed the microscopy images of pollen identified in each honey sample. Pollen grain identification could be done by observing the shape of pollen grains. Clearly, the pollen shape of stingless bee honey C was triporate and this indicated that it belongs to the family of *Turnera subulata* (Sharma and Bhat, n.d.). The shape of pollen obtained for Tualang honey D was tetragonal tetrad. With this shape, the flora family of honey D was Fraxinus with reference to Kiew and Muid (1991). Next, the shape of pollen for multi-floral honey E was perprolate which reflected the species of coconut (Rosdi et al., 2016). Lastly, the pollen shapes for raw unprocessed honey F and G were the same. Although the shapes were irregular, but they could be identified to be the floral family of *Pinus* after comparing with the result obtained by Hayley (2019). Unfortunately, pollen type of honey sample A and B were undetectable due to the low resolution of image.

(a)



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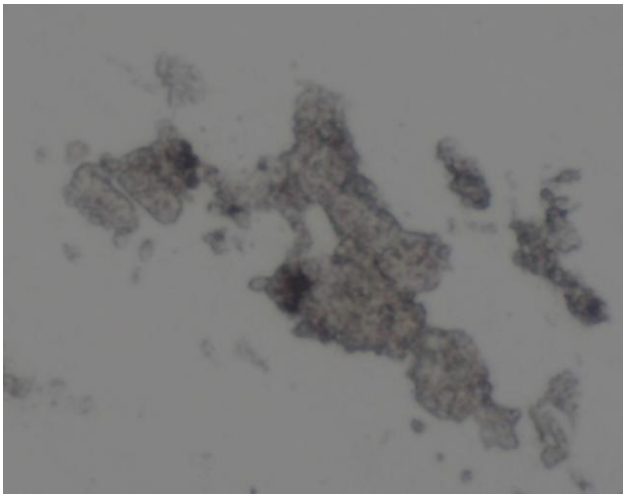

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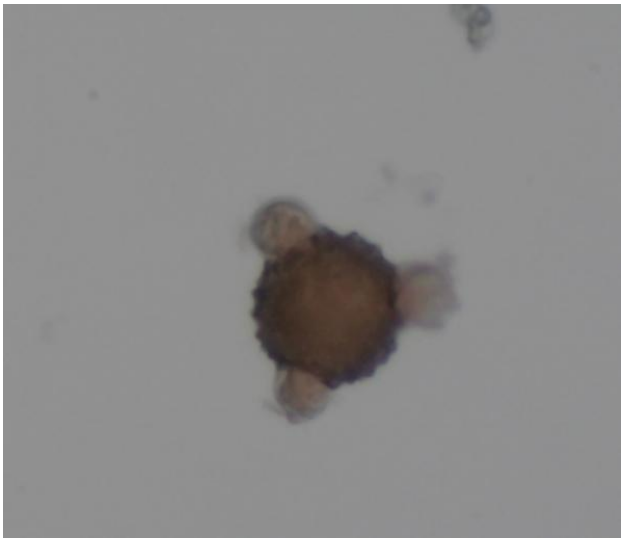

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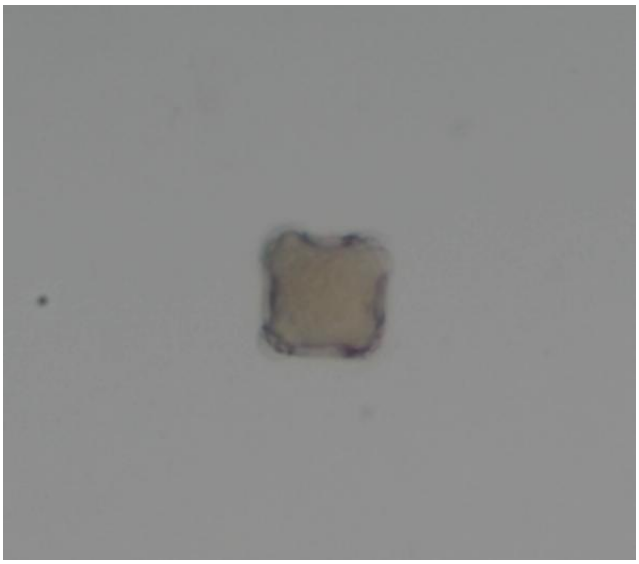

