CHAPTER 2: HONEY PURITY TESTS

2.1 INTRODUCTION

Honey is gathered by honey bees from two different sources: nectar or honeydew. Nectar is the most common source of honey worldwide, while honeydew is only common in European countries such as Greece and Austria (Bodganov, 2010b). Nectar is a sugar solution of varying concentration secreted by flower nectary. The sugar composition in nectar, with principal sugars being frucrose, glucose and sucrose, is typical from the plant species. Most nectar consists mainly of fructose and glucose. Its sugar concentration actualy depends on the different climatic factors such as temperature, soil, humidity and season (Office of Complementary Medicines, 1998).

Honey is carbohydrate rich syrup. It is not only a popular sweetener but also a folk medicine used since ancient time (National Honey Board, 1996; Bodganov, 2010a). The increasing demand of honey in the market nowadays leads to the phenomenon of the dishonest act of honey adulteration and production of synthetic honey. This is so because of the high profit seek by the seller, as the price of adulterated honey or synthetic honey is much lower than the cost of pure honey.

"Honey adulteration" refers to the act of adding some foreign substances into pure honey. This incident had existed since hundreds of years ago. It was recorded in "*The Virtues of Honey*" the first book of Sir John Hill (1957) which alerted readers: "Beware of honey with dishonest mixture or flour and other ingredients." (Sanford, 2003). It had become a common problem in the market nowadays due to the difficulties in identifying the adulterated honey (Korth and Ralston, 2002).

Over the years, many foreign substances such as glucose, dextrose, molasses, corn syrup, sugar syrup, flour and paraffin had been used as adulterants in honey adulteration. Starch and similar products might lead to some changes in physical characteristic, such as samples with no flavor, only sweet in taste, extremely light or dark in color or with strange aroma.

In the early 1970s, adulterants can be easily detected until the development of high fructose corn syrups. High Fructose Corn Syrup (HFCS) is a product which has similar characteristics to pure honey. It contains most of the major constituents of pure honey and routinely available at a lower price compared to pure honey. Honey adulteration by using HFCS has since then become extremely profitable because of the possibility of mixing 80 to 90% of HFCS without being detected (Sanford, 2003).

Fortunately, a lot of laboratory techniques and tests for honey adulteration have been developed to identify the purity of honey samples. However, not all of the tests may meet the wish of all consumers around the world because the standard for honey purity varies between countries.

Several laboratory techniques had been developed and adapted by Honey Research Group, Department of Molecular Medicine, Faculty of Medicine, University of Malaya for determination of honey purity in Malaysia. These techniques of honey purity tests include:

1. Determination of HMF level by spectrophotometer.
2. Determination of sugar profile by gas chromatography
3. Determination of water content by hand held refractrometer.
4. Determination of hydrogen peroxide.
5. Determination of pH value by pH meter.

Honey purity can be determined and identified based on the Malaysian Pure Honey Standard from Department of Standards Malaysia. The honey samples are identified as pure honey if they meet all the characteristics of pure honey. Generally, honey from tropical countries such as Malaysia contains minimum 60% of reducing sugar (fructose, glucose and maltose).The ratio of fructose to glucose ranges from 0.9 to 1.35. It usually contains less than 5% of sucrose. The HMF level in honey from tropics is slightly higher but is not more than 80mg/kg.

Results obtained from the purity test for the sample of adulterated honey was similar to the pure honey, but the amount of sucrose level found is more than 5%. While for the sample of the synthetic honey, it contains a high level of HMF which exceeds 80mg/kg.

2.2 RESEARCH OBJECTIVES

As the usage of pure honey has been rediscovered, there has been a rapid increase in the demand of honey in the market all over the world, as well as our country. The situation of limited availability and increasing price of pure honey has lead to the problem of honey adulteration and site of synthetic honey in the market. Understandably, many consumers are worried about the quality and purity of the commercial honey in the market.

Due to the increase problem of having honey adulteration and synthetic honey in the market, the objective of this part of the research project were done to identify the purity and quality of the selected Malaysian Commercial honey. This was done by comparing several basic biochemical characteristics and properties of the honey

samples. These characteristics include HMF level, sugar profile, pH value, hydrogen peroxide level and water content.

Tests were conducted to ensure the two selected honey (Gelam Honey and Nenas Honey) used as wound treatment in the animal study of this project are pure samples. Besides that, identification adulterated honey and synthetic honey from the 19 randomly selected honey samples were also done.

