DISCRIMINATION OF PURE AND ADULTERATED *Heterotrigona itama* HONEY WITH HIGH FRUCTOSE CORN SYRUP USING SELECTED VIBRATIONAL SPECTROSCOPY AND CHEMOMETRIC TECHNIQUES

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A dissertation submitted in partial fulfilment of the
requirements for the award of the degree of
Master of Science (Forensic Science)

Faculty of Science
Universiti Teknologi Malaysia

SEPTEMBER 2020

ACKNOWLEDGEMENT

Alhamdullilah, praise to God that only by His Will I have completed this dissertation. In preparing this thesis, I was in contact with many people, researchers, academicians, and practitioners. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation to my main dissertation supervisor, Dr Mohamad Afiq bin Mohamed Huri for encouragement, guidance, critics and friendship. I am also very thankful to my co-supervisor Dr Arniza Khairani Binti Mohd Jamil for her guidance, advice and motivation. Without their continued support and interest, this thesis would not have been the same as presented here.

I am also indebted to the laboratory staff of Faculty Science, Universiti Teknologi Malaysia (UTM) for their assistance throughout the execution of this research project. My fellow postgraduate colleagues, Sumathy, Syafiqa, Farah Zafira, and Anis Norsyazleen, should also be remembered for their support. My sincere appreciation also extends to all my colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. Last but not least, I am very grateful to all my family members for their continuous support and understanding during my duration of completing this dissertation.

ABSTRACT

Honey is one of the natural food products available since in the olden days and are always in demand due to its numerous nutritional value and therapeutic properties. However, increasing in environmental pollution, spreading of diseases and world climate changes have led to a dwindling in global honeybee populations. The difficulty of this issue is becoming more complex due to increased application of sugar-based adulterants which shows similar physical appearance and carbohydrate composition to pure honey. This study presents the data from Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) and Raman spectroscopy coupled with Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) to differentiate pure and adulterated stingless bee honey, *Heterotrigona itama* (*H. itama*). The pure *H. itama* honey was obtained in Skudai, Johor and adulterated honey samples were attained with High Fructose Corn Syrup (HFCS) in five different percentages (w/w) namely 5%, 10%, 20%, 40% and 50%. All samples were firstly subjected to physical properties (density and refractive index) analysis. Welch-ANOVA results concluded that there were significant differences ($p < 0.05$) among groups for both density and refractive index analyses. Next, characterization of the pure and adulterated *H. itama* were done in full region for ATR-FTIR (4000-600 cm^{-1}) and Raman (1430-200 cm^{-1}) spectroscopy. For chemometric analysis, fingerprint region (1700-400 cm^{-1}) for both spectroscopic spectra were analysed. PCA was done to understand the organization of data, while LDA was done to predict the major factors for grouping and prediction. PCA scores for first three components for both ATR-FTIR and Raman were 79.1% and 72.8% respectively. Employment of LDA had improved the groupings of the honey samples when compared with PCA, which resulted in LDA cross-validation for correct classifications rate at 97.6% for ATR-FTIR while for Raman was 100%. While PCA and LDA models appear satisfactory, utilization of ATR-FTIR can be the more preferable means of analysis for discriminating the pure and adulterated honey samples, considering its simplicity and effectiveness.