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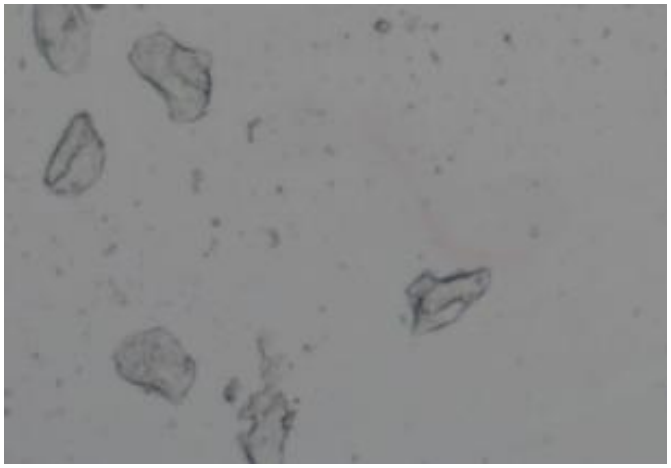

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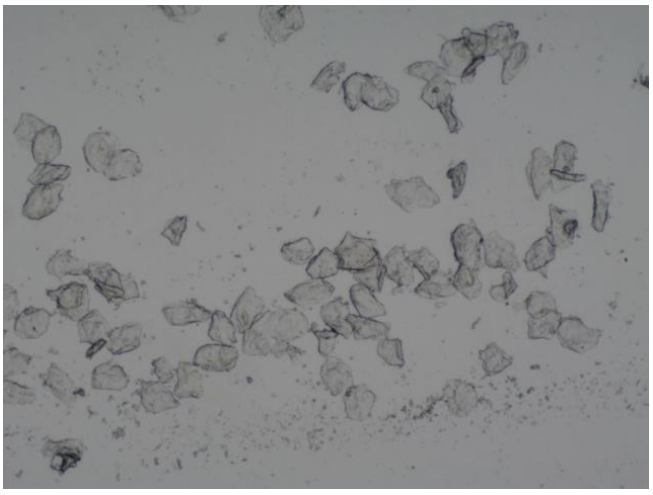

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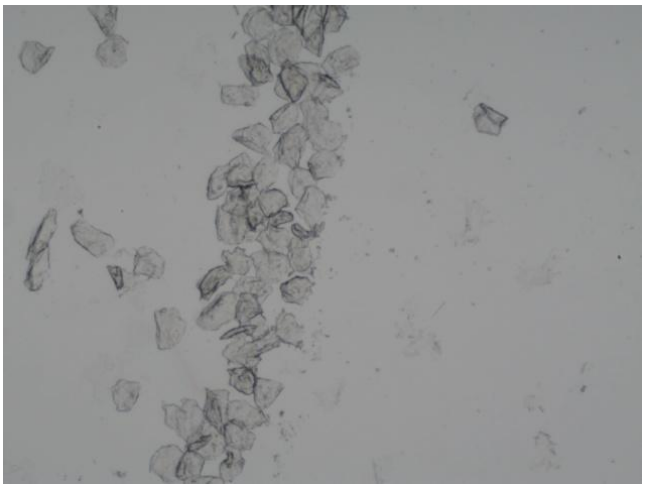

Figure 4.22: Light Micrographs of (a) Taiwan Multi-floral honey A, (b) *Apis Cerena* honey B, (c) Stingless bee honey C, (d) Tualang honey D, (e) Local Multi-floral honey E, (f) Dark raw unprocessed honey F and (g) Light raw unprocessed honey G.

The botanical origins of other honey samples were summarized and tabulated in Table 4.4.

