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FULL PAPER

Analysis of trehalulose in kelulut honey samples via HPLC-MS

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Abstract

Over recent years, there had been many studies by Malaysian researchers focusing on kelulut (stingless bee) honey chemical profile. One of the ultimate purposes of carrying out the chemical profiling activity was to search for suitable unique biomarkers that can be used as quality as well as authenticity indicators. Both aspects are crucial in order to maintain the premium status of kelulut honey as it has always been regarded as one of the best multifunctional local health supplements. An interesting finding on a chemical component of kelulut honey was recently published. Trehalulose was identified as a major component in kelulut honey sourced from both *Geniotrigona thoracica* and *Heterotrigona itama* which are common species in Malaysian stingless bee farms. Since trehalulose is not commonly available from other natural sources, it can be a good quality marker candidate. To qualify as a good quality marker, a biochemical component must fulfil some criteria. This article reports on the potential of trehalulose as a quality indicator or marker in *H. itama* honey. Our preliminary studies focused on the analytical aspect of trehalulose based on analysis using high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD). Our findings showed that all kelulut honey samples collected contained trehalulose and the HPLC analysis can be carried out using a simple method provided that a pure commercial standard is available.

1. Introduction

In Malaysia, kelulut (stingless bee) honey has been regarded as one of the best multifunctional health supplements and is hence sold at a premium price. There have been many claims attached to it such as antioxidant, antitumour, antidiabetes, anti-inflammation, antibacterial and wound healing promoter. These activities could be due to the presence of many different bioactive components such as flavonoids, phenolic acids and minerals (Silva *et al.*, 2013; de Sousa *et al.*, 2016; Ranneh *et al.*, 2018; Ávila *et al.*, 2019; Biluca *et al.*, 2019). However, some of these components are not unique to stingless bee honey and are also commonly found in other food products while some of them are not consistently found in different stingless bee honey sourced from different locations. Therefore, they could not be considered to be good quality marker candidates. A good quality marker or indicator must be uniquely and consistently present in a product (irrespective of its sources), stable, exhibits certain biological activities and can be analysed qualitatively as well as quantitatively.

Trehalulose, a rare disaccharide, is a sucrose isomer. However, unlike sucrose, trehalulose is acariogenic and exhibits a lower glycemic index (Fletcher *et al*., 2020). The acariogenic property is mainly associated with the α-1,1-glycosidic bond between its glucose and fructose subunits which cannot be easily broken by *Streptococcus mutans*, a significant caries-causing oral bacteria. The stronger bond also contributes to its lower glycemic index (GI) which is in contrast to the weaker α-1,2-glycosidic bond in sucrose which has a higher GI. Wach *et al*. (2010) reported that trehalulose also has an advantage over another low GI sucrose isomer, isomaltulose, with α-1,6-glycosidic bond. This is due to the fact that trehalulose triggers a lower insulin response than isomaltulose. These facts support the traditional claim that kelulut honey is suitable to be taken by diabetic patients and can help in reducing blood sugar.

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This study focused on the development of a simple analysis of trehalulose and verifies the method using mass spectrometry analysis. Kelulut honey samples sourced from different locations (*Heterotrigona itama* species) were analysed for trehalulose to find out whether trehalulose is consistently present in all kelulut honey. Two different monofloral kelulut kinds of honey (coconut and acacia) were also compared against multi floral kelulut honey as well as tualang Apis bee honey. This is to find out whether trehalulose is consistently present in different types of kelulut honey. The absence of trehalulose in certain monofloral kelulut honey may indicate that certain components required for trehalulose production might be absent in the plants of nectar source. It is also interesting to know whether trehalulose can also be found in tualang *Apis dorsata* honey which is normally kept in its comb to mature or ripen before being harvested. The ripening process normally takes about 3 weeks before harvesting (Chua and Adnan, 2014). Fermentation or other enzymatic reaction may take place within such period of time and this may result in the production of trehalulose provided that the required enzyme and substrate are present (Tian *et al.*, 2019). This can be expected since trehalulose has been previously found in small quantities in honey (Low and Sporns, 1988; Nakajima *et al.*, 1990). However, both studies did not specify the name of *Apis* species involved.

