Jurnal Teknologi

Full paper

Thermal Treatment Effect on Free Amino Acids in Honey Samples

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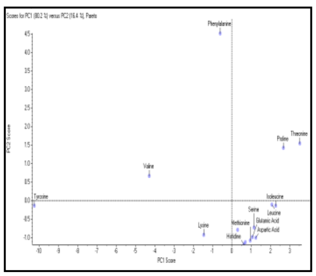
Article history

Received :4 October 2013
Received in revised form :
13 May 2014
Accepted :22 May 2014

Graphical abstract



Abstract

Free amino acids are minor constituents in honey which are responsible for the determination of botanical origin of honey. However, the composition of free amino acids was likely to be altered upon thermal treatment. Therefore, this study was conducted to investigate the profile of free amino acids before and after thermal treatment at 90°C for 30 minutes. This study revealed that phenylalanine (101.84-139.74 mg/kg), tyrosine (28.71-138.36 mg/kg) and proline (23.93-83.21 mg/kg) were found abundantly in all honey samples such as Tualang, Gelam and Acacia honey samples. After the honey were heated, it was found that proline and threonine were significantly reduced, while tyrosine, valine and lysine were increased in all honey samples. The proteolytic digestion was responsible for the increase of tyrosine, valine and lysine concentration after thermal treatment. The decrease could be attributed to the denaturation of proline and threonine themselves, apart from the reaction between the carbonyl group of reducing sugar and the amino acids upon thermal treatment.

Keywords: Thermal treatment; honey; free amino acids

1.0 INTRODUCTION

Amino acid is the building block of peptide and protein. Amino acids can be classified into essential and non-essential amino acids. They present in honey as minor constituent which approximately account for 1% of the total weight of honey. However, the composition of free amino acid can be used as a marker to characterize honey based on its botanical origin. The profile of free amino acids is crucial for honey characterization, besides melisopalinological analysis. Doner (2003)[1] reported that honey contained 11 to 21 types of amino acids. Among the amino acids, proline is the predominant amino acid which usually covers for 50-85% of the total amino acids.

Possibly, thermal treatment on honey could alter the profile of amino acids, and thus affecting for the classification of honey origin. Thermal treatment is usually applied to prevent honey from fermentation and crystallization, as well as to facilitate bottling process. Different thermal intensities are applied in order to reduce the moisture content to less than 20% for storage[2].

The process of thermal treatment usually associates with the structural changes of protein, and thus might increase or decrease the concentration of free amino acids in honey. The concentration of free amino acids in honey can be accurately determined by using a liquid chromatography coupled to mass spectrometer (LC-MS/MS). This hybrid system was found to be more accurate than reversed phase liquid chromatography, particularly for trace amount of compound. Furthermore, UV- or fluorescence-based detector of liquid chromatographic method requires derivatization before analysis[3]. Therefore, this study was focused on the effect of thermal treatment on the profile of free amino acids by using LC-MS/MS method and the data was interpreted by multivariate data analysis approach.

2.0 EXPERIMENTAL

2.1 Sample Preparation and Storage

Three types of honey samples were collected from various locations in Malaysia. The floral honey samples; Tualang and Gelam honey were purchased from the Federal Agriculture Marketing Authority (FAMA), Kedah and the honeydew honey; Acacia honey was collected from a bee farm located at Kota Tinggi, Johor. All honey samples were stored in clean airtight glass bottles at temperature between 18-24°C until analysis.

2.2 Thermal Treatment on Honey Samples

Thermal treatment was applied to reduce honey moisture to less than 20%. The honey samples of Tualang, Gelam and Acacia were heat treated at 90°C for 30 minutes[4]. The honey samples (10 g) were conventionally heated in a water bath without stirring and then cooling to room temperature before measurement of moisture content. The moisture content of honey samples was measured according to the standard method from the Association of Official Analytical Chemists (AOAC) Official Method 969.38 (2000)[5] using a handheld honey refractometer (Atago, Tokyo Japan).

2.3 Analysis of Free Amino Acids

The composition of free amino acids in honey samples was determined by using a liquid chromatography coupled to mass spectrometer (LC-MS/MS) according to the method described by Nimbalkar *et al*. (2012)[6] with minor modification. A mixture of amino acid standard (AA-S-18) from Sigma (Missouri, USA) was used to build the calibration curve of each amino acid. A working standard mixture (0.2-1.0 mg/l) was prepared by diluting the stock standard solution.