Table 4.4: Botanical Origin of each Honey Sample.

Honey Sample	**Type of Honey**	**Botanical Origin (flora family)**
A	Multi-floral Honey (Taiwan)	Unknown
B	*Apis Cerena* Honey	Unknown
C	Stingless Bee Honey	*Turnera subulata* (passion flower)
D	Tualang Honey	*Fraxinus* (olive and lilac)
E	Multi-floral Honey (Malaysia)	*Cocos nucifera* (coconut)
F	Raw Unprocessed Honey (dark)	*Pinus* (Pine)
G	Raw Unprocessed Honey (light)	*Pinus* (Pine)

CHAPTER 5

CONCLUSION

5.1 Conclusion

The core of this study was to investigate the physiochemical parameters of six honey samples from Malaysia and one sample from Taiwan. Through various analysis techniques, different content of sugar, antioxidants and heavy metals, different values of conductivity and pH as well as their pollen types were observed in the tested honey samples. Composition of honey changes from one location to another.

The sugar content of seven types of honey samples was estimated by two methods which are Fehling Test and UV/Vis Spectroscopy. From the result of Fehling Test, highest amount of reducing sugar was found in market-source honey while the least was in multi-floral honey A from Taiwan. According to Codex Alimentarius Standard, total reducing sugars in pure honey should be more than 60% (Codex Alimentarius Commission, 1981). Only local multi-floral honey E, dark and light raw unprocessed honey F and G met the standard of at least 60% reducing sugars. However, this method might not be accurate enough to determine the content of sugar in honeys due to the presence of non-reducing sugar, sucrose. Fehling Test only evaluated the content of reducing sugars in honeys. Therefore, UV/Vis spectroscopy was introduced as the second stage to further determine the content of individual sugars in honeys. The contents of glucose, fructose and sucrose as well as fructose/glucose ratios were evaluated. Fructose affects the sweetness of honey. Source of nectar affects the content of glucose in honey. Adulterated honey tends to have higher sucrose level. The contents of each individual sugar were in the standard range specified in Codex Standard or other journal articles. Apart from that, fructose-to-glucose ratio was determined as it acts as the most important quality evaluation parameter. Lower ratio increases the chances of crystallisation in honey. Lowest ratio was found in the local multi-floral honey E and crystallisation was observed during storage. This implies that the quality of honey sample E is not as good as other honey samples.

Current study showed that all honey samples contain heavy metals with different concentration among different regions. ICP-OES was applied in this study for elemental analysis. Findings indicated that Zn and Cu had the most and least concentrations in each sample. The differences of metal concentrations might be due to types of soils and fertilizers used, way of growing the plants and distance between the floral origin and industries. All examined honeys give good source of minerals. Overall, lowest level of elements was found in dark raw unprocessed honey F from Malaysia which implies its high purity. Highest amount of trace element was found in multi-floral honey A from Taiwan. As a summary, results from this study showed that the contents of trace elements were within the allowable limit set by WHO/Codex standard on daily intake by human.

The antioxidant properties of honey were resulting from the presence of antioxidant compounds like carotenoids, flavonoids and polyphenolics. These antioxidant compounds help in wound healing, slowing down aging, preventing cardiovascular diseases and cancer. In this study, the antioxidant compounds in honeys were analyzed by UV/Vis Spectroscopy. Antioxidants analyzed include lycopene, β-carotene and phenolics. Among the analyzed antioxidants, phenolic compounds play the most important role in affecting antioxidant properties of honeys although the content is very low. Overall, dark raw unprocessed honey F had the highest concentration of antioxidants while honey A had the least content. To be specific, highest amount of lycopene and β-carotene were found in honey F but *Apis Cerena* honey B is rich in phenolic compound. Hence, it could be concluded that honey F is a better source of antioxidants than other tested honeys.

