Quality of stingless bee honey based on volatile organic compounds and gas released

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Abstract. The aim of this study is to investigate the volatile organic compound released by stingless bee honey stored at room temperature (~24°C) for 10 days and how yeast can influence the stingless bee honey quality. A newly harvested stingless bee honey is divided into 8 samples with each containing 100 ml. Each sample is filled into a glass bottle and 8 samples are considered for 8 different days of evaluation. Gas produced by the samples are collected and analyzed using FTIR coupled with White Gas Cell. The spectrum analysis is done by comparing the spectrum of volatile compound with the infrared fingerprint spectral characteristics from Infrared Analysis Inc. database library via Perkin Elmer's spectrum software. Ethanol, methanol, methane, carbon dioxide, water vapour, and ethyl acetate were found to be released by the stingless honey samples. The bands area were identified in order to calculate the concentration of volatile compounds in the honey's gas. The trend of each of the volatile gasses can be seen from Day-1 until Day-8 of evaluation. To study the presence of yeast, 10 g of stingless bee honey is incubated overnight in nutrient broth followed by centrifuging 1.5 ml of the culture broth repeatedly to obtain cell pellet for VPSEM analysis. VPSEM results indicated that morphology of yeast found in the honey sample. Ethanol concentration found to decrease by time however detailed analysis revealed that sample with the presence of yeast shows an increase of ethanol deliberated by the stingless bee honey. This result affirmed that the presence of yeast may facilitate the further alcoholic fermentation in stingless bee honey by which affecting the sensory value of the stingless bee honey.

1. Introduction

Stingless bee becoming more popular since the honey produced is revealed to have higher nutritional value compared to honey produced by honey bee. Stingless bee stores its honey inside cerumen pots making it distinctive from honey bee that stores its honey inside honeycomb. Beside, stingless bee takes longer time to produce high amount of honey resulting to limited market supply. This problem becomes another reason on why stingless bee honey's market price is reported 2 times higher than honey of honey bees. In Malaysia, two common species of stingless bees found are *Heterotrigona itama* and *Geniotrigona thoracica*. Kelly et al., (2014) reported that almost 90% of stingless bee honey sold in local market was harvested from *Heterotrigona itama*. The focus of demand is now on authentic and high quality stingless bee honey due to the nutritional quality and therapeutic ability. However, this situation give rise to the increasing in risks of fraud as there is lacking in quality standard measurement

technique prior to limited knowledge on the stingless bee honey. Moreover, stingless bee honey quality can be varied according to several factors including type of stingless bee species, type of pollen used and the environmental condition. Postharvest components especially storage condition also can be responsible in constraining the accuracy in determining the authenticity of stingless bee honey in market (Shadan et al., 2017). For sting bee honey, Sanz et al., (1994) mentioned there is less focus on microbial contamination as sting bee honey is known to have higher antimicrobial activity due to its low pH and low water activity. However, for stingless bee honey, moisture content is reported to be higher. High moisture content is believed to be the one that induce the fermentation process in honey. Although honey is commonly low in water content as it is high in sugar, the hygroscopic characteristic of honey making water uptake from environment easier and helping the growth of osmophilic yeast. Amada et al., (2014) also affirmed that with the high moisture condition in honey, fermentation in honey can be induced by some fungi and yeast genera, such as *Penicillium*, *Mucor*, *Saccharomyces*, *Schizosaccharomyces* and *Torula*. Rosa et al., (2003) stated in their study that yeast can be metabolically active and grow in honey even in high concentration of sugar by using sugar as food source. Yeast in honey can be favourable and unfavourable in honey depends on the end use of honey. Yeast is favourable in honey that will be used during the production of mead, honey beverages that containing 8-18% of ethanol (v/v) via fermentation process aided by yeast (Pereira et al., 2015). Alcoholic fermentation in honey will produce ethanol as it's by product together with carbon dioxide. Despite that, for normal honey consumption, the presence of high concentration of ethanol in honey may affect the sensory quality of honey. Amada et al., (2014) mentioned in their study that the sugar content, maturity or ripeness of honey and the presence of active compounds such as aliphatic and aromatic alcohols, aldehydes, acids and their ester are the components that responsible for organoleptic quality of honey. So, in this study, 8 bottles of stingless bee honey representing 8 different days of analysis are stored at room temperature (~24°C) for 10 days before the volatile compounds in the samples including ethanol, methanol, ethyl acetate, and methane are identified using long optical path Fourier Transform Infrared Spectroscopy (FTIR). At the end of experiment, yeast is suspected to affect the ethanol concentration. So, the objective of this study is to investigate the effect of yeast on the stingless bee honey quality by determining the volatile compounds using FTIR coupled with White Gas Cell. The presence of yeast is proven by using VPSEM images.