Honey sample (1 g) was weighed and mixed with a solvent system consisted of 5 ml of 20% (v/v) methanol with 0.1% (v/v) formic acid. The mixture was vortexed for 10 minutes followed by centrifugation at 10 000 rpm for 20 minutes. The supernatant was filtered through 0.2 µm membrane filter and 5 µl sample was injected to Ultra Performance Liquid Chromatography (UPLC, Waters Acquity Milford, MA) system coupled with a triple quadrupole-linear ion tandem mass spectrometer (Applied Biosystems 4000 QTrap; Life technologies Corporation, Carlsbad, CA) with an electrospray ionisation (ESI) source. The hybrid system was UPLC-ESI-MS/MS. The C18 Acquity column (100 mm x 2.1 mm, 1.7 µm) was used and the mobile phase consisted of (A) ultra-pure water with 0.1% (v/v) formic acid and (B) 50% methanol with 0.1% (v/v) formic acid at a flow rate of 0.15 ml/min. The gradient of the mobile phases were as follows; 0-1.0 min, 10 %B; 1-10 min, 10-80 %B; 10-12 min, 80 %B; 12-13 min, 80-10 %B and 12-18 min, 10 %B. In a two-component gel, it is easy to modify the molecular structure of either of the two components.

■3.0 RESULTS AND DISCUSSION

3.1 Moisture Content of Honey Samples after Thermal Treatment

The initial moisture content of Tualang, Gelam and Acacia honey samples were ranged from 21.36-28.17% which could accelerate the fermentation and crystallization of honey. The maximum moisture content of honey allowed in order to prevent fermentation process is 21%[7]. The European regulation also indicates that honey exceeding 20% of moisture content is prone to rapid fermentation and crystallization[8]. This explains the need of thermal treatment for local honey samples because honey from tropical countries are high in moisture content. Thermal treatment at 90°C for 30 minutes was found to be effective in reducing moisture of Tualang, Gelam and Acacia samples to less than 20% (17.0-19.8%) as presented in Table 1. The percentage of reduction in water content for the honey samples ranged from 20.4 to 32.6%. This treatment condition at 90°C for 30 minutes was selected for the subsequent study to investigate the heating effect on free amino acids of honey samples.

Table 1 Moisture content of honey samples after heat treated at 90°C for 30 minutes

Honey Sample	Moisture (%)			t-test value
	Fresh Honey	Heat Treated Honey	Reduction	($t_{critical\ two\text{-}tail}$ 12.7)
Tualang	28.17±0.09[a]	18.98±0.12[b]	32.62	15.8
Gelam	25.69±0.21[a]	19.78±0.14[b]	23.01	103.0
Acacia	21.36±1.83[a]	17.00±0.07[a]	20.40	109.0

[a, b]: different letter in the same row of data indicated that the values were significantly different using paired t-test.

In the present study, the amino acids of phenylalanine (101.84-139.74 mg/kg), tyrosine (28.71-138.36 mg/kg) and proline (23.93-83.21 mg/kg) were found to be present in the significant amount in Tualang, Gelam and Acacia honey samples. Acacia honey samples contained the highest amount of total amino acids compared to Tualang and Gelam honey samples. Tualang and Gelam honey samples are blossom honey from plant nectars, whereas Acacia is a honeydew honey which is mainly from plant exudates, fruits and excretions of plant-living insects[9].

Thermal treatment at 90°C for 30 minutes resulted to both increment and reduction of some amino acids in Tualang, Gelam and Acacia honey samples (Figure 1). The proteolytic digestion might be responsible for the increase of the aforementioned amino acids after thermal treatment. Proteolytic digestion could break down the protein into smaller unit of polypeptides and amino acids during heating[9]. The detection of amino acids from proteolytic digestion could change the profile of free amino acids in honey. This explains certain amino acids were increased after thermal treatment.

The decrease of some amino acids in Tualang, Gelam and Acacia honey samples were associated with denaturation process. As described by Kauzmann (1959)[10], denaturation caused the loss of activity through the unfolding or aggregation of protein at high temperature. As the protein unfolds, more amino acids were exposed to denaturation[11]. Not limited to that, the reaction of carbonyl group from reducing sugar with amino acid during thermal treatment could also decrease the concentration of free amino acids in honey samples.

For better visual illustration, the statistical multivariate analysis was applied in this study. The multidimensional data sets were analyzed by using pattern recognition tool such as the principle component analysis (PCA) to cluster the amino acids resulted from thermal treatment with respect to the honey samples. The illustration of PCA in the form of score and loading plots are presented in Figure 2.