Acidity and pH value of honey are useful parameters for evaluating honey quality. However, determination of pH has less complexity than acidity. Hence, pH is measured instead of acidity in current study. Variety of pH could be affected by source of nectar and pH of soil. Findings indicated that all investigated honey samples in this study had pH value within the standard limit of 3.40 to 6.10 which is set by Codex standard. Low pH value could help in inhibiting the growth of microorganisms.

Electrical conductivity of honey is correlated to the amount of mineral and organic acids in honey. Higher amount of minerals resulted in higher

conductivity. Besides, honey dark in colour will have higher conductivity than light colour honey. Among the seven honey samples, Taiwan multi-floral honey A and *Apis Cerena* honey B had the uppermost conductivity readings but did not exceed standard limit of 800 μS/cm. Smallest conductivity value was observed in light raw unprocessed honey G with only 107.17 μS/ cm. It is also one of the light colour honey among seven samples. This confirmed the statement of lower conductivity in light colour honey than dark honey.

This study ended with the determination and characterisation of pollen present in honey. Pollen was collected by honey bees while they extract nectar from flowers. Therefore, the shape and characteristic of pollen found in honey can be used to identify the floral source of honey. Light microscopy was applied in identifying the pollen. Unfortunately, pollen in honey sample A and B could not be used to identify the floral source due to the low resolution of image. However, the floral source of the remaining honey samples could be identified successfully based on the shapes and characteristics of the pollen observed.

In conclusion, no contamination was identified in all tested honey samples. All tested honeys are safe to consume. Meanwhile, dark raw unprocessed honey F has the most excellent quality among seven tested honey by having least heavy metals, highest amount of antioxidant compounds, low pH and low fructose-to-glucose ratio. On the other hand, local multi-floral honey E and Taiwan multi-floral honey A are the honeys with lower quality due to highest fructose/glucose ratio and highest heavy metal concentration & lowest antioxidants respectively.

5.2 Limitation and Recommendation

Measurement of experimental results in term of precision and reliability is achievable by proper operating procedure and recommendations. Uncertainties and contextual factors in current study indeed require few improvements to be suggested.

In the analysis of sugar content of honey, although UV/Vis spectroscopy is widely used in determination of individual sugars due to its simplicity, but it does reduce the accuracy of results due to the overlapping of

spectra of sucrose, glucose and fructose. UV/Vis method spectrophotometer will not distinguish between the interest compound and contaminants which absorb at the certain wavelength. If the uninterested compound reflects light to the detector, the reading is still recorded. Chromatographic techniques are still the most powerful analytical method for the analysis of carbohydrates in food. High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) are commonly applied to separate and identify carbohydrates with high accuracy and precision. Carbohydrates can be separated significantly for better analysis result. For the analysis of carbohydrates, HPLC with refractive index detector or pulsed amperometric detector and amino columns are best suited for the analysis of sugars.

Pollen identification was done after simple sample preparation using centrifugation. Pollen analysis was performed on the supernatant itself without acetolysis method. In fact, for better extraction of pollen from honey, acetolysis method is more recommended and suitable. After first time of centrifugation, glacial acetic acid and potassium hydroxide is needed to add to the residue. Then, boil the residue for 5 minutes and undergo second stage of centrifugation. The final residue is then coats on glycerine jelly before microscopy observation (Rosdi et al., 2016). The images of pollen arise from acetolysis method are clearer and more distinctive. Pollen identification and characterization can be done with ease.

Ideally, succeeding recommendations are proposed to improve validity of results.

