Classification and Adulteration Detection of Honey from Different Floral and Geographical Origins – Case of Zambian and Botswana Honey

By

Tumelo Padiso

University of Botswana

Faculty of Science -Department of Chemistry

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Supervisor: Prof.K. Sichilongo, PhD

CERTIFICATION

The undersigned certify that they have read and here by recommends or acceptance by the Faculty of Science a dissertation titled *"Classification and Adulteration Detection of Honey from Different Floral and Geographical Origins – Case of Zambian and Botswana Honey."*, in fulfilment of the requirements for the Master of Science Degree in Analytical Chemistry at the University of Botswana.

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DECLARATION AND COPYRIGHT

"I hereby declare that the dissertation submitted for the degree of Master of Science in Chemistry (MSc Analytical Chemistry) to the University of Botswana, is my own original work and has not previously been submitted to any other institution and any work quoted is indicated and acknowledged by means of a comprehensive list of references."

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DEDICATION

I am dedicating this work to my family.

LIST OF UNCOMMON SYMBOLS AND ABBREVIATIONS INCLUDING NOTATIONS OF SI UNITS

Analysis of variance	(ANOVA)
Apiary-Disease Research and Control Laboratory	(ADRCL)
Automated Mass Spectral Deconvolution and Identification System	(AMDIS)
Carbowax- divinylbenzene	(CW–DVB)
Carboxene-Polydimethylsiloxane	(CAR–PDMS)
Direct insertion	(DI)
Divinylbenzene	(DVB)
Divinylbenzene Polydimethylsiloxane Carboxene	(DVB–PDMS–CAR)
Electron Ionization	(EI)
Fourier Transform Infrared spectroscopic with Attenuated Total Reflectance	(FTIR-ATR)
Fourier Transform Infrared Spectroscopy	(FTIR)
Gas Chromatography	(GC)
Gas chromatography-mass spectrometry	(GC-MS)
Gross Domestic Product	(GDP)
Headspace	(HS)
Headspace- Solid-Phase Micro Extraction	(HS-SPME)
High Pressure Liquid Chromatography	(HPLC)

International Community for Bee Botany	(ICBB)
International Honey Commission	(IHC)
Linear Discriminant Analysis	(LDA)
Liquid Chromatography Mass Spectrometry	(LC-MS)
Liquid-Liquid Extraction	(LLE)
Mass Spectral Library	(MSL)
Mass Spectrometry	(MS)
Multivariate analysis of variance	(MANOVA)
Negative Chemical Ionization	(NCI)
Partial Least Squares	(PLS)
Perfluorotributylamine	(PFTBA)
Pharmaceutical honey volatile metabolites	(PHVMs)
Polyacrylate	(CAR–PDMS)
Polydimethylsiloxane	(PDMS)
Polydimethylsiloxane-divinylbenzene	(PDMS-DVB)
Polytetrafluoroethylene	(PTFE)
Positive Chemical Ionization	(PCI)
Principal Component Analysis	(PCA)
Principal Factor Analysis	(PFA)
Solid-Phase Extraction	(SPE)

Solid-Phase Micro Extraction	(SPME)
Southern African Development Community	(SADC)
Stepwise Discriminant Analysis	(SDA)
Supercritical Fluid Extraction	(SFE)
Ultra-Pressure Liquid Chromatography – Mass Spectrometry	(UPLC-MS)
Ultra-Pressure Liquid Chromatography – Mass Spectrometry	(UPLC-MS)
United States Pharmacopeial Convention	USP)
Universal Attenuated Total Reflectance	(UATR)

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ABSTRACT

The physiochemical properties of three commercial and three real natural honeys from Zambia and Botswana were analysed using methods specified by the International Honey Commission. Some of the parameters were specific conductivity, water and ash contents, pH, and acidity. One commercial honey from Zambia i.e. ZAM 1 had a moisture content of 18.56% and electrical conductivity of 0.725 mS/cm. Furthermore, the honeys were also analysed for adulteration with sucrose, fructose, maltose and glucose. Partial validation of the FTIR method used for adulteration detection gave limits of quantification (LOQ) values that ranged between 1.80 and 5.12% w/v, with calibration curves that were linear indicated by R^2 values that ranged between 0.9858-0.9989. The concentration ranges for the sugars were as follows (% in brackets): sucrose (LOQ-6.01), fructose (34.69-43.66), maltose (5.38-12.88) and glucose (25.52-32.57). The volatile classification of the same honeys based on geographic and floral origins was also done forthwith. Classification using gas chromatography – mass spectrometry/solid phase micro extraction (GC-MS/SPME) was accomplished on three commercial and three unprocessed organic honeys ($p<0.05$). The Automated Mass spectral Deconvolution and Identification System (AMDIS), *Metab* R, an R platform application and MINITAB version 14 were used for data processing. 17 volatile metabolites in three commercial and 42 in three unprocessed organic honeys were identified, confirmed and formed the basis for differentiation. Database search showed that, the honeys were polyfloral with major ingredients coming from common flowering plants, conifers and other gymnosperms such as *Carica papaya L.* (Papaya), *Monstera deliciosa* (Ceriman) and fruits i.e. Guava, melon and pineapple endemic in the areas from which the honeys originated from.

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1. INTRODUCTION

1.1. What is Honey?

'Honey is the natural sweet substance produced by honey bees from the nectar of plants which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature' (Alimentarius, 2001). Honey has been reported to consist of water, fructose, glucose, sucrose, maltose, minerals, organic acids, amino acids, vitamins, flavonoid, proteins and acetylcholine (Doner, 1976; Mesallam & El-Shaarawy, 1987). Honey can be classified as either blossom honey or honeydew honey. Blossom is produced by bees from nectar collected from flowers of blossoming plants. Honeydew honey is obtained from excretions of plant-sucking insects (Pita-calvo & Vázquez, 2018). The composition, taste and smell of honey vary according to floral source, geographical origin, climate of the region and harvest season. The best way authenticate honey by classification of based on it's the physiochemical properties and through identification of bio-markers present in a given honey. Honey production has been documented to have immense health, economic and environmental benefits (Gannabathula et al., 2011; Tonks et al., 2007).

1.2. Literature Review and Justification

1.2.1. Physiochemical properties of honey

The International Honey Commission compiled analytical methods used for the determination of the geographic origin of honey as well as the quality. The floral source of a given honey has a great impact on its chemical composition and characteristics (Gok, Severcan, Goormaghtigh, Kandemir, & Severcan, 2015). It can then be inferred that honeys from similar botanical and geographic origins will have similar physicochemical properties. This will allow classification of honey according to botanical and geographical origins, using their physicochemical properties.

The Physicochemical properties investigated in this study included moisture, electrical conductivity, pH and free acidity by titration to pH 8.3, ash content and determination of sugars by FTIR (fructose, Glucose, and sucrose). These techniques are outlined in the harmonized methods of the International Honey Commission (IHC) (Bogdanov et al., 1999).

The percentage of moisture content refers to the amount of water present per 100 grams of honey. It is one of the most important indicators of a honey's quality. This is because a honey's moisture content displays its resistance to yeast fermentation and therefore its stability. The percentage moisture of content of honey can naturally be as low as 13% or as high as 23%, depending on the its floral source, climatic conditions of the region and other factors (Bogdanov, Martin, & Lullmann, 2002). Values greater than 20% have a high probability of the honey fermenting. Karabagias, Badeka, Kontakos, Karabournioti, & Kontominas, 2014a noted that commonly honeydew will pose lower values than blossom and heather honey generally have higher water content values. High moisture content also indicates adulteration by dilution however, it is important to know that the moisture can be altered intentionally or unintentionally during honey processing. Determination of the percentage moisture content by refractive index is anticipated to be used in this project since it gives an indication of adulteration. This property can be used as a parameter to classify honey and additional can be used to infer the quality of a honey.

The pH and free acidity values indicate the freshness of the honey. All honeys are acidic and, most commonly have pH values ranging from 3.5-5.5. This is owing to the presence of organic acids. These acids add to honeys distinctive flavour as well as improving its stability by resisting fermentation. Free acidity measures how resistant honey is to spoilage and is measured in units of milliequivalents. The lower the value for free acidity the more resistant the honey is to fermentation. Free acidity increases with time. The lower the value, the fresher the honey, and thus can be used

as a quality criterion parameter (Karabagias et al., 2014a). 50 milliequivalents are the maximum acidity in honey permitted by the IHC. Honey fermentation causes an increase of acidity and because of this, a maximum acidity value has proven useful although there is considerable natural variation. The experiments for pH and free acidity by titration to pH 8.3 have poor reproducibility. This is owing to the fact the endpoint of the titration is not well defined (Bogdanov & Martin, 2002). The pH value will also be used to classify honey.

Ash content is a measure of the total amount of minerals within food in this instance honey. Ash is the inorganic residue remaining after the water and organic matter has been removed by heating in the presence of oxidizing agents (Marshall, 2010). The ash content of honey generally ranges from 0.02-1%. The ash content has been used as a criterion to determine the botanical origin of a honey, the blossom honey has a lower ash content than the honeydews. This value is dependent on the type of nectar as well as its botanical and geographical origin. Electrical conductivity provides a faster and easier alternative method for distinguishing the floral and geographical origin of honey when compared to ash content determinations.

Electrical conductivity gives a measure of the amount electrical current honey can carry (Subbiah, Stembridge, & Morison, 2015). This parameter has been used to distinguish honeydew and blossom honey, as honeydew has a high mineral content, as a result, its electrical conductivity is generally greater than 0.8 $mScm^{-1}$ (Olga, María, & Carmen, 2012). Thus electrical conductivity depends greatly on the honey's mineral content (Karabagias et al., 2014a). Electrical conductivity has been found to be influenced by honey's botanical and geographical origin (Pita-calvo & Vázquez, 2018). Table 1 shows the IHC determined values of electrical conductivity of honeys from different botanical and geographical origins.

Table 1. Electrical Conductivity (mS/cm), floral and geographical origin of honey

Floral Source	Geographical origin	mS/cm
Arbutus	Europe	0.63-0.90
Heath	Australia	0.74-1.02
Brassica	Europe	0.09-0.27
Chestnut	Europe	0.80-2.07
Centaurea	Europe	0.21-0.75
Citrus	Europe	0.10-0.35
Heather	Europe and Asia	0.42-1.40
Eucalyptus	Europe, America and Australia	0.19-1.33
Leatherwood	Australia	0.46-0.89
Cotton	Europe	0.45-0.76
Honeysuckle	Europe	0.09-0.30
Sunflower	Europe	0.20-0.60
Manuka	Asia	0.31-1.07
Lavender	Europe	0.12-0.60
Tea tree	Australia	0.40-1.12
Lucerne	Europe	0.11-0.23
Phacelia	Europe	0.09-0.44
False acacia	Europe and Asia	0.09-0.30
Alpine rose	Europe	0.15-0.45
Rosemary	Europe	0.10-0.35
Dandelion	Europe	0.29-0.65
Thyme	Europe	0.24-0.72
Lime	Europe and Asia	0.33-1.15
Clover	Europe	0.13-0.25
Blossom, blend	Europe, Asia, America and Australia	0.10-0.70
Honeydew	Europe, Asia, America and Australia	0.80-2.11

This prescribed data compiled by the IHC. The minimum and maximum values for conductivity were obtained at 99% confidence level. (Bogdanov et al., 2000).

Thus, electrical conductivity is an important criterion for determining the botanical origin of honey. Electrical conductivity determinations are generally very fast, the results produced are reproducible while requiring relatively inexpensive instrumentation. As a result, the parameter is used in routine honey quality control.

In a study conducted by Karabagias et al., 2014a Greek pine honey from 4 different regions in Greece was classified based on their geographical origin, according to their physicochemical properties using Multivariate analysis of variance (MANOVA) and Linear Discriminant Analysis (LDA). The physicochemical parameter determined included pH and free acidity, laconic and total acidity, electrical conductivity, moisture content, ash content, lactonic/free acidity ratio and colour parameters. The finding showed the honey was successfully classified according to its geographical origin using physicochemical properties with 79.5% correct prediction.

In the northwest region of Spain, a study conducted by Olga et al., 2012 investigated if honey's of different botanical origins, namely honeydew and blossom, could be differentiated from one another. Physicochemical parameters of honeydew and blossom honey were found as well as the melissopalynological analysis of the honey's. Cluster analysis was applied followed by a t-test and the results indicated that the two honeys were significantly different therefore they could be distinguished from each other. The physiochemical properties that had a substantial impact on the differences between the honeys included the enzymatic content, electrical conductivity, moisture content, colour, mineral content. The study by Olga et al., 2012 showed that the honey could be classified according to its botanical origin.

Similarly, a study by Serrano, Villarejo, Espejo, & Jodral, 2004 characterized two monofloral Andalusian honeys specifically Citrus and Eucalyptus. The honeys were characterized using their physicochemical properties, moisture, hydroxymethylfurfural, diastase, pH, free acidity, lactone

acidity, electrical conductivity, glucose, fructose, sucrose, proline, invertase, glucose-oxidase, water activity and insoluble solids. Statistical tools such as analysis of variance, principal factor analysis (PFA) and stepwise discriminant analysis (SDA) were successfully used to classify and distinguish the two honeys. These multi-variant statistical tools were employed to determine six physicochemical parameters that caused the most significant discrimination between the honeys. These were free acidity, moisture content, invertase, total sugars, electrical conductivity and insoluble solids. The findings likewise demonstrated that electrical conductivity is the most closely associated to the botanical origin of honey.

In Uruguay (Corbella & Cozzolino, 2006), researchers investigated the physicochemical properties of honeys of different botanical origins (Pasture, Eucalyptus, Citrus, Baccharis, and polyfloral) in order to differentiate them using multi-variant statistical tools. The physiochemical parameter such as moisture content, pH, electric conductivity, the concentration of hydroxymethyl-2-furaldehyde and colour composition were determined. The sorting was performed using principal component analysis (PCA) and linear discriminant analysis. The results from PCA indicated that the parameters that greatly influenced the botanical sources of the honey were moisture content, pH and electrical conductivity. The honeys were correctly sorted according to their botanical origin without the role of pollen analysis.

These above studies indicate that it is feasible to classify honey according to its botanical and geographical origins based on the honeys physiochemical properties with the application of multi-variant statistical tools.

Table 2 shows expected value for physicochemical properties of blossom and honeydew (Bogdanov et al., 2000).

Table 2. Summary of physicochemical properties

	Blossom honey	Honeydew
	Range	Range
moisture content	15-20	15-20
pH-value	3.5-4.5	4.5-6.5
electrical conductivity	>0.8mS/cm	<0.8mS/cm
Ash content	0.1-0.5	0.6-2.0

1.2.2. The honey industry

Honey production is a very lucrative industry owing to the fact that honey is five times more expensive than oil and demand for it continues to rise not only in Africa but globally (Jiwaji, 2007). According to the United Nations Food and Agricultural Organization (FAO) data, the world's bee hive stock escalated from 50 million in 1961 to about 83 million in 2014, representing 1.3% average annual growth (UNFAO, 2017). The average annual growth rate increased to 1.9% since 2009. The global trade of honey reached a value of US$2. 4 billion in 2017 (Workman, 2018). The honey industry is booming with global exports that grew by 5.8% between 2016 and 2017. Honey production is dominant in developing countries, while the developed countries provide the market. The Beekeeping sector in Botswana displays steady growth, however, accelerated development is required for it to become an economically viable venture. The Government of Botswana is keen to oversee the development of the honey production industry. This is demonstrated by the recent selection of Botswana to host a state-of-the-art Apiary-Disease Research and Control Laboratory (ADRCL) (Botswana Standard, 2017). This laboratory will cater to the rest of Africa in research involving disease that affects the African bee. This is in addition to the fact the bee honey

production is rapidly growing in the SADC region (Jiwaji, 2007). Honey production provides rural employment. Bees are useful in cross-pollination and oblige beekeepers to grow trees as sources of nectar (Turner & Makhaya, 2014). Thus honey production is beneficial to the environment since bees contribute to biodiversity and can add to the gross domestic product (GDP) of any country. The price of commercial honey is greatly influenced by its floral source and geographical origin, with monofloral honey demanding a higher price than its polyfloral counterpart (Schuhfried et al., 2016). Akin to all foodstuffs honey is subject to fraud. The high economic value of honey makes it an even more alluring target for fraudsters. According to the United States Pharmacopeial Convention (USP) the term food fraud defines the "deliberate substitution, addition, tampering or misrepresentation of food, food ingredients or food packaging, or false or misleading statements made about a product for economic gain." Adulteration refers to the intentional addition of a fraudulent component to a food product (Pustjens, Weesepoel, & Ruth, 2016). Notwithstanding, trade-in honey has been riddled with fraud which is a threat to the market distortion due to uncertainties in the authenticity, geographical and botanical origin of the honey as well as its quality.

1.2.3. Challenges

Fraudulent activities in honey production manifest themselves in many forms such as dilution of honey with sugar and polysaccharide syrups (Daniele & Casabianca, 2012). As well as feeding honey bees' with saccharides or invert saccharide derivatives in order to stimulate increased production. In addition, fraud occurs in the form of mislabelling this is done in order to imitate honeys with high prices from certain geographical and/or botanical origins (Moore, Spink, & Lipp, 2012). The media have made concerning reports that more Mānuka honey was sold worldwide than was produced in New Zealand (Burns, Dillon, Warren, & Walker, 2018). These activities have become an international issue due to the concern of the authenticity and quality of the honey

(Moore et al., 2012). Honey fraud is predominantly financially motivated and has potentially negative consequences, such as the loss of consumer confidence this can impact the honey industry at large. Some consumers use honey as a dietary substitute for sugar and adulteration with sugars could negatively impact their health. Beekeepers lose out from the profits made by fraudsters when they imitate their honey and may also gain a bad reputation as a result (Kennedy, 2012). Food fraud is a global concern and is driven by the low probability of being detected. Thus, there is a great need for a systematic classification database for honey's based on geographical and floral origin. These efforts should be complemented with adulteration detection, which is a result of the addition of sugar syrups in order to increase the volume and thus the income therefrom.

During the process of production honey bees will naturally deposit pollen from plants into the resultant honey. This pollen originates from the floral source of the honey which the nectar is collected from and thus, by study the pollen species present in a honey sample, it is possible to determine its botanical origins. This method of identifying pollen present in honey by microscopic examination, in order to determine its botanical origin is known as Pollen analysis or melissopalynology. The standard for this method is the pollen of the suspected plant species. Pollen analysis is still used as a part of quality control in the European Union and has been of immense aid in combating honey fraud. This technique was proposed by the International Community for Bee Botany (ICBB) in 1970 and updated in 1978 (Louveaux, Maurizio, & Vorwohl, 1978).

In a study conducted by Sniderman, Matley, Haberle, & Cantrill, 2018, Australian honeys were analysed using Pollen analysis. It was determined that these honeys have a high number of Myrtaceae pollen per sample with a mean of 4.6. The value is higher than honeys found outside

of Australia and may be used as a criterion to authenticate Australian honey (Sniderman et al., 2018).

Portuguese honey was studied in a similar fashion (Silva, Sousa, & Taveira, 2017). 16 honey sample of different floral origins were collected from the Castelo Branco region of Portugal and Pollen analysis was carried out on the samples. The results for pollen analysis were expressed in terms of percentages of total pollen was determined used Pollen analysis. Generally monofloral honeys consisted predominantly of a single pollen type in amount greater than 45% of the total pollen content. Using this technique 12 of the honey samples were found to be monofloral from plant species such as Erica, Calluna vulgaris, Lavandula sp., Echium spp. and Campanula. The pollen present in polyfloral honey samples was also identified. Using this method it is possible of authenticant pollen present in a honey and determine it botanical origins.

Both these studies show that characterizing honey by using pollen indigenous to a region is a good method to protect it from fraudulent activities. This makes the method ideal for identifying the floral source of honey. Notwithstanding, this technique has its own shortcomings which render it less accurate. One of the limitations is that it is difficult to find purely monofloral honey because bees often collect nectar from different plants and thus different pollen varieties. Another shortcoming of this technique is that since pollen analysis is based on microscopic observation the method tends to be laborious and time-consuming. Specialized knowledge is also a prerequisite to apply this method. Ultrafiltration of honey to remove pollen makes melissopalynology unsuitable in many instances where the beekeepers depend on bees that collect nectar from polyfloral sources. The method also does not indicate if the honey has been adulterated. Due to these issues, it is not feasible to use pollen analysis on a routine basis, especially in the African context and was thus not explored further in this project. This raises the need to explore more suitable techniques to

determine the quality and geographical origin of honey. An alternative to pollen analysis has been the identification of DNA markers present in pollen to confirm the botanical origin of honey. This method is novel and promising (Soares, Amaral, Beatriz, Oliveira, & Mafra, 2014).

Despite the IHC providing guidelines on the quality control of organic honey, many African countries that already produce or are in the process of producing honey have not taken advantage to ensure these standards are met. There are not many African countries that have classified their honeys according to their botanical and geographical origins based on their physicochemical properties. Such a classification of honey also gives an indication of the quality of the honey. This project was conceived as a pilot project to demonstrate the feasibility of such studies in Botswana, where literature showed that these studies have not been done. At conception, it was envisaged that results from this project would inspire its expansion to create databases for varietal honey from many regions within Botswana and even beyond its borders. The establishment of such a database would help protect beekeepers from the effects of fraudulent or counterfeit honey. A database will also instil a sense of confidence in honey consumers.

1.3.1. Methods of analysis

1.3.2. Principals of FTIR

The high concentration of sugars such as fructose, glucose, maltose and especially sucrose may indicate the presence of adulteration of honey. According to the IHC (Bogdanov et al., 2002), the amounts of sugars prescribed in some European honeys are given in Table 3.

Table 3. Suggested Sugar content in honey

Sugar content	Honey	Proposed value

Sum of fructose and glucose	Blossom	≥ 60g/100g
	Honeydew	≥ 45g/100g
Sucrose	Not listed below	≤5g/100g
	Banksia, Citrus, Hedysarum, Medicago, Robinia, Rosemarinus	≤ 10 g/100 g
	Lavandula	≤ 15

Thus, it is of utmost importance to determine the concentration of these sugars. Several instruments have been used to measure the amount sugars and detect adulteration in unprocessed food such as honey, which include paper chromatography (Stoffyn & Jeanloz, 1954) High Pressure Liquid Chromatography (HPLC) (Brons & Olieman, 1983) thin-layer chromatography (Vomhof & Tucker, 1965) gas-liquid chromatography (Sweeley, Bentley, Makita, & Wells, 1963) and High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (Hanko & Rohrer, 2000) and Nuclear magnetic resonance spectroscopy (Ertelli et al., 2010) and carbon isotope ratio analysis (Padovan, Jong, Rodrigues, & Marchini, 2003). However, these techniques require sample prep, storage and require the use of organic solvents. Fourier Transform Infrared Spectroscopy (FTIR) offers a rapid method for analysing samples that is non-destructive, inexpensive and green.

The infrared spectroscopy is the study of how matter responds to and interact with infrared radiation. When molecules absorb infrared radiation the chemical bonds binding them to tend to vibrate. Thus infrared spectroscopy gives the measure of a sample vibrational frequency after absorbing energy from an infrared beam (Putzig et al., 1994). The infrared spectrum of a sample

is the plot of measured infrared intensity (absorbance or transmittance) versus wavelength (or the frequency at which the absorbance or transmittance occurs).

The infrared spectrum is obtained by exposing a sample to an infrared beam and measuring the absorption or transmittance of the light at each frequency. These measurements are achieved with the use of an instrument called the Fourier Transform Infrared Spectroscopy (FTIR) (Putzig et al., 1994) . The peaks of a spectrum correspond to the frequency of absorbed radiation and are called the wavenumber. The spectrum of a given sample will give information about the structure of the compound. For example, functional groups in the compound have a distinct vibrational frequency, this can then be used to identify which functional groups are present in the compound, as shown in Table 4 (Luykx & Ruth, 2008). Therefore the infrared spectrum of a sample under investigation will represent its chemical fingerprint (Luykx & Ruth, 2008).

Table 4. Shows the peaks that have been observed in honey using FTIR-ART

Wavenumber range	**Functional group**	**Reference**
3000–2800 cm^{-1}	C–H stretching (carbohydrates)	(Gallardo-Velázquez, Osorio-Revilla, Zuñiga-de Loa, & Rivera-Espinoza, 2009)
	O–H stretching (carboxylic acids)	(Movasaghi, Rehman, & Rehman, 2008)
	NH_3 stretching (free amino acids)	(Gallardo-Velázquez et al., 2009)

1700–1600 cm^{-1}	O–H stretching/bending (water) C=O stretching(mainly from carbohydrates) N–H bending of amide I (mainly proteins)	(Cai & Singh, 2004) (Gallardo-Velázquez et al., 2009) (Philip, 2009)
1540–1175 cm^{-1}	O–H stretching/bending C–O stretching (carbohydrates) C=O stretching of ketones C–H stretching (carbohydrates)	(Gallardo-Velázquez et al., 2009) (Tewari & Irudayaraj, 2004) (Tewari & Irudayaraj, 2005)
1175–940 cm^{-1}	C–O & C–C stretching (carbohydrates) Ring vibrations (mainly from carbohydrates)	(Subari, Mohamad Saleh, Md Shakaff, & Zakaria, 2012) (Gallardo-Velázquez et al., 2009)
940–700 cm^{-1}	Anomeric region of carbohydrates C–H bending (mainly from carbohydrates) Ring vibrations (mainly from carbohydrates)	(Subari et al., 2012) (Gallardo-Velázquez et al., 2009) (Tewari & Irudayaraj, 2004)

FTIR has been employed in the food industry for the purpose of quality control to detect adulterants in foodstuffs (Hug, Chalmers, & Griffith, 2002; Li-Chan, Chalmers, & Griffiths, 2010). This method of adulteration detection has been applied to honey (Liu, Qu, Luo, Xu, & Zhong, 2019). Vibrational spectroscopy has been used to distinguish honey samples from the different botanical and geographical origin. Fourier Transform Infrared spectroscopic with Attenuated Total Reflectance (FTIR-ATR) has demonstrated the ability to quantify the concentration of fructose, glucose, sucrose, maltose in honey thus detecting adulteration (Anjos, Campos, Ruiz, & Antunes, 2015). The application of chemometric method such as partial least squares (PLS) and PCA in

conjunction with vibrational spectroscopy offer means of both adulteration detection as well as geographical classification (Anjos et al., 2015).

The infrared range can be divided into three regions, namely the near-infrared region (12800-4000 cm^{-1}), the mid-infrared region (4000-200 cm^{-1}) and far-infrared region (50-1000 cm^{-1}). In our discussion of the quality control of honey, only the mid-infrared region will be investigated this is credited to the type of data that can be pulled from this region. This is information about the functional groups present in honey and the ability to find instinctive peaks in the fingerprint region (1500 to 500 cm^{-1}). That will greatly go towards authentication of a honeys botanical origin as well as detection of adulterants.

In a study conducted by Irudayaraj & Sivakesava, 2001 the use of FTIR-ATR was employed as a screening tool to determine the level of sugar adulteration in honey are discussed. In the study the honey samples were deliberately adulterated using glucose, fructose, sucrose and corn syrups than scanned in the mid-infrared region this is approximately 4000-200 cm^{-1}. The mid-infrared region gave information about the functional groups present in the honey. This further facilitated the identification of the adulterants by identifying its characteristic peak of glucose, fructose, sucrose and corn syrups. After the mid-infrared spectrum of the adulterated honey was obtained multi-variant statistical analysis was applied to the data. Statistical tools used on the spectral data, i.e. the data was subjected to PCA followed by LDA and canonical variant analysis. These tools allowed the compression of the data identification of the sugar used as the adulterant and percentage to which the honey was adulterated. This model is promising as it identifies corn syrup and its concentration with an accuracy of 90%. To improve this model more honeys from different floral origins need to be investigated since this will create a more robust model.

