Quality Assessment and Detection of Adulteration Using Several Analytical Approaches in Commercially Available Honey Samples from Addis Ababa, Ethiopia

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DECLARATION

I declare that this thesis is submitted to the Graduate Program of College of Natural and Computational Sciences, of Addis Ababa University for the Master's Degree in Food Science and nutrition. I would like to prove through my signature below that it is my independent work and has not earlier been submitted elsewhere by me or anybody else. All authors of the references cited in the current study were duly acknowledged.

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This is to certify that, this thesis entitled "**Quality assessment and detection of adulteration using several analytical approaches in commercially available honey samples from Addis Ababa, Ethiopia**" submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in **Food Science and nutrition** to the Graduate Program of College of Natural and Computational Sciences, Addis Ababa University, through the Center of **Food Science and nutrition**, done by **Teferi Damto Tolcha** (I.D.No. GSR/7994/11) is an authentic work carried out by him under our guidance. The matter embodied in this project work has not been submitted earlier for the award of any degree or diploma to the best of our knowledge and belief.

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LIST OF ABBREVIATIONS AND ACRONYMS

AACA ……. …..Addis Ababa City Administration

ASCII…….American Standard Code for Information Interchange

AEAC……Ascorbic Acid Equivalent of Antioxidant content

ANOVA……Analysis of Variance

ARSD…….Apiculture Research Strategy Document

CSA ……...Central Statistics Agency

EHC……European Honey Commission

ESA…….Ethiopian Standard Agency

EU……European Union

FAO …….Food Agriculture Organization

FTIR ……Fourier-transform Infrared Spectroscopy

GAE…….Gallic acid Equivalent

HFCS……High Fructose Corn Syrup

HBRC……Holeta Bee Research Center

HPLC……High Performance Liquid chromatography

ILCA…….International Livestock Center for Africa

IHC……. International Honey Commission

M.a.s.l. ……The Meter above sea level

MIR……..Mid-Infrared

MoARD ……Ministry of Agriculture and Rural Development

OARI…….Oromia Agricultural Research Institute

PCA……. Principal Component Analysis

PLS…….Partial Least Square

QE ……. …Quercetin Equivalent

TFC……....Total Flavonoid Content

TPC ……Total Phenol Content

USDA……United State Department of Agriculture

UV-VIS ……....Ultraviolet-visible spectroscopy

AUTHOR BIOGRAPHY

The author was born on September 19, 1992, Yaya Gulale Woreda of North Shoa Zone, Oromia region from his father Damto Tolcha and his mother Shukkarre Nannessa. He attended elementary school education at Qare Tokke Elementary school from 2000 to 2007 G.C. Then he continued with secondary and Preparatory School education at Dire Dalati Secondary from 2008 to 2009 G.C and at Fital from 2010 to 2011 G.C respectively. Then he joined Jimma University on October 5, 2012, and graduated with a BSc degree in Post-Harvest Management and Food Technology on June 21, 2014, G.C. After graduation, he was employed at Addis Ababa city administration as a technical supporter and advisors of the manufacturing sector at Small and Micro Enterprise Development Bureau from November 16, 2015, to December 30, 2016, G.C. Starting from January 15, 2017 G.C he was employed by Oromia Institute of Agricultural Research and served as Assistant Researcher in bee product quality improvement and value addition at Holeta Bee Research Center until he joined Addis Ababa University for MSc study at the Center for Food Science and Nutrition on April 01, 2019. The author will serve the research center after completing his MSc study as per the agreement between the institute and the author.

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ABSTRACT

Honey is one of the most commercialized bee products. The study was carried out keeping in view the recently emerging concern of low quality and adulteration of natural honey with various substances to increase volume on the honey markets. This research was designed to evaluate the quality and detect adulteration from commercial honey available in the Addis Ababa market. For this purpose commercially available and deliberately adulterated honey produced by direct incorporation of different proportions of commercial adulterants were used. Preliminary quick test, characterization of physicochemical parameters, biochemical properties, and screening of spectrum by FTIR coupled with multivariate analysis were tested as alternative analytical methods for honey authentication and adulterations detection. The HMF, free acidity, and ash content of all commercial honey samples were found within the limit of the standard for honey. Except for the honey samples collected from processers (19.48±0.4%) and retail stores (20.49±0.13), the moisture content of other commercial honey samples didn't fulfill standards (≤ 21%). The level of proline for honey samples collected from the street (67.1±0.52 mg/kg) was found below the required limit. The fructose, glucose, sucrose, maltose content of the commercial honey samples were found to be in the range of 33.85±0.65 to 48.61±0.51, 33.07±1.58 to 44.3±0.82, 0.91±0.05 to 6.23±2.49, and 0.51±0.14 to 2.4±0.44 respectively. The TPC, TFC, and antioxidant activities of commercial collected samples are in a good quality. Deliberately adulterated honey has lower proline (213±9.43mg/kg) than marketed honey (270.83±14.18mg/kg) and pure honey (381mg/kg). Sucrose (5.23±2.23) and maltose (4.09±1.00) for deliberately adulterated honey was higher than pure and marketed honey. Preliminary quick test methods were used to detect adulterated honey, but these methods were found specific to adulterants materials. The proline and pH levels decreased as molasses, sugar, and banana adulterants increased, while increased as melted candy and shebeb adulterants increased. Moisture content decreased as sugar, melted candy, and shebeb adulterants were increased, while decreased as molasses and banana adulterants increased. HMF content increased as molasses, melted candy, and shebeb adulterants were increased. The sugar compositions are key differential criteria to detect the adulteration of honey with sugar. Another scope of the study was to differentiate adulterated honey samples from the natural one using FT-IR spectra coupled with principal component analysis (PCA) and cluster analysis (CA) and discrimination of sample groups was achieved successfully with clustering. In conclusion, it was observed that the results were

in agreement with standard values (Codex Alimentarius, EU, and Ethiopia Standard agency). But, some of the quality parameters of honey has deviated from national and international standards. This study successfully demonstrated a method to rapidly and accurately classify and authenticate honey. Accordingly, it is recommended that frequent training for stakeholders on adulteration detection methods should be carried to avoid adulteration of honey from the markets.

Keywords: Biochemical, Commercially available honey, Deliberately adulterated, Detection of adulterations, Multivariate analysis, Physicochemical, Quick test.

1. INTRODUCTION

1.1. Background

Natural honey is a very nutritious food product, which contains water (17%), saccharides (75%), amino acids, minerals, vitamins, enzymes (invertase, catalase, amylase), phenols, organic acids, pigments, volatile oils, and aromatic substances (Dela Fuente *et al.*, 2011; Ouchemoukh *et al*., 2010). The quality of honey is mainly determined by its sensorial, chemical, and physical, characteristics (Grigoryan, 2016; Finola *et al*., 2007**)**. Honey specifications developed by Codex Alimentarius Commission (2001) and Ethiopian Standard (2005) have used as a physicochemical criterion for honey characterization: namely, water content, ash, pH, electrical conductivity, hydroxymethylfurfural aldehyde (HMF), reducing sugar and sucrose as well as diastase activity and proline. The comparison of these honey quality standards with the naturally occurring values (Persano & Piro, 2004; Bogdanov *et al*., 2003) can hint about a possible adulteration. On the other hand, sensory evaluation enables us to identify qualified defects, such as fermentation, impurities, off-odors, and flavors (Piana *et al*., 2004).

Commercially available honey samples differ in quality on account of various factors like geographical, floral source, and processing conditions (Sulieman *et al*., 2013). People think that honey is a raw product extracted from the honeycombs, which can be consumed directly, but most of the honey available in the market is the processed one (Akpabli, 2015).In commercial honey processing, the applications of heat are an important operation and are known to have a potential for eliminating spoilage microorganisms, facilitating packaging, and delaying crystallization (Subramanian *et al.,* 2007). However, heating honey to higher temperatures of more than 70 ^{o}C is not recommendable, because it causes alteration of flavor, color, granulation of honey and produces HMF; also degrades bioactive compounds including antioxidants which result in product quality deterioration (Visquert, 2004).

The adulteration of honey is a serious, widespread problem and is a global concern. In developing countries, it has a substantial economic and nutritional, and health impact on consumers (Guler *et al*., 2014). Due to a lack of detection methods, policies, and awareness of peoples, the adulteration is not obvious for common users. Adulterated honey is commonly labeled as natural and the priced is identical to pure honey, which is dishonorable and unfair to the customers (Chen *et al.*, 2011) and had been common among local honey production and

retail in Ethiopia(Yeserah *et al*.,2019). Adulteration with table sugar, water, molasses, shebeb, ripened banana, coca-cola, wheat flour syrup, potato, sweet potato, maize syrup, pollen, empty combs, and melted candy during processing is reported in Ethiopia (Ambaw & Teklehaimanot, 2018; Gemeda & Negera, 2017). Generally, consumers are unaware of the quality of honey they buy, so it is necessary to monitor the quality of commercial honey, due to the aforementioned cases of adulteration (Iftikhar *et al*., 2014). For this reason, detection of adulterant materials from honey has become a very important issue for processors, retailers, and consumers as well as regulatory authorities (Chen *et al*., 2011).

At present, a variety of analytical techniques have been developed to detect adulteration in honey, but three approaches can be distinguished for the utilization of these techniques (Cordella *et al.,* 2002). The first approach to is detect significant deviations from expected values in the concentration of naturally occurring components. An alternative approach to demonstrating that a foreign component (a marker) is present in the honey, which could be a chemical constituent (complexes, molecules) that proves either the adulteration or authenticity of the honey. The third possibility to the detection of adulteration method consists of using analytical techniques derived from a physical and chemical analysis by considering the whole of the sample to show the effects of the adulteration on the properties of the sample (Cordella *et al*., 2003). Detection of adulteration is typically supported on composition and structure (molecular mobility) or physical properties measured by molecular refraction, differential thermal analysis, chromatography, stable isotope methodology, trace elements techniques spectroscopy (Yimaz, 2014; Tomaszewska & Kijowski, 2010). Although these methods are useful to assess the adulteration of honey, some of them are time-consuming, and can be expensive, requiring the development of fast, non-destructive, easy to use, and sensitive analytical methods.

Due to its varied ecological and climatic conditions, Ethiopia is home to a number of the foremost numerous flora and fauna in Africa. This has favoured the existence of 10 million honey bee colonies of which 7.5 million are confined in beehives and the remaining exist in the forest (Adgaba, 2007). As a result, the country is the leading honey producer in Africa (Demewez *et al.,* 2012). According to CSA (2011), the total annual honey production was estimated to be 53000 metric tons. However, the bulk of honey is crude and poorly managed. Honey has good quality as long as it is in the hive, but faulty handling from the time of its harvest until it reaches to market is responsible for its inferior quality (Crane, 1975).

Ethiopia is an advantageous country known for high production and exporting honey, Addis Ababa is the capital city of the country where different sources of honey found. Numerous supermarkets, shops, local markets, small, and retail stores (big honey verandas), in the city serve as a place for honey markets. Even if the central honey markets are Markato (one of the largest open markets in Africa) and Shola market, which are not well organized; a large volume of honeys are marketed in the different areas of the sub-city. Most of these honey samples are traded without quality sign or reference to their origins and this may lead to honey adulteration and marketing of non-standard honey. There are reports of a decline in the quality of the honey in the open market, since honey being sold commercially as genuine products. It is important to do the quality testing of commercial honey due to increasing problems related to adulteration and tampering with natural honey sold in the market. To date, there has not been any information on the quality and adulteration status as well as the mechanism of detection of adulteration of the honey sold in the open-air markets in Ethiopia especially in Addis Ababa, in which this study covered.

1.2. Statement of the problem

According to Krell (1996), the quality of honey is an important aspect for both domestic and international markets, as it helps to achieve competitive premium prices and promote human health. There have been serious reports of poor quality honey on the market and their safety is also increasingly being questioned (Kugonza &Nabakabya, 2008). There is a common perception that honey which is sold in markets of Ethiopia is of poor quality and is suspected of adulteration with different adulterants to increase quantity thereby maximizing profit from sales in the market. Such kinds of honey may have poor physicochemical, organoleptic, and hence poor functionality. Reports have indicated that such adulterated honey does not have any nutritional and medicinal values (Bogdanov & Gallmann, 2008). Recent evidence by Legesse (2014) suggested that actors of honey value chains are likely to unhygienically handle the process of honey, intentionally adulterate, and possibly contaminate the honey, making them unsafe and also of low quality.

Unfortunately, not much work has been reported on the quality and adulteration detection techniques especially for commercially available honey in Ethiopia, with exception of few studies on local traditional tests practiced by beekeepers and customers (Ambaw & Teklehaimanot, 2018; Gemeda & Negera, 2017; Gebremariam & Brhane, 2014). The studies

done on the Ethiopian honey were focused on the physicochemical and botanical origins of the honey sample (pH, free acidity, ash content, color, electrical conductivity, HMF, sugar content) (Ambaw & Teklehaimanot,2018; Mulugeta *et al.*, 2017; Belay *et al.*,2016; Tesfaye.,2016; Gebru,2015; Gebremariam & Brhane,2014; Yadata,2014 ;Gobessa *et al*. 2012; Kebede *et al*.,2012; Kinati *et al*.,2011; Alemu, 2010; Tsegga,2009). At present, there is no enforcement by authorities to prevent adulterated honey from regulating in the market in Ethiopia as there is a lack of data on the honey to identify, characterize and differentiate between natural and adulterated honey**.** Beekeepers, honey processors, and customers heavily rely on their own experience and observation to determine whether the honey meets the quality requirement. For the above and many other reasons, it is very important to investigate and understand the detection of honey adulterants by comprehensive multicomponent analysis approach. To this effect, this study aimed at the authentication of quality and determination of adulteration in honey based on the quick test method, physicochemical, biochemical markers and using analytical techniques like HPLC, UV-VIS, and FTIR combined with multivariate analysis for the consumers or regulatory authorities to check before buying or prior approval of honey to be sold in the Addis Ababa markets areas in Ethiopia.

1.3. Objective of the study

1.3.1. General objective

To characterize the quality and detect adulteration of honey samples collected from the Addis Ababa market using several analytical approaches.

1.3.2. Specific objective

The specific objectives of these thesis were to:

- ✓ Assess the physicochemical and biochemical properties of commercially available honey.
- ✓ Compare the physicochemical and antioxidant properties of pure, marketed, and deliberately adulterated honey samples.
- ✓ Detect the adulteration of honey samples based on quick test methods.
- ✓ Detect the adulteration of honey samples based on their physicochemical and biochemical markers.
- ✓ Detect the adulteration of honey based on FT-IR conjugated with multivariate techniques.

2. LITERATURE REVIEW

2.1. Definition of honey

According to Codex (2001), honey is the natural sweet substance, produced by honeybees from the nectar of plants or secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature. Honey consists essentially of different sugars, predominantly fructose and glucose as well as other substances such as organic acids, enzymes, and solid particles derived from the honey collection and shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey (EU, 2002). Honey shall not have any objectionable matter, flavor, aroma, or taint absorbed from foreign matter during its processing and storage. The EU directive for honey (2002) has a similar definition, on honey produced by *Apis mellifera* and listing similar analytical criteria. Some analytical criteria regarding the composition are moisture, sugar content, water-insoluble solids, free acidity, diastase activity, hydroxymethylfurfural, sugar, and electrical conductivity.

2.2. Composition of honey

Honey is an excellent source of energy-containing approximately 80 g/100 g carbohydrates (35 g/100 g glucose, 40 g/100 g fructose, and 5 g/100 g sucrose) and 20 g/100 g of water which is quantitatively the second most important component of honey (Rodriguez *et al.*, 2004). Besides, honey contains more than 200 substances including organic acids such as acetic acid and gluconic acid (Rodriguez *et al.*, 2004). Vitamins and minerals are present in a very small quantity, particularly iron and copper which are responsible for the redox properties of honey and potassium, being the most abundant. Honey also contains trace amounts of niacin, calcium, copper, riboflavin, iron, magnesium, and zinc (Kumar *et al*., 2010). Among honey, constituents are also amino acids, hydroxymethylfurfural, and phenolic compounds. Flavonoids present in honey are comprised of flavanones, flavones, and flavonols while phenolic acids are substituted cinnamic acids and benzoic acids. These compounds are the main contributors to the color, taste, and aroma of honey (Karabagias *et al*., 2014; Ferreira *et al*., 2009). The diversity of the physical and chemical properties of honey depends on the

nectar and pollen of the original plant, color, flavor, moisture, and contents of protein and sugars (White & Maher, 1980; White, 1978).

Table 1.Chemical composition of honey (g/100 g)

Constituents (%)	Mean	Range
Water	17.9	13.21–26.50
Fructose	39.44	37.07–42.65
Glucose	28.15	18.20–32.10
Sucrose	3.19	0.36–16.57
Other sugars	8.5	0.1–16.0
Minerals	0.36	0.11–0.72
Total protein	1.13	0.22–2.93
Acids (gluconic acid)		0.17–1.17
Vitamins, enzymes, aromas	<0.1	
Phenolic compounds	0.1	0.02–0.2
pH value	3.9	
Ash	0.169	

(adapted from Solayman *et al.* 2016; Bradbear, 2009; Bogdanov *et al.*, 2008; Perez, 2002; Terrab *et al.*, 2002).

2.3. Honey quality

According to the European Council (2002) concerning honey, the general and specific characteristics which are important in the assessment of quality and indirectly its authenticity are related to moisture, free acidity, electrical conductivity, diastase activity, sugar, and hydroxymethylfurfural (HMF) content. In these regulations, sensory and physicochemical properties of honey are established by setting the minimum or maximum amount related to maturity, purity, and deterioration parameters for honey. Concerning maturity, the regulation evaluates sugar content and moisture; for purity, it analyses ash content, electrical conductivity, and insoluble solids in water (Madejczyk *et al*., 2011).

The quality of honey and its specific character are determined by the specific flora and vegetation in the area from which the honey originates and the diversity of the ecosystem in which the bees are kept, specifically in non-industrial areas (Zabrodska, & Vorlova., 2014). The quality and biochemical properties of honey are related to honey maturity, production methods, climatic conditions, processing and storage conditions, as well as the nectar source of the honey (Oddo & Bogdanov, 2004; Bogdanov, 1999, Crane, 1980). However, the quality and composition of honey are negatively affected by other factors such as overfeeding with

sucrose, harvesting before maturity, unhealthy storage conditions, and overuse of veterinary drugs (Oddo and Bogdanov, 2004, Sahinler *et al*., 2004).

Honey is generally evaluated by a physicochemical analysis of its constituents. These constituents influence the storage quality, granulation, texture, flavor, and the nutritional quality of the honey and are also responsible for the medicinal quality of honey. The identity and quality parameters of honey are considered useful for detecting these possible adulterations and also for confirming the hygiene conditions for the manipulation and storage of honey (Puscas *et al,* 2013). Ethiopian honey differs in color, taste, quality, and quantity produced (Beyene & David, 2007). According to a report by Beyene & Verschuur (2014), inadequate production knowledge and poor post-harvest handling system often result in a poor quality of honey. Excessive using of smoking materials during honey harvesting and inappropriate storage containers are the main problems in honey quality.

2.4. Honey market

In Ethiopia, a high portion of honey is sold for income generation. The domestic honey market starts at the smallholder bee keeper's level, which majorly sells crude honey to collectors in the nearest town/village markets (Abebe, 2009). Beekeepers of the country sell the largest proportion of their honey during harvest at low prices mainly to meet their demand for cash to pay taxes, debts, and other social obligations. Similar authors notified that the price of honey is also governed by different factors such as distance from the market (28%), quality of honey (25%), consumer's preference (20%), the color of honey (15%), and test of honey (12%). About 90% of honey is sold for income generation and of this, about 70% is used for 'tej' brewing.

The bulk honey market is characterized by low-priced honey of fair to good quality, but distinct quality differences exist (Beyene & David, 2007). Moreover, Tadesse and Gebregziabher, (2014) revealed that all honey samples obtained from apiary sites and many commercial samples collected from local markets in northern parts of Ethiopia are of good quality and met the limits of the national and international standard. The physicochemical test results for some honey samples collected from local markets had a higher level of certain parameters than recommended suggesting some level of adulteration is practiced by few honey traders. Molan, (1996) reported that most of the honey available in the market has been processed. Usually, it has been pasteurized to destroy microorganisms that occur in honey and

prolong the shelf-life, or to delay crystallization and in some cases process honey by microfiltration, because consumers prefer honey to be liquid and clear.

The quality of Ethiopian honey available on the market varies due to various factors, such as geographical, floral sources, seasonal, processing, and storage conditions (Teferi, 2018). The market for honey in Ethiopia is generally not well developed, mainly due to a limited number of buyers relative to the number of producers (suppliers), poor market infrastructure, and information (Amanuel,2011). Honey and other apiculture products (i.e. beeswax, propolis, pollen, royal jelly, and bee venom) are among the growing export commodities with good potential for several African countries. But, the global honey market offers huge opportunities for Ethiopian honey (MoARD, 2007).

2.5. Production of honey

Honey production is one of the direct contributions of beekeeping practices (ARSD, 2000). In terms of economic contribution and export commodities, honey is one of the marketed livestock products of Ethiopia. According to Bogdanov *et al*. (2008) on average, the total honey production per year in the world is estimated to be 1.3 million tonnes. About 47% of the total world‘s honey production is produced by developing countries (Gebretsadik & Negash,2016). With the huge resources in the country coupled with the ancient culture of the people of the country, Ethiopia is the largest honey-producing country in Africa and one of the top ten countries in the world (CSA,2012; MoARD, 2007) and varieties of honey are shipped all over the world. According to the CSA (2011), the annual total production of honey accounts for 53000 metric tons. Ethiopia's honey production accounts for approximately 2.5 % of world production and 21.7% of African honey production (Fikru, 2015). The majority which is above 80% of the honey produced is used for honey winemaking and the rest 30 % and less is used for local consumption and little export (CSA, 2012; Amanuel, 2011; Beyene, & David, 2007).

The honey produced in Ethiopia is expected to become a major commodity for acquiring foreign currency to improve the Ethiopian economy. The country already earns an average of 420 million ETB annually from the sale of honey (Gidey & Kibrom, 2010). The major destination market for Ethiopian honey and beeswax includes Germany, Norway, Sudan, USA, UK, Japan, etc (Eshete, & Eshetie, 2018). Productivity from traditional hives is very low, with an average of 5-6 kg per year; while production from improved hives (including

transitional hives) reaches levels of 18- 30 kg per year (MoARD, 2007). Honey production is declining due to high labor costs and low profits from the honey business. Therefore, to overcome this decline pure honey is adulterated with different adulterant materials (Anthony & Balasuriya, 2016).

2.6. Physicochemical properties of Ethiopian honey

The physicochemical properties of given honey are influenced by the nectar types that the honeybee used, bee species, agroecology (climatic and soil), and post-harvest honey handling practices. Honey is generally evaluated by physicochemical analysis of its constituents, including moisture, reducing sugar, sucrose, water-insoluble, ash, free acidity, hydroxymethylfurfural, pH, diastase activity, and electrical conductivity (Khalil & Suhaliman, 2010; Mairaj *et al*., 2008). Therefore, analysis of the physicochemical properties of honey is used to verify the genuineness of the product and to tell the possible presence of artificial components or adulterants (Belay *et al*., 2013; Meda *et al*., 2005). Some of the physicochemical properties of honey are indicated in Table 2.

2.6.1. pH

Honey pH has great importance during the storage of honey, as they influence the texture, stability, and shelf life of honey (Terrab *et al*., 2004). In Ethiopia, the pH of most honey samples is studied by many authors as shown in Table (2). Based upon the research pH of Ethiopian honey samples was found to be acidic. The variations observed in the honey samples may be attributed to the presence of different acids found in different floral types (Gebreegziabher *et al.*2013). The storage factor and temperature contribute to the low pH value in honey. The pH determination could be also correlated with other authenticity parameters to verify the adulterations and index of possible microbial contamination (Yadeta & Kebede, 2014). Therefore, the low pH of Ethiopian honey confirms that it well inhibits the presence and growth of microorganisms.

2.6.2. Free acidity

Free acidity may be explained by taking into account the presence of organic acids, which are proportional to the corresponding lactones, or internal esters (Finola *et al*., 2007). The high acidity of honey is an indication of the fermentation of sugars present in the honey into organic acid (Ouchemoukh *et al*., 2007). The free acidity of the Ethiopian honey sample is shown in

(Table 2). The reported data indicated that the majority of free acidity in the different parts of Ethiopia shows a good characteristic. However, the variation observed between honey samples may be due to the presence of different organic acids, geographical origin, harvest, and storage conditions as well as adulteration with water (Tornuk *et al.,* 2013). Although the acidity of honey is desirable, when the acidity increases very much, the honey becomes sour. The acceptable limits for free acidity values (≤40meq/kg) set by ESA and CAC, whereas the limit for honey acidity is ≤ 50 meq/kg according to EU (2002). Free acidity below the acceptable limit indicates the freshness of the honey samples and the absence of unwanted fermentation in the analyzed honey samples.

2.6.3. Ash content

Honey normally has low ash content and it depends on the materials collected by the bees foraging on the flora (Gairola, 2013). The ash content of honey from Ethiopia is reported by different authors as shown in Table 2. At the national level, the mineral content of honey ranges from 0.01 to 1.16% with a mean value of 0.23% (Adgaba, 1996). The maximum limits set for ash content of the honey by EU, CA, and ESA is (0.6%). Although the majority of the ash content value of Ethiopian honey conforming to the requirements set by Ethiopian standard, EU, and Codex, the values of ash content above standard limits are also reported. The mineral content variation is related to the geographical areas, seasons, and botanical origin of the honey (Gobessa *et al*., 2012). Many authors reported the variation in ash content comes from the natural property of soil and protein. In addition, the variation comes from improper handing from farm to fork in addition to environmental biodiversity. There is usually a positive correlation between the color, mineral content, and electrical conductivity of honey (Karabagias *et al*., 2014). A high value of electrical conductivity also indicative of environmental pollution across the industry with heavy metals, pesticides, herbicides, insecticides, and sometimes the addition of substance contain high mineral to honey (Nanda *et al*., 2003).

2.6.4. Hydroxyl methyl furfural (HMF)

HMF is a break-down product of fructose formed slowly during storage and very quickly when honey is heated. The HMF content of Ethiopian honey samples is presented in (Table

2). According to ESA (2005) and Bogdanov (2002), the maximum limit of HMF content in honey is 40 and 80 mg/kg respectively. HMF is present only in trace amounts and its concentration increases with storage and prolonged heating of honey (Bogdanov, 2011). Doner (1977) indicated that the higher values of HMF also point towards the possibility of honey adulteration by invert syrup. Therefore, HMF level is not only indicative of honey freshness but also of storage conditions, overheating and it is also a major honey quality factor that indicates adulteration.

Table 2. Physicochemical properties of Ethiopian honey

Place	RS (%)	S (%)	MC	FA(meq kg -1)	pH	EC (µs cm^{-1})	Ash(%)	HMF(mg/ kg-1)	Reference
Adigrat and surrounding areas	50.31-79.56	2.24-12.2	17.5-22.6	3.99 -45.17	3.4-4.65	0.13 -0.56	0.09 -0.54	8.32-45.26	(Gebremariam & Berhane,2014)
Bale Natural Forest	51.28 -69.2	3.01-7.62	14.6-22.8	13 -46	3.3 -4.85	0.22 -1.34	0.14 -0.3	27.10-40.8	(Tesfaye,2016)
Homesha,Western Ethiopia	62.0-71.0	4.4 -12	15.0-18.16	18.0-36.54	3.52 -4.46		0.014-0.31	0.5 -3.2	(Gobessa *et al* 2012)
West Shewa Zone, Oromia Region	61.38-72.87	6.84-15.9	16.6-18.6	7.42-13.8	3.77-4.22	0.384-0.65	0.03-0.095		(Mulugeta *et al.*, 2017)
Sekota, Northern Ethiopia	63.4-71.7	1.0 -5.2	13.9-17.7	10-38.98	3.55-4.75		0.01-0.52	0-2.5	(Alemu *et al*., 2013)
Gomma Woreda of South-Western Ethiopia	61.15-77.41	0.75-6.96	15.6-23.5	0.30-57.30	3.45-4.18		0.05-0.60	0.05-17.70	(Kinati.,2011)
Ethiopian Monofloral	-	1.1-2.8	14.4-20.5	20-55	3.4-4.6	0.14-0.58	0.20-0.39	40 max	(Belay *et al., 2016)*
Ethiopian Standard	65	10	21 max	40		≤0.8	≤0.6		EQSA (2005)

RS (%)=Reducing sugars; S(%)=sucrose; MC=moisture content ; FA (meq/kg^{-1})=Free acidity, EC=Electrical content($ms\ cm^{-1}$), W.I(%)=Water-insoluble; HMF(mg/kg^{1})= Hydroxymethyl furfural aldehyde; SNNP=Southern Nation Nationalities of people

2.6.5 Moisture content

The moisture content of honey is the most important quality parameter because the rate of fermentation and its shelf life span is greatly determined by moisture content (Perez-Arqillue *et al*., 1994). Honey only with less than 18% water can be stored with little or no risk of fermentation, however, honey with over 19% water will ferment (White *et al.,* 1962). As shown in (Table 2) the moisture content of Ethiopian honey is reported in the different previous studies. The maximum and minimum limit of moisture content for Ethiopian honey so far analyzed is 32% and 23% respectively (Adgaba, 1999). However, the maximum acceptable moisture content of honey reported by the International Honey Commission is 20% (Bogdanov, 2002). This parameter is a major problem in Ethiopia honey because the majority of honey is harvested from traditional beehive by traditional harvesting and processing technique with a lack of beekeepers knowledge of honey quality. The problem does not only arise from beekeepers, collectors and retailers were also lack knowledge on how to handle the honey and how to protect the quality collected from beekeepers. Within these factors, the moisture content of Ethiopian honey becomes high as compared to the world standard.

2.6.7 Reducing sugar

The reducing commonly found in honey are fructose and glucose. The reducing sugar content of Ethiopian honey is shown in Table 2. Adgaba (1996) reported reducing sugar in Ethiopian honey accounted for about 70% on an average. Silici (2002) also reported the average for reducing sugar was 67.60 % and 69.47% in Turkey and US honey samples respectively. The variation in the percentage of reducing sugar in Ethiopian honey might be because of the ripeness and adulteration of honey (Belay *et al*., 2013). Maurizio (1959) reported that the sugar profile of honey depends upon the sugar and enzymes present in the nectar. Total reducing sugar contents in all honey samples are within quality requirement limits (≥65%) (ESA; CAC; EU).

2.6.8. Sucrose content

Sucrose is non-reducing sugar found in honey. As shown in Table 2 the sucrose content of Ethiopian honey varied across data reviewed and some values shown above the requirement set by Ethiopian standard, Codex, and EU. The slight excess value of sucrose content of honey might be due to adulteration by the addition of commercial sugar to honey and harvesting of

unmatured honey. There is an indication that the amount of sucrose in honey differs also according to the nectar compound. Adgaba (1999) reported the mean values of sucrose content average are 3.6%. According to the international standard, it should not exceed 5g/100g, and above these levels indicates that honey is adulterated with sugar or sucrose.

Table.3 Compilations of articles from different continents regarding the identity and quality of honey parameters.

