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Classification of Phenolic Compounds in Honey Using Gas Chromatography Mass Spectrometry (GCMS) by Chemometrics Method

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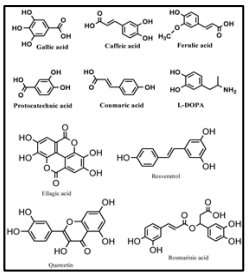
GRAPHICAL ABSTRACT



Structure of phenolic compounds

ABSTRACT

Honey is known as one of natural health foods which contain antioxidant properties that help cure many diseases. The level of antioxidant is based on the presence of phenolic compound contain in honey. This study aims to identify the phenolic compounds contain in two different honey: Kelulut honey and Tualang honey. The data of phenolic compounds analysis were interpreted for classification of antioxidant compounds in honey samples by chemometrics tools, Principal component analysis (PCA) and Hierarchical Cluster Analysis (HCA). Five samples of each types of honey were taken from different places in and out of Malaysia. The samples were extracted using Hydrophilic-Lipophilic Balance (HLB) Oasis cartridges by Solid Phase Extraction (SPE) method and gas chromatography mass spectrometry (GCMS) was used to analyse the sample. Based on the results, the phenolic compound detected in both honey samples were benzoic acid, phenol, cinnamic acid, coumaric acid, ferulic acid, caffeic acid, gentisic acid, protocatechuic acid, salicylic acid and quercetin. However, salicylic acid and gentisic acid only identified in Tualang honey and not detected in Kelulut honey. PCA and HCA were applied to classify the honey samples according to their origin and similarities. The data showed that Kelulut honey have high antioxidant because it has higher concentration of phenolic compounds than Tualang honey and can be discriminate using PCA and HCA method. This suggests that both honeys were good for health, the study of both honeys should extend to broader in order to know more benefits of the honeys. Furthermore, Kelulut honey is useful in promoting better nutrition and preventing some related diseases.

Keywords: Phenolic compound, stingless bee honey, apis honeybee, antioxidant, chemometrics analysis

1. INTRODUCTION

The stingless bee is a natural type of bee that exists in almost every continent with the majority located in Latin America, the mainland of Australia, Africa, and Eastern and Southern Asia [1]. Kelulut honey is produced by stingless bee of Trigona spp. The bees produce honey by storing it naturally in the pot (cerumen) which used across time and space. The storing honey in the pot may have many beneficial properties especially for health. Because of its healing properties honey is famous in food industry and traditional medical product.

For tualang honey is known well as Malaysian multifloral jungle honey which is produced by the rock bee (Apis dorsata). The name of this honey is come from the name of a tree called Tualang. The bee builds hive high up in the branches of Tualang tree (Kompassia excelsa). Tualang trees are usually found mainly in tropical rain forests and can reach up to 250 feet in height. About 30 000 bees can live in the hive that can be up to 6 feet across. Mostly Malaysian used Tualang honey in traditionally as a health and anti-ageing supplement [2].

Honey is recognised as natural antioxidant property [8]. The components such as phenolic acid, enzymes, vitamins, flavonoids and a small amount of mineral content, particularly copper and iron have made honey become the health and important products or foods. Most chronic diseases such as cancer, neurological degeneration and coronary are a consequence of oxidative damage. This is reported by numerous studies and also proven that antioxidant capacity against reactive oxygen species always associated by therapeutic potential of honey. Phenolic compounds in honey contain phenolic acids (caffeic, chlorogenic, coumaric, ellagic, ferulic, gallic, homogentisic, phenyl lactic, syringic and vanillic acids) which act as antioxidant in honey and can give benefits in would healing, treatment of skin ulcer a well as gastrointestinal disorder [4].

Other than that, the development of statistical software has authorized researcher to perform a wide variety of statistical analysis and solve problem. PCA and HCA are some of the chemometrics tools that used for classification, calibration and exploratory issues [5]. They are widely used to determine the similarities and the results are easily to interpret. Chemometrics can improve the understanding of chemical information and to correlate quality parameters or physical properties to analytical instrument data by mathematical and statistical methods. This method also can be used to classified data of phenolic compound.

In this study, gas chromatography mass spectrometry (GCMS) method is used to determine phenolic compounds in honey. The method can analyse the phenolic compounds from honey by identification and quantification. Then, the result

data will use to do classification according principal component analysis (PCA) and hierarchical cluster analysis (HCA) method.