In a similar study (Wang, Kliks, Jun, Jackson, & Li, 2010) FTIR-ATR was used to determine the concentration of selected sugars in honey and thereby detecting adulteration. This was done by preparing a calibration mixture of standard sugars, which included standards for glucose, fructose, sucrose, and maltose, they were then analysed with FTIR-ATR in the mid-infrared region. The distinct peaks of the four sugars were found in the fingerprint within 1500 and 800cm^{-1}. The calibration was successfully achieved with 7 sets of the sugars. Commercial honeys from different geographical regions around the world were then analysed using FTIR-ATR and the concentration of the sugars were determined then validated using HPLC. The spectral information of the honeys was applied to partial least square 2nd-derivative model this showed a high level of predictive accuracy with the highest R^2 value of 0.999. This work showed that FTIR-ATR can be utilized for both qualitative and quantitative analyses of honey from different geographical origins to determine sugars. This can be used to draw conclusions about the adulteration status of the honey. FTIR analysis is fast and can enable greater quality control of honey. The model was only tested on commercial honey, which may have already been adulterated or altered during the processing of the honey. It is recommended to test the method with honey samples collected directly from beekeepers.

In another study (Başar & Özdemir, 2018), FTIR-ATR was used to detect the presence of beetroot sugar and corn syrup, known adulterants in Turkish honey. These honeys were from different botanical and geographic origin. The adulterated honey was prepared by mixing varying amounts of beet-root sugar, corn syrup and water with honey collected from beekeepers. The infrared spectra in this case 4000-600 cm^{-1} was obtained for the adulterated and unadulterated honey samples and multi-variant statistical analysis were applied to the results. With the use of a genetic algorithm-based inverse least squares and (PLS) the honey content and amount of adulterants was determined. This method is of interest because it was applied to honeys from different botanical

origins. The honey samples were obtained within turkey and it would be imperative and interesting to test these techniques on honeys from different geographic origins. This model only gave information on two adulterants but did not provide information on the sugar content or the extent of adulteration. The authentication of the botanical origin of honey is another important issue.

In another investigation involving (Kasprzyk, Depciuch, Grabek-Lejko, & Parlinska-Wojtan, 2018) FTIR-ATR was used to authenticate Brassica honey using Brassica pollen as a reference standard. This was done in order to determine the amount of Brassica pollen in the honey under study. The spectral data for Brassica pollen, Brassica honey and non-Brassica honey were obtained. Using discriminate analysis the distinctive peaks that characterized Brassica honey were determined. The investigation did not only authenticate Brassica honey, but it also discriminated it from non-Brassica honey. This study demonstrated that FTIR-ATR with the assistance of chemometric tools can be used to classify honey based on its botanical origin to allow authentication of honey. This method could be very valuable in route quality control of food. However, it requires the presence of pollen in honey to facilitate its authentication. This can be problematic if the pollen has to be filtered out. There is a need for a method that does not require pollen in the classification and authentication of a honeys the botanical origin.

In a different study (Gok et al., 2015), Turkish honey of different botanical origins was classified using spectral data obtained from FTIR-ATR. The Honeys investigated consisted of the following botanical origins polyfloral, anzer, organic, Taurus flower, pine, chestnut, cedar and rhododendron honey. This included honey adulterated with either maple syrup, fructose syrup or gBrassica molasses. This was done in order to see if FTIR-ATR could be used to discriminate the botanical origins of honey, but also it allowed the detection of adulteration of honey. The spectrum of the honeys was obtained in the range of 650-4000 cm^{-1}. The spectral difference of the honey was used

in conjunction with chemometric tools, i.e. Cluster analysis and PCA. The method successfully classified honeys base on their botanical origins. This method was also capable of adulteration detection in the honey samples. The limitation was that the study did not investigate and classify honeys from different geographical sources.

The physicochemical properties of honeys from different geographical and floral origins have not been studied in Botswana. The determination of the physicochemical properties, as well as the spectral data of local honeys, could be used to classify honeys according to the botanical and geographical origin as well as identify the presence of adulterants. This would function as a building block to establish a database that would protect beekeepers, from fraud. Such a database would also provide a means of quality control. Another means of determining the botanical origins of honey is by the use of volatile profiles.

1.3.3. The volatile profile of honey

The volatile profile of a foodstuff is most commonly used to evaluate its organoleptic quality for the purpose of quality control as well as authentication (Careri et al., 1993). In the case of honey volatile organic compounds are responsible for its aroma. The aroma is also a factor in the flavour of honey. Honeys aroma is primarily composed of volatiles. In total, more than 600 volatile compounds have been reported in honey, which belongs to different chemical classes (Castro-Vazquez, Perez-Coello, & Cabezudo, 2003). The chemical classes to which the volatiles identified in honey are hydrocarbon; aldehyde; alcohol; ketone; acid; ester; aromatic and its derivatives, furan and pyran; norisoprenoids; terpenes and its derivatives and sulphur; and cyclic compounds (Barra, Ponce-Díaz, & Venegas-Gallegos, 2010).

Not all of these volatiles found in honey emit a fragrance. For instance linalool has a spicy floral scent (Montenegro et al., 2009), whilst fumaric acid is odourless (Silici, 2011). These volatile compounds primarily originate from the plant nectar or honeydew collected by bees. As a result, the type of volatile compounds present in honey is greatly dependent on the plant from which the nectar was collected. Hence, the volatile profile of a honey is highly influenced by its floral origin. (Kaškonienė, Venskutonis, & Čeksterytė, 2008). Some of the compounds originating from plants remain unchanged during the honey-making process. Whilst other compounds are modified by being metabolized by honey bees or from handling and during honey processing or from environmental contamination (Jerković & Marijanović, 2009). Nevertheless, due to the number of volatile organic compounds present in honey, it is possible to determine its botanical origin by analysing its volatile profile (Radovic et al., 2001). Thus, the volatile profile of honey is an important characteristic criterion. It has also been reported that some volatile organic compounds for certain honeys are characteristic of its botanical origin (Bouseta, Collin, & Dufour, 1992).

Therefore, it is possible to determine the botanical origin of honey by analysing its volatile profile and determining the floral source of certain volatiles. It has been observed that honeys from Acacia floral source will have volatiles such as cis-linalool oxide and heptanal present in their volatile profiles (Wardencki, Chmiel, Dymerski, Biernacka, & Plutowska, 2009). Volatiles have been used to classify honey from a specific geographical origin (Radovic et al., 2001). These investigations have been conducted in the Netherlands, Spain and Portugal (Radovic et al., 2001). It has been suggested the English honey can be distinguished from others by the presence of 1-penten-3-ol (Radovic et al., 2001). This shows that by using the volatile profile of honey, it is possible to authenticate the botanical and geographical origin.

VOCs can't be used to differentiate between floral and geographical origin however it is possible to do a library search on the VOCs present in a honey and find its floral source. If a floral source is indigenous to a region it is probable to assume it geographical origin. Furthermore, by determining VOCs that act as bio marks for a honey from a certain floral or geographical region it possible to authenticate the honey sample. It has been proven that the volatile profile of honey can be applied in the certification of the floral source and geographical origin of honey. In order to analyse these volatiles it is first necessary to extract them from honey, then to identify the compounds using Gas chromatography-mass spectrometry (GC-MS).

1.3.4. Applications of gas chromatography mass spectrometry (GC-MS) in the characterisation of honey

GC-MS is a modern instrumental analytical technique that is used for both separation and identification of volatiles. GC-MS couples the feature of gas chromatography with those of a mass spectrometer. This is managed in such a way that the gas chromatography (GC) is responsible for separating the constituents of a volatile or semi-volatile mixture. Whilst the mass spectrometry (MS) characterizes each component by measuring the mass to charge ratio of ions. Thus the structural information of the compounds are obtained this facilitates the identification and quantification of the compound. Therefore the GC provides separation and the MS enables identification of the compounds in a sample as well as quantification (Sahil, Prashant, Akanksha, Premjeet, & Devashish, 2011). Techniques such as GC-MS and liquid chromatography mass spectrometry (LC-MS) have been employed in the classification of honey on the basis of geographical and botanical origin in order to forestall malpractices in honey trade. Recently, other advanced techniques such as ultra-pressure liquid chromatography – mass spectrometry (UPLC-

MS) in conjunction with multivariate statistics have also been reported for discriminating honeys based on their floral and geographical origins (Jandrić, Frew, Fernandez-Cedi, & Cannavan, 2017). These methods are based on the non-targeted volatile approach coupled with identification of known characteristic markers.

GC-MS has been utilized to identify then quantify volatile and semi-volatile species in honey, which could function as bio markers (Karabagias, Badeka, Kontakos, Karabournioti, & Kontominas, 2014b; Seisonen, Kivima, & Vene, 2015). These bio markers can serve as fingerprints and play a central part in authenticating the floral origin of a honey. GC-MS methods can be used as critical discrimination tools of honey with different floral and geographical origin. With this technique, it can be inferred with reasonable confidence that honey discrimination based on floral and geographical origin using GCMS and chemometric statistical tools is possible. These methods should, if utilized properly arrest the problem of fraud through counterfeits in the honey industry. As a matter of confirmation, discrimination of honey from different botanical and floral origins has been reported in Europe (Piazza & Oddo, 2004) including botanical species that give unifloral honey (Piazza & Oddo, 2004), in Brazil (Ribeiro et al., 2014), in China (Chen et al., 2012), in Uruguay (Corbella & Cozzolino, 2006), in Argentina (Fechner, Moresi, Díaz, Pellerano, & Vazquez, 2016), in Greece and Turkey (Tananaki, Thrasyvoulou, Giraudel, & Montury, 2007), in Morocco (Chakir, Romane, Marcazzan, & Ferrazzi, 2016), in Spain (Manzanares, García, Galdón, Rodríguez-Rodríguez, & Romero, 2017), in Finland (Kortesniemi et al., 2016), in Tunisia (Boussaid et al., 2018). In order to classify honey based on botanical and geographical origins, using GC-MS it is first necessary to extract the volatiles from the honey. One of the most efficient methods of volatile extraction and pre-concentration is accomplished with the utilisation of solid-phase micro extraction (SPME).

1.3.5. Principles of solid-phase micro extraction (SPME) and its application to honey characterization

Traditional analytical methods for analyte extraction require the use of organic solvents. These methods include liquid-liquid extraction (LLE) (Pirard et al., 2007), simultaneous distillation-extraction (Maignial, Pibarot, Bonetti, Chaintreau, & Marion, 1992), supercritical fluid extraction (SFE) (Rissato, Galhiane, Knoll, & Apon, 2004), solid- phase extraction (SPE) (Rissato et al., 2004) and ultrasound extraction (Fontana, Camargo, & Altamirano, 2010). Some of these methods have been used to extract volatiles of honey and have been shown to have several shortcoming. The experimental procedure for these techniques require the use of large volumes of organic solvents, which may be hazardous to the health of the analyst and the environment. Since the methods require the use of large quantities of organic solvents for successful extraction. This has the potential to make the method expensive and scientifically dilute the concentrations of the extracted analytes. The analytes obtain may be in such low concentration that this creates the need for pre-concentration. This adds an additional step and introduces the potential of contamination. The extraction methods alluded to also tend to be laborious and time consuming. Since honey is a complex matrix. This further complicates extraction of the volatiles due to the presence of sugars that can form artifacts due to thermal degradation.

SPME is a simple and rapid extraction technique. Generally speaking the experimental method consists of just two steps, (i) extraction onto the fibre and (ii) desorption in the GCMS inlet. SPME can be coupled with GCMS and can be easily automated. This method is also cheaper since the SPME fibre can be reused. The technique requires very little to no solvent for extraction of volatile compounds and thus the method can be regarded as green. SPME combines isolation of volatiles and pre-concentration in one step through adsorption onto the fibre (Chen, Jin, Fan, & Wang, 2017).

This method also has the advantage of producing reproducible results once all the steps in the extraction procedure are optimized. Figure 1 shows a schematic of the SPME procedure.

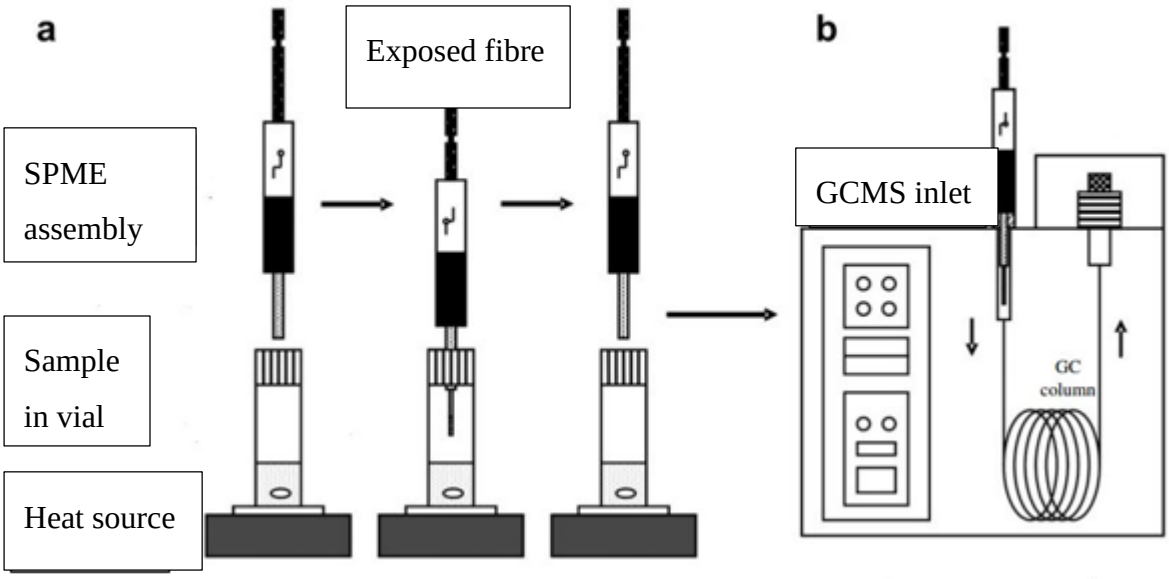


Figure 1. (a) Headspace (HS)-SPME extraction onto fibre and (b) thermal desorption into GCMS inlet (Cuevas-Glory, Pino, Santiago, & Sauri-Duch, 2007)

SPME functions by absorption of volatile organic compounds from their matrix (samples can be liquid, solids, or gases) in this case honey onto a silica fibre with polymeric coating. The analyte extracted is then transferred into the GCMS injector from the fibre through thermal desorption for analysis. SPME extraction can be performed in two modes as described:

(i) Direct insertion (DI) - whereby the fibre is submerged in the sample matrix or

(ii) Headspace (HS) this is whereby the fibre is only exposed to the vapour phase of the sample (the sample must be volatile).

HS is most effectively used in a complex matrix like honey. This is because the matrix does not come into contact with the fibre and thus it is a natural way to protect it. Since the analytes of interest are volatile, this also makes headspace extraction more popular. The driving force of SPME extraction is the polarity of the fibre and that of the volatiles. Studies have been performed

to investigate the best SPME fibre for the volatile extraction of honey. Table 5 shows a list of commercially available SPME fibres, their polarities and the analyses they most effectively extract.

Table 5. List of commercially available SPME fibres for GC

Fibre coating	Film thickness (µm)	Polarity	Coating method	Compounds to be analysed
Polydimethylsiloxane (PDMS)	100	Non-polar	Non-bonded	Volatiles
PDMS	30	Non-polar	Non-bonded	Non-polar semi volatiles
PDMS	7	Non-polar	Bonded	Medium- to non-polar semi volatiles
PDMS–divinylbenzene (DVB)	65	Bipolar	Cross-linked	Polar volatiles (amines and nitro aromatics compounds)
Polyacrylate (PA)	85	Polar	Cross-linked	Polar semi volatiles (phenols)
Carboxene(CAR)–PDMS	75	Bipolar	Cross-linked	Gases and volatiles
CAR–PDMS	85	Bipolar	Cross-linked	Gases and volatiles
Carbowax(CW)–DVB	65	Polar	Cross-linked	Polar analytes (alcohols) and volatile compound
CW–DVB	70	Polar	Cross-linked	Polar analytes (alcohols)
DVB–PDMS–CAR	50/30	Bipolar	Cross-linked	Odours and flavours

Source: (Vas & Vekey, 2004)

(Verzera, Campisi, Zappalà, & Bonaccorsi, 2001) reported that the PDMS-DVB fibre gave better results than the PA fibre when evaluating the volatile extracts of honey. This could have been due to the fact that PDMS-DVB is a bipolar fibre while the PA is non polar. In this instance, the PDMS-DVB was able to extract both polar and non-polar volatiles from the honey. The honeys under

study were eucalyptus, orange, wild flowers and chestnut. A classification of the honeys was accomplished on the basis of their floral origins.

In Spain (Pérez, Sánchez-Brunete, Calvo, & Tadeo, 2002) analysis of volatile compounds of honey using HS-SPME for extraction and GCMS for determination has been reported. Orange, eucalyptus, rosemary, lavender and thyme honeys were investigated in this study. Two SPME fibres CAR-PDMS and PDMS-DVB were compared. CAR-PDMS was found to extract more volatiles and at a higher concentration than PDMS-DVB. This was attributed to the greater fibre thickness of CAR-PDMS as it could accommodate a higher variety of volatile compounds and to a higher degree in the analysed honeys. The volatiles in the honeys were different from each other and thus the method could potentially be used to characterize and differentiate honeys of different botanical origins. This method also had potential to be improved and adapted to study honeys from different geographical regions in conjunction with other chemometric tools.

In Italy researchers reported the volatile profile of honeys (Piasenzotto, Gracco, & Conte, 2003) of varying botanical origins with the application of HS-SPME-GC-MS. The honeys investigated were all from different hive sites within Italy. The honeys included orange, chestnut, eucalyptus, lime tree, thyme and dandelion. This study also evaluated the performance of two SPME fibres namely CAR-PDMS (75 µm thick) and PA (85 µm thick). The CAR-PDMS provided poor results and peak distortion in the honey profile. The most optimal results were found with the PA fibre. This was attributed to the volatiles found in the honey samples which were very polar and their extraction was favoured by a polar fibre. A significant finding in this study was that honeys with similar botanical origins produced similar volatile profiles. This study suggested that by identifying volatiles only present in a specific floral source, it was possible to use it as a marker to authentic a particular honey. All the honeys analysed had the same geographical origin and thus

this technique could be used to evaluate honeys from different geographical origins even without the use of analytical statistical tools.

In Spain, a group of researchers reported the volatile profiles of commercially available honeys obtained with the use of HS-SPME-GCMS (Soria, Martínez-Castro, & Sanz, 2003). The floral origins of the honeys were rosemary, heather, honeydew, orange blossom, lavender including a polyfloral honey. The extraction efficiencies of four SPME fibres were studied which included PDMS (100 µm thick), PA (85 µm thick), CW-DVB (65 µm thick) and CAR-PDMS (75 µm thick). The PDMS fibre showed the lowest extraction efficiency of the volatiles. The CAR-PDSM fibre had the most satisfactory recovery for most compounds of high and medium volatility. This was attributed to the fibre's bipolar properties. However, for polar compounds better extraction was performed by both PA and CW/DVB fibres which are both polar. Overall the PA fibre was used in the final method since it showed the highest precision. This is the primary requirement for classification of honey based on floral origins. However, if the volatility content of the honey under investigation is low CAR-PDSM fibre is recommended (Cuevas-Glory et al., 2007). From literature, this is because of its higher overall sensitivity and can characterize honey, even if it's volatile concentration in low. This method has been used to study a wide variety of fibres and indicates that the volatile extraction of honey depends on the polarity of the fibre as well as the polarity and concentration of volatiles found in the honey. This method also has potential to characterize honey based on its floral origins. The geographical origin of the honey in this study was not stated. It is also possible that the volatiles in the commercial honey may have been lost during processing. The more useful information could have been gathered if the honey was collected directly from beekeepers.

The volatile profiles of Mexican honeys has also been reported (Cuevas-Glory, Ortiz-Vázquez, Pino, & Sauri-Duch, 2012) using HS-SPME-GCMS and a PDMS-DVB (65 µm thick) fibre to analyse them. The honey samples were collected from different geographical regions around the Yucatan Peninsula in Mexico. The honeys investigated were Tahonal, Dzidzilche´ or Haabin. Approximately 70 volatile compounds were identified in each honey type in conjunction with Principle Component Analysis (PCA) which was applied to distinguish between the different honey types. HS-SPME-GCMS with the aid of chemometric statistical tools was successfully used to characterise and discriminate the honeys based on their floral origins.

Good results have been obtained using DVB-CAR-PDMS (50/30 µm thick) to extract volatiles of honey. In Greece, (Alissandrakis, Kibaris, Tarantilis, Harizanis, & Polissiou, 2005) characterized honeys from different botanical origins using their volatile profiles obtained by HS-SPME-GCMS. The volatile profiles of Greek honeys for different floral origins were successfully determined. The honeys were cotton, orange, thyme, pine, fir, eucalyptus, chestnut, strawberry, sage and prostrate knotweed. In this study, 15 compounds were identified as potential bio markers for cotton honey. This was done by comparing volatiles of the honeys under investigation and identifying the compounds that were only present in cotton honey. The compounds that were detected were cinnamaldehyde, cinnamyl alcohol, cinnamic acid, neryl and geranyl nitrile, benzene propanol, homovanillyl alcohol, (*E*) - and (*Z*)-*p*-methoxy-cinnamic acid, 2-methyl-*p*-phthalaldehyde, coniferaldehyde, *p*-coumaric acid, ferulic acid, scopoletin and scoparone. This method also allowed the quantification of identified compounds using *β*-ionone as the internal standard. The techniques described enabled classification of honeys based on their floral origins, as well as quantification of the identified volatile compounds with the use of an internal standard. This method has the potential to also be used to identify potential markers for honeys of a specific floral origin. However to confirm these markers more honeys of varying floral and geographical region

needed to be studied. Another potential advantage is that this method did not use multi variant statistical tools to distinguish the honeys from each other.

In another study, 50/30 µm DVB-CAR-PDMS SPME fibres were used to extract volatiles of honey in China (H. Chen et al., 2017). Non-targeted volatile profiles of Chinese honeys were obtained in order to characterize them based on their floral origins. HS-SPME-GC and chemometric statistical tools were used to analyse the data. The honey samples were collected from different regions around China. The samples included acacia, linden, vitex and Brassica honey. Methyl decanoate was used as an internal standard and this enabled quantification of the volatiles. Statistical tools, i.e. PCA was used to do data filtration technique to reduce the number of variables from 2734 to 70. Based on the filtered volatiles Partial least squares discriminant analysis in conjunction with other predictive models were used to classify the honeys based on their botanical origins. This method was shown to accurately authenticate the floral origins of the sample honeys with 100% certainty. In the case of the characterisation of linden honey, eight volatile compounds were identified as potential markers. This method demonstrated that the classification of honey can be enhanced with the aid of chemometric statistical tools. Using this technique honeys can be characterised and potential markers can be detected. However honeys from different geographical origins were not classified. If this was done, the method could have been demonstrated be more robust. It is recommended here that, more honeys of varying floral origins should be studied using this method to confirm potential biomarkers. Once these markers are identified, authentication of the floral origin of honey could be simplified and this could be used in the routine quality control of honey.

A similar study that used 50/30 µm DVB-CAR-PDMS SPME fibres for the purpose of honey volatile isolation has been reported in Greece (Karabagias, Nikolaou, & Karabagias, 2019). Non-

targeted volatile profiles of honeys with varying botanical origins were obtained using HS-SPME-GCMS. The samples analysed included orange, fir, honeydew, pine, thyme, asfaka, strawberry tree, chestnut, and cotton honeys. The volatiles were identified using benzophenone as an internal standard in an effort to quantify the isolated volatiles. Pharmaceutical honey volatile metabolites (PHVMs) are volatile that have been found to be biologically active. The PHVMs identified in this study included: terpenes, norisoprenoids and benzene derivatives. Among these compounds terpenoids have shown biological activities against cancer, malaria, inflammation, and a plethora of infectious diseases (Wang, Tang, & Bidigare, 2005). The identification and quantification would be of great interest to the pharmaceutical community as well as the health conscious consumer. The identification of PHVM in honey could greatly increase the value of the honey. This method can be used as a key stepping stone in creating a database of honeys which show the biologically active volatiles present in them. This method didn't include classification of a search for biomarkers in the honeys which could dramatically improve the quality of the results.

In another study by (Wang et al., 2019) that does not use SPME but some of the principles only were applied. Headspace gas chromatography-ion-mobility (HS-GC-IMS) was employed to obtain non-targeted 3-D imaging of honey's volatiles profiles. The honey samples were collected from China, which included winter, sapium as well as deliberately adulterated honey. Statistical tools, i.e. PCA and PLS-DA, were used to classify and determinate the floral origins of the honeys. This method was also used to identify potential biomarkers. By using the triple locked strategy, the adulterated honeys were successfully discriminated from the other two honeys. A similar technique could be implemented in HS-SPME-GCMS in order to detect adulteration, and determine if a honey poly or mono floral.

Thus HS-SPME-GCMS provides a great deal of valuable information about a honey. The volatile organic compounds of honey can be applied in efforts to classify honeys on the basis of floral source. Classification of honey according to geographical and floral origin can be employed to deter fraud. For instance the floral sources of a polyfloral honey could be determined be analysing the volatiles followed by classification using appropriate statistical tools. By identifying volatiles with known biological activity, extra value could be placed in the honey. The non-targeted volatile analysis of honey provides the classification of botanical origins and authentication of floral origins of honeys.

1.4. Problem statement

Honey production in Africa has potential pitfalls among which is honey authenticity which has two major aspects. Firstly fraud which comes in the form of adulteration of honey with corn syrups, high fructose corn syrups, invert syrups or high fructose inulin syrups. Secondly, fraud in the form of mislabelling of honey produces to imitate those from a different floral or geographical origin. This is possible due to the lack of a systematic classification database for honeys based on geographical and botanical origin as has been done in other parts of the world. These activities have the potential to ruin the SADC regions access to advanced markets. This may result in a loss of consumer confidence in their honey products. To demonstrate how important this problem is, recently, many instances of so-called 'honey laundering' on a large-scale were reported (Leeder, 2011). Honey laundering involves the ultra-filtration of honey to remove pollen or soil that could be used to trace its origin. It is of utmost importance to protect honey producer from fraud. To ensure this, honey products from the Botswana are to be classified on the basis of geographical/botanical/floral origin and their authenticity registered using analytical techniques and the available expertise.

1.4.1. Research problem

The classification of honey products in Botswana will clearly distinguish the botanical/geographical origins of the honeys and will reveal the floral characteristics from which the bees collect the nectar. Adulteration detection methods also need to be improved to combat the fraudulent honey. This will address both, honey products with labels indicating wrong sources and or countries of production, and also counterfeits which in most cases are authentic honey products adulterated with mixtures of syrups. Morden analytical instrumentation such as GC-MS are capable of doing this classification in conjunction with characterization based on the physicochemical parameters as prescribed by international standards and the International Honey Commission (IHC). This will protect honey producers from losing income in this growing industry.

1.4.2. Objectives

The main aim of this project is to classify honeys from selected regions around Botswana based on their geographical and botanical/floral origins, using GC-MS, physicochemical characterization and multivariate statistical tools.

1.4.3. Specific Objective

1. To collect honey samples from selected areas surrounding Gaborone specially Tlokweng, Ramotswa and Pelotshetla directly from beekeeper as well as commercially available honeys from Zambia and Botswana.
2. To use data collected during honey sample collection from Bee Keepers to classify botanical species that give polyfloral honey in the Botswana.
3. To analyse the samples using GC-MS and classical analytical methods for physicochemical properties as prescribed by international standards.

4. To perform multivariate statistical data analysis on the data.
5. To classify the honeys and develop a mini database.
6. To publicize the results.

1.4.4. Hypothesis

- Honey from Botswana has not been classified based on botanical and/or geographic origins.
- Due to this there is a potential for fraudulent activities when Botswana starts to export its honey.
- Adulteration and mislabelling of the origin of honey is rampant resulting in loss of income by Apiarists and honey producers in the SADC region.