Physicochemical parameters									**Country**	**Reference**
Sugar(g/ 100g)			pH	F.A(meq/kg)	Ash (g/100g)	EC(ms/cm)	HMF(mg/kg)	MC(g/100g)		
Fru	Glu	Sucr								
			3.8	6.25	0.2	0.488	ND	18.5	Ethiopia	Andualem, 2014
			3.75	32.43	0.21	0.69	36.35	18.80	Ethiopia	Tesfaye *et al*., 2016
			4.24	18.67	ND	0.207	13.75	11.74	Nigeria	Nweze *et al*., 2017
			3.33-5.54	16.05-34.1	ND	0.143- 2.006	0.58-4.25	15.56-18.15	Turk	Kivrak *et al.,* 2017
37.75-41.40	28.8-37.30	0.15-1.43		20.10 - 35.20		0.24- 0.99	5.36 – 15.00	15.4– 17.38	Spain	Manzanares *et al*., 2014
39.44-42.42	29.3-33.0	0.47 1.86		10.69 – 30.74		0.31– 1.12	7.16 – 30.43	14.6– 18.59	Morocco	Chakir *et al.,* 2011
33.30 38.60	21.0– 26.35	0.12-0.50		23.60 – 45.50			2.80 – 7.40	17.1-20.50	Brazil	Moreira *et al.,* 2007
		5-10	3.2-4.5	50	0.6-1	0.26-0.84	40-80	18-23	World standard	Bogdanov(1999)

EC (ms/cm): electrical conductivity in ms/cm; HMF (mg/kg): Hydroxymethyl furfural in milligram per kilogram of honey; MC (g/100g): moisture content in gram per 100 gram of honey, Free acidity (meq/kg): mill equivalent of free acidity per kilogram of honey, Ash (g/100g): Ash contents in gram per 100g of a honey sample, Suc (g/100g): sucrose in gram per 100 gram of honey sample, Gluc g/100g: Glucose content in gram per 100 gram of honey sample, Fru g/100g: Fructose content in gram per 100gram of honey sample

2.6.9. Proline

Proline originates mainly from the salivary secretions of honeybees during the conversion of nectar into honey. Proline represents a total of 50 to 85% of amino acids (Truzzi *et al*., 2014; Iglesias *et al*., 2006). It has been used as a criterion for the evaluation of the maturation of honey, and in some cases, adulteration with sugar. A minimum value of 180 mg kg^{-1} of proline is accepted as the limit value for pure honey (Manzanares *et al*., 2014; Hermosin *et al*., 2003). Even though this quality parameter is a good indication for adulteration and ripeness of honey during harvesting, there is no reported proline content of Ethiopian and standard requirements for this parameter.

2.7. Biochemical properties of honey

Honey contains a variety of biochemical that is recognized in food for health encouraging actions (Saranraj *et al*., 2016; Gheldof & Engeseth, 2002). Among the different compounds, many components are known to act as antioxidants, including vitamin C, vitamin E, amino acids, proteins, α-tocopherol, flavonols, catechins and carotenoids, and enzymes, such as catalase and peroxidase (Khalil *et al*.,2010; Aljadi *et al*.,2004). Honey also contains a variety of phenolic compounds, which are good sources of antioxidants (Beretta *et al*., 2005; Aljadi *et al*., 2004).

2.7.1. Antioxidant activity

Unlike other sweeteners, honey has strong antioxidant activity that provides food with nutritional and technological advantages. Honey has proved to prevent or delay food spoilage due to oxidative reactions, protecting meats against lipid oxidation (Gheldof *et al*., 2002; Antony *et al*., 2000), and vegetable products against enzymatic browning (Chen *et al*.,2000; Oszmianski & Lee,1990). Therefore, honey has great potential to be used as a natural antioxidant for foods (Nagai *et al*., 2006; Gheldof *et al*., 2002). Phenolic compounds (flavonoids, phenolic acids), as well as melanoidins (Maillard reaction products), appear to be the most important constituents of honey responsible for its antioxidant activity (Sancho *et al.,* 2016 ; Bogdanov, 2012). These compounds, together with glucose oxidase, catalase, carotenoids, organic acids, ascorbic acid, amino acids, and proteins have been described as honey antioxidants (Blasa *et al*., 2006; Nagai *et al*., 2006; Beretta *et al*., 2005).

The antioxidant activity of honey depends on its botanical source (Kucuk *et al*., 2007; Vela *et al*., 2007), as well as the packaging material, processing ways, and storage conditions (Wang *et al*., 2004; Gheldof *et al.,* 2002). Many researchers found that honey with dark color has a higher total phenolic content and consequently a higher antioxidant capacity (Beretta *et al*., 2005; Frankel *et al*., 1998). Research shows that honey's antioxidant capacity is reduced upon storage at room temperature (Saric *et al*., 2012). Concerning raw and processed honey, the antioxidant activity of both of them after storage was described as analogous (Wang *et al*., 2004). Some of the antioxidant components of honey from different areas of the world are shown in (Table 4).

Table 4: Antioxidant properties of honey

Parameters			Country	Reference
TPC mgGAE/100 g	TFC mgQE/100g	AEACmgAEAC /100g		
110.39- 196.50	18.51- 32.88	9.650- 50.169	Malaysia	Chua *et al*., 2012
54.30 ± 7.19	2.68 ± 0.38	31.06±2.19	Cuba	Alvarez-Suarez *et al*., 2018
459.83 ± 1.92	54.23 ± 0.62	236.8-315.9	Algeria	Khalil *et al*., 2012
757.2±30.8	43.1±2.1	-	Bangladesh	Islam *et al*., 2017
243.01 ± 74.91	37.70±19.75	276.96-324.47	Malaysia	Moniruzzaman *et al*., 2013

TPC: total phenol content, mgGAE/100g: milligram of gallic acid equivalent per 100 gram or kilogram of honey, TFC: total flavonoid content, mgQE/100g: milligram of Quercetin equivalent per 100 gram or kilogram of honey, mg CE/kg): milligram of Catchein equivalent per 100 gram or kilogram of honey, mg AEAC/g: milligram of Ascorbic acid equivalent of antioxidant content per 100 gram or kilogram of honey.

2.7.2. Total phenol and flavonoid contents

Honey's phenolic phytochemicals are important aromatic secondary metabolites derived from plants, whose nectars or honeydew are sipped by bees, as well as from pollen or propolis (Ferreres *et al*., 1992). Their range in honey is about 5-1300 mg/kg (Al-Mamary *et al*., 2002; Gheldof & Engeseth, 2002). Polyphenols are divided into several classes, according to the phenolic structural features (Grassi *et al.,* 2010). In honey, they are mainly flavonoids, phenolic acids, and phenolic acid derivatives (Anklam, 2001). They are non-flavonoid polyphenolic compounds derivatives of benzoic acid (gallic, ellagic, protocatechuic acids) and cinnamic acid

(caffeic, sinapic, ferulic, coumaric acids) (Amiot *et al*., 1989). The total phenolic content in honey is strongly correlated with its antioxidant activity (Bertoncelj *et al*., 2007; Beretta *et al*., 2005; Meda *et al*., 2005), thus can it be used as a reliable parameter to indicate antioxidant activities in honey.

Flavonoids are a large family of plant phenolic pigments. They contain several phenolic hydroxyl functions attached to ring structures (Evans *et al.,* 1997). Many flavonoids (such as apigenin, pinocembrin, kaempferol, quercetin, galangin, chrysin, and hesperetin), phenolic acids (such as ellagic, caffeic, p-coumaric, ferulic acids, and ascorbic acid) have a synergistic antioxidant effect (Rakha *et al*., 2008; Turkmen *et al*., 2006). The quantity of these components varies widely according to the floral and geographical origin of honey. In Ethiopia, there is limited information on bioactive compounds and antioxidant capacity of commercially available and adulterated honey. However, studies elsewhere indicated the presence of bioactive compounds (total phenol, total flavonoid, total carotenoids, vitamin C, total free amino acids, total protein, and folic acid content) and the antioxidant capacity of honey samples (Alvarez-Suarez *et al*., 2018; Alvarez-Suarez *et al*., 2012).

2. 8.Adulteration of honey

Honey is a natural substance of relatively high commercial value and limited supply; it is more prone to adulteration and fraudulent practices such as selling it under a false name or origin (Zabrodska & Vorlova, 2014). Honey adulteration can be seen from three different point of view:(1) Public health, because it may involve ingredients which are not allowed due to their toxic or allergenic potential (Everstine *et al*., 2013), (2) legal, because according to the European Union (Europe, 2010) the addition of any compound into honey is prohibited and (3) economic, by unfair competition involving the industry, distributors and the livelihood of beekeepers, leading to a destabilization of markets (Sobrino, *et al*., 2017). Honey adulterants are mainly banana, molasses, sugar, shebeb, inverted syrup fed to bees, and low quality honey added to high priced honey.

Adulteration methods of honey can be direct or indirect (Zabrodska & Vorlova, 2015). Direct adulteration of honey is accomplished by the direct addition of external substances to the honey. The addition of adulterants after honey harvesting, which can lead to higher honey production through direct adulteration (Zabrodska & Vorlova, 2014). Indirect adulteration is attributed with the manual feeding of bees with artificial sugar at the stage during the dearth period. This type of adulteration can be unintentional, such as when caused by mistakes made by the beekeepers, or misuse of their technology, particularly when there is the need to feed the honey bee colonies

before winter or before the honey flow season (Solayman *et al.,* 2016; Ozcan *et al*., 2006). The study by Solayman *et al.* (2016) suggested that external honey bee feeding with artificial syrup in improper protective measures affects the final sugar composition. Dumté (2010) suggested that adulteration might have occurred through the feeding of the bees with sugar syrup. In Ethiopia, although there is a sugar feeding program during the dearth period, there is no research conducted on the effect of this feeding on honey quality.

2.9. Common type of adulterant materials used in Ethiopia

Sources of adulterants in honey can vary from one region to another, depending on the price and easy availability of the various sugars or sweeteners. In Ethiopia, some authors reported different types of adulterant materials from different parts of the country as shown in (Table 5 & 6). Ambaw & Teklehaimanot (2018) survey of the Arsi zone honey products is showed that 95.5% of the actors of adulteration were retailers. Adulterating honey most commonly taken place by mixing, homogenizing, heating, or melting adulterant materials with pure honey.

Table 5. Commonly used adulterant materials in Ethiopia

Place/area	**Name of adulterant materials**	**Reference**
Arsi	sugar, ripened banana, wheat flour, potato, maize flour, pollen, empty combs, melted candy, molasses, and hot water	Ambaw & Teklehaimanot (2018)
Eastern Tigray region	sugar syrup, maize and/or wheat flour syrup, banana and sweet potato	Gebremariam& Brhane (2014)
Oromia region	sugar, candy, molasses, banana, orange, and cumber	Gemeda & Negera (2017)
Bahir dar	sugar or invert	Ambaw & Teklehaimanot (2018)
Gedo (SNNP)	sugar syrup, maize and/or wheat flour, banana and sweet potato	Sebeho (2015)
Adigrat and surrounding area	Sugar, water, banana	Gebremariam & Brhane (2014)

Table 6. Techniques of adulterating honey and ways of identification of it in Ethiopia

Adulterant material	Ways of adulteration	Ways of identification	Reference
Sugar	Hot water 1lit+1kg	Sticky by palpation,	(Ambaw & Teklehaimanot, 2018; Gemeda & Negera, 2017)
	sugar+1/2kg honey	sweaty by testing, poor viscosity during dropping, low price, tasting and smelling, continuous flow	
Banana	Crashed banana+honey (1:1)	Soily by observation and palpation no soft feeling,	(Ambaw & Teklehaimanot, 2018)
Banana +sugar	a mixture of sugar and banana,	low price, cast formation testing and smelling continues to flow, coca-cola, using fire test	(Gemeda & Negera, 2017)
Banana + molasses	a mixture of sugar and molasses	testing and smelling, continues flow, the thickness of honey, coca-cola, using fire test	(Gemeda & Negera, 2017)
Wheat flour	Simply mixing with honey and hot water (1:1)	Observation, testing viscosity, oily appearance, price	(Ambaw & Teklehaimanot, 2018)
Molasses	Mixing with the ratio of 1:1	No specific identification methods	(Ambaw & Teklehaimanot, 2018)
Sweet potato and potato	Mixing after being roasted by fire	By testing	(Ambaw & Teklehaimanot, 2018)
Brood comb and pollen	Mixing during harvest	Observation	(Ambaw & Teklehaimanot, 2018)

2.10. Effect of adulteration on honey properties

Adulteration alters the quality and safety of honey. Honey adulterated with water will deteriorate faster and adulterants can lower the medicinal value as well as may have a health effect on the consumers (Anthony & Balasuriya, 2016). However, honey adulterated by sugar addition changes in some chemical and biochemical parameters, such as enzymatic activity, electrical conductivity, and contents of specific compounds (HMF, glucose, fructose, sucrose, maltose, isomaltose, proline, ash) when compared to a control. Moreover, some chemical parameters, such as HMF content, formerly suggested as a test to detect the addition of invert syrups, maybe ambiguous because HMF and enzymatic activity vary in different kinds of

honey and can spontaneously change in honey when subjected to heat or abusive storage in warm environments (Ajlouni & Sujirapinyokul, 2010). In addition to flavor and nutritional value, one of the most valuable qualities of honey is the therapeutic potential given by low molecular biologically active compounds like phenolic compounds, organic acids, volatile compounds, vitamins, amino acids which are altered due to adulteration(Anthony & Balasuriya, 2016; Bogdanov & Gallmann, 2008).

2.11. Method for detection of adulterant in honey

Detecting adulterated honey is not just limited to the direct addition of sugars into natural honey, but is extended to indirect feeding of honey bee colonies with concentrated sugar solutions during the main nectar flowing season (Guler *et al*., 2014). This widespread irresponsible act not only jeopardizes consumer welfare (Guler *et al*., 2007; Oddo *et al.,* 2004) but also can potentially incur health problems, especially in kids and the elderly. Testing honey adulteration can be done by analyzing different physicochemical parameters like melissopalynological pattern, sensory analysis, sugar profile, amino acid profile, enzyme activities (diastase, invertase), HMF, and proline (Zabrodska & Vorlova, 2014; IHC, 2009; Cotte *et al*., 2004).

Honey adulteration can also be detected using several modern techniques. Modern methods used for detection of adulterants from honey were summarized by Yilmaz *et al* (2014) as electrochemical analysis, enzymatic methods, thin-layer chromatography (TLC), carbon isotope, flow injection analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC), anion-exchange liquid chromatography (LC), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), mid-infrared, near-infrared (NIR) transfectance spectroscopy, gas chromatography-mass spectrometry (G.C.-MS), high performance (HP) anion-exchange chromatography with pulsed amperometric detection method (HPAEC- PAD), high-performance thin-layer chromatography (HPTLC), isotope ratio mass spectrometry coupled with an elemental analyzer, and low field nuclear magnetic resonance (Yilmaz *et al*., 2014). Because these sophisticated methods are required high technology and are generally not economical as well as none of the methods could be used to identify all the adulterants in the honey simultaneously, there is a need for the development of a more practical and less costly method to detect honey adulteration. This section describes a set of different tests normally used nowadays by beekeepers, at the marketplace for consumers,

and at a laboratory for an expert where there is no sophisticated equipment easily to identify honey adulteration.

2.11.1. Quick tests used to assess the adulterated honey

There is a preliminary assessment to be conducted to know whether honey is adulterated or pure commonly at home and local markets in Ethiopia as shown in Table 7 (Ambaw & Teklehaimanot, 2018; Gemeda & Negera, 2017; Gebremariam & Berhane, 2014).

Table 7. Simple preliminary tests to identify adulterated honey

Test type	Known pure honey	Intentionally adulterated honey	Reference
Glass-filled water test	100%settled	100% dissolved	Ambaw & Teklehaimanot, 2018; Gebremariam & Brhane, 2014
Thumb test	100% intact	100% spread	Ambaw & Teklehaimanot, 2018; Gebremariam & Brhane, 2014
Match-lighting test	100% give light	100% smokes	Ambaw & Teklehaimanot, 2018; Gebremariam & Brhane, 2014
Shelf life (1year)	100% crystalize		Ambaw & Teklehaimanot, 2018

2.11.2. Physicochemical marker to assess honey adulteration

Knowledge of the chemical characteristics of honey, as one of the respected health-promoting natural products, is of general interest in terms of their protection against adulteration (Arvanitoyannis *et al*., 2005). Testing honey adulteration can be done by analyzing different physicochemical parameters like sugar profile (glucose, fructose, sucrose), enzyme activities (diastase, invertase), hydroxymethylfurfural, and proline (IHC, 2009; Cotte *et al*., 2004). The comparison of the results with the naturally occurring values can hint at a possible adulteration (Persano Oddo & Piro, 2004; Bogdanov *et al.,* 2003). Adulterated honey loses some of the nutritional and medicinal benefits, compared with pure honey, and may vary greatly

concerning its physicochemical properties, e.g. refractive index, moisture content, total soluble solids, density, specific weight, capillary action, surface tension, and pH value. The physical characteristics of pure honey are different from adulterated honey particularly inconsistency, water content, ash content, and water-insoluble materials (Ambaw & Teklemedhin, 2018). Adulteration of honey by crystallized cane sugar, invert sugar syrup, and cane sugar syrup can be detected with chemical determinations including HMF, glucose, sucrose, fructose, and diastase (Codex, 1989; White, 1979). HMF content is a good indicator of adulterated honey as it can demonstrate the incorporation of inverted sugars (Pasias *et al*., 2017; Paradkar & Irudayaraj, 2002). Carbohydrate analysis is also a common approach to detect honey adulteration from chemical analysis. It separates the component of reducing and non-reducing sugar which indicates the sign of adulteration. Therefore, honey that contains more than 5% sucrose may be unripe; sucrose is not converted completely into glucose and fructose by the invertase enzyme (Ouchemoukh *et al*., 2007). Gebremariam & Berhane (2014) reported that the higher sucrose content of honey is an indication for the addition of commercial sugar to honey and there was possible adulteration with sugar syrup in some of the samples purchased from local markets.

2.11.3. Detection of adulteration based on biochemical properties

Adulteration procedures change the biochemical composition of honey, particularly the proline, vitamin, mineral, and enzyme content (Guler *et al*., 2017). Biochemical markers of honey-like test protein, phenolic, flavonoids, and antioxidants of the honey samples are used to determine whether the honey samples are adulterated or not (Guler e*t al.*,2017; Guler *et al*.,2014; Kropf *et al*.,2010). Besides, total phenolic content, flavonoid concentration, and antioxidant activity of honey may be useful indicators for the determination of the authenticity of honey and the management practices of honey producers (Lianda *et al*.,2018; Nibset *et al*.,2018; Cimpoiu *et al*.,2013; Khalil *et al*.,2010). The biochemical activity of honey is also affected by processing, handling, and storage (Turkmen *et al*., 2005; Gheldof & Engeseth, 2002). Nibset *et al* (2018) demonstrated that the protein content of honey is a factor that can be reliably used for the detection of adulterated honey with <30% added sugar. Some researchers reported that the use of excessive sugar with the intent of producing greater yields adversely affected the proline content of the honey (Ruof *et al*., 2006; Sahinler *et al*., 2004). Nibset *et al.* (2018) suggested that the proline content may be a more suitable indicator for the differentiation of natural and adulterated honey. Some researchers analyze proline as quality

criteria for honey ripeness, and as an indicator of sugar adulteration, especially when the values of this amino acid are significantly lower than 180 mg/kg, the minimum value that has been agreed for genuine honey (Bogdanov *et al*., 1999).

2.12. Fourier transform infrared spectroscopy (FTIR)

Infrared (IR) spectroscopy has become almost indispensable in the chemistry laboratory as it is ideally suited for carrying out qualitative and quantitative analysis, particularly of organic compounds (Khandpur, 2005). IR spectroscopy is a fast, accurate, and non-destructive technique that can detect a range of functional groups through the interaction between infrared radiation and a sample that can be solid, liquid, or gaseous (Amir *et al.,* 2013). The IR region of the electromagnetic spectrum spans from 14,000–50 cm^{-1} and is divided into three areas, namely: near IR (14,000–4,000 cm^{-1}), mid-IR (4,000–400 cm^{-1}), and far IR (400–50 cm^{-1})(Guillen & Cabo, 1997; Diem 1993). The sensitivity and accuracy of FTIR detectors along with a wide variety of software algorithms have dramatically increased the practical use of infrared for quantitative analysis. The narrower bands of the fingerprint region reduce the problem of overlap, allowing the use of some simple mathematical treatments, such as calibrations of peak heights or areas plotted directly against concentration (Lichtenberg *et al*., 2002). However, in complex systems such as adulterated honey samples, the spectra of the individual components are very similar, and the effect of the overlap requires more sophisticated approaches. The development of an FTIR spectroscopic procedure will help the honey industry for the rapid detection of critical adulterants, which otherwise is not possible by existing methods. FTIR methods, often in combination with multivariate statistical analyses, have been developed for honey authentication about determining botanical and geographical origin (Gok *et al*., 2015; Cozzolino *et al*.,2011) and also honey adulteration (Cozzolino *et al*., 2011, Kelly *et al*., 2006).

2.12.1. Infrared spectrum of bio-molecules

Molecular vibrations characterizing different molecules can be found along the entire IR spectrum (Figure 1). As most of the functional groups have relatively sharp absorption bands in the fingerprint region, the quantification of the components can be determined using data handling techniques. FT-IR spectroscopy probes the biochemical composition of a sample and determines the precise position and amplitude of infrared (IR) absorption by biochemical

bonds. However, the visual interpretation of IR spectra is very complex as the IR spectra result from various vibrational modes of molecules. Despite the complex molecular structure of cells, the major vibrational modes of molecules belong to lipids, proteins, nucleic acids, and polysaccharides (carbohydrates).

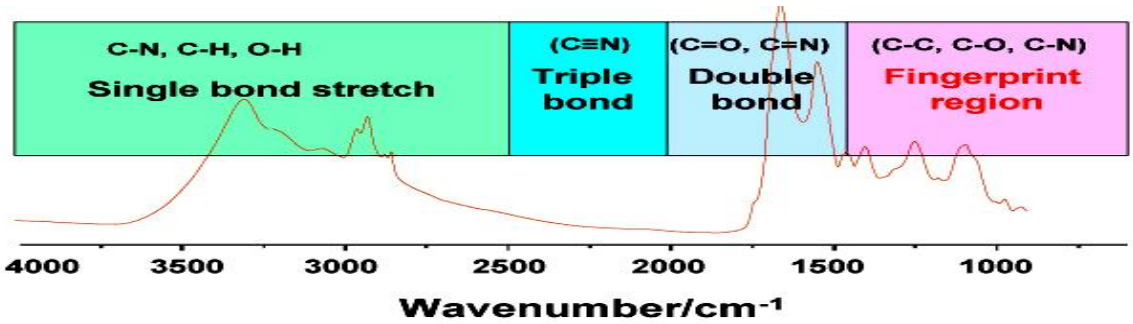


Figure 1: Correlation chart with functional groups in the IR fingerprint region ranging between 4000–650 cm^{-1}. Adapted from www.biomedcentral.com

Proteins are composed of individual units called amino acids. In the spectra of honey, intensive bands due to amide I (1620-1690 cm^{-1}) have been observed (Gasparri, & Muzio, 2003; Lasch *et al.,* 2002). These correspond to the C=O stretching coupled to the N-H bending and the C-H stretching modes of peptide bonds (Mourant *et al.*, 2003). It is well documented that absorbance in the range from 1645 to 1662 cm^{-1} is generally associated with the presence of α-helices (Byler & Susi, 1986). The amide II bands between 1530-1570 cm^{-1} arise from the vibrational modes that involve the C-N-H bending and C-N stretching of peptidic bonds (Gasparri, & Muzio, 2003). The amide III bands between 1240-1340 cm^{-1} are contributed by proteins arising from C-N stretching and N-H bending (Mourant *et al*., 2003; Gasparri, & Muzio, 2003).

Major absorption of carbohydrates peaks are observed at 1028 cm^{-1} (C-OH, C-O stretch), 1080 cm^{-1} (C-OH, C-O stretch), 1095 cm^{-1} (CH_2, C-C stretch), and 1455 cm^{-1} (CH_2, C-H bending) (Lasch *et al*.,2002). The absorbance obtained between 950 and 750 cm^{-1} known as the anomeric region, which was frequently preferred for the spectral analysis of carbohydrates in IR spectroscopy (Gok *et al*., 2015). Kacurakova *et al*. (2000) reported that the region at 1200–850 cm^{-1} is dominated by stretching vibrations of C – O, C– C, ring structures, and deformation of CH2 groups' vibration characteristic for polysaccharides. The bands ranged between 1500 and 750 cm^{-1} are very significant for honey or similar products since this zone is attributed to

the absorption of the major monosaccharides (fructose and glucose) and disaccharides (sucrose)(Gallardo-Velázquez *et al*.,2009). The vibrations in the regions 940–1175 cm^{-1} are due to the C–OH group as well as the stretches C=C and C=O in the carbohydrate structure (Masek *et al*., 2014).

2.13. Multivariate analysis for spectral analysis

Univariate analytical methods are the simplest ways of displaying the biochemical information and determining peak positions. Although this is a simple approach to spectral analysis, it only utilizes a small part of the biochemical data. As the intricacy of the IR spectrum increases within and between sample types, the unambiguous assignment of the spectral bands to specific molecular groups becomes more difficult. Therefore, in complex biological samples, multivariate statistical techniques are employed as they utilize the entire spectra and all of the biochemical information contained within the spectra. Many groups have used principal component analysis (PCA) for the separation of FT-IR and Raman spectral data (Lyng, 2007; Kendall, 2003; Stone, 2000). Raw data acquired through FT-IR spectroscopy requires spectral manipulation to extract qualitative and quantitative information. Spectra are subjected to pre-processing methods to effectively suppress or eliminate unnecessary facts and noise. This approach monitors vibrations due to pre-assigned specific functional groups (e.g. C=O, N-H, C-H, etc) and explores changes in these functional groups as potential biomarkers.

2.13.1. Principal component analysis (PCA)

PCA is an unsupervised classification method, where the spectra are separated solely on the measure of their variance. It combines the advantages of both unequivocal classification and outlier detection (Bossart, 2003). It is a well-established method ideally suited to distinguish and visualize small, reoccurring spectral variations in large datasets. These can include both spectral data and pseudo-color principal component (PC) score images. PCA reduces the number of parameters needed to represent the variance in the spectral dataset. It resolves a complete spectral dataset into a few key spectral components and can thus identify and isolate important trends within the dataset (Lasch *et al.,* 2002). The first PC represents the highest variance in the spectral dataset; the second PC represents the next highest variance. The most significant PC scores for each spectrum in the dataset were plotted against one another to enable the natural clustering with cell line type or blood group to be visualized. This approach

identifies natural clusters or grouping with no prior knowledge of their classification. The application of PCA resulted in a data grouping of each of the different samples used, working as a discriminative screening tool of pure and adulterated samples.

2.13.2. Hierarchical cluster analysis (CA)

The main goal of the hierarchical cluster analysis was to spontaneously classify the data into groups of similarity (cluster), searching objects in the n-dimensional space located in the closest neighborhood and to separate a stable cluster from other clusters (Simeonov *et al*.,2007). Cluster analysis was displayed to find similarities between the honey samples and also between the variables. Then we were able to check if on that basis they create clusters and we could observe any similarities. Using cluster analysis it was possible to display the object similarity reliably and make the initial interpretation of the data set structure.

3. MATERIALS AND METHODS

3.1. Study design

3.1.1. Samples collection

The present study was carried out in Addis Ababa, which is the capital city of Ethiopia. Currently, there are ten sub-cities in Addis Ababa city administration delineated based on geographical setup, population density, asset, service providers' distribution, and convenience for administration (AACA, 2004). Addis Ababa is situated at a latitude of 9°3′ North and 38°43′ East (ILCA, 1994). It lies in the central highlands of Ethiopia at an altitude of 2500 m.a.s.l. It has an average rainfall of 1800 mm per annum. The annual average maximum and minimum temperatures are 26 °C and 11 °C, respectively; with an overall average of 18.7 °C. The highest temperatures are reached in May. The main rainy season extends from June to September. Addis Ababa has a relative humidity varying 70% to 80% during the rainy season and 40% to 50% during the dry season. The human population is estimated at 4 million inhabitants (UN-Habitat, 2007). In Addis Ababa, there are huge honey market challenges including, Marketo one of the biggest open markets in Africa, which is suspected of low quality and adulteration. Many companies are engaged in producing, processing, and exporting honey in active and non-active participation (Fresenbet, 2019).

3.1.2. Sampling and sampling techniques

This study had two components: the first part was the collection of honey samples available for customers purchased from the supermarkets, street, local markets, small shops, and retail stores (big honey verandas) to assess the quality and status of adulteration practices. The second part has involved the addition of the sample set by adulterating raw honey samples with sugar, ripened banana, molasses, melted candy, and shebeb (salt-like substances): a commonly used adulterants in Ethiopia. A honey sample of approximately 500 g was randomly purchased from different locations of eight conventionally selected sub-cities of Addis Ababa. From one sub-city three honey samples from different market types were collected according to availability. The study involved a total of 30 samples consisting of 16 randomly selected supermarkets, four from honey traders,1 from the street, one local market, three samples from processors, two small shops, and three retail stores (honey verandas) in Addis Ababa. Detailed information for these samples was summarized in Table 8.

The raw honey sample was obtained from the HBRC bee farm colonies with no feeding which was used as a control as well as for deliberate adulteration. Samples were properly identified by date of collection, sources, and sample type (Table 8). The honey samples were immediately transported to the HBRC bee product laboratory and stored at room temperature until they were analyzed.

Table 8. Description of study area sample collection.

Sample code	Area	Sub-city	Sample source
1	Gojjam beranda	Addis Ketema	Street areas
2	Gojjam beranda	Addis Ketema	Retail store(veranda)
3	Gojjam beranda	Addis Ketema	Retail store(veranda)
4	Gojjam beranda	Addis Ketema	Retail store(veranda)
5	Gojjam beranda	Addis Ketema	Honey trader/retailer
6	Gojjam beranda	Addis Ketema	Honey trader/retailer
7	Amade gebeya	Addis Ketema	Local market
8	Piasa	Areda	Supermarket
9	Piasa	Areda	Supermarket
10	Churchill road	Areda	Supermarket
11	Atlas	Kirkos	Supermarket
12	Bole madinalem	Bole	Supermarket
13	Bole madinalem	Bole	Supermarket
14	Bole (Japan Embassy)	Bole	Supermarket
15	Bole rewanda	Bole	Honey processors
16	Walo sefer	Kirkos	Honey processors
17	Walo sefer	Kirkos	Honey processors
18	Sarbet	Nefassilklafto	Supermarket
19	Sarbet	Nefassilklafto	Supermarket
20	Bisrat Gabriel	Nefassilklafto	Supermarket
21	Lideta	Lideta	Supermarket
22	Kolfe keraniyo	Kolfe keraniyo	Supermarket
23	Kolfe keraniyo	Kolfe keraniyo	Supermarket
24	Kebena	Yeka	Small shop
25	Shola market	Yeka	Honey trader/retailer
26	Shola market	Yeka	Honey trader/retailer
27	Maganagna	Yeka	Small shop
28	Guard shola	Bole	Supermarket
29	Guard shola	Bole	Supermarket
30	Guard shola	Bole	Supermarket

3.2. Sample analysis of market honey

3.2.1. Preliminary quick test assessment

A preliminary assessment was conducted for commercially available sold at markets.

Flame Test honey: sample was ignited for a second or minutes using a candle flame or match stick showed whether smokeless flame or smoky flame and/or cracking sound (Tadesse & Berhane, 2014).