2. EXPERIMENTAL

2.1. Sample Preparation

Two types of honeys, which were Tualang honey (*Apis dorsata*) and Kelulut honey (*Trigona sp*) were collected from different places (Table 1). Each of the samples was prepared in duplicate.

Table 1. The types of honey samples with their regions

No	Traditional name	Sample Code	Honey type	Regions	Source
1	Tualang Honey	TH1	*Apis dorsata*	Perlis, Malaysia	Commercial
2		TH2	*Apis dorsata*	Pahang, Malaysia	
3		TH3	*Apis dorsata*	Kedah, Malaysia	
4		TH4	*Apis dorsata*	Pahang, Malaysia	
5		TH5	*Apis dorsata*	Riau, Indonesia	
6	Kelulut Honey	KH1	*Heterotrigona Itama*	Johor, Malaysia	Bee farm
7		KH2	*Geniotrigona Thoracica*	Johor, Malaysia	
8		KH3	*Honeydew Honey*	Johor, Malaysia	
9		KH4	*Tetragonula Sirindhornae*	Johor, Malaysia	
10		KH5	*Tetrigona Binghami*	Johor, Malaysia	

The samples of honey will be stored under temperature of refrigeration (5 °C ± 2 °C). The samples then will be placed in the 50 mL polypropylene tube with its screw cap before storing it at cool temperature until the time of use [12]. 10 g of honey samples were dissolved in 50 mL of deionised water adjusted to pH 2 with 0.1 M of hydrochloric acid. The mixtures were stirred in a magnetic stirrer for 15 minutes before filtered through cotton wool to remove the solid particles. The solutions were passed through Oasis HLB column, then washed with 50 mL of deionised water adjusted to pH 2 to remove all sugars and other polar constituents of honey. Then, the solutios were eluted with 50 mL methanol. The methanolic solutions were evaporated to dryness at 40 °C until the volume decreased to 5 mL. The solutions then were flushed with nitrogen drying until the sample concentrated. The residues were filtered through 0.45 µm syringe filter. The samples were stored at -20 °C until analysis [7].

2.2. Gas chromatography mass spectrometry (GCMS) instrumentation

Gas Chromatograhy mass spectrometer measurements were carried out on Agilent Technologies gas chromatograph mass spectrometer model 7820A equipped and Shimadzu GCMS-TQ8040 with the capillary column HP-5MS column (30 m, 0.25 mm i.d., thickness 0.25 µm). Chromatographic conditions were as follows: gas carrier used were helium gas at 1 mL min-1, the detector temperature was 300 °C and injector temperature was 250 °C. The temperature used included the follow settings: 50°C isothermal for 2 min, increased to 200 °C at a rate of 8 °C min -1, held isothermal for 5 min, then increased to 290 °C at a rate of 7 °C min -1 held for 5 min. The injected volume was 1 µL and splitless mode was used. Mass spectra were recorded in electron ionization mode at 70 eV with ion source temperature 300 °C and scanning the 70-200 m/z range.

2.3. Statistical analysis

The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were both performed using the Solo (Eigenvector Research Incorporated) software. Dataset was prepared using Microsoft® Excel spreadsheet (Microsoft Corporation) to transfer the data to the Solo software. The collected data were classified by applying pattern recognition methods which was PCA as an unsupervised classification method and HCA as an unsupervised learning method. The data of phenolic compound is classified among the honey samples based on the chemical and physical characteristics. PCA will be applied to the data set of 20 samples of honey after standardisation (the mean of the values for each variable is subtracted from each variable value and the result is divided by the standard deviation of the values for each variable). After

standardisation, each parameter contributes equally to the data set variance and carries equal weight in principal component calculation [9].

3. RESULTS AND DISCUSSION

3.1. Phenolic compounds in honey samples

Based on GCMS chromatograms results, there are ten phenolic compounds were identified in TH and eight phenolic compounds in KH samples as shown in Table 2. The identified and quantified phenolic compounds in the both honey types were benzoic acid, phenol, cinnamic acid, hydrocinnamic acid, ferulic acid, caffeic acid, gentisic acid, protocatechuic acid, salicylic acid and quercetin. However, salicylic acid and gentisic acid only detected in TH because the compound does not presence in the KH. Benzoic acid, phenol, cinnamic acid, coumaric acid, ferulic acid, caffeic acid, gentisic acid, protocatechuic acid, salicylic acid is categorised as phenolic acids while quercetin is a flavonoid.