2. EXPERIMENTAL

2.1. Material and Methods

Acetone of analytical grade was obtained from Chem city (Botswana). The Deionised water (18mΩ) used throughout this study was obtained from Millipore water system. Reagent grade Bezophenone was obtained from Acros Organics(USA), glucose, D (+) and maltose were obtained from Saarchem Pty Ltd (Germany) , fructose and sucrose, D (+)were obtained from Fluka Chemika (Germany). All reagents used were of reagent grade.

2.1.1. Honey Samples

Commercial honey samples were randomly purchased from food supermarkets in Lusaka, Zambia and Gaborone, Botswana. In addition real honey samples were collected from selected regions surrounding the Gaborone area, namely Tlokweng, Ramotswa and Pelotshetla directly from beekeepers. These honey samples were code named TLOK, RAMO and PELO respectively. The commercial samples on the other hand were code named as ZAM, ZAM1 and BOT. These samples were stored at room temperature in silica bottles capped with tops underlined with Teflon.

2.1.2. Determination of Physiochemical Properties

The physiochemical properties of honey, which were analysed included moisture content, electrical conductivity, pH and free acidity and ash content. These properties were determined based on the methods outlined by the international honey commission (Bogdanov et al., 2002). Five replicates analyses were performed for each sample this was done to enable the implementation of statistical tools.

2.1.3. Measurement of moisture by refractometric method

The thermostated refractometer used was obtained from Atago (Japan) model RX-5000. The procedure was adopted based on those outlined by the IHC (Bogdanov et al., 2002). Firstly, to dissolve sugar crystals in the honey sample about 5g was transferred into a stoppered flask, then placed in a water bath set to 50°C (±0. 2). After the crystals dissolved the solution was allowed to cool to room temperature. Enough honey sample was then placed on the surface of the prism of the refractometer to completely cover it. The chamber of the refractometer where the prism is located was shut and the thermostat was set to 20°C. The honey sample was then allowed to acquire this temperature before the refractive index and temperature readings were taken. This procedure was repeated to obtain 5 measurements for each of honey samples and the prism was cleaned, the dried after recording every reading. The refractive index readings were adjusted for 20°C then the moisture content was determined using Equation 1 (Sesta & Lusco, 2008):

$$\boldsymbol{W = \frac{-0.2681 - \log(R.I - 1)}{0.002243}} \qquad (1)$$

Where R.I is the refractive index corrected for 20 °C.

W is the water content in grams per 100g of honey, thus it is referred to as the percentage moisture content of the honey.

2.1.4. Measurement of electrical conductivity

The conductivity meter used was obtained from Crison instruments (S.A) conductimeter model basic 30, with a voltage of DC 12V and current of 200mA. The conductivity meter was calibrated with O.147 mS/cm and 12.88 mS/cm at 25 °C standards. The experimental procedure adopted was based on those drafted by the IHC (Bogdanov et al., 2002). Deionised water was used to dissolve 50g of the honey sample this solution was then quantitatively transferred into a 250 ml volumetric flask and made up to volume the mark with deionised water. 40 ml of the sample solution was

decanted into a beaker. The conductivity cell was rinsed thoroughly with the remaining part of the sample solution. The electrical conductivity was obtained by immersing the conductivity cell in the 40 mL honey sample solution. Owing to the want of a thermostated conductivity cell these determinations were conducted out at room, therefore the correction formula was applied to the readings to correct for 20°C. This is for every degree over 20°C 3.2% of the electrical conductivity value is taken off from itself and for temperatures below 20°C 3.2% of the conductivity value is added to itself. This was repeated to obtain 5 readings for each of the honey samples using different component parts of the honey sample the results were expressed in milliSiemens per centimeter ($mScm^{-1}$).

2.1.5. Measurement of pH and free acidity by titration to pH 8.3

The pH meter used was obtained from HANNA Instruments Inc. (Woonsocket RI USA MADE in ROMANIA) model HI 2209. A standard solution of 0.1M sodium hydroxide was prepared. The pH meter was calibrated using buffer solutions for pH 4.0 and 7.0. The procedure for determining pH and free acidity by titration to pH 8.3 of honey was adopted from and outlined by the IHC (Bogdanov et al., 2002). 75 mL of deionized water was used to dissolve 10 g the honey sample in a 250 ml beaker. A magnetic stirrer was used to stir the solution while the pH electrode was immersed in the solution and the pH value was recorded. The solution was titrated rapidly using 25 mL burette against 0.1M NaOH to pH 8.30. This procedure was repeated to obtain 5 readings for each of the honey samples.

2.1.6. Measurement of ash content

An electric furnace adjustable to 1300°C (± 25°C) was used for the purposes of ashing. The procedure for determining the ash content of honey was adopted from (Bogdanov et al., 2002) with the only modification being the preliminary evaporation and the furnace temperature used. 5g

of accurately weighted honey sample (m_0) was placed into a ceramic crucible. Preliminary evaporation was performed using a hotplate to charring the honey in order to remove smoke. After the preliminary charring, the dish was placed in the preheated furnace at 550°C for 3 hours. This temperature is higher than the recommended temperature of 400°C. The honey samples did not ash at the recommended temperature, thus a higher temperature was adopted as well as a longer ashing time this may be due to the furnace used being obsolete. The ceramic crucible as allowed to cool in a desiccator. The weight of the cool ash and ceramic crucible was accurately measured (m_1). The *WA* represents the ash content of honey sample and is calculated using Equation 2:

$$\boldsymbol{WA} = \frac{(\boldsymbol{m1}-\boldsymbol{m2})}{\boldsymbol{mo}} * 100 \qquad (2)$$

Where:

m_0 = weight of a honey sample taken,

m_1 = weight of dish + ash,

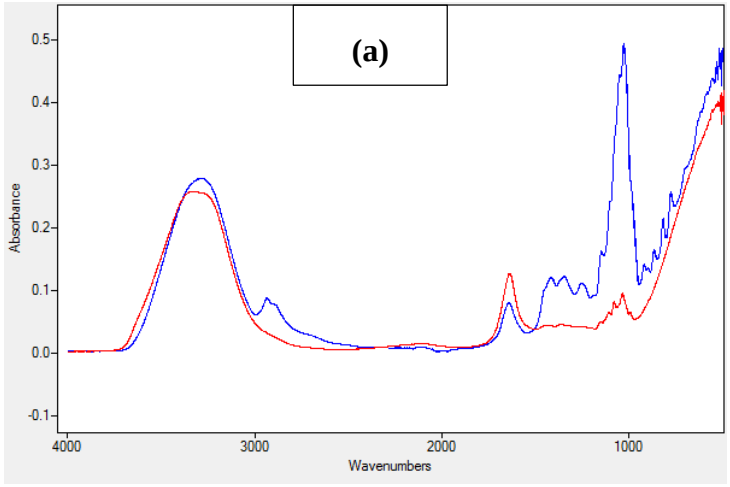
m_2 = weight of the dish

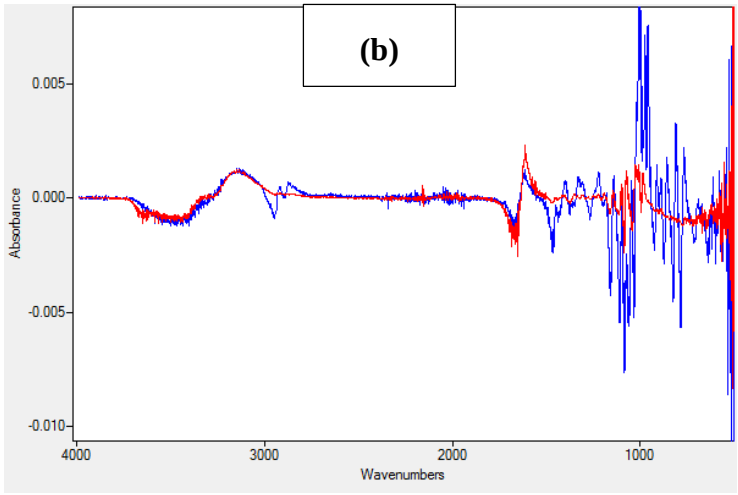
This procedure was repeated to obtain 5 readings for each of the honey samples

2.1.7. Determination of sugars (fructose, glucose D (+), sucrose D (+) and maltose) in honey by FTIR- UATR

The infrared spectral data was obtained with the use of an FTIR acquired from PerkinElmer required with a Universal Attenuated Total Reflectance (UATR) Accessory for Spectrum Two. The spectra was analysed in the MIR namely 400-4000 cm^{-1} (J. Wang et al., 2010). Before each spectrum determination the background spectrum of the clean crystal surface was found and suppressed in order increase the accuracy of the sugar and honey spectral data. Five standards for

each sugar was prepared with known concentrations of sucrose, D (+) (0.20, 2.04, 4.1, 6.03 and 8.01%), glucose, D (+) (4, 6.25, 8.5, 13.02 and 15.26 %), fructose (4.01, 6.25, 8.52, 13.03 and 15.26%), and maltose (0.20, 2.01, 4.05, 6.05 and 8.01%) they were prepared using deionized water and the concentration was expressed in weight per volume. These sugar spectral data were obtained as well as spectrum for all the honey samples.





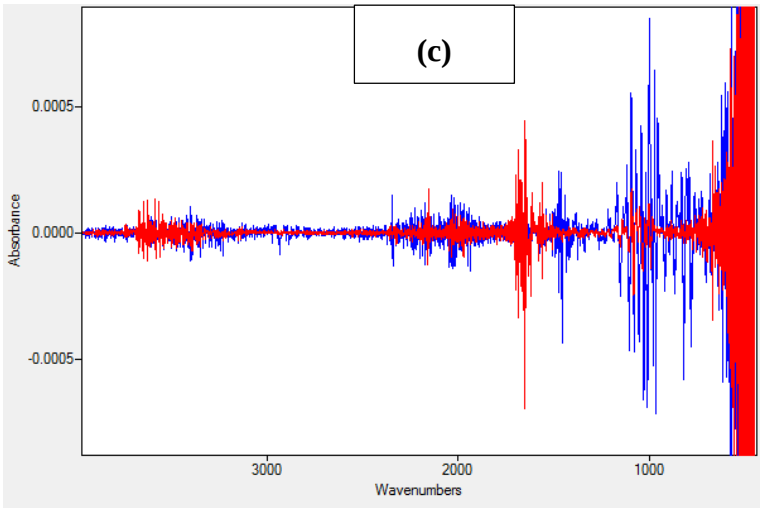


Figure 2. (A) Normal FT-IR spectra of 15.26 % glucose solution (red) and RAMO honey (blue), (B) first derivative (C) second derivative spectra respectively.

Essential FTIR Version 3.50.185 was used to convert the FT-IR spectra of the sugars and the honey samples from the percent transmittance versus wavenumber in cm^{-1} values to absorbance versus wavenumber in cm^{-1} units. The spectra was then subjected to second order derivatives this is done to increase the sensitivity of the peaks these operations are demonstrated in Figure 2 (a-c). Using this data the key peaks for each sugars were identified along with their corresponding absorbance. The sugar standards were used to construct a calibration curve of absorbance versus percent w/v sugars for each of the individual sugars. The sugars were identified in the honey samples by using the key peaks of the corresponding sugars. The concentration of each sugar in the honey sample was determined by using the calibration curve that was constructed and comparing the absorbance of honey with that of the sugar at the key peaks. The spectral data of the honey samples were used to classify the honey samples.

2.2. The non-targeted volatile profile of Commercial and Botswana honey by HS-SPME-GCMS

2.2.1. Sample Preparation

Sample preparation was adopted from (Piasenzotto et al., 2003) and (Špánik, Pažitná, Šiška, & Szolcsányi, 2014) with minor modifications. The modifications were that instead of using 3 g of honey sample, 6 g were used. Experiments were scaled up by doubling all other parameters i.e. into the 20 mL amber vial, 1.0 g of anhydrous sodium sulphate and 1.0 mL of internal standard solution were added. The internal standard was benzophenone (grade 99.9%); the solution (0.6 $mgml^{-1}$) was prepared by dissolving 30 mg of benzophenone in 16 ml of acetone in a 50 mL volumetric flask and diluting to the mark with distilled water. All other conditions were as described by (Piasenzotto et al., 2003).

2.3.2. Solid Phase Micro-Extraction

Conditioning of the vial was done at 30 ˚C for 30 minutes in a water bath before performing SPME headspace sampling. The SPME needle was then inserted into the vial septum, and exposed to the headspace for 30 min while the solution was stirred with a polytetrafluoroethylene (PTFE) coated magnetic stir bar at 700 rpm. Headspace sampling was done at 80 ^{o}C. Solid phase micro extraction (SPME) was done using the Supelco SPME holder for manual sampling (Supelco Co, Bellefonte, PA, USA) part number 57330-U fitted with an SPME fused silica fibre assembly coated with 65 µm polydimethylsiloxane-divinylbenzene (PDMS-DVB) 23 Ga, manual holder (Part Number 57346-U) (Bellefonte, PA, USA). The PDMS/DVB fibre was adopted because it has been used successfully for sampling honey tested by (Verzera et al., 2001) who found that it was best suited for analysing honeys from orange, eucalyptus, wild flowers, and chestnut. The PDMS/DVB is a bipolar fibre so it allows the extraction and investigation of polar and non-polar volatiles.

2.4. Apparatus

2.4.1. Mass Spectrometry

An Agilent 5975 C inert XL EI/CI MSD equipped with a Triple axis detector (Agilent Technologies, Palo Alto, CA, USA) was used. The acquisition software was Agilent MSD Productivity Chemstation. The system is equipped with an auto sampler and can be operated in the EI/CI modes with CI further being operated in positive chemical ionization (PCI) and negative chemical ionization (NCI) modes. The mass spectrometer was operated in electron ionization (EI) mode with the electron multiplier voltage setting at 1965 V, a solvent delay time of 2.50 minutes, a scan mass range of *m/z* ratio 50 – 500. The mass spectrometer (MS) source and MS quadrupole mass analyzer were held at 200 and 150^{o}C respectively while the transfer line was maintained at 250^{o}C. Trace ion detection was off with the filament emission current auto-set at 34.610 µA. The voltages

in the ion source were auto-set using perfluorotributylamine (PFTBA) and the auto tune function in the acquisition software.

2.4.2. Gas Chromatography

Agilent GC 7890A coupled to the MSD was used. Sample introduction was accomplished by manual SPME sampling followed by direct insertion via the injector septum on the injector port. The injector was split/splitless heated to 260^{o}C and in this instance, the splitless mode was used. Helium with 99.995 % purity was used as a carrier gas at a flow rate of 1.5 mL/ min. A 30 m HP5MS-UI column (Agilent J & W Column, Agilent Technologies, USA) with an internal diameter of 0.25 mm and film thickness of 0.25 µm was used throughout. The oven program was adopted from (Piasenzotto et al., 2003) as follows: the initial temperature was 50 ^{o}C and was held at this level for 4 minutes, then ramped to 230^{o}C at 10^{o}C/min and held there for 10 minutes. It was ramped again to 250^{o}C at 10 ^{o}C/min and held there for 10 minutes. The total run time was 44 minutes.

2.4.3. Data Analysis

Metab R was used, an R Package for high-throughput analysis of metabolomics data generated by GC-MS. *Metab* R is an R (R Development Core Team, 2008) package developed by the R Development Core Team in 2008. This software uses the R programming language for data correction, filtering and reshaping of datasets initially produced by the Automated Mass Spectral Deconvolution and Identification System (AMDIS) i.e. a freeware for deconvoluting chromatograms generated by GC-MS in order to produce refined datasets for further analysis. Datasets produced by AMDIS require these manipulations in order to create data matrices that can be fed directly into other statistical analyses software such as MINITAB, SPSS etc. *Metab* R can be downloaded from Biconductor, which is a repository of open source software for bioinformatics

at: http://www.bioconductor.org/packages/release/bioc/html/Metab.html. Bioconductor is an open source, open development software project that serves as a repository for tools earmarked for the analysis and comprehension of high-throughput genomic data. The core of Biconductor software development is the R programming language. *Metab* R can be used to recalculate peak intensities, display peak areas, remove false-positives, normalize by internal standard, normalize by biomass as well as carry out the htest i.e. analysis of variance (ANOVA) and the t-test on data generated by AMDIS for volatiles both targeted and non-targeted. While AMDIS is popular for untargeted metabolomics of biological samples, it has three major shortfalls i.e. (i) The assignment of different *m/z* ratios for quantification of the same metabolite, (ii) attachment of the same retention times to different metabolites and results and (iii) production of datasets that do not make them amenable to further analysis.

2.3.4. AMDIS/METAB R Data Processing

In this study, the raw data files acquired using the Agilent 5975C Series GC-MS Productivity Chemstation acquisition software were converted to the DCF format also known as AIA format as described by (Aggio, 2018). They were further processed using the "Batch Job" function embedded in the AMDIS software to create text file outputs in TXT format. Initially a library was created based on one honey type using the AMDIS Build One Library function after searching the entire NIST 05 library. Later, one raw data file of each honey type was also searched and a library built. Finally, all the libraries were combined using the "Library Transfer" function on AMDIS. The process was repeated for the blank honey raw data files acquired using Productivity Chemstation and a separate library was built in the same way. These steps resulted in the construction of two libraries in i.e. one for all metabolites found in all honey sample types including benzophenone as the internal standard and the other for the blank and the internal standard in the

mass spectral library (MSL) formal used by AMDIS. Once this was done, processing of the data was ready using *Metab* R. The syntax of the commands on the R platform are all given by (Aggio, 2018). Data was processed using the "MetReport ()" command that requires the use of the library MSL file, the CDF files and the TXT file generated by the AMDIS batch job function. Once the csv file output was generated giving peak intensities for all the CDF files processed, normalization using the internal standard intensity was done with the "normalizeByInternalStandard" command on the R platform. This enabled all peak intensities of the metabolites to be divided by the peak intensity for the internal standard. In this way, fluctuations that could have been caused by temperature/pressure fluctuations and injection volumes on the GC-MS system was corrected. This was done for the blank CDF file too, and then the metabolites in the blank sample run were subtracted from the honey sample runs. Since five extractions and runs were done per sample, a metabolite that was identified in three runs was taken as positive identification and confirmation for further data analysis. The *Metab* R software package c speeded up the data mining process in this case because there was no limit as to how many CDF files could be processed at one instance. The flow chart in Figure 3 shows the steps taken to further process the GC-MS Chemstation/AMDIS raw data files using *Metab* R software package.

AMDIS

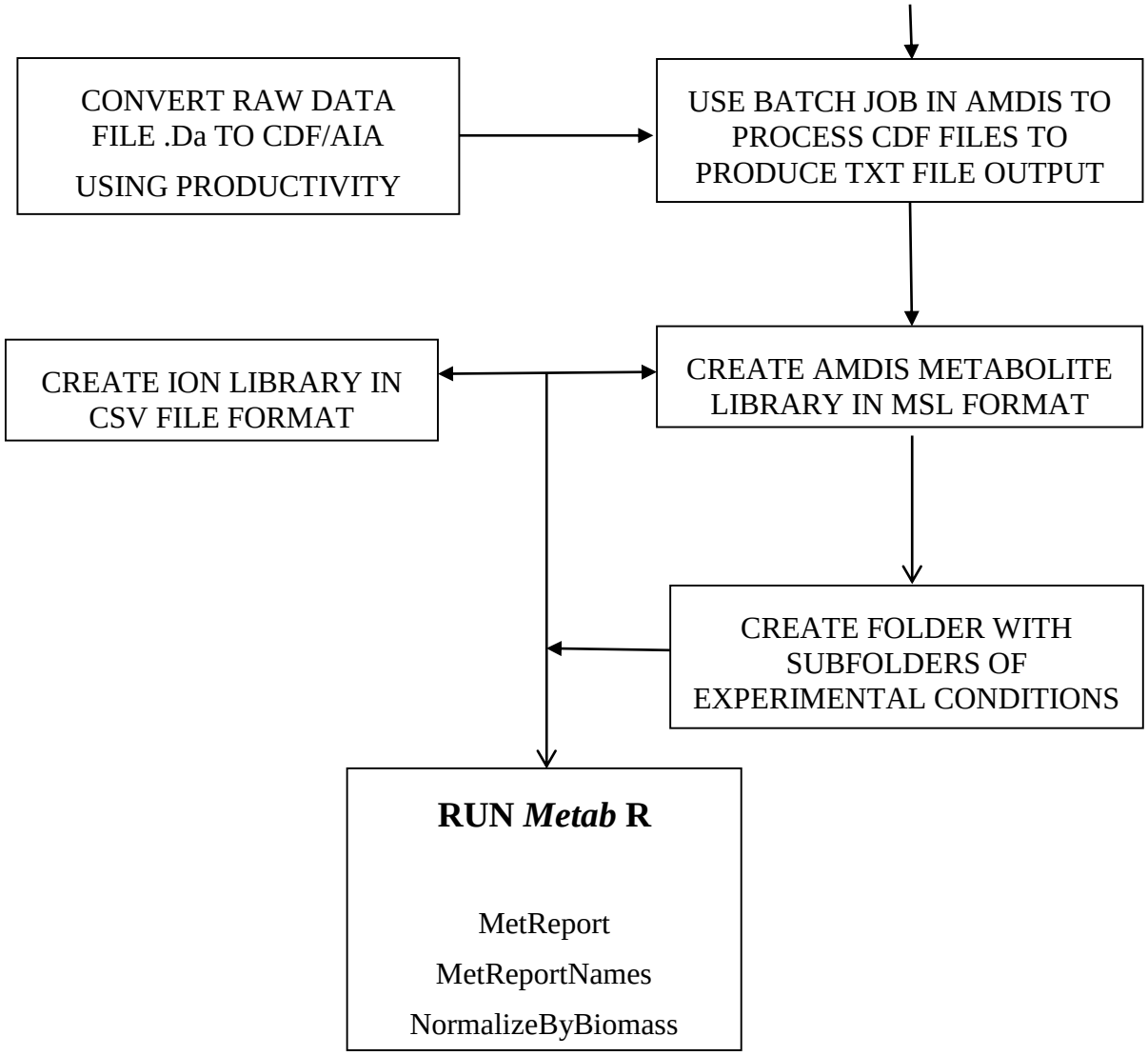


Figure 3. Flow chart for data processing of the GC-M S Chemstation/AMDIS raw data files using *Metab* R software.

3. Results and discussion

Results are subdivided into three sub-sections. These are the results from the physicochemical evaluation of the honeys, the adulteration detection and classification of the honeys according to

geographical origin based on physicochemical characteristics and volatile metabolites identified by SPME-GCMS.

3.1. Physicochemical Parameters of the Honeys

3.1.1. Moisture by refractometric method

Table 6. Honey samples refractive index Adjusted for 20℃

	Commercial honeys			Real Honey		
	ZAM	**ZAM1**	**BOT**	**PELO**	**TLOK**	**RAMO**
	1.49262	1.49008	1.48697	1.48558	1.49849	1.48964
	1.49224	1.49136	1.48753	1.48503	1.49909	1.4886
	1.49172	1.49178	1.4879	1.4852	1.49793	1.48847
	1.49217	1.4918	1.48716	1.48471	1.49774	1.48876
	1.49304	1.49173	1.48711	1.48513	1.4978	1.48887
Mean	1.49236	1.49135	1.48733	1.48513	1.49821	1.48887

The values of refractive index are only needed to calculate the values of moisture content. Refractive index give no additional value about the honey. Equation 1 requires the use of refractive index values shown in Table 6 and the values for the moisture content adduced are shown in Table 7. Equation 3 was used to describe the precisions expressed as % relative standard deviation (RSD) and values greater the 10% are indicative of poor reproducibility as prescribed by the IHC (Bogdanov et al., 2002).

$$\boldsymbol{RSDr}\ \% = \mathbf{100} * \left(\frac{\boldsymbol{r}}{\boldsymbol{x}*\mathbf{2.8}}\right) \qquad (3)$$

r is the range of the results for a honey sample.

x is the mean of the results for a honey sample.

Table 7. Percentage moisture content of honey samples (water content g/100 g of honey)

	Commercial honeys			Real Honey		
	ZAM	**ZAM1**	**BOT**	**PELO**	**TLOK**	**RAMO**

	17.56	18.56	19.79	20.25	15.27	18.74
	17.71	18.06	19.57	20.57	15.03	19.15
	17.91	17.89	19.42	20.50	15.49	19.20
	17.73	17.88	19.72	20.69	15.56	19.08
	17.4	17.91	19.74	20.53	15.54	19.04
Mean (Sd)	17.66±0.19	18.06±0.29	19.65±0.15	20.51±0.16	15.38 ±0.23	19.04±0.18
95% confidence UL	17.85	17.87	19.84	20.70	15.57	19.23
95% confidence LL	17.47	18.25	19.46	20.32	15.19	18.85
RSD% (n=5)	1.031	1.344	0.6724	0.7662	1.231	0.8628

All the honey samples had good precision shown by the RSD values which were less than 10 %. For the commercial honeys, the Zambian honeys had the lowest reproducibility. The IHC prescribes that for natural and unadulterated honey, the maximum value of moisture content should not exceed 21% (Bogdanov et al., 2002). PELO honey displays the highest moisture content, this could be influenced by its floral source, as well as, the environmental conditions of its origin. It is important to note at higher values for moisture content have a higher probability to ferment thus, a shorter shelf life. TLOK honey possesses the lowest moisture content, indicating long shelf life. However, it is important to take into consideration the free acidity of the honey in order to determine if a honey is likely to ferment and to elucidate its freshness. From Table 7, none of the honeys exceed the prescribed moisture content of 21% at the 95 % confidence level. This indicates that the likelihood that the honeys were diluted with water is extremely low or none.

Table 8. One-way ANOVA for percentage moisture content of honey samples (water content g/100 g of honey)

SUMMARY

Groups	Count	Sum	Average	Variance
ZAM	5	88.31	17.662	0.03687
ZAM1	5	90.3	18.06	0.08345
BOT	5	98.24	19.648	0.02297
PELO	5	102.54	20.508	0.02602
TLOK	5	76.89	15.378	0.05117
RAMO	5	95.21	19.042	0.03232

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	81.02163	5	16.20433	384.5963	4.39E-22	2.620654
Within Groups	1.0112	24	0.042133			
Total	82.03283	29				

Using one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means were not significantly different. From Table 8, the calculated F value was 384.6 and was greater than the critical F value i.e. of 2.621. Thus the null hypnosis was rejected and the alternative hypothesis was accepted that all the mean values for the honeys were significantly different. Tukey Pairwise Comparisons was used then used to determine which honeys, if any, were significantly similar to each other and the output is shown in Table 9.

Table 9. Grouping Information Using the Tukey Method and 95% Confidence

Honey sample	Grouping
PELO	A
BOT	B
RAMO	C
ZAM1	D
ZAM	D
TLOK	E

The means of honey samples that do not share a letter are significantly different. Samples grouped with the same letters are significantly similar to each other.

What is interesting to note is that the two Zambian commercial honey samples were significantly similar to each other in terms of moisture content, this may be due to honeys being from similar

climatic regions or similar vegetation. Another possibility is that the two honeys are from the same source and were processed in a similar manner.

3.1.2. Electrical conductivity

Table 10. Conductivity (mS/cm) values of honey for Adjusted 20℃

	Commercial honeys			Real Honey		
	ZAM	**ZAM1**	**BOT**	**PELO**	**TLOK**	**RAMO**
	0.648	0.725	0.823	0.874	0.511	1.111
	0.646	0.727	0.831	0.876	0.511	1.109
	0.644	0.732	0.8393	0.876	0.512	1.110
	0.641	0.725	0.8329	0.877	0.512	1.111
	0.645	0.722	0.8425	0.875	0.513	1.113
Mean (Sd)	0.645±0.003	0.726±0.004	0.834±0.008	0.876±0.001	0.512±0.001	1.111 ±0.001
95% confidence UL	0.6482	0.7296	0.8372	0.8790	0.5152	1.114
95% confidence LL	0.6414	0.7228	0.8303	0.87218	0.5084	1.107
RSD% (n=5)	0.3878	0.4923	1.268	0.1390	0.1390	0.1288

All the honey samples had good precisions reflected in the RSD values which were less than 10 %. The IHC states that, electrical conductivity is an important criterion that can be used to classify honey based on their floral and geographical origins. Using Table 1 and Table 10 as a guide it is possible to infer the possible floral sources honeys from those geographical origins indicated. The Zambian and TLOK honey from Botswana falls in the blossom honey type, while, BOT, PELO and RAMO honeys all natural honeys from Botswana could be grouped as Honeydew. It is important to note that all the honeys in this study are polyfloral and this information may only be

applicable to mono-floral honeys. This classification system is also only applicable to honeys from Europe, Asia, America and Australia. This illustrates the importance of creating a database for African honeys. According to (Bogdanov et al., 2002) electrical conductivity is influenced by free acidity and ash content. Thus, it can be predicted the RAMO natural honey from Botswana should have high ash content, while TLOK natural honey should have the lowest ash content when using the values given in Table 10.

