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Research Article

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Glycerol and Myo-Inositol as Marker Compounds for Determination of Freshness in Malaysian Stingless Bee Honey

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ABSTRACT

Fresh honey is always seen as better than the use of old or stored honey for medicinal purposes. Long storage of honey can degrade certain compounds with increased HMF content, which can lower the quality of the honey itself. The present study investigates the variability of the content of two possible markers in Malaysian stingless bee (G. thoracica, H. itama and T. apicalis), namely glycerol (GCR) and myo-inositol (MYI) as a function of its storage time. In this work, GCR and MYI were detected and evaluated in all eight honey samples using the GC-MS method based on the NIST standard reference database. The average peak area abundance value for GCR at initial measurement was at 447,758,853+114,869,619 and decreased to 271,164,640+120,593,470 after 2 months of storage, whilst for MYI, initial measurement was 166,193,862+59,833,930 and reduced to 91,795,248+28,421,379 after 2 months of storage. Results showed that the samples kept at 4ºC decreased in the content of GCR and MYI about 24.56% and 28.84% respectively after two months of storage. The changes of GCR and MYI contents in honey can be used as possible marker compounds for honey freshness as a function of storage time.

Keywords: Glycerol; Myo-inositol; GC-MS; Stingless bee; Honey

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INTRODUCTION

Honey contains many compounds, which are important for many biological activities [1-3]. Honey possesses strong antimicrobial [2], anti-inflammatory [4], antioxidant activities [5] and is able to boost immune system [6]. Its role in enhancing wound healing and regeneration is well known due to the honey properties [7]. Stingless bee honey has different physical and chemical properties compared to *Apis mellifera* honey. Stingless bee honey has higher values of moisture, water activity, electrical conductivity, free acidity, low enzyme activity and a distinct sugar spectrum

[8-12]. Stingless bee honey also shows higher antimicrobial activity, antioxidant and excellent wound healing activity [10,13-18] due its high organic compounds present in honey. It is valued as medicinal substance by certain tribes in South America, Asia, Africa and used for treatment of certain diseases [19]. The use of fresh honey is always seen as better than the use of old or stored honey. Stored honey for a long period of time has been shown to reduce its antioxidant activities [20] and degrade certain compounds (including phenolic acid and flavonoid) [20,21] which lead to an increase of HMF content [22], thus, lowering the quality of the honey [21]. The aim of this study is to investigate the possible compounds that can be utilized as markers for honey freshness in Malaysian stingless bee honeys specifically in the most common domesticated stingless bee, *Geniotrigona thoracica*, *Heterotrigona itama* and *Tetrigona apicalis*. The selected compounds, glycerol and myo-inositol could be potentially served as alternative freshness markers for the Malaysian stingless bee honey samples. The contents of two possible marker compounds are determined in the abundance of peak area value in GC-MS analysis to support their marker profiles qualitatively.

MATERIALS AND METHODS

Sample Collection

Eight honey samples from different hives of three stingless bee species were collected from Syamille Agrofarm, a stingless bee farm located in the northern part of Malaysia, Kuala Kangsar, Perak. The three species which include *Geniotrigona thoracica* (3 samples), *Heterotrigona itama* (2 samples) and *Tetrigona apicalis* (3 samples), were identified by the Entomology Section, Malaysian Agriculture and Research Development Institute, Serdang, Malaysia. The collected samples were kept in a minimal light condition, refrigerate at 4 ºC and stored for 2 months.

Reagents

Helium was purchased from a local supplier with high grade purity (99.999%). Derivatization reagents; pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma Aldrich (St. Louis, MO, USA) and Supelco (Bellefonte, PA, USA) respectively.

Derivatization and GC/MS Analysis of Stingless Bee Honey

The samples were derivatized based on previously reported work by Graikou [23] with slight modifications, prior to GC-MS analysis. In brief, about 4 mg of sample was mixed with 40 µL of pyridine and 60 µL BSTFA and heated in the water bath for 20 min at 80 °C. The clean silylated extract was analyzed by GC-MS. A portion (2 µL) of the derivatized sample was injected into the GC-MS system with a split ratio of 1:20. The analyses were performed using an Agilent 7890A gas chromatograph (Santa Clara, CA, USA) equipped with an Agilent mass selective detector (MSD) 5975C (Santa Clara, CA, USA) and split injector. Chromatographic separations were performed on an Agilent HP5-MS (30 m × 0.25 mm I.D × 0.25 µm film thickness) using helium as a carrier gas at 1.0 mL/min in a constant flow rate mode. The temperature of the inlet was set at 200 ºC. The GC oven temperature was initially held at 100 ºC and then increased to 300 ºC at a rate of 5 ºC/min. The identification of the compounds was based on the comparison of their retention time (RT) and mass spectra with those from NIST Revision 2005 (D.05.02) standard reference database.