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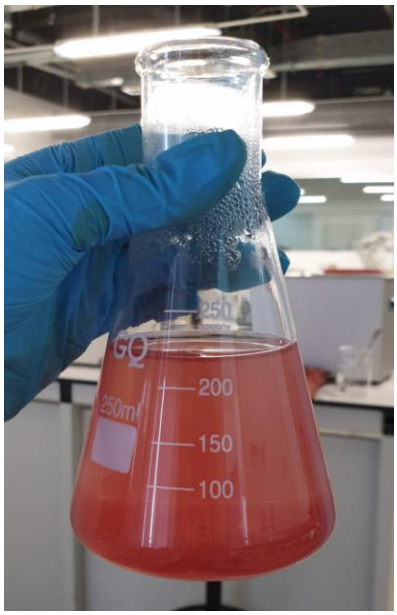
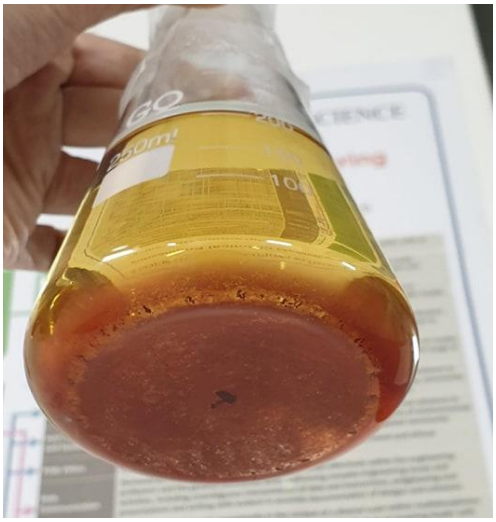
APPENDICES

APPENDIX A: Colour Changes of Honey Sample in Fehling Test

Deep blue colour disappeared and rust red precipitate, Cu_2O appeared



End point of titration with yellowish solution and rust red precipitate



APPENDIX B: Calculations and Detail Results for Fehling Test.

First procedure				Second procedure				Total reducing sugar %
Sample	**W1 (g)**	**Y1 (mL)**	**C1(g/100g honey)**	**Sample**	**W2 (g)**	**Y2 (mL)**	**C2(g/100g honey)**	
A	1.010	60.60	408.46	**A**	1.010	22.60	87.62	**21.45**
B	1.010	58.00	426.77	**B**	1.010	16.60	119.29	**27.95**
C	1.000	49.00	510.20	**C**	1.000	13.20	151.52	**29.70**
D	1.010	74.00	334.49	**D**	1.010	18.20	108.80	**32.53**
E	1.010	57.00	434.25	**E**	1.010	4.80	412.54	**95.00**
F	1.005	85.60	290.60	**F**	1.005	7.80	255.13	**87.79**
G	1.005	80.40	309.40	**G**	1.005	9.20	216.31	**69.91**

Sample Calculation of Total Reducing Sugars (%) in Honey (Aljohar et al., 2018)

W1 = weight of honey sample used in first procedure
W2 = weight of honey sample used in second procedure
Y1 = volume of diluted honey sample solution consumed in first procedure
Y2 = volume of diluted honey sample solution consumed in second procedure
C1 and *C2* = gram reducing sugar/ 100g of honey

Honey Sample A

$$C1 = 25 \times 1000/W1Y1$$
$$= 25 \times 1000/(1.010)(60.60)$$
$$= 408.46 \frac{g}{100g} honey$$

$$C2 = 2 \times 1000/W2Y2$$
$$= 2 \times 1000/(1.010)(22.60)$$
$$= 87.62 \frac{g}{100g} honey$$

$$Total\ reducing\ sugars\% = {}^{C2}/_{C1} \times 100\%$$
$$= {}^{87.6194}/_{408.4567} \times 100\%$$
$$= 21.45\%$$

APPENDIX C: Calibration Data for Sucrose, Fructose and Glucose.

Sucrose

Concentration (mg/L)	Absorbance 1	Absorbance 2	Absorbance 3	Average
0	0.0000	0.0000	0.0000	0.0000
1000	0.1047	0.1046	0.1046	0.1046
2000	0.3224	0.3221	0.3221	0.3222
3000	0.6085	0.6089	0.6086	0.6087
4000	0.7686	0.7687	0.7688	0.7687
5000	0.9274	0.9279	0.9250	0.9268