Many researchers used GCMS method to identify volatile compounds in honey for characterisation [10]. According to the them, the phenolic compounds such as gallic acids, benzoic acid, caffeic acid, cinnamic acid, ferrulic acids, coumaric acids, and syringic acids does not present in TH but present in other honey samples likes gelam honey and coconut honey [3]. But, other researcher stated that phenolic acids such as 2-hydroxycinnamic acid, caffeic acid, cinnamic acid, gallic acid, p-coumaric acid, syringic acid and flavonoids likes apigenin, catechin, chrysin, kaempferol, luteolin were found in the TH [6]. Compared to the result (Table 3), benzoic acid, phenol, ferullic acid, protocatechuic acid, salicylic acid, gentisic acid and quercetin were the new phenolic compounds found in TH. But, the low total peak area at benzoic acid (2.42), protocatechuic acid (2.51) and salicylic acid (3.17) were detected and the compounds only identified in TH3 and TH4 samples. The highest total peak area of phenolic compound detected in TH samples was cinnamic acid (115.68) and the compound was identifed in mostly TH samples accept in TH5 samples.

Based on Table 3, benzoic acid, phenol, caffeic acid, cinnamic acid, ferullic acid, protocatechuic acid, salicylic acid and quercetin were the phenolic compounds that had been identified using GCMS. The data showed that phenol have the highest total peak area (160.77) followed by hydrocinnamic acid (134.13). In the data, phenol, caffeic acid and cinnamic acid were identified in all KH samples. In other study have found phenolic compounds such as gallic acid, caffeic acids, syringic acid, catechine, apigenin, chrysin, 2-hydroxycinnamic acid, kaempferol, p-coumaric acid were previously identified in kelulut honey by using LCMS/MS method [11]. Benzoic acid, phenol, ferullic acid, protocatechuic acid and quercetin were the new phenolic compounds that were analysed in KH samples. However, the total peak area in benzoic acid (7.10), ferullic acid (3.01) and protocatechuic acid (7.10) were found very low in KH samples as the compounds were only identified in KH2, KH5 and KH4 respectively.

TH has a greater number of phenolic compounds than KH because the presence of salicylic acid and gentisic acid found in TH samples. Based on result (Table 3), cinnamic acid was the most phenolic compounds identified in the both honey samples followed by phenol and caffeic acid. Salicylic acid is the least phenolic compound that was identified in the both honey samples because the compound was only detected in TH4. The phenolic compounds in KH samples mostly have higher peak area than TH samples. KH samples also have the higher total of peak area in benzoic acid, phenol, caffeic acid, hydrocinnamic acid, cinnamic acid, protocatechuic acid and quertin. This shows that phenolic compounds in KH are more concentrated to be detected by GCMS. In this study, we determined the antioxidant of honey based on the phenolic content in the honey samples. TH samples contain more phenolic compounds than KH samples but KH have higher peak area than TH. Since antioxidant level of honey have been attributed to its phenolic content, KH have higher antioxidant level since the samples have higher peak area detected in GCMS compared to KH. In previous study also prove that KH have higher antioxidant properties due to their high concentration of phenolic substances in the honey samples [11]. Moreover, different regions of honey contain different phenolic compounds and their composition. However, antioxidant activity cannot be determined by phenolic compounds alone because there are also non phenolic compounds that can high the level of antioxidant even the higher number of phenolic compounds in a molecule have low antioxidant.

There are only a few researches determining phenolic compounds in TH and KH using GCMS because many of them used HPLC and LCMS to analyse the compounds. This is due the volatile compounds of honey such as ester, alcohol, aldehyde, fatty acid that also showed in GCMS and made phenolic compounds difficult to identify and quantify. In this study, gallic acid connot be identifying by GCMS because of their low concentration to detect. Another limitation in this study was the peaks of GCMS were determined by comparing directly with mass spectra in NIST library since no internal standard were included in the samples

Table 2. Retention time of phenolic compounds in honey samples

Tualang honey	Kelulut honey	R.T. (min)
Benzoic acid	Benzoic acid	4.63
Phenol	Phenol	15.08
Caffeic acid	Caffeic acid	21.69
Ferullic acid	Ferullic acid	24.87
Hydrocinnamic acid	Hydrocinnamic acid	12.96
Cinnamic acid	Cinnamic acid	20.94
Protocatechuic acid	Protocatechuic acid	17.33
Salicylic acid	-	8.45
Gentisic acid	-	35.74
Quercetin	Quercetin	23.47