ABSTRAK

Madu adalah salah satu produk makanan yang boleh didapati sejak zaman dahulu dan digemari kerana banyak nilai pemakanan dan sifat-sifat terapeutiknya. Tetapi, peningkatan pencemaran alam, penyebaran penyakit, dan perubahan iklim dunia telah menyebabkan kemerosotan populasi lebah madu sedunia. Kesukaran permasalahan ini semakin kompleks dengan peningkatan penggunaan bahan lancung berasaskan gula yang menyerupai rupa fizikal dan komposisi karbohidrat madu asli. Kajian ini membentangkan data daripada spektroskopi Pantulan Keseluruhan Dikecilkan- Inframerah Transformasi (ATR-FTIR) dan spektroskopi Raman dengan gabungan analisis komponen prinsipal (PCA) dan linear diskiminasi analisis (LDA) untuk membezakan madu asli lebah tidak bersengat, *H. itama* dengan madu lebah tidak bersengat *H. itama* lancung. Madu *H. itama* asli telah diperolehi di Skudai, Johor dan madu lancung telah diperolehi dengan sirap jagung berfruktos tinggi (HFCS) pada lima peratusan (w/w %) yang berbeza iaitu 5%, 10%, 20%, 40% dan 50%. Kesemua sampel telah melalui analisis untuk penentuan ciri-ciri fizikal (ketumpatan dan indeks biasan) dahulu. Keputusan dari Welch-ANOVA menyatakan bahawa terdapat perbezaan statistik yang ketara ($p < 0.05$) antara sampel untuk kedua-dua ketumpatan dan indeks biasan. Seterusnya, pencirian bagi *H. itama* asli dan lancung telah dilakukan di lingkungan penuh untuk kedua spektroskopi ATR-FTIR (4000-600 cm^{-1}) dan Raman (1430-200 cm^{-1}). Lingkungan cap jari (1700-400 cm^{-1}) untuk kedua-dua spectrum telah dijalankan untuk kemometrik analisis. PCA telah dilakukan dengan menggunakan perisian Minitab untuk memahami organisasi set data manakala LDA pula telah dilakukan dengan penggunaan perisian Minitab dan SPSS untuk menjangkakan faktor-faktor utama bagi pengelompokan. Pencapaian skor PCA untuk 3 komponen pertama bagi kedua ATR-FTIR dan Raman adalah 79.1% dan 72.8% untuk masing-masing. Penggunaan LDA telah meningkatkan pengelompokan sampel madu apabila dibandingkan dengan PCA sahaja, dengan memberikan kadar klasifikasi pengesahsahihan silang yang betul iaitu 97.6% untuk ATR-FTIR manakala Raman adalah 100%. Walaupun terdapat model PCA dan LDA yang memuaskan, penggunaan ATR-FTIR sebagai kaedah analisis dalam membezakan sampel madu asli dengan madu lancung lebih digemari dengan mengambil kira kesederhanaan dan keberkesanannya.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
ATR-FTIR	-	Attenuated Total Reflectance-Fourier Transform Infrared
CV	-	Coefficient of Variation
GC-MS	-	Gas Chromatography Mass Spectrometer
HF05	-	5% adulterated honey
HF10	-	10% adulterated honey
HF20	-	20% adulterated honey
HF40	-	40% adulterated honey
HF50	-	50% adulterated honey
H. itama	-	*Heterotrigona itama*
HFCS	-	High Fructose Corn Syrup
HMF	-	5-*hydroxymethylfurfural*
IRMS	-	Isotope-ratio mass spectrometry
LDA	-	Linear Discriminant Analysis
MARDI	-	The Malaysian Agricultural Research and Development Institute
PCA	-	Principal Component Analysis
PH	-	Pure Honey
PLS-DA	-	Partial Least Squares Discriminant Analysis
PLSR	-	Partial Least Squares Regression
PCR	-	Principal Component Regression
RMSEP	-	Root Mean Squared Error of Prediction
SCIRA	-	Stable Carbon Isotope Ratio Analysis
SIMCA	-	Soft Independent Modelling of Class Analogy
SPSS	-	Statistical Package for the Social Sciences
UV-Vis NIR	-	UltraViolet- Visible Near Infra-Red
ZnSe	-	Zinc Selenide

LIST OF SYMBOLS

ρ	-	Density
^{o}C	-	Degree celcius
% w/w	-	Percentage weight/weight
^{12}C	-	Carbon-12
^{13}C	-	Carbon-13
C3	-	Calvin cycle/ C3 cycle
C4	-	Hatch-Slack Pathway/ C4 cycle

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Honey has been one of the natural food products available. It contains immeasurable health benefits that are even recorded in the Holy Al-Quran (Omar & Omar, 2018). In Malaysia, rearing stingless bees is more favoured to the bees' farmers due to their populations that are less vulnerable to harsh environment and seasonal changes (Kelly *et al*., 2014). Moreover, Malaysia has various species of stingless bees ranging from 17 to 32 species, and among these, *Heterotrigona itama* bees are currently estimated for 90% of stingless bee honey sold in Malaysia (Kelly *et al.,* 2014).

Studies have shown that stingless bee honey has antimicrobial, anti-inflammatory and antioxidant properties, as well as propitious for wound stimulation, healing burns and treatment of gastric ulcers, (Martinotti & Ranzato, 2018; Rhodes & Molan, 2015; Mohd Yusoff *et al.,* 2012). Such benefits have made honey as one of the most valuable food products in the market. Unfortunately, rising in environmental pollution and transmission of diseases have led to a declination in global honeybee populations (Zabrodska & Vorlova, 2014). Such situation (when combined with increasing in demand for its production) has made pure honey existence in the market become scarcer and consequently resulted in increasing of honey adulteration activities (Se *et al.,* 2018), including Malaysia (Kamaruddin *et al*., 2006)..