Fructose

Concentration (mg/L)	Absorbance 1	Absorbance 2	Absorbance 3	Average
0	0.0000	0.0000	0.0000	0.0000
1000	0.7853	0.7851	0.7877	0.7860
2000	1.2044	1.2073	1.2104	1.2074
3000	1.5767	1.5690	1.5712	1.5723
4000	1.9760	1.9834	1.9645	1.9750
5000	2.2703	2.2551	2.2560	2.2605

Glucose

Concentration (mg/L)	Absorbance 1	Absorbance 2	Absorbance 3	Average
0	0.0000	0.0000	0.0000	0.0000
1000	0.2026	0.2024	0.2025	0.2025
2000	0.4921	0.492	0.492	0.4920
3000	0.7902	0.7905	0.7904	0.7904
4000	0.9454	0.9454	0.9458	0.9455
5000	1.3292	1.3298	1.3304	1.3298

APPENDIX D: Calculations and Detail Results for Determination of Sucrose, Fructose and Glucose

Sucrose

Sample	Absorbance 1	Absorbance 2	Absorbance 3	Average	Sucrose concentration	Percentage (%)
A	0.2477	0.2475	0.2475	0.2476	2455	**4.909**
B	0.253	0.2533	0.2533	0.2532	2483	**4.966**
C	0.2409	0.2409	0.2409	0.2409	2421	**4.842**
D	0.2518	0.2521	0.2524	0.2521	2478	**4.955**
E	0.2404	0.2405	0.2409	0.2406	2419	**4.839**
F	0.2307	0.2306	0.2303	0.2305	2368	**4.736**
G	0.2233	0.2241	0.2236	0.2237	2333	**4.667**

Sample Calculation: Honey Sample A

By using the equation from calibration curve:

$$y = 0.197x - 0.236 \quad (1)$$

Substitute $y =$ Average absorbance of honey sample A at 580nm into the equation to get *x* which is the concentration of sucrose in the sample.

$x = (y + 0.236) \div 0.197$

$x = 2455\ mg/L$

Given sample solution used is 50mL,

$$\%\ of\ sucrose\ content = \frac{2455mg}{L} \times \frac{1L}{1000mL} \times \frac{100mL}{50mL}$$

$$= 4.909\ \%$$

Fructose

Sample	Absorbance 1	Absorbance 2	Absorbance 3	Average	Fructose concentration	Percentage (%)
A	1.3994	1.4005	1.4021	1.4007	3733	**37.33**
B	1.3861	1.3878	1.3907	1.3882	3704	**37.04**
C	1.3676	1.3675	1.371	1.3687	3659	**36.59**
D	1.3498	1.3501	1.35	1.3500	3616	**36.16**
E	1.3289	1.3272	1.3291	1.3284	3566	**35.66**
F	1.298	1.2988	1.2973	1.2980	3497	**34.97**
G	1.2882	1.2865	1.2884	1.2877	3473	**34.73**

Sample Calculation: Honey Sample A

By using the equation from calibration curve:

$$y = 0.435x - 0.223 \quad (2)$$

Substitute $y =$ Average absorbance of honey sample A at 540nm into the equation to get *x* which is the concentration of sucrose in the sample.

$x = (y + 0.223) \div 0.435$

$x = 3733\ mg/L$

Given sample solution used is 10mL,

$$\%\ of\ sucrose\ content = \frac{3733mg}{L} \times \frac{1L}{1000mL} \times \frac{100mL}{10mL}$$

$$= 37.33\ \%$$

Glucose

Sample	Absorbance 1	Absorbance 2	Absorbance 3	Average	Glucose concentration	Percentage (%)
A	0.8719	0.8729	0.8737	0.8728	4438	**36.99**
B	0.8573	0.8578	0.8585	0.8579	4381	**36.51**
C	0.8314	0.8313	0.8316	0.8314	4280	**35.67**
D	0.8456	0.8464	0.8467	0.8462	4337	**36.14**
E	0.8355	0.8362	0.8368	0.8362	4298	**35.82**
F	0.7977	0.797	0.7988	0.7978	4152	**34.60**
G	0.7637	0.7642	0.7647	0.7642	4024	**33.53**

Sample Calculation: Honey Sample A

By using the equation from calibration curve:

$$y = 0.262x - 0.290 \quad (3)$$

Substitute $y =$ Average absorbance of honey sample A at 490nm into the equation to get *x* which is the concentration of sucrose in the sample.