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

2.3.1.1 HONEY SAMPLES

In addition of Gelam Honey and Nenas Honey, 19 samples were randomly selected for this research (Figure 2.1 and Table 2.1). Some of these honey samples were obtained commercially. The selected pure honey samples (Gelam Honey and Nenas Honey) were obtained from the Department of Agriculture, Parit Botak, Johor, Malaysia.

Figure 2.1: Selected samples for honey purity test.

Table 2.1: Labeling of 21 selected honey samples for the honey purity test.

Label	Honey Sample	Label	Honey Sample
A	Honey contained Royal Jelly	L	White honey
B	HFCS	M	Capilano honey
C	Carrefour brand honey	N	Imitation honey
D	Yu Yan Sang longan honey	O	Sarawak honey
E	Melaka honey	P	Black honey
F	Giant Bee pure honey	Q	Honey A
G	Vpure 850	R	Madu hutan
H	Vblack 850	S	Madu hitam
I	Vroyal 850	T	Gelam honey
J	Rosebee honey	U	Nenas honey
K	Mr Kang's honey		

2.3.1.2 CHEMICALS AND REAGENTS

All chemicals and reagents used in the experiments were analytical grade:

100ml of Carrez solution I made by dissolving 15 g of potassium hexacyanoferrate (II), $K_4Fe(CN)_6.3H_2O$ in water. 100ml of Carrez solution II made by dissolving 20g of zinc acetate, Zn $(CH_3COO)_2.2H_2O$ was dissolved in water and make up to 100 ml. 100ml of 0.2% Sodium Bisulphite solution made by dissolving 0.20g of solid hydrogen sulfite, NaHSO3, (metabisulphite, Na2S2O5) in water. This solution should be prepared on the day of use.

Others chemicals used were pyridine, Hexamethyldisilazane (HMDS), Trifluoroacetic acid (TFA), buffer solutions to calibrate the pH meter at 3.7 (or 4.0), 7.0 and 9.0, 0.1 Sodium hydroxide solution that is accurately standardized and hydrogen peroxide test kit. Carbon dioxide free, distilled water, carbon dioxide free was used to in this study.

2.3.1.3 INSTRUMENTS AND APPARATUS

A few instruments were used in this study, e.g. spectrophotometer, operating in a wavelength range 284nm and 336 nm, vortex mixer, Gas Chromatography (GC-14A, Shimadzu Coorporation, Japan), water bath, hand-held honey refractometer (ATAGO, Japan) HHR-2N Cat No. 2522, pH meter with accurate to 0.01 units and magnetic stirrer.

Apparatus such as filter paper for general purpose, 50ml beaker, 1cm Quartz cell, 50ml volumetric flask, 18mm x150mm test tubes, 5ml screw cap vials, pipette, 10ml burette, 25 ml automatic titrator and 250ml beaker were used in this study.

2.3.2 METHODS

2.3.2.1 DETERMINATION OF HYDROXYMETHYLFURFURAL (HMF) LEVEL

This method was established in Honey Research Lab, Department of Molecular Medicine, Faculty of Medicine, University of Malaya based on the original work of White, 1979. This method was used to determine the concentration of 5-(hydroxymethyl-) furan -2-carbaldehyde (HMF). The result of HMF level is usually expressed in milligrams per kilogram (mg/kg).

Figure 2.2: Spectrophotometer for HMF level test.

5.0g from each honey sample was weight accurately into a beaker to test the HMF level. 25.0 ml of distilled water was then added and mixed well until the 5.0g honey samples was completely diluted dissolved. The mixed solution was then transferred into a 50ml volumetric flask.

0.5ml of Carrez solution I was added into the volumetric flask and mixed well by vortex. The mixed solution was then added with Carrez solution II and mixed thoroughly by vortex. Then, distilled water was added into the volumetric flask up to the mark. A drop of ethanol might be needed to suppress the foam that form during mixing. This mixture was then filtered using a filter paper. The first 10ml of filtered solution was rejected while the remaining solution after filtration was collected. 1.0ml of the filtrated solution was pipetted into two separate test tubes with the volume of 1.0ml each.

For sample solution, 1.0 ml of the distilled water was added to the test tube that contained honey solution. This solution was mixed well by vortex followed by the addition of 1.0ml of 0.2% sodium bisulphate and mixed well by vortex for reference.

Table 2.2: Guide for preparation of dilution for sample solution and reference solution.