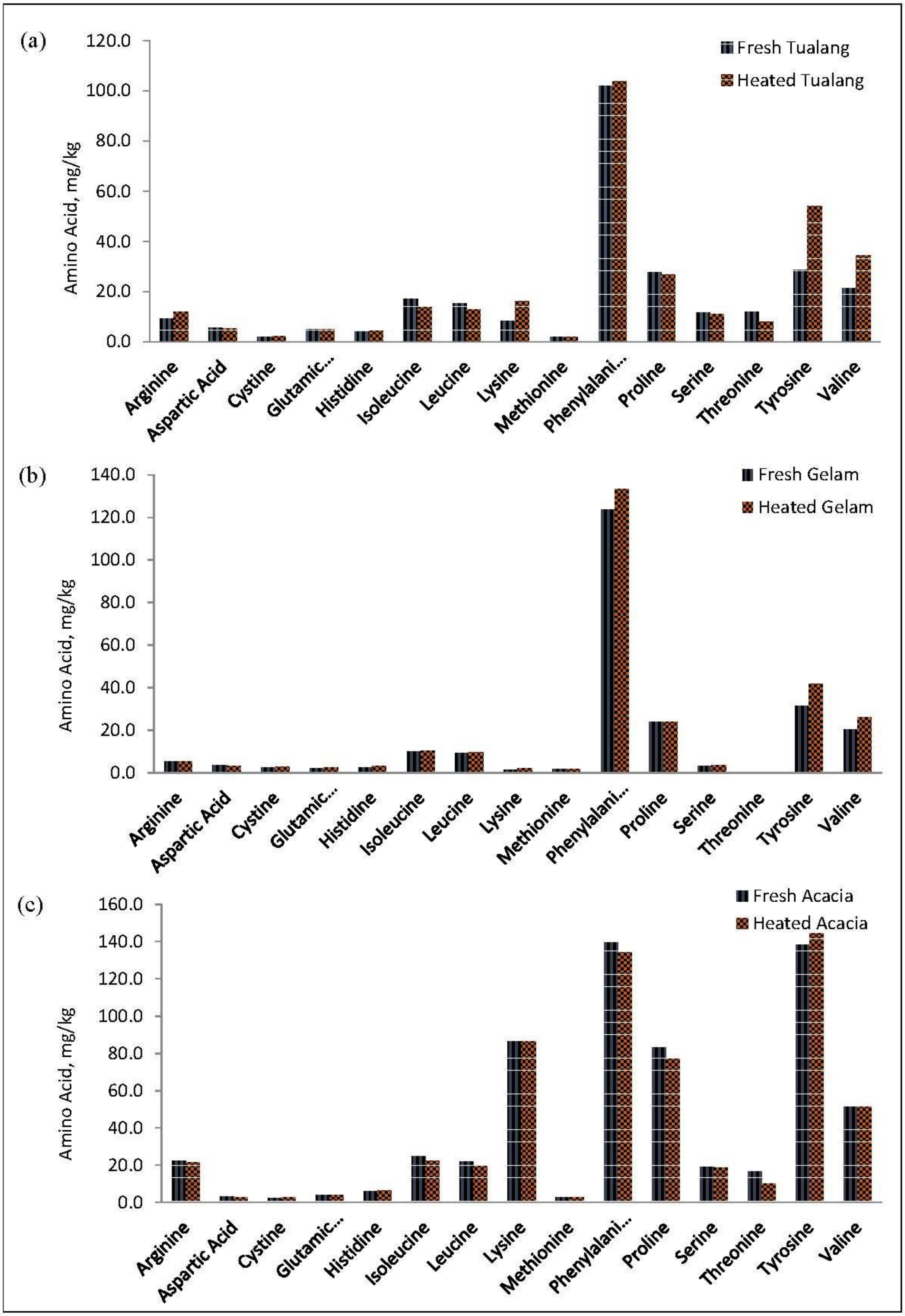


Figure 1 Free amino acid composition in (a) Tualang, (b) Acacia and (c) Gelam honey samples before and after thermal treatment