Dissolving methods: Honey samples were added into a glass of water and observation of the added honey showed either dissolved or settled at the bottom of the glass (Krell, 1996).

Thumb Test: A gentle compressing was given to a drop honey sample on the thumb to know the ability to stick or not (Tadesse & Berhane, 2014).

Coca-cola Test. The honey samples were added to a baker. Then a 4 ml of coca-cola was added to the sample.

3.2.2. Determination of physicochemical analysis

The following physicochemical properties of commercially available honey were analyzed based on International Honey Commission methods (IHC, 2009) compared with pure honey (control) and standard set requirements.

3.2.2.1. Determination of moisture content

The moisture content of honey samples was determined using an Abbe refractometer (ABBE-5 Bellingham Stanley. Ltd, United Kingdom) that can be thermostated at 20 $^{\circ}$C and regularly calibrated with distilled water according to International Honey Commission (Bogdanov, 2009). Honey samples were homogenized and placed in a water bath until all the sugar crystals were dissolved. After homogenization, the surface of the prism of the refractometer was covered with honey and after 2 minutes refractive index for moisture was determined. The value of the refractive index of the honey sample was determined using a standard table designed for this purpose.

3.2.2.2. Determination of pH and free acidity

Ten grams of honey was dissolved in 75 ml of distilled water in a 250 ml beaker and stirred using a magnetic stirrer (Bogdanov,2009).. The electrode of the pH meter (METTLER TOLEDO, CHINA) was immersed in the solution and the pH of honey was recorded. For the measurement of free acidity, the solution was further titrated with 0.1 M sodium hydroxide (NaOH) solution at a pH of 8.30. For precision, the reading to the nearest 0.2 ml was recorded using a 10 ml burette. Free acidity is expressed as mill equivalents or a mill mole of acid/kg honey and is equal to ml of 0.1M NaOH x 10. The result is expressed to one decimal place following the procedure of Bogdanov (2009).

Acidity =10 V, Where: V = the volume of 0.1N NaOH in 10 g of honey.

3.2.2.3. Determination of total ash content

Determination of ash content was carried out by incinerating honey samples at 600 °C in a muffle furnace (BioBase JKKZ.5.12GJ, Shandong, China) to constant mass according to International Honey Commission (Bogdanov, 2009). First, the crucible was heated in an electrical muffle furnace at an ashing temperature and subsequently cooled in a desiccator to room temperature and weighted to 0.001 g (M_2). Then 5 g ($M_{0)}$ of each honey sample was weighed to the nearest 0.001 g and taken into a platinum crucible and two drops of olive oil were added to prevent foaming. Water was removed and started ashing without loss at a low heat rising to 550- 600 ^{o}C using electrical devices. After the preliminary ashing, the dish was placed in the preheated furnace and heated for at least 1 h. The ash crucible was cooled in the desiccators and weighed. The ashing procedure was continued until a constant weight was reached (M_1). Lastly, the weight of ash in g/100 g honey was calculated using the following formula: -

$$WA = \frac{M1 - M2}{M0}$$

Where M0= Weight of honey taken.

M1= Weight of ash + dish ; M2= Weight of a dish.

3.2.2.4. Determination of sugars profile

Honey sugars were determined using high-performance liquid chromatography (HPLC-1260 Infinity Series Agilent Technologies, USA) according to the International Honey Commission (Bogdanov, 2009). Five grams of honey was dissolved in 40 ml distilled water. A 25 ml of acetonitrile was pipetted into a 100 ml volumetric flask and the honey solution was transferred to a flask and filled to the mark with distilled water and the solution of each honey sample was filtered using a syringe filter (0.45 µm) before chromatographic analysis. The HPLC separation system was composed of an analytical stainless steel column, 4.6 mm in diameter, 250 mm in length, containing amine-modified silica gel with 5-7 µm particle size. The flow rate of 1.3 ml/min, mobile phase acetonitrile: water (80:20, v/v), and sample injection volume 10 µl. The sugars were detected by a Refractive Index Detector thermostated at 30 ^{o}C temperature. The identification of honey sugars was obtained by comparison of their retention times with those of the standard sugars (Bogdanov, 2009). Standard sugars with their percent of purity level used were glucose (> 99.5%), sucrose ((> 90%), maltose (> 90%), and fructose (>99.5%) made in Germany, Sigma Aldrich. A five of series serial dilution of the standard of fructose, glucose, sucrose, and maltose mixture containing 2 g, 1.5 g. 1 g, 0.5 g, and 0.15 g were weighed and dissolved in a mixture of solvents containing in 40 ml distilled water and 25 ml acetonitrile according to International Honey Commission (Bogdanov, 2009) to draw a calibration curve.

3.2.2.5. Determination of hydroxyl methyl furfural (HMF)

HMF was determined using 6800 UV– Vis spectrophotometer (JENWAY, United Kingdom). A 5 g honey sample was weighed in a small beaker and mixed in 25 ml distilled water and transferred into 50 ml volumetric flask (Bogdanov, 2009). A 0.5 ml carrezz solution I (15 g $K_4Fe(CN)_6$. $3H_20$ /100 ml distilled water) was added and mixed with 0.5 ml carrezz solution II (30 g Zn acetate /100 ml distilled water). The solution was mixed with the honey solution. A droplet of alcohol was added to the solution. The solution was filtered through a filter paper and the filtrate (10 ml) was discarded. A 5 ml filtrate was added into each of two test tubes and 5 ml distilled water was added into the first test tube (sample solution), while 5 ml sodium

bisulfite solution (0.20% of 0.20 g $NaHSO_3$/100 ml distilled water) was added into the other test tube (reference). The contents of both test tubes were well mixed by vortex mixer and their absorbance was recorded spectrophotometrically. By subtracting the absorbance measured at 284 nm for HMF in the honey sample solution against the absorbance of reference (the same honey solution treated with sodium bisulfite, 0.2%) at 336 nm, and the result was calculated and expressed according to the International Honey Commission (Bogdanov, 2009). Hydroxyl methyl furfural (HMF)/100 g honey = $(A_{284} - A_{336}) \times 14.97 \times 5$/g sample.

Where A_{284}= absorbance at 284, A_{336} = absorbance at 336, 14.97= constant, 5= theoretical nominal sample weight and g = mass of honey sample.

3.2.3. Determination of Antioxidant properties analysis

3.2.3.1. Total phenolic contents

To analyze and compare the total phenol content between honey samples, the Folin-Ciocalteu method was used (Chua *et al*., 2013). The honey stock solution was prepared by mixing 5 g of honey sample in 50 ml of distilled water and filtered through Whatman no.1 filter paper. From this stock solution, 0.5 mL aliquot was mixed with 2.5 ml of 0.2N Folin- Ciocalteu reagent and incubated for five min. A 2 ml of 75 g/l sodium carbonate solution was added to the solution. Finally, after the solution was incubated for 2 h at 25°C, the absorbance of the reaction mixture was measured at 765 nm using a UV-Vis spectrophotometer (PerkinElmer Lambda 950 UV /VIS/NIR spectrophotometer). Gallic acid (0-200 mg/L) was used as a standard chemical to produce a calibration curve and finally, the total phenol content was reported mean ± standard error and expressed as milligram of gallic acid equivalent (GE) in 100 gram of honey from the mean value of triplicate data using the calibration equation derived from a calibration curve.

3.2.3. 2. Total flavonoid content

The total flavonoid content of honey was estimated by aluminum chloride (AlCl3); Quercetin was used as the reference which was expressed as QE. The total flavonoid content of each honey samples was determined (Chua *et al.,* 2013). The stock solution was prepared by diluting five grams of honey sample in fifty milliliters of distilled water and filtered through Whatman no.1 paper. Five milliliters from honey stock solution was pipetted and mixed in five milliliters of 2% aluminum chloride ($AlCl_3$) solution. After incubation for ten min, the absorbance of the reaction mixture was measured at 415 nm by using a spectrophotometer (Perkin Elmer Lambda 950 UV/VIS/NIR spectrophotometer). Quercetin (0-200 mg/L) was used as a standard chemical to produce a calibration curve and finally, the total flavonoid content was reported as the mean value of triplicate assays and expressed as milligram of Quercetin equivalent (QE) per100 gram of honey from the mean value of triplicate data using the calibration equation derived from a calibration curve.

3.2.3..3. The antioxidant activity of honey

The radical scavenging activity of the honey extract was determined using assay according to Chang *et al* (2001). The method involves reducing an alcoholic solution of purple DPPH radicals, which, upon receiving an electron or hydrogen radical, changes color from violet to yellow (diphenyl-picryl hydrazine), accompanied by a decrease in absorbance at the wavelength observed. Antioxidant compounds in honey samples were evaluated by measuring the ascorbic acid equivalent antioxidant capacity (AAEAC) following standard methods (Islam *et al*., 2012). Ascorbic acid 10 mg/ml was used as a reference. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution (20 mg/l) was prepared by dissolving 0.5 mg of DPPH in 25 ml of methanol. The honey solution was prepared by mixing 30 mg honey in 1 ml methanol and 0.75 mL of methanolic honey solution was added to 1.5 ml of DPPH solution. The decrease in the absorption of the DPPH solution after the dilution of an antioxidant was measured at 517nm. The blank was composed of 0.75 ml of a methanolic honey solution mixed with 1.5 ml of methanol. Ascorbic acid (0-200 mg/l) was used as a standard chemical to produce a calibration curve. Finally, the measurements were replicated three times and the mean value was expressed as milligram of ascorbic acid equivalent antioxidant content per

100 g of honey from the mean value of triplicate data using the calibration equation derived from the calibration curve.

3.3. Sample analysis of deliberately adulterated honey

3.3.1. Collection of adulterant materials and preparations of deliberately adulterated honey

Five adulterants were considered in this study: melted candy, molasses, table sugar, ripened banana, and shebeb. The adulterant materials were obtained from the market and shop. Shebeb is a white, salt-like adulterant substance widely used in northern Ethiopia mixed with honey by melting the shebeb (1 ratio) into water contain (4 ratios) (1:4) ratio. After melting shebeb, honey was mixed with melted shebeb. The physical appearance of shebeb is shown on (Appendix 6). For table sugar adulteration, table sugar was changed into powder form and mixed with honey. For banana adulteration ripened banana was chopped into pieces and mixed. The intentionally adulterated honey sample was produced in this study simulated the actual adulteration process in a real environment by adulterating raw honey sample with sugar, ripened banana, molasses, melted candy, and shebeb in ratios 1:20 (5%),1:10 (10%), 1:5 (20%), and 1:2.5 (40%),1:2 (50%) w/w and pure honey samples free of adulteration (0%) as control. In the adulteration, the honey proportion was 100%–X%, where X is the percentage of the adulterant solution. Before mixing, the honey samples were warmed up to 50 ^{o}C to dissolve solids and obtain an adequate viscosity. The resulting adulterated sample was vigorously stirred to homogenize the mix. The sum mass of honey and adulterants were 500 g. All the mixed sample solutions were stored overnight at room temperature to further homogenize the components of the mixture before analysis.

Table 9. Preparation of deliberately adulterated honey and its proportions

Sample code	Type of adulterant materials	Percent of adulterant materials
DA 1	Molasses	5% molasses,95 % honey
DA 2	Molasses	10 % molasses, 90 % honey
DA 3	Molasses	20 % molasses,80 % honey
DA 4	Molasses	40 % molasses,60 % of honey
DA 5	Molasses	50 % molasses,50 % honey

DA 6	Banana	5% Banana,95 % honey
DA 7	Banana	10 % Banana, 90 % honey
DA 8	Banana	20 % Banana,80 % honey
DA 9	Banana	40 % Banana,60 % honey
DA 10	Banana	50 % Banana,50 % honey
DA 11	Melted Candy	5% Candy,95 % honey
DA 12	Melted Candy	10 % Candy, 90 % honey
DA 13	Melted Candy	20 % Candy,80 % honey
DA 14	Melted Candy	40 % Candy,60 % honey
DA 15	Melted Candy	50 % Candy,50 % honey
DA 16	Sugar	5% Sugar,95 % honey
DA 17	Sugar	10 % Sugar, 90 % honey
DA 18	Sugar	20 % Sugar,80 % honey
DA 19	Sugar	40 % Sugar,60 % of honey
DA 20	Sugar	50 % Sugar,50 % honey
DA 21	Shebeb	5% Shebeb,95 % honey
DA 22	Shebeb	10 % Shebeb, 90 % honey
DA 23	Shebeb	20 % Shebeb,80 % honey
DA 24	Shebeb	40 % Shebeb,60 % of honey
DA 25	Shebeb	50 % Shebeb,50 % honey

Note, DA=Deliberately adulterated

3.3.2. Determination of a preliminary quick test in a deliberately adulterated honey

The preliminary quick test assessment of deliberately adulterated honey samples was mixed based on Table 9, and compared with pure honey (control).

3.3.3. Determination of physicochemical content in deliberately adulterated honey

The physicochemical properties of deliberately adulterated honey were analyzed based on International Honey Commission methods (IHC, 2009) and, compared with pure honey (control) and standard set requirements as described in the section above.

3.3.4. Determination of biochemical properties in deliberately adulterated honey

The biochemical properties of deliberately adulterated honey were analyzed based on the methods described in the section above and compared with pure honey (control).

3.4. Fourier-Transform infrared spectroscopy (FTIR) analysis

The FT-IR spectrum of the honey samples was obtained using 65 FT-IR spectrometers Perkin Elmer (USA) in the chemistry department of Addis Ababa University and functional groups were determined with the help of IR correlation charts and the functional groups were noted. The wavenumber region for the analysis was 4000-400 cm^{-1}(in the mid-infrared range) operated at transmittance with a 4 cm^{-1} nominal resolution, and the average scanning speed of 4 mm/sec for the sample and background. The IR spectra were reported in % transmittance. Honey samples using (exactly 1 mg) were weighed using electronic digital balance and mixed with 250 mg KBr powder. The mixture was powdered further to mix well and then put in a sample holder of the instrument for a solid sample. The spectral data for each sample was collected as ASCII file and manually transferred to an Excel.

3.5. Multivariate analysis

To understand the distribution of the analyzed samples, based on the assessed parameters, principal component, and cluster analysis were used. The Unscrambler 10.1 (Camo Software AS., Norway) was used for performing PCA and cluster analysis. For the determination of spectral differentiation among studied groups, cluster analysis was performed in the spectrum in the range of 1800–650 cm^{-1}. Moving average smoothing was applied with 15 spectral

points before standard normal variate transformation (Barnes *et al*., 1989) to correct the scatter effect. Second derivative spectra were calculated using the gap-segment method (Norris, 2001). Each wavelength of FT-IR was treated as a variable and they were weighted by dividing by standard deviation. Clustering the samples by their similarities in terms of physicochemical properties, antioxidants, and spectral features were done.

3.6. Statistical analysis

SPSS for Windows Version 20 software package was used for analyzing the data. Determination of the significant differences between honey samples was done using one-way ANOVA and, Independent-Sample T-Tests. Means and standard errors of the recorded data were calculated. Multivariate analysis (principal component analysis and cluster analysis) was performed using Unscrambler software (Camo).To classify kinds of honey according to the physicochemical and antioxidant parameters, cluster analysis was applied.

4. RESULTS AND DISCUSSION

4.1. Summary of Quick test for commercially marketed honey

These preliminary assessments were conducted to know whether the honey was adulterated or pure commonly at home and local markets. Table 10 summarized the quick test results of commercially collected honey samples. From Table 10, it can be seen that 100% (3 samples out of 3) of honey samples collected from street areas was settled at the bottom of the glass in the water, followed by 75% (9 samples out of 12) samples from honey traders and 56.23% (27 samples out of 48) a sample from a supermarket. 3 samples out of 3 (100%) of honey collected from the local market were dissolved in the water, followed by 66.67 % (6 samples out of 9) for processors and 50% (3 samples out of 6) for honey collected from a small shop.. This result highlights, it can be seen that the honey collected from the market contains a foreign additive substance and is dissolved in the water. The dissolving methods result highlights that the honey samples collected from the local honey market have foreign materials added to it.

Flame test methods were conducted for marketed honey collected as shown in (Table 10). The majority of the marketed honey sample did not give light which indicates the sign of impurities of the honey. All samples collected from the local market, street honey, small shops honey samples produced smoky flame and cracking sound during the flame test.36 samples out of 48(75%) of a supermarket and honey traders, six samples out of 9 (66.67%) of honey samples collected from processors, and veranda produce smokes and no give light during the flame test. The majority of the marketed honey sample did not give light which indicates the sign of impurities of the honey. Thumb methods of the quality assessment showed that, if there is no foreign matter the honey sticks or intact to the thumb and spreads otherwise. In this regard, honey samples collected from small shops (6 samples out of 6) and processors (9 samples out of 9) intact to the surface of the thumb, followed by the retailer store (veranda) 66.67% (6 samples out of 9), supermarket 62.5% (30 samples out of 48). On the other hand, 3 samples out of 3 (100%) street areas honey spread around the thumb followed by 50% (6 samples of 12) of honey traders, 33.33% (3 out of 9 samples) from retail stores (veranda).

These findings suggest that, thumb test methods were used to detect adulterated honey which has solid particles and very low viscosity.

During the Coca-cola test, 40% (25 samples out of 45) of honey collected from the supermarket, 3 samples out of 9 (33.33%) honey from processors, and 3 samples out of 12 (25%) from honey trader no produced bubbling sound and foam during mixing. On the other hand, 3 samples out of 3 (100%) from the local market, and 100%(3 samples out of 3) from small shops produced bubbling sound and foam during mixing with coca-cola followed Honey mixed with foreign substances forms foam and bubbling sound. According to this finding, the Coca-cola test method is used to detect honey adulteration which is done at the local level. During the coca-cola test, it was observed that pure honey that contains solid particles like wax, pollen, and dead bees sometimes produced bubbling sound and foam. As shown in Table 10, honey obtained from supermarkets showed no fermentation except 12 samples out of 48 (25%) of honey obtained from the supermarket which showed fermentation on the top because of handling problems during storage (humidity, storage temperature, and equipment). Honey fermentation leads to spoilage of honey and also reduces its shelf life span (Perez-Perez et al., 2007). Many consumers still assume that if honey has fermented it has gone bad or has been adulterated with sugar. Although fermentation is one of the important factors that affect the quality of honey, there is a few information on the fermentation status of Ethiopian honey.

Table 10. The Quick test results for commercially marketed honey.

Test type	Supermarket	Small shops	Street areas	Retail(veranda) store	Honey trader	Processors	Local Market
Dissolving Method	56.25% (27/48)settled 31.25% (15/48) dissolved 12.5 % (6/48) of 50% is dissolved	50% (3/6) settled 50% (3 /6) dissolved	100%(3 /3) settled	33.3%(3/9) settled 33.3% (3/9)dissolved 33.3%(3/9) of 50 % of the sample dissolved	75% (9/12) settled 25%(3/12) dissolved	66.67%(6 /9) settled 33.33 % (3/9) dissolved	100% (3 /3) dissolved
Thumb test	62.5%(30/ 48) intact 25% (12/48) spreads or spills 12.5% (6 /48) intact but contain a foreign substance	100% (6 /6) intact	100% (3 /3) spreads	66.67% (6/9)intact 33.33% (3/9) spreads or spills	50% (6/12)intact 50%(6/12) spreads or spills	100%(9/ 9) intact	100%(3/3) intact but have foreign substance
Flame test	75% (36 /48)smokes 25%(12/48) give light	100% (6/6) smokes	100% (3 /3)smokes	66.67% (6/9)smokes 33.33% (3/9)give light	75% (9/12) smoke 25%(3/12) give light	66.67% (6/9) smokes 33.33%(3/9) give light	100%(3/3) smokes
Coco cola test	53.33% (18/45) no produce bubbling and sound 40% (24/45) produce bubbling and foam	100%(3/3) produce bubbling and foam	100% (3/3) a little bubbling and foam	66.67% (6/9) produce bubbling and foam 33.33% (3/9) a little bubbling and foam	75%(9/12) produce bubbling and foam 25%(3/12) no produce bubbling and foam	66.67% (6/9) produce bubbling and foam 33.33% (3/9) no bubbling and foam	100%(3/3) bubbling and foam
Fermentation	75%(36/48) not fermented 25%(12/48) fermented	100%(6/6) not fermented	100% (3/3) not fermented	100%(9 /9) not fermented	100% (12/ 12) not fermented	100%(9/9) not fermented	100% (9/9)not fermented

4.2. Levels of physicochemical properties of commercially available honey

The results of the analysis of the honey samples that were collected from commercially available market sources are presented in Table 11.

4.2.1. Proline

Different commercially available honey types were analyzed for their proline contents (Table 11). A statistically significant difference ($p< 0.05$) was observed among the honey samples collected from the local market, processors, supermarkets, retail stores, and street areas. The highest proline content was recorded for local market honey types (423.05±11.9 mg/kg) and the lowest was recorded for the street samples (67.1±0.52 mg/kg). However, there was no statistically significant difference between the mean values of proline the contents of the honey samples collected from small shops and honey traders (Table 11). The present study agreed with other studies, where similar proline values (453.09-470.54 mg/kg) were reported for other Portuguese commercial honey samples, with a higher value (1044.36 mg/kg) being reported for the heather honey (Aazza *et al.,* 2013). Higher values, above 2,000 mg/kg, were exhibited by Burkina Faso (Meda *et al*., 2005) and Algerian (Khalil *et al.*2012) honey while proline values lower than 180 mg/kg have been reported for Tunisian honey (Boussaid *et al*., 2018). Jilani *et al.* (2008) reported proline contents in the range of 465- 828 mg kg^{-1} for some Tunisian honey. The level of proline has been reported to vary according to the honey flora, however, this is often additional closely related to the bees' performance (Cotte *et al.,* 2004). The proline content of honey is measured as a criterion for estimating the quality of honey(ripeness and genuineness) (Bogdanov, 2002) and the antioxidant activity of the honey (Saxena, *et al*.,2010; Meda, *et al.,*2005) and it may be used also for characterization based on botanical origin (Bogdanov *et al.,* 2004; Soria *et al.,* 2004).

The mean proline content of all samples was in agreement with international (Codex, 2001, & EU, 2002) parameters recommended for *Apis mellifera* honey which should be above 180%, except for means of proline from the street honey types. The smaller amount of proline of the analyzed in street honey samples indicates the honey's unripeness and that there is a high probability for sugar adulteration. Experimental studies have reported that

honey from bees fed on sugar water exhibits low proline values and honey has been adulterated with sugar (Cavrar *et al.,* 2013). The importance of proline has been emphasized to discriminate natural and artificial honey samples in previous studies (Sorkun *et al*., 2002, White, 1979). These differences might be attributed to beekeeping conditions (feeding with more syrup), plant species, and environmental factors (White, 1979). This study indicated that the standard value for proline suggested by Codex Alimentarius is very low; therefore, it was suggested that the standard value for proline should be examined with more honey samples.

Table 11. The mean and standard error (mean ± SE) values for the physicochemical content of commercially available honey collected from study areas.

Market type	Proline(mg/kg)	Moisture content (g/100g)	pH	HMF (mg/kg)	Free acidity(meq/kg)	Ash(g/100g)
Super market	234.45±16.9^{c}	21.36±0.31^{ab}	3.62±0.07^{ab}	20.15±2.94^{a}	29.28±3.1^{ab}	0.26±0.03^{a}
Small shops	349.79±53.4^{abc}	22.56±1.19^{a}	3.44±0.04^{ab}	14.72±1.91^{a}	26±1.71^{ab}	0.24±0.04^{a}
Street honey	67.1±0.52^{d}	21.77±0.48^{ab}	3.46±0.02^{ab}	16.26±3.63^{a}	12±1.15^{c}	0.15±0.1^{ab}
Retail store(veranda)	281.98±36.01^{bc}	20.49±0.13^{ab}	3.32±0.04^{ab}	14.95±0.44^{a}	16.5±1.3^{bc}	0.13±0.03^{bc}
Honey trader	398.27±29.73^{ab}	23.04±0.62^{a}	3.38±0.04^{ab}	21.23±3.67^{a}	28.13±1.1^{ab}	0.21±0.1^{ab}
Processors	236.18±44.69^{c}	19.48±0.4^{b}	3.77±0.05^{a}	17.5±5.95^{a}	33.83±5.15^{a}	0.26±0.05^{a}
Local market	423.05±11.91^{a}	22.3±0.53^{a}	3.22±0.00^{b}	10.66±1.24^{a}	17.5±0.29^{abc}	0.07±0.01^{c}

Means with different superscript (a, b, c) column are significantly different at P<0.05 assessed by Duncan's multiple ranges.

4. 2.2. Moisture content

Moisture content is the key criterion that determines the ability of honey to remain fresh and free of fermentation (Silva *et al*., 2009; Bogdanov *et al*., 1999). Table (11) illustrates the main characteristics of the commercially available honey. A statistically significant difference ($P< 0.05$) was observed among honey types (local market, honey trader, and small shop) from processors honey types. The highest moisture content was recorded for honey collected from honey traders (23.04±0.62%) and the lowest was recorded from the processors (19.48±0.4%).

The moisture content of honey collected from processors of this study was comparable with a study done by Fikru *et al*. (2012), (17.2 ± 0.86%), Tesfaye *et al*., (2016), (18.80±0.36%), Gebreegziabher *et al*., (2013), (18.4±0.8%). However higher than the study done by Tewodros *et al*. (2013) with 16.0 ± 1.25% from Ethiopian honey that was directly harvested from the hive. Results are also similar to Adgaba,(1999) who expressed that the mean result of Ethiopian honey moisture content found to be 20.5% but higher than those of Latif, *et al.,* (1956) who have reportable the moisture content of Pakistani honey to be within the range of 14.3 and 18.6%. Crane (1980) suggested that a low moisture content recorded could be due to honey harvested having reached maturity and advantageous, as it can promote a longer shelf life during storage (Terrab *et al*., 2003). The mean moisture content of processors and retail store (veranda) was in agreement with the national standard (ESA, 2005) and international (Codex, 2001 & EU, 2002) parameters recommended which should be a maximum of 20%, while the moisture content of honey obtained from a honey trader, small shops, supermarket, and street areas honey was found to be slightly above the national and international standards. The moisture content of honey could vary within the chain under the same ecological zone due to improper handling practice between harvesters, retailers & merchants. Similar to this study, Gebremariam & Berhane (2014) revealed that some honey samples collected from local markets had higher moisture content than national and international standards. The variation occurred between the moisture content of honey is mainly due to harvesting, handling, processing, and adulteration condition.

4. 2.3. Ash content

The average ash contents of different honey samples are summarized in Table 11. The ash contents of the samples collected from a supermarket, processors, and small shops were significantly different ($P<0.05$) from the honey samples collected from a local market and retail store (honey veranda). The highest mean ash content was recorded for a supermarket (0.26±0.026 g/100 g) and processors (0.26±0.05 g/100 g) and the lowest was recorded for the local market (0.07±0.01 g/100 g). The variability in the ash content of pure honey could be due to harvesting processes, beekeeping techniques, and the material collected by the bees during foraging (Finola *et al*., 2007). The ash content of this study was lower than the result (0.46 ± 0.03) reported by (Alvarez-Suarez *et al*., 2018). Similar values were reported by Kayode & Oyeyemi (2014) in Nigeria honey ranged from 0.004 to 0.440. Rodriguez *et al*. (1994) suggested the ash content of honey depends on the material contained in the pollen collected by the bees throughout foraging on the flora. The maximum limits set for ash content of the honey by EU, CAC, and ESA is (0.6%). None honey samples in this study exceed the standard limits. Higher mineral content could be due to the difference in botanical origin, materials gathered by the bees, soil composition, and environmental conditions. Rodriguez *et al*. (1994) suggested that the ash content of honey depends on the material contained in the pollen collected by the bees throughout foraging on the flora. Moreover, the same authors reported that the variation in the ash content comes from the natural property of soil and plants.

4. 2.4. Hydroxymethyl furfural aldehyde (HMF)

The HMF values of honey samples obtained from commercial areas (Table 11) ranged between 21.23±3.67 mg/kg mean value for the honey trader and 10.66±1.24 mg/kg mean value for the local market. However, they were not statistically ($P>0.05$) different from each other. None of the investigated samples exceeded the allowed limit of national (ESA, 2013) and international quality standards (Codex, 2001, and EU, 2002). Similar values reported by different scholars in Ethiopia reported HMF include 6.3 mg/kg in the Jimma Zone (Kinati *et al*., 2011), 15 mg/kg in the Tigrai Region (Gebru *et al*., 2015), and 6.3 mg/kg in the Gonder (Getu, & Birhan, 2014). But higher HMF value was reported by different scholars as

compared to this study like the one reported by Fredrick *et al*. (2013) and Muthui, (2012) from Kenya, and Kugonza and Dorothy (2008) from Uganda. They reported honey HMF content in samples from supermarkets as 85.4 ± 0.15 mg/kg, 3.7-389.4mg/kg and 103.2 ± 40.5mg/kg, respectively. Kugonza and Dorothy (2008) reported the HMF content of honey from brands varied from 7.1 ± 2.1 to 267.5 ± 183.2; while honey from Stail markets and hawkers were found the HMF as 60.1 ± 12.5 and 45.5 ± 5.7, respectively. Such high HMF concentration value is due to the effect of honey samples taken from market stress, poor handling of honey which affects the quality of the product. The natural levels of HMF are expected to tolerate its increases to some extent (10 mg/kg) due to handling, extracting, and conditioning or storing operations; and also due to liquefaction and pasteurization to improve manageability and destroy the crystallization nuclei (Visquert *et al*., 2014; Crane, 1990).

4. 2.5. pH

Table 11 shows the pH values for commercial market honey samples. The pH ranges from 3.22 to 3.62. As indicated in Table 11 there was a significant difference ($P<0.05$) between the mean pH values of honey samples collected from processors and the local market. The differences in the pH values of the samples might be due to the differences in the source of honey such as botanical, and processing methods (Alvarez-Suarez *et al*., 2018). If there is a low pH (high acidity), it can inhibit the presence and growth of microorganisms, and it can extend shelf-life. It also makes good taste for consumption. This type of honey would be more compatible with many food products for consumption in domestic and international markets (Kinati *et al.*, 2011). This is usually achievable because honey is acidic by nature. After all, it contains different organic acids like gluconic acid, formic acid, oxalic acid, and lactic acid (Nanda *et al*., 2003). European Honey Commission has set a range of 3.6-4.3, so pH values obtained in the present study were within the range specified by EU (2002) and the Codex (2001).The mean pH value from (3.77±0.05 to 3.22±0.00) of this study low enough to inhibit microorganism's development (Gomes *et al*., 2010; Cavia *et al.,* 2002). The results are in good agreement with a previous study in Malaysian (3.78 ± 0.21) (Moniruzzaman *et al*., 2013) but lower than the pH value from the Istanbul market 4.32 (Uran *et al*., 2017). Besides honey from Burkina Faso had pH values ranging from 3.5 to 4.7 (Meda *et al*., 2005), while honey from Nigeria had pH values ranging from 3.1-6.1 (Adebiyi *et al*., 2004). In

general, pH variability is due to the foraged plants, enzymatic process, and fermentative conversion of raw materials (Louveaux, 1985). Honey pH can provide a good indication of its origin and can also predict honey degradation during storage (Jeanne, 2005).