2. Materials and methods

2.1 Sample collection

A total of ten multi floral kelulut honey samples were collected from different locations in Negeri Sembilan, Sarawak, Pahang, Johor and Selangor. Nectar sources in these areas are fruit trees such as durian (*Durio zibethinus*), rambutan (*Nephelium lappaceum*) and duku (*Lansium domesticum*) apart from 'air mata pengantin' (*Antigonon leptopus*). These samples would be used for qualitative analysis of sucrose, maltose and trehalulose by comparing the mass fragmentation pattern of the disaccharide standards and the components present in the honey samples.

In order to compare the sugar profiles between different monofloral and multi-floral kelulut honey, coconut and acacia monofloral kelulut honey were collected from Selangor Fruit Valley kelulut farm. Another multi-floral kelulut honey was sourced from Mantin, Negeri Sembilan. All kelulut honey samples were collected in August. The only kelulut species involved in this study was *Heterotrigona itama*. Tualang honey (wild honey) was purchased from a trusted seller who collected it from an *Apis dorsata* hive on a tualang tree (*Koompassia excelsa*).

2.2 Sugar standards

Standard trehalulose (>90% purity) was purchased from Biosynth Carbosynth (Staad, Switzerland). Fructose, glucose, sucrose and maltose (>98% purity) were purchased from Nacalai Tesque (Kyoto, Japan).

2.3 High-performance liquid chromatography analysis

Sample preparation was carried out according to Malaysian Standard MS2683:2017. The quantitative assay was performed using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). The HPLC mobile phase used was a premixed 85% acetonitrile in deionized water. All samples were eluted through a GL Sciences NH2 column (150 mm × 4.6 mm, oven temperature of 40^{o}C) and the flow rate was fixed at 0.8 ml/min. Since the highest purity commercial standard of trehalulose available is specified as >90% pure, quantitative analysis of trehalulose can be less accurate.

2.4 High-performance liquid chromatography-mass spectrometry analysis

In order to confirm the identity of each sugar peak, each sample was analysed using the same HPLC setup as above except for the detector which was replaced with Bruker Amazon SL ion trap mass spectrometer (ITMS). The ITMS with electrospray ionization (ESI) was run in negative mode with SmartFrag which ramps the excitation voltage during fragmentation. The mass spectrum data is important to ensure that the sugars are correctly identified since the retention time of sucrose, maltose and trehalulose are very close to each other.

2.5 Statistical analysis

Means and standard deviations were calculated based on triplicate experiments. The data were analyzed using ANOVA and Duncan's multiple range test at a significance level of $p<0.05$ using SAS System (SAS for Windows version 9.4).

3. Results and discussion

3.1 Differentiation between sucrose, maltose and trehalulose based on mass spectral analysis

The optimized method successfully separated the 5 sugar peaks (Figure 1). In order to verify the identity of the peak, the standards were analysed using a mass spectrometer which can provide structural data based on the daughter fragments produced. It is therefore a superior method compared to ELSD and refractive index detectors (RID) which cannot provide any structural data. Despite the similarities in molecular weight of the

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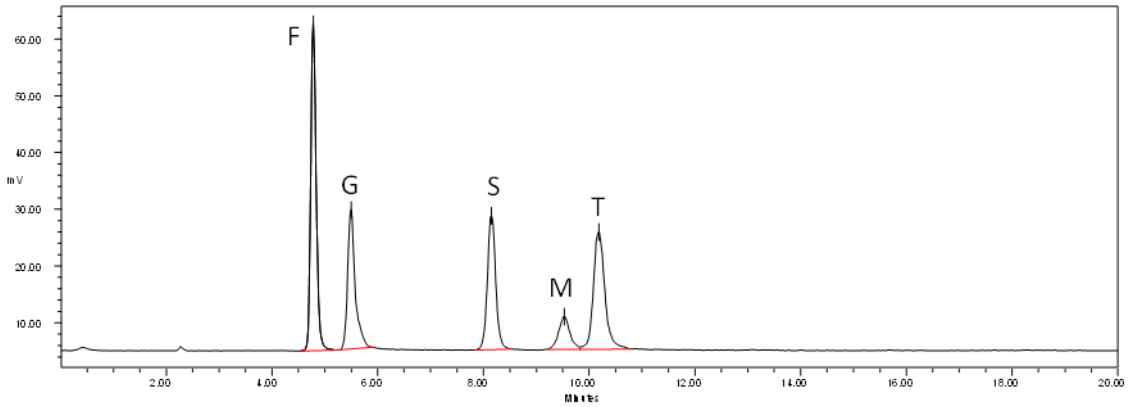