Additions to test tube	**Sample solution**	**Reference solution**
Initial honey solution	1.0 ml	1.0ml
Water	1.0ml	-
0.2% sodium bisulphite solution	-	1.0ml

The absorbance of the sample solution against the reference solution at 284nm and 336nm were obtained within an hour by using 1cm quartz cell. If absorbance at 284nm was more than the value of 0.6, the sample had to be diluted with distilled water and for the reference with 0.2% bisulphite to obtain the sample absorbance that is low enough for accuracy of results. Each honey sample was prepared and tested for three times. Results were recorded as Mean ± Standard Error Mean (S.E.M).

Calculation of HMF level content in mg/kg.

The HMF content (mg/kg)= $\frac{A_{284}-A_{336} \times 149.7 \times 5 \times D}{W}$

A_{284} = Absorbance at 284nm.
A_{336} = Asorbance at 336 nm.
149.7 = Factor $\frac{126 \times 1000 \times 1000}{16830 \times 10 \times 5}$
D = Dilution Factor
W = Weight of sample

126 = Molecular weight of HMF.
16830 = Molar absorpticity of HMF at 284 nm.
1000 = Conversion g into mg.
10 = Convertion 5 into 50 ml.
1000 = Conversion g of honey into kg.

2.3.2.2 DETERMINATION OF SUGAR PROFILE

This method was established in Honey Research Lab, Department of Molecular Medicine, Faculty Medicine, University of Malaya by adapting and modifying methods from Doner, White and Philips which is published in 1979 (Aljady, 2003). This method determined the sugar profile (glucose, fructose, sucrose and maltose) in the honey samples.

In the preparation of sample for standard, 1.0mg of each standard sugar (fructose, glucose, maltose and sucrose) was weighed accurately into a 5ml screw-top

glass vial. For honey sample preparation, 5.0 mg of honey samples were weighed accurately into a 5ml screw-top glass vial. The samples prepared were then added with 0.45ml of pyridine. These vials were immersed into the waterbath at 70℃ for at least 10 minutes. 0.5ml of Hexamethyldisilazane (HMDS) was added to each vial and mixed well. Then, 0.05ml of Triflouroacetic acid (TFA) was carefully added drop by drop into the mixture. The vial was shaken occasionally during the adding of TFA at room temperature.

These clear solutions were allowed to stand for at least 15 to 30 minutes. Homogenous clear solution was obtained. If the solutions turn cloudy, new samples preparation were needed. The solutions were left for 24 hours at room temperature before injecting into the gas chromatography (GC-14A). 1.0µl of each sample solution was required for the injection into the capillary of gas chromatography column.

Calculation for weight and percent of sugar in honey.

$$\text{Weight of sugar in honey (mg)} = \frac{\text{Peak of sugar in honey}}{\text{Area peak in standard}} \times \text{weight of standard (mg)}$$

$$\text{Percent of sugar in honey (\%)} = \frac{\text{mg sugar in honey}}{\text{Weight of honey sample}} \times 100\%$$

2.3.2.3 DETERMINATION OF WATER CONTENT IN HONEY

The hand held honey refractometer is specially designed to determine the percentage of water in honey (Figure 2.3). The method using this honey refractometer was determination of water content in honey was easy.

Figure 2.3: ATAGO HHR-2N hand held refractometer.

The prism of the hand held refractometer was cleaned and dried before used. Followingt that, the prism of the refractomer was covered evenly with a honey sample evenly (Figure 2.4). The reading of the refractive index was recorded. Each sample was measured twice and the average value was taken. After used, the prism of the refractometer was cleaned carefully.

Figure 2.4: Prism of refractometer covered with honey.

2.3.2.4 DETERMINATION OF HYDROGEN PEROXIDE

This is the easiest test to perform and determines the existence of hydrogen peroxide in honey sample.

A 30 % (w/v) concentration of honey was prepared by weighing 3g of honey and diluted in 10 ml of distilled water. The mixture was then incubated in a waterbath

at 37 ℃ for 30 minutes. The test strips from the test kit was dipped in the mixture and the color developed was read against the colour code to obtain the concentrations of the H_2O_2 formed. Each sample was tested three time for accuracy purpose and the results were recorded.

2.3.2.5 DETERMINATION OF pH LEVEL

pH reading of the crude honey was very unstable and hard to determine. Therefore honey was diluted into 10 %solution (w/v). The pH meter was calibrated at pH 3.7 (4.0), 7.0 and 9.0 before used. 10 g of honey sample was dissolved in 75 ml of carbon dioxide-free distilled water. This solution was mixed by using magnetic stirrer. The pH electrodes were immersed into the solution. pH value of the honey solution was recorded in two decimal places.