Table 11. One-way ANOVA for conductivity (mS/cm) values of honey for adjusted 20℃

SUMMARY

Honey sample	Count	Sum	Average	Variance
ZAM	5	3.224	0.6448	6.7E-06
ZAM1	5	3.631	0.7262	1.37E-05
BOT	5	4.1687	0.83374	5.78E-05
PELO	5	4.378	0.8756	1.3E-06
TLOK	5	2.559	0.5118	7E-07
RAMO	5	5.554	1.1108	2.2E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.072365	5	0.214473	15616.4	2.57E-41	2.620654
Within Groups	0.00033	24	1.37E-05			
Total	1.072695	29				

Using one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means for electrical conductivities were not significantly different. From Table 11, the calculated F value was 15616 and was greater than the critical F value i.e. of 2.621. This meant that the mean values for electrical conductivity of the honeys were significantly different from each other. Tukey Pairwise Comparisons was then used to determine which honeys, if any, were significantly similar to each other in terms of their electrical conductivities and the output is shown in Table 12.

Table 12. Grouping Information Using the Tukey Method and 95% Confidence

Honey sample	Grouping
RAMO	A
PELO	B

BOT	C
ZAM1	D
ZAM	E
TLOK	F

The means of honey samples that do not share a letter are significantly different. Samples grouped with the same letters are significantly similar to each other.

All the honeys were significantly different from each other shown by the grouping in Table 12. Since, all the honey samples were polyfloral it is probable to deduce that they originate from different floral sources.

3.1.3. pH and free acidity by titration to 8.3

Table 13. pH values for honey

	Commercial honeys			**Real Honey**		
	ZAM	**ZAM1**	**BOT**	**PELO**	**TLOK**	**RAMO**
	4.77	4.14	4.07	4.09	4.01	4.32
	4.82	4.13	4.10	4.09	4.07	4.20
	4.83	4.12	4.10	4.08	4.05	4.12
	4.83	4.12	4.08	4.09	4.06	4.04
	4.83	4.12	4.10	4.09	4.05	3.99
Mean (Sd)	4.82±0.03	4.13±0.01	4.09±0.01	4.09±0.004	4.05±0.02	4.13±0.1
95% confidence UL	4.868	4.178	4.142	4.140	4.010	4.186
95% confidence LL	4.765	4.075	4.039	4.037	3.9965	4.083
RSD% (n=5)	0.4452	0.1735	0.2615	0.0874	0.5293	2.857

All the honey samples had good precisions because all the RSD values Table 13 were less than 10%. RAMO had highest RSD value this may be due to the composition of the honey, which was not consistent throughout as indicated by the pH values obtained. This could be indicative of the inhomogeneity of the honey, which could stem from it having multiple floral sources. The IHC describes all natural honeys as being acidic with pH values ranging between 3.5-5.5 due to the presence of organic acids (Bogdanov et al., 2002). The mean pH values for all the honeys fell within that range with ZAM honey being the least acidic.

Table 14. One-way ANOVA for pH values for honey

SUMMARY						
Honey sample	**Count**	**Sum**	**Average**	**Variance**		
ZAM	5	24.08	4.816	0.00068		
ZAM1	5	20.63	4.126	8E-05		
BOT	5	20.45	4.09	0.0002		
PELO	5	20.44	4.088	2E-05		
TLOK	5	20.24	4.048	0.00052		
RAMO	5	20.67	4.134	0.01718		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	2.17651	5	0.435302	139.8186	6.19E-17	2.620654
Within Groups	0.07472	24	0.003113			
Total	2.25123	29				

Using one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means were not significantly different. From Table 14, the calculated F value was 139.8 and was greater than the critical F value i.e. of 2.621. Thus the null hypnosis was rejected and the alternative hypothesis was accepted that all the pH mean pH values for the honeys were significantly different. Tukey Pairwise Comparisons was then used to determine the similarities of the honeys.

Table 15. Grouping Information Using the Tukey Method and 95% Confidence

Honey sample	**Grouping**
ZAM	A
ZAM1	B

BOT	B
PELO	B
TLOK	B
RAMO	B

The means of honey samples that do not share a letter are significantly different. Samples grouped with the same letters are significantly similar to each other.

ZAM differed from all the other honeys because of its high pH values as seen in Table 15. All the other honey samples were significantly similar to each other based on their pH values. These honeys may consist of similar organic acids.

Table 16. One-way ANOVA for pH values for honey excluding ZAM honey

SUMMARY						
Honey sample	**Count**	**Sum**	**Average**	**Variance**		
ZAM1	5	20.63	4.126	8E-05		
BOT	5	20.45	4.09	0.0002		
PELO	5	20.44	4.088	2E-05		
TLOK	5	20.24	4.048	0.00052		
RAMO	5	20.67	4.134	0.01718		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	0.023704	4	0.005926	1.646111	0.201932	2.866081
Within Groups	0.072	20	0.0036			
Total	0.095704	24				

Using one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means were not significantly different. From Table 16, the calculated F value was 1.646 and was less than the critical F value i.e. of 2.866. This meant that there was no significant difference between the sample honeys.

Table 17. Free acidity values for honey

Commercial honeys	**Real Honey**

	ZAM	ZAM1	BOT	PELO	TLOK	RAMO
	51.2	59.8	58.5	37.8	31.2	43.2
	42.9	54.9	54.6	36.6	31.4	42
	48.3	58.9	54	36.4	30	41.2
	45.4	65.9	52.7	37.8	30	40.4
	49.2	54.7	54.2	37.2	27.8	39.9
Mean (Sd)	47.4±3.3	58.8±4.6	54.8±2.2	37.2±0.6	30.1 ± 1.4	41.3±1.3
95% confidence UL	49.80	61.24	57.20	39.56	32.48	43.74
95% confidence LL	45.00	56.44	52.40	34.76	27.68	38.94
RSD% (n=5)	6.250	6.811	3.750	1.339	4.273	2.857

All the honey samples had good precisions because all the RSD values Table 17 were less than 10%. The PELO honey had the best reproducibility. The IHC prescribes that for fresh and unfermented honey, the maximum value of moisture content should not exceed 50 milliequivalents (Bogdanov et al., 2002). From Table 17, ZAM1 and BOT exceeded the prescribed free acidity of 50 at the 95 % confidence level. This indicated that both honeys were not fresh or may have undergone fermentation. The real honey samples did not exceed 50 milliequivalent indicating their freshness and the high resistance to fermentation.

Table 18. One-way ANOVA for free acidity values for honey

SUMMARY				
Groups	**Count**	**Sum**	**Average**	**Variance**
ZAM	5	237	47.4	10.685
ZAM1	5	294.2	58.84	20.858
BOT	5	274	54.8	4.785
PELO	5	185.8	37.16	0.428

TLOK	5	150.4	30.08	2.052
RAMO	5	206.7	41.34	1.718

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2953.946	5	590.7891	87.46816	1.32E-14	2.620654
Within Groups	162.104	24	6.754333			
Total	3116.05	29				

With the help of one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means were not significantly different. From Table 18, the calculated F value was 87.47 and was greater than the critical F value i.e. of 2.621. Thus the null hypnosis was rejected and the alternative hypothesis was accepted that all the mean free acidity values for the honeys were significantly different.

Table 19. Grouping Information Using the Tukey Method and 95% Confidence

Honey sample	Grouping
ZAM1	A
BOT	A
ZAM	B
PELO	C
RAMO	C
TLOK	D

The means of honey samples that do not share a letter are significantly different. Samples grouped with the same letters are significantly similar to each other.

The BOT and ZAM1 honeys were similar to each other as seen in Table 19. PELO and RAMO were also similar to each other. This was in terms of the freshness and the likelihood of the honeys to ferment. However, African honeys were not studied when the IHC prescribed the maximum value. The maximum values for free acidity were set using European honeys only. This shows that baseline measurements for fresh honeys from Africa must be recorded as well as for fermented

honey in order to make appropriate comparisons and create a database. The free acidity of a honey indicates the freshness of a honey and thus, cannot be used to classify honey.

3.1.4. Ash content

Table 20. Ash content for the honeys in this study

	Commercial honeys			Real Honey		
	ZAM	**ZAM1**	**BOT**	**PELO**	**TLOK**	**RAMO**
	0.5246	0.4910	0.3180	0.4436	0.3398	0.6499
	0.4544	0.4700	0.3194	0.4358	0.3332	0.6349
	0.4583	0.4590	0.4382	0.4382	0.3598	0.6436
	0.4063	0.4281	0.2734	0.4546	0.3531	0.6398
	0.4052	0.4350	0.2686	0.4528	0.3590	0.6921
Mean (Sd)	0.4498± 0.05	0.4566±0.0 3	0.3235±0.0 7	0.4450±0.00 8	0.3490±.0 1	0.6521± 0.02
95% confidence UL	0.4845	0.4913	0.3582	0.47970	0.38368	0.6868
95% confidence LL	0.4151	0.4219	0.2888	0.41030	0.31428	0.6174
RSD% (n=5)	9.48	4.920	18.73	1.509	2.711	3.133

All the honey samples had good precisions because all the RSD values Table 17 were less than 10% except for BOT honey. BOT honey had lowest reproducibility compared to the other honeys. This high RSD values could be indicative of the method having poor precision. May be owing to the furnace not being state of the art or moisture still being present after ashing. The IHC has no prescribed value for the ash content of honey. However, generally ash content ranges from 0.02-1 % (Marshall, 2010). All the honeys fell within this range. It has also been noted that, honeys with a high ash content have a higher mineral content. Thus RAMO honey could be recommended for people with mineral deficiencies.

Table 21. One-way ANOVA for ash content for the honeys in this study

SUMMARY

Honey sample	Count	Sum	Average	Variance
ZAM	5	2.2488	0.44976	0.002392
ZAM1	5	2.2831	0.45662	0.000662
BOT	5	1.6176	0.32352	0.004682
PELO	5	2.225	0.445	7.15E-05
TLOK	5	1.7449	0.34898	0.000142
RAMO	5	3.2603	0.65206	0.000531

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.335014	5	0.067003	47.4045	1.16E-11	2.620654
Within Groups	0.033922	24	0.001413			
Total	0.368937	29				

Using one way ANOVA, the null hypothesis was tested, i.e. that all the honey sample means were not significantly different. From Table 21, the calculated F value was 47.40 and was less than the critical F value i.e. of 2.621. Thus the null hypnosis was rejected and the alternative hypothesis was accepted that all the mean values for the honeys were significantly different.

Table 22. Grouping Information Using the Tukey Method and 95% Confidence

Honey sample	Grouping
RAMO	A
ZAM1	B
ZAM	B
PELO	B
BOT	C
TLOK	C

The means of honey samples that do not share a letter are significantly different. Samples grouped with the same letters are significantly similar to each other.

What is interesting to note is that the two Zambian commercial honeys as well as PELO honey were similar to each other in terms of ash content as seen in Table 22. In the case of the Zambian honeys, this may be on account that they were from similar geographical origins. What is surprising is that the honey from PELO was similar to the Zambian honeys. These honeys may be from similar vegetation. With the TLOK and BOT honey being similar to each other. This may be due to honeys being from similar climatic regions or similar vegetation. Another possibility is that the two honeys are from the same floral source as they share the same geographical origin.

3.2. Adulteration Detection by FT-IR

3.2.1 Detection of sugars (fructose, glucose, D (+), sucrose, D (+) and maltose) by FTIR

Using Table 4 i.e. a summary of fundamental peaks for sugars identified in the literature as a reference and comparing it to the FTIR spectrum of all the commercial and real honeys, the wavenumber ranges corresponded to those expected. Figure 4 is an example of a FT-IR spectrum of BOT honey. A comprehensive set of FT-IR spectra data for all the honeys is given in the Appendices. From the FTIR spectrum of honeys given in Figure 4 and in the Appendices, all the samples had similar peaks with broad bands around the 3200 cm^{-1} region from OH group stretching's due to water and sugars (carbohydrates), 2900 cm^{-1} C–H **s**tretching (carbohydrates) most likely from sugars, 1600 cm^{-1} O–H bending from the deformation of water present and peaks from 1544-700 cm^{-1} were due to the presence of sugars (carbohydrates).

Comparing the FTIR spectrum of syrup in Appendix A, figure A13 to that of real honeys Figure 4 and Figures A1-5 almost all the peaks were identical except for peaks of syrup found at 989.47 and 923.06 cm^{-1}. The presence of these peaks could indicate presence of unusual sugars found in syrup. Thus, when investigating the authenticity of honey if these peaks were present at a high intensity (absorbance) this was concluded to indicate some form of adulteration. It is important to analyse other syrups commonly used to adulterate honey and identify their characteristic peaks who could serve as fingerprints for adulteration identification.

All the peaks from the real natural honeys were predominately due to the presence of water and sugars in the form of sucrose, fructose, glucose and maltose. The key peaks for maltose, fructose, sucrose and glucose were identified at wavenumbers 1083.8, 1063, 1046 and 1037.1 cm^{-1} respectively. These peaks were identified in honey and used for the purpose of classification.

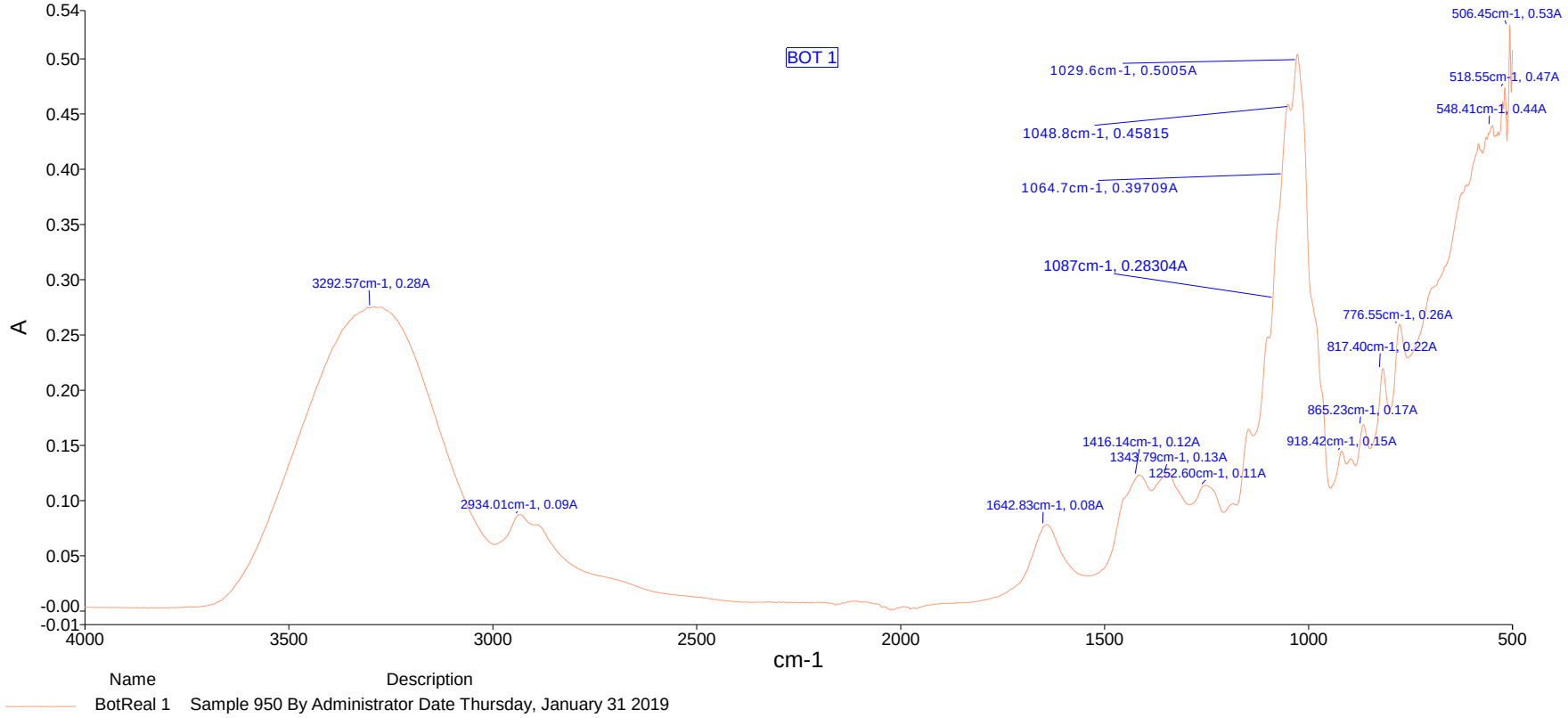


Figure 4. FTIR spectrum of BOT 1 honey.

3.2.2. Data analysis for quantification of sugars

Essential FTIR Version 3.50.185 was used for the purpose of quantification. The second order derives of the sugar standards were used to construct calibration curves. 5 measurements per sample of honey were determined from the produce calibration curve. The quantification summary for fructose is given below while for the rest of the sugars they are given in the Appendices.

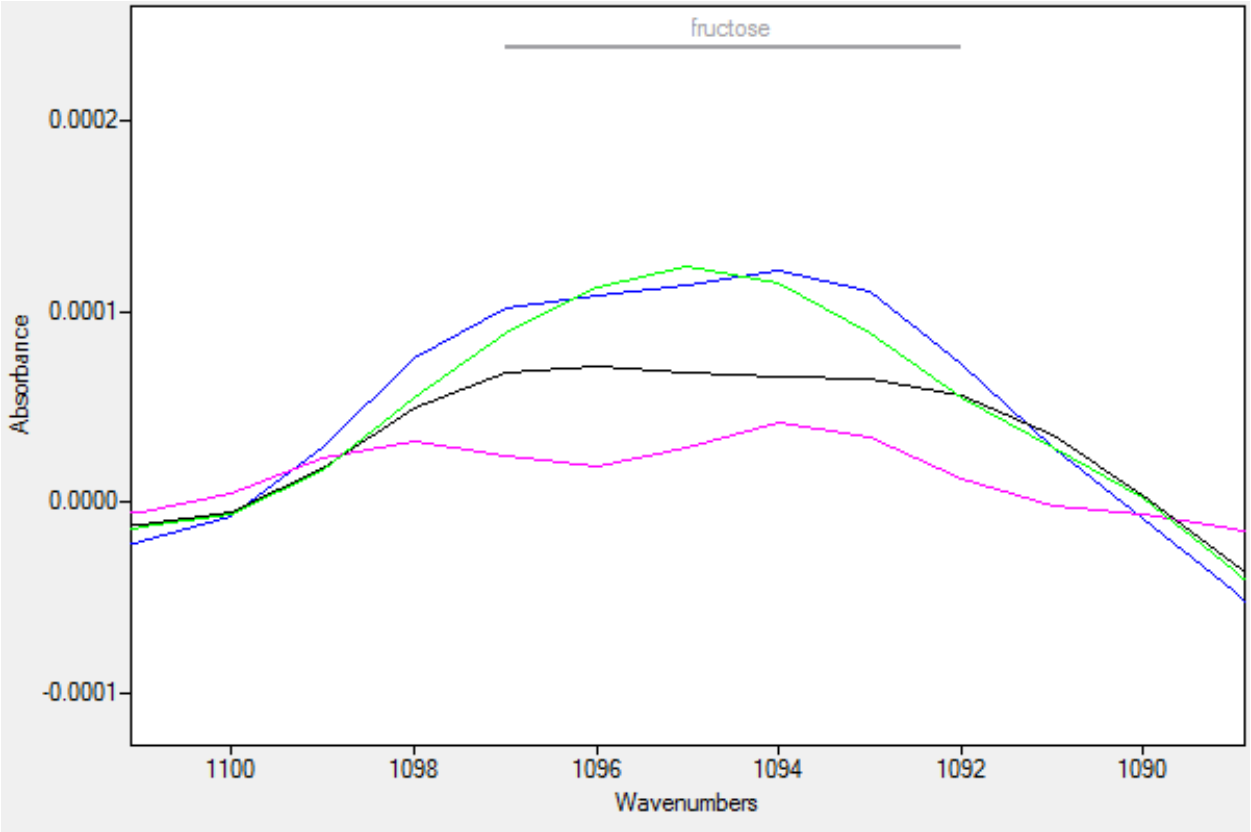


Figure 5. Illustrates the peak used to calibrate fructose.

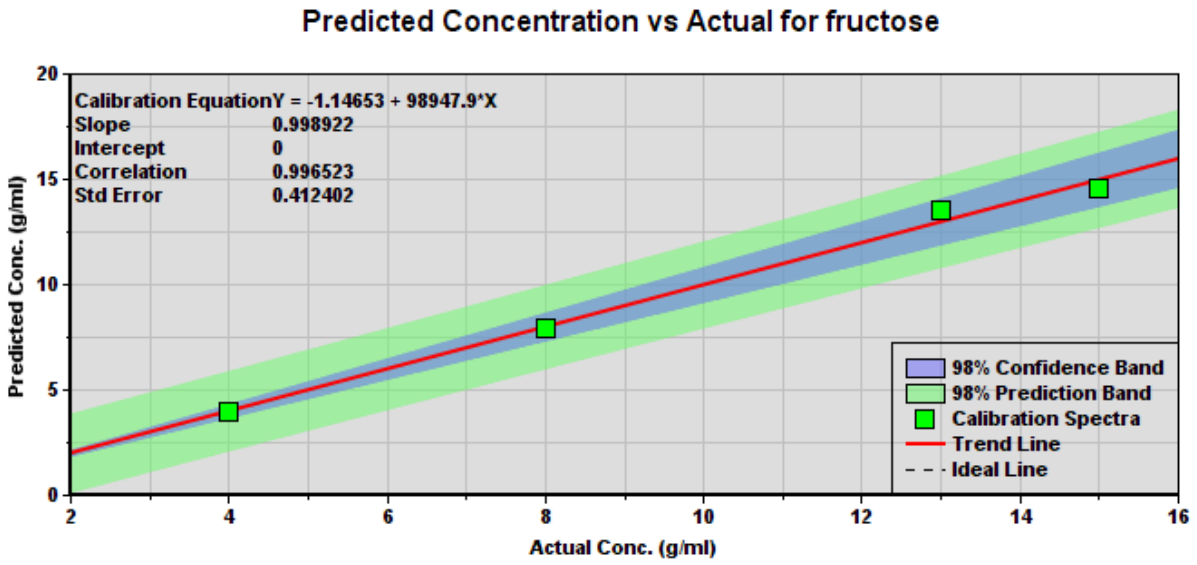


Figure 6. Calibration curve that is used to predict the concentration of the sugar in the honey.

Table 23 shows some partial validation parameters for the procedure while Table 24 shows the actual values for the sugar contents of the honeys.

Table 23. Validation parameters for the quantification of sugars in the honey samples

Sugar	**R^2**	**Equation of the line**	**Standard Error (n=5)**	**LOQ (g/mL) In solution**	**Range of sugar in honey samples**
Sucrose	0.9858	$y = 1111053x - 0.65218$	0.65	3.96	LOQ – 6.01
Fructose	0.9989	$y = 98948x - 1.14653$	0.41	2.46	34.69-43.66
Maltose	0.9964	$y = 67505x - 0.208989$	0.30	1.80	5.38-12.88
Glucose	0.9967	$y = 264384x - 4.344$	0.85	5.12	25.52-32.57

Table 24. Sugar contents of the honeys by FT-IR

Sample Code	Sucrose (g/100 g)	Maltose (g/100 g)	Glucose (g/ 100 g)	Fructose (g/100 g)	Total (Glucose + Fructose) (g/100g)
PELO	ND	5.38	25.63	34.69	60.32
RAMO	ND	11.87	26.13	35.11	61.24
TLOK	8.89	8.64	31.33	34.95	66.28
ZAM	3.98	12.87	32.57	36.70	69.27
ZAM1	6.01	11.08	25.52	35.81	61.33
BOT	4.26	10.21	31.28	43.66	74.94

ND not determined below limit of quantification.

A comparison of literature values from two studies for the sugar contents in natural honeys was done as seen in Table 25 (Pascual-Mate et al., 2008) captured from the cited reference and Table 86 (Anjos et al., 2015).

Table 25. Sugar contents of the honeys from the northern Iberian Plateau by FT-IR

Sample Code	Sucrose (g/100 g)	Maltose (g/100 g)	Glucose (g/ 100 g)	Fructose (g/100 g)	Total (Glucose + Fructose) (g/100g)
Clover (n=3)	(0.18-0.27)	(3.89- 6.11)	(28.87-32.40)	(37.12-39.17)	(65.99-71.57)
Heather (n=10)	(0.01-0.73)	(2.32-5.96)	(26.26-35.10)	(35.55- 42.45)	(61.81-77.55)
Honeydew (n=18)	(0.07-0.85)	(2.91- 6.56)	(24.75-28.83)	(34.56- 38.93)	(59.59-67.76)
Multifloral (n=15)	(0.05-1.22)	(2.93-5.54)	(24.14-35.31)	(35.81- 42.60)	(62.60-77.24)

Table 26. Mean and standard deviation (Sd) of sugar content of honey samples from Portugal

Sample Code	Sucrose (g/100 g)	Maltose (g/100 g)	Glucose (g/100 g)	Fructose (g/100 g)	Total (Glucose + Fructose) (g/100g)
Eucalyptus (n=5)	1.23 ± 0.47	1.30 ± 0.43	23.34 ± 1.58	35.82 ± 1.51	67.26 ± 2.21
Multifloral (n=11)	1.06 ± 0.44	1.28 ± 0.69	23.21 ± 4.53	35.10 ± 4.15	68.55 ± 5.02
Rosemary (n=6)	1.05 ± 0.16	1.42 ± 0.94	25.27 ± 3.15	37.57 ± 3.19	70.01 ± 4.88
Heather (n=5)	1.04 ± 0.36	1.01 ± 0.48	23.15 ± 2.43	35.97 ± 1.17	66.27 ± 2.88

From Tables 25 and 26 i.e. literature values from other studies, the values obtained in this study for the total content of glucose and sucrose were comparable although the values for sucrose and maltose seemed a bit higher than those obtained in the literature. The values prescribed by the IHC for sucrose as seen in Table 3, precluded the possibility of adulteration for the natural and commercial honeys in this study.

Using Table 3 and 24 it can be inferred that all the honey samples analysed are most probably blossom honeys based on their values for total sugars. Most of the sugar values had high LOQ owing to the low reproducibility of the results. These values were only applicable to honey samples studied by the IHC namely in Europe. However, these values can be used to give an indication of adulteration by sugar.