$x = (y + 0.290) \div 0.262$

$x = 4438\ mg/L$

Given sample solution used is 12mL,

$$\%\ of\ sucrose\ content = \frac{4438mg}{L} \times \frac{1L}{1000mL} \times \frac{100mL}{12mL}$$

$$= 36.99\ \%$$

APPENDIX E: ICP-OES Results for Zinc, Nickel, Copper and Chromium Analysis

Concentration (ppm)					
Samples	**Elements**	**Triplicate**			**Average**
		1	**2**	**3**	
A	Zn	0.161	0.161	0.161	**0.161**
	Cu	0.034	0.034	0.034	**0.034**
	Ni	0.123	0.123	0.123	**0.123**
	Cr	0.042	0.042	0.043	**0.043**
B	Zn	0.150	0.149	0.150	**0.150**
	Cu	0.037	0.037	0.036	**0.037**
	Ni	0.045	0.045	0.045	**0.045**
	Cr	0.039	0.039	0.039	**0.039**
C	Zn	0.130	0.130	0.130	**0.130**
	Cu	0.031	0.032	0.031	**0.032**
	Ni	0.030	0.030	0.030	**0.030**
	Cr	0.034	0.034	0.035	**0.035**
D	Zn	0.154	0.155	0.155	**0.154**
	Cu	0.031	0.031	0.031	**0.031**
	Ni	0.033	0.032	0.032	**0.033**
	Cr	0.035	0.036	0.036	**0.036**
E	Zn	0.159	0.159	0.160	**0.159**
	Cu	0.030	0.030	0.030	**0.030**
	Ni	0.042	0.042	0.042	**0.042**
	Cr	0.037	0.037	0.038	**0.037**
F	Zn	0.087	0.087	0.087	**0.087**
	Cu	0.039	0.039	0.039	**0.039**
	Ni	0.028	0.028	0.028	**0.028**
	Cr	0.037	0.037	0.037	**0.037**
G	Zn	0.151	0.150	0.151	**0.151**
	Cu	0.030	0.030	0.031	**0.031**
	Ni	0.035	0.035	0.035	**0.035**
	Cr	0.037	0.037	0.037	**0.037**

APPENDIX F: Detail Results and Calculation for Determination of Total Phenolic Content (TPC)

Sample	Absorbance				TPC (mg GAE/L)	TPC (mg GAE/ kg honey)
	Reading 1	Reading 2	Reading 3	Average		
A	0.0655	0.0721	0.0702	0.0693	2.521	50.41
B	0.1570	0.1571	0.1572	0.1571	4.895	97.89
C	0.0480	0.0481	0.0479	0.0480	1.946	38.92
D	0.1322	0.1321	0.1320	0.1321	4.219	84.38
E	0.0468	0.0470	0.0465	0.0468	1.913	38.25
F	0.1458	0.1460	0.1459	0.1459	4.592	91.84
G	0.0990	0.0986	0.0988	0.0988	3.319	66.38

Sample Calculation: Honey Sample F

By using the equation from calibration curve:

$$y = 0.037x - 0.024 \quad (4)$$

Substitute $y =$ Average absorbance of honey sample F at 760nm into the equation to get *x* which is the concentration of total phenolic content as in mg/L of gallic acid equivalent (GAE) in the sample.