3.2. Principal component analysis (PCA)

Principal component analysis (PCA) was performed over the data for 10 compounds in 20 honey samples which were tualang honey and kelulut using autoscaling. This analysis was for distribution of honey samples from different botanical origins and antioxidants. The first two components of scatter plot were estimated with PCA, and the data structure visual was obtained in reduced dimension. The plot separation of honey separation was occurred in 2-dimensional plot and 3-dimensional plot of the sample scores (Figure 1). The number of principal component (PCs) is same with either the smallest number of samples or number of variables from mathematics point of view [13]. In this study, the number of samples is 20 while the number of variables is 10. Therefore, 10 PCs can be computed. According to principle of PCA, PCk is less important than PC (k + 1) for all consecutive PCs and more important than PC (k + 1). Thus, PC 1 is more important than PC 2 and other PCs. The total PCs can explain 68.77 % of the total variance, which are 26.84 % of the variance, was represented as the first principal component (PC1), 15.59 % of variance was represented as PC 2 and PC 3 represented 14.00 % of the variance. As shown in the PCA score plot (Figure 1), the honey samples were discriminated into two types of groups which corresponded with their phenolic compounds and region. The discrimination was considered as medium due to the percentage of total PCs was lower than 70-80 %.

The sample plots were scattered and only some of the samples that can be seen grouping. Most of the TH samples were indicated by the negative axis of PC1 and negative of PC3. For the KH were mostly distributed in the positive axis and negative of PC2. This showed that the number of phenolic compounds were effective in classifying the different species of honeybees. The level of antioxidant can be related to the content of phenolic compounds in the honey samples. Although KH samples from the same location while TH samples came from different location, in reality the honey properties also affected by the seasons and weather. PCA is an unsupervised technique, meaning that it shows the main structure in the data without considering a special direction or type of information. It was already clear in the PCA score plot that the two types of honeys were discriminated.

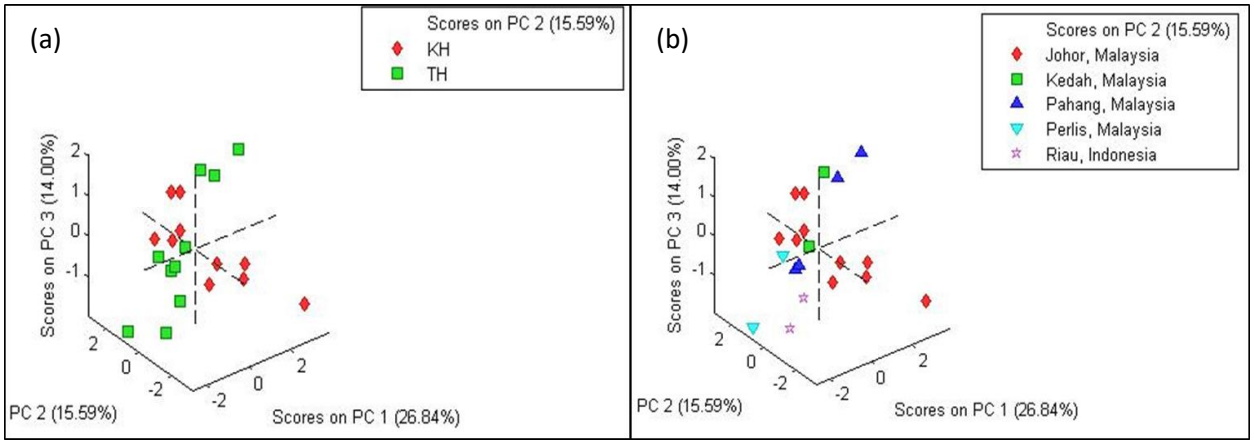
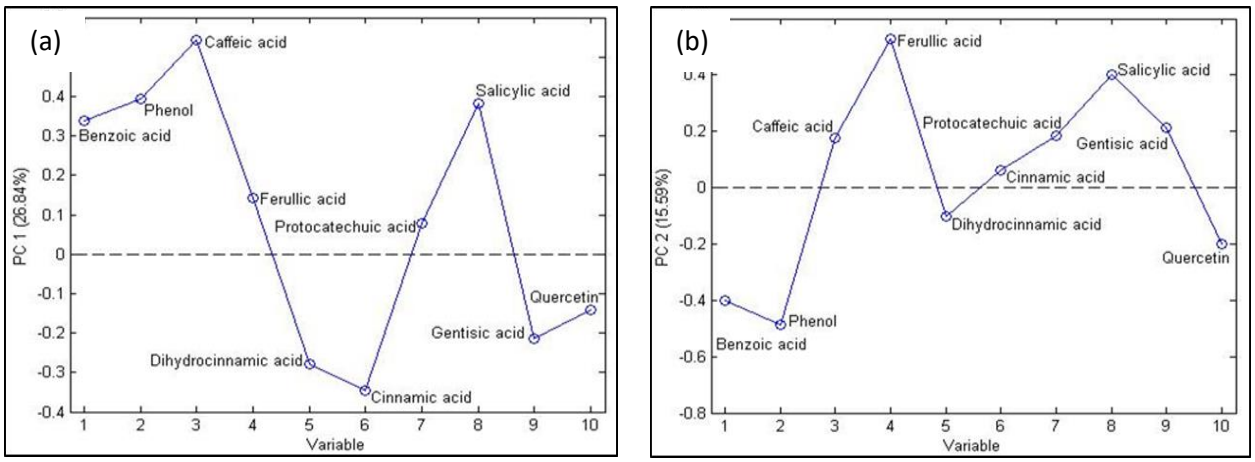