Figure 1. HPLC chromatogram showing the peaks of fructose (F), glucose (G), sucrose (S), maltose (M) and trehalulose (T)

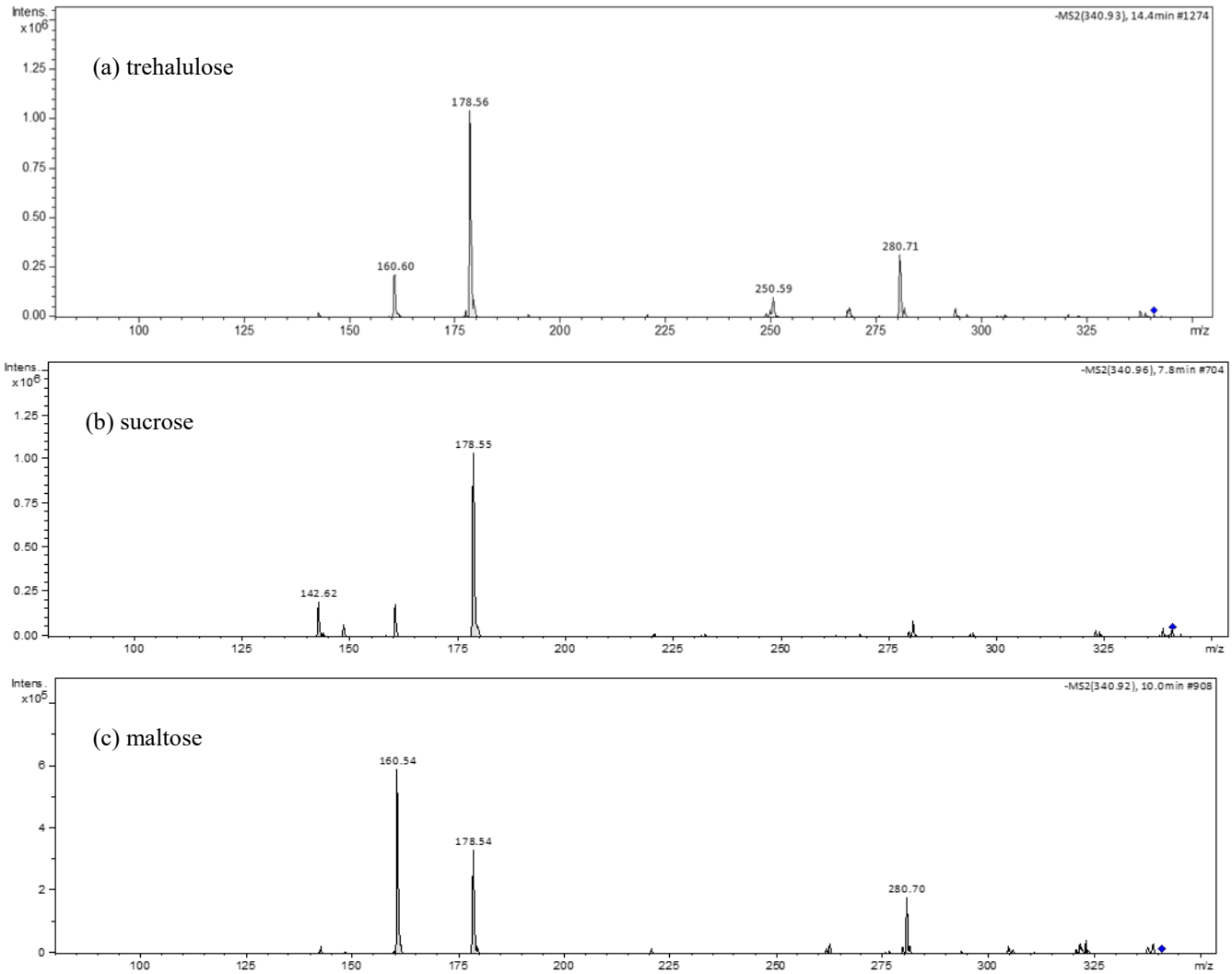

Figure 2. Mass spectra of trehalulose (a), sucrose (b), and maltose (c) ESI-MS negative mode