4. 2.6. Free Acidity

Honey acidity is related to having organic acids, especially gluconic acid, in equilibrium with their corresponding lactones or internal esters, and inorganic ions, mainly phosphate, sulfate, and chloride (Silva *et al*., 2009; Terrab *et al.,* 2002). The free acidity from processors samples (33.83±5.15meq.kg^{-1}) was found to be significantly different ($p < 0.05$) from the street honey types (12±1.15 meq.kg^{-1}) and retail stores (16.5±1.3meq.kg^{-1}) (Table 11). However, a statistically significant difference ($P>0.05$) was not observed in the free acidity among samples from the supermarket, small shops, and honey traders. The highest mean free acidity value of 33.83±5.15meq.kg^{-1} was recorded for the processor's honey types. The mean free acidity of all honey samples fits the national ($\leq$ 40 meq/kg) (ESA, 2005) and international (Codex, 2001, and EU, 2002) quality standards which should be a maximum of 50 meq/kg.

The present results compared well with the study done by Kamal *et al.,* (2002) (6.7-22.9 meq/kg^{-1}) of acidity in different honey samples. Free acidity values of honey close to the result obtained in the current study were reported from Tigray (29.89±5 meq/kg; Gebreegziabher *et al*., 2013) and Amhara (27.34 ± 5.06 meq/kg^{-1}; Alemu *et al*., 2013). The mean free acidity of honey from this study was higher than the free acidity of honey reported from Nigeria (18.67 ± 0.64 meq/kg) Nweze *et al*., 2017) and, the Polish market (14.40 ± 0.58 meq/kg^{-1}) (Makarewicz *et al*., 2017). A study conducted by Gebremariam & Brhane (2014) stated that market samples were found to have higher free acidity value than recommended for the authentic (pure) honey samples. A study report by Muli *et al.* (2007) from Kenya, indicated that the content of free acidity of honey was in the range of 8 to 71.9 meq/kg^{-1} for honey samples collected from traditional processors, beekeepers, and honey traders. The report by Fredrick *et al.* (2013) showed that the free acidity of honey samples collected from a local market (supermarket) and brands of honey was 56.7 meq/kg^{-1} which is much higher than the current study. When the free acidity becomes high, the honey is

fermented at some point, and the resulting alcohol is converted into organic acid. Thus, the honey becomes sour to taste, and hence, it is less acceptable (da Silva *et al*., 2016; Kugonza & Dorothy, 2008). The considerable variation in the amount of free acids in honey perhaps reflects the time required for nectar to be completely converted into honey under differing conditions of the environment, colony strength, and the sugar concentration of the nectar. Different management, harvesting, and processing techniques can also influence the final quality of honey in terms of free acid levels (Krell *et al*., 1996).

4.3. Levels of sugar composition in commercially available honey

The carbohydrate composition of bee honey is one of the key factors in establishing its botanical origin and, indirectly, contributes to its proper classification and trace adulteration (Muthui, 2012). The sugar content of honey is mainly fructose, glucose, and sucrose (Aljohar *et al*., 2018). Although the determination of the individual sugar content of monosaccharide (glucose, fructose) or disaccharides (sucrose) is essential, qualification of honey samples and can be accomplished by further determination of fructose to glucose ratio (Rybak-Chmielewska,2007).

4. 3.1. Fructose

Fructose is the dominant sugar in honey samples and responsible for the sweetness of honey (Crane, 1990). The mean fructose content in the honey from the local market (48.61±0.51%) was significantly different ($P<0.05$) from those of honey traders (39.42±1.01%), small shops (33.85±0.65%), and supermarket (39.13±0.98%) samples (Table 12). However, a statistically significant difference ($P>0.05$) was not observed in the fructose content between the samples collected from the local market, processors, veranda stores, and street areas. Table 12 showed that the highest fructose content was recorded for the local market and the lowest was recorded for small shops (33.85±0.65%). This study obtained a fructose content in the range of 25.80-49.48% with a mean of 40.08±0.67%; and the values agree well with the EHC range (31.2- 42.4%) except that few of the samples had low fructose values. The low value for fructose content indicates honey heated or adulterated. Honey sample collected from the

street (47.2±0.24) has contained high fructose content compared to others. This slight increase is probably due to the nectar richness in fructose (Louveaux, 1985). The results of most of the study samples were similar to the values reported by Al-Arrify (2002) (43.19%), Joshi *et al* (2000) (45.93%), Ismaeil (1972) reported 42.81%, White *et al* (1962) reported 38.19%, Kucuk *et al.,* 2007 reported 7.7 to 43.9%, Amri *et al*. (2008) reported with a range of 30.6-45.1 %, Amri,(2013) reported 27.2-44.3% and Belay *et al*, (2017) reported (43.1+ 0.4 g/100g) for Ethiopian monofloral honey. These values agree with the values obtained in the present study.

Table 12. The mean and standard error (mean± SE) of the sugar profile of commercially collected honey

Type of honey	Fructose (%)	Glucose (%)	Sucrose (%)	Maltose (%)	Reducing Sugar (%)	F/G ratio
Super market	39.13 ± 0.98^{cd}	37.9 ± 0.9^{abc}	2.87 ± 0.4^{bc}	1.17 ± 0.18^{b}	77.02 ± 1.1^{bcd}	1.07 ± 0.04^{b}
Small shops	33.85 ± 0.65^{d}	34.2 ± 3.34^{bc}	1.51 ± 0.1^{bc}	0.84 ± 0.08^{b}	68.01 ± 3.64^{d}	1.04 ± 0.10^{b}
Street honey	47.2 ± 0.24^{ab}	33.07 ± 1.58^{c}	13.4 ± 0.77^{a}	0.51 ± 0.14^{b}	80.3 ± 1.34^{abc}	1.43 ± 0.08^{a}
Retail store(veranda)	43.58 ± 1.9^{abc}	44.3 ± 0.82^{a}	6.23 ± 2.49^{b}	0.56 ± 0.15^{b}	87.88 ± 1.22^{a}	0.99 ± 0.06^{b}
Honey traders	39.42 ± 1.0^{bcd}	41.53 ± 2.7^{ab}	5.74 ± 1.5^{bc}	0.57 ± 0.10^{b}	80.95 ± 3.4^{abc}	0.99 ± 0.07^{b}
Processors	41.45 ± 1.6^{abc}	33.64 ± 1.7^{bc}	2.08 ± 0.5^{bc}	2.4 ± 0.44^{a}	75.10 ± 1.4^{cd}	1.27 ± 0.09^{ab}
Local market	48.61 ± 0.51^{a}	37.1 ± 0.04^{abc}	0.91 ± 0.05^{c}	0.79 ± 0.26^{b}	85.65 ± 0.55^{ab}	1.31 ± 0.01^{ab}

Note: Means with different superscript (a, b, c, d) within the columns are statistically different at P<0.05, F/G= Fructose to Glucose ratio

4.3.2. Glucose

The mean glucose values obtained from veranda honey types (44.3±0.82%) was significantly different (P<0.05) from processors (33.64±1.68%), street honey (33.07±1.58%), and small shops (34.17±3.34%) (Table 12) samples. Honey obtained from a veranda

store had the highest mean glucose value (44.3±0.82 g/l00g). This is due to the honey from certain plant species or honey sourced from feeding bees with syrups or honey in which artificial substances containing high values of glucose were added (Abdel-Aal *et al*., 1993). In this study, glucose content ranged from 25.57-49.57 % with a mean value of 37.35%. Some of these values agreed well with the EHC (23-32%). There are samples which had glucose content above stated by the Codex (2001). Kucuk *et al*. (2007) reported a similar study on the glucose content range of 34.9-40.2% except on the higher range. Amri *et al*. (2008) reported honey glucose levels to a range of 20.3-40.2%, which also agrees with the present study. The results obtained in this study are also in line with those reported by Jeanne (2005), who mentioned glucose content values ranging from 22.0 to 40.8%. Moreover, Belay *et al*. (2017) reported the glucose content (37.2+0.4g/100g) in the Ethiopian monofloral honey. The sample obtained from veranda stores (44.3±0.82%) and honey traders (41.53±2.65%) have glucose content above 40 % as compared to other studies. These findings suggest that the extremely high values of glucose in this study showed the addition of additives (Muthui *et al*., 2012).

4. 3.3. Reducing sugars

The reducing sugars of commercially available honey are presented in (Table. 12). The honey samples from the retail store (veranda) recorded the highest (87.88±1.22 %) reducing sugar and the lowest (68.01±3.64%) was recorded from small shops. Significance difference ($P<0.05$) was observed between retail stores from the veranda, local market, and small shops. The lowest reducing sugars were recorded for small shops (68.01±3.64%). Fructose and glucose constitute the primary sugars in all honey samples; in the honey of good quality, the percentage of fructose should exceed that of glucose. Reducing sugar in honey attributes to predetermine stages of honey ripeness nectar sources in different geographical locations (da Silva *et al*., 2016). Sohaimy *et al.* (2015) reported that the glucose content was lower than the fructose content which indicated the natural feeding of honey colonies in Saudi, Egyptian, and Yemeni honey. These results supported the previous several studies on different honey types (EL-Metwally, 2015; Buba *et al*., 2013; Manzoor *et al.,* 2013). The result is also in agreement with the studies done by Birhanu (2015) who reported (60.5%) for honey samples collected from farmers' hives and local honey market, but higher than studies conducted by

Gebremariam & Berhane,(2014) who reported (42.4%) for honey samples collected from local markets, pure honey, beekeepers, and adulterants.

The possible reasons for lower reducing sugar in honey is due to adulterations with other products; since the addition of other products decreases the ratio (%) of reducing sugar from the components in the honey. The lower reducing sugar in honey content can be also due to the effect when honey is harvested during hot conditions, or when the honey sample is taken from hot areas (Aljohar *et al.,*2018). Similarly, if it is hotter during honey harvesting, or processing, the reducing sugar content in honey will be lower. Generally, from this study, fructose and glucose sugars are the dominant sugars found in all sources of honey samples, while fructose was more abundant than glucose. The sum of fructose and glucose (F+G) values corresponded with the value (>60 g/100 g) given by Codex (2001).

4.3.4. Sucrose

From the present analysis (Table 12), a statistically significant difference ($P<0.05$) in the sucrose concentration was recorded between street honey, honey from the veranda, and the local market. The highest (13.4±0.77%) was produced by street honey samples followed by veranda stores honey samples (6.23±2.49%) and honey traders (5.74±1.52%). The difference could be due to different honey maturity levels since honey from the different areas were foraged and harvested. The highest mean sucrose was, found from street honey types, which could be due to honey from a certain floral origin, method of preparing honey by beekeepers overfeeding the bees with sugar and addition with sugar and syrups (Kelly *et al*., 2006). The study result indicated that a sucrose honey range of 0.07-16.46% and these result was higher than those previously reported for honey samples from Malaysia (3.02±1.33; Moniruzzaman *et al*., 2013), Bangladesh honey (6.1±0.1; Isalm *et al*., 2017), and from Nigeria (2.36 ± 0.05; Nweze *et al*., 2017) except for some few samples. The result of this study is in line with studies by Kinati *et al*. (2011) (5.68-9.45%), Tadesse and Gebregziabher (2014) (3.8-9.8%) and Getu, & Birhan (2014) (7.6%) who carried out analysis on honey samples collected from the local market, pure honey, adulterated one and from forest areas, respectively. Similarly, a study was done in Sudan, by Hana'a (2007) (5.5%) on honey samples collected from different floral sources beekeepers and by Mohammed *et al.* (2014) (5.8-8.7%) for honey

samples. The results of this study indicated that the honey samples obtained from street areas ,retail stores (veranda) ,and honey from a trader had sucrose values above 5 g/l00g limit proposed by the EHC (Bogdanov & Martin, 2000; Codex, 2001) and Ethiopia standard (ESA,2005).

4. 3.5.Maltose

The maximum and minimum mean ± standard error value of maltose was recorded from processors (3.04 ± 0.97) and street honey samples (0.73±0.14), respectively. The maltose content of honey samples collected from processors was significantly different ($P<0.05$) from other types of honey samples, but the concentration of maltose from the supermarket, small shops, street honey, trader honey, and the local market was not significantly different from each other (Table 12). The results of this study were found lower than the one reported by (Makarewicz *et al*., 2017) (1.88±0.11 to 6.64±0.15) for the Polish market honey. A study report for the Lithuanian honey collected from the market showed that maltose concentration varied from 0.29±0.01 to 1.41±0.01%. Kaškonienė (2011) suggested that artificial honey was distinguished from natural honey by a high amount of maltose. This finding suggested that the maltose content of commercially collected honey samples were low as compared to other countries.

4. 3.6.Fructose and glucose (F/G) ratio

The F/G ratio obtained for commercially marketed honey in this study ranged from street 1.43±0.08 to 0.99±0.07 (honey traders and honey from veranda). Street honey showed a significant difference ($P<0.05$) from other types of honey (Table 12). Krell (1996) reported that honey with a fructose: glucose ratio lower than 1.2 may be adulterated. The F/G ratio in various honey varied from 0.84 to 1.89 (Persano Oddo *et al*., 1995). The F/G ratio gives information about the crystallization state of honey, when the F/G ratio is 1.14 or less there is a tendency to crystallize, while values over 1.58 are associated with no such tendency (Tosi *et al*., 2004). Therefore, the F/G ratio is a good criterion for determining the ease with which honey crystallizes. Honey analyzed in Nepalese had F/G ratio which ranged between 0.85-1.49 (Surendra, 1999) and these results agree with the present results except on the upper part

where some samples seem to have high F/G ratios. White & Sicilliano (1980) reported F/G ratio range of 0.76-1.86. According to Moreira & Maria (2001), honey samples with F/G ratios smaller than one (1) may be adulterated, which probably represents an addition of some kind of syrup. Results of the honey analysis obtained in the current study also agree with the result from the honey produced in Spain in which the F/G ratios range from 1.00-1.43 (Bonvehi & Coll, 1994). However, the values obtained were higher than the values reported by A-Arrify (2002) which was 1.09, and Joshi *et al.* (2000) for Nepalese honey which was 1.1 and is closer to the values obtained by White *et al*. (1962) (1.22), Serra & Ventura (1995) (1.19) and less than the value obtained by Ismaeil (1972) which was 1.75.

4. 4. Levels of antioxidant activity in commercially available honey

4.4.1. Total phenolic content (TPC)

The total phenolic content of honey samples collected from honey traders showed statistically ($P<0.05$) significant difference from all samples except from supermarkets (Table 13). The highest TPC was recorded from the processors honey sample with a mean value of 533.39±28.9 mg GAE/100 g followed by the supermarket (343.27±33.45 mg GAE/100 g) and honey traders (263.02±10.06mg GAE/100 g). Total phenolic compounds ranged from 142.58 to 808.76 $mg \cdot kg^{-1}$ and this in agreement with the study done by Sime *et al* (2015) who reported that the total polyphenol content of the honey samples ranged from 330±38 to 610±5 mg GAE/100g from the Southern region of Ethiopia. Many researchers found that honey with dark color has a higher amount of total phenolic compounds (Gheldof *et al.,* 2002) and this result is in agreement with some commercial Ethiopian honey samples analyzed in this study. Compared to the TPC values from the result of other studies, the mean values of TPC for a honey of this study was higher than that of Malaysian samples (186.70±0.84 mg GAE/kg from acacia honey) and (226.29±1.18mg GAE/kg from pineapple honey) (Moniruzzaman *et al*., 2013). For Slovenian honey, the phenolic content ranged from 448 to 2414 mg GAE/100g, (Bertoncelj *et al*., 2007) which is higher than the TPC of honey from the present study. Besides, Ouchemoukh *et al* (2007) found a high TPC in the range of 79.00 and 1304.00 mg GAE/100g. Also, Rebiai *et al* (2015) investigated total phenolic compounds ranged from 179.2 to 1831.8 $mg \cdot kg\text{-}^{1}$. This is in line with the finding of Alvarez-

Suarez *et al*., (2010) who noted on factors that represent its chemical composition and phenolic content include bee species, bee forage, and geographical origin of honey.

Table 13. Mean and standard error (mean ± SE) values for total flavonoids, total phenolics, and antioxidant concentrations honey samples collected from market

Sample type	TPC (mgGAE/100g)	TFC (mgQE/100g)	DPPH (mg AEAC) /100 g)
Super market	343.27±33.45^{ab}	1252.15±142.35^{abc}	836.07±146.10^{a}
small shops	158.26±1.23^{b}	303.89±9.27^{bc}	959.65±9.79^{a}
street honey	176.24±0.09^{b}	256.91±60.50^{c}	130.54±9.68^{b}
Retail store (big veranda)	201.05±14.49^{b}	881.22±26.89^{bc}	290.04±13.21^{ab}
honey traders/collectors	263.02±10.06^{b}	295.40±111.37^{bc}	380.11±44.69^{ab}
Processors	533.39±28.9^{a}	2013.63±136.89^{a}	766.52±22.39^{a}
Local market	212.08±2.25^{b}	1420.07±77.46^{ab}	1327.17±909.29^{a}

Means with the same letter (a, b, c, d) within the columns are not statistically significant ($p \leq 0.05$). Notice: SE: Standard Error, TPC (mgGAE/100g of honey): Total phenolic content in milligram of GE per one hundred gram of honey sample, TFC (mgQE/100g of honey): Total flavonoid content in milligram of Quercetin equivalent per one hundred gram of honey sample, AOC (mg AAE/100g of honey): Antioxidant content in mg of Ascorbic acid equivalent in one hundred of a honey sample.

4.4.2. Total flavonoid content (TFC)

Total flavonoid content of the honey samples was measured using the calibration equation ($y = 2.487x + 0.036$; $R^2 = 0.9925$), derived from a calibration curve. The highest TFC was recorded from the local market with a mean of (1327.17±909.29 mg QE /100g) followed by small shops (959.65±9.79 mg QE /100g) and supermarket (836.07±146.10mg QE /100g) (Table 13). All honey samples in this study showed a significant difference ($P<0.05$) from street honey samples. The TFC from this study was higher than the result obtained from Turkish honey (1.12-9.24 mg QE/100g) (Kivrak *et al.,* 2017) and commercial Portuguese honey (1.73 ± 0.80 mg QE /100 g) but comparable with Rebiai *et al.* (2015) for Algeria honey with a range of 159.42 to 497.56 mg QE/100g. Phenolic acid, flavonoid, and tannins

are the common classes that are found under polyphenol (Keckes *et al.,* 2013) and show wide structural differences. The variation in total flavonoid content can be attributed due to geographical location, environmental factors, and the treatment used in the present study (Nayik *et al*., 2018).

4.4.3 Ascorbic acid Equivalent Antioxidant Capacity (AEAC) assay

The determination of total antioxidant content was done using ascorbic acid equivalent antioxidant capacity (AEAC) assay. Statistically, the processor's honey sample is shown significantly ($P<0.05$) different result from the street honey, honey trader, veranda honey, and small shops honey samples. However, there is no significant difference ($P>0.05$) between a supermarket, small shops, veranda honey, honey trader (Table 12) samples. The highest concentration of AEAC (2013.63±136.89 mg AAE /100 g) was recorded for the processors samples while the lowest (256.91±60.50 mg AAE /100 g) for street honey (Table 13). The mean values of antioxidants in these honey samples were above the results reported from Burkina Faso honey sample 11.27 ± 0.02 to 65.86 ± 0.1 mg AAE /100g (Meda *et al*., 2005), a honey sample from Bangladesh 18.4 ± 0.7 to 34.1±1.4 mg AAE /100g (Islam *et al*., 2012), and Indian honey samples ranging from 15 to 30 mg AAE /100g (Saxena *et al*., 2010). But the AEAC levels from this study were similar to the result obtained from Malaysian honey samples (276.96 to 324.47 mg AEAC/kg) (Moniruzzaman *et al*., 2013), Indian honey (151 to 295 mgAEAC/kg) (Saxena *et al*., 2010), and Burkina Faso honey (270.40 ± 146.8 mg/kg) (Meda *et al.*, 2005) samples.

4.5. Detection of adulterations using several techniques

4.5.1. Quick test for deliberately adulterated honey

Quick technique for identifying the adulterated and pure honey samples were presented in Table 14. In this study, the observations of physical tests were used useful to spot pure and impure honey samples.

Dissolving methods were conducted to know the characteristics of honey adulterated with different adulterant substances. The result demonstrated that 15 samples out of 15(100%) banana adulterated, 75% (11.25 samples out of 15) of molasses adulterated and 60% (9 samples out of 15) of shebeb adulterated honey was dissolved quickly in water as compared to pure honey which of 100% (3 samples out of 3) settled at the bottom (Table 14). Based on the study result, the dissolving method was suitable to detect honey adulterated with banana at home, local markets, as well as at the industrial level, but not suitable for sugar adulterated honey detection since 100 % (15 out of 15 samples) settled as pure honey. The results of this study are in line with Gebremariam & Berhane (2014) report in Adigrat and its surrounding areas in the Tigray region and Ambaw & Teklehaimanot (2018) in Arsi zones. The thumb test result showed that, 25 % (3 samples out of 12), 80 % (12 samples out of 15), and 100 % (3 samples out of 3) samples of honey adulterated with molasses, melted candy, and control honey intact on the thumb respectively. Also, 15 samples out of 15 (100%) of banana and 9 samples out of 12 (75%) of molasses adulterated honey spread around the thumb during the thumb test. 100% (15 samples out of 15) of sugar and shebeb adulterated honey showed sticky to the thumb which indicates a sign of purity, but they have a solid foreign particle. Based on the study result, the thumb test methods were suitable to detect banana and molasses adulterated honey efficiently. However, using a thumb test it is difficult to detect honey samples adulterated with sugar and shebeb, but it is possible to differentiate from pure honey based on the solid particles. The results of this study are in line with Gebremariam& Brhane,(2014) report in Adigrat and its surrounding areas in the Tigray region and Ambaw & Teklehaimanot (2018) in Arsi zones.

All samples (15 samples out of 15) of molasses, shebeb, and banana adulterated samples showed smoky flame and cracking sound followed by sugar (12 samples out of 15) 80% and 9 samples out of 12 (75%) of melted candy which indicates the sign of addition of foreign substances in the honey (Table 14). The obtained result showed that the flame test was the best traditional method to detect honey adulterated with molasses, banana, and shebeb adulterants. During the Coca-cola test method, all adulterant materials (15 samples out of 15) produced a bubbling sound and foam. The present study also confirmed that the coca-cola test methods were a good choice to detect honey mixed with sugar and sugar-related

products. The results obtained suggested that the ability of detection of the preliminary quick test was found specific and depend on the physical properties of adulterant materials

Table 14.The quick test results of deliberately adulterated honey samples

Test type	Molasses A.Honey	Banana A.Honey	Melted candy A.Honey	Sugar A.Honey	Shebeb A.Honey	Control(pure honey)
Dissolving method	75%(11.25/15) dissolved 25% (3.75/15) 50% dissolved	100% (15/15)dissolved	60% (9/15) settled 40% (6/15) dissolved	100%(15/15) settled	60 %(9 /15) dissolved 40 % (6/15) settled	100%(3/3) settled
Thumb test	75% (11.25/15) spread or spills 25 % (3.75/15) intact	100% (15/15)spread or spills	80% (12 /15) intact 20%(3/15) intact but have a foreign substance	100% (15/15) intact but have foreign substance	100% (15/15) intact but have foreign substance	100%(3/3) intact
Flame test	100% (15/15) smokes	100% (15/15) smokes	75%(11.25/15) smokes 25%(3.75/15) give light	80% (12/15) smokes 20%(3/15) give light	100%(15/15 smokes	100%(3/3) give light
Coco cola test	100% (15/15) produce bubbling and foam	100%(15/15) produce bubbling and foam	100% (15/15)produce bubbling and foam	100%(15/15) produce bubbling and foam	100%(15/15) produce bubbling and foam	100% (3/3) produce no bubbling and foam

(A=Adulterated)

4.5.2. Effect of molasses adulteration on honey physicochemical properties

4.5.2.1. Proline

In deliberately adulteration with molasses (5 to 50%) the proline contents ranged between 292.58±41.58 mg/kg (5%) to 206.51±2.97 mg/kg (50%). The proline content of pure honey was 381.5±1.15 mg/kg (Table.15). Thus, increasing molasses mixture in honey decreases proline contents. The mean values of proline for control honey samples showed a significant difference ($P<0.05$) from the mean proline values of all honey samples adulterated with molasses. The 50% adulteration level possessed a proline content of which is within the critical level (180 mg/kg). This results in agreement with Cavrar *et al*. (2013) which showed proline (mg/kg) content of honey adulterated with saccharide syrup was 258±66.52 mg/kg. Previously, proline was reported by several authors as the most important biochemical component for monitoring adulteration (Guler *et al*., 2007; Rouff *et al*., 2007). The study done by Czipa *et al*. (2012) suggested that the invert sugar and saccharide syrup contained proline in lower quantities 47 and 28 mg/kg, respectively. The same authors also indicated that the proline content decreased also when the heat was treated. Proline is produced by the salivary secretion of honey bees throughout the conversion of nectar into honey. Proline content is considered an important quality parameter for honey which will function as a further determinant of purity and maturity of honey. For the present study, the proline values of all samples were found to be within accepted ranges. But the pure honey sample contains the highest proline content than deliberately adulterated honey samples Czipa *et al.* (2012). So we may not draw any meaningful conclusion using proline content in deliberate adulteration using molasses because all the reading for deliberate adulteration is all within the proposed proline content of 180 mg/kg.

4.5.2.2. Moisture content

In deliberate adulteration with molasses (5 to 50%), the moisture content values ranged between 17.28±0.00 (5%) to 23.5±0.03 (50%). The mean values of moisture content for 50% adulteration level showed a significant difference ($P<0.05$) from others and pure honey samples. The 50% molasses adulterated honey was shown a significant difference ($P<0.05$)

between others. The moisture content of pure honey was 19.65±0.0 (Table 15). Thus, increasing the molasses mixture in honey does increase the moisture content. These results agree with the results obtained by Abdel-Aal *et al*. (1993) which was reported as an increase of HFCS adulterant in honey gave an increase in moisture content. But, Muthui (2012) investigated that increasing molasses mixture in honey does not give a clear cut trend; these results contradicted to present study. El-Bialee & Sorour (2011) reported that adulterating pure honey with the addition of four different materials (starch solution 3%, glucose syrup 20%, molasses, and distilled water) can result in a slight difference in refractive index values. Some moisture content above or below the range was recorded in the deliberate adulteration, but the results of this study indicated that moisture content increase as concentration of molasses increases. 40% and 50 % molasses adulteration level possessed critical value above the limit proposed by Codex for moisture content of 21.0g/100g. A higher moisture content could lead to undesirable fermentation, causing the honey to spoil and lose flavor and decreasing quality (Costa *et al*., 1999).

Table 15. Mean and standard error (mean± SE) physic-chemical content for the results obtained adulterated honey with molasses.

Treatments	Proline (mg/kg)	Moisture content (g/100g)	pH	HMF(mg/kg)	Free acidity (meq/kg)	Ash (g/100g)
Control	381.5±1.15^{a}	19.65±0.0^{c}	3.75±0.03^{d}	16.89±0.16^{b}	26.5±0.87^{c}	0.19±0.01^{c}
5% mollases	292.58±41.58^{b}	16.19±0.00^{e}	4.52±0.14^{c}	18.62±0.07^{b}	29±0.58^{c}	0.45±0.00^{a}
20% mollases	237.98±1.20^{bc}	17.28±0.63^{d}	5.16±0.01^{b}	57.96±15.80^{a}	48±1.73^{b}	0.43±0.06^{ab}
40% mollases	236.36±0.63^{bc}	22.47±0.03^{b}	5.46±0.01^{a}	59.67±1.07^{a}	73±4.04^{a}	0.43±0.02^{ab}
50% mollases	206.51±2.97^{c}	23.5±0.03^{a}	5.59±0.05^{a}	74.4±13.91^{a}	77±10.39^{a}	0.35±0.01^{b}

Means with different superscript (a, b, c) column are significantly different at P<0.05 assessed by Duncan's multiple ranges

4.5.2.3. pH

In deliberate adulteration with molasses (5 to 50%), the pH values ranged between 4.52±0.14 (5%) to 5.59±0.05 (50%). The mean values of pH for 40% and 50% adulteration levels showed a significant difference ($P<0.05$) from others and pure honey samples. The pH value in pure honey was 3.75±0.03. Increasing of molasses in honey leads to an increase in pH (pH was directly proportional to the percentage of molasses adulterant) as shown in (Table 15). 50% adulteration level showed significantly different from others. The 20%, 40%, and 50% adulteration level possessed a pH value above the critical value of (3.60-4.60) proposed by (Bogdanov & Martin, 2002). A similar study was reported by Muthui (2012). Ribeiro *et al*. (2014) observed a rise in the percentage of the adulterant resulted in significantly increased pH values for the honey adulterated with corn syrup. Similary, Rehman *et al*. (2008) suggested the pH of adulterated honey samples was higher than that of pure samples in honey samples analyzed from Pakistan. Oroian *et al*. (2018) also reported that the adulteration of honey with fructose increased the pH values of the samples, while the adulteration of honey with hydrolyzed inulin syrup decreased the pH values of the samples. Trifiro *et al*. (1990) hypothesized that the condensation between free amino group of amino acids and carbonyl groups of glucose present in molasses caused a decrease in pH. This is due to the water content of samples resulting in increased fermentation with a further decrease in the pH value (Aljohar *et al*., 2018). Relatively more acidic values ($pH < 3.24$) indicate improper storage or impure samples.

4.5.2.4. Free acidity

In deliberate adulteration with molasses (5 to 50%), the free acidity ranged between 29±0.58 meq/kg (5%) to 77±10.39 meq/kg (50%). The free acidity of pure honey was 26.5±0.87 meq/kg, thus increasing molasses mixture in honey leads to an increase in free acidity (free acidity was directly proportional to the percentage of molasses adulterant) (Table 15). The 40% and 50% adulteration levels possessed a free acidity value which is above the international critical value set by Codex (2001) and showed a significant difference ($P<0.05$) from other samples. A similar finding was reported by Muthui (2012) that the free acidity ranged between 42.0 meq/kg to 102.0 meq/kg for honey samples adulterated with molasses.

The high free acidity might be as a result of the fermentation of honey sugars to alcohol by microorganisms and further oxidation to carboxylic acids by the enzyme glucose oxidase in the honey. Ozhan (2008) and Wang *et al*. (2006) reported on the fact that reaction of amines forms compounds presented of in molasses lower basicity and degradation of sugars into acids. These findings are almost similar to the values reported in Burkina Faso where a range of 20.3 to 60.8 meq/kg was obtained (Meda *et al*., 2005).

4.5.2.5. Hydroxymethyl furfural aldehyde (HMF)

In deliberate adulteration with molasses (5 to 50%), the HMF values ranged between 18.62±0.07 mg/kg (5%) to 74.4±13.91 mg/kg (50%). Except for the 5% adulteration level, other molasses adulterated honey showed a significant difference ($P<0.05$) from pure honey samples. The HMF value in pure honey was 16.89±0.16 mg/kg. Therefore the increase of molasses in honey leads to an increase in HMF (HMF was directly proportional to the percentage of molasses adulterant) as shown in (Table 15). Molasses not only contain organic acids (*e.g*., formic acid) but also divalent and trivalent cations and anions (*e.g*., Cl-) all of which should auto-catalytically convert the sugars to HMF (Howard e*t al*., 2018).As well known, HMF is formed in two ways; (a) it is formed as an intermediate product in the Maillard reaction (Ames, 1992) and (b) it is formed from direct dehydration of sugars under acidic conditions during heat treatments of the foods (Kroh, 1994). 20%,40%, and 50% molasses adulteration level showed above 40 mg per kg honey of national (ESA, 2005) and international (Codex, 2001, & EU, 2002) quality standards. The high values of HMF also indicated that the honey samples might have heated and adulterated with processed sugar (Gebremariam & Berhane, 2014). HMF is an important indicator for honey purity, as HMF content is widely recognized as a parameter that indicates the freshness of honey, its content increases during conditioning and storage (Terrab *et al*., 2002).