2. Materials and methods

2.1 *Sample preparation*

Freshly harvested stingless bee honey was divided into 8 glass bottles each containing 100 ml of stingless bee honey. Every bottle is equipped with the gas tube on the top of sealer. The samples then were stored in room temperature which around 24°C for 10 days allowing the gas accumulation in the bottles.

2.2 *FTIR coupled with White Gas Cell*

A cyclone C5 gas cell (Specac Ltd., UK) with 2 to 8 meters adjustable path length was connected to the Frontier FTIR spectrometer (Perkin Elmer) with a deuterated triglycine sulphate (DTGS). The air in the cyclone glass cell was pumped out by an external pump connected to the outlet of the glass cell until the pressure inside the glass cell reaches -1 bar. After that, the background spectrum was taken by using the spectrum software with an average of 8 scans. The range of the spectrum was set from 4000 cm^{-1} to 500 cm^{-1}. During the background scan, 150 ml of the gas from the honey's sample was taken out by using a 60 ml syringe. The gas was transferred to a Tedlar bag. The amount of the gas transferred was around 150 ml. The Tedlar bad filled with volatile gasses of the honey was connected to the inlet of the gas cell. Absorbance spectra of volatile gasses were measured using spectrum software recorded in vacuum as the reference with an average of 8 scans. The spectrum of each samples was taken in 8 different days of evaluation.

2.3 *Analysis of yeast via VPSEM*

Overnight culture broth was prepared by mixing 1 ml of stingless bee honey into 10 ml nutrient broth. The broth was incubated for 24 hours in 37°C of temperature with 200 rpm shaker. The overnight broth culture was then transferred into 1.5 ml eppendorf tube repeatedly to obtain adequate amount of cell pellet via centrifuge. The centrifuge was done for 10 minutes per session using 10 000 rpm rotation. Since sample for VPSEM need to be solidified, the cell pellet was soaked with HMDF for 24 hours and was centrifuged again using ethanol and distilled water. The cell pellet then was air dried before ready to be scanned under VPSEM.

3. Result and discussion

3.1 *Spectral characteristic analysis of volatile gases from stingless bee honey*

As resulted after comparing with the standard spectral library, in addition to ethanol and ethyl acetate, the bands at 3080 - 2820 cm^{-1} are also due to the absorption of methanol and methane. Se et al., (2016) reported the presence of ethanol and ethyl acetate in their stingless bee honey sample but there was no methanol and methane are found. Besides, ethanol, ethyl acetate and methanol also shared the same absorption band with wavenumber ranges from 1150 - 950 cm^{-1}. However, the differentation between all of them can still be done because each of them shows an absorption band where the others was not be observed. Ethyl acetate shows an absorption band at 1820 – 1280 cm^{-1} that could not be observed for ethanol while methane shows an absorption band at 1380 – 1250 cm^{-1} which was not observed for methanol. In the other hand, the standard spectra need to be multiplied with a constant in order to compare the shape with the volatile gases. This is because these bands is differ in term of its absorbance intensity and shape. Peak matching has to be done to confirm the presence of O-H bond and C-H bond inside the gases released by the stingless bee honey samples.

Table 1. Summary of observed infrared characteristics absorption band of ethanol, ethyl acetate, methanol and methane

Volatile gases	**Absorption band (cm^{-1})**
Ethanol	**3080-2820**
	1300-1160
	1150-950
Ethyl acetate	**3080-2820** **1300-1180** **1820-1280** **1150-950**
Methanol	**3080-2820** **1150-950**
Methane	**3080-2820** **1380-1250**

3.2. *Quantitative analysis of present volatile gases*

As presented on Table 2, the concentration of ethanol was calculated by choosing a band from the fingerprint that does not observed for other elements which was 1300 – 1180 cm^{-1}. The ethanol concentration is higher on day 1 of analysis but steadily decreasing from 17.42 Nppm until 0 Nppm on day 6. However, on day 7, the concentration of ethanol was recorded as highest with 320.89 Nppm. Due to that, Ethanol was reported as main volatile compound that commonly found in honey due to the fermentation process. According to Se et al., (2016), lactic acid bacteria is responsible in production of ethanol in honey as lactic acid bacteria is able to produce lactic acid in two condition of fermentation which are homo-fermentative and hetero-fermentative. During, hetero-fermentative, the lactic acid bacteria can produce lactic acid together with other compounds including ethanol, carbon dioxide and lactate (Muller, 2001). The decreasing concentration of ethanol from day 1 to day 6 is believed due to the reducing in number of lactic acid bacteria hence reducing the homo or hetero fermentation in the honey sample. As reported by Olofsson et al., (2008), the viability of lactic acid bacteria is declining throughout the days of storage due to the surrounding becoming unsuitable for its growth. The sudden inclining of ethanol concentration on day 7 is believed to be caused by the presence of yeast in the sample which can initiate alcoholic fermentation that producing ethanol as by-products. To make sure the presence of yeast in the sample of stingless bee honey, VPSEM image is obtained and presented in Figure 5.