3.3. Classification of the Honeys Based on Physicochemical Characteristics and their Volatile Metabolites by SPME-GC-MS

3.3.1. Classification of Honeys using their physicochemical characteristics

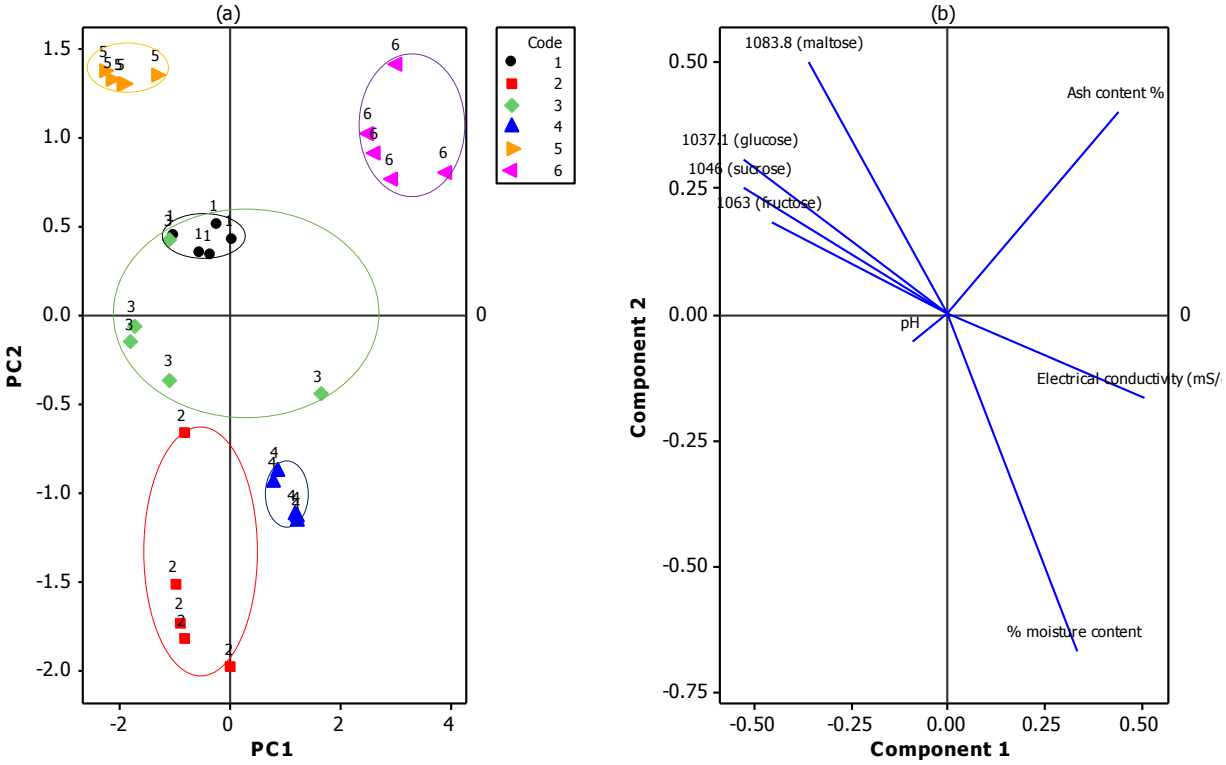


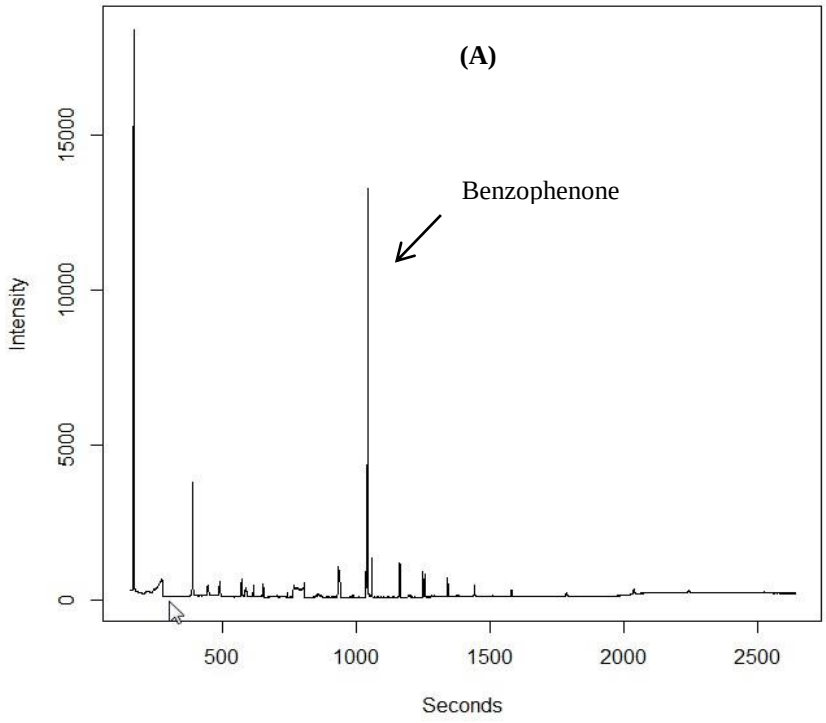
Figure 7. (a) Scores (b) loadings plots for the physiochemical properties of honey samples. 1 = ZAM; 2 = BOT 1; 3 = ZAM1; 4 = PELO; 5 = TLOK; 6 = RAMO

Using an 8 component PLS model with a p value of 0.019 which was less than 0.05, a variance of 100% and an R^2 value of 0.537852. The two honeys from Zambia were similar in terms of the physicochemical parameters that were determined. The PELO and BOT honeys were also found to be similar to each other in terms of their moisture content values. From examination of figure 7 the loadings plot in (b) and the scores plot in (a), the honey from PELO showed the highest moisture content, the honey from Ramotswa had the highest ash content indicating a high mineral content, the ZAM 1 honey had the highest pH value. TLOK honey had the highest absorbance values for

the maltose peak. The pH of BOT 1 and ZAM 3 were similar but the physicochemical property that had the biggest impact on the honeys was the amount of sucrose with a standardized coefficient of 2.071. A very interesting observation from the classification was the distinctiveness of all the honeys apart from the two honeys from Zambia i.e. ZAM 1 and ZAM which were very similar or the same.

3.3.2. The non-targeted volatile profile of commercial and Botswana honey by SPME – GCMS Chromatographic selectivity

Figure 8 shows representative chromatograms captured during *Metab* data processing. (A) is a chromatogram of the SPME of the head space of one of the commercial honeys i.e. ZAM1, (B) of one of the real honeys and (C) of a blank extract. From the blank chromatograms, the selectivity and absence of matrix interferences were demonstrated. The benzophenone internal standard peak was very prominent in all the extracts.



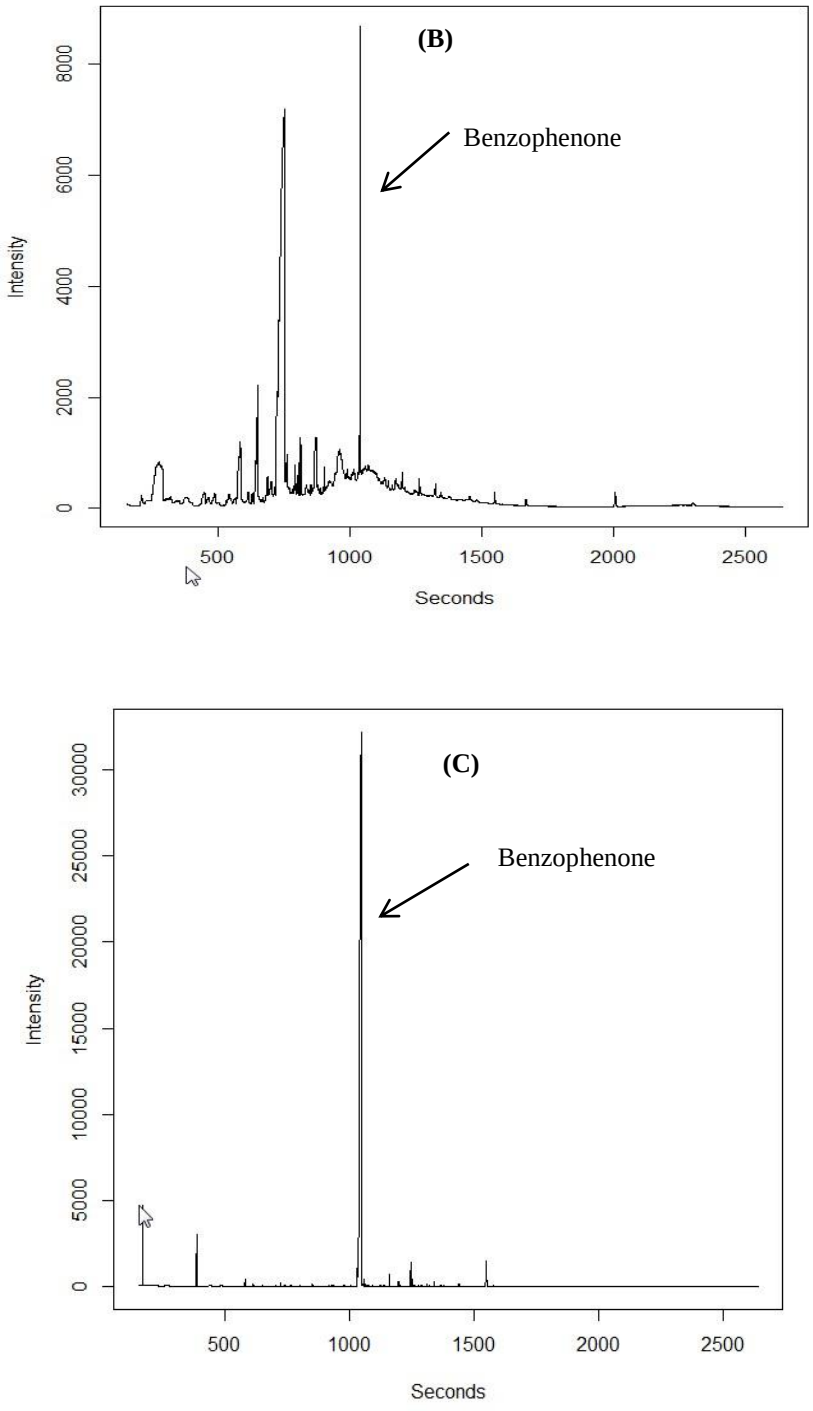


Figure 8. The Metab output of a chromatogram during the data processing showing (A) a commercial honey (B) a real sample from Tlokweng and (C) blank using benzophenone as an internal standard.

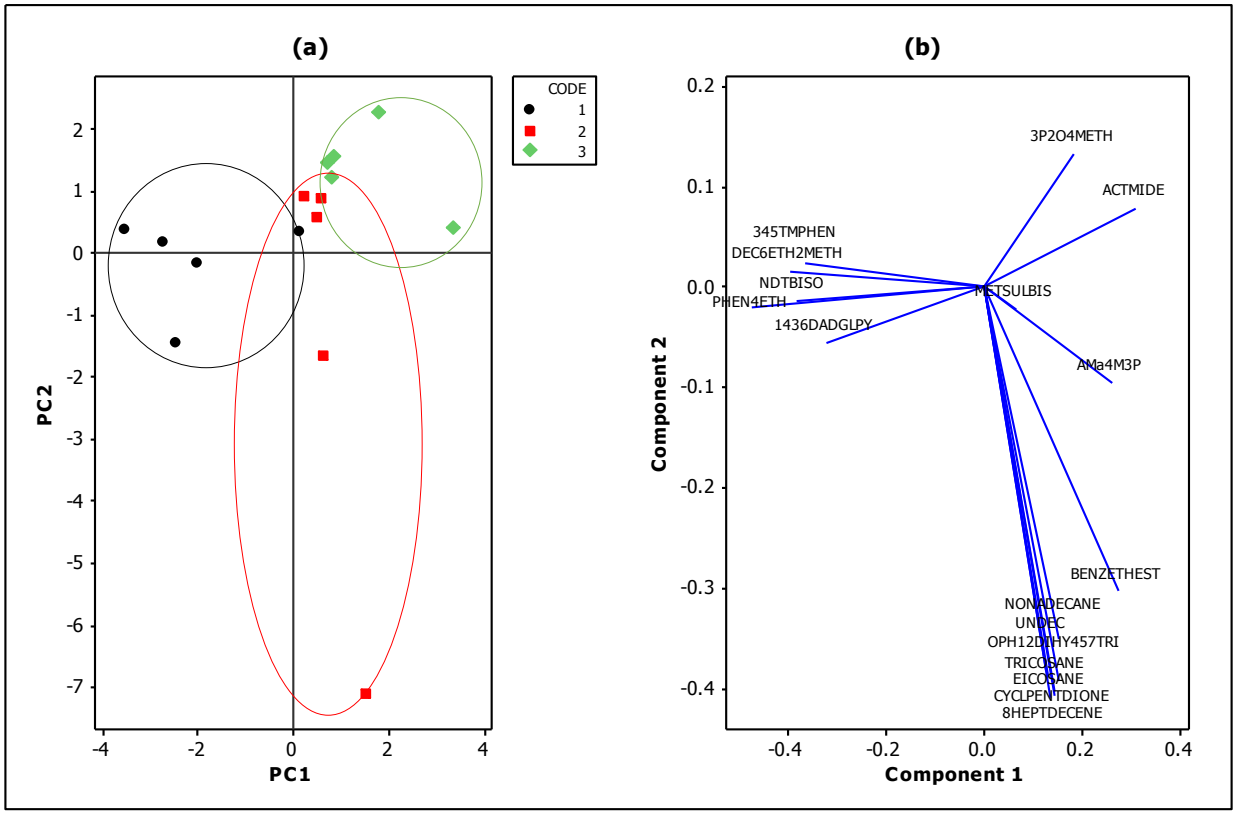
Table 27. Sample data matrix of volatile identified from three commercial honeys after normalization using benzophenone as internal standard

METABOLITE	BOT1	BOT1	BOT1	BOT1	BOT1	ZAM2	ZAM2	ZAM2	ZAM2	ZAM2	ZAM1	ZAM1	ZAM1	ZAM1	ZAM1
Acetamide	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0030	0.0012
Methane, sulfonylbis-	0.0000	0.0000	0.0021	0.0008	0.0014	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
enol, 4-ethyl-	0.0012	0.0009	0.0008	0.0005	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3,4,5-Trimethylenol	0.0069	0.0016	0.0000	0.0025	0.0000	0.0000	0.0000	0.0003	0.0001	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000
Methyl-6-deoxy-6-fluoro-2,3,4-tri-O-methylád-galactopyranoside	0.0000	0.0000	0.0006	0.0004	0.0000	0.0000	0.0170	0.0000	0.0005	0.0021	0.0000	0.0000	0.0000	0.0000	0.0000
Undecane	0.0000	0.0000	0.0012	0.0006	0.0004	0.0093	0.0109	0.0000	0.0000	0.0000	0.0000	0.0011	0.0000	0.0009	0.0000
1,4:3,6-Dianhydro-à-d-glucopyranose	0.0000	0.0016	0.0036	0.0018	0.0010	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3-Penten-2-one, 4-Methyl-	0.0013	0.0031	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0016	0.0000	0.0000	0.0063	0.0063
8-Heptadecene	0.0000	0.0000	0.0000	0.0000	0.0000	0.0354	0.0761	0.0100	0.0012	0.0075	0.0000	0.0000	0.0000	0.0000	0.0000
α-Methyl-α-[4-methyl-3-pentenyl]oxiranemethanol	0.0460	0.0277	0.0282	0.0187	0.0171	0.0000	0.1468	0.0086	0.0000	0.0000	0.0145	0.0612	0.0012	0.4265	0.0247

Benzene, 1-isocyano-3-methyl-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0042	0.0000	0.0009	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Benzeneaceticacid, ethyl ester	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0017	0.0040	0.0000	0.0201	0.0000
Cyclopentadecanone	0.0000	0.0000	0.0000	0.0000	0.0000	0.2366	0.6599	0.0710	0.0213	0.0683	0.0000	0.0000	0.0000	0.0000	0.0000
Decane, 6-ethyl-2-methyl-	0.0011	0.0005	0.0000	0.0008	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Eicosane	0.0000	0.0000	0.0000	0.0000	0.0000	0.0598	0.2148	0.0352	0.0000	0.0016	0.0000	0.0000	0.0000	0.0000	0.0000
Ethanol, 2-(2-ethoxyethoxy)-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0011	0.0000	0.0043	0.0012
N-Dimethylaminomethyl-tert.-butyl-isopropylosine	0.0078	0.0166	0.0078	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Othalene,1,2-dihydro-4,5,7-trimethyl-	0.0000	0.0005	0.0000	0.0000	0.0008	0.0000	0.0171	0.0008	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Nonadecane	0.0000	0.0000	0.0000	0.0000	0.0000	0.2088	0.2050	0.0335	0.0036	0.0300	0.0000	0.0000	0.0000	0.0000	0.0000
Tricosane	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1459	0.0136	0.0023	0.0183	0.0000	0.0000	0.0000	0.0000	0.0000

3.3.3. Characterization of Commercial Honeys by Partial Least Squares (PLS)

Table 27 was obtained by following the flow chart from Figure 3. The volatiles were quantified in a semi quantitative manner using benzophenone to normalize for variations in the amounts extracted. The data in Table 27 as were transposed to make volatiles variables i.e. predictors and the geographical origins as the responses. It was then fed into the MINITAB version 14 spreadsheet followed by (PLS) analysis. The model employed had 6 components with an R^2 value of 0.9285 and a *p* value of 0.0000 i.e. less than 0.05 thus authenticating its validity. Figure 9 (a) shows the scores plot and (b) the corresponding loadings plot and (c) the PLS standardized coefficient plot. The variance carried by the predictors i.e. metabolites was 94.1%. The classification of the honeys was very distinct as seen in Figure 9 (a) the scores plot and (b) the loadings plot showing the two commercial honeys from Zambia coded 2 for ZAM1 and 3 for ZAM and the commercial honey from Botswana coded 1 for BOT1. The data in the loadings plot was decoded and the volatile compounds in the honeys identified and tabulated in Table 21.



1=BOT1; 2 = ZAM1; 3 = ZAM

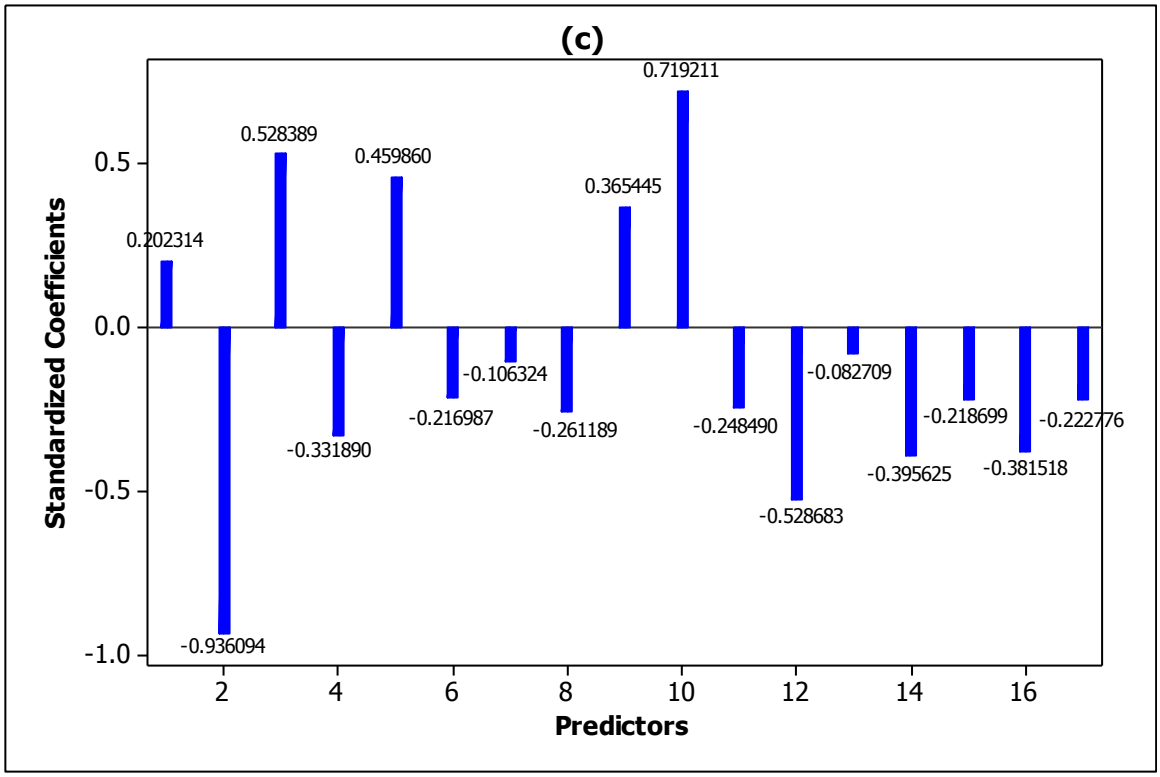


Figure 9. (a) Scores (b) loadings and (c) standardized coefficient plots for all the identified metabolites in commercial honey samples.

In a semi-quantitative and qualitative approach, the retention times, amounts expressed as percent, the AMDIS net match factors and the one way ANOVA *p*-values calculated using *Metab* and the MINITAB standardized regression coefficients for the most important volatile compounds that distinguished the three honeys extracted from Figure 9 (c), were recorded in Table 28. In a nutshell, the amount expressed as (%) represents the area of the deconvoluted component relative to the total ion count for the entire chromatogram expressed as a percentage. The net match factor determines the final match quality, value for the match between the deconvoluted component and the library spectrum. A value of 100 represents a perfect match (D'Arcy & Mallard, 2004).

Table 28. Metabolites and their associated parameters from AMDIS, Metab and MINITAB data analysis

No.	HONEY	METABOLITES	RT (Min)	Amount (%)	Match Factor (Net)	One-Way ANOVA	Std. Coeff.
	BOT1					***p*-value**	
1		N-Dimethylaminomethyl-tert.-butyl-isopropylosine	5.4931	0.06	83	0.0000	0.39563
2		1,4:3,6-Dianhydro-α-d-glucopyranose	11.5574	0.01	85	0.0000	-0.21699
3		Sulfonylbis-Methane	11.9114	0.01	100	0.0000	-0.93609

4		3,4,5-Trimethylenol	13.4462	0.01	90	0.0000	-0.384828
5		4-ethylenol	15.4075	0.0001	98	0.0000	0.528389
6		6-ethyl-2-methyl-decane	24.4039	0.11	75	0.0000	0.52868
	ZAM1						
1		α -Methyl- α -[4-methyl-3-pentenyl]oxiranemethanol	9.4997	0.26	90	0.0000	0.365445
2		1,2-dihydro-4,5,7-trimethyl-nathalene	14.0189	0.01	83	0.0000	-0.2187
3		8-Heptadecene	17.7791	0.08	92	0.0000	-0.26119
4		Cyclopentadecanone	17.8062	3.30	92	0.0000	0.24849
5		Undecane	20.1446	0.0001	92	0.0000	0.45986
6		Nonadecane	20.1499	0.08	99	0.0000	0.38152
7		Eicosane	20.1996	0.23	85	0.0000	
8		Tricosane	24.3169	0.31	89	0.0000	0.22278
	ZAM						

1	4-Methyl-3-penten-2-one	3.3126	0.02	93	0.0000	-0.10632
2	Acetamide	6.1873	0.01	77	0.0000	0.202314
3	Ethyl ester benzeneacetic acid	12.3317	0.03	95	0.0000	0.719211

Std. Coef. = Standardized Coefficient

As seen in Table 28, all except tricosane of the volatile components of BOT1 made a very significant contribution to the differentiation of the honey from ZAM1 were only three components with the highest standardized coefficients were of significance. ZAM1 contained ethyl ester benzene acetic acid, which made the most significant positive contribution to the volatility of the metabolites of all the three honeys. In ZAM 1, seven out of eight volatile constituents made the honey different from the other two with two making a negative impact and five a positive one. ZAM 1 was also unique in that it contained a high amount of cyclopentacanone of 3.3. %, α - Methyl- α - [4-methyl-3-pentenyl] oxiranemethanol of 0.26 %, eicosane of 0.23 % and tricosane of 0.31 %. This outcome was not a surprise since from Table 28, which was constructed out of a database and literature search, these compounds are major compositions of flowering plants, conifers and other gymnosperms which are common species in Zambia that the bees collect honey from in Zambia. In BOT, there was quite a significant amount of 6-ethyl-2-methyl-decane at 0.11 %, which made a significant contribution to the differentiation of the honey. According to Table 28, this is a major constituent of *Guiera senegalensis*, *Hibiscus rosa-sinensis L* and many other flowers very common in the tropics including Botswana. The volatile metabolites in ZAM were not predominant, although two out of three made a positive impact towards the differentiation of the honey. Table 28 gives the occurrence of other metabolites that have not been mentioned.

3.3.4. Data Base Search for Metabolite Sources

Several databases were searched for the identified metabolites which as seen from Table 29, confirmed the three commercial honeys as being polyfloral.

Table 29. Sources of volatile metabolites after database search for commercial honeys

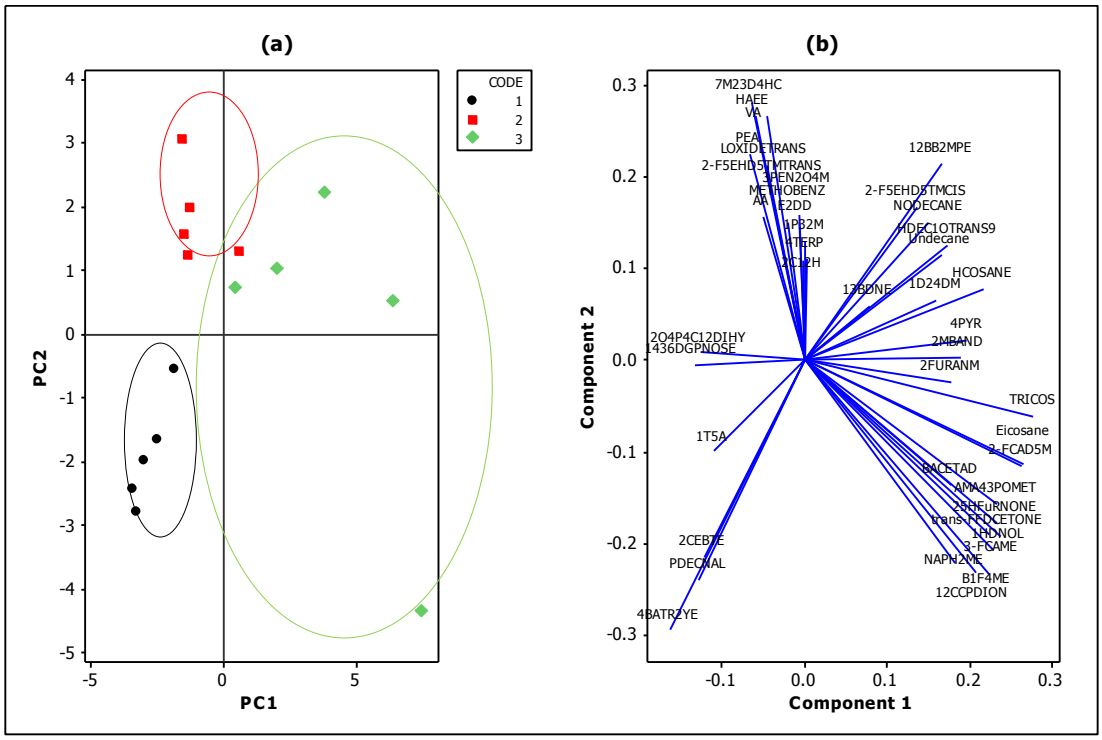
HONEY	METABOLITES	Botanical nomenclature for plant and/or species derived from	Ref.
BOT			
	N-Dimethylaminomethyl-tert.-butyl-isopropylosine	*Zanthoxylum tetraspermum wight & arn*	30
	1,4:3,6-Dianhydro-α-d-glucopyranose	*Barleria noctiflora L.f.*	31
	Sulfonylbis-Methane (DMSO)	Primitive plants	www.justice.gov
	3,4,5-Trimethylenol	Derivative of 1,2,4-trimethylenol form *Centaurium erythraea*	ChEBI
	6-ethyl-2-methyl-decane	*Guiera senegalensis, Hibiscus rosa-sinensis L* and many other flowers	10.7324/JAPS.2013.30328
	4-ethylenol	Spolage yeast *Brettanomyces* on skins of fruits	HMDB
ZAM1			
	α -Methyl- α -[4-methyl-3-pentenyl]oxiranemethanol	*Coriandrum sativum L* (Coriander)	
	1,2-dihydro-4,5,7-trimethyl-nathalene		
	8-Heptadecene	*Chrysoyllum albidum G. Don* (White star apple), *Camellia oleifera, Citrus sinensis*	10.17265/2161-6256/2017.04.002

	Cyclopentadecanone	Trail eromone from musk like animals	10.1016/j.fct.2011.07.040
	Nonadecane	Pimenta dioica (Allspice)	HMDB
	Eicosane	flowering plants, conifers and other gymnosperms	ChEBI
	Tricosane	flowering plants, conifers and other gymnosperms	ChEBI
ZAM			
	4-Methyl-3-penten-2-one	*Osmanthus fragrans*	HMDB, PUBCHEM
	Acetamide	Red Beetroot	
	Ethyl ester benzeneacetic acid	*Monstera deliciosa* and . Aegle marmelos Odoriferous constituent of many plants i.e. apple, gBrassicafruit, guava fruit, papaya, melon, pineapple, wheat bread, crispbread, wines, fruit brandies, shoyu, bael	PUBCHEM

3.4. Characterization of Real Honey by Partial Least Squares (PLS)

Using AMDIS and *Metab*, the raw data was treated as explained for the commercial honeys and fed into MINITAB version 14 and (PLS) analysis performed on it. The model employed 10 components with an R^2 value of 0.9978 and a *p* value of 0.000 which was less than 0.05 thus authenticating its validity. Figure 10 (a) shows the scores plot and (b) the corresponding loadings plot while (c) shows the standardized regression coefficient plot. The variance carried by the predictors i.e. metabolites was 94.5%. The differences in the composition of the honeys were very

distinct as seen in Figure 10 (a) and (b) with the honey from Pelotlhetla differing from that from Ramotswa and also from Tlokweng.



