DISCRIMINATION OF STINGLESS BEE HONEY ADULTERATION BY CANE SUGAR SYRUP USING CHEMOMETRIC-INTEGRATED SPECTROSCOPIC TECHNIQUES

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ABSTRACT

Stingless bee, a type of bee which is stingless, is one of the bee species which has the ability to produce honey and locally known as *kelulut* in Malaysia. Increase of demand for its production, has contributed to the scarcity of the pure honey in market and resulted in adulteration of the pure honey for economic gain by unethical individuals. In addressing such an issue, numerous physicochemical and instrumental analyses were developed to detect and discriminate adulterants in honey samples. This study presents the discrimination analyses of adulterated stingless bee honey using rapid, non-destructive, simple, and greener spectrometric methods; Attenuated-Total-Reflectance-Fourier-Transform Infrared (ATR-FTIR) and Raman spectroscopy integrated with Principle Component Analysis (PCA) and Linear Discriminant Analysis (LDA). The pure stingless bee honey was obtained from Skudai, Johor. Adulterated honey samples were attained by adding cane sugar syrup in five different percentages (w/w) namely 5%, 10%, 20%, 40% and 50%. All samples were initially subjected to physical properties (Brix, refractive index and density) analysis. Using one-way ANOVA, density of pure stingless bee honey has no significance differences ($p>0.05$) between adulterated samples, except between adulterant ($p<0.05$). Meanwhile, brix value and refractive index of pure honey shows significant difference between adulterant and adulterated honey. Next, characterization of the pure and adulterated stingless bee was 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 95.2% and 65.7% 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 100% for both ATR-FTIR and Raman. The protocol proposed could be an alternative option as opposed to the laborious, time consuming, destructive and involve hazardous chemicals, chromatographic analyses such as GC and HPLC, considering that Raman spectroscopy, like ATR-FTIR is simple, fast, non-destructive and greener analytical techniques.

ABSTRAK

Lebah tidak bersengat, yang dikenali sebagai kelulut di Malaysia, adalah salah satu spesies lebah yang mempunyai kemampuan untuk menghasilkan madu. Peningkatan permintaan untuk pengeluarannya, telah menyumbang kepada kekurangan madu tulen di pasaran dan mengakibatkan pemalsuan madu tulen sering dilakukan untuk menjana ekonomi oleh individu yang tidak beretika. Dalam menangani masalah seperti itu, banyak analisis fizikokimia dan instrumentasi dibangunkan untuk mengesan dan membezakan bahan campuran dalam sampel madu. Kajian ini melibatkan analisis diskriminasi madu kelulut yang dicampur menggunakan kaedah spektrometrik yang cepat, tidak merosakkan, sederhana, dan lebih hijau; specktroskopi inframerah transformasi Fourier dengan pantulan total dilemahkan (ATR-FTIR) dan Raman yang digunakan bersama dengan Analisis Komponen Utama (PCA) dan Analisis Diskriminan Linear (LDA). Madu lebah yang tulen diperoleh dari Skudai, Johor. Sampel madu dicampur dicapai dengan menambahkan sirap gula tebu pada lima peratus yang berbeza (b/b) iaitu 5%, 10%, 20%, 40% dan 50%. Semua sampel pada awalnya menjalani analisis sifat fizikal (Brix, indeks biasan dan ketumpatan). Dengan menggunakan satu cara ANOVA, ketumpatan madu kelulut tulen didapati tidak mempunyai perbezaan yang signifikan ($p > 0,05$) antara sampel yang dicampur, kecuali antara sampel gula tebu ($p < 0,05$). Sementara itu, nilai brix dan indeks bias madu tulen menunjukkan perbezaan yang signifikan antara madu yang tidak dicampur dan yang telah dicampur. Seterusnya, pencirian madu kelulut asli dan tidak tulen dilakukan di lingkungan penuh untuk spektroskopi ATR-FTIR (4000-600 cm-1) dan Raman (1430-200 cm-1). Untuk analisis kemometrik, *finger print region* (1700-400 cm-1) untuk kedua-dua spektroskopi dianalisis. PCA dilakukan untuk memahami organisasi data, sementara LDA dilakukan untuk meramalkan faktor utama pengelompokan dan ramalan. Skor PCA untuk tiga komponen pertama untuk kedua-dua ATR-FTIR dan Raman masing-masing adalah 95.2% dan 65.7%. Penggunaan LDA telah meningkatkan pengelompokan sampel madu jika dibandingkan dengan PCA, yang menghasilkan pengesahan silang LDA untuk kadar klasifikasi yang betul pada 100% untuk kedua-dua ATR-FTIR dan Raman. Protokol yang diusulkan ini boleh menjadi pilihan alternatif berbanding dengan analisis kromatografi seperti GC dan HPLC, dimana kaedah spektroskopi Raman dan ATR-FTIR dapat menjimat masa analisis dan tidak memusnahkan sampel.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
ATR-FTIR	-	Attenuated Total Reflectance-Fourier Transform Infrared
AHCS05	-	5% w/w/ adulterated honey – with cane sugar
AHCS10	-	10% w/w/ adulterated honey – with cane sugar
AHCS20	-	20% w/w/ adulterated honey – with cane sugar
AHCS40	-	40% w/w/ adulterated honey – with cane sugar
AHCS50	-	50% w/w/ adulterated honey – with cane sugar
AHCS	-	Adulterated honey samples (with cane sugar syrup)
AUD	-	Australian Dollar
CCD	-	cooled front-illuminated charge-coupled device
CS	-	cane sugar
DF	-	discriminant function
LDA	-	Linear discriminant analysis
LED	-	Light-emitting Diode
MIR dTGS	-	mid-infrared deuterated triglycine sulfate
PC	-	principle component
PCA	-	principle component analysis
PH	-	pure honey
RI	-	refractive index
RM	-	Ringgit Malaysia
sd	-	standard deviation
TE	-	Thermoelectric
UATR	-	universal attenuated total reflectance
UTM	-	University Teknologi Malaysia