Statistical Analysis

Statistical data analysis was performed by using Microsoft Excel software and data was presented in mean ± standard deviation. Student t-test was used to determine the significant difference between the two parameters.

RESULTS AND DISCUSSION

Screening of Marker Compounds in Pure Stingless Bee Honey

Under the optimized GC-MS derivatization conditions, all eight stingless bee honey samples were analyzed in replicates (at least n=3) using GC-MS to identify the possible marker compounds present in all the honey samples. Based on the GC-MS NIST standard reference database, two compounds were detected consistently in all samples, namely glycerol (GCR) and myo-inositol (MYI). Thus, GCR and MYI were selected as possible marker compounds for the three species of stingless bee, for further investigations in this study. Typical GC-MS chromatograms of the prepared pure stingless bee honey of three different species (a) *H. itama* (b) *G. thoracica* and (c) *T. apicalis* are shown in Figure 1.

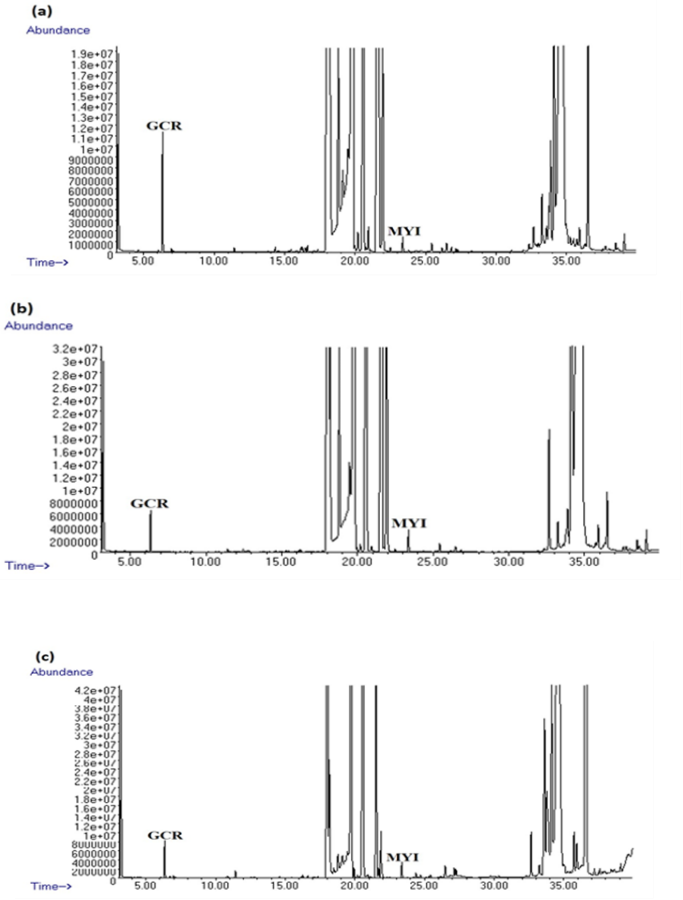

Figure 1. GC-MS chromatogram of volatile compounds in (a) *H. itama* (b) *G. thoracica* and (c) *T. apicalis*

Glycerol content is commonly found in honey but it is present as a minor constituent [24]. It is produced by the activity of microorganisms that exists in the nectar and honey dew which are collected by bees from plants and flowers. The average glycerol content in honey bee was 172 mg/kg, whereby the maximum value was 601 mg/kg. Most honey had glycerol content less than 100mg/kg [25].

Myo-inositol is known as valuable nutrient for every animal species and in plants, in which it exists in the form of cylitols and methyl inositols [26]. Derivatives of myo-inositol and its phosphate are necessary as a secondary messenger molecule for cellular activities (such as for cell growth, apoptosis, cell migration, cell differentiation, stress response, seed germination, gene expression, hormone function, protein trafficking) [27]. The study on 28 examined honey samples collected from various Spanish regions revealed that myo-inositol was found in the range of 0.14 to 2.78 mg/g [28].

Based on the obtained results, the average peak area abundance of GCR in fresh pure stingless bee honey samples was in the range of 151×10^6 to 606×10^6. Meanwhile, for MYI, the average peak areas obtained were in the range of 52×10^6 to $151 \times 10\text{^}6$. These ranges were then used to analyze the degradation of GCR and MYI in pure stingless bee honey samples stored up to two months.