1 = PELOTLHETLA; 2 = RAMOTSWA; 3 = TLOKWENG

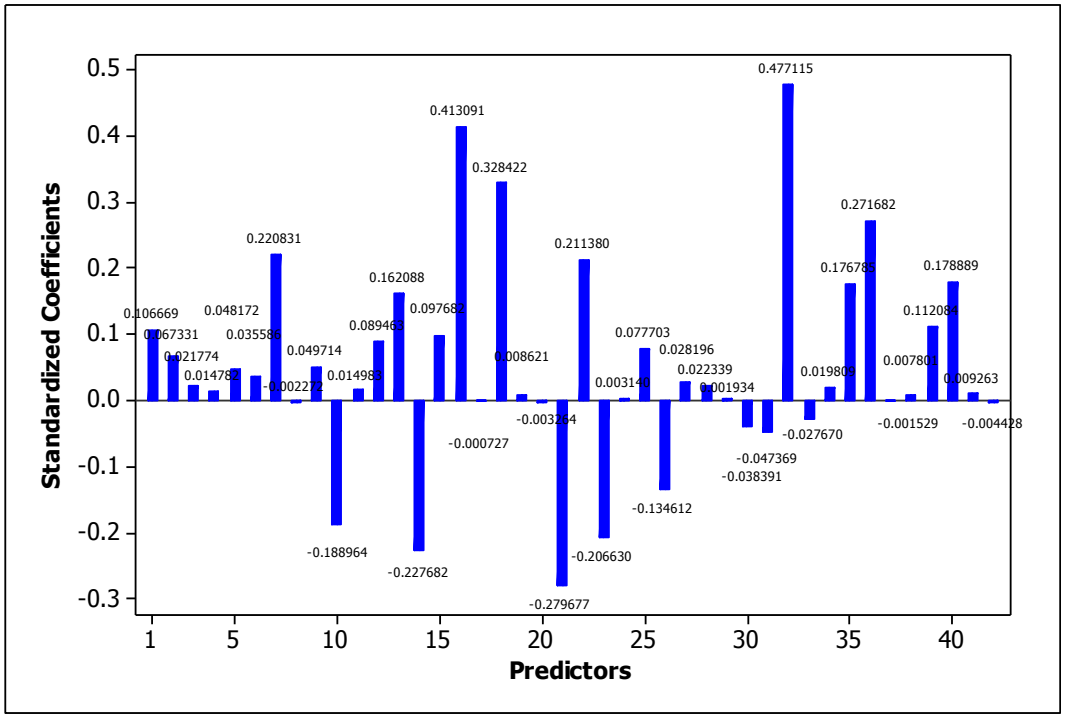


Figure 10. (a) Scores (b) loadings and (c) standardized coefficient plots for all the identified metabolites real honey samples

Data from AMDIS, *Metab* and Minitab was collated in Table 30 showing retention times, amounts (%), net match factors, one way ANOVA and standardized coefficients. The *p* values for all the metabolites were less than 0.05. Interestingly, the major differences between the three honeys were made by the honeys from Tlokweng and PELOTLHETLA as seen from the significant standardized coefficients. Where the AMDIS net match factors were below 70%, all the metabolites did not contribute to the differentiation of the three honeys with correspondingly low amounts registered. The low amounts were speculated to have been responsible for this. Interestingly, 2-Furanmethanol, 5-ethenyltetrahydro-α,α-5-trimethyl-, trans was the most abundant in honey from Ramotswa at 0.01%. This is also called trans-Linalool oxide, which is a major constituent of *Camellia sinensis* (tea) *Passiflora mollissima (Kunth) L. H. Bailey* i.e. passion fruit which is very common in Botswana. The honey from Tlokweng was dominated by 1,2-cyclopentanedione at

0.08 % derived from *Carica papaya L*. i.e. Papaya; 2-furancarboxaldehyde, 5-methyl- at 0.01% from *Capsicum annuum* i.e. pepper; furfural at 0.03% from many fruits, nuts and agricultural byproducts. Plants that blossom into these flowers are widespread in the area that the honey was collected from. Others were 1-hexadecanol at 0.03%, 1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester at 0.05% and eicosane at 0.01%, which are major constituents of plants/fruits/flowers and other sources indicated in Table 31. From Table 31, benzene, 1-fluoro-4-methyl was speculated to be a product of fungal cometabolism of toluene by *Cunninghamella echinulata* and *Aspergillus niger* and other forms of bacteria. Fluorinated natural products are very rarely encountered and play significant armacological roles in living biological systems.

Table 30. Identified compounds in the three real honeys

No.	Honey	Compound	R.T. (min)	Amount (%)	Match Factor (Net)	One-Way ANOVA	Std. Coeff.
						***p*-value**	
1		2-Cyclopenten-1-one, 2-hydroxy-	4.4444	4.2×10^{-4}	65	0	
2		Vinyl acetate	6.6771	1.7×10^{-5}	62	0	
3		3-PENTEN-2-ONE, 4-METHYL-	7.3237	5.2×10^{-5}	71	0	
4		1-Penten-3-one, 2-methyl-	8.0283	1.1×10^{-5}	85	0	
5	RAMO	2-Oxo-4-enyl-6-(4-chloroenyl)-1,2-dihydropyrimidine	8.1465	4.3×10^{-4}	75	0	-0.13461
6		Linalool oxide trans	9.5006	5.5×10^{-4}	71	0	
7		4-TERPINEOL	9.5142	1.5×10^{-4}	69	0	
8		enylethyl Alcohol	10.2711	8.8×10^{-4}	81	0	0.162088
9		Methanol, oxo-, benzoate	11.2149	4.2×10^{-4}	94	0	

10		2-Furanmethanol, 5-ethenyltetrahydro-α,α-5-trimethyl-, trans	11.2155	1.0×10^{-2}	61	0	
11		1,4:3,6-Dianhydro-à-d-glucopyranose	11.5413	1.4×10^{-4}	78	0	
12		Acetic acid	12.0055	6.4×10^{-5}	77	0	0.106669
13		7-Methoxy-2,3-dienyl-4H-chromen-4-one	12.4619	9.0×10^{-6}	62	0	
14		Hexadecanoic acid, ethyl ester	21.0284	6.5×10^{-4}	90	0	
1		Pentadecanal-	13.1541	2.1×10^{-4}	90	0	
2	PELO	4-Ethylbenzoic acid, tridec-2-ynyl ester	14.03	1.9×10^{-5}	62	0	
3		1H-Tetrazol-5-amine	16.8607	6.2×10^{-4}	67	0	

1		1,3-Butadiene	2.8177	7.2×10^{-5}	73	0	0.328422
2		2(5H)-Furanone	4.8226	2.9×10^{-4}	86	0	
3		2-Furanmethanol	4.8673	4.7×10^{-4}	71	0	0.21138
4		1,2-Cyclopentanedione	6.7279	8.0×10^{-2}	93	0	
5		2-Furancarboxaldehyde, 5-methyl-	7.1566	1.0×10^{-2}	83	0	-0.27968
	TLOKWENG						
6		Benzene, 1-fluoro-4-methyl-	7.5052	9.0×10^{-4}	83	0	
7		1-Decene, 2,4-dimethyl-	8.7244	1.1×10^{-4}	100	0	0.220831
8		Benzeneacetaldehyde	8.9621	2.8×10^{-3}	99	0	
9		α-Methyl-α-[4-methyl-3-pentenyl]oxiranemethanol	9.591	4.2×10^{-5}	86	0	0.477115
10		Furfural	9.7306	3.0×10^{-2}	98	0	

11	2-Furanmethanol, 5-ethenyltetrahydro-α,α-5-trimethyl-, cis-	9.7695	1.9×10^{-3}	80	0	-0.20663
12	3-Furancarboxylicacid, methyl ester	10.031	1.7×10^{-4}	91	0	
13	trans-Furfurylideneacetone	11.4209	2.0×10^{-5}	89	0	
14	2-Methylbutanoic anhydride	11.7073	6.6×10^{-4}	83	0	
15	Undecane	13.0383	1.0×10^{-2}	93	0	-0.22768
16	Nathalene, 2-methyl-	13.0395	5.3×10^{-5}	94	0	0.112084
17	1-Hexadecanol	14.5311	3.0×10^{-2}	67	0.00013	0.115144
18	Nonadecane	20.0944	6.6×10^{-4}	91	0	0.178889

19	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	20.7834	5.0×10^{-2}	95	0	0.413091
20	Heneicosane	22.0136	3.5×10^{-4}	96	0.0199	0.176785
21	Tricosane	24.2452	2.1×10^{-4}	90	0	
22	Hexadecen-1-ol, trans-9-	27.6158	4.7×10^{-5}	91	0	0.271682
23	Eicosane	27.803	1.0×10^{-2}	61	0	-0.18896

Std. Coef. = Standardized Coefficient

3.4.1. Data Base Search for Metabolite Sources

As for commercial honeys, several databases were searched for metabolite sources which confirmed that the three real honeys from Botswana were polyfloral varieties.

Table 31. Sources of volatile metabolites after database search for real honeys

Honey	Compound	Botanical nomenclature for plant and/or species derived from	Ref.
RAMO			
	2-Cyclopenten-1-one, 2-hydroxy-	Coffee, odor: maple caramel	HMDB
	Vinyl acetate	Watercress plants	
	3-Penten-2-one, 4-Methyl-	*Osmanthus fragrans* (Capsicum species) herbs and spices	HMDB
	1-Penten-3-one, 2-methyl-	Cloudy apple juice	10.1021/jf0715727
	2-Oxo-4-enyl-6-(4-chloroenyl)-1,2-dihydropyrimidine	Antibacterial agents	Int. J. Universal arm. Bio Sci., 2(3) 469-476
	Linalool oxide trans	*Camellia sinensis* (Tea*) Passiflora mollissima (Kunth) L. H. Bailey* etc	HMDB
	4-Terpineol	*Pimenta dioica* (Allspice)	HMDB
	enylethyl Alcohol	*Prunus dulcis*, syn. *Prunus amygdalus* (Almond)	HMDB

	Methanol, oxo-, benzoate	*Monstera deliciosa* (ceriman), *Annona cherimola* (cherimoya); various flower oils, banana, cherry, pimento berry	HMDB
	2-Furanmethanol, 5-ethenyltetrahydro-α,α,5-trimethyl-, trans- ALSO trans-Linalool oxide	*Camellia sinensis* (Tea) Passiflora *mollissima (Kunth) L. H. Bailey* etc	HMDB
	1,4:3,6-Dianhydro-α-d-glucopyranose	*Barleria noctiflora L.f.*	31
	Acetic acid	*Clostridium acetobutylicum* (bacteria)	HMDB
	7-Methoxy-2,3-dienyl-4H-chromen-4-one	Favone i.e. flavonoids group mainly from spices, and red–purple fruits and vegetables	
	Hexadecanoic acid, ethyl ester	*Monstera deliciosa* (Ceriman) also in various fruits, e.g. apricot, sour cherry, gBrassicafruit, bilberry, guava fruit, melon, pineapple	HMDB
PELO			
	Pentadecanal-	*Citrus limon* (Lemon oil) and *Cinnamomum micranthum*	HMDB
	4-Ethylbenzoic acid, tridec-2-ynyl ester	*Gauzuma ulmifolia lam*	10.5530/pj.2018.1.33
	1H-Tetrazol-5-amine	*Pleurotus sp.* Oyster Mushroom	10.22207/jpam.11.2.28
TLOK			
	1,3-Butadiyne	*Hedera rhombea*	Z. Naturforsch. 61c, 536-540 (2006)
	2(5H)-Furanone	*Aspergillus sp*	34

	Sambucus nigra (black elderberry) Coffee	HMDB
2-Furanmethanol	Arabica etc	
1,2-Cyclopentanedione	*Carica papaya L.*	10.1016/j.talanta.2010.10.064
2-Furancarboxaldehyde, 5-methyl-	*Capsicum annuum* (Pepper)	HMDB
	Product of bacteriological metabolism of	10.1128/AEM.67.3.1030–
Benzene, 1-fluoro-4-methyl-	toluene	1034.2001
	Capsicum annuum (pepper), *Ampelopsis*	10.7506/spkx1002-6630-
1-Decene, 2,4-dimethyl-	*grossedentata*	201416038
	Major active ingredient of cooked pine	PMID: 16910727 , 7818768 ,
	mushroom i.e. *Tricholoma matsutake* = syn. *T.*	15606130
Benzeneacetaldehyde	*nauseosum* = syn. *Armillaria ponderosa*	
α-Methyl-α-[4-methyl-3-	*Coriandrum sativum L* (Coriander)	HMDB
pentenyl]oxiranemethanol		
Furfural	Many fruits, nuts and agricultural byproducts	PUBCHEM
2-Furanmethanol, 5-ethenyltetrahydro-	*Camellia sinensis* (Tea) Passiflora *mollissima*	HMDB
α,α,5-trimethyl-, cis- ALSO cis-Linalool	*(Kunth) L. H. Bailey* etc	
oxide		
	Hibiscus esculentus (Okra) and peanut,	HMDB
3-Furancarboxylic acid, methyl ester	tomatoes etc	
trans-Furfurylideneacetone	Alcoholic beverages i.e. rum and coffee	HMDB
	Flavor component of apple and strawberry	PBMED
2-Methylbutanoic anhydride	found in many fruits	

	Undecane	Pimenta dioica (Allspice)	HMDB
	Nathalene, 2-methyl-	*Juglans nigra* (Black walnut) family	HMDB
	1-Hexadecanol	Palm oil and coconut oil	HMDB
	Nonadecane	*Pimenta dioica* (Allspice)	HMDB
	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	*Syzygium aromaticum* (Clove)	HMDB
	Heneicosane	*Periploca laevigata* and *Carthamus tinctorius*	PUBCHEM
	Tricosane	Flowering plants, conifers and other gymnosperms	ChEBI
	Hexadecen-1-ol, trans-9-	intermediate in the biosynthesis of medium chain wax esters	Lipids 45, 263-273
	Eicosane	Flowering plants, conifers and other gymnosperms	ChEBI

4. Conclusion

Commercial and natural honey samples from selected regions around Botswana were classified based on their geographical and botanical/floral origins, using GC-MS, physicochemical characterization and multivariate statistical tools. Classification was accomplished using a combination of Productivity Chemstation, NIST AMDIS, Metab and MINITAB version 14 for volatile metabolites. A database search of the volatiles showed the floral sources of the honeys which confirmed that all the honeys were polyfloral. Physicochemical parameters were adduced using classical analytical methods of analysis prescribed by the IHC while adulteration detection which involved analysis of four sugars i.e. glucose, maltose, sucrose and fructose was accomplished using FTIR and data processed by Essential FTIR software version.

From the values obtained for sugars i.e. glucose, fructose, maltose and sucrose, the natural honeys compared well with commercial honeys and were well within acceptable error prescribed by the IHC and this confirmed that all the honeys were not adulterated

4.1. Recommendations

When properly implemented it is possible to use FTIR with the aid of PLS to predict the physiochemical properties of honey in addition to the extent to which a honey is adulterated. If a robust and extensive database were to be created, it would be possible to forestall feud, in the form of adulteration. This would be done via PLS by calibrating the instrument and samples data are outside the norm can be further analysed this would act as an early warning system for adulteration. LCMS determinations of the flavonoids in honey could provide valuable information about the bioactivity of honey from Botswana and hence the potential health benefits. Mineral content determinations could be incorporated to provide nutritional information of honey samples, this would positive impact the monetary value of the analysis honey.

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APPENDIX A

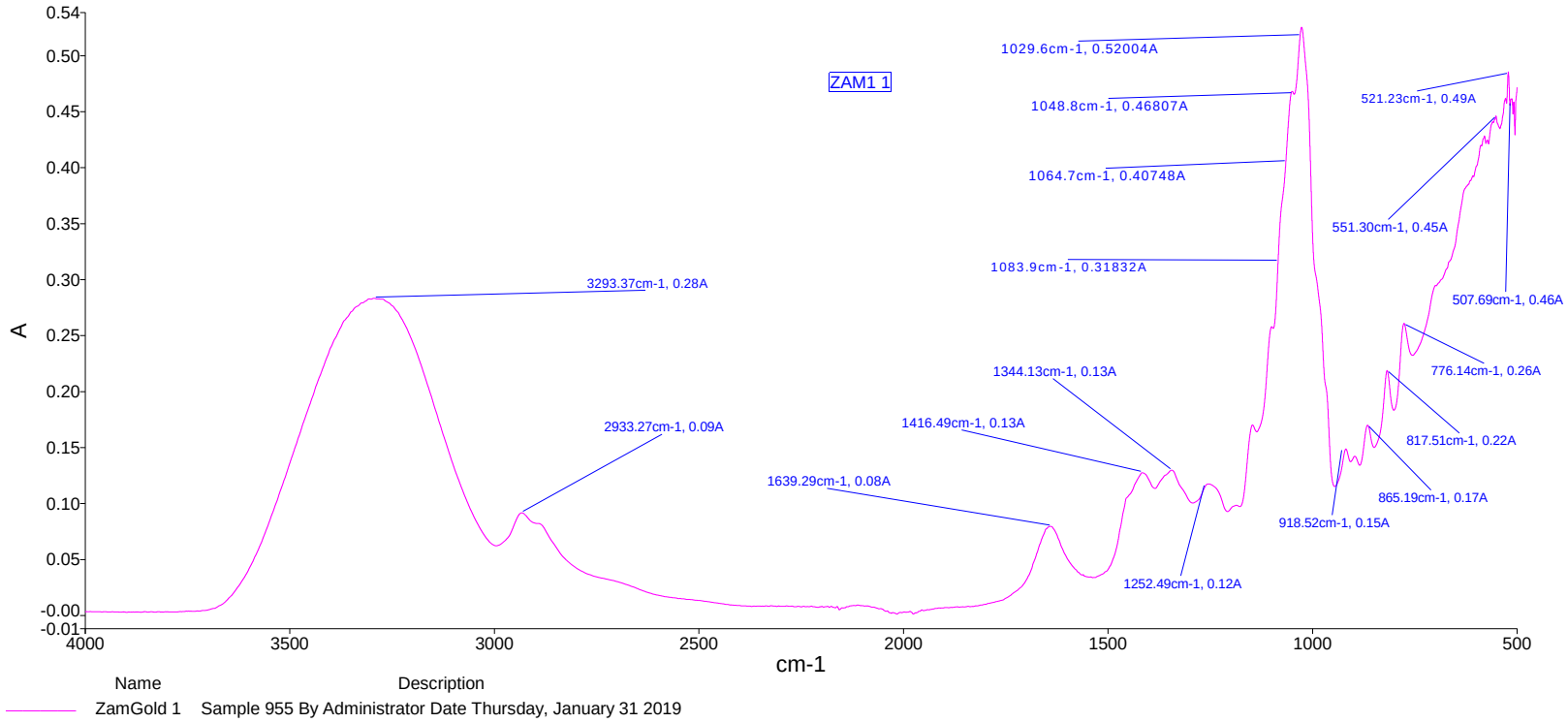

Figure A1. FTIR spectrum of ZAM1 1 honey**.**

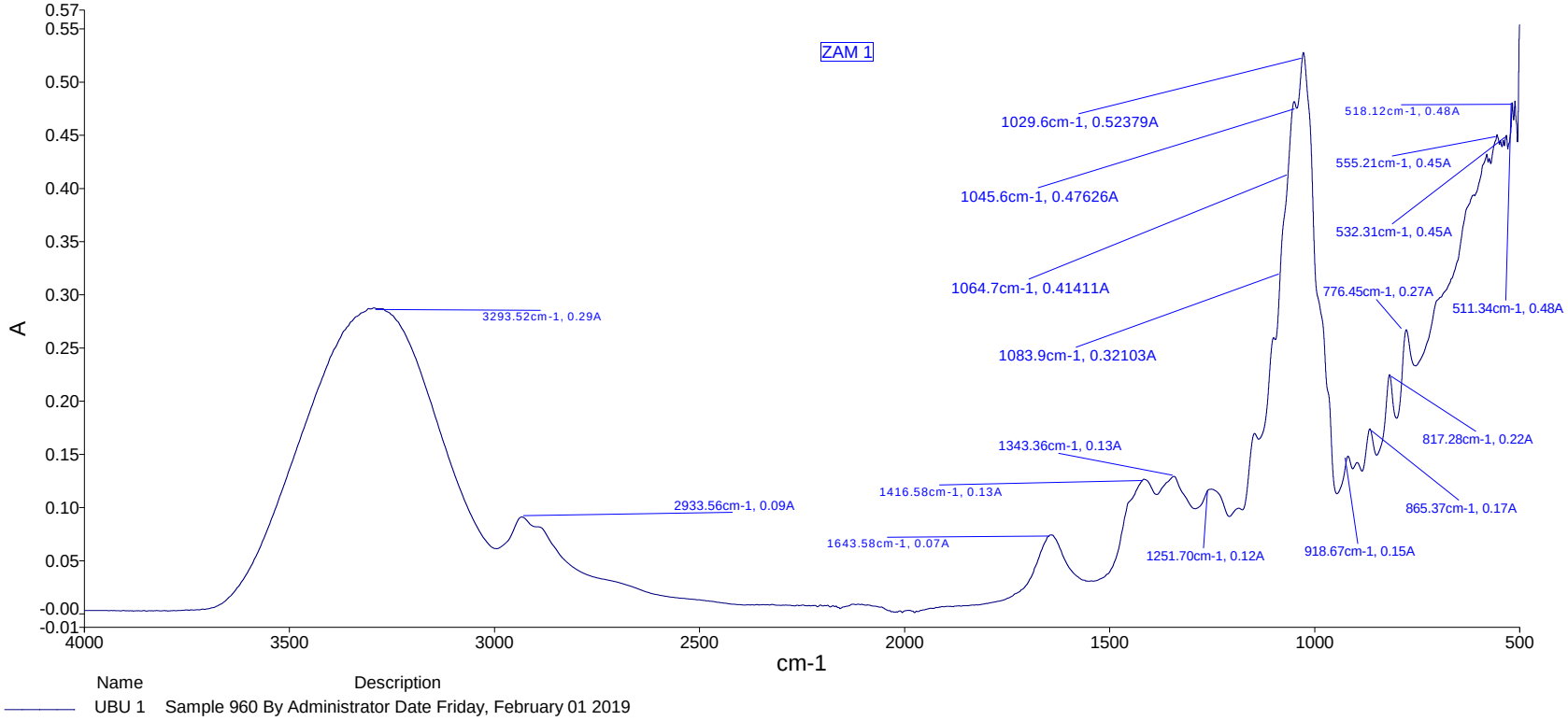

Figure A2. FTIR spectrum of ZAM 1 honey.

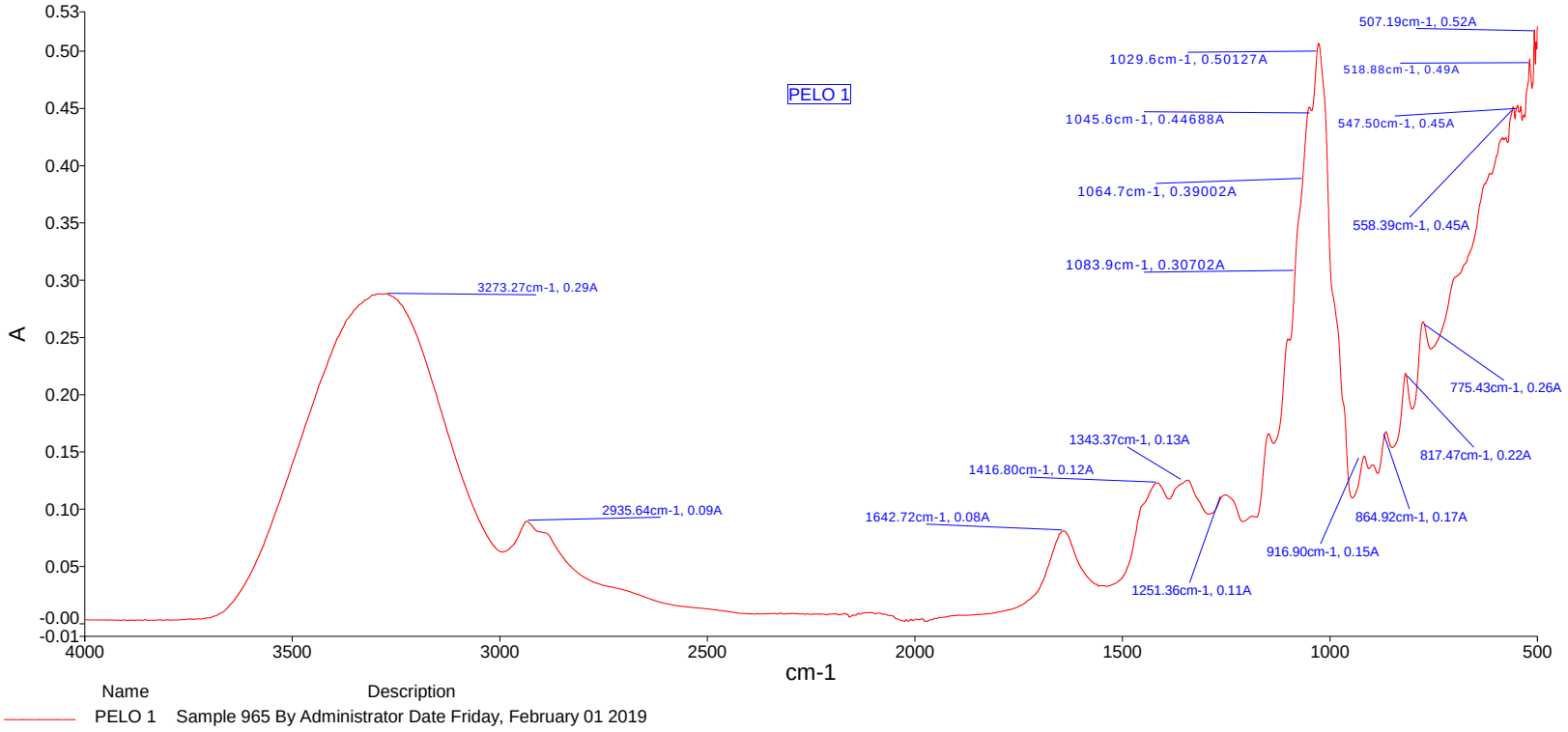

Figure A3. FTIR spectrum of PELO 1 honey.