4.5.2.6. Ash content

In deliberate adulteration with molasses (5 to 50%) the ash values ranged between 0.62±0.00 g/100g (5%) to 0.35±0.01g/100g (50%). Ash content in pure honey was 0.19±0.01g/100g

and showed a significant difference (P<0.05) from other molasses adulterated honey samples. Therefore, the increase of molasses in honey leads to an increase in ash content value (Ash content was inversely proportional to percentage to molasses adulterant). Abdel-Aal *et al*. (1993) examined the detection of adulteration honey deliberately adulterated with 10, 20, 30, 40, and 50% HFCS and suggested that the increases in the ash can be considered as simple and rapid tests for adulteration levels ranging between 10% and 50%. In addition, Ribeiro *et al* (2014) reported that the ash content of pure blossom honey increased gradually as the percentage of adulterant increased and they mentioned the increases of ash content were observed in all samples adulterant. The present study indicated that, all honey samples adulterated with molasses are with national and international standards (0.6g/100g) specified by ESA (2005), Codex (2001), and EU (2002).

4.5.3. Effect of molasses adulteration on honey antioxidant activity.

In deliberate adulteration with molasses (5 to 50%), the total phenolic content (TPC) values ranged between 373.43±40.5 mgGAE/100g (5%) to 814.33±35.40 mgGAE/100g (50%) (Table 16). The TPC value in pure honey was 264.92±7.52 mgGAE/100g, so increasing molasses mixture in honey leads to an increase of TPC value (TPC was directly proportional to the percentage of molasses adulterant). The mean TPC value of 20% adulteration level showed a significant difference (P<0.05) from pure honey samples. Previously, Blasa *et al.* (2006) and Bertoncelj *et al.* (2007) reported that darker honey tended to have higher phenolic contents when compared to lighter colored honey and our findings agree with these results. These investigations provide further support for the hypothesis that molasses adulterated honey has high TPC. While in deliberate adulteration with molasses (5 to 50%) the total flavonoid content (TFC) values ranged between 1405.44±36.96 mgQE/100g (5%) to 8867.85±129.59 mgQE/100g (50%).The mean value of TFC for 40% adulteration level showed a significant difference (P<0.05) from the 5% adulteration level and pure honey samples. The TFC value in pure honey was 719.47±93.81 mgQE/100g, so increasing molasses mixture in honey leads to an increase of TFC value and then decreased at 50% adulteration level which does not give a clear cut trend (but we can say that TFC was directly proportional to the percentage of molasses adulterant). The industrial molasses reaction

matrix contains additional components including lipids, proteins, oligosaccharides and polysaccharides, amino acids, organic acids and phenolics (Mee *et al*.,1979) .For deliberate adulteration with molasses (5 to 40%), the ascorbic acid equivalent antioxidant capacity (AAEAC) values ranged between 934.62±238.91 mgAAE/100g (5%) to 1894.29±184.05 mgAAE/100g (40%) (Table 16). The AAEAC value in pure honey is 329.65±128.46 mgAAE/100g, so increasing molasses mixture in honey leads to an increase of AAEAC value (AAEAC which was directly proportional to the percentage of molasses adulterant). Because a lower AAEAC value indicates stronger antioxidant properties, pure honey may contain the strongest antioxidant properties which are significant differences from other honey types.

Table 16. Mean and standard error (mean ± SE) values for the total flavonoids, total phenolic, and antioxidant concentrations honey adulterated with molasses.

Treatments	TPC (mgGAE/100g)	TFC (mgQE/100g)	AOC (mgAAE/100g)
Control	264.92±7.52^{d}	719.47±93.81^{c}	329.65±128.46^{b}
5% molasses	373.43±40.5^{c}	1405.44±36.96^{c}	934.62±238.91^{b}
20% molasses	830.79±26.54^{a}	4566.95±96.8^{b}	938.45±127.01^{b}
40% molasses	734.04±2.82^{b}	8867.85±129.59^{a}	1894.29±184.05^{a}
50% molasses	814.33±35.40^{ab}	6420.86±2105.0^{ab}	N.D

Means with the same letter (a,b,c,d) within the column are not statistically significant (P<0.05). Notice: - SD: Standard error, TPC (mgGAE/100g of honey): Total phenolic content in milligram of gallic acid equivalent per one hundred gram of honey sample, TFC (mgQE/100g of honey): Total flavonoid content in milligram of Quercetin equivalent per one hundred gram of honey sample, DPPH (mgAAE/100g): 1,1-diphenyl-2-picrylhydrazyl Scavenging activity in milligram of Ascorbic acid equivalent in 100g of a honey sample, N.D=non-detected.

Different scholars reported that both the total phenolic content and antioxidant activity of honey may be useful indicators for the determination of the naturalness of honey and by inference, the management practices of honey producers (Nisbet *et al*.,2018; Cimpoiu *et al.,*2013; Das *et al.,*2013; Khalil *et al*.,2010; Lianda *et al*.,2012). A high level of antioxidants, in terms of total phenol and total flavonoid contents, can be measured in pure honey. In this study, molasses exhibited a significantly high value of phenolic and flavonoid contents than

pure honey. This might be due to antioxidant value was contributed from molasses adulterants. Based on this finding, honey adulterated with molasses cannot be detected based on the total phenol and total flavonoid content.

4.5.4. Effect of table sugar adulteration on honey physicochemical content

4.5.4.1. Proline

In deliberate adulteration with sugar (5 to 50%), the proline content ranged between 214.31±5.59 mg/kg. (5%) to 212.42±4.30 mg/kg. (50%). The proline value of pure honey was 381.5±1.15 mg/kg and showed a significant difference ($P<0.05$) from all table sugar adulterated honey samples (Table 17). Proline, an essential free amino acid used for quality control of honey samples (Paramás *et al*., 2006). Important biological degradation occurred in proline contents of honey, when the colonies were fed with commercial sugars (20 L/colony and over) in the main nectar flow period (Guler *et al*., 2017).Values below 180 mg/100 g may indicate the unripeness of honey samples and adulteration (Bogdanov *et al*., 1999). All of the sugar adulterated honey samples presented were found within the acceptable limit of international standards. However pure honey samples contain higher proline content than all sugar adulterated levels. Thus, sugar adulterants decrease the proline content of honey samples.

4.5.4.2. Moisture content

In deliberate adulteration with table sugar (5 to 50%), the moisture content ranged between 20.37±0.00 (5%) to 16.76±0.00 (50%). Increasing the table sugar mixture in honey, decreasing the moisture content (moisture content was inversely proportional with the level of sugar adulteration) (Table 17). The pure honey had 19.65±0.00% moisture content. Abdel-Aal *et al.* (1993) reported an increase of HFCS adulterant in honey gave an increase in

moisture content. Similar findings obtained by Gebremariam, & Berhane (2014) indicated that, the addition of commercial sugar products to honey increase moisture content from 17.92 +0.55 for pure honey to 19.39 + 0.93 for a mixture of honey and sugar. Joshi *et al*. (2000) suggested that sugar-fed honey is low in water content. The values of moisture content for deliberate adulteration are lower than the value (21.0 g/100g) set by the Codex and EU. The 50% adulteration level possessed moisture content of which was below the critical values of 21.0% (Bogdanov, 1999) indicating the fermentation ability of honey is low. Furthermore, the moisture content of all the samples was below 21% as specified by the Codex and the European Union standards (Codex, 2001). The low moisture content observed may have been due to the increase in the sugar content of the samples. Thus, the values obtained from this study showed that the honey samples adulterated with table sugar, contain low moisture content and not suitable to detect honey adulterated with sugar, based on moisture content.

Table 17: Mean and standard error (mean ± SE) of physicochemical content for the results obtained for honey adulterated with table sugar.

Treatments	Proline(mg/kg)	Moisture content (g/100g)	pH	HMF(mg/kg)	Free acidity (meq/kg)	Ash(g/100g)
Control	381.5±1.15^{a}	19.65±0.0^{b}	3.75±0.03^{b}	16.89±0.2^{a}	26.5±0.87^{a}	0.19±0.01^{b}
5% table sugar	214.31±5.59^{b}	20.37±0.0^{a}	3.76±0.07^{b}	12.78±0.1^{b}	16.5±3.18^{b}	0.86±0.2^{a}
10% table sugar	215.31±0.37^{b}	19.27±0.3^{c}	3.84±0.00^{ab}	11.3±0.12^{b}	16.25±1.3^{b}	0.79±0.1^{a}
40% table sugar	241.33±18.90^{b}	18.93±0.1^{d}	3.89±0.06^{ab}	11.28±0.8^{b}	16±1.15^{b}	0.13±0.03^{b}
50% table sugar	212.42±4.30^{b}	16.76±0.0^{e}	3.92±0.02^{a}	5.04±0.64^{c}	13±0.58^{b}	0.03±0.01^{c}

Means with different superscript (a, b, c) column are significantly different at P<0.05 assessed by Duncan's multiple range

4.5.4.3. pH

In deliberate adulteration with sugar (5 to 50%), the pH values ranged between 3.84±0.00 (5%) to 3.92±0.02 (50%). The pH value of table sugar was 6.67 (Muthui ,2012) while that of pure honey was 3.75±0.03 (Table 17). The increase in table sugar mixture content in honey leads to a slight increase in pH of the honey (pH was directly proportional to the percentage of sugar adulterant). Although the mean pH values of 50% adulteration level showed a significant difference ($P<0.05$) from others, the pure honey sample has a low pH value as compared to table sugar adulterated honey samples. However, the value of acidity was evidently low in the adulterated honey. A similar study by Gebremariam, & Berhane (2014) investigated that the addition of commercial sugar to honey increase pH content from 3.83 ± 0.143 % for pure honey to 4.92 ± 0.67 for a mixture of honey and sugar. The 50% adulteration level possessed a pH value which is within the range of critical value of 3.60-4.30 proposed by (Bogdanov & Martin, 2002). The study done by Czipa *et al*. (2019) reported that the honey samples adulterated with sugar contain higher pH content than that of pure acacia honey samples. This finding indicates that pH values were not important to discriminate pure honey samples from table sugar adulterated one.

4.5.4.4. Free acidity

In deliberate adulteration with table sugar (5 to 50%), the free acidity values ranged between 16.5±3.18 meq/kg (5%) to 13±0.58 meq/kg (50%). The free acidity of pure honey was 26.5±0.87 meq/kg and showed a significant difference ($P<0.05$) from all table sugar adulterations level. Thus, increasing the table sugar mixture in honey leads to a decrease in the free acidity values (free acidity was inversely proportional to the percentage of sugar adulterant) (Table 17). The 50% adulteration level possessed a free acidity below the critical value of 50 meq/kg free acidity. This finding suggested that, table sugar adulterants reduce the free acidity of the honey samples. A decrease in the free acidity of honey produced by feeding honey bees with industrial sugar syrup is reported also by Ozcan *et al.* (2006) and Ross *et al*. (2009). Important biological degradation occurred in free acidity contents of honey, when the colonies were fed with commercial sugars (20 L/colony and over) in the main nectar flow period (Guler *et al*., 2017). Gebremariam & Brhane (2014) also showed the

addition of commercial sugar products to honey decrease free acidity content from 15.44 ± 4.93 meq/kg for pure honey to 10.54 ± 2.38meq/kg for a mixture of honey and sugar. Puteri *et al* (2018) stated adulterated honey samples had high free acidity. None of the samples adulterated with sugar exceeded the permitted acidity limit (50 meq/kg) indicating the absence of an undesirable fermentation process during table sugar adulteration. Free acidity indicates one of the quality parameters of honey samples and it reveals whether the honey is fermented or not (Silvano *et al*., 2014) and corresponds to the presence or absence of organic acids in the product (Bogdanov *et al.* (2008).

4.5.4.5. Hydroxymethyl furfural aldehyde (HMF)

In deliberate adulteration with sugar (5 to 50%) the HMF values ranged between 12.78±0.08 mg/kg (5%) to 5.04±0.64 mg/kg (50%). The HMF value of pure honey was 16.89±0.16 mg/kg and showed a significant difference ($P<0.05$) from all table sugar adulterations level. Thus, increasing sugar mixture in honey leads to a decrease in the HMF values (HMF was inversely proportional to the percentage of sugar adulterant)(Table 17). The 50% adulteration level possessed HMF of which is below the critical value of 40 mg/kg set by Codex (2001). Therefore, HMF content should not important for the identification of adulterated honey produced by adulterating honey with industrial commercial sugars

4.5.4.6. Ash content

In deliberate adulteration with sugar (5 to 50%), the ash contents ranged between 0.19±0.01 g/100g (5%) to 0.03±0.01g/100g (50%). The ash content of pure honey was 0.19 ±0.01 g/100g. Thus increasing the sugar mixture in honey, the ash content decreased (ash content showed inversely proportional to the level of sugar). These results agree with the findings of Gebremariam & Berhane, (2014), in which the addition of commercial sugar products to honey decreases ash content from 0.22 ± 0.14 for pure honey to 0.07 ± 0.05 for a mixture of honey and sugar. Important biological degradation occurred in mineral contents of honey, when the colonies were fed with commercial sugars (20 L/colony and over) in the main nectar flow period (Guler *et al*., 2017). Furthermore, the results revealed that the honey

produced from colonies fed with sugar syrup was showed low ash content (Buba *et al*., 2013; Sahinler *et al*., 2004). In the study, the 50% adulteration level possessed ash content below the critical value of 0. 6 % ash content (Bogdanov *et al.,* 1999).

4.5.5 .Effect of table sugar adulteration on honey antioxidant activity.

In deliberate adulteration with table sugar (5 to 40%), the TPC values ranged between 179.40±18.58 mgGAE/100g (5%) to 117.41±2.25 mgGAE/100g (50%) (Table 18). The TPC value in pure honey is 264.92±7.52 mgGAE/100g and showed a significant difference ($P<0.05$) from all table sugar adulterated honey samples. The increasing the table sugar mixture in honey leads to a decrease of TPC value (TPC was inversely proportional to the percentage of table sugar adulterant). Czipa *et al*. (2018) confirmed that honey samples adulterated with sugar additives had reduced TPC which was agreed with the present study. The amount phenolic substances existing in honey rely upon the nectar source, beekeeping practices, climatic conditions and biochemical changes in honey constituents (Kucuk *et al*.,2007; Nayik & Nanda, 2016). For deliberate adulteration with table sugar (5 to 50%), the TFC values ranged between 302.77±19.8 mgQE/100g (5%) to 480.63±57.77 mgQE/100g (50%). The TFC value in pure honey is 719.47±93.81 mgQE/100g and showed a significant difference ($P<0.05$) from all table sugar adulterated honey samples. Increasing table sugar mixture in honey does not give a clear-cut trend (but we can say that TFC was directly proportional to the percentage of sugar adulterant). But, the mean value of TFC for all table sugar adulteration levels is lower than the mean TFC of pure honey. The results of this study showed that TFC is suitable for detecting honey adulterated with table sugar.

In deliberate adulteration with table sugar (5 to 40%), the ascorbic acid equivalent antioxidant activity (AAEAC) values ranged between 460.04±162.68 mgAAE/100g (5%) to 844.20±125.10 mgAAE/100g (40%). The AEAC value in pure honey is 329.65±128.46 mgAAE/100g and showed a significant difference ($P<0.05$) from all table sugar adulterated honey samples. An increasing sugar mixture in honey does not give a clear-cut trend (but we can say that AEAC was directly proportional to the percentage of table sugar adulterant). But, the mean value of AEAC for all table sugar adulteration level is greater than the mean AEAC of pure honey. Because a lower AEAC value indicates stronger antioxidant

properties, pure honey contains the strongest antioxidant properties which significant differences from other honey types. Nisbet *et al*.,(2018) confirmed that the TPC, TFC and antioxidant activities of honey adulterated with sugar is lower than the pure one.

Table 18. The mean and standard error (mean ± SE) values for total flavonoids, total phenolics, and antioxidant concentrations for honey adulterated with table sugar.

Treatments	TPC (mgGAE/100g)	TFC (mgQE/100g)	AOC (mgAAE/100g)
Control	264.92±7.52^{a}	719.47±93.81^{a}	329.65±128.46^{c}
5% table sugar	179.40±18.58^{b}	302.77±19.8^{bc}	460.04±162.68^{ab}
10% table sugar	196.30±14.96^{b}	328.24±41.40^{bc}	570.72±60.84^{ab}
40% table sugar	168.83±10.58^{b}	154.40±47.16^{c}	457.27±125.32^{ab}
50% table sugar	117.41±2.25^{c}	480.63±57.77^{b}	844.20±125.10^{a}

Means with different superscript (a, b, c) column are significantly different at P<0.05 assessed by Duncan's multiple ranges

4.5.6. Effect of banana adulteration on honey physico chemical content

4.5.6 1.Proline

In deliberate adulteration with banana (5 to 50%) the proline contents ranged between 192.65±18.7 mg/kg (5%) to 146.29±2.7mg/kg (50%) (Table 19). The proline of pure honey was 381.50±1.15 and showed a significant difference (P<0.05) from all banana adulterated honey samples. Thus, increasing the banana mixture in honey leads to a decrease in proline value .The highest proline was found in the pure honey, whereas there was a considerable decline in the adulterated with 5%, 10%, 20%, 40%, and 50% level of adulteration. The 20%,40%, and 50% adulteration level possessed a proline of which is below the critical value of 180 mg/kg set by EU (2002).. A similar study was reported by Czipa *et al*. (2012) that the proline content of honey glucose syrup was 213 mg/kg which is closer to the mean proline concentration supermarket and processors honey. The proline content of honey decreased considerably with increasing syrup levels of all sugar. A certain ratio between concentrations of proline could be used to separate natural and adulterated honey (Alvarez-Suarez *et al*., 2010; Krell, 1996).

The importance of proline has been emphasized to discriminate natural and artificial honey samples in previous studies (Sorkun *et al.,* 2002; Basoglu *et al.,* 1996). The results of this study confirmed that proline could be used for the detection of honey adulterating with, mainly 20%, 40%, and 50 % level of banana adulterants most deliberately adulterated honey has a mean of proline above the standard required by Codex. Therefore, we suggest that the amount of proline given as a standard by Codex is too low to discriminate adulterated honey samples.

Table 19. Mean and standard error physicochemical content for the results obtained honey adulterated with banana

Treatments	Proline(mg/kg)	Moisture content (g/100g)	pH	HMF(mg/kg)	Free acidity((meq/kg)	Ash(g/100g)
Control	381.50±1.15^{a}	19.65±0.00^{d}	3.75±0.03^{c}	16.89±0.16^{b}	26.5±0.87^{c}	0.19±0.01^{c}
5% banana	192.65±18.70^{b}	19.44±0.00^{d}	4.02±0.04^{c}	14.63±0.1^{cd}	22±0.00^{d}	0.13±0.02^{c}
10% banana	186.86±8.65^{bc}	26.2±0.00^{c}	4.00±0.07^{c}	13.20±0.58^{d}	22±0.58^{d}	0.16±0.01^{c}
20% banana	167.58±6.38^{bc}	41.65±0.51^{b}	4.44±0.10^{b}	13.50±0.12^{d}	38.5±2.02^{b}	0.33±0.03^{b}
40% banana	151.8±5.19^{bc}	42.35±0.05^{b}	4.51±0.04^{b}	15.45±.06^{bc}	40±1.15^{ab}	0.4±0.03^{ab}
50% banana	146.29±2.71^{c}	43.26±0.70^{a}	4.91±0.24^{a}	20.2±0.35^{a}	42.5±0.87^{a}	0.46±0.03^{a}

Means with different superscript (a, b, c) column are significantly different at P<0.05 assessed by Duncan's multiple range

4.5.6. 2.Moisture content

In deliberate adulteration with banana (5 to 50%), the moisture values ranged between 19.65±0.00 (5%) to 43.26±0.70 (50%), while the moisture content in pure honey was 19.65±0.00. Thus, increasing the banana mixture in honey leads to increases in moisture value (moisture content was directly proportional to the percentage of banana adulterant)(Table 19). The mean value of moisture content of 50% adulteration level showed a significant difference ($P<0.05$) from other banana adulterated and pure honey samples. More than 20 % moisture confirms irregularities concerning the level of maturity reached in the hive, manufacturing and storage conditions, climatic conditions, and adulteration attempts (Nozal *et al.,* 2005; Codex, 2001;). The current study found that significantly the highest moisture content was found in 50%, whereas a considerable decline in the 40%, 20%, 10%, control, and 5% adulteration level ($P<0.05$). The 20%, 40%, and 50% adulteration levels possessed moisture content which is above the critical value of 21.0% reported by Codex (2001) and EU (2002). An increase in moisture content is indicative of adulteration and low moisture content protects honey from the attack by microorganisms (Adenekan *et al,* 2012).

4.5.6. 3.pH

In deliberate adulteration with banana (5 to 50%), the pH values ranged between 4.02±0.04 (5%) to 4.91±0.24 (50%). The pH of pure honey was 3.75±0.03, so increasing banana mixture in honey leads to an increase in pH (pH was directly proportional to the percentage of banana adulterant) (Table 19). The results of this study indicated that the highest pH was found in the 50%, whereas there was a considerable decline in the 40%, 20%, 5%, 10%, and control and showed a significant difference ($P<0.05$). The 20%, 40%, and 50% of adulteration levels possessed above the critical value of 3.6-4.30 pH value proposed by (Bogdanov & Martin, 2002). Ribeiro *et al*.(2014) observed a rise in the percentage of the adulterant significantly increased pH values for the honey adulterated by corn syrup. The pH can inhibit the growth of microorganisms, contributing to the stability of honey since a decrease in pH values may indicate honey fermentation (Cavia *et al*., 2007; Terrab *et al.,*

2004). These findings suggest that the substantial variation among the pH value of the adulteration level of bananas could be used as detection parameters.

4.5.6. 4.Free Acidity

In deliberate adulteration with banana (5 to 50%), the free acidity values ranged between 22±0.00 meq/kg (5%) to 42.5±0.87meq/kg (50%). The free acidity of pure honey was 36.0 meq/kg, therefore increasing banana mixture in honey leads to an increase in free acidity (free acidity is directly proportional to the percentage of banana adulterant) (Table 19). The significantly highest free acidity was found in 50%, whereas a considerable decline in 40%, 20%, control 10%, and 5% ($P<0.05$). The 50% adulteration level possessed a free acidity below the critical free acidity value of 7.0-46.8 meq/kg (Bogdanov, 1999). In most of the samples tested, the values for free acidity were within the established limits (50 meq/kg) set by (EU, 2002; Codex, 2001). However, banana adulterants caused the increment of free acidity which is a sign of fermentation and deterioration. Thus, using free acidity it might be possible to detect honey adulterated with banana adulterants. Increased acidity of honey is an indicator of a fermentation process and the transformation of alcohol into organic acid (Prica et al. 2014).

4.5.6. 5 Hydroxymethyl furfural aldehyde (HMF)

In deliberate adulteration with banana (5 to 50%), the HMF values ranged between 14.63±0.1 mg/kg (5%) to 20.2±0.35 mg//kg (50%). HMF of pure honey was 16.89±0.16 mg/kg, therefore, the increasing banana mixture in honey leads to a slight increase in HMF (HMF is directly proportional to the percentage of banana adulterant) (Table 19). The significantly highest HMF was found in 50% ($P<0.05$). In this study, the 50% adulteration level possessed HMF contents below the critical value of 40 mg/kg set by national and international standards. Capuano & Fogliano (2011) and Yucel & Sultanoglu (2013) concluded that high HMF content in honey may also be an indication of falsification by adding invert syrup because HMF can be produced by heating sugars in the presence of an acid to the inversion of sucrose. This study confirmed that banana adulterant doesn't increase the HMF content of

honey samples. The HMF content in honey formed from carbohydrates is an indicator of thermal treatment, mainly from fructose that disintegrates at approximately 60 °C, which is thermally more labile than sucrose and glucose (Dimins *et al*. 2006).HMF is considered as one quality parameter in evaluating honey freshness and honey deterioration.

4.5.6. 6.Ash content

In deliberate adulteration with banana (5 to 50%), the ash content ranged between 0.13±0.02 (5%) to 0.46±0.03 (50%). Ash content of pure honey 0.19±0.01g/100g, therefore increasing banana mixture in honey leads to an increase in ash content (Ash content is directly proportional to the percentage of banana adulterant) (Table 19). The highest ash value was found in 50%, whereas there was a considerable decline in 40%, 20%, 10%, 5%, and control and showed a significant difference ($P<0.05$) from others. For this study, the 50% adulteration level possessed ash value below the Codex standard (2001). This finding suggested banana adulterated honey could not be detected based on ash contents. Ribeiro *et al*. (2014) reported the ash content increased gradually as the percentage of HFCS adulterant increased. The high ash content may indicate an excess of inorganic material from external contaminants (such as handling and equipment) as well as from environmental pollution (Anklam, 1998). For these reasons, it is considered an important quality parameter in honey samples.

4.5.7. Effect of banana adulteration on honey antioxidant activity

In deliberate adulteration with banana (5 to 40%), the TPC values ranged between 1100.55±32.72 mgGAE/100g (5%) to 955.62±112.85 mgGAE/100g (40%) (Table 20). The TPC value in pure honey was 264.92±7.52 mgGAE/100g, so increasing the banana mixture in honey does not give a clear cut trend. However, the TPC value increased because of mixing banana into honey. In deliberate adulteration with banana (5 to 40%), the TFC values ranged between 123.44±19.51 mgQE/100g (5%) to 249.43±69.33 mgQE/100g (40%). The TFC value in pure honey is 719.47±93.81 mgQE/100g and showed a significant difference ($P<0.05$) from the others. The total phenolic content of supplement fed honey was found in the range of 158.04–174.87 mg GAE/100g (Kamal *et al.,* 2000). Thus, increasing the banana

mixture in honey does not give a clear cut trend. But, the value of TFC decreased because of mixing banana into honey comparing to pure honey. For deliberate adulteration with banana (5 to 40%), the ascorbic acid equivalent antioxidant capacity (AAEAC) values ranged between 184.81±3.91 mgAAE/100g (5%) to 232.68±11.17 mgAAE/100g (40%). The AEAC value in pure honey is 329.65±128.46 mgAAE/100g, and showed a significant difference ($P<0.05$) from the others. Thus, increasing the banana mixture in honey does not give a clear-cut trend; however, the value of AEAC decreased because of mixing banana honey comparing to pure honey. Because a lower AEAC value indicates stronger antioxidant properties, honey adulterated at a 10 % level may contain the strongest antioxidant properties which significant difference from other honey types. The increment of TPC and AEAC may be attributed from banana adulterants. Similar results were reported by (Nisbet *et al.,*2018; Cimpoiu *et al*.,2013; Das *et al*.,2013; Khalil *et al*.,2010; Lianda *et al*.,2012) regarding the effect of adulterant materials on antioxidant value and reported that adulterant materials caused reduced the antioxidant activity of honey samples and reported that adulteration substance could alter the antioxidant activities.

Table 20. The mean and standard error (mean ± SE) values for total flavonoids, total phenolics, and antioxidant concentrations for honey adulterated with banana.

Treatments	TPC (mgGAE/100g)	TFC (mgQE/100g)	AOC (mgAAE/100g)
Control	264.92±7.52^{a}	719.47±93.81^{a}	329.65±128.46^{c}
5% banana	1100.55±32.72^{a}	123.44±19.51^{cd}	184.81±3.91^{c}
10% banana	725.23±37.89^{b}	277.17±37.87^{b}	152.47±8.51^{d}
20% banana	304.97±11.01^{c}	124.25±49.08^{d}	215.65±13.63^{b}
40% banana	955.62±112.85^{ab}	249.43±69.33^{bc}	232.68±11.17^{b}

Means with the same letter (a, b, c, d) within the column are not statistically significant ($p\leq0.05$). Notice: - SD: Standard error, TPC (mgGAE/100g of honey): Total phenolic content in milligram of gallic acid equivalent per one hundred gram of honey sample, TFC (mgQE/100g of honey).Total flavonoid content in milligram of Quercetin equivalent per one hundred grams of honey sample, DPPH (mgAAE/100g):1,1-diphenyl-2-picrylhydrazyl Scavenging activity in milligram of Ascorbic acid equivalent in 100g of a honey sample.

4.5.8. Effect of melted candy adulteration on honey physicochemical content

4.5.8. 1.Proline

In deliberate adulteration with melted candy (5 to 50%) the proline content values ranged between 283.11±0.66 mg/kg (5%) to 194.19±12.14 mg/kg (50%). The proline content of pure honey was 381.5±1.15 mg/kg and showed a significant difference ($P<0.05$) from the all melted candy adulterated honey samples (Table 21). Increasing melted candy mixture in honey decreases proline (proline content was inversely proportional to melted candy adulterant level). The 50% adulteration level possessed a proline content of 194.19±12.14 mg/kg which is within the acceptable limit is 180mg/kg. These results in agreement with Cavrar *et al* (2013) findings which showed proline content of honey adulterated with sucrose syrup was 258±66.52 mg/kg. The proline contents of all melted candy adulterated honey samples were found to be within accepted ranges of 180mg/kg. So we may not draw any meaningful conclusion using proline content in deliberate adulteration. But the control sample has high proline content than all melted candy concentrations.

4.5.8. 2.Moisture content

In deliberate adulteration with melted candy (5 to 50%), the moisture content values ranged between 20.32±0.62 (5%) to 10.82±0.02 (50%). 50% melted candy adulterated was shown a significant difference between others (40%, 20%, 10%, 5%) adulteration level and control honey samples. The moisture content of pure honey was 19.65±0.0%. The mean value of moisture content for pure honey samples and 5% adulteration level showed a significant difference ($P<0.05$) from the other samples. These findings suggest that increasing melted candy mixture in honey does decrease the moisture content (moisture content was inversely proportional to the percentage of melted candy adulterant) as shown in (Table 21). The moisture content obtained in the deliberate adulteration honey samples is below the range, but the results of this study indicate that moisture content was reduced as the concentration of melted candy increases. Very low moisture content was also recorded for 40% and 50 % melted candy adulteration below the limit proposed for moisture content of 21.0g/100g by

Codex alimentations and EU. The moisture content of honey is most important in the assessment of ripeness and shelf life. The moisture content of honey samples is important because it contributes to their ability to resist fermentation and granulation during storage (Singh & Bath, 1997).

Table 21.The mean and standard error of physicochemical content for the results obtained for honey adulterated with melted candy.