The most regular form of honey adulteration is by the addition of common sugar syrups (e.g. High Fructose Corn Syrup (HFCS), maltose syrup, and inverted syrups) which have lower economic values aside from having similar physical appearance and carbohydrate composition with the pure honey (Parker *et al.,* 2010;

Rios-Corripio *et al.,* 2012). HFCS is an artificially composed sugar syrup, genetically modified enzymatic hydrolysis of corn starch (Zabrodska & Vorlova, 2014). Conversely, HFCS possess high concentrations of fructose and glucose which may lead to detrimental health effects such as diabetes if consumed in long-term duration (Parker *et al.,* 2010; Samat *et al.,* 2018; Goncalves *et al.,* 2019).

In Malaysia, stingless bees are largely farmed for its honey, generally priced at approximately RM335 per litre, contributing about RM200 mil annually to the Malaysia's economy (Basrawi *et al.,* 2017; Bernama, 2020).Thus, HFCS- adulterated honey in the market not only would cause adverse effect in human health, as well as affecting the market growth by damaging the consumer trust. (Dong *et al.,* 2018). Therefore, having a robust means for discriminating pure and HFCS adulterated honey samples using selected physical properties and spectroscopic analysis combined with chemometric analysis appears relevant.

In order to discriminate between pure and adulterated honey, numerous physicochemical parameters and instrumental techniques have been developed to determine and quantify the presence of adulterants in the claimed pure honey sold in different premises. As the refractive index is inversely proportional to the water content in honey, it has been used for moisture content determination in honey that can lead to adulteration determination in honey (Adebiyi *et al.,* 2004). Meanwhile for density, previous study has shown that adulterated honey has low density compared to a good natural honey (Lullah-Deh *et al.,* (2018)). Therefore, taking into account on time, expenses and instrument liability, density and refractive index were selected as physical characteristics parameters observed in this study.

Combination of rapid and non-destructive of both Raman and ATR-FTIR spectroscopy were selected since both of the techniques have shown their potential in this study as powerful characterization and profiling techniques. This was due to both of these spectroscopy techniques complimenting each other in terms of principle of analysis. ATR-FTIR utilized on the light absorption and changes in dipole moment of the molecule, meanwhile Raman employed light scattering and changes in polarizability of the molecules (Skoog *et al.,* 2016). Moreover, ATR-FTIR is

sensitive towards hetero-nuclear functional group vibrations and polar group (e.g. OH group) while Raman is more to homo-nuclear molecular bonds (e.g. C-C and C=C) (Wu *et al.,* 2017). In terms of spectral interference, fluorescence samples may interfere with the capability of Raman in taking spectra, but not particularly an issue for ATR-FTIR. ATR-FTIR is prone in detecting water, while Raman has no problem in this issue (Skoog *et al.,* 2016). Hence, in this study, both data from ATR-FTIR and Raman were put alongside to compare its effectiveness to differentiate between pure and adulterated honey samples.

However, fingerprinting analyses such as vibrational spectroscopy produced a large amount of data to process, and are usually combined with chemometric analysis for better data presentation. Based on Sivakesava & Irudayaraj (2001), chemometric analysis provides the application of mathematical and statistical approaches for further understanding of the chemical information and to relate the quality parameters to the data from the analytical instrument. For food adulteration cases, statistical approaches that are commonly used are exploratory (e.g. Principal Component Analysis, PCA) (Efenberger-Szmechtyk *et al.,* 2017) and classification analyses (Linear Discriminant Analysis, LDA) (Wu *et al.,* 2017). Both PCA and LDA were used in this study to reduce the data dimensionality from a large dataset of ATR-FTIR and Raman spectra, as well as for prediction purposes, respectively.

PCA is known as the unsupervised pattern recognition and would represent the reduced data into a new orthogonal variable of principal component (PC) (Jamaludin *et al.,* 2017). The pattern can be used for comparing the differences and visualizing the variations between pure and adulterated honey samples (Dramicanin *et al.,* 2018). Meanwhile for LDA, also known as the supervised pattern recognition, can be used to find one or more linear functions of the data for enabling better separation between pure honey, HFCS and adulterated samples (Dramicanin *et al.,* 2018).

1.2 Problem Statement

With the improvement of science and technology, the fact that a pure product with high commercial value and available in scarcity can be repackaged into a cheaper and bulk product by the addition of adulterants appear worrying. The counter-measure for pure honey adulteration has been carried out on an ongoing basis by various authorities around the globe. Despite having an established Honey Standard for Malaysia (Standard Malaysia, 2017), the activity of the adulteration is increasing whereby 80% of stingless bee honey products sold in the market are adulterated with sugar, starch and corn flour (Lisut *et al*., 2017). This unwanted situation can be attributable to cheaper adulteration and production processes, as well as availability of adulterant in bulk quantity and similarity in its carbohydrate composition with that of pure honey.