$x = (y + 0.024) \div 0.037$

$x = 4.592 \frac{mg\ GAE}{L}$

Given sample solution used is 1mL with 0.05g of honey,

$Total\ phenolic\ content\ \left(\frac{mg\ GAE}{kg\ honey}\right)$

$= \frac{4.592 mg\ GAE}{L} \times \frac{1L}{1000mL} \times \frac{1mL}{0.05g\ honey} \times \frac{1000g\ honey}{1kg\ honey}$

$= 91.84\ \frac{mg\ GAE}{kg\ honey}$

APPENDIX G: Detail Results and Calculation for Determination of Carotenoids

Sample	Absorbance			Lycopene (mg/100 mL honey)	Beta-carotene (mg/100mL honey)	Lycopene (mg/kg honey)	Beta-carotene (mg/kg honey)
	453nm	505nm	663nm				
A	0.0013	0.0012	0.0003	0.0033	0.0029	**32.79**	**28.76**
B	0.0054	0.0046	0.0030	0.0114	0.0169	**113.9**	**169.0**
C	0.0056	0.0045	0.0031	0.0108	0.0183	**108.1**	**183.3**
D	0.0046	0.0036	0.0021	0.0087	0.0144	**87.23**	**143.8**
E	0.0048	0.0022	0.0010	0.0039	0.0172	**38.57**	**171.7**
F	0.0079	0.0058	0.0030	0.0138	0.0246	**138.3**	**245.6**
G	0.0035	0.0021	0.0006	0.0047	0.0107	**47.16**	**107.3**

Concentrations of Lycopene and β-carotene

Both carotenoids can be calculated using equations below:

$$lycopene\left(\frac{mg}{100ml}\right) = -0.0458A_{663} + 0.372A_{505} - 0.0806A_{453} \quad (2)$$

$$\beta - carotene\left(\frac{mg}{100ml}\right) = 0.216A_{663} - 0.304A_{505} + 0.452A_{453} \quad (3)$$

Sample Calculation: Honey Sample F

$$lycopene\left(\frac{mg}{100ml}\right) = -0.0458(0.0030) + 0.372(0.0058) - 0.0806(0.0079)$$

$$= 0.0138mg/100mL$$

$$lycopene\left(\frac{mg}{kg\ of\ honey}\right) = \frac{0.0138\,mg}{100\,mL} \times 100 \times \frac{mL}{0.001\,kg}$$

$$= \mathbf{138.3}\,\frac{\boldsymbol{mg}}{\boldsymbol{kg\ of\ honey}}$$

$$\beta - carotene\left(\frac{mg}{100ml}\right) = 0.216(0.0030) - 0.304(0.0058) + 0.452(0.0079)$$

$$= 0.0246mg/100mL$$

$$\beta - carotene\ \left(\frac{mg}{kg\ of\ honey}\right) = \frac{0.0246\,mg}{100\,mL} \times 100 \times \frac{mL}{0.001\,kg}$$

$$= \mathbf{245.6}\,\frac{\boldsymbol{mg}}{\boldsymbol{kg\ of\ honey}}$$

APPENDIX H: Results for pH Analysis.

Sample	Reading 1	Reading 2	Reading 3	Avg Reading	Mass of honey used (g)
A	3.98	3.84	3.89	**3.90**	5.03
B	4.79	4.69	4.69	**4.72**	5.02
C	3.36	3.39	3.38	**3.38**	5.02
D	4.85	4.75	4.73	**4.78**	5.02
E	3.52	3.57	3.56	**3.55**	5.02
F	4.26	4.32	4.28	**4.29**	5.01
G	4.33	4.25	4.25	**4.28**	5.00

APPENDIX I: Results for Electrical Conductivity Analysis.

Sample	Reading 1 (µS/cm)	Reading 2 (µS/cm)	Reading 3 (µS/cm)	Avg Reading (µS/cm)	Mass of honey used(g)
A	647.00	655.00	654.00	**652.00**	10.02
B	661.00	667.00	656.00	**661.33**	10.02
C	187.50	191.10	186.70	**188.43**	10.03
D	359.00	368.00	367.00	**364.67**	10.03
E	231.00	228.50	229.00	**229.50**	10.02
F	486.00	460.00	466.00	**470.67**	10.03
G	106.90	105.20	109.40	**107.17**	10.01