2.4 RESULTS AND DISCUSSIONS

2.4.1 HMF LEVEL IN HONEY

The results obtained were compared to the value accepted by the European Standard of Pure Honey. According to the European Standard of the pure honey, the HMF level of the pure and fresh honey from tropical countries should not exceed 80mg/kg. For pure honey from temperate country, HMF level should not be more than 40mg/kg.

Table 2.3: Reading of HMF level in 21 selected samples (mg/kg).

Samples	Mean HMF Level (mg/kg)	S.E.M
A	132.20	0.76
B	9.49	0.62
C	112.99	0.79
D	117.13	1.22
E	72.00	0.82
F	138.74	0.59
G	2.62	0.96
H	138.99	1.01
I	112.89	1.01
J	181.24	0.85
K	65.57	0.72
L	115.85	0.77
M	36.81	0.29
N	4.65	0.20
O	214.75	0.96
P	126.70	0.63
Q	66.90	0.18
R	98.03	0.55
S	67.71	0.50
T	**36.90**	**0.11**
U	**48.00**	**0.08**

From the results obtained in the HMF level determination by spectrophotometer, 52.38 % of the samples tested in this study contained the HMF level which exceeded 80mg/kg (Figure 2.5 and Table 2.3). In other words, 11 out of 21 samples in this study showed HMF level exceeding the value for honey from tropical country. The samples that exceeded 80mg/kg of HMF level were Samples A, C, D, F, H, I, J, L, O, P and S. 47.62 % of the samples showed results that met the European Standard for pure honey; i.e. HMF level did not exceed 80 mg/kg (Figure 2.5). 10 out of 21 samples in this study showed that its HMF level as lesser than 80mg/kg. These samples were samples B, E, G, K, M, N, Q, S, T and U. These 10 samples might be pure honey or adulterated honey. However, samples which contained HMF level exceeding 80mg/kg could be classified as synthetic honey. HMF levels alone could not certify the honey purity.

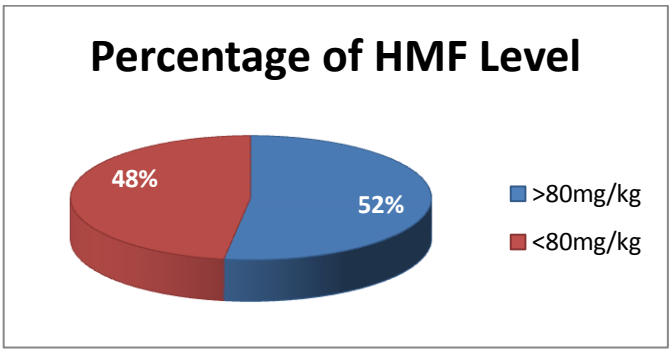

Figure 2.5: Percentage of HMF level in 21 honey samples.

According to Bogdanov (2010c), HMF is a deposition product of fructose. It is always present in a very little amount but rarely exceeds 10 mg/kg in pure honey samples. The level of HMF in honey samples varies in different countries. For honey from tropical country, the maximum acceptable value of the HMF level is 80 mg/kg. This is because the formation of HMF is dependent on pH and higher temperature. Due to the high level of HMF level in honey from the tropics which is similar to the adulterated honey, it is hard to differentiate between pure and adulterated honey by using the HMF level determination method alone.

The factors that cause the increase of HMF level in honey include prolonged storage at high temperature and overheating of honey samples. These lead to the increase of HMF level in honey to 30-40 mg/kg or might even exceed 100 mg/kg. Thus, one could only assume that the high level of HMF in honey sample only implied that the sample is old or synthetic honey. The storage condition of honey might also increase HMF level in honey sample because higher temperature might lead to the formation of HMF and also lead to the increase level in HMF. Thus, storing honey in refrigerator can prevent the increase of the HMF level in honey. HMF content in honey is one of the indicators to determine freshness of pure honey. Thus, HMF level of determination is the method that is always used in determining the freshness, overheating and pureness of honey (Bogdanov, 2010c).

Determination of HMF level by spectrophotometric method is one of the easiest and simplest procedures used in laboratories. The protocol of this method is much simpler and it is cheaper compared to the HPLC method. This method is easier to repeat and not time consuming. However, the purity of honey sample cannot be judged by the result on HMF levels done. Thus, other experimental methods to test the other characteristics of honey are needed to certify its purity.