While several analytical methods using Gas Chromatography (GC) and Stable Carbon Isotope Ratio Analysis (SCIRA) for discriminating pure and adulterated honey samples have been reported (Da Silva *et al.,* 2017; Soares *et al.,* 2017), they are of destructive in nature and would require long analysis time. Nevertheless, similar approaches for dealing with the Malaysian honey samples remain unreported. In addition, specific analytical protocol/ procedure for analysing the purportedly adulterated honey samples for forensic purposes is lacking.

Considering that ATR-FTIR and Raman spectroscopy have been utilized for forensic provenance, largely attributable to non-destructive nature and specificity, their suitability for categorically discriminating the pure and adulterated honey samples proves forensically interesting. While combination of ATR-FTIR and Raman spectroscopy data with chemometric techniques like PCA and LDA for several matrices has been reported (Oroian & Ropciuc 2018; Salvador et al., 2019) the same for stingless bee honey remains lacking so far.

1.3 Objectives

With reference to the pure stingless bee honey samples and adulterated ones containing HFCS, the objectives of the study were:

a) To compare the density and refractive index between the two categories of samples.
b) To analyse the region characteristics of the two categories of samples using Raman spectroscopy and ATR-FTIR spectroscopy.
c) To perform the classification prediction of the two categories of samples using the unsupervised PCA and supervised LDA.

1.4 Hypothesis

It was hypothesized that the density and refractive index of the pure and adulterated honey samples would differ significantly.

1.5 Scope of Study

A bulk sample of pure stingless bee (*H. itama)* honey was obtained from a local producer at Universiti Teknologi Malaysia (UTM) Skudai, Johor (without filtration) and stored in an air-conditioned room (25 ± 2^{o}C) before any analysis was conducted. Next, the pure honey was adulterated with HFCS at five different percentages (5, 10, 20, 40 and 50 % w/w). For each assay, a sample was analysed in six replicates in order to get an accurate result as well as sufficient sample sizes for chemometric analysis.

Physical characteristics (density and refractive index) of pure *H. itama* honey, HFCS and five (% w/w) of adulterated *H. itama* honey samples were determined. Density test was done according to a method modified from Gomez-Diaz *et al.*,

(2012) while the refractive index was measured through a refractometer at wavelength 589 nm adapted from Fatima *et al.,* (2017). The results obtained were further analysed with statistical analysis of Welch-ANOVA and Games-Howell's Post-Hoc test in order to determine statistical significance.

For discriminating the pure and adulterated honey samples, utilization of vibrational spectroscopy (ATR-FTIR and Raman spectroscopy) was done. In order to obtain the chemical information, wavenumber and intensities of the samples, full region from wavenumber of 4000 – 600 cm^{-1} were selected for ATR-FTIR with 16 scans, meanwhile for Raman, the region used were from Raman shift of 1430 – 200 cm^{-1} with 20 scans.

The chemometric analyses of the fingerprint region (1400 – 700 cm^{-1}) for ATR-FTIR and Raman spectroscopy were employed. Pre-processing step was done to all the spectra prior to pattern recognition technique. For ATR-FTIR the pre-processing step was done using *Spectrum* (version 10, Perkin-Elmer, USA) software, while for Raman, Origin Pro (version 2018, OriginLab corp., USA) software was used for adjusting the baseline and normalization of the raw data.

Next, the data were further processed using Minitab (version 17, LLC, USA) software in order to organize the datasets and to determine the main contributing factor for grouping using PCA. Next, predictive models for unknown samples were established using the supervised LDA through SPSS (version 20, IBM, USA) software. The visualization for a three-dimensional plot for PCA and LDA was also done using Minitab software.

1.6 Significance of Study

This present research would provide the specific analytical protocol/ procedure for analysing the pure and adulterated honey samples using the non-destructive ATR-FTIR and Raman spectroscopy, in combination with the pattern recognition techniques viz. PCA and LDA. While the ATR-FTIR- LDA revealed

97.6% of correct classification for the pure and the different adulterated honey samples, the same was 100% for Raman-LDA. Considering such high accuracies, the use of these two approaches appears forensically supported.

In this context it is pertinent to indicate that analysis using ATR-FTIR- LDA can be relatively easier and cheaper although slightly lesser in accuracy when compared with that of Raman-LDA. Hence, utilization of ATR-FTIR- LDA along with assessment of physical properties can be a feasible qualitative screening approach for discriminating the pure and adulterated honey samples. The results of which can be confirmed using the more specific Raman-LDA approach. This proposed protocol may prove useful at eliminating the needs for the laborious analytical methods involving GC and HPLC that are currently in place for analysing honey samples. This approach would benefit the relevant authorities in need for verification of pure honey products.

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