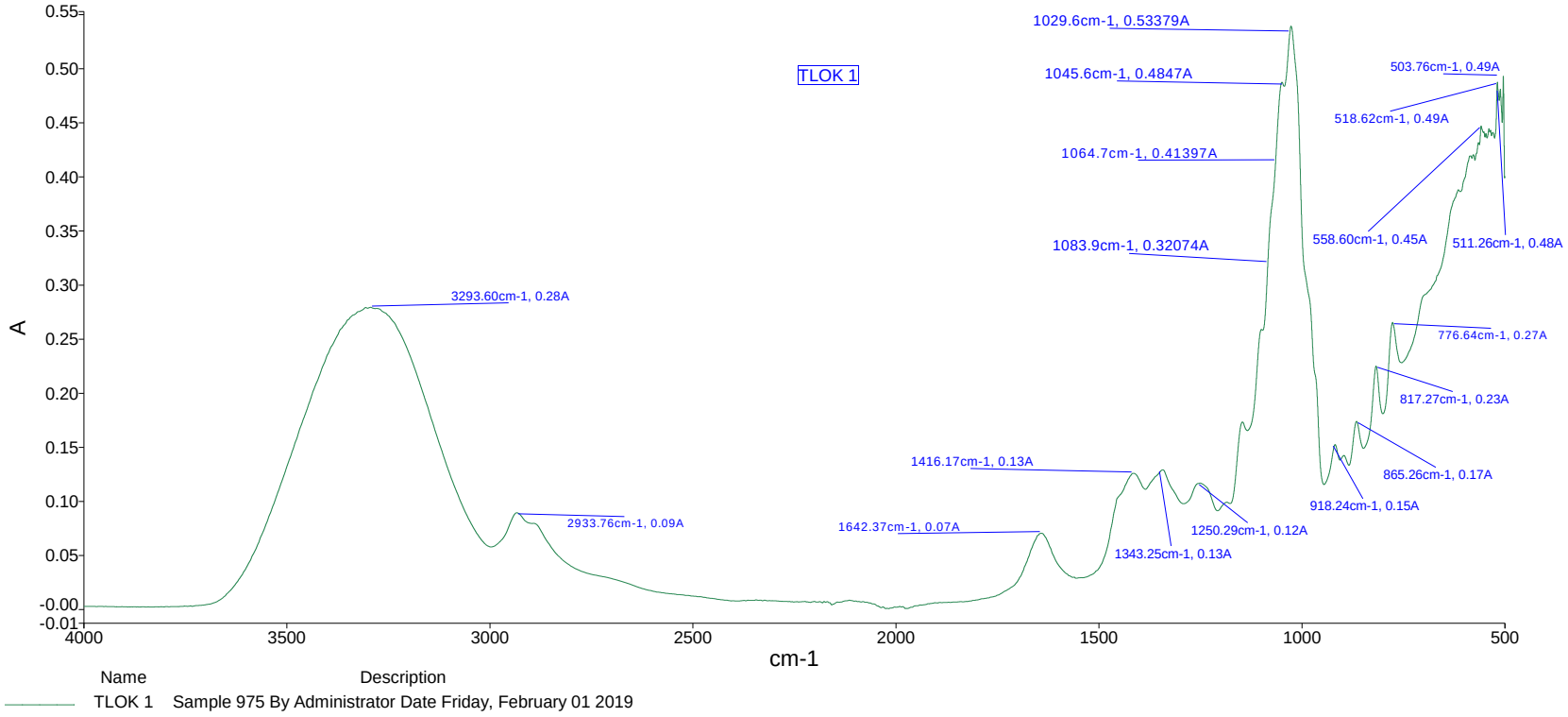

Figure A3. FTIR spectrum of TLOK 1 honey.

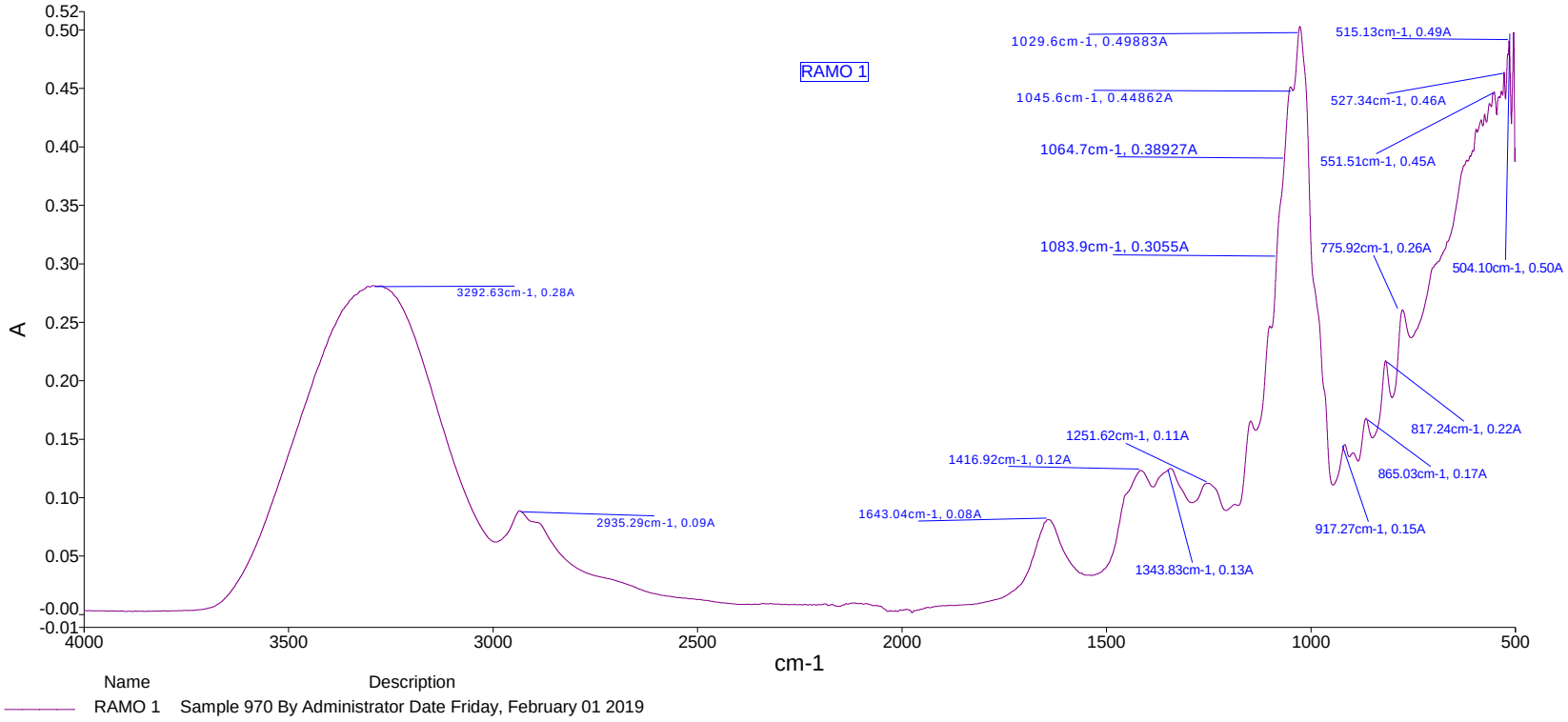

Figure A4. FTIR spectrum of RAMO 1 honey.

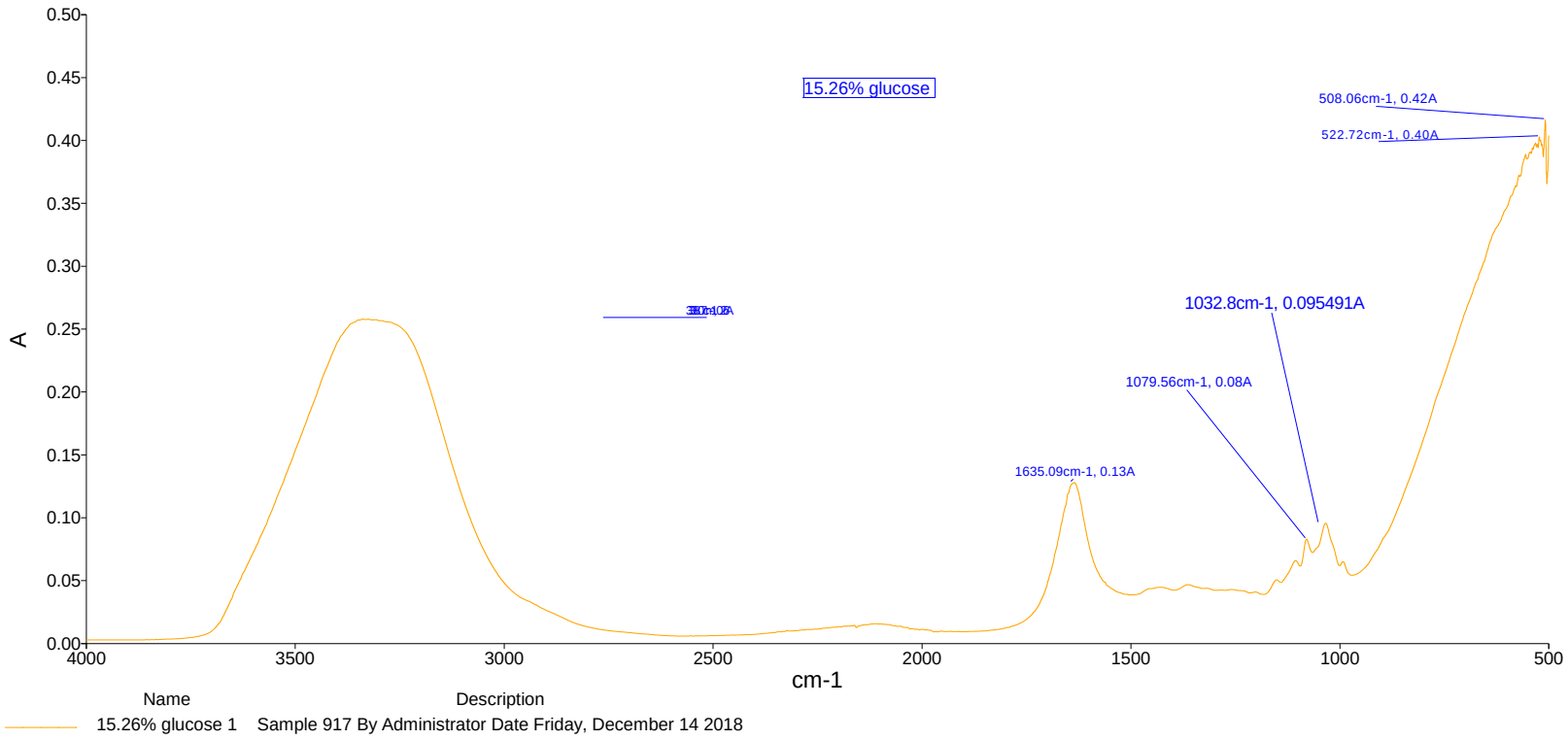

Figure A5. FTIR spectrum of 15.26% glucose.

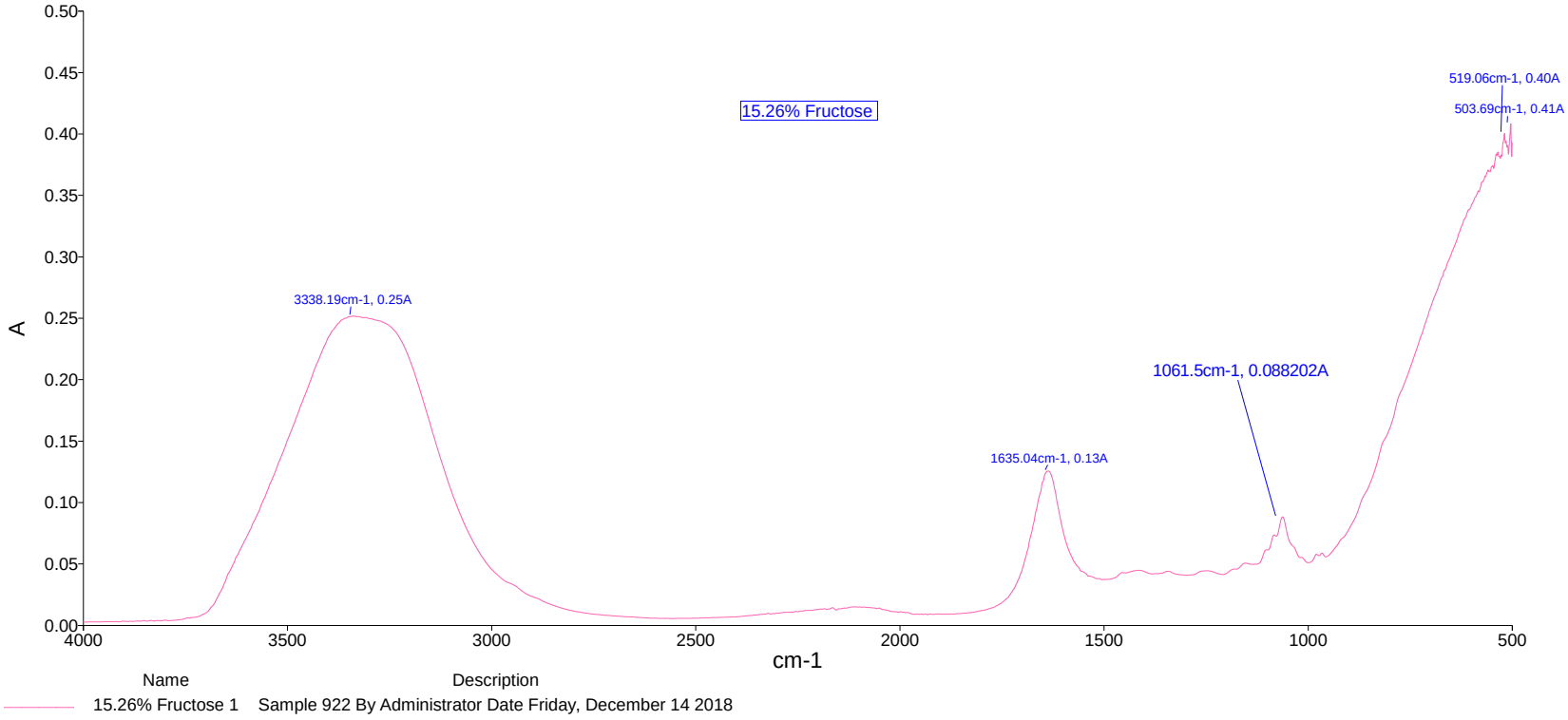

Figure A6. FTIR spectrum of 15.26% Fructose.

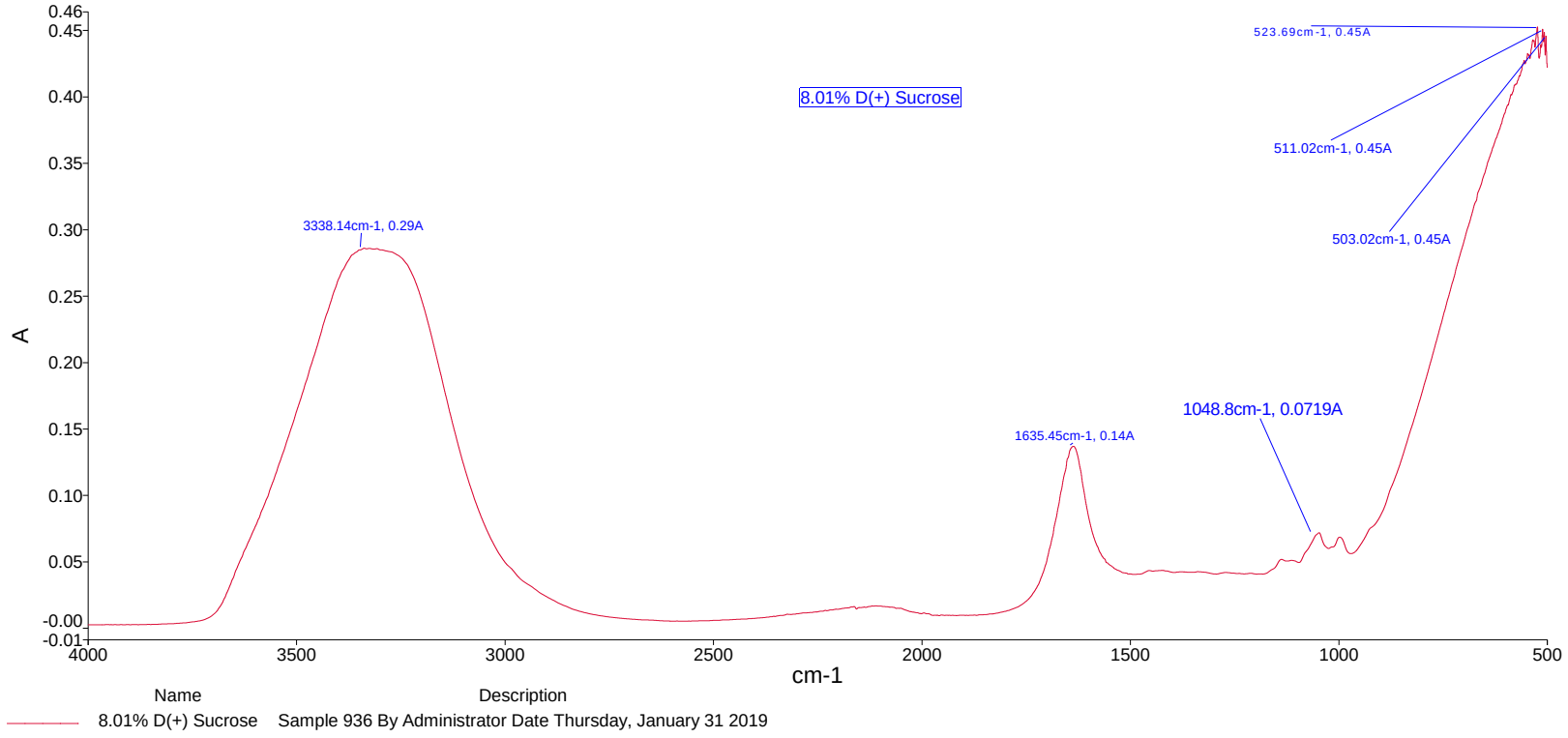

Figure A7. FTIR spectrum of 8.01% Sucrose.

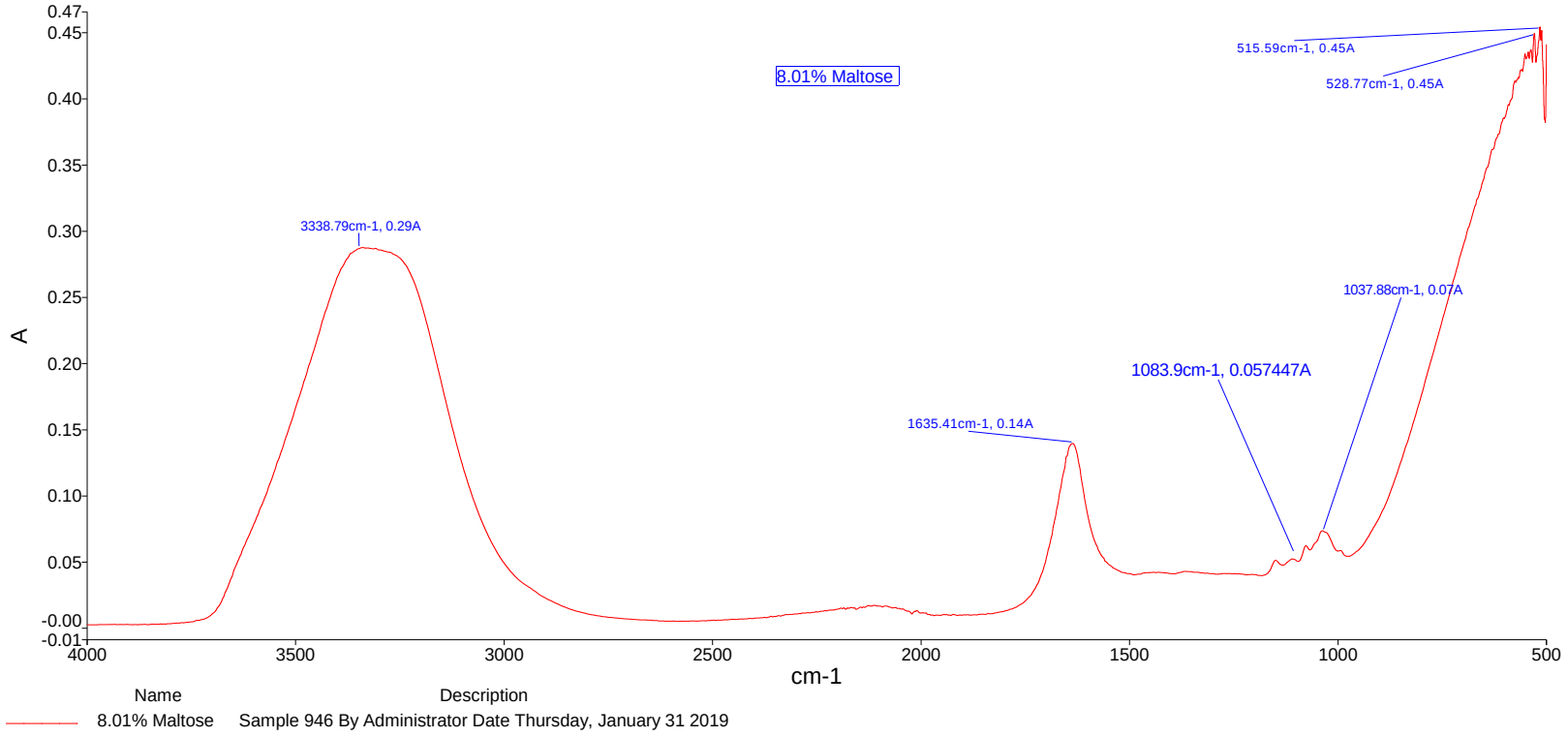

Figure A8. FTIR spectrum of 8.01% Maltose.

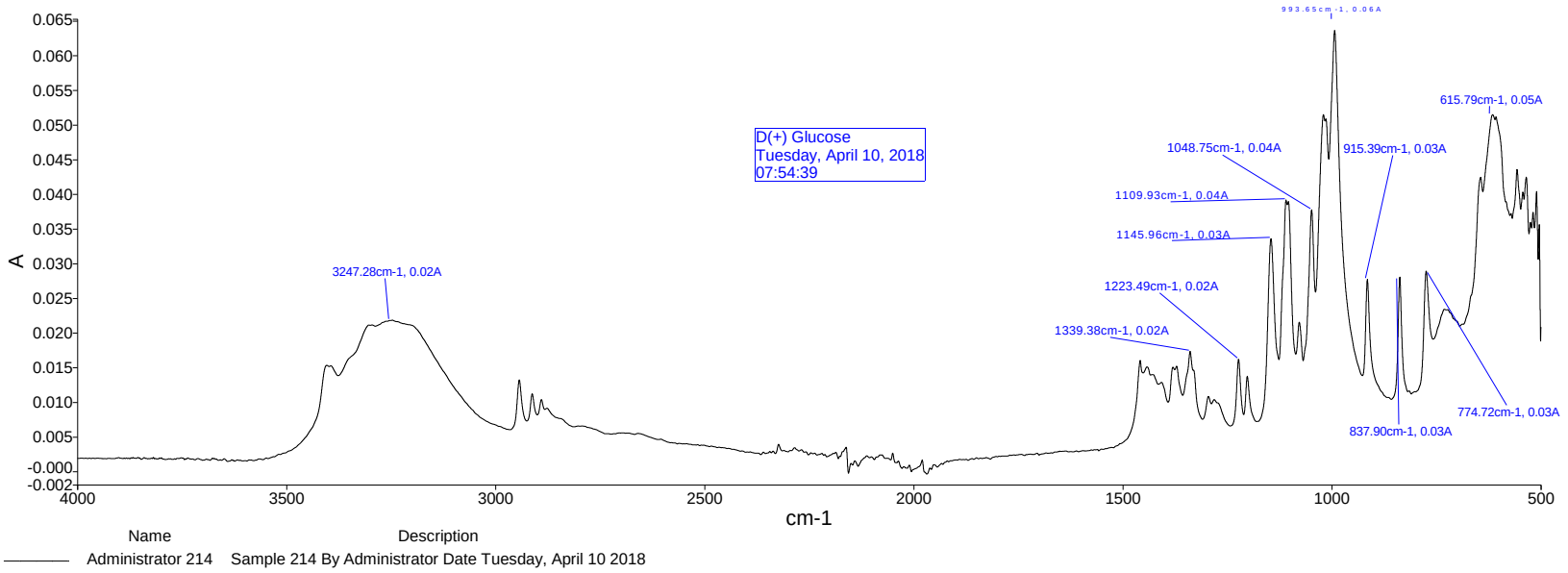

Figure A9. FTIR spectrum of Glucose.