Treatments	Proline(mg/kg)	Moisture content(g/100g)	pH	HMF(mg/kg)	Free acidity (meq/kg)	Ash(g/100g)
Control	381.5 ± 1.15^{a}	19.65 ± 0.00^{a}	3.75 ± 0.03^{c}	16.89 ± 0.16^{d}	26.5 ± 0.87^{ab}	0.19 ± 0.01^{bc}
5% melted candy	283.11 ± 0.66^{b}	20.32 ± 0.62^{a}	3.81 ± 0.03^{ab}	4.69 ± 0.12^{e}	28 ± 2.31^{a}	0.13 ± 0.04^{c}
10% melted candy	249.78 ± 8.35^{b}	16.76 ± 0.00^{b}	3.75 ± 0.07^{c}	53.65 ± 1.42^{c}	21 ± 1.15^{bc}	0.70 ± 0.28^{a}
20% melted candy	240.27 ± 30.51^{bc}	14.26 ± 0.0200^{c}	3.88 ± 0.03^{ab}	94.61 ± 0.00^{b}	19 ± 0.58^{cd}	0.57 ± 0.16^{ab}
40% melted candy	240.54 ± 11.89^{bc}	13.49 ± 0.06^{c}	3.89 ± 0.00^{ab}	235.03 ± 4.32^{a}	17.5 ± 1.44^{cd}	0.23 ± 0.04^{bc}
50% melted candy	194.19 ± 12.14^{c}	10.82 ± 0.02^{d}	3.92 ± 0.04^{a}	240.34 ± 84.40^{a}	16 ± 2.31^{d}	0.06 ± 0.02^{c}

Means with different superscript (a, b, c) column are significantly different at $p\leq0.05$ assessed by Duncan's multiple ranges

4.5.8. 3.pH

In deliberate adulteration with melted candy (5 to 50%), the pH values ranged between 3.81±0.03 (5%) to 3.92±0.04 (50%). The pH value of pure honey was 3.75±0.03, therefore the increase of melted candy in honey leads to an increase in pH (pH was directly proportional to the percentage of melted candy adulterant level) as shown in (Table 21). This study confirms that 50% adulteration level showed a significant difference from other samples. Yadata (2014) confirmed that the honey adulterated with sugar syrup has very low acidity (high pH) while that adulterated with inverted sugar has higher acidity (low pH). The pH values of all melted candy honey samples were within the acidic specified limit of pH of 3.75 to 3.92 reported by many scholars. Honey pH is related to microbial growth, due to bacterial growth occurring in a neutral and mildly alkaline environment; however, yeasts and moulds are found in an acidic environment (pH= 4.0 -4.5) and do not grow well in alkaline media (Conti, 2000; Silva *et al*., 2009).

4.5.8. 4.Free acidity

In deliberate adulteration with melted candy (5 to 50%), the free acidity ranged between 28±2.31 meq/kg (5%) to 16±2.31 meq/kg (50%). The free acidity value for pure honey was 26.5±0.87meq/kg, thus increasing the melted candy mixture in honey leads to a decrease in free acidity (free acidity was inversely proportional to the percentage of melted candy adulterant) (Table 21). The recommended acidity of honey is usually less than 40 meq/kg of honey (ESA 2005). Free acidity is a parameter that can assist in assessing the deterioration level of honey and its limit is established as 50 meq kg^{-1} (Codex, 2001; EU, 2002). These results suggested that the free acidity of honey samples was affected by adulteration with melted candy.

4.5.8. 5. Hydroxymethyl furfural aldehyde (HMF)

In deliberate adulteration with melted candy (5 to 50%), the HMF values ranged between 4.69±0.12 mg/kg (5%) to 240.34±84.40 mg/kg (50%). A significant difference ($P<0.05$) was

observed among the means value of HMF content of all melted candy adulterated and pure honey samples. The HMF value in pure honey was 16.89±0.16, therefore the increase of melted candy in honey leads to an increase in HMF (HMF was directly proportional to the percentage of melted candy adulterant) as shown in (Table 21). The results of this study indicated that 20%, 40%, and 50% adulteration level contain HMF above national (ESA, 2005) 40 mg/ kg HMF and international 40 mg/ kg(EU, 2002); 60 mg/kg (Codex, 2001); 80 mg/kg for tropical countries (Codex, 2001) quality standards. HMF can be formed by the Maillard reaction (heating of reducing sugars in the presence of proteins), or by dehydration under acidic conditions (Bogdanov *et al*., 2004). HMF amounts exceeding this maximum limit are considered the main indicator of honey deterioration (Bogdanov & Martin 2002), either through heating or adulterations. Doner (1977) reported that higher values of HMF point towards the possibility of honey adulteration by invert syrup.

4.5.8.6. Ash content

In deliberate adulteration with melted candy (5 to 50%), the ash values ranged between 0.13±0.04 g/100g (5%) to 0.06±0.02 g/100g (50%) (Table 21). Ash content in pure honey was 0.19±0.01g/100g; therefore the increase of melted candy in honey leads to a decrease in ash content value (Ash content was inversely proportional to percentage to melted candy adulterant). The present study agrees with the report of Ribeiro *et al*.(2014). The result showed that melted candy decreased the ash content of honey. Normally pure honey has low ash content and may be different from one sample to another because it depends on the material added to honey samples.

4.5.9. Effect of melted candy adulteration on honey antioxidant activity.

In deliberate adulteration with melted candy (5 to 40%), the TPC values ranged between 309.84±5.90 mgGAE/100g (5%) to 422.01±15.04 mgGAE/100g (40%) (Table 22). The TPC value in pure honey is 264.92±7.52 mgGAE/100g, so increasing the melted candy mixture in honey leads to an increase of TPC value (TPC was directly proportional to the percentage of melted candy adulterant. In deliberate adulteration with melted candy, the TFC values ranged between 1118.75±56.19 mgQE/100g (5%) and 542.15±41.55 mgQE/100g (40%). The TFC

value in pure honey is 719.47±93.81 mgQE/100g, so increasing the melted candy mixture in honey leads to a decrease of TFC value (TFC was inversely proportional to the percentage of melted candy adulterant).

In deliberate adulteration with molasses, the AEAC values ranged between 1209.58±66.58 mgAAE/100g (5%) to 726.34±92.35 mgAAE/100g (40%). The AEAC value in pure honey is 329.65±128.46 mgAAE/100g, so increasing the melted candy mixture in honey leads to a decrease of AEAC value (AEAC was inversely proportional to the percentage of melted candy adulterant). Because a lower AEAC value indicates stronger antioxidant properties, pure honey contains the strongest antioxidant properties which significantly differed from other honey types. Similar results were reported by (Nisbet *et al.,*2018; Cimpoiu *et al*.,2013; Das *et al*.,2013; Khalil *et al*.,2010; Lianda *et al*.,2012). The amount and form of phenolic substances existing in honey rely upon the nectar source, beekeeping practices, climatic conditions, and biochemical changes in honey constituents (Kucuk *et al*., 2007; Nayik & Nanda, 2016).

Table 22. The mean and standard error (mean ± SE) values for total flavonoids, total phenolics, and antioxidant concentrations for honey adulterated with melted candy.

Treatments	TPC (mgGAE/100g)	TFC (mgQE/100g)	AOC (mgAAE/100g)
Control	264.92±7.52^{c}	719.47±93.81^{b}	329.65±128.46^{b}
5% melted candy	309.84±5.90^{b}	1118.75±56.19^{a}	1209.58±66.58^{a}
10% melted candy	301.08±8.51^{b}	667.33±93.03^{b}	1511.79±70.07^{a}
20% melted candy	407.06±10.02^{a}	737.84±13.95^{b}	1345.30±241.24^{a}
40% melted candy	422.01±15.04^{a}	542.15±41.55^{b}	726.34±92.35^{b}

4.5.10. Effect of shebeb adulteration with honey on physico chemical content

4.5.10.1. Proline

In deliberate adulteration with shebeb (5 to 50%), the proline ranged between 176.96±84.21 mg/kg (5%) to 52.01±15.92 mg/kg (50%). The proline value of pure honey was 381.5±1.15 mg/kg and showed a significant difference ($P<0.05$) from the others (Table 23). But, there is no significant difference ($P>0.05$) between the shebeb adulterated honey samples, except for the 50% adulteration level. Increasing the shebeb mixture in honey, the proline content decreased (proline content was inversely proportional to the shebeb adulterant level). All adulteration level of shebeb adulterated (50%, 40%, 20%, 10%, and 5%) has proline value below internationally recommended (180 mg/kg). Bogdanov (1999) suggested that honey that contains less than 180 mg /kg of honey is not pure. The results of this finding showed that proline content was the best parameter to detect honey adulterated with shebeb adulterants.

4.5.10.2. Moisture content

In deliberate adulteration with shebeb (5 to 50%), the moisture ranged between 18.91±0.15 (5%) to 15.547±0.22 (50%). Thus increasing the shebeb mixture in honey, does not give a clear cut trend, the moisture content increased, and then it decreased (Table 23). In pure honey moisture content was 19.65±0.00%. The 50% adulteration level possessed a moisture content of 15.547±0.22 % which was below the critical values of 21.0% of moisture according to Codex (2001) standard. All adulteration level is within the acceptable limit of internationally recommended parameters (≤21%). Moisture content determines the amount of water present in honey. Adulterant materials can alter the moisture content or refractive index of honey samples. Samat *et al*.(2018) reported that moisture content in pure honey was slightly higher but is not significant compared to adulterated honey. The same authors also suggested that the moisture content of adulterated honey can be engineered according to the Codex and stable because of lacking hygroscopic behavior even at room temperature (Samat

et al., 2018). Overall, these results indicate that mixing shebeb into honey causes moisture content to decrease in honey samples.

Table 23.The mean and standard error of physicochemical content for the results obtained for pure honey and honey adulterated with shebeb.

Treatments	Proline(mg/kg)	Moisture content(g/100g)	pH	HMF	Free acidity (meq/kg)	Ash(g/100 g)
control	381.5±1.15^{a}	19.65±0.0^{a}	3.75±0.03^{a}	16.9±0.16^{e}	26.5±0.87f	0.19±0.01^{e}
5% shebeb	176.9±84.2^{b}	18.9±0.15^{b}	3.1±0.02^{b}	9.48±0.37^{f}	297.5±1.4c	0.6±0.17d^{e}
10% shebeb	150.89±6.5^{b}	19.86±0.3^{a}	2.85±0.05^{c}	39.12±2.2^{d}	165±18.48^{e}	0.84±0.08^{d}
20% shebeb	161.66±3.8^{b}	19.4±0.1^{ab}	2.86±0.01^{c}	60.18±0.1^{c}	1045±25.9^{c}	2.86±0.03^{c}
40% shebeb	123.4±17.2^{b}	19.5±0.1^{ab}	2.91±0.01^{b}	83.16±0.5^{b}	1185±14.4^{b}	5.60±0.18^{b}
50% shebeb	52.01±15.9^{c}	15.55±0.2^{c}	2.83±0.01^{b}	95.51±0.1^{a}	1245±2.89^{a}	6.77±0.25^{a}

Means with different superscript (a, b, c) column are significantly different at p≤0.05 assessed by Duncan's multiple ranges.

4.5.10.3. pH

In deliberate adulteration with shebeb (5 to 50%), the pH values ranged between 3.07±0.02 (5%) to 2.83±0.01 (50%). The pH value of pure honey was 3.75±0.03 and showed a significant difference (P<0.05) from the others (Table 23). It is interesting to note that increasing shebeb mixture content in honey leads to a decrease in the pH of the honey (pH was inversely proportional to the percentage of shebeb adulterant. Another important finding was that shebeb adulterated honey contain a very low pH value (high acidity) in contrast to the pH of deliberately adulterated honey, which is contradicted and questionable. This indicated that shebeb adulterated honey is highly acidic and makes it unique from other adulterants materials. These results are consistent with those of other studies which suggest that the adulteration of honey with fructose increased the pH values of the samples, while the adulteration of honey with hydrolyzed inulin syrup decreased the pH values of the samples (Oroian *et al*., 2018). Samat *et al*.(2018) also reported the same results. Puteri *et al (2018*)

also stated adulterated honey samples had the lowest pH. The implication of this is the possibility that shebeb adulterant increases the acidity of honey during mixing.50% adulteration level possessed a pH value of 2.83±0.01 which was below the critical value of 3.60-4.30. The low pH value of honey determines its microbiological stability and may indicate high contents of minerals (El Sohaimy *et al*., 2015). This study confirmed that honey adulterated with shebeb had the highest pH value and the highest total ash content. The pH level affects the keeping quality of honey as it influences its texture, stability, and shelf-life (Terrab *et al*., 2002).

4.5.10.4. Free acidity

In deliberate adulteration with shebeb (5 to 50%) the free acidity values ranged between 297.5 ±1.44 meq/kg (5%) to 1245±2.89 meq/kg (50%). The free acidity value of pure honey was 26.5±0.87 meq/kg, thus increasing shebeb mixture in honey leads to an increase in the free acidity values (free acidity was directly proportional to the percentage of shebeb adulterant)(Table 23). The free acidity of shebeb adulterated and pure honey samples showed a significantly different ($P<0.05$) from each other. The results of this study confirm that shebeb adulterated honey was characterized by high free acidity value. In this study, 10%, 20%, 40%, and 50% adulteration level possessed a free acidity which is above the critical value of 50 meq/kg set by Codex (2001). Free acidity is a measure of honey deterioration. This parameter is linked with the natural presence of organic acids in honey, which remain in equilibrium with internal esters, lactones, and some inorganic ions like phosphates, sulfates, and chlorides. In addition, a high value of total acidity may imply that at some point the honey began to ferment and that the produced alcohol can be transformed into organic acids (Grigoryan, 2016).

4.5.10.5. Hydroxymethyl furfural aldehyde (HMF)

In deliberate adulteration with shebeb (5 to 50%) the HMF values ranged between 9.48±0.37 mg/kg (5%) to 95.51±0.09 mg/kg (50%). The HMF value of pure honey was 16.89±0.16 mg/kg, thus increasing the shebeb mixture in honey leads to an increase in the HMF values (HMF was directly proportional to the percentage of shebeb adulterant) (Table 23). The HMF

of shebeb adulterated and pure honey samples showed significantly different (P<0.05) from each other. The process of HMF can be accelerated by the heating process, which is a common practice in adulterated honey production (Khalil *et al*. 2010). Furthermore, Lee and Nagy (1990) reported that HMF can form under acidic conditions even at low temperatures. The process can be observed deliberately adulterated honey samples with shebeb used in the study. Honey samples adulterated with shebeb at 20%, 40%, and 50% adulteration level possessed a high value of HMF, which is above the acceptable limit recommended by Codex (2001) fix at 40 mg/kg. This study has shown that honey adulterated with shebeb adulterant could be detected by HMF content.

4.5.10.6 Ash content

In deliberate adulteration with shebeb (5 to 50%), the ash contents ranged between 0.61±0.17 g/100g (5%) to 6.77±0.25 g/100g (50%) (Table 23). The ash content of pure honey was 0.19±0.01 g/100g, thus increasing the shebeb mixture in honey, the ash content increased (ash content showed directly proportional to the level of shebeb adulterant). The ash content of shebeb adulterated and pure honey samples showed a significantly different (P<0.05) from each other's, except for control and 5% adulteration level. All shebeb adulteration level possessed ash content of which is above the critical value of 0. 6 % set by Codex (2001). Abdel-Aal *et al*.(1993) suggested that the increases in ash content of honey samples with HFCS can be considered as simple and rapid tests for adulteration levels ranging between 10% and 50%. In addition, Ribeiro *et al*. (2014) reported, the ash content of pure blossom honey increased gradually as the percentage of adulterant increased. Therefore, shebeb could be a major factor for the increment of ash content in honey and could be used as the detection method for natural honey from adulterated one.

4.5.11. Effect of shebeb adulteration with shebeb on honey antioxidant activity.

In deliberate adulteration with shebeb (5 to 50%), the TPC values ranged between 202.78±11.96 mgGAE/100g (5%) to 506.60±131.85 mgGAE/100g (50%)(Table 24). The

TPC value in pure honey is 264.92±7.52 mgGAE/100g, so increasing shebeb mixture in honey leads to an increase of TPC value (TPC was directly proportional to the percentage of shebeb adulterant). This study has shown that, except for 40% and 50 % shebeb adulteration levels, other adulterated samples possessed TPC below the pure honey samples. This indicates that TPC could be used to detect the low level of honey adulterated with shebeb. In deliberate adulteration with shebeb (5 to 50%), the TFC values ranged between 581.02±195.93 mgQE/100g (5%) and 115.67±10.30 mgQE/100g (50%). The TFC value in pure honey was 719.47±93.81 mgQE/100g, so increasing shebeb mixture in honey leads to an increase of TFC value (TFC was directly proportional to the percentage of shebeb adulterant). The results of this research support the idea that TFC could be used to differentiate pure and adulterated honey.

Table 24.The mean and standard error (mean ± SE) values for total flavonoids, total phenolics, and antioxidant concentrations for pure honey, and honey adulterated with shebeb.

Treatments...	TPC (mgGAE/100g)	TFC (mgQE/100g)	AAEAC (mgAAE/100g)
Control	264.92±7.52^{bc}	719.47±93.81^{a}	329.65±128.46^{e}
5% shebeb	202.78±11.96^{c}	581.02±195.93^{a}	2037.94±62.5^{a}
10% shebeb	202.87±6.08^{c}	286.15±8.78^{b}	1430.94±101.91^{b}
20% shebeb	228.06±8.16^{c}	208.95±32.23^{b}	956.08±30.46^{d}
40% shebeb	419.79±36.34^{ab}	202.65±12.16^{b}	1359.30±56.84^{bc}
50% shebeb	506.60±131.85^{a}	115.67±10.30^{c}	1140.52±42.42^{cd}

In deliberate adulteration with shebeb (5 to 50%), the AEAC values ranged between 2037.94±62.5 mgAAE/100g (5%) and 1359.30±56.84 mgAAE/100g (50%). The AEAC value in pure honey is 329.65±128.46 mgAAE/100g, so increasing shebeb mixture in honey leads to a decrease of AEAC value (AEAC was inversely proportional to the percentage of shebeb adulterant). Because a lower AEAC value indicates stronger antioxidant properties, pure honey contains the strongest antioxidant properties which significant differences from other honey types. Similar results were reported by (Nisbet *et al.,*2018; Cimpoiu *et al*.,2013; Das *et al*.,2013; Khalil *et al*.,2010; Lianda *et al*.,2012;).

4.5.12. Effect of adulterant substances on the sugar profile of honey.

4.5.12.1 Fructose content

The fructose content of pure honey samples significantly different ($p<0.05$) from shebeb and sugar adulterated honey. However, a significant difference was not observed among the mean value of fructose content in molasses, melted candy, and banana adulterated honey samples (Table 25) The highest fructose content was recorded for pure honey (48.75±0.00) followed by molasses (36.63±4.48) and banana (35.55±6.60). The lowest mean of fructose value was recorded for sugar adulterated (20.92±5.33) samples. Some adulterated honey contains low fructose values (Garcia *et al*., 2002). These results suggest that fructose content is used to detect honey adulterated with banana, melted candy, molasses, shebeb, and sugar because the mean fructose content of those adulterant substances was low compared to pure honey. Therefore, it can be suggested that the amount of fructose given as a standard was used to discriminate adulterated honey.

4.5.12.2. Glucose content

The effect of adulterant materials on the glucose content of honey is summarized in (Table 25). A significant difference was not observed between adulterant materials on glucose content. However, numerically the mean value glucose for pure (control) 39.04±4.8 was the highest followed by shebeb (39.04±0.11) and banana (36.25±4.91) adulterated honey. The lowest mean value of glucose was recorded by molasses adulterated honey (27.40±0.7). These results agree with the ones obtained by Abdel-Aal *et al.* (1993) where a decrease of glucose was observed with an increase of HFCS adulterant in honey. The studies done by Muthui (2012) and Abdel-Aal *et al.(* 1993) was found that the extremely high values of glucose were indicative of additives added honey samples. The results of this study indicate that the glucose content of honey could be affected by adulterants materials and used to detect adulteration.

Table 25. Mean and standard error (mean ± SE) values for sugar profile adulterated honey samples.

Treatments	Fructose (%)	Glucose (%)	Sucrose (%)	Maltose (%)	F/G ratio (%)	Reducing sugar(%)
Molasses	36.63±4.5^{ab}	27.40±0.7^{a}	2.86±0.80^{b}	2.11±0.8^{ab}	1.33±0.13^{a}	64.04±5.20^{ab}
Banana	35.55±6.6^{ab}	36.25±4.9^{a}	1.09±0.88^{b}	3.73±0.04^{ab}	1.12±0.4^{ab}	71.80±2.72^{ab}
Melted candy	30.28±6.2^{ab}	34.23±5.5^{a}	5.74±3.08^{ab}	3.20±1.8^{ab}	0.86±0.1^{ab}	64.51±11.7^{ab}
Sugar	20.92±5.33^{b}	33.75±4.6^{a}	20.12±12.3^{a}	9.35±5.82^{a}	0.60±0.1^{b}	54.67±9.91^{c}
Shebeb	25.72±3.39^{b}	39.05±0.1^{a}	1.30±0.57^{b}	5.28±0.3^{ab}	0.66±0.02^{b}	64.77±7.65^{ab}
Control	48.75±0.00^{a}	39.04±4.8^{a}	0.76±0.04^{b}	0.88±0.00^{c}	1.25±0.0^{ab}	87.68±0.11^{a}

Note: Means with different superscript (a, b, c, d) within the columns are statistically different at P<0.05, F/G= Fructose to Glucose ratio

4.5.12.3. Reducing sugar

The results of the analysis showed that the mean value of reducing sugar content of honey ranged from 54.67±9.91 for sugar adulterated and 87.68±0.11 for pure honey samples (Table 25). Comparison between honey samples showed that pure honey and banana honey met the quality standard for reducing sugar while other samples (sugar, shebeb, molasses, and melted candy adulterated samples) were lower than the standard permissible limit. According to the proposed Ethiopian standard (2005) and Codex (2001), a minimum reducing sugar content of 65%. These results are in agreement with that of Nauta (1983) who also reported that reducing sugars in honey was in the range of 60 to 65%. Gebremariam& Berhane (2014) investigated the addition of commercial sugar products to honey decrease reducing sugars (%) content from 69.81 ± 4.79 for pure honey to 42.38 ± 7.93 for a mixture of honey and sugar. Samat *et al*.(2018) confirmed that the reducing sugar of adulterated honey is low compared to pure honey. The addition of water into

the honey decreased the sugar content is reduced after water is added. Oshomah & Agbaji (2015) obtained sugar content in the range of 71.48% and 83.18%, with a mean value of 79.04%. Nearly in all honey samples, two important monosaccharides glucose and fructose predominate, which are defined as reducing sugars.

4.5.12.4. Sucrose content

The mean value of sugar adulterated honey (20.12±12.31) showed a significantly different from control and other deliberately adulterated samples (molasses, banana, and shebeb) except melted candy adulterated honey (Table 25). Sugar and melted candy adulterated honey samples possessed sucrose content above the maximum amount in honey as 5% (Bogdanov *et al*., 2000). These results agree with the findings of other studies, in which the addition of commercial sugar to honey increases sucrose content from 3.81 % for pure honey to 9.80 % for a mixture of honey and sugar (Gebremariam & Berhane, 2014). This implies that the higher sucrose content of honey is an indication for the addition of commercial sugar to honey and there was possible adulteration with sugar syrup in some of the samples purchased from commercially available honey. The low level of sucrose content is an indication of low or no sugar feeding to the bees as well as the absence of adulteration (Abu-Tarboush *et al*., 1993). Therefore, such sugars may be used as markers since they are present in natural honey at remarkably lower concentrations, usually to 3% (Cotte *et al*., 2003; Joshi *et al.,* 2000), or in some kinds of honey to 5% (Costa *et al*., 1999).\

4.5.12.5. Maltose content

The highest mean value of maltose content was recorded for sugar adulterated honey (9.35±5.82) followed by shebeb (5.28±0.25) and banana (3.73±0.04) adulterated honey (Table 25). The lowest maltose content was recorded for pure honey (0.88±0.00). Sugar adulterated honey showed significantly different from control honey samples ($P < 0.05$). However, a significant difference was not observed between other deliberately adulterated honey samples. No oligosaccharide of more than 5 degrees of polymerization (DP) was found in honey samples. But a large amount of these high oligosaccharides was present in starch syrups as the intermediate product of syrup producing process, enzymolysis of starch

(Low, 1998; White, 1978). Therefore these high oligosaccharides may be taken as an indicator of starch syrups in honey adulteration detection (Morales *et al*., 2008). This result confirmed that sugar adulterated honey has a high maltose content which is suitable for detecting honey adulterated with sugar.

4.5.12.6. F/G (Fructose/Glucose) ratio

The highest mean value of the F/G ratio was recorded for molasses (1.33±0.13) and the lowest was recorded for sugar (0.60±0.07) adulterated honey (Table 25). Molasses adulterated honey samples showed a significantly different ($P<0.05$) from shebeb and sugar adulterated honey samples. The F/G ratio was found to the lower in adulterated honey (Manzanares, *et al.,* 2011; Tosi *et al*., 2004). These results are consistent with those of other studies, which suggested that the F/G ratio should be taken into account to evaluate honey adulteration (Manzanares, *et al*., 2011; Kolayi *et al*., 2010; Tosi *et al*., 2004). For comparison, F/G ratios of honey from different studies were reported to be 1.11-1.36 in Algerian (Oucemoukh *et al.,* 2010) and 1.19–1.34 in Venezuelan multifloral honey (Rodríguez *et al.,* 2004). It is reported that the F/G ratio of 1.14 or less would indicate fast granulation, while values over 1.58 are associated with no tendency to granulation (Tosi, *et al*., 2004; White, 1979). Overall, these results indicated that all deliberated adulterated honey samples have a low F/G ratio of less than 1.14 except molasses and pure honey.

4.5.13. Comparison of physicochemical and biochemical characteristics of marketed and deliberately adulterated honey samples

The physicochemical and biochemical properties of commercially collected and deliberately adulterated honey samples are listed in Table 26. Statistical analyses showed that there are significant differences between the pure and deliberately adulterated honey samples based on proline, ash content, pH, free acidity, HMF, glucose, maltose, reducing sugar, and TFC ($p<0.05$). However, a significant difference was not observed on moisture content, sucrose and F/G ratio of the market collected and deliberately adulterated honey samples ($P>0.05$).

The proline content of honey samples collected from marketed was found significantly higher than deliberately adulterated honey ($p < 0.05$). However; pure honey samples from the HBRC bee farm had a proline content of 381 mg/kg, which is higher than the two types of honey, indicating the sign of purity. Proline content is considered an important quality parameter for honey that can serve as an additional determinant of purity and maturity of the honey sample. The highest moisture content was found in adulterated honey, but the differences were not statistically significant ($P>0.05$) and do not fulfill the standard requirement set by Codex (2001) and EU (2002). But, control (pure) honey samples from the HBRC bee farm had a moisture content of 19.65±0.00% which fulfills the standard requirements. This result showed that by determining the moisture content of honey it is possible to differentiate the natural and adulterated honey. The ash content of the deliberately adulterated honey samples was found significantly higher than marketed honey types. Control (pure) honey samples from the HBRC bee farm had an ash content of 0.19±0.01 g/100g. This suggested that the honey from deliberately adulterated honey contain high impurities and unwanted particles which make ash content higher and also honey was sourced from the different species and geographical region. Also, harvesting honey under different conditions, processed under different conditions, adulterating with different adulterants materials raises the ash contents.

Table 26: The mean ± standard error (mean ± SE) for marketed and deliberately adulterated honey

Parameter	**Market honey**	**Adulterated honey**
Proline(mg/kg)	270.83±14.18[a]	213±9.43[b]
Ash(g/100g)	0.23±0.02[a]	0.98±0.20[b]
pH	3.54±0.04[a]	3.98±0.09[b]
Free acidity(meq/kg)	27.12±1.83[a]	188.3±44.26[b]
Moisture content(g/100g)	21.43±0.23[a]	21.51±1.01[a]
HMF(mg/kg)	18.70±1.76[a]	99.50±13.58[b]
Fructose (%)	40.08±0.67[a]	32.37±2.64[b]
Glucose (%)	38.15±0.71[a]	34.39±1.79[b]
Sucrose (%)	3.70±0.46[a]	5.23±2.23[a]
Maltose (%)	1.09±0.12[a]	4.09±1.00[b]
F/G ratio	1.09±0.03[a]	0.98±0.09[a]
Reducing sugar (%)	78.23±0.91[a]	66.75±3.43[b]

AEAC(mg AAE/100g)	1103.50±106.03[a]	1052.50±66.64[a]
TPC(mg GAE/100g)	313.66±22.31[a]	341.83±27.86[a]
TFC (mg QE/100g)	763.42±109.58[a]	1322.00±301.98[a]

Note: Means with different superscript (a, b) within the rows are statistically different at P<0.05, F/G ratio =fructose to glucose ratio, TPC=Total phenolic content (mg GAE/100g), TFC=Total flavonoid content(mg QE/100g),AEAC= Ascorbic acid Equivalent Antioxidant Capacity mg AAE/100g

The mean value of HMF of deliberately adulterated honey does not fulfill the standard requirement set by Codex (2001) and EU (2002). If HMF amounts exceed 40mg/kg; or 80mg/kg for Tropic, it is considered as an indicator of honey deterioration (Bogdanov & Martin, 2002) through heating, or storing for long periods or due to adulteration with inverted sugar (Codex, 2001).The highest pH value was found in adulterated honey, in which the differences were statistically significant (P<0.05). However, control (pure) honey samples from the HBRC bee farm had a pH value of 3.75±0.03 which is lower than adulterated honey samples. This finding suggested that the adulteration of honey can change the pH of honey samples. Different authors also suggest that adulteration may alter the pH value (Rehman *et al.,* 2008; Oroian *et al*., 2018). The mean free acidity contents of deliberately adulterated honey samples were found higher than marketed honey. But, control (pure) honey samples from the HBRC bee farm had free acidity of 26.5±0.87 meq/kg^{-1} which is lower than both commercially available and adulterated honey. Pure and marketed honey samples meet the standard requirement set by Codex (2001) and EU (2002) which is less than 50 meq/kg.

The highest fructose content was found in marketed honey samples with a significant difference existed between the two sources of honey samples (P<0.05) (Table 26). But, control (pure) honey samples from the HBRC bee farm had fructose content of 48.75±0.00 % which is higher than both types of honey. The results indicate that pure honey contains high fructose content. A similar study was reported that authentic honey contains more fructose than glucose (White *et al*., 1962). Comparing the mean glucose content of honey samples from the market collected and deliberated adulterated, it was found out that, the marketed honey contains significantly higher glucose content than deliberately adulterated ones

(P<0.05) (Table 26). But, pure honey samples from the HBRC bee farm had glucose content of 27.40±0.7% which is lower than both types of honey. The reducing sugar of marketed honey was found significantly higher than deliberately adulterated honey (P<0.05). Pure honey samples had a higher reducing sugar (87.68±0.11%) than both types of honey samples. The results of this study indicated that pure honey has high reducing sugar. A significant difference has not existed between the two mean values of sucrose content (P>0.05). But, pure honey samples from the HBRC bee farm had sucrose content of 2.86±0.80 % which is lower than both types of honey. Deliberately adulterated honey does not fulfill the standard requirement set by Codex (2001) and EU (2002) which is less than 5 g/100g. The findings showed that the probability of Ethiopia marketed honey samples mixed with adulterants and harvested unripe honey. A high sucrose concentration in honey usually reflects an early harvest because the sucrose has not been fully transformed into glucose and fructose by the action of invertase (Kucuk *et al*., 2007). The maltose content was found to the higher in adulterated honey (P<0.05). Pure honey samples had a maltose content of 2.11±0.79 which is lower than adulterated types of honey. The F/G(Fructose/Glucose) ratio was found to be lower in adulterated honey, with no significant difference exists between the two mean values of F/G ratio content (P.>0.05) (Table 26). But, pure honey samples from the HBRC bee farm had F/G ratio content of 1.25±0.00 which is higher than both types of honey. Honey, which contains less glucose than fructose can stay fluid (Ouchemoukh *et al*., 2007). Krell (1996) reported that honey with a fructose: glucose ratio lower than 1.2 may be adulterated; this also depends on the type of honey.

