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Untargeted Small Metabolite Identification from Thermally Treated Tualang Honey

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***Abstract*—**This study investigated the effects of thermal treatment on Tualang honey sample in terms of honey colour and heat-induced small metabolites. The heating process was carried out in a temperature controlled water batch at 90°C for 4 hours. The honey samples were put in cylinder tubes with the dimension of 1 cm diameter and 10 cm length for homogenous heat transfer. The results found that the thermal treatment produced not only hydroxylmethylfurfural, but also other harmful substances such as phthalic anhydride and radiolytic byproducts. The degradation of honey protein was due to the detection of free amino acids such as cysteine and phenylalanine in heat-treated honey samples. Sugar dehydration was also occurred because fragmented di-galactose was identified based on the presence of characteristic ions in the mass fragmentation pattern. The honey colour was found getting darker as the heating duration was increased up to 4 hours. Approximately, 60 mm PFund of increment was noticed for the honey colour with the colour change rate of 14.8 mm PFund per hour. Based on the principal component analysis, the score plot clearly shows that the chemical profile of Tualang honey was significantly altered after 2 hours of heating at 90°C.

***Keywords*—**Honey colour, hydroxylmethylfurfural, thermal treatment, Tualang honey.

I. Introduction

HONEY is a natural sweet substance which has been orally consumed for its high nutritive value and topically applied for its medical purposes since ancient time [1]. Raw honey is usually filtered to remove pollen and other suspended materials prior to heating and bottling. Heating is the most important processing step for honey to reduce the water content to prevent fermentation [2], dissolve the sugar crystal nuclei to retard granulation [3], homogenize honey colour for the preference of consumers [4] and destroy the sugar tolerant osmophilic yeasts to prolong the shelf life of honey [5]. However, heating may darken the honey colour because of oxidation on furanic compounds.

Hydroxymethylfurfural (from hexoses) belongs to the furan family, which is also one of quality indicators for honey freshness. The other compounds that probably arising from thermal treatment on sugar-rich foodstuffs include furfural (from pentoses), 2,5-dimethylfuran, 5-methylfurfural, furfuryl alcohol, dihydro-5-methyl-2(3H)-furanone, dihydro-2-methyl-3(2H)-furanone and 1-(2-furanyl)-ethanone [3], [6]. Possibly, additional synthesis routes will be promoted and it produced short chain alcohols such as 3-methyl-1-butanol and 3-methyl-3-buten-1-ol [7]. Nevertheless, the metabolites produced after thermal treatment is highly relied on the biochemical composition of honey, which is botanical and geographical origins dependence.

With the advancement of analytical technique nowadays, compounds with a wide range of chemistry can be analyzed with minimum sample pre-treatment. A large data can be generated in a short period and metabolite identification can be performed at high confident level with the assistance of statistical approach. To the best of our knowledge, chemometric method such as principal component analysis is useful for untargeted data mining to obtain information from highly complex datasets [8]. This mathematical technique analyzes the entire data without compromising any dimensional factor.

In the present study, thermal treatment was carried out on the Tualang honey samples at 90°C for 4 hours. The heating temperature 90°C was used to avoid sugar caramelization during treatment. Tualang honey is the most widely consumed honey in Malaysia. The honey samples were sampled for colour measurement and metabolite identification by high mass accuracy and sensitivity of hybrid analytical tool, namely liquid chromatography coupled with quadrupole-time-of-flight mass analyzer. The huge datasets were then analyzed by an unsupervised pattern recognition technique of principal component analysis for data mining.

II. Materials and Methods

A. Honey Samples and Chemicals

Tualang honey sample was purchased from Federal Agriculture Marketing Authority (FAMA), Kedah. The honey sample was stored in amber glass bottle at 20°C before analysis. HPLC-grade of acetonitrile, chloroform, and formic acid (98-100%) were obtained from Merck (Darmstadt, Germany).

B. Thermal Treatment on Honey Sample

Honey sample (20 g) was put in a cylinder tube (1 cm diameter x 10 cm length). The honey sample in the tube was heated in a water bath at 90°C for 4 hours. A 2 g of honey sample was drawn for furanic compound extraction and identification at an hour time interval.

C. Extraction of Furanic Compounds by Chloroform

The furanic compounds in heat-treated honey samples were extracted according to the method described by [9] with modification. The honey sample (2 g) was diluted with 2 ml of

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deionized water and sonicated for 5 minutes. A 0.5 ml of chloroform was added into the solution and mixed by a vortex mixer vigorously for 5 minutes. The solution was centrifuged at 13,000 rpm for 10 min and the upper aqueous layer was discarded. The bottom chloroform layer was vacuum dried and reconstituted with methanol for LC-QTOF MS analysis.

D.Measurement of Honey Color

The colour of honey samples was measured by using colorimetric method as described by [10]. The measurement of colour is expressed in the unit of light transmittance (mm PFund) at 635 nm as presented in (1).

$$\text{Honey colour (mm PFund)} = -38.7 + 371.39 \times \text{Absorbance} \quad (1)$$

E. Small Metabolite Identification by LC-QTOF MS

A capillary liquid chromatography (Dionex Corporation Ultimate 3000; Sunnyvale, CA) system was integrated with a diode array detector (Dionex Ultimate 3000) to fingerprint the samples. A C18 reversed phase XSelect HSS T3 column (2.1 × 100 mm, 2.5 µm) with a flow rate of 150 µl/min was used for separation and detected at 276 nm. A binary gradient system consists of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile). The LC gradient was 0–5 min, 10 % B; 5–15 min, 10-80 % B; 15-20 min, 80 % B; 20-21 min, 80-10 % B; 21-25 min, 10 % B. The injection volume was 5 µl. All samples were filtered with 0.2-µm nylon membrane filter prior to injection.

The QTOF mass spectrometer was used for the small metabolite screening from m/z 100–1000. It was calibrated by using 1 pmol of reserpine (m/z 609.2807) before use to ensure the mass accuracy. A single Information Dependent Acquisition (IDA) method was created to acquire both TOF MS and three dependent runs of product ion scan with rolling collision energy. Nitrogen gas was used for nebulizing (40 psi) and curtain gas (25 psi). Collision gas was set at 3, the accumulation time was 1 s for TOF MS and 2 s for each product ion scan. The voltage of ion spray was 5500 V for positive ion mode and -4500 V for negative ion mode. The declustering potential was 40 V and the focusing potential was set at 200 V.

F. Statistical Analysis

The mass spectra of honey samples were analyzed by an unsupervised statistical approach; principal component analysis (Marker View 1.2.1.1, ABSciex, USA) using Pareto scaling with the minimum spectral peak width, 0.3 amu; retention time tolerance, 0.1 min and mass tolerance, 0.1 amu.. This multivariate data analysis was used to cluster the huge datasets at different heating duration for pattern recognition.

III. Results and Discussion

Honey is believed as the most nutritious food, which can also provide instant energy because of its high reducing sugars composed primarily of glucoses (25–45%) and fructoses (25-37%) [11]. Thermal treatment is given to honey to deactivate microorganisms, as well as to delay fermentation and crystallization by reducing the water content to less than 20%. This treatment is important for honey with high water content from tropical countries [12]. The heating temperature was monitored at 90°C to avoid sugar caramelization [13]. However, the heating process was still found to make the honey color darker than untreated honey samples. Fig. 1 shows the increase of honey colour as the heating duration was increased up to 4 hours. Approximately, the colour of Tualang honey increased for 15 mm PFund in an hour of heating time at 90°C.

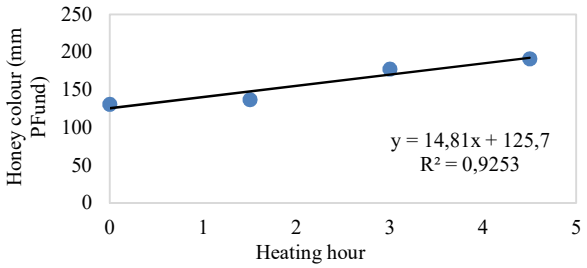

Fig. 1 Honey colour at different heating duration

Indeed, thermal treatment accelerates the process of Maillard reaction (non-enzymatic browning reaction) and acid-catalyzed dehydration of hexoses [14]. The acceleration of the reactions consequently will produce furanic compounds such as hydroxymethylfurfural and its congeners. These furanic compounds are reactive and unstable [15], as well as known to have cytotoxic [16], genotoxic [17] and tumoral (colon-rectum, hepatic and skin cancers) [18] effects. In the present study, thermal treatment on Tualang honey also detected the formation of hydroxylmethylfurfural at m/z 127 with its characteristic ions such as m/z 105 (-H_2O) as the base peak and m/z 81 (-CO) as presented in Table I [19]. Hydroxylmethylfurfural could be the most intense compound after heating for 4 hours at 90°C because the peak signal is the highest in the chromatogram at 275 nm (Fig. 2). The chromatogram also indicates that additional peaks at the retention time between 2 and 4 min were produced upon heating. The signal intensity of the peaks become greater as the heating duration was increased. Therefore, this study was focused on the metabolites that possibly detected within this range of retention time.

The chemical components in Tualang honey upon thermal treatment were investigated by using high mass accuracy hyphenated analytical tool, namely LC-QTOF MS. The detection of free amino acids such as cysteine (m/z 122) and phenylalanine (m/z 166) in thermal-treated honey samples explains that heating can degrade honey protein into smaller units of amino acids. Only these two amino acids could be extracted from honey solution by chloroform and detected at the earlier retention time, namely 3.6 and 2.6 min for cysteine and phenylalanine, respectively. Free amino acids have fast elution time in a reversed phase liquid chromatographic system [20]. Cysteine was identified because it was fragmented into the base peak of m/z 105 by the loss of NH_3. The protonated aromatic phenylalanine was fragmented into m/z 149 as phenonium ion after the loss of NH_3. Dookeran

and co-workers [25] suggested that the loss of NH_3 from protonated phenylalanine was due to the phenyl migration. Further fragmentation would produce ion, m/z 131 because of the loss of a water molecule from the phenonium ion, and subsequently ion at m/z 103 due to the loss of CO. The immonium ion at m/z 120 is the most abundant ion, which is most likely corresponding to the loss of H_2O and CO from the protonated phenylalanine resulted from in-source fragmentation [22].

TABLE I
PUTATIVE COMPOUNDS THAT IDENTIFIED FROM MASS SPECTRA BETWEEN RETENTION TIME 2 AND 4 MINUTES

Rt (min)	Mode	Parent ion (m/z)	Product ion (m/z)	Putative compound	Reference
3.6	+	122	105/79/73	Cysteine	[20]
3.0	+	127	109/81/53	Hydroxymethylfurfural	[19]
2.4	+	149	121/103/93/65	Phthalic anhydride	[21]
2.6	+	166	149/131/120/103/93/77	Phenylalanine	[22]
3.4	+	266	248/176/146/134/105/79	Radiolytic byproducts	[23]
3.2	+	284	266/248/206/176/134/105	Radiolytic byproducts	[23]
2.3	+	328	310/292/282/264/246/166/120	Fragment of di-galactose	[24]

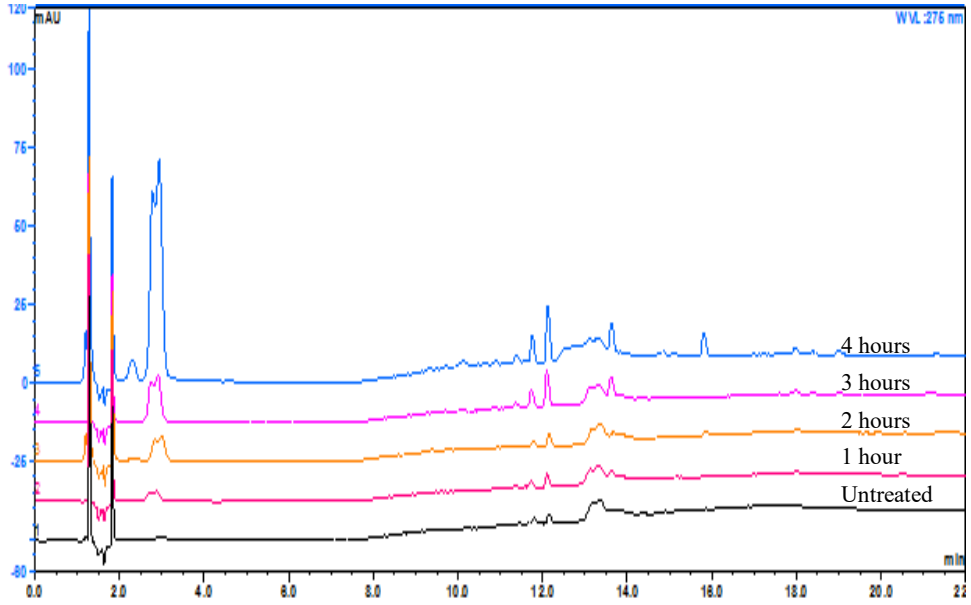

Fig. 2 Chromatogram of Tualang honey samples heated at 90 °C for 4 hours at 276 nm

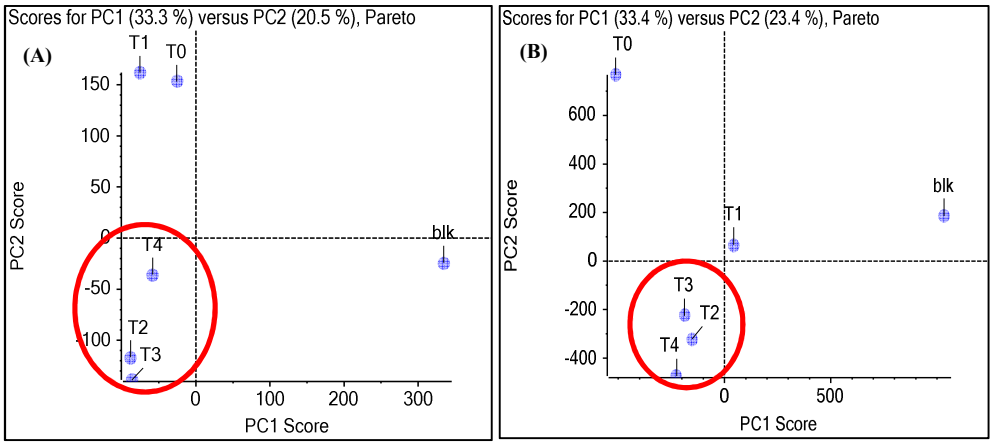

Fig. 3 Score plots of principal component analysis for mass spectra at positive (A) and negative ion (B) modes; T0, untreated Tualang honey; T1-T4, heat-treated Tualang honey for 1-4 hours

Not only honey protein, thermal treatment could also cause sugar degradation because m/z 328 was detected at the retention time of 2.3 min. This ion was found to be produced from di-galactose degradation in line with the finding of Stahl [24] who reported that the bond breaking of di-galactose would produce fragment ions such as m/z 310, 292, 264 and 246 because of the loss of water and carbonyl groups.

Phthalic anhydride is another possible furanic compound that was produced during thermal treatment on honey. The formation of phthalic anhydride could be attributed to the hydrolysis of phthalic acid during heating. Indeed, phthalic acid was reported in Turkish honey samples and the compound was likely to be one of the chemical indicators for the geographical origin of Turkish honey [26]. Previous studies showed that phthalic anhydride could cause rhinitis, chronic bronchitis and asthma [27], [28]. The detection of compounds at m/z 266 and 284 are believed as radiolytic byproducts of honey thermal treatment [23].

The mass spectra of honey samples at different heating duration were statistically analyzed by principal component analysis. This multivariate clustering technique was used to group the large datasets into 5 principal components. The first two principal components represent about 54 and 57 % of the total variance for positive and negative ions, respectively (Fig. 3). The score plots clearly show that the honey profile was significantly changed after 2 hours of heating at 90 ^{o}C. The chemical profile of untreated honey appears to be closer to an hour treated honey sample, but far away different from the honey samples heated for 2 hours and above. The statistical interpretation is in line with the graphical observation in which the additional peaks were found to be significantly increased after 2 hours of heating (R_t 2-4 min).

IV. Conclusion

A high throughput technique was used to identify small metabolites, which were produced from thermal treatment on Tualang honey samples. The study found that prolong thermal treatment, specifically more than 2 hours of heating at 90 ^{o}C could significantly change the chemical profile of Tualang honey. Chloroform was able to extract hydroxylmethylfurfural and other toxic substances such as phthalic anhydride and radiolytic byproducts from the thermally treated honey samples. The honey colour was getting darker as the heating duration was increased, most probably due to the oxidation of dehydrated sugar.

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