Effect of Storage Time on Marker Compounds

To evaluate the effect of storage time on the content of marker compounds (GCR and MYI), pure stingless bee honey samples were analyzed by GC-MS at initial points of storage and after two months storage. The changes in the content of markers compounds were observed. Our study however is not focusing on the quantitative analysis of the two marker compounds, GCR and MYI, but rather to propose their usefulness as possible markers and indicators for honey freshness since they degrade significantly over time. In this work, we suggest the use of the peak area abundance to determine the GCR and MYI contents in fresh stingless bee honey samples. The changes in the peak area abundance of GCR and MYI was used to estimate the percentage of degradation of the two selected markers. The average peak area values for GCR and MYI in all samples are shown in Table 1.

Table 1. The average changes of peak area abundance value of GCR and MYI at initial and after 2 months of storage

Compound	Peak area value	
	Initial measurement	After 2 months of storage
Glycerol	447,758,853 + 114,869,619	271,164,640 + 120,593,470
Myo-Inositol	166,193,862 + 59,833,930	91,795,248 + 28,421,379

The mean of peak area value from all the samples analyzed for GCR was 447,758,853 + 114,869,619 and for MYI, the mean of peak area value was 166,193,862 + 59,833,930 at initial measurement but both compounds degraded after two months of storage with GCR reduced to 271,164,640 + 120,593,470 and MYI reduced to 91,795,248 + 28,421,379. The average peak area value of GCR was significantly reduced to about 24.56% and MYI was reduced up to 28.84% after two months of storage ($p<0.001$) (Figure 2). The changes of GCR content in honey was somehow expected as the study of Olaitan, Adeleke and Ola [30] showed that the microorganism found in honey could convert GCR into several GCR intermediates. The microorganism such as bacteria and yeast of contaminated bee can cause contamination of the nectar [29] and thus affecting GCR in honey. Bacteria such as *Enterobacter sp.* and *Klebsiella sp.* found in honey can convert GCR into several metabolic intermediates such as 1,3-propanediol, succinate, dihydroxyacetone, propionic acid, pigments, ethanol and butanol [30]. Furthermore, some of GCR converting microorganisms are able to grow anaerobically and utilize glycerol as an energy source. Higher

degradation of GCR may suggest microorganism contamination which is possibly affecting the quality and freshness of honey.

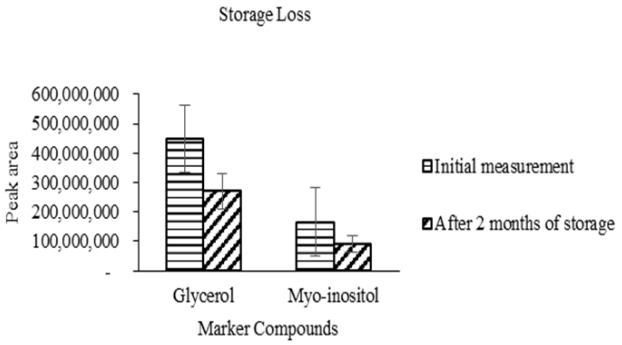

Figure 2. The Peak area values of GCR and MYI at initial and after 2 months of storage. The differences at initial and after two months measurement was significant ($p<0.001$) for both GCR and MYI

MYI, is an important polyol that exists in all living organisms (from bacteria to human) which are involved in cellular metabolism [31]. MYI exists in its free form and is covalently bound to phospholipid as phosphatidylinositol (PI) which is particularly related to the biosynthesis and degradation of the myo-inositol-containing phospholipids associated with biological membranes [32]. The cleavage of PI by phospholipase C creates inositol phosphate (IP) and diacyglycerol which are molecules that are involved in central cell signalling [33]. Myo-inositol was shown to be catalized and degraded by bacteria. According to Morinaga, Ashida and Yoshida [34], *Bacillus subtilis*, which is commonly present in the gut of the bees [35] was able to reduce myo-inositol level due to the reaction of conversion of myo-inositol into scyllo-inosose (SIS or 2-keto-MI) [34].

Interestingly, our data showed the reduction of GCR and MYI contents, upon storage up to two months at 4 ºC. The degradation of the compounds is observed up to two months of storage and estimated to be around 24.56% for GCR and 28.84% for MYI. Study on the factors that could contribute to the degradation of the two markers should be carried out in future.

CONCLUSION

The results obtained in this study enable us to suggest that glycerol and myo-inositol can be taken into account as eligible freshness markers for three common domesticated species (*G. thoracica*, *H. itama* and *T. apicalis*) of Malaysian stingless bee honey samples. Within 2 months of storage, the glycerol and myo-inositol content were decreased to about 24.56% and 28.84%, respectively. Based on the results obtained, the two selected compounds, glycerol and myo-inositol could be potentially served as alternative freshness markers (as a function of the storage time) for the Malaysian stingless bee honey samples.

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