2.4.2 SUGAR PROFILE IN HONEY

According to the European Standard for pure honey, the content of reducing sugars (fructose, glucose and maltose) in pure honey sample should not be less than 60 %. However, the percentage of reducing sugars in honey from tropical country such as Malaysia might be slightly lower than 60 % due to the high humidity. Besides that, the ratio of the fructose/glucose obtained for pure honey may vary from 0.9 to 1.35 (Kamaruddin, pers. com). Adulterated honey and pure honey might have similar characteristics of total percentage of reducing sugars. Thus, other characteristic in the sugar profile is needed to identify adulterated honey. One of the characteristics that the adulterated honey showed is having sucrose level exceeding 5%. Sucrose level of not more than 5% has been established by the European Standard for pure honey.

Table 2.4: Sugar profile of the 21 samples.

Samples	Fructose (%)	Glucose (%)	Maltose (%)	Sucrose (%)	F/G (%)	F+G+M (%)
A	38.07±0.06	36.28±0.26	0.64±0.05	0.79±0.10	1.05±0.01	75.03±0.37
B	23.66±0.78	21.49±0.57	0.00	37.26±0.70	1.10±0.01	45.10±1.38
C	53.06±0.95	26.24±0.43	0.78±0.10	0.68±0.09	2.02±0.02	80.25±1.45
D	37.22±0.97	20.62±0.32	0.55±0.37	0.47±0.12	1.81±0.03	64.62±1.14
E	36.13±0.96	27.94±0.10	3.53±0.24	0.76±0.14	1.75±0.03	60.47±1.33
F	42.62±0.17	33.62±0.94	2.67±0.03	0.46±0.03	1.52±0.00	73.15±0.17
G	36.13±0.58	32.44±0.90	1.18±0.19	0.74±0.04	1.07±0.02	71.67±0.86
H	33.31±0.82	43.95±0.91	0.79±0.18	0.84±0.29	1.03±0.03	66.98±1.87
I	26.76±0.51	29.17±0.85	0.46±0.04	0.73±0.11	0.61±0.02	70.56±1.06
J	28.55±0.62	25.25±0.50	1.37±0.22	0.82±0.25	0.98±0.01	59.25±1.44
K	19.16±0.67	24.78±0.79	0.00	40.65±0.47	0.75±0.01	44.15±0.71
L	32.25±0.85	24.38±0.82	0.97±0.07	0.32±0.06	1.30±0.01	57.50±0.85
M	33.00±0.33	24.38±0.82	10.89±1.03	0.25±0.08	1.35±0.03	68.66±1.83
N	0.20±0.01	8.37±0.91	37.13±0.50	0.58±0.17	0.02±0.00	45.04±0.75
O	25.15±1.38	31.83±0.62	1.17±0.04	0.62±0.04	0.79±0.04	38.53±1.11
P	23.50±0.98	36.01±0.68	0.78±0.03	0.68±0.06	0.65±0.02	60.56±1.15
Q	3.72±0.13	9.34±0.51	11.04±0.99	30.49±0.35	0.40±0.01	23.48±0.61
R	19.89±0.52	30.48±0.66	1.08±0.12	1.38±0.18	0.65±0.02	51.07±0.69
S	4.30±0.28	6.49±0.46	10.96±0.09	33.93±0.64	0.66±0.01	21.58±0.77
T	**25.15±0.04**	**24.05±0.38**	**0.59±0.56**	**0.97±0.01**	**1.04±0.05**	**50.76±0.08**
U	**28.03±0.18**	**26.43±0.29**	**0.92±0.03**	**0.60±0.20**	**0.94±0.02**	**55.98±0.80**

Results obtained were recorded in Table 2.4. 47.62 % of samples in this study contained more than 60 % of total reducing sugars (Figure 2.6). In other words, 10 out of 21 samples in this study contained more than 60% of reducing sugars of their sugar profiles. The samples that contained more than 60% of reducing sugars were Samples A, C, D, E, F, G, H, I, M, and P. 23.81% of samples in this study contained less than 60 % but more than 50 % of reducing sugars of the sugar profile (Figure 2.6). Samples J, L, R, T and U might be adulterated honey or pure honey from tropical country which contain around 50 to 60 % of reducing sugar. 28.57 % (6 out of 21 samples) selected for this study contain less than 50 % of reducing sugars. This showed that these samples (Samples B, K, N, O, Q and S) did not meet the standard requirement for pure honey.