Figure 1. Analysis of PCA (a) principal component number (b) PCA plot according type of samples (c) PCA plot according sample region

Figure 2 showed the loading plot analysis of PC 1, PC 2 and PC 3 against the variables. For PC 1, the plot showed that caffeic acid was the most significant variables as it had the highest plot while cinnamic acid was the lowest plot at between -0.3 and -0.4 and gave the least significant variables. The significant variables for PC 2 and PC 3 were ferullic acid and quercetin respectively while the least were phenol and gentisic acid. The highest the data plot, the most significant variables contributed to each PC. Between the PCs, quercetin in PC 3 was the highest plot at 0.6 while cinnamic acid in PC 1 was the lowest plot. The data also showed that caffeic acid, protocatechuic acid, and salicylic acid were plotted at positive axis against all the PCs.



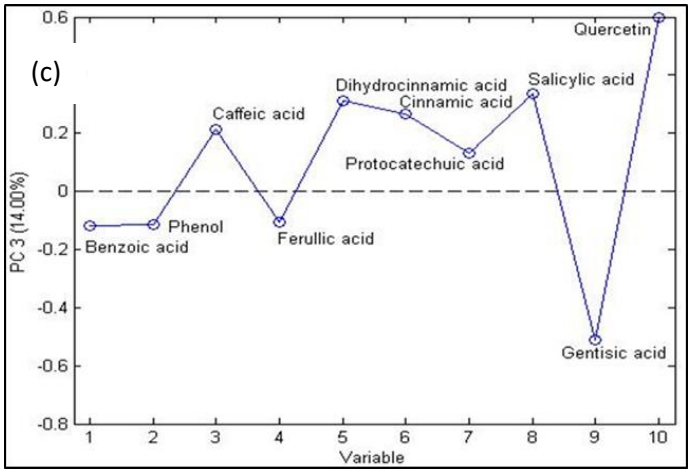


Figure 2. Loading plot analysis PCs against variables a) PC 1 b) PC2 c) PC 3

3.3. Hierarchical Cluster Analysis (HCA)

This technique was applied to describe the overall nearness among the honey samples by using the phenolic compounds data and antioxidant competence studied. The similarities of honey samples was calculated by the measuring the distance in Euclidean distance. Ward's and hierarchical values were set as the parameters of the clustering algorithm and linkage rule. Figure 3 shows the dendrogram of HCA results which the heights of the clusters are proportional to the Euclidean distance between the clusters. The vertical line in the dendrogram represented honey samples and the horizontal line in the dendrogram represented linkage distance between samples. The small linkage between the samples distance means the high of characteristics similarity. From the dendrogram, the data can be classified into three main clusters. From the top, the first cluster of honey samples (a) comprised of KH2, KH3 and KH5. For the second clusters (b) were KH1, KH4 and KH5. The third clusters (c) were TH1, TH2, TH3 and TH5. Cluster (a) and (b) contain KH samples while cluster (c) contain TH samples. The dendrogram second last line showed cluster (b) for KH samples and cluster (c) for TH samples were clustered together. For the last dendrogram line, the data had been clustered into two, the first was KH and the second cluster was TH and KH. This means that KH samples in cluster (b) have many similarities with TH samples in cluster (c) due to their botanical origin and their concentration content. The similar floral and geographical origin also can be one of the factors the samples have similar of phenolic content and antioxidant. Based on Kek (2018), the different species of honey bees have different physicochemical and antioxidant properties by obtaining the different clusters of honey bees and stingless honey bees through HCA result.