LIST OF SYMBOLS

%	-	percentage
ºBrix	-	degree Brix
ºC	-	degree Celsius
w/w	-	weight per weight
ρ	-	density
α	-	significance level
A	-	absorbance
cm^{-1}	-	wavelength
cm^{-3}	-	cubic centimetre
nm	-	nanometre
µm	-	micrometre(s)
mL	-	millilitre(s)
g	-	gram(s)
kg	-	kilogram(s)
sec	-	second(s)
rpm	-	rotation per minute
W	-	walt

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Honey is a sweet and nutritious substance converted from flower nectar naturally by honeybees (Se *et al.*, 2019; Paradkar & Irudayaraj, 2001), using its secretary products (Chalhoub *et al.*, 2005). It has complex and variable matrix components (Li *et al.*, 2012) in aqueous form, comprising approximately 18% water, 80% carbohydrates and also minimal quantities of vitamins, minerals, proteins and amino acids (Dzolin *et. al.*, 2019; Anjos *et al.*, 2015; Oliveira *et al.*, 2002). Honey carbohydrates are composed of monosaccharides, mainly fructose and glucose (about 70%), disaccharides such as sucrose and maltose and minimal amount of trisaccharide (Anjos, Santos, Paixão, & Estevinho, 2018). Owing to its nature of composition, natural honey is being one of the oldest sweetening agent that can be consumed by humans without processing (Oroian *et al.*, 2017) and has been gaining considerable popularity in the last two decades because of its high nutritional value and healing properties (Wu et al., 2017).

In Malaysia, apart from being naturally harvested, honey is obtained commercially from two types of beekeeping, namely Apiculture and Meliponiculture using honey bees (*Apini*) and stingless bees (*Meliponini*) respectively (Siok *et al.*, 2017). Between these two tribes, stingless bee (locally known as kelulut (Amin *et al.*, 2018)) are more preferred for commercial beekeeping for many reasons, among others; morphology of stingless bee with reduced sting and the absence of venom (Omar *et al.*, 2019), the population is less vulnerable to extreme environments and seasonal changes (Se *et al.*, 2018). It also has been reported to have higher medicinal and nutritional values compared to honey bees honey (Zhao *et al.*, 2016). Stingless bee honey is reported to contain high antioxidant properties that promotes health in human (Amin *et al.*, 2018; Wan Ismail, 2016).