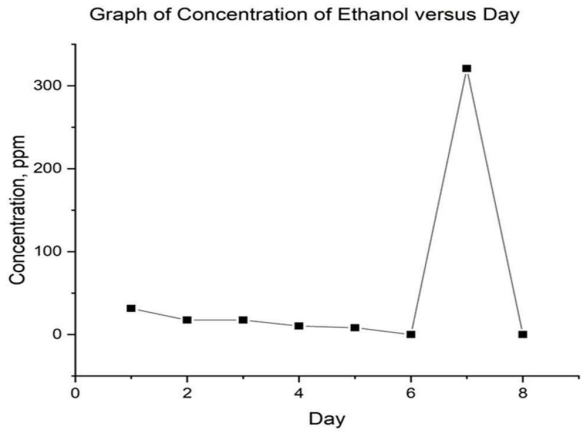

Figure 1: Graph of ethanol trend

For methanol, band 1159 - 950 cm^{-1} is used for calculation of methanol concentration. Table 3 indicated that the concentration of methanol is highest on first day of analysis with 10.58 Nppm was recorded. The methanol concentration started to decrease from day 2 untill remained at 0 Nppm from day 4 to day 8. However, the presence of methanol in stingless bee honey is yet to be reported. However, methanol is a potent toxicant that can occur naturally at low level in alcoholic beverages due to the ethanol fermentation (Paine and Davan, 2011). Ohimain (2016) reported that methanol production in fermented beverages can be linked to the activities of pectinase producing yeast, fungi and bacteria. The crucial point exposed by Ohimain (2016) is fermenting microbes including Saccharomyces cerevisiae and Lactobacillus can be another possible source of methanol in fermented alcoholic beverages. In related to the declining trend of methanol concentration as shown on Graph 2 below, the decreasing ethanol fermentation rate due to the reducing of lactic acid bacteria viability can be the possible reason to explain

why this situation happened. This is because, the methanol is suspected to be released from ethanol fermentation. So, with the slower rate of fermentation, the lesser the release of methanol.

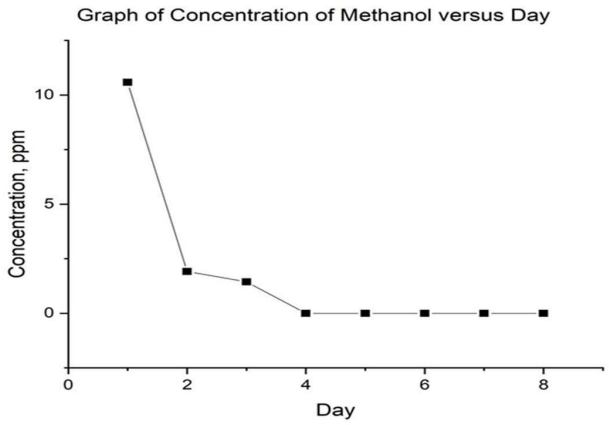

Figure 2: Graph of methanol trend

Using the band of 1310 – 1295 cm^{-1}, the concentration of methane in stingless bee honey for 8 days was calculated and presented on Table 4. Initially, 0 Nppm of methane was detected but on day 4, 2.27 Nppm of methane was detected before was found increased to 10.23 Nppm on day 5. 0 Nppm of methane was able to be detected on day 6 and 7. However, on day 8, 6.81 Nppm of methane was observed. The trend of methane concentration can be observed on the Graph 3 below. The presence of methane in stingless bee honey also has not been reported yet. There are a few suggestions on the source of methane production including from bacterial contamination and also from the further conversion of the fermentation by-products.

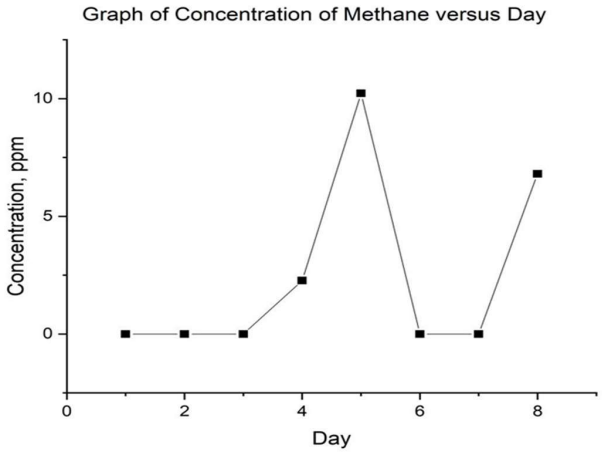

Figure 3. Graph of methane trend

Using the band range of 1820 – 1720 cm^{-1}, as shown in Table 5, the concentration of ethyl acetate recorded for 8 different days was calculated. Ethyl acetate was found only on day 7 with the

concentration 14.70 Nppm. Based on Graph 4, the trend of ethyl acetate is almost similar with ethanol trend as shown on Graph 1. Ethyl acetate in stingless bee honey is believed to be produced due to an esterification reaction between ethanol and acetic acid (Se et al., 2016).