disaccharides, the base peak and fragmentation patterns observed were different (Figure 2). The base peak for both sucrose (Figure 2b) and trehalulose (Figure 2a) is 179 m/z while trehalulose spectrum shows an extra 281 m/z major fragment. In contrast, the base peak for maltose (Figure 2c) is 161 m/z.

Based on the retention time and mass spectra, all ten kelulut honey samples from different locations showed the presence of trehalulose and a very low amount of maltose. Sucrose could not be detected in all samples. Since the trehalulose standard available was approximately 90% pure, the trehalulose content could only be estimated which ranges approximately between 5 to 30 g per 100 g of honey. However, more samples need to be analysed to give a more significant statistic.

Recently, trehalulose had been identified in kelulut honey (Fletcher *et al.*, 2020). Based on a comparison between our unpublished data on sugar profile of kelulut honey studies and other earlier reports (Kek *et al.*, 2017; Shamsudin *et al.*, 2019; Shamsudin *et al.*, 2019; Tuksitha *et al.*, 2018; Wong *et al.*, 2020), trehalulose may have been previously mistaken for sucrose or maltose as the

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high-performance liquid chromatography (HPLC) retention time for all three sugars are very close to each other or even overlap without optimization. Without confirmation using mass spectrometry or nuclear magnetic resonance, such confusion may have led to the error in sucrose and maltose analyses results. Our finding concurs with the view of Fletcher *et al.* (2020). In contrast to those reports, our sugar profiling study which involved more than 200 samples showed that both sucrose and maltose contents in kelulut are lower than 10%(w/w). This data was used to develop the Malaysian Standards MS2683:2017 (Malaysian Standard, 2017)

Most laboratories perform sugar analyses by employing HPLC with ELSD or refractive index (RI) detectors due to the much lower cost of maintenance. However, these detectors only provide retention time as a means for component identification. Most commercial laboratories would not consider purchasing trehalulose standards since it is relatively expensive (USD1,400 per g) and only very few clients request trehalulose quantification. Hence, without the availability of trehalulose chemical standards, there is a tendency to misidentify trehalulose for the more common sucrose or maltose. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) on the other hand could provide further details on the molecular structure of the components detected. The analyses based on mass spectra and NMR spectra (Popova *et al.*, 2021) are therefore more reliable in distinguishing the 3 sucrose isomers.

Malaysian Standard MS2683:2017 specifies that a high quality kelulut honey should contain no more than 8% of sucrose and not more than 10% of maltose. It is interesting to observe that sucrose and maltose contents exceeded the specified limit in some studies which employ ELSD and RID detectors, while values reported in two studies using gas chromatography-mass spectrometry (GCMS) and NMR were well within the limits specified (Table 1). However, the number of studies using GCMS and NMR were too small to be regarded as a fair comparison. More quantitative studies using MS and NMR are required for a better comparison. Therefore, it is suggested that if kelulut honey samples are found to contain very high sucrose or maltose, which exceeds the limit specified, further analysis should be carried out using MS and NMR to reconfirm the identity of disaccharides present.

3.2 Sucrose, maltose and trehalulose content in multifloral and monofloral kelulut honey in comparison with Apis bee honey

The sum amount of fructose and glucose in all four types of honey complied with the MS2683:2017 parameters which state that the sum amount of fructose and glucose must not exceed 85%. In terms of the disaccharides, the results of sugar analysis showed that maltose could not be detected in all three kelulut kinds of honey while sucrose was present in coconut and acacia kelulut honey at 3.31±0.01% and 3.27±0.09% respectively. These are well within the limit and therefore all three types of kelulut honey conform to the parameters specified in the MS2683:2017. Those kelulut honey samples also contained trehalulose which was estimated between 5 to 30 g per 100 g of honey, based on a standard curve plotted using 90% pure trehalulose standard. This is slightly lower than reported by Fletcher *et al.* (2020), which ranges between 13 to 44 g per 100 g of honey, who studied stingless bee honeys from *Tetragonula carbonaria, Tetragonula hockingsi,*

Table 1. Sucrose and maltose content in *H. itama* honey.