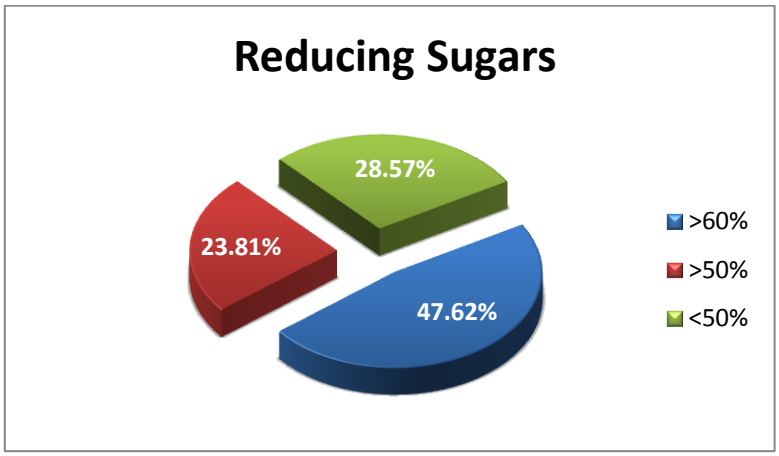

Figure 2.6: Percentage of reducing sugars in 21 samples.

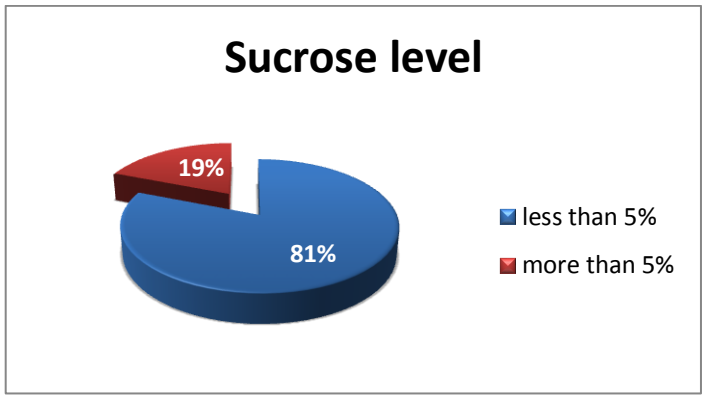

Figure 2.7: Percentage of sucrose level of 21 samples.

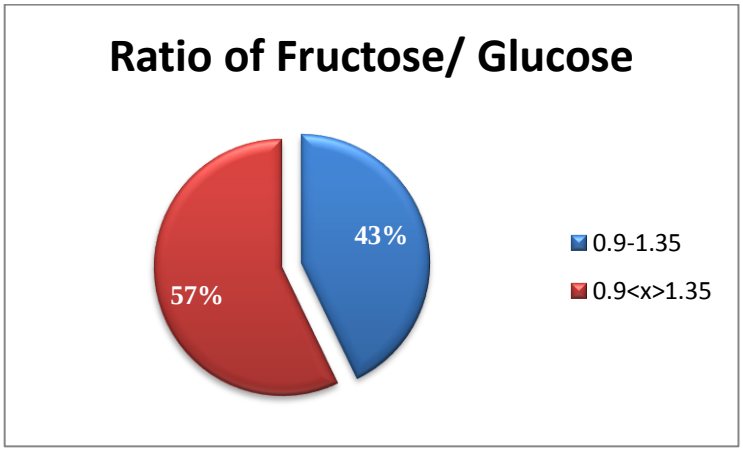

Figure 2.8: Ratio of fructose/glucose of 21 samples.

National Honey Board and Codex Alimentations Commission (CAC) as well as the European Honey Standard suggested that the minimum total of reducing sugars in pure honey should be 60%. The content of reducing sugars might vary due to the storage factor, enzyme activity and acid reversion in honey. Besides that, time of collection for honey also affected the total amount of reducing sugars in honey. For honey collected in flowering season, the total amount of reducing sugars is expeted to be higher.

From the results obtained, only 19 % of the 21 samples selected for this study contained sucrose level that is more than 5 % of total sugar profile (Figure 2.7). 17 samples (81 %) out of 21 samples contained the sucrose level which was less than 5 % of total sugar. Thus, Sample B, J, Q and S were considered as impure honey.