As seen from Table. 26, the highest TPC and TFC value was determined in adulterated honey, with no significant difference exists between the two sources of honey (P>0.05) samples. Pure honey samples from the HBRC bee farm had TPC 264.92±7.52 *mg GAE/100g,* which is lower than both types of honey but had TFC 719.47±93.81 mg QE/100g which is lower than the deliberately adulterated honey. This is maybe due to the source difference coming from the different nectars and geographical regions. Statistically, no significant difference existed between the two mean values of AEAC content (P>0.05). But, pure honey samples had AEAC (329.65±128.46 *mg AAE/100g*) content which is lower than both types of honey samples. Because a lower AEAC value indicates stronger antioxidant properties, pure honey contains high antioxidant properties which significantly differrent from other honey

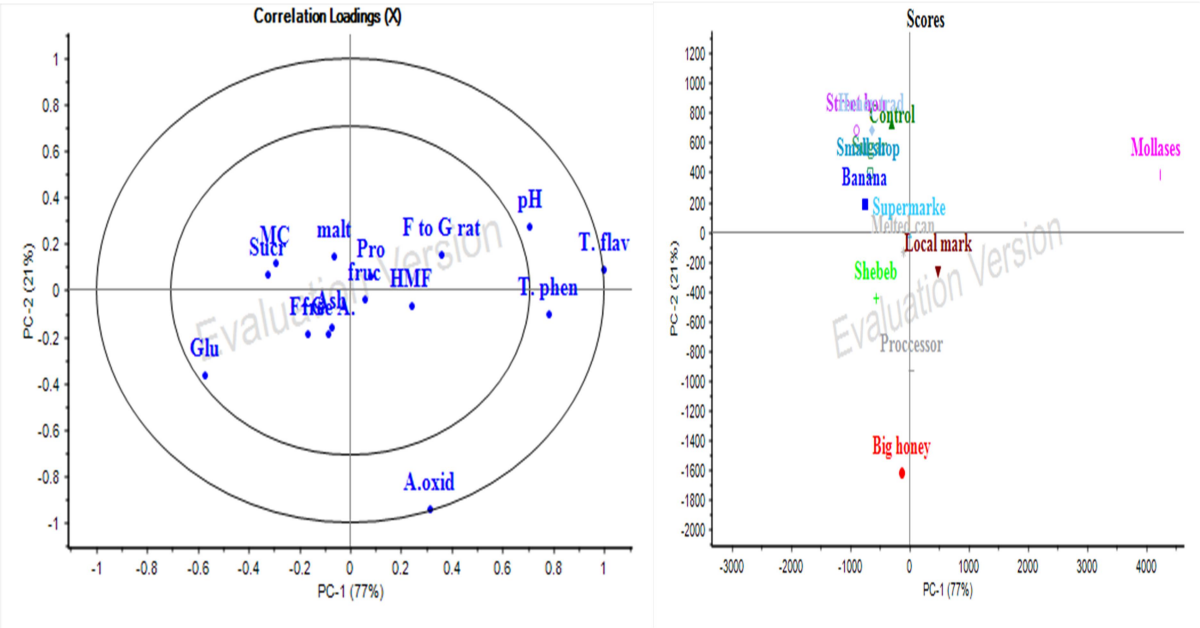
types. The overall analysis showed that the substantial difference in HMF, pH, ash content, free acidity, fructose, glucose, sucrose, maltose, reducing sugar and F/G ratio parameters between honey types (pure, marketed, and deliberately adulterated) may enable us to distinguish between authentic and adulterated honey samples.

4.5.14. Principal component analysis and Cluster analysis for adulterated honey

The scatter plot of the first two PCs (PC1 and PC2) for the classification of honey samples according to the honey similarity is shown in Figure (2a & b). The angle between the lines, or, to be more precise, the cosine of the angle between the lines, approximates the correlation between the variables they represent. Angles close to the right angle confirms the strong independence of random variables. The closer the angle is to 90, or 270 degrees, the smaller the correlation. An angles close to 0 or 180 degrees reflects strong a correlation of 1 or −1, respectively. The first principal component (PC-1) accounted for 77 % of the variance, while the second principal component (PC-2) accounted for 21 % of the variance. Together, the first two principal components accounted for 98% of the initial variability. Considering the given data, 77% was conserved in the first principal component. Accordinly, sucrose, moisture, glucose, reducing sugar, ash were explained in the negative direction; and TPC, TFC, pH, F/G ratio, and HMF of honey were explained in the positive direction. For the second component, 21% of the given data was retained and represented, mainly the maltose, proline, fructose, and F/G ratio in the positive direction; and AEAC, reducing sugar ash and free acidity in the negative part. The loadings indicated that glucose, sucrose, and moisture content were negatively correlated with the pH, TPC and TFC, AEAC, HMF, and F/G ratio. This indicates that honey quality increasing as the value of PC1 became increasingly positive (Fig.2a & b). TPC was positively related to TFC, pH, and F/G ratio, and negatively related to glucose, sucrose, and moisture content. The results from PCA demonstrate that the higher glucose, sucrose, and moisture content indicates the lower the quality of honey samples. This finding indicates that those parameters were important to differentiate pure honey samples from adulterated ones.

Molasses adulterated honey samples showed different from the other types of honey samples and clustered on the positive side of PC1 and overlapped in pH, TPC, and TFC direction.

This shows that molasses adulterated samples can be detected by having high TPC, TFC, and pH (low acidity). Besides, molasses adulterated honey characterized by low glucose, sucrose, and moisture content. In contrast, banana, shebeb, melted candy, sugar adulterated honey samples, and small shops on the negative side of PC1 and overlapped in the high glucose, sucrose, moisture content, ash, free acidity, and maltose direction as shown previously in Figure (2 a & b). This signifies that banana, shebeb, melted candy, sugar, and small shops honey samples showed low quality according to the European Community regulation than other types of honey samples. Moreover, the honey sample collected from the veranda store and processors was the only honey type displayed on the negative side of PC2. This finding overlapped with higher total AEAC values as those were shown in Figure (2a & b). On the positive side PC2 street areas honey, trader honey, control, and supermarket displayed and shared high maltose, proline, and fructose contents.



Pro=Proline=,MC=moisture content, Ash=Ash content, HMF=hydroxyl methyl furfural aldehyde, pH=pH of honey, FA=Free Acidity, Fr=Fructose, Glu=Glucose, Malt=Maltose, Su =Sucrose, F/G ratio=Fructose/glucose ratio TF=Total flavonoid, TPn =Total phenol, AO=Antioxidant activity. The degree of proximity between variables and the narrower angle between diagonal lines indicated a strong association.

Figure 2. PCA Correlation loadings of marketed and pure honey samples(a), PCA scores plot of marketed and pure honey samples(b)

PCA is known to be a good tool for information extraction from multivariate matrices and concentrate it in only a few components (Bevilacqua*, et al*., 2013)

The cluster analysis (CA) conducted with the Ward method indicated the similarity of honey samples depending on their physicochemical and antioxidant parameters. The generated dendrogram (Fig.3), which depicts the similarity of the analyzed physicochemical and antioxidant parameters, allowed to distinguishing the clusters of parameters. The first graphical evidence is that samples can be divided into four main groups. The first cluster is a subdivision in the bottom cluster that places sugar adulterated honey samples as a unique sample in one cluster. Sugar adulterated honey is characterized by having low fructose, reducing sugar and F/G ratio content, and having high glucose, maltose, and sucrose. Shebeb adulterated honey samples characterized by having high moisture content, ash content, HMF, free acidity, and pH, while having low fructose and F/G ratio would have appeared in the second cluster. The third cluster included molasses as unique honey types. This finding confirmed molasses adulterated honey having high HMF, free acidity, ash content, F/G ratio, TPC, TFC, and pH. The dendrograms of variables (Figure 3) showed that the banana adulterated, melted candy adulterated, street areas honey, veranda store honey, small shops, local market, honey traders, control, processors, supermarket formed one cluster, and becomes the fourth cluster. The dendrograms for this study indicated that it is easily possible to classify honey based on its quality and discriminate honey adulterated samples from the pure one.

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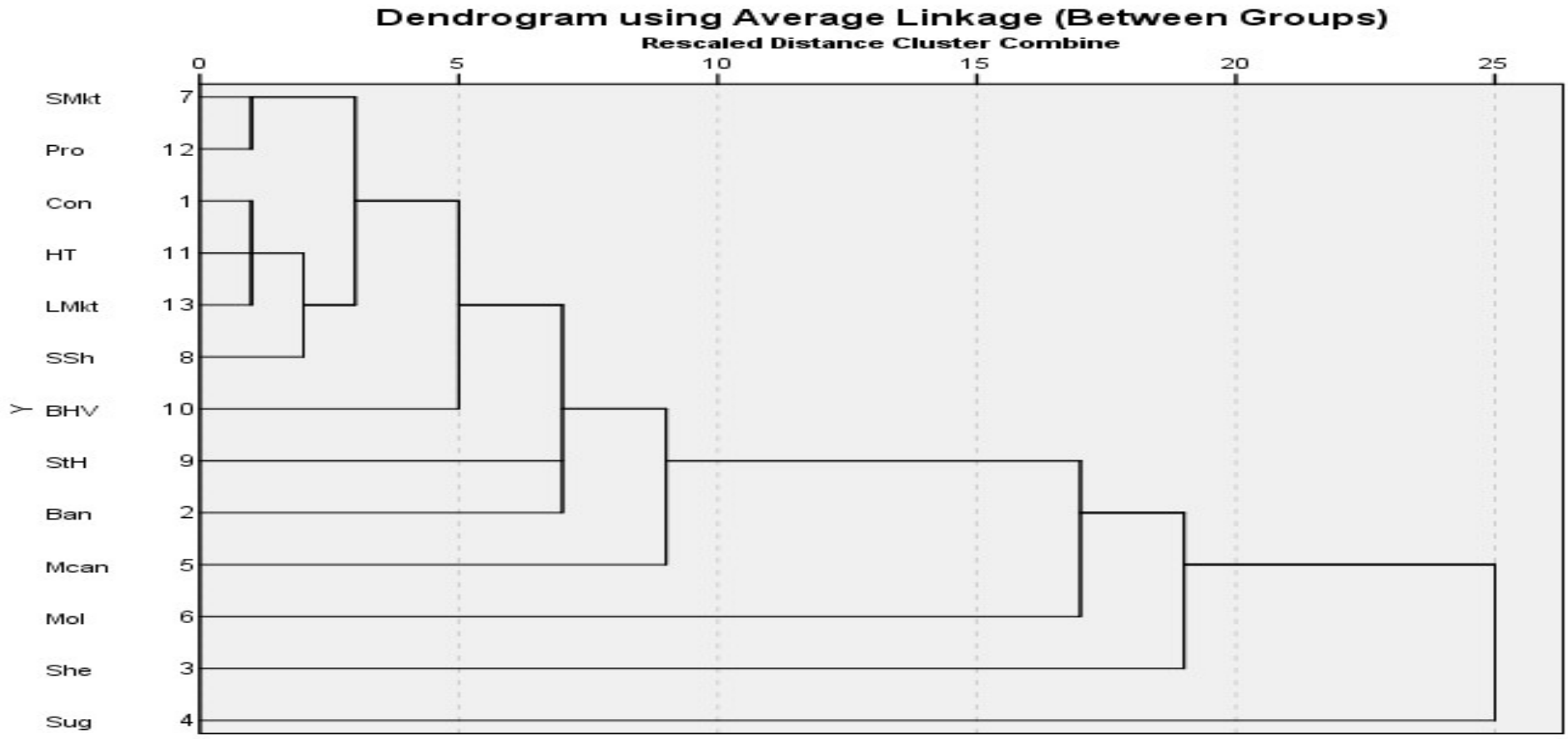


Smkt=supermarket, Pro=processors, Con=Control, HT=honey trader, Lmkt=Local market, SSh=small shop, BHV=Honey from veranda, StH=street honey, Ban=Banana, Mcan=melted candy, Mol=Molasses, She=shebeb, Sug=sugar

Figure 3. Hierarchical clustering of marketed and deliberately adulterated honey samples based on physicochemical and biochemical properties

4.6. Detection of adulteration based on Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectrum is a technique to measure the wavelength of a sample and find out the peak value, characteristic bands of different functional groups. Figure 4 showed the FT-IR spectra of pure, adulterated, and marketed honey. Although there was some overlap observed between the peaks in the spectrum of the honey and the peaks in the spectra of the adulterants, visually there are observable differences in the fingerprint region from 650 to 1800 cm^{-1} and 2300-3000 cm^{-1}.

4.6.1. FTIR Spectra of authentic and adulterated honey sample

In this study, FTIR was used to compare honey samples based on their spectral differences in the 4000-650 cm^{-1} spectral region. A representative of the spectrum of honey is shown in Figure 4.Table 27 represents the band assignment along with corresponding modes of vibrations in the FTIR spectrum honey sample as well as shifted wave number from pure among sample scanned by FTIR. There are a total of ten authentic honey samples analyzed using FTIR Spectrometer. For the adulterated honey samples, there were ten spectra obtained from the FTIR for adulterated samples of honey (with molasses, banana, melted candy, sugar, and shebeb) with different proportions. The spectrum of authentic honey samples together with spectra of the five adulterant solutions is shown in (Fig 5 and Annex 13-18). The adulterated honey sample's spectral showed a shift to longer wavelength and a broadening of the absorption bands as the concentration of adulterants increases

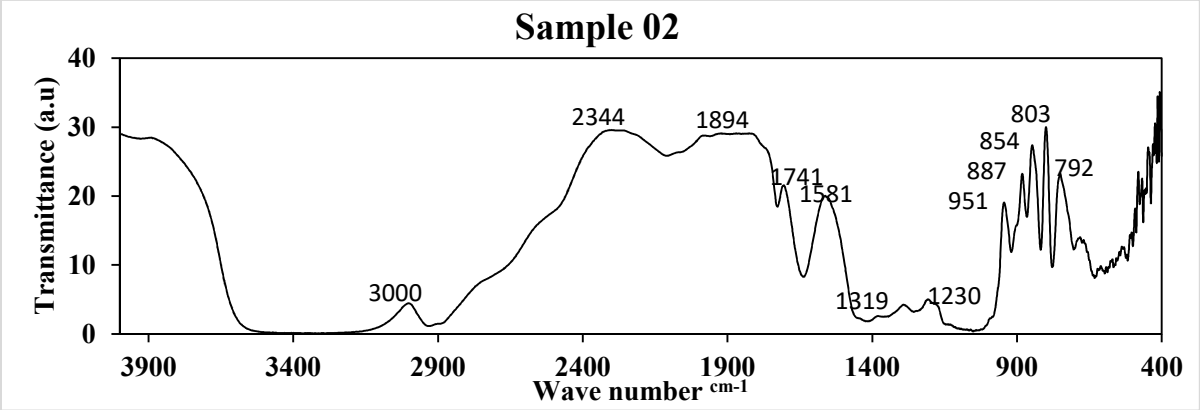
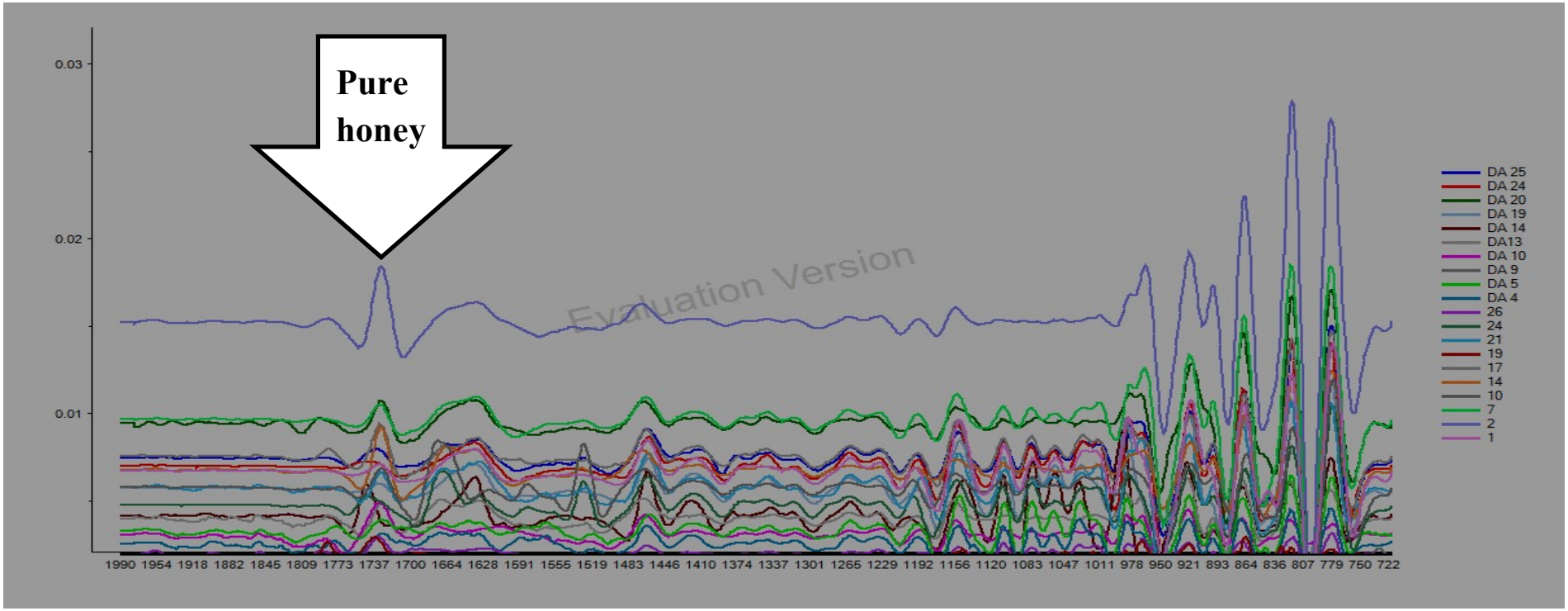


Figure 4.FT-IR spectra of pure honey



DA25=shebeb adulterated honey; DA24=shebeb adulterated honey; DA20=sugar adulterated honey; DA19=sugar adulterated honey; DA14=Melted candy adulterated honey; DA13=Melted candy adulterated honey; DA10=Banana adulterated honey; DA9=Banana adulterated honey; DA5=Molasses adulterated honey; DA4=Molasses adulterated honey;2=pure honey samples;1=street areas honey;24= small honey shops; supermarket samples (10,14, 17,19 & 21) and honey trader (7 & 26)

Figure 5. FT-IR spectrum of transformed data

Table 27. Summary of FT-IR band Assignments for pure marketed and adulterated honey and shifted wave numbers.

Wavenumber(cm^{-1}) of pure honey	**Shifted Wavenumber (cm^{-1})**	**Molecular vibrations of functional groups and their biochemical contributor**	**Reference**
792 803 854 887	761,765, 793, 804, 805, 809, 813 843, 855, 857 878, 890,891, 934	Anomeric region of carbohydrates -C–H bending (mainly from carbohydrates) -Ring vibrations (mainly from carbohydrate)	(Mathlouthi &Koenig, 1986; Subari *et al*., 2012) Gallardo-Velázquez *et al*., 2009; Kelly *et al.,* 2004; Tewari & Irudayaraj, 2004)
951	950, 952, 953, 954,955,956, 957, 958, 959, 960, 973.	C-O & C–C stretching (carbohydrates) -Ring vibrations (mainly from carbohydrates)	(Subari *et al*., 2012; Tewari & Irudayaraj, 2005) (Gallardo-Velázquez *et al.,* 2009; Tewari & Irudayaraj, 2004)
1230	1210, 1212,1222, 1226,,1233, 1236 1239, 1252,1255, 1259, 1280	O–H stretching/bending -C–O stretching (carbohydrates) -C–H stretching (carbohydrates) -C=O stretching of ketones	(Gallardo-Velázquez *et al*., 2009; Tewari & Irudayaraj, 2004)
1319	1312, 1321, 1322,,1329,1330, 1365	-C=O stretching of ketones	
1581	1548,1552,1569,1574,1576,1580, 1582, 1585, 1586, 1588, 1591,1594	-Bending primary and secondary amine and amides(N-H), C≡C,Amide group	Dovbeshko *et al*., 2000 Parker (1983)
	1604, 1607,1612	O–H stretching/bending (water) C=O stretching (mainly from	Philip, 2009 Gallardo-Velázquez *et al.*,

		carbohydrates) N–H bending of amide I (mainly proteins)	2009) (Cai & Singh, 2004; Stuart, 1996)
1741	1738	C=O stretch in unconjugated ketones, carbonyls in ester groups (frequently of carbohydrate origin)	Faix (1991), Pandey & Pitman (2003)
1894	1892,1896, 1897, 1903, 1906, 1918,1920,1924, 1931,1940, 1948, 1959, 1967, 1995, 1997	Aromatic overtone rings, multiply bonded nitrogen compounds, such as cyanides (nitriles),cyanates, isocyanates, thiocyanates, and diazo compounds,C-C stretch,phenyl ring substance	
2344	2214, 2335, 2342, 2346, 2359,2362,2385, 2387, 2390, 2394,2399,,2405, 2409,2481	NH Amino-related component	Nandiyanto *et al*., 2018)
3000	3000	C-H stretching vibrations	

In this study, spectra of the honey sample were investigated in two regions varied between 3000–2300 cm^{-1} and 1800–650 cm^{-1}, and the best differentiation was achieved only in the 1800–750 cm^{-1} region. The bands ranged between 1500 and 750 cm^{-1} are very significant for honey or similar products since this zone is attributed to the absorption of the major monosaccharides (fructose and glucose) and disaccharides (sucrose) (Gallardo-Velázquez *et al*., 2009). A peak obtained between 950 and 750 cm^{-1} was known as the anomeric region, which was frequently preferred for the spectral analysis of carbohydrates (Gok *et al*., 2015). 1474–1199 cm^{-1} band responsible for bending of O–C–H, C–C–H, and C–O–H (Tewari & Irudayaraj, 2004). The band varying between 1700–1600 cm^{-1} corresponds to O–H stretching/bending in water (Cai *et al*., 2004) and C=O stretching in carbohydrates (Gallardo-Velázquez, *et al*.,2009). The band within the region of 3000–2800 cm^{-1} is responsible for C–H stretching of carboxylic acids and NH_3 stretching band of free amino acids, which are found in honey in very low amounts (Anjos *et al*.,2015).

The spectrum of pure honey of sample 02 shows peaks at 792,803,854,887,951,1230,1319,1581, 1741,1894,2344,3000 cm^{-1} (Figure.4).In this work; the qualitative detection of adulterants in honey according to the infrared spectrum has been attempted. Some specific peaks that could distinguish the adulterated honey from the pure one are observed in honey adulterated with different adulterant materials used in this study. Shebeb adulterated honey showed clearly observed peaks at 2214 and 2704 cm^{-1}, sugar adulterated at 765,878,1967,2481 cm^{-1}, banana at 2346 and 2409 cm^{-1},melted candy at 934,973,1252,1365,1604 and 1906 cm^{-1}, molasses at 1920,1931,and 2405 cm^{-1} peak that could distinguish the adulterated honey from the natural ones. For commercially available honey, sample 17 showed at 1252,1903 and 1940 cm^{-1}, sample 10 showed an observed peak at 1259,1607 and 1997 cm^{-1}, sample 14 showed at 1612 and 1955 cm^{-1} peak that could distinguish from the others samples. The region between 940–700 cm^{-1} due to C–H bending and ring vibrations (mainly from carbohydrates) (Gallardo-Velázquez *et al*., 2009; Kelly *et al.,* 2004; Tewari & Irudayaraj, 2004). The vibrations in the regions 940–1175 cm^{-1} are due to the C–OH group as well as the stretches C-C and C-O. According to Gok *et al*. (2015), the vibration in the region 1540–1175 cm^{-1} is the result of deformations of O-H, C-O, C-H, and C=C in the carbohydrate structure (Subari *et al*., 2012; Gallardo-Velázquez *et al*., 2009; Tewari & Irudayaraj, 2004). The region between 1700–1600 cm^{-1} could be due to O–H

stretching/bending (water) (Cai & Singh, 2004), C=O stretching (mainly from carbohydrates) (Gallardo-Velázquez *et al*., 2009), N–H bending of amide I (mainly proteins) (Philip, 2009).The vibration at 3000 cm^{-1} due to C–H stretching (carbohydrates) (Gallardo-Velázquez *et al*., 2009). This FTIR analysis shows that the adulteration of honey can also be detected by analyzed the spectrum shape.

4.6.2. Multivariate analysis

In this study, the best differentiation was achieved only in the 1800–650 cm^{-1} region. The bands at 1800–650 cm^{-1} spectral region were selected for successful discrimination of clusters. For the calculation of sample similarities, the Euclidean distance was used indicating the complete linkage clustering values. The results obtained are represented in (Figure. 6) in the form of dendrograms. Clear cut classes were gathered over the range of 1800–650 cm^{-1} with high heterogeneity values (up to 10). Pure honey samples (02) collected from the HBRC bee farm formed one cluster as unique and different from others in terms of its spectral features. The second cluster is formed a unique arm of the sample of DA9, which is banana adulterated honey. Shebeb and sugar adulterated honey samples (DA25, DA24, DA20 & DA19) are aggregated in one cluster become the third cluster. Also within this group, the honey sample was collected from a trader (7), the supermarkets (10 & 17), from the street areas (01), and the small honey shops (24) clustered. Thus, it can be suggested that commercially available honey in the study area had the probability to adulterate with sugar and shebeb. The arm of the fourth cluster is composed of honey samples adulterated with banana (DA 10), molasses (DA 5 & DA 4), and melted candy (DA 14 & DA 13). In addition, the commercially available honey samples collected from a supermarket (14, 19 & 21 samples) and honey trader (26) were clustered in the fourth arm. These finding highlights that honey samples collected marketed may have the probability of adulterating with banana, molasses, and melted candy. Honey is a complex food material, and the adulteration agents have a high degree of similarity with the authentic samples. Keeping into account these findings, the classification of the adulteration agent could be separated using chemometrics based on spectral differences.

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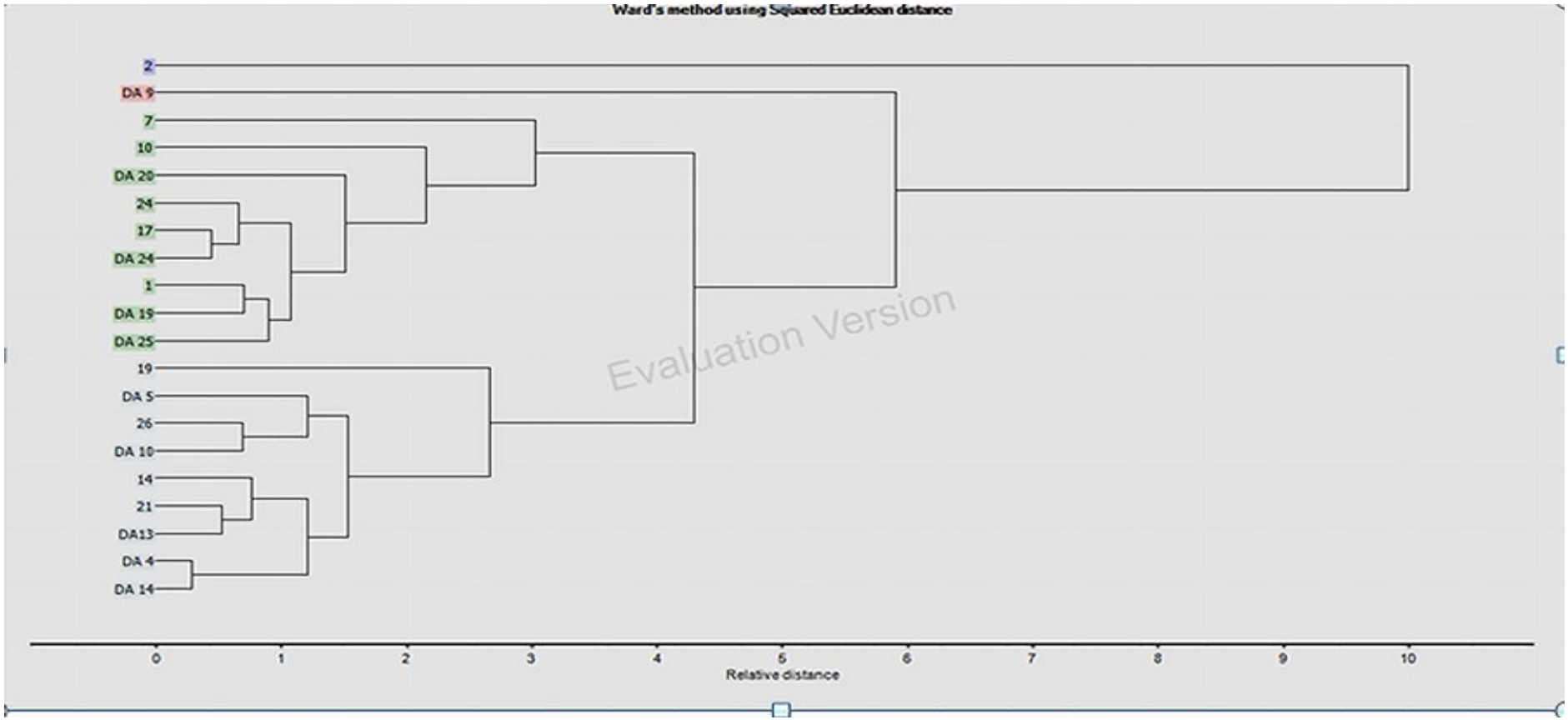

DA25=shebeb adulterated honey; DA24=shebeb adulterated honey; DA20=sugar adulterated honey; DA19=sugar adulterated honey; DA14=Melted candy adulterated honey; DA13=Melted candy adulterated honey; DA10=Banana adulterated honey; DA9=Banana adulterated honey; DA5=Molasses adulterated honey; DA4=Molasses adulterated honey;2=pure honey samples;1=street areas honey;24= small honey shops; supermarket samples (10,14, 17,19 & 21) and honey trader (7 & 26).

Figure. 6. Hierarchical clustering of all samples in the 1800–650 cm^{-1} (fingerprint) spectral region

PCA was applied to FTIR spectra of all groups, obtaining evident discrimination (score plot) of the adulterated (molasses, banana, melted candy, sugar, and shebeb) and marketed honey samples (Figure. 7), to obtain the adulteration directions. A clear splitting of the data can be observed as depicted in (Figure 7) by the first two principal components in the 4000- 650 cm^{-1} region. This describes 94 % of the total variance for PC1 and 2 % for PC2 and that the first two factors account for more than 96% of the total variance. To study the wavenumber that had the most influence on these two PCs, the loading values were represented. As depicted in Figure 8, the PC1 described by sugar adulterated (DA 20 & DA19) and sample 21(supermarket honey) in the positive direction and molasses adulterated (DA 4 & DA 5) and sample 26 in the negative direction (honey trader). The pure honey sample (02) is displayed in the positive direction of PC1 separately. PC1 showed high loading values at 1747-1713,1643,1384,960,950,919,882,864,882,864,,816,801,782,718-651 cm^{-1}. The bands appearing in the region of 1600-1700 cm^{-1} originated as a result of the carbonyl group (C=O) and C≡C stretching, and this region was found to be related to phenolic molecules (Tahir, *et al.,* 2017). This region contains a common set of spectral information responsible for the separation of sugar, shebeb adulteration, and marketed from pure honey along PC1 scores direction. PC1 describes why sugar, shebeb, 21, 26, 14, and 02 samples are different from each other along PC1. The results of this study confirmed that honey samples collected from market areas are not pure honey based on the grouping that formed because it is grouped in the adulterated honey.