Authors	Method	Sucrose content (%)	Maltose content (%)
Tuksitha *et al.* (2018)	HPLC-RID	ND	33.7±2.1
Kek *et al.* (2017)	HPLC-ELSD	32.33	ND
Wong *et al.* (2020)	HPLC-ELSD	0.04±0.18[1]	24.89±10.07[1]
	HPLC-ELSD	0.04±0.30[2]	29.57±5.63[2]
Shamsudin *et al.* (2019b)	HPLC-RID	28.44±0.89[3]	ND[3]
	HPLC-RID	37.32±1.14[4]	0.89±0.43[4]
	HPLC-RID	17.36±1.09[5]	ND[5]
Shamsudin *et al.* (2019a)	HPLC-RID	ND[3]	22.56±1.22[3]
	HPLC-RID	ND[4]	37.35±0.58[4]
	HPLC-RID	ND[5]	31.50±2.11[5]
Abdul Malik *et al.* (2020)	HPLC-RID	51.885±16.083	ND
Mustafa *et al.* (2019)	NMR	1.4	7
Omar *et al.* (2019)	GCMS	0.45±0.50	5.73±3.62

[1]Nanga Dap area, [2]Tanjung Manis area, [3]monofloral acacia honey, [4]monofloral starfruit honey, [5]monofloral gelam honey.
ND: Not detected

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Table 2. Sugars content in honey samples.

Honey samples	Fructose (F) (g/100 g)	Glucose (G) (g/100 g)	F and G (sum) (g/100 g)	Sucrose (g/100 g)	Maltose (g/100 g)	Trehalulose
H. itama (multiflora)	9.98±0.64[a]	6.31±0.36[a]	16.29±1[a]	ND	ND	Present
H. itama (coconut)	12.09±1.02[b]	8.43±0.34[b]	20.52±1.36[b]	3.31±0.01[a]	ND	Present
H. itama (acacia)	19.26±4.62[c]	15.35±4.03[c]	34.62±8.63[c]	3.27±0.09[a]	ND	Present
A. dorsata (tualang)	43.12±0.85[d]	32.00±9.38[d]	75.12±8.53[d]	ND	7.55±1.23	ND

Values are presented as mean±SD. Values with different superscript within the same column are significantly different (P<0.05). ND: Not detected

Heterotrigona itama, Geniotrigona thoracica and *Tetragonisca angustula*. However, this is much higher than the study reported by Nakajima *et al*. (1990), between 0.5 to 2.5% of total solid, which involved 9 different samples of *Apis* bee honey. Since water content was not included in the results by Nakajima *et al*. (1990), the trehalulose content per 100 g of raw honey in those honey would be lower. In contrast, only maltose could be detected in tualang *A. dorsata* honey and no trehalulose could be detected.

The results also showed that all the kelulut samples (monofloral and multifloral) relatively high amount of trehalulose (Table 2). In conclusion, trehalulose can be used as a quality indicator of kelulut honey. A minimum amount of trehalulose can be specified as a requirement for high quality kelulut honey. However, as this study only involved *H. itama,* more studies involving more stingless bee species and nectar sources should be carried out in order for such specifications to be applied to kelulut honey in general.

4. Conclusion

The presence of fake and adulterated honey in the market led to suspicion among consumers regarding the authenticity of the honey. Kelulut honey is known for the sourness and sweetness of acacia kelulut honey might give a wrong impression on its authenticity and whether its sugar content can still conform to the MS2683:2017. This study has proven that the sugar content of both coconut and acacia monofloral kelulut honey conforms to the requirements set out in the standard.

Conflict of interest

The authors declare no conflict of interest.

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