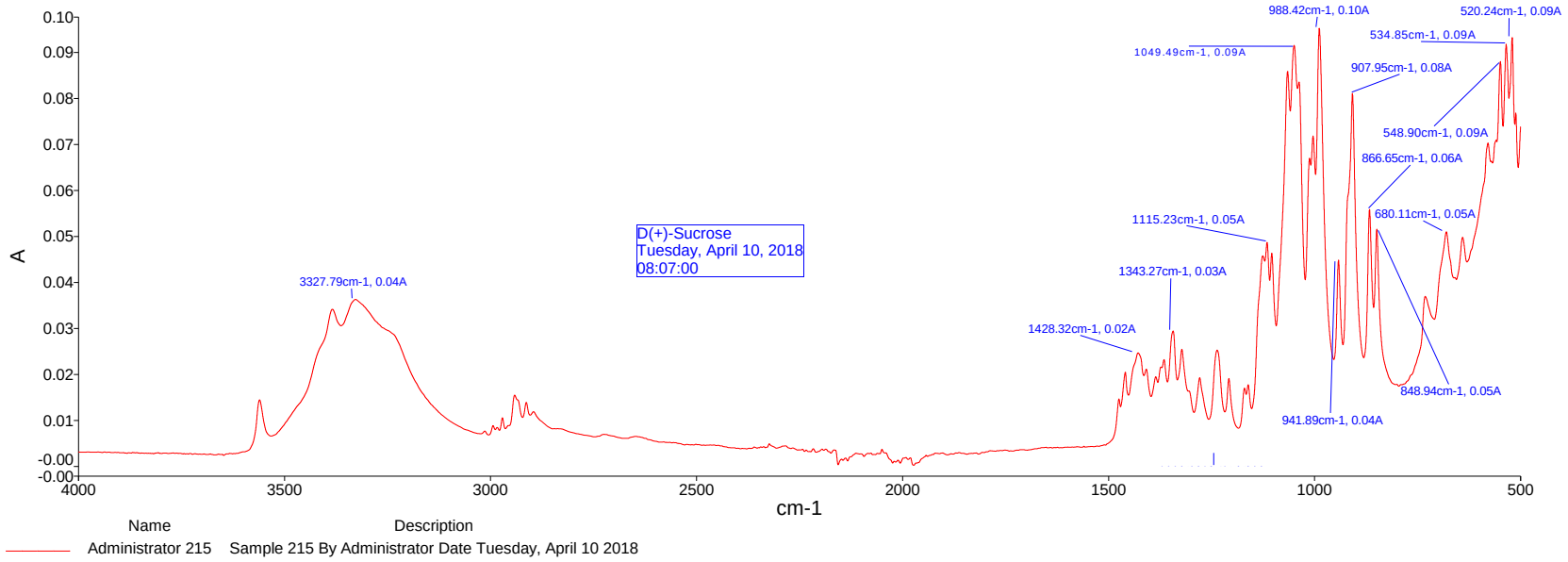

Figure A10. FTIR spectrum of Sucrose**.**

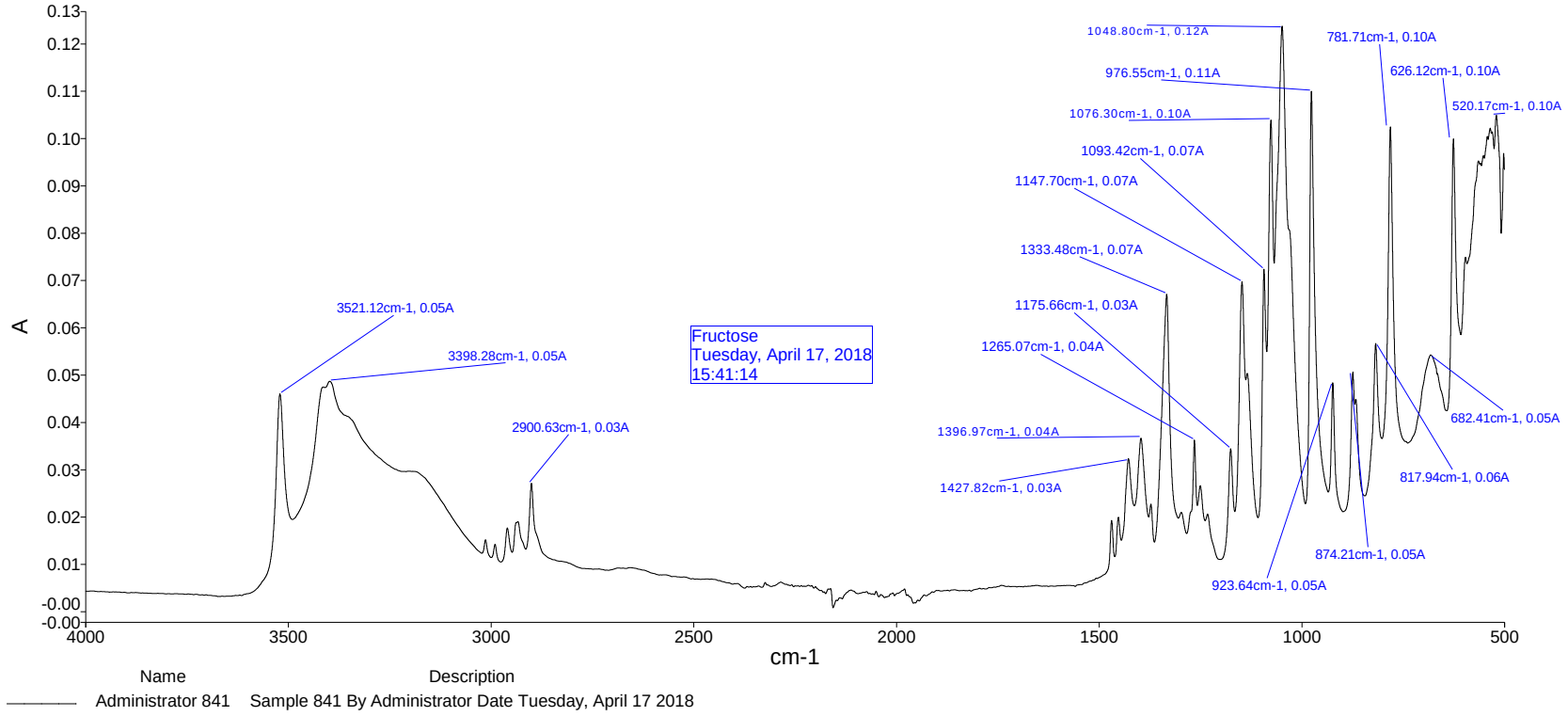

Figure A11. FTIR spectrum of Fructose.

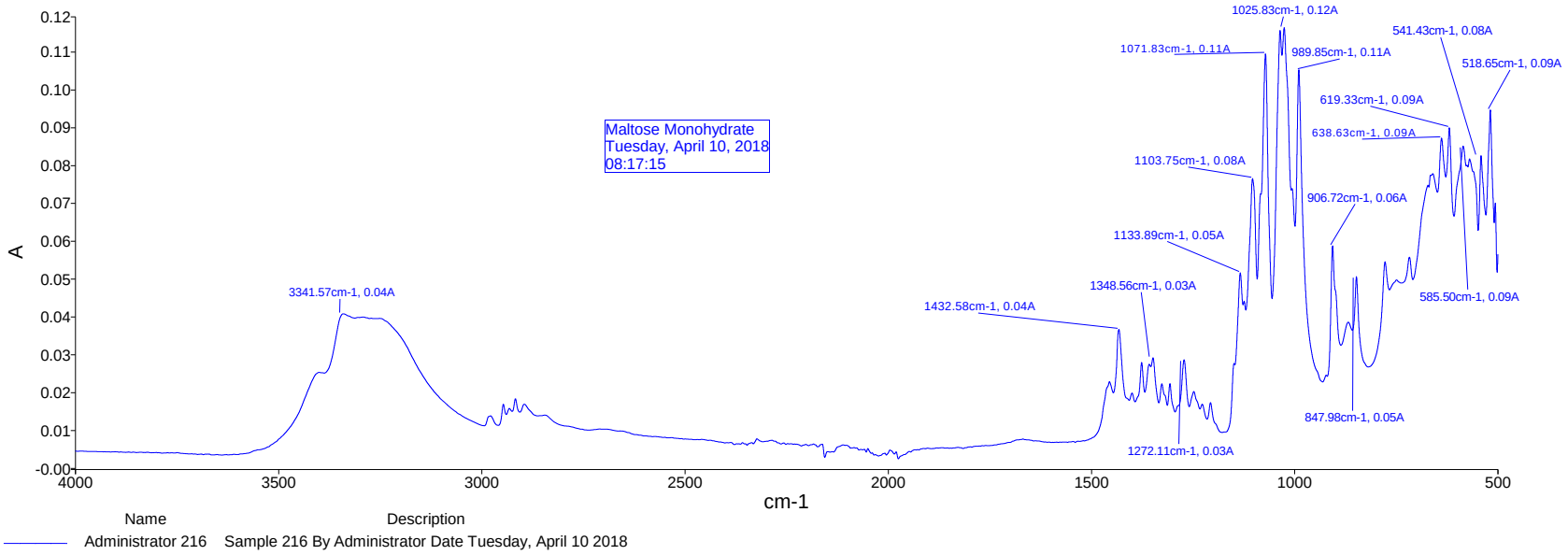

Figure A12. FTIR spectrum of Maltose**.**

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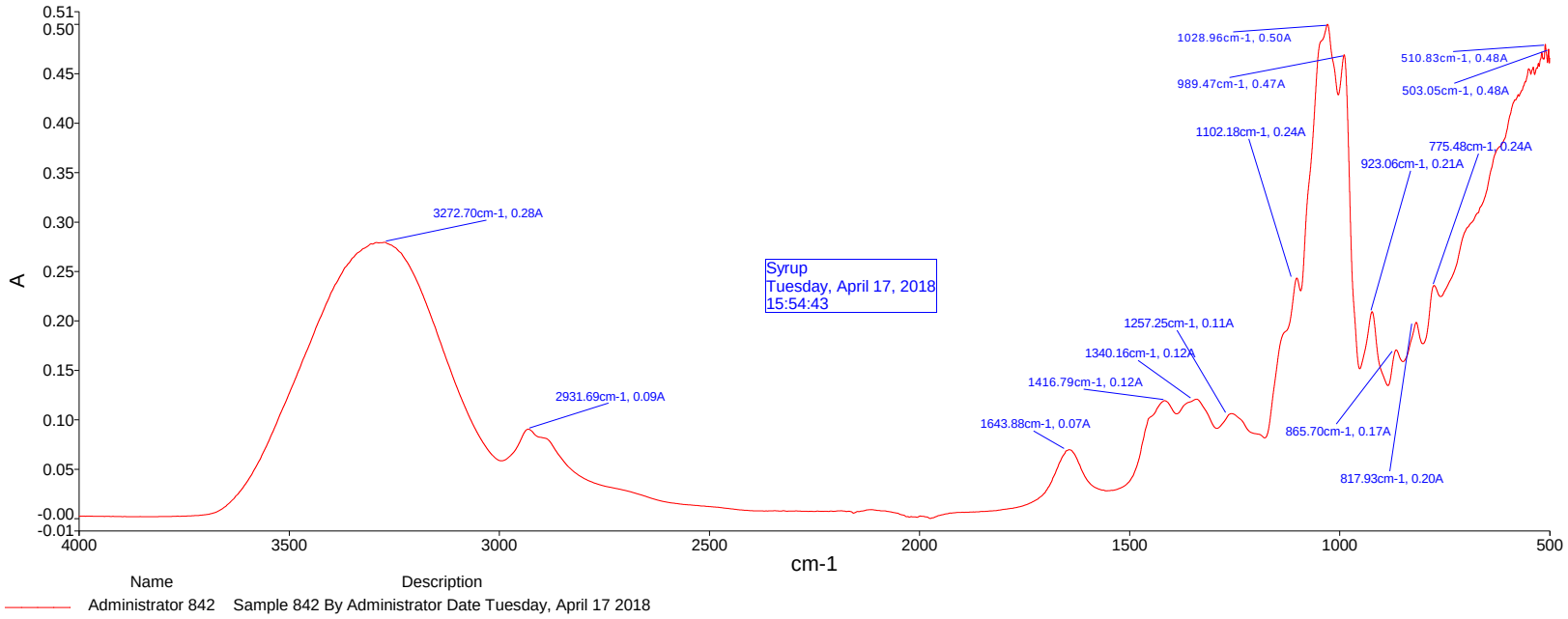

Figure A13. FTIR spectrum of Syrup.

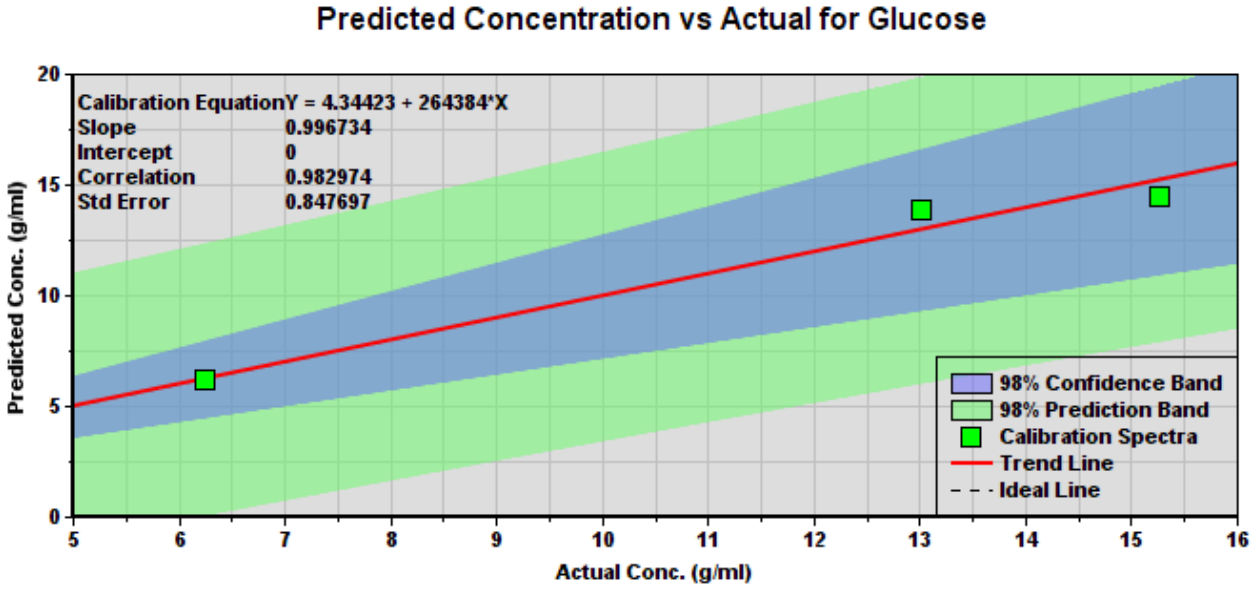

Figure A14. Calibration curve that is use to predict the concentration of glucose in honey.

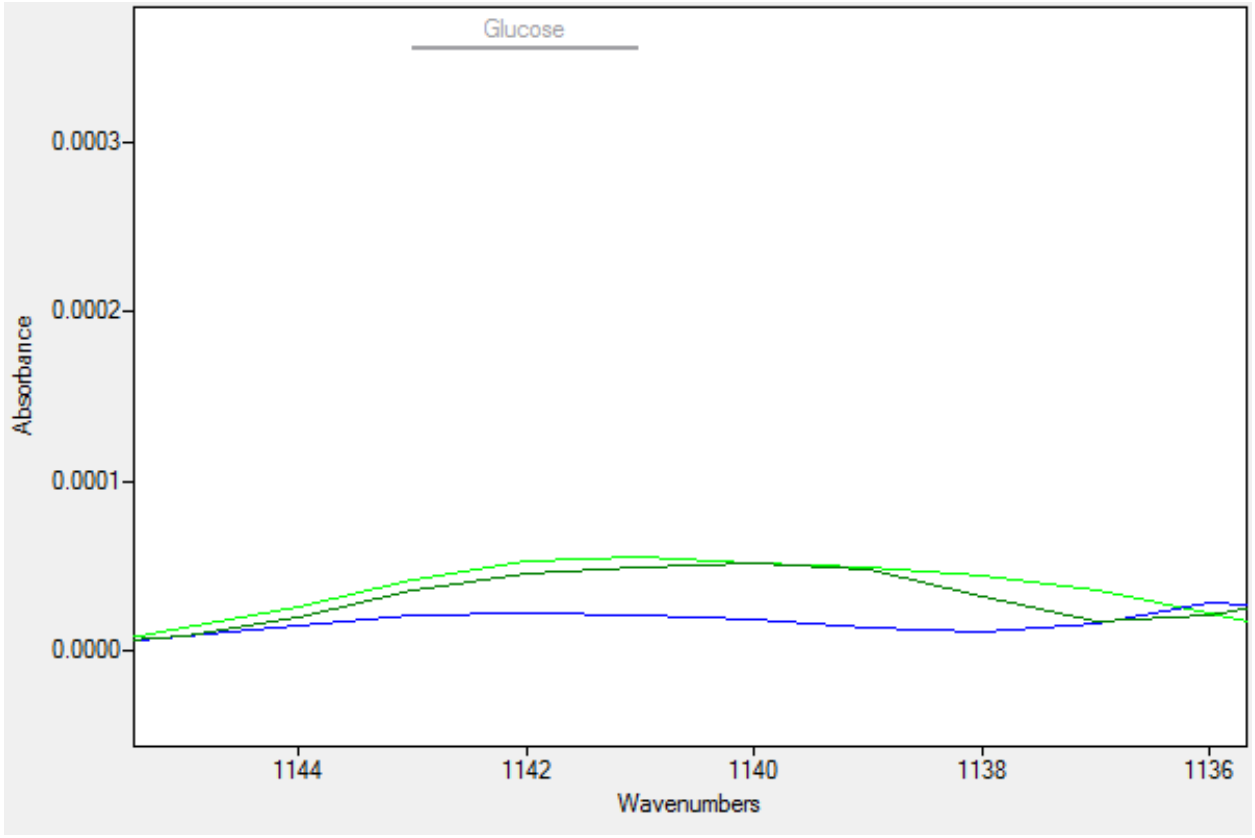

Figure A15. Illustrates the peak used to calibrate glucose.

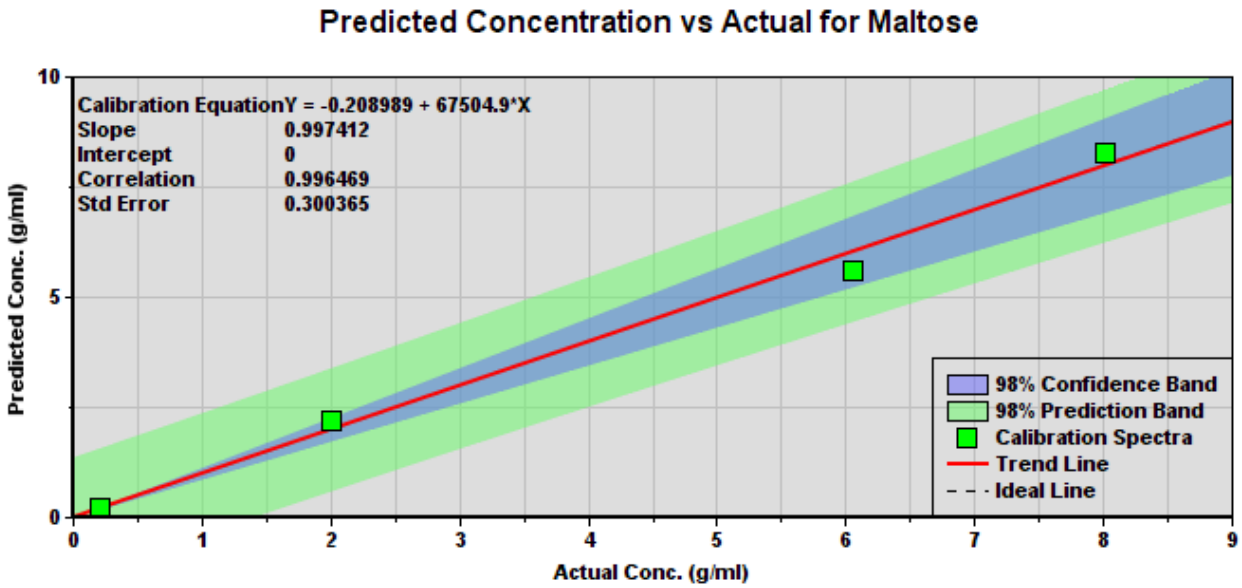

Figure A16. Calibration curve that is use to predict the concentration of maltose in honey.

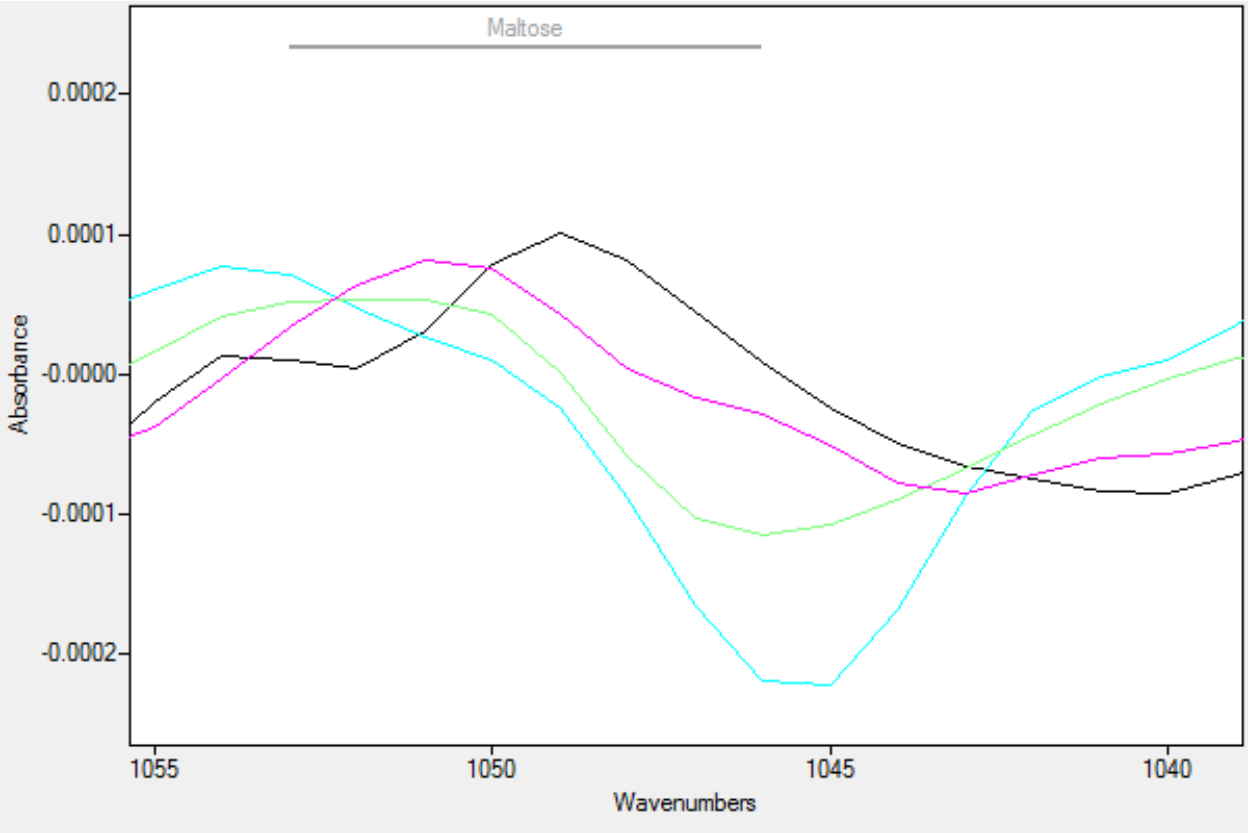

Figure A17. Illustrates the peak used to calibrate maltose.

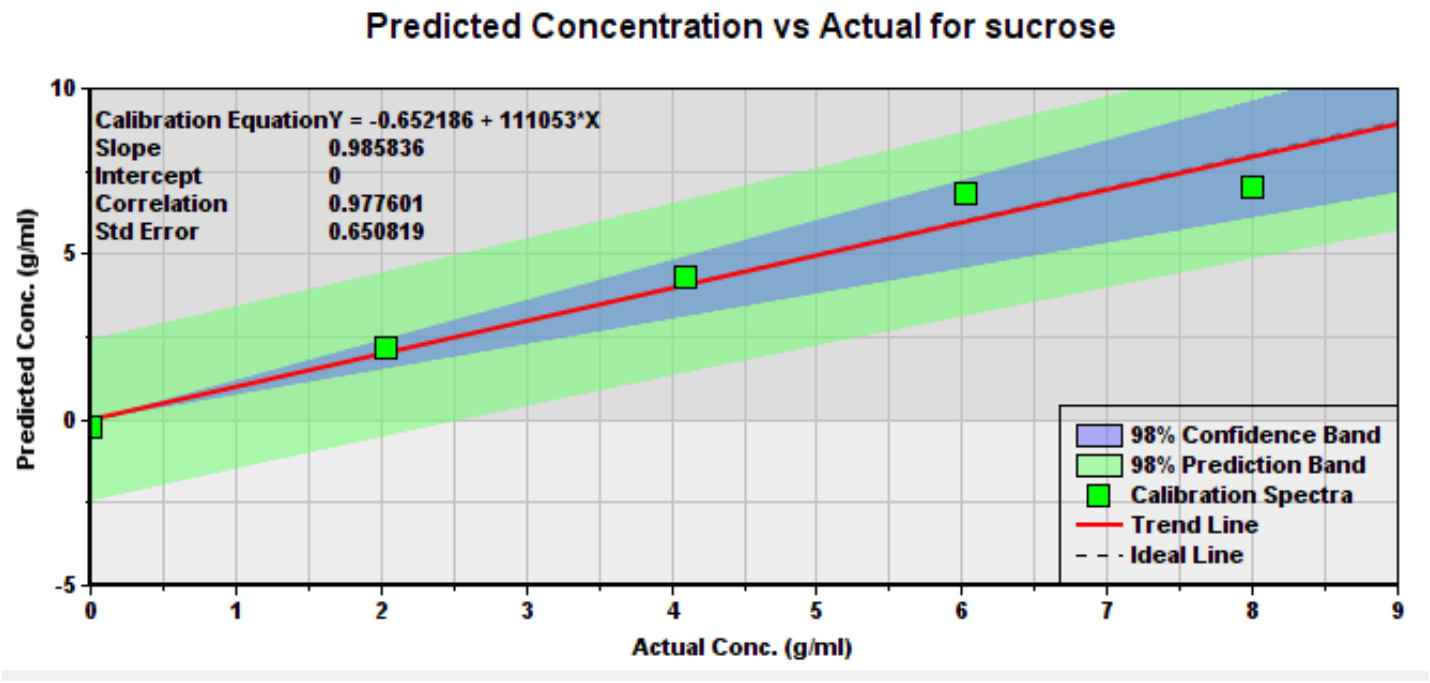

Figure A18. Calibration curve that is use to predict the concentration sucrose in honey.

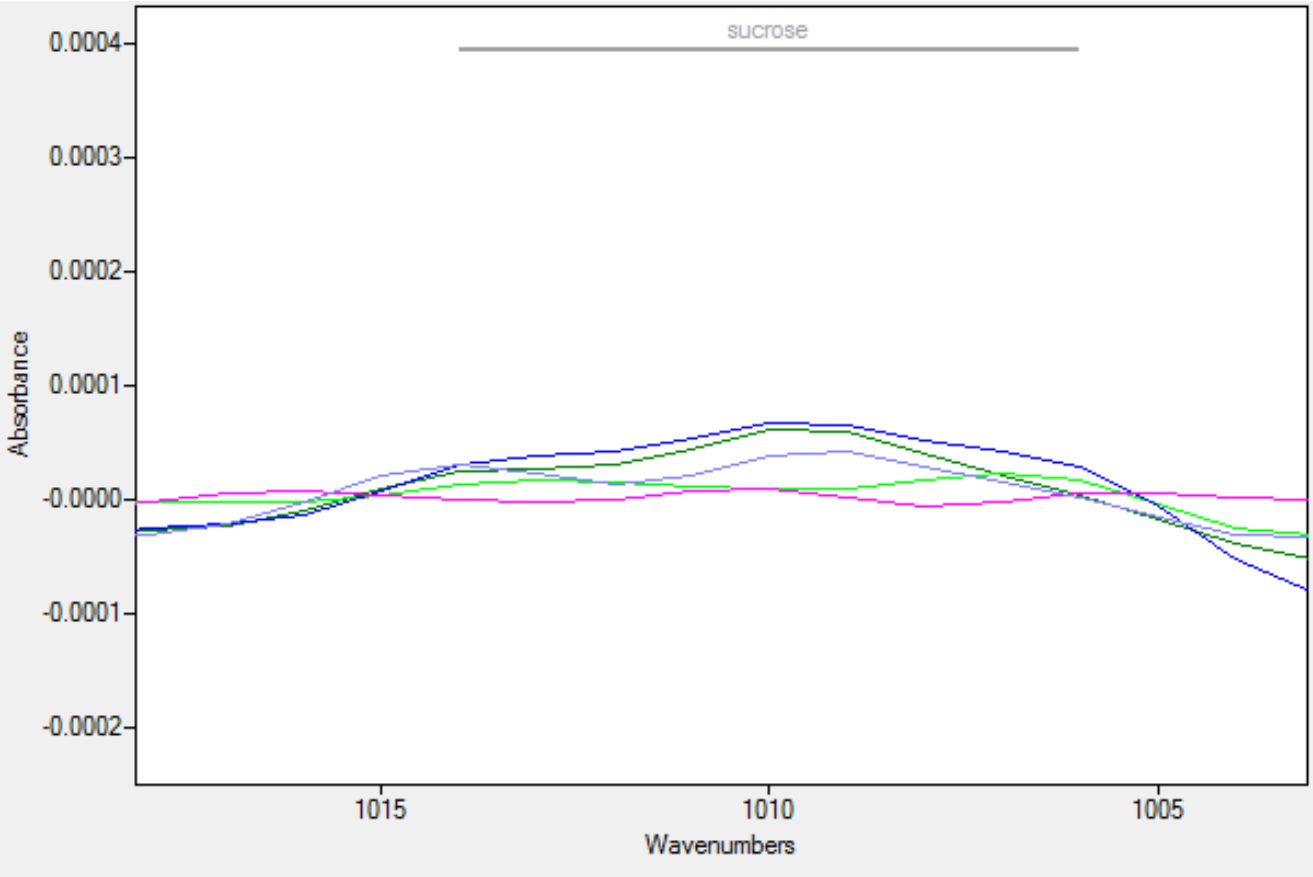

Figure A19. Illustrates the peak used to calibrate sucrose.