For the second component, 2 % of the given data was retained and represented, mainly by banana (DA 9), melted candy (DA 14 & DA 13), sample 19 and 10 from the supermarket in a positive direction. Shebeb adulterated (DA 24 & DA 25) and 17 represented on the negative direction of PC2.The major loading in the PC2 is the peak at 1756-1671,1748-1674,1460,1384,1190,1038,1009,994,978,961,952,933,896,865, 840,818,798,780,762,707 and 665 cm^{-1}.The region which is very distinctive in the evaluation and description of honey is the band from 890 to 810 cm^{-1} characteristic for the vibration anomeric region of carbohydrates or C–H deformation (Tulchinsky *et al*., 1976). The bands in the region 1700–1600 cm^{-1} had been previously assigned as amide I vibrations of the honey proteins (Philip, 2009). The second PC loading obtained in the present study shows many of the features that can be attributed to C-O and C-C stretching modes (Hineno,1977) in the 900 to 1153 cm^{-1}

and the bending modes of O–C–H, C–C–H, and C–O–H angles in 1199 to 1470 cm^{-1} region. The bands 1434–1436, 1476, 1502, and 1580 cm^{-1}, representing water with less or more H–bonds (Rambla *et al.*, 1997, Segtnan *et al.*, 2001). The score plot shows that the purity of honey in the market generally can not be trusted as some of the market honey samples were grouped in the adulterated honey samples.

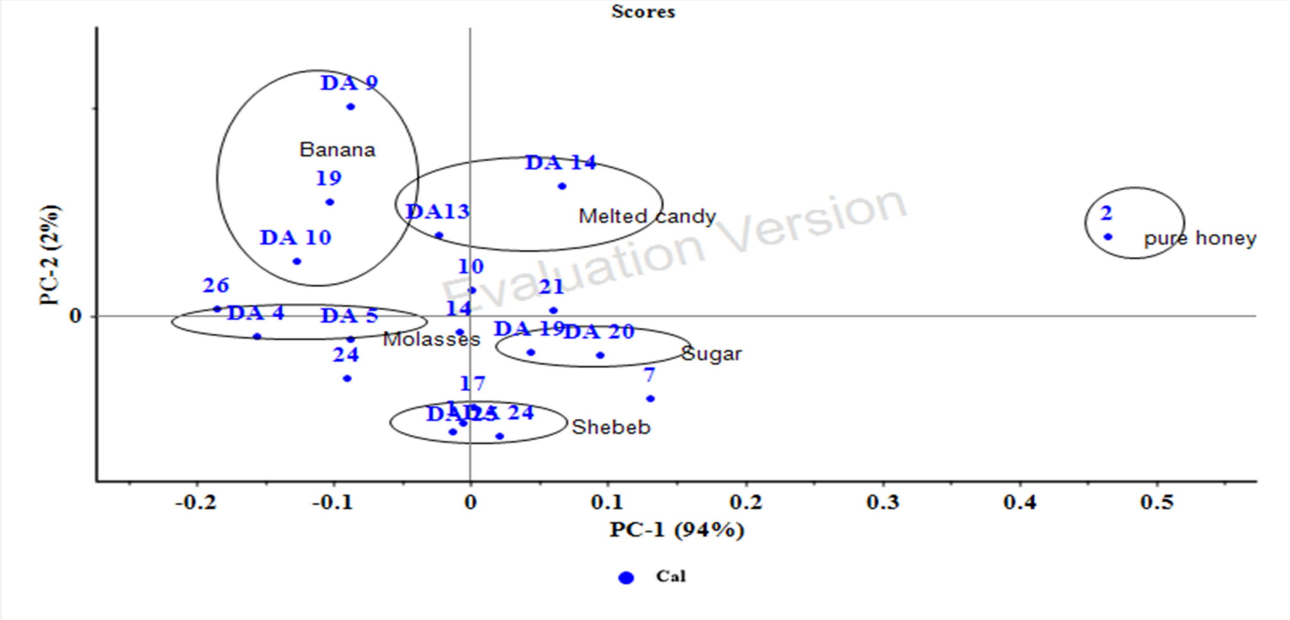

Figure 7 Score plot obtained from the principal component analysis (PCA) applied to the FTIR spectra

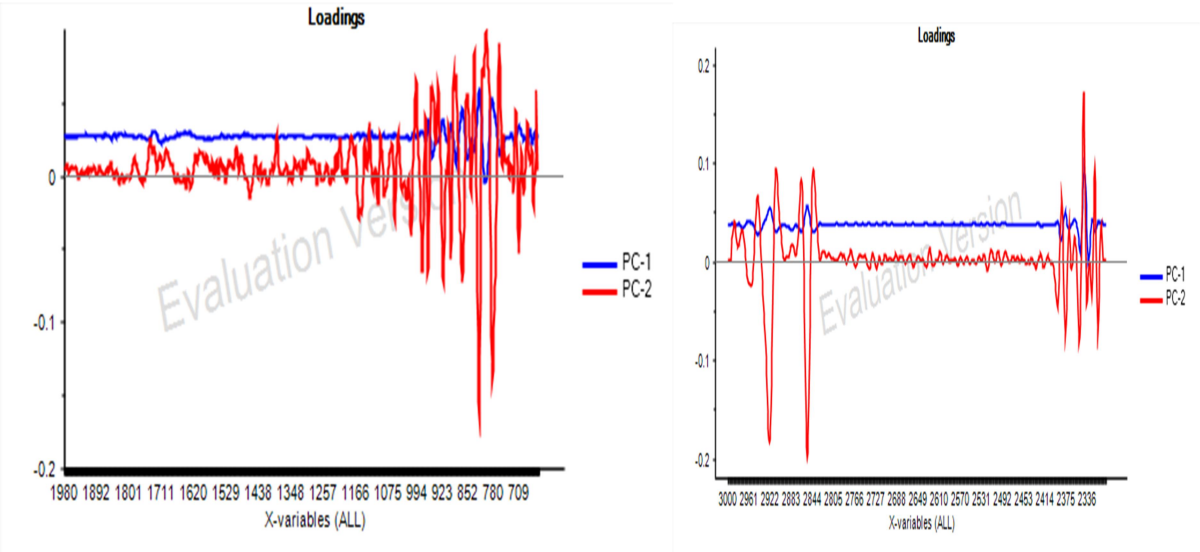

Figure 8. Loading obtained from the principal component analysis (PCA) applied to the FTIR spectra.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Despite the popularity of multiple nutrition, therapeutic uses, and high cost of honey, only a few reports have been conducted in Ethiopia dealing with the commercial quality and adulteration detection of honey samples. This is extremely important since the authenticity of honey is of great importance both from commercial and health points of views. In this work, quick assessment test, basic physicochemical, and antioxidant properties of different commercially available honey samples were determined. The physicochemical properties of commercially available honey samples were mostly at good quality. But some samples from the honey market had constituent levels either below or above the recommended levels.

This study investigated parameters that are potentially useful in differentiating between pure and adulterated honey samples with common honey adulterants in Ethiopia. Four types of quick test methods were applied to the honey samples. The preliminary test method depends on the physical properties of adulterants materials. Dissolving methods were found as important methods to detect banana and molasses adulterated honey, while a thumb test could be used to detect banana and melted candy adulterated honey. Flame tests were found to detect honey adulterated with molasses, banana, and shebeb. All adulterant materials used in this study was showed different properties when mixed with honey samples. Honey quality parameter like proline, moisture content, HMF, free acidity, and ash, TPC, TFC, and antioxidant activity were decreased with sugar mixture increase in honey. Except for pure and banana adulterated honey samples, a reducing sugar of others adulterated honey didn't meet national and international honey specifications. Physicochemical properties of deliberately adulterated honey did not meet the quality criteria set by codex (2001), EU (2002), and ESA (2005). The determination of: ash content, free acidity, HMF, moisture content fructose, sucrose, maltose, and F/G ratio were potentially useful in the differentiation of pure and adulterated honey samples. Based on the results obtained, it is possible to discriminate between honey types by multivariate analysis (PCA & cluster analysis). In the present study, the FTIR technique and multivariate analysis were successfully used to determine honey adulteration. PCA analysis showed that the cumulative

variance of the two first PCs was 96%, which indicated that the studied parameters were able to distinguish the honey samples. Overall, this finding suggested that the techniques used for honey quality monitoring was effective detecting honey adulteration and quantity estimation of honey contents by correlating with physical and chemical analysis. Honey adulteration is the issue of all aspects of honey value chains to maintain its quality and safety.

5.2. Recommendations

Based on the results of this study, the following recommendations are made for future consideration.

- ✓ Given the results of the study, using quick test methods, physicochemical analysis, biochemical analysis, and FT-IR spectroscopy analysis conjugated with multivariate analysis feasible to detect adulteration of honey.
- ✓ Training of beekeepers and other stakeholders on rapid test assessment to keep quality, identify the adulterated honey, and avoid adulteration
- ✓ Updating the Ethiopian honey's standards and specifications is required. The Moisture content, sucrose, and proline content of some of the deliberately adulterated honey samples were similar to the pure honey. Besides, there is no standard specified for fructose, glucose, and maltose as well as need for updating for reducing sugar and sucrose.
- ✓ Strong legal framework and enforcement is required to reduce honey adulteration
- ✓ Further research on the effect of honey adulteration on human and alternative control systems should be encouraged.

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APPENDIX

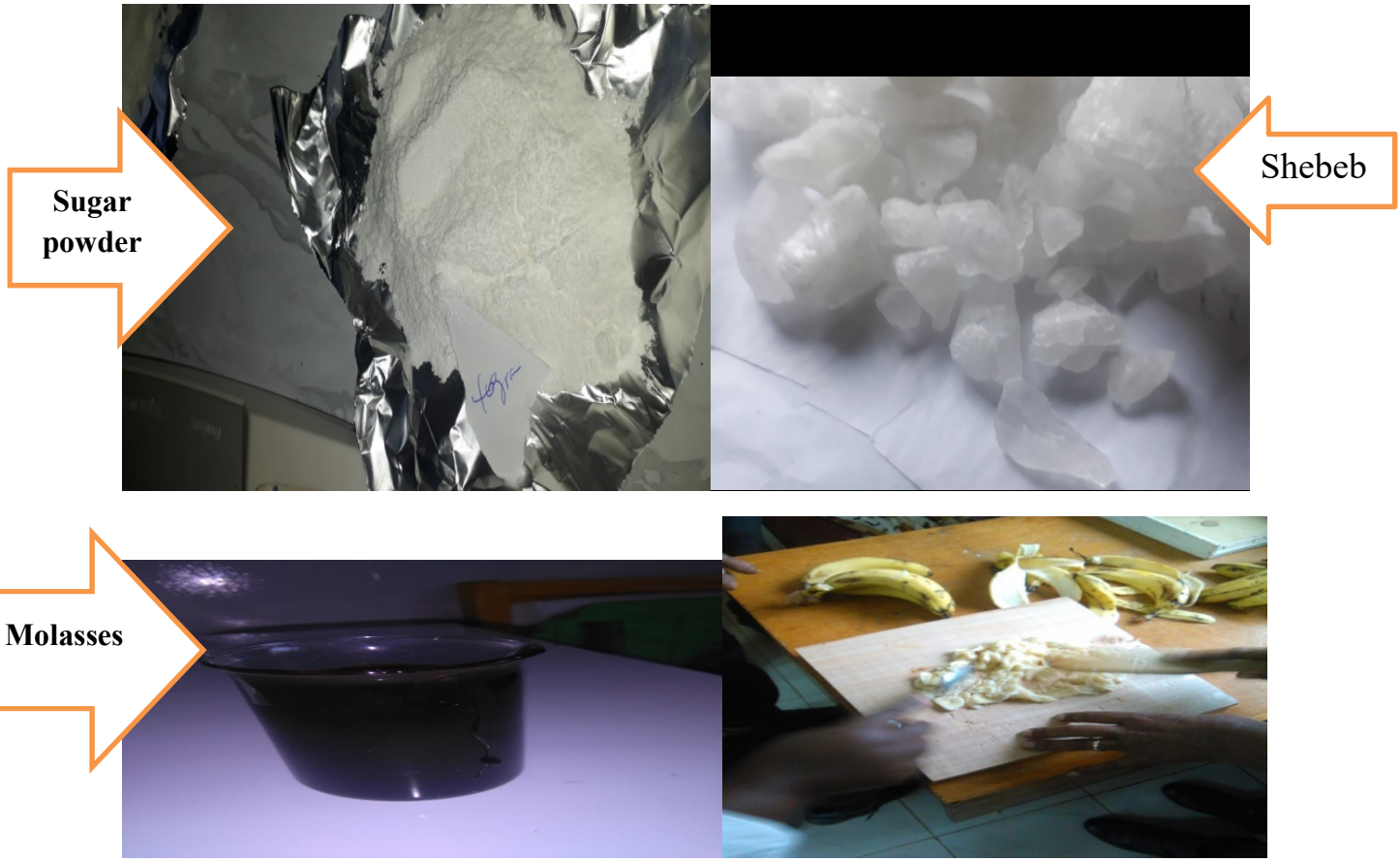
Annex 1. Sample collected from commercially available in Addis Ababa

Annex 2: Analyzing free acidity and pH content of the honey samples by using a pH meter

Annex 3: Analyzing sugar the honey samples by HPLC

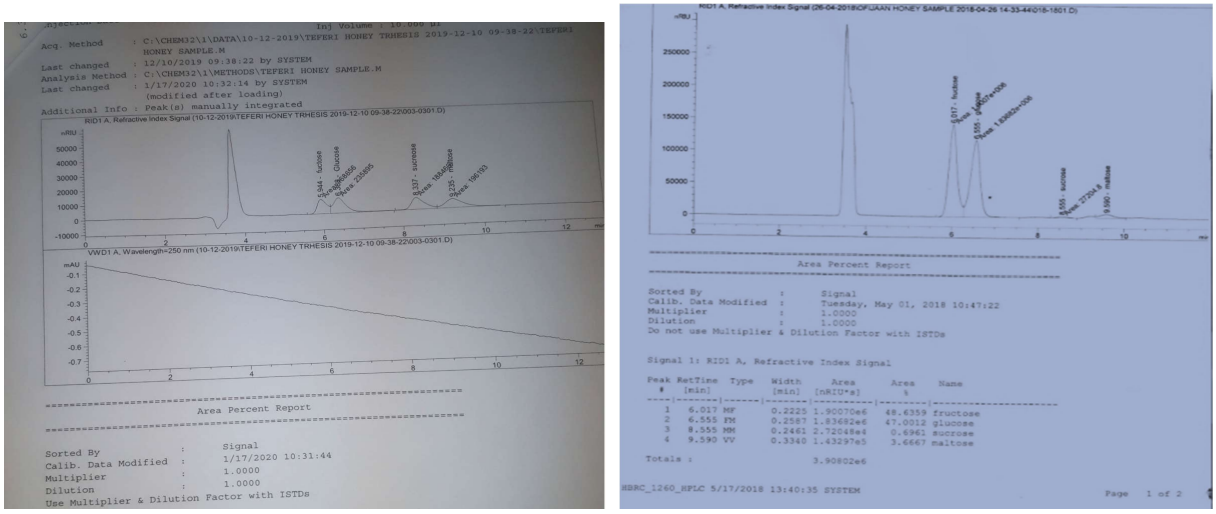
Annex 4: Ash Analysis using muffle furnace (carbonation of honey samples using hot plate)

Annex 5: Analyzing HMF the honey samples by UV-VIS spectroscopy

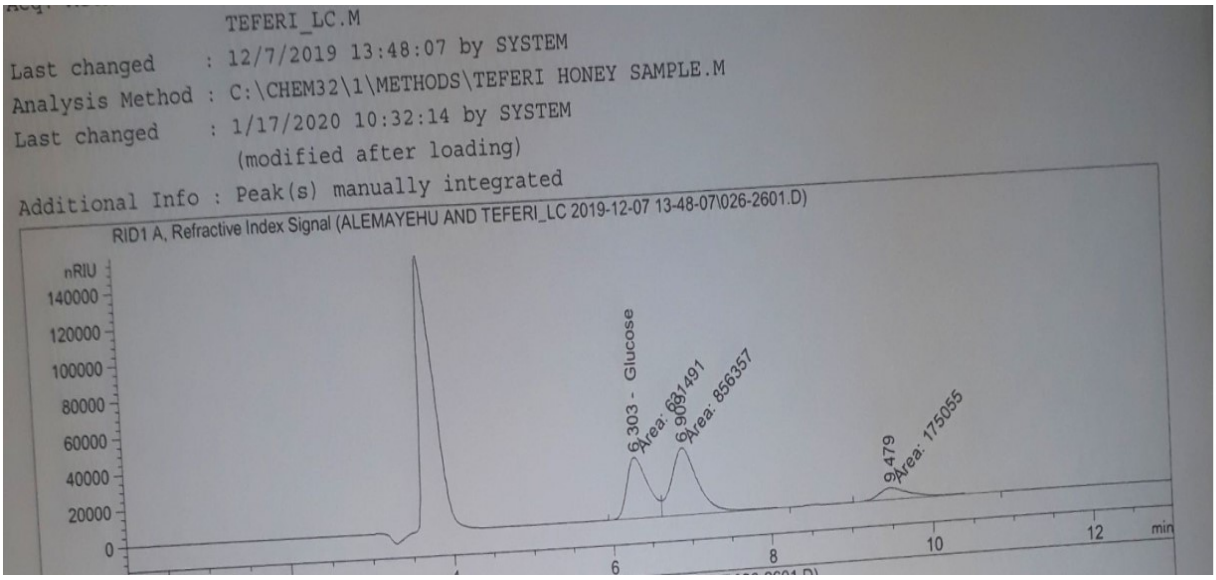


Annex 6: Adulterant materials used in this study

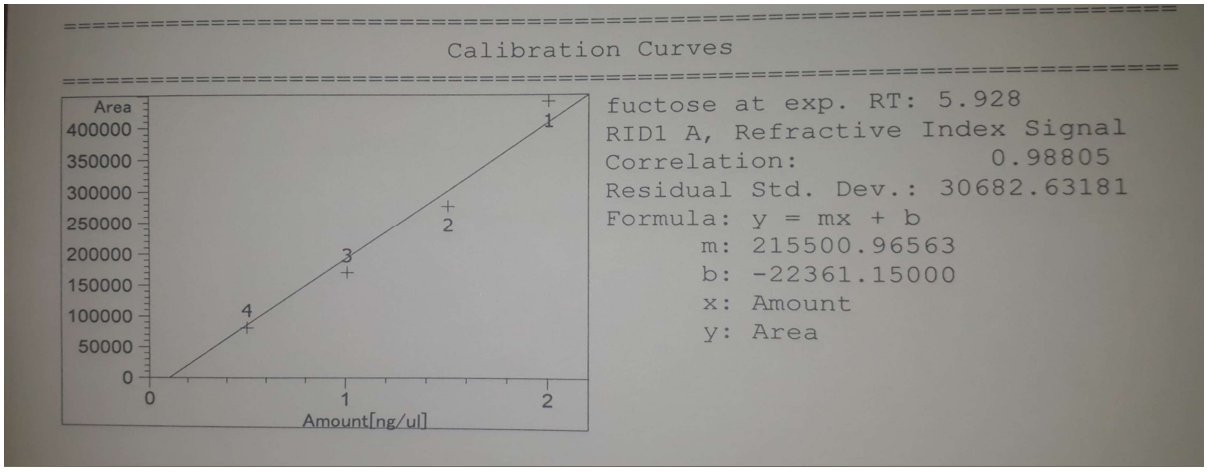
Annex 7: Deliberately adulterated honey prepared in a laboratory

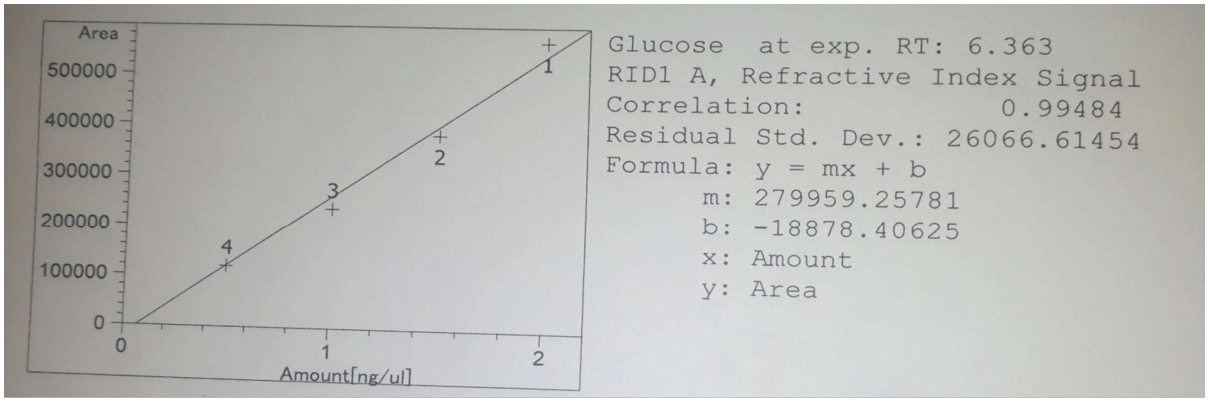


Annex 8: Chromatogram by HPLC, of fructose, glucose, sucrose, and maltose of market honey

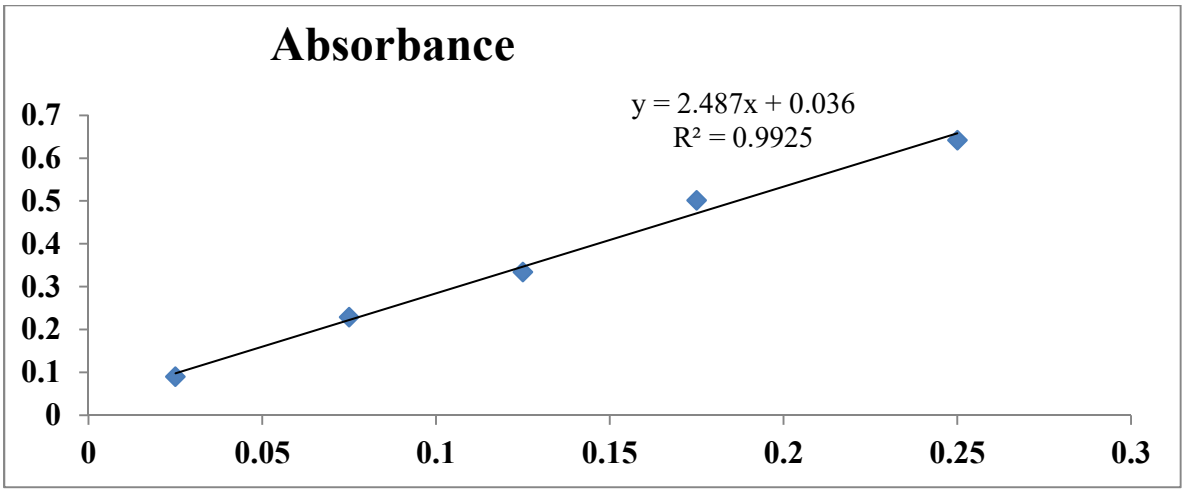


Annex 9: Chromatogram by HPLC, of fructose, glucose, sucrose, and maltose of adulterated honey

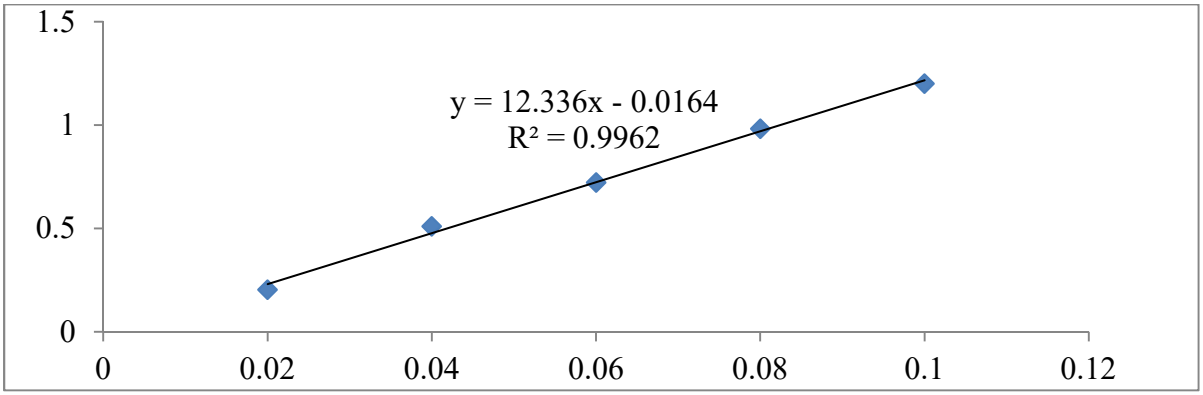




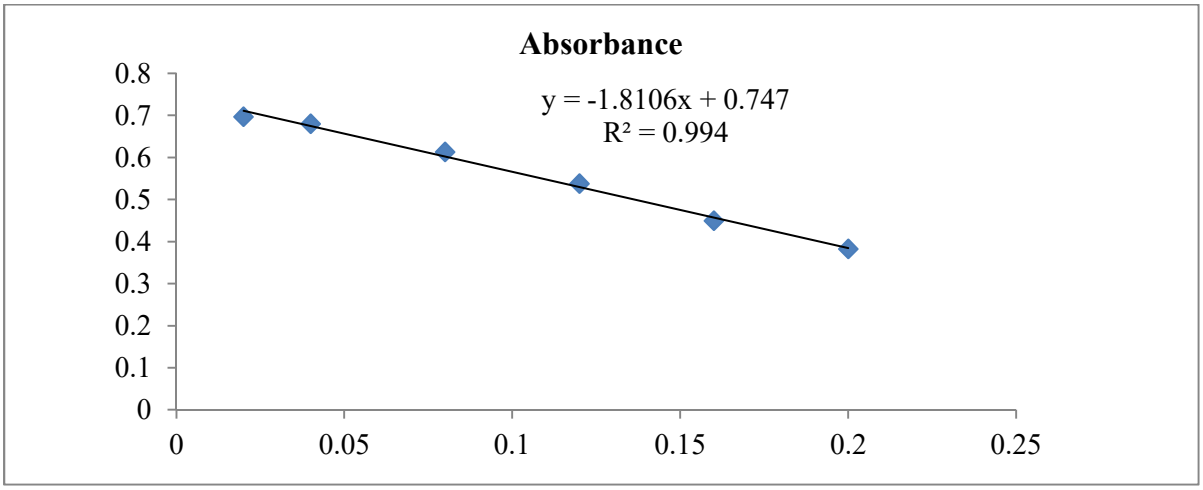
Annex 10: Calibration curve of fructose and glucose



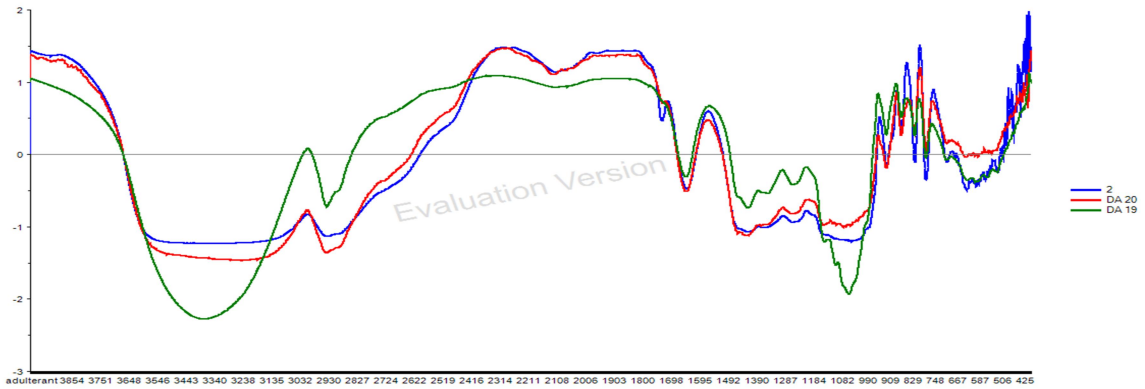
Annex 11.Calibration curve of flavonoid



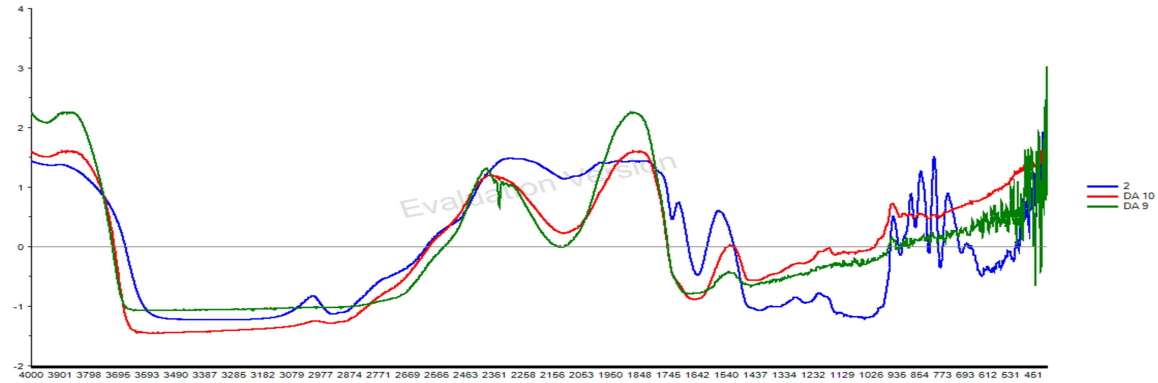
Annex 12: Calibration curve of phenol



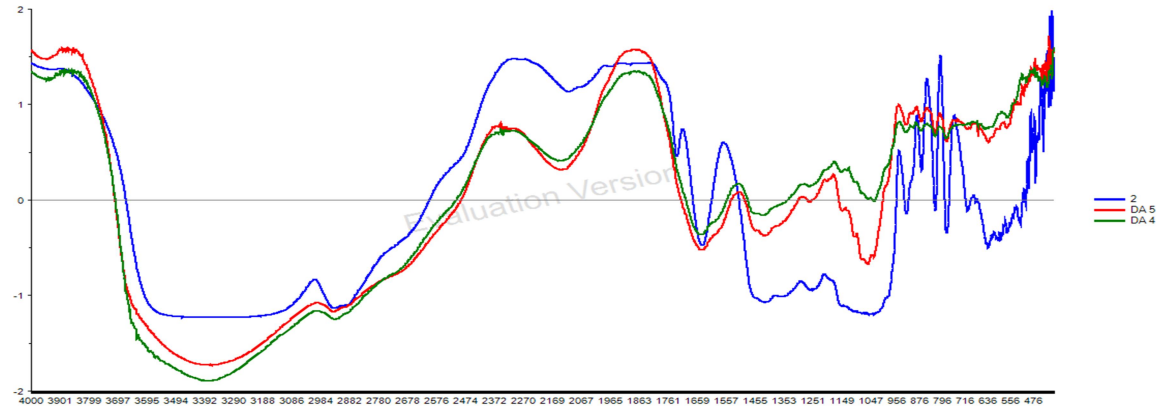
Annex 13: Calibration curve of Ascorbic acid