However, the production of stingless bee honey is limited (five times less compared to honey produced by other members of genus Apis) mainly due to their small size (Omar *et al.*, 2019) and is estimated to have been priced much higher than *Apis mellifera* honey (Shamsudin *et al.*, 2019), costing around RM116 per kilogram in 2014 (Kelly *et al.*, 2014) and increased to more than RM400 per kilogram in 2018 (Shadan *et al.*, 2018). Such situation, when combined with increase of demand for its production, has contributed to the scarcity of the pure honey in market. Consequently, this has rendered substantial attention of unethical individuals in adulterating the pure honey in aiming to maximize their economic gain by selling them at premium prices (Salvador *et al.*, 2019; Samat *et al.*, 2018; Se *et al.*, 2018).

Honey is commonly adulterated directly or indirectly (Se *et al.*, 2019; Soares *et al.*, 2017; Wu *et al.*, 2017; Zábrodská & Vorlová, 2014). The most regular form of honey adulteration is by directly adding various cheap and easily available sweeteners such as refined cane sugar, jaggery, beet sugar, high fructose corn syrup, corn sugar syrup, and maltose syrup (Oroian *et al.*, 2017; Salvador *et al.*, 2019; Se *et al.*, 2019). The most known adulterants are invert beet and cane syrups, which are usually difficult to detect due to the ability of mimicking the natural fructose-glucose-sucrose profile of honey (Paradkar & Irudayaraj, 2001). High sucrose content makes cane sugar as an ideal adulterant of honey. Zakaria *et al.*, 2011 reported on honey adulterated with cane sugar which was selected as an adulterant base on the cost-effectiveness and easy availability within Malaysia (Se *et al.*, 2019).

Unfortunately, prolonged consumption of adulterated honey could result in many serious health issues such as induce obesity, increase blood glucose level and demonstrate toxicity effects (Samat *et al.*, 2018), subsequently damage the reputation of genuine producers and violate trust and rights of consumers of natural honey. Therefore, having a simple yet reliable means for discriminating pure and cane sugar syrup (CS) adulterated honey samples for high throughput routine analysis appears relevant.

In addressing such an issue, numerous physicochemical and instrumental analyses were developed to detect and discriminate adulterants in honey samples.

Adulteration process could affect the physical properties of honey (El-Bialee & Sorour, 2011). The amount of total soluble solids and water present in honey are an important parameters of honey quality (Moniruzzaman *et al.*, 2013) and have been used for the determination of honey adulteration, often represented by the Brix value and refractive index (RI) of honey samples respectively (Adebiyi *et al.*, 2004; El-Bialee & Sorour, 2011). Meanwhile, previous study has shown that density of honey samples has correlation to both Brix and RI value depending on the degree of adulteration of the sample (El-Bialee & Sorour, 2011; Lullah-Deh *et al.*, 2018). Thus, physical characteristics namely Brix value, refractive index and density were selected to be observed in this study, taking into consideration the expenses, time and instrument liability.

In the perspective of instrumental analysis, although chromatographic analyses such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) have been reported extensively in studying the discrimination of adulterated honey samples, they suffers from drawbacks in handling huge amounts of samples because time-consuming, destructive and tedious besides often uses hazardous chemicals (Jamaludin *et al.*, 2017; Razali *et al.*, 2018; Se *et al.*, 2018). Therefore, the need to develop rapid, non-destructive, simple (no sample preparation needed), and greener (uses less or no chemicals) screening as well as confirmatory methods to evaluate honey authenticity are of utmost concern.