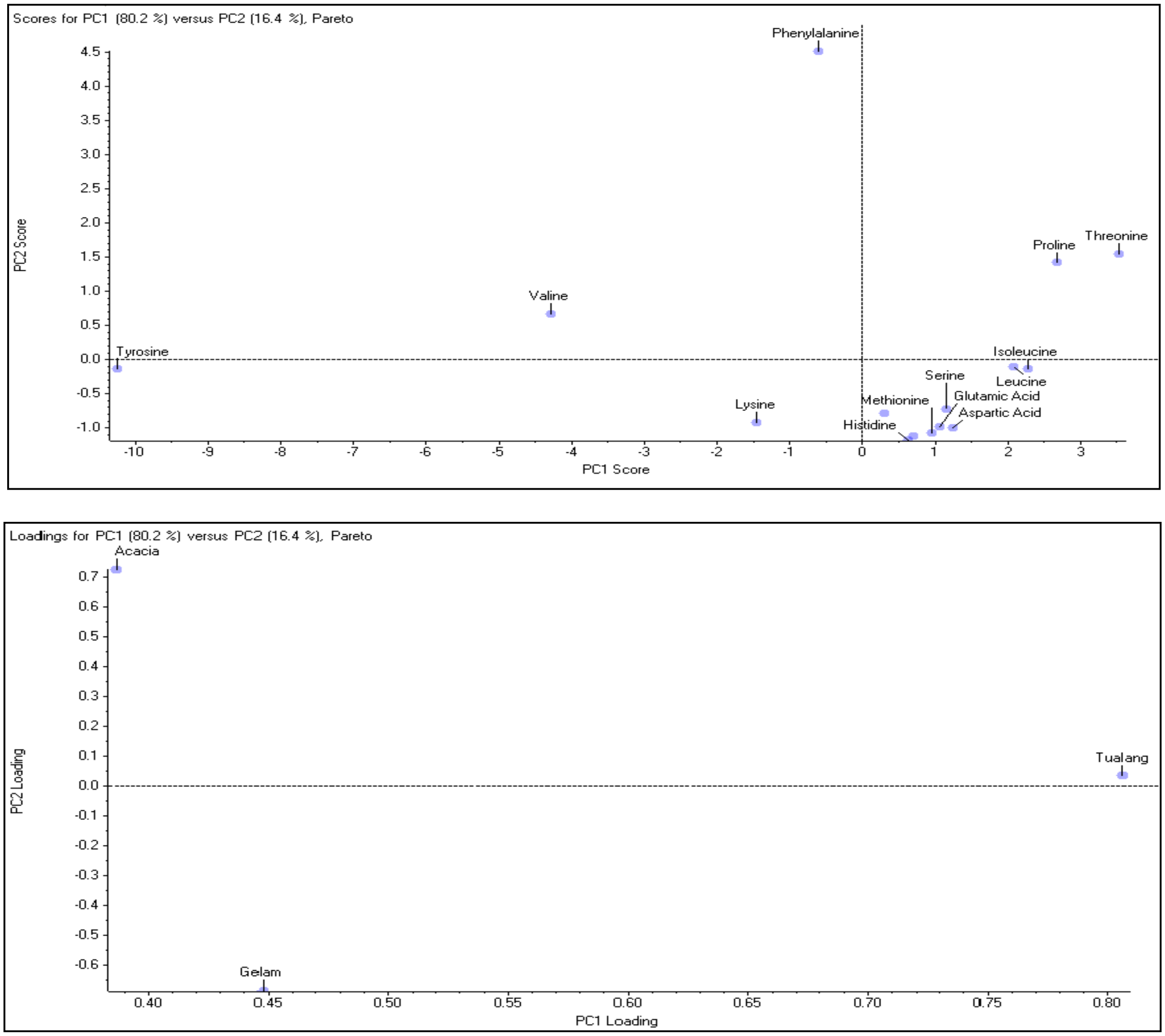


Figure 2 Principle component analysis of biochemical components in Tualang, Gelam and Acacia honey samples

The first principle component (PC1) explains 80.2% of the total variance, mainly contributed by proline and threonine. The respective amino acids experienced the significant reduction after thermal treatment with the highest scores in both PC1 and PC2. The amino acids of tyrosine, valine and lysine located at the highest negative score in PC1 indicated that the amino acids were significantly increased in Tualang, Gelam and Acacia honey samples after thermal treatment (Figure 2a). From the loading plot (Figure 2b), it is clear that all honey samples are located at the positive region of PC1, which indicated that all honey samples did not have large difference in their biochemical components.

■4.0 CONCLUSION

Thermal treatment at 90°C for 30 minutes could cause both increment and reduction of free amino acid composition in Tualang, Gelam and Acacia honey samples. The amino acids of proline and threonine experienced the significant reduction after thermal treatment, whereas tyrosine, valine and lysine were significantly increased in all honey samples after thermal treatment. The results reveal that thermal treatment can alter the profile of free amino acids, which is used to characterise the botanical origin of honey. The high sensitivity of LC-MS/MS approach was successfully determined the free amino acid profile without the laborious derivatization procedures.

Acknowledgement

This research work was granted under GUP (Tier 1), Research Alliance Biotechnology (7125.00H05). The authors would like to thank Ms. Norfahana Talib and Ms. Norul Liza A. Rahaman for their support and assistance.

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