Sucrose content in the honey sample is a significant criterion to determine the honey purity. This is because the sucrose present in natural or pure honey is little because of the activity of invertase enzyme present in honey. These enzymes are responsible for the breakdown of sucrose. Thus, the content of sucrose in pure honey is low. In other words, high sucrose level in a honey sample indicates that the honey may be adulterated. Over heating of the honey sample might denature invertase, stopping the enzyme activity that breaks down the sucrose into glucose and fructose. Thus, sucrose level remains high in the adulterated honey. Besides that, sucrose content which is higher than 8 % is generally associated with sugar feeding to the bees.

According to the results obtained, 43 % (9 out of 21 samples) of the selected samples met the Malaysian pure honey standard ratio of fructose/glucose (0.9-1.35) (Figure 2.8). These samples were Sample A, B, G, H, J, L, M, T and U. 57 percent (12 out of 21 samples) of the samples did not meet the standard ratio. Thus, Samples C, D, E, F I, K, N, O, P, Q, R and S might not be pure honey.

The overall results of HMF level test and sugar profile test by gas chromatography (GC), showed that only few samples out of the 21 selected samples met the European Standard for pure honey. These samples were Sample G, M, T and U. But these results did not confirm the purity of honey. Other characteristics were still needed to confirm the purity of honey.

Fructose (approximately 38 %w/w) and glucose (~31%) are the two major sugars present in honey lesser amount of sucrose (~1 %) and other disaccharides and oligosaccharides could also be found. Fructose predominates and tends to give honey the taste which is sweeter than sugar while glucose level in honey is dependent on its source of nectar (White, 1957). According to the data obtained, Sample G, M, T and U met the characteristics of the sugar profile in pure honey.

GC method was chosen to be used to determine the sugar profile this study due to its high sensitivity and reliability. This method is simple and easy to handle. It is also less costly compared to the other methods for determining sugar profile in honey samples.

2.4.3 WATER CONTENT IN HONEY

From results obtained, 21 selected honey samples showed that the percentage of water content varies from 16.70 to 26.50 % (Table 2.5). Results of the four honey samples (Samples G, M, T, and U) that were assumed to be pure honey according to the HMF level and sugar profile test were 20.40 %, 16.70 %, 26.50 % and 23.90 % respectively.

Table 2.5: Mean of percentage of water content in 21 samples.

HONEY SAMPLES	MEAN OF WATER CONTENT (%)
A	20.02
B	18.10
C	18.50
D	19.90
E	18.90
F	20.90
G	20.40
H	20.50
I	20.50
J	17.50
K	23.70
L	24.50
M	16.70
N	17.60
O	20.50
P	21.30
Q	22.60
R	21.50
S	17.40
T	**26.50**
U	**23.90**

Water is the third highest component of honey (National Honey Board, 1996). Water content of honey may vary from 15 to 20% (Bogdanov, 2010c). This is the main criteria used to determine the keeping quality and storage condition of honey. Besides that, the origin of honey might also relate to the water content of honey. Honey from tropics might contain more water due to the humidity. Water content of honey is also closely related to its fermentation. The higher the water content of the honey, the higher the is possibility of fermentation happening in the honey samples. According to Eva Crane (1975), honey that contains more than 18 % of water is likely to get fermented during prolonged storage. The higher the water content in honey sample, the higher the survival rate of the yeast in honey. Thus, the higher the fermentation activity which occurs in honey and indirectly leads to the increase of acidity of honey. Other than that, the water content of honey might also be due to the time of honey collection. Water

content of honey that was collected during raining season is higher compared to the honey collected in the dry season.

2.4.4 pH LEVEL OF HONEY

Pure honey normally contains relatively small amount of acid which is important for the honey taste. It contains a number of amino acids (0.05 to 0.1%) and organic acid (0.57%) (National Honey Board, 1996). Thus, honey is mildly acidic and pH value lesser than 7. The average pH value for most honey is 3.9. The typical range of pH value of honey varies between 3.4 and 6.1 (Bogdanov, 2010c).