On the other hand, vibrational spectroscopy techniques namely ATR-FTIR and Raman spectroscopy have been reported as powerful characteristics and classification techniques especially when involving complex matrices such as honey samples (Lullah-Deh *et al.*, 2018; Wu *et al.*, 2017). These two techniques complement each other in terms of principle of analysis (Gautam *et al.*, 2015; Se *et al.*, 2019), whereby to fully assess molecular vibrational modes of a molecule, ATR-FTIR and Raman spectroscopy are commonly requested for asymmetric vibrations of polar groups and symmetric vibrations of nonpolar groups, respectively (Bunaciu et al., 2020). In terms of spectral interference, fluorescence samples may interfere in obtaining Raman spectra, but not particularly an issue for ATR-FTIR (Wu *et al.*, 2017). Raman spectroscopic however has some distinct advantages over ATR-FTIR such as: no interference from water content (Anjos *et al.*, 2018; Hackshaw *et al.*, 2020; Oroian *et al.*, 2017; Wu *et al.*, 2017) and the samples can be readily analysed through polymer or glass packaging (Bunaciu *et al.*, 2020; Salvador *et al.*, 2019). Thus, in this study, datasets from both ATR-FTIR and Raman were put alongside to study their effectiveness to distinguish pure honey samples from the adulterated ones.

However, ATR-FTIR and Raman spectroscopic data consists of huge variables (wavenumbers) and usually need to be integrated with chemometric (multivariate statistical analysis) techniques such as principle component analysis (PCA) and linear discriminant analysis (LDA) to utilize the complete information of the complex spectra datasets (Arvanitoyannis *et al.*, 2005; Gautam *et al.*, 2015; Se *et al.*, 2019). With this, patterns of pure and adulterated stingless bee honey samples could be modelled and these models can be used routinely to predict the authenticity of newly acquired samples of similar type (Gautam et al., 2015)(Salvador et al., 2019). In this study, PCA was used to reduce the data dimensionality and for better visualization of large dataset of ATR-FTIR and Raman spectra, while LDA was used for classification of pure and adulterated stingless bee honey.

1.2 Problem Statement

The occurrence of adulterated honey in Malaysian market cannot be ruled out and has come to prominence in recent years despite of having an established Standard for Malaysia (Standard Malaysia, 2017). In a study conducted over a period of ten years, Kamaruddin and Mohd.Nuruddin, (2006) reported that 77% of honey in Malaysian market was adulterated, which is well supported by Lisut *et al.*, (2017) in recent year. Considering both commercial and health aspects, the counter-measure for the authenticity of honey has been of great importance and has been carried out on an ongoing basis by various authorities worldwide.

With current advancement in technology, doctored or fraudulent adulterated honey becomes challenging to be identified, leading to misidentification even for experts (Samat *et al.*, 2018). In regards with that, the association between multiple fast and simple analyses are essential to produce reliable findings for high-throughput routine analysis of honey adulterations. This could also be an alternative to the analysis using conventional chromatographic techniques such as GC and HPLC that are time consuming. Nevertheless, specific analytical protocol/ procedure using such multiple analyses for analysing the purportedly adulterated Malaysian stingless bee honey samples for forensic purposes is lacking.

Other than being known as fast and simple techniques, spectrometry techniques such as ATR-FTIR and Raman are also considered for being non-destructive and adhering green chemistry. Hence, these techniques could be good alternatives for studying their suitability in categorically discriminating the pure and adulterated honey samples in the aspects of forensic. However, due to the nature of spectroscopic of having a large number of datasets, analyses using these techniques usually combined with chemometrics for better data presentation (Sivakesava & Irudayaraj, 2001). While discrimination of stingless bee honey adulteration by ATR-FTIR spectroscopy combined with chemometric techniques has been reported (Se *et al.*, 2018), the same for the combination of both ATR-FTIR and Raman integrated with chemometrics techniques like PCA and LDA remains unreported in Malaysian context.

The development of such protocol that combines ATR-FTIR and Raman is vital as these techniques are complementary to each other (Larkin, 2011; Gautam *et al.*, 2015; Se *et al.*, 2019), although ATR-FTIR is seen as inexpensive compared to Raman (Se *et al.*, 2018; Zhu *et al.*, 2010). To the best of the author's knowledge, to date, study of stingless bee honey authenticity in terms of adulteration, using ATR-FTIR along with Raman spectroscopy in Malaysia, remains scarce. Review of literature also reveals very limited information pertaining to this aspects, especially that which involving study of cane sugar syrup (CS), which is reported as the widely used adulterant in Malaysia (Se *et al.*, 2019).

1.3 Objectives of Study

Referring to the pure stingless bee honey (PH) samples and intentionally adulterated ones containing sugar cane syrup as adulterant (AHCS), this study was aimed at:

a) Comparing the physical characteristics (Brix value, density and refractive index) between the two categories of samples.
b) Analysing the spectral characteristics of the two categories of samples using ATR-FTIR and Raman spectroscopy.
c) Performing the discrimination analysis of the two categories of samples using unsupervised PCA and supervised LDA chemometrics multivariate analysis.

1.4 Hypothesis

It was hypothesised that the PH and AHCS samples would have significant different of Brix value, density and refractive index. It was also hypothesised that the adulteration of honey using CS could be discriminated using chemometric-integrated spectroscopic techniques (ATR-FTIR and Raman).

1.5 Scopes of Study

For this study, the PH sample were obtained from a local beekeeper farm in University Teknologi Malaysia (UTM) Skudai, Johor, meanwhile the adulterant was purchased in the form of granulated cane sugar (CS) from a local store and prepared into cane sugar syrup in a laboratory condition. The adulteration of the honey samples was done at five different percentages (5, 10, 20, 40 and 50 % (w/w)). In order to get an accurate result as well as sufficient sample sizes for chemometric analysis, six replicates of samples were prepared and analysed for each assay.

For the physical properties study, the analyses which were performed includes Brix value (representing total soluble solid), refractive index and density. Brix analysis was performed using hand-held digital refractometer employing method suggested by (Shamsudin *et al.*, 2019). Refractive index was measured through a refractometer at wavelength 589 nm using the method adapted from (Moniruzzaman et al., 2012; Moniruzzaman et al., 2013). Meanwhile, density was performed according to the method modified from Gómez-Díaz *et al.*, (2012) and Kinoo *et al.*, (2012). The results obtained were further analysed with statistical analysis of one-way ANOVA and Turkey Post-Hoc test in order to determine statistical significance.

To analyse spectral characteristics of PH, CS and AHCS, samples were subjected to vibrational spectroscopy analyses. For ATR-FTIR spectroscopy analysis, samples were scanned at the full region from wavenumber of 4000 – 600 cm^{-1} with 16 scans and spectral data were pre-processed (baseline correction) using PerkinElmer Spectrum (version 10.4.3) software. Meanwhile for Raman spectroscopy analysis the region from Raman Shift of 1430 – 200 cm^{-1} were selected with 20 scans and spectral data were pre-processed (baseline correction and normalization) using OriginPro (version 2018) software.

For chemometric analysis for both instrumental analyses, the pre-processed spectral data at the fingerprint region of 1400-700 cm^{-1} were employed as the variables. Datasets were organized and processed using unsupervised PCA in order to determine the main contributing factors for grouping, by employing Minitab software. Next, supervised LDA were applied to establish predictive models for sample discrimination using the IBM SPSS Statistics software. The PCA and LDA three-dimentional visualization plots were also obtained using Minitab software.

1.6 Significance of Study

In a matter of forensic significance, this present research would provide specific analytical procedures/protocols using chemometric-integrated ATR-FTIR and Raman spectroscopy, which are expected to be useful in discriminating pure and adulterated stingless bee honey samples. Hence, the outcomes of this research would pave the way in developing a fast, simple yet as an accurate alternative protocol to the existing laborious analytical protocol involving HPLC and GC in validating authenticity of stingless bee honey in routine monitoring of this food commodity by the enforcement agencies. Moreover, this proposed protocol could entrust the forensic approach of chemometrics-integrated spectroscopic techniques in consumer chemistry related caseworks in Malaysia as an established method and to be presented as convincing evidence for testimony in the court of law.

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