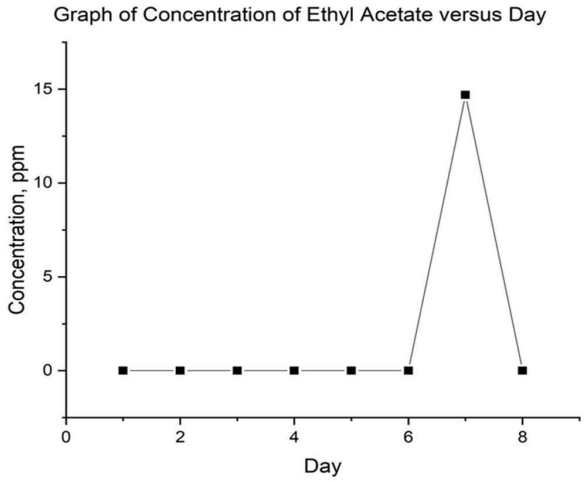

Figure 4: Graph of ethyl acetate trend

Table 2. Concentration of ethanol, methane, methanol and ethyl acetate evaluated in 8 days of evaluation.

Day of evaluation	Ethanol	Methane	Methanol	Ethyl acetate
	Concentration (Nppm)	Concentration (Nppm)	Concentration (Nppm)	Concentration (Nppm)
1	31.44	0	10.58	0
2	17.42	0	1.92	0
3	17.42	0	1.44	0
4	10.23	2.27	0	0
5	8.33	10.23	0	0
6	0	0	0	0
7	320.89	0	0	14.70
8	0	6.81	0	0

3.3. *Presence of yeast via VPSEM*

Based on Figure 5, yeast was observed under VPSEM image and proving that the yeast is present in the sample of stingless bee honey that had been stored for more than a month at room temperature. Abramovic et al., (2008) stated that fermentation caused by osmophilic yeasts such as *Saccharomyces* spp. will occur easily with water activity higher than 0.6. Abramovic et al., (2008) also found that water activity for stingless bee honey is commonly higher than 0.6 even with the storage at 4°C. This fermentation is known as alcoholic fermentation where ethanol and carbon dioxide are released as by-products. This can be the reason why the concentration of ethanol highly increase reaching 320.83 Nppm recorded on day 7 of analysis.

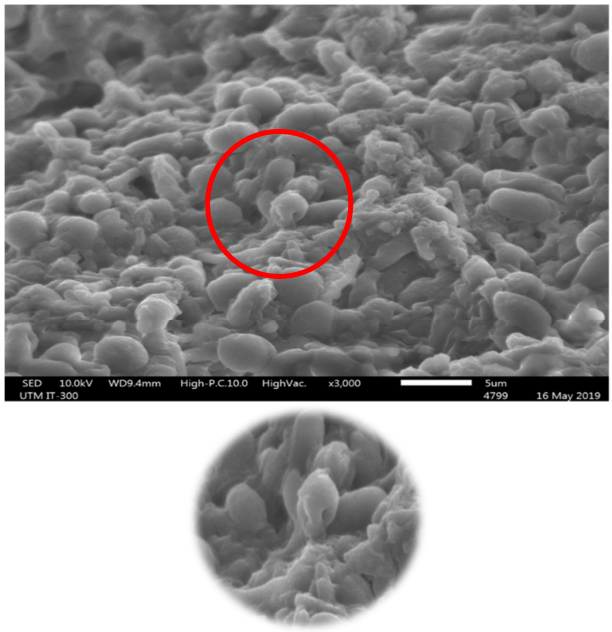

Figure 5: Image of yeast under VPSEM

4. Conclusion

In this study, two new volatile compounds, methanol and methane were found in stingless bee honey apart from ethanol and ethyl acetate. These compounds are probably derived from fermentation by-products and also could be from sample contamination. The presence of yeast is believed to affect the concentration of volatile compounds by facilitating the fermentation process. In regards to that, 320.89 Nppm of ethanol gas was found together with 14.70 Nppm of ethyl acetate on Day-7 of evaluation. Hence, volatile organic compounds are potentially can be an indicator of honey quality as it can be related to the presence of yeast. However, further study is needed to investigate the reason behind the occurrence of these volatile compounds with the presence of yeast.

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