Table 2.6: The reading of pH level of 21 samples (10 percent w/v)

SAMPLES	pH LEVEL
A	3.20
B	5.13
C	4.09
D	3.92
E	4.11
F	3.79
G	3.26
H	3.25
I	3.22
J	3.37
K	4.12
L	3.46
M	4.39
N	3.52
O	3.22
P	3.19
Q	3.79
R	3.86
S	3.12
T	**3.84**
U	**3.51**

According to results, pH value of 21 samples ranged from 3.12 to 5.13 (Table 2.6). Only 4 samples (Samples G, M, U and T) had the value of pH that meets the pure honey characteristics. pH level of Samples M, T and U are 4.39, 3.84 and 3.51 respectively (Table 2.6). However, only three honey samples met the characteristics of pure honey. pH level of Sample G was 3.26 and its value was lower than the typical range of pure honey, Sample G was an adulterated honey although it was highly similar to the pure honey's characteristic. In conclusion, based on these test there were only 3 out of a total of 21 samples obtained commercially that met the characteristic of pure honey. These 3 samples are Sample M, Sample U and Sample T.

Honey from the tropical countries has lower acidity due to their high level of water content. Water content in honey might lead to the activity of fermentation that could lead to the increase of acidity level in honey. The impure or adulterated honey might have low pH level that which do not demonstrate pure honey criterion. The storage factor and temperature also contributes to the low pH value in honey.

2.4.5 HYDROGEN PEROXIDE CONTENT IN HONEY

The content of the glucose oxidase in honey can lead to the formation of hydrogen peroxide. The higher the activity of glucose oxidase, the higher the level of hydrogen peroxide produced.

According to the results, only 19 % of the selected samples contain glucose oxidase enzymes. Four samples showed the existence of glucose oxidase which is responsible for the formation of hydrogen peroxide. Sample F, Sample M and Sample T showed the hydrogen peroxide level of 2.5 while Sample U contain higher reading of hydrogen peroxide which iwas 10 (Table 2.7).

Table 2.7: Hydrogen peroxide level of 21 selected samples.

SAMPLES	H_2O_2
A	0
B	0
C	0
D	0
E	0
F	2.5
G	0
H	0
I	0
J	0
K	0
L	0
M	2.5
N	0
O	0
P	0
Q	0
R	0
S	0
T	**2.5**
U	**10**

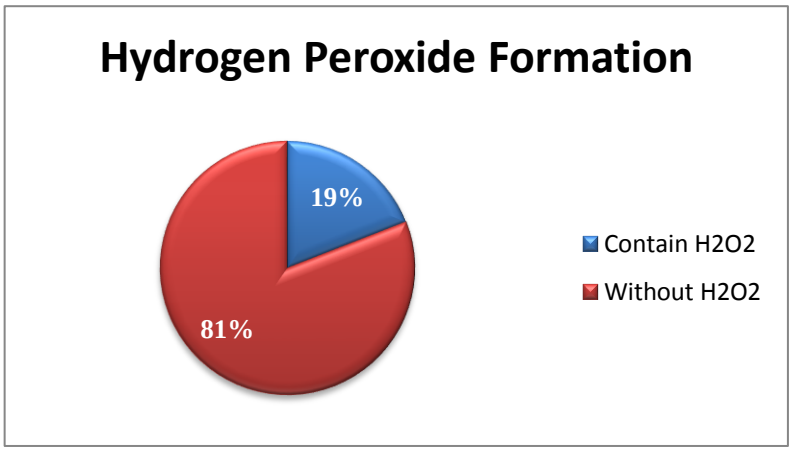

Figure 2.9: Hydrogen peroxide formation in 21 samples.

The presence of glucose oxidase enzyme is also another character of pure honey that has not been process. It is an indicator of honey freshness. Although hydrogen peroxide production of pure honey is not currently accepted, its use is helpful in honey purity assessment (Kamaruddin, Pers. Com).

2.5 CONCLUSION

The different tests performed verified that the two Malaysian honey (Gelam honey and Nenas honey) selected are pure honey. These two samples met all the characteristics of pure honey from tropical country. They contained a level of reducing sugar more than 60%; fructose/glucose ratio in the range of 0.9 to 1.35. They also contained sucrose level which less than 5 % from the total sugar profile. Other than that, HMF level was also less than 80mg/kg. Thus, the two Malaysian honey samples (Gelam and Nenas honey) selected for the animal study are confirmed as pure honey.

Table 2.8: Summary of the characteristics on both selected Malaysian honey.

	HMF Level (mg/kg)	F/G (%)	F+G+M (%)	Water Content (%)	pH Level	H2O2 Content
Gelam Honey	36.90	1.04	50.76	26.50	3.84	2.5
Nenas Honey	48.00	0.94	55.98	23.90	3.51	10
Standard	<80.00<	<5.00	<60.00	<30	<3.4-6.1	Present