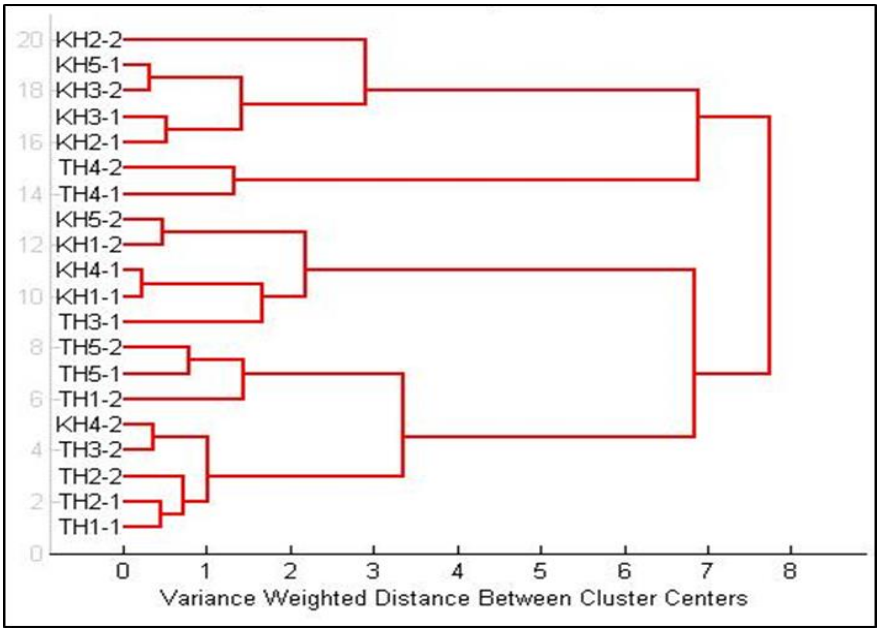


Figure 3. Dendrogram HCA for honeys samples (a) and (b) cluster of KH samples (c) cluster of TH samples

4. CONCLUSION

There were only a few studies identified phenolic compounds in TH and KH using GCMS compared other methods. The combination between GCMS and chemometrics analysis in this study was proposed in order to determine phenolic compounds in tualang honey and kelulut honey with their antioxidant. GCMS was performed to authenticate TH and KH samples via identification and quantification of phenolic compounds such as benzoic acid, phenol, cinnamic acid, coumaric acid, ferulic acid, caffeic acid, gentisic acid, protocatechuic acid, salicylic acid and quercetin to know their antioxidant based on the phenolic compounds. The solid phase extraction method was the excellent extraction for GCMS identify the phenolic compounds contain in the honeys. For the first time, benzoic acid, phenol, ferullic acid, protocatechuic acid, salicylic acid, gentisic acid and quercetin were the new phenolic compounds identified in both honeys even the studied using HPLC and LCMS did not detect these compounds. Based on GCMS results, TH have a greater number of phenolic compounds than KH however, KH have higher peak area which showed that KH have more concentrated phenolic compounds in the honey. This factor can affect the level antioxidant in the honey. In this study, because KH have the high peak area, it showed that KH have higher antioxidant compared to TH. However, antioxidant activity cannot be determined by phenolic compounds alone because there are others compounds that can higher antioxidant activity. As an alternative approach, PCA and HCA methods were employed by analysing the variable amounts of phenolic compounds in honeys indicated correlations with discriminative antioxidant competences. In PCA analysis, the total of PCs was 68.77 %. The discrimination was medium because PCs is lower than 70-80 %. For HCA anaysis, TH samples have more similarity. KH samples in cluster (b) and TH samples cluster (c) are similar due to their similarity in botanical origin and their concentration content. Based on chemometrics analysis, HCA showed better to discriminate the honey sample compared to PCA. For the futher exploration of PCA and HCA, a bigger scale of type of samples and different variables need to be used for the better discrimination. Moreover, other than GCMS, LCMS also can be use to determine phenolic compounds in honey samples. As both honeys were good for health, the study of both honeys should extend to broader in order to know more benefits of the honeys. Moreover, KH is useful in promoting better nutrition for the future as KH have high antioxidant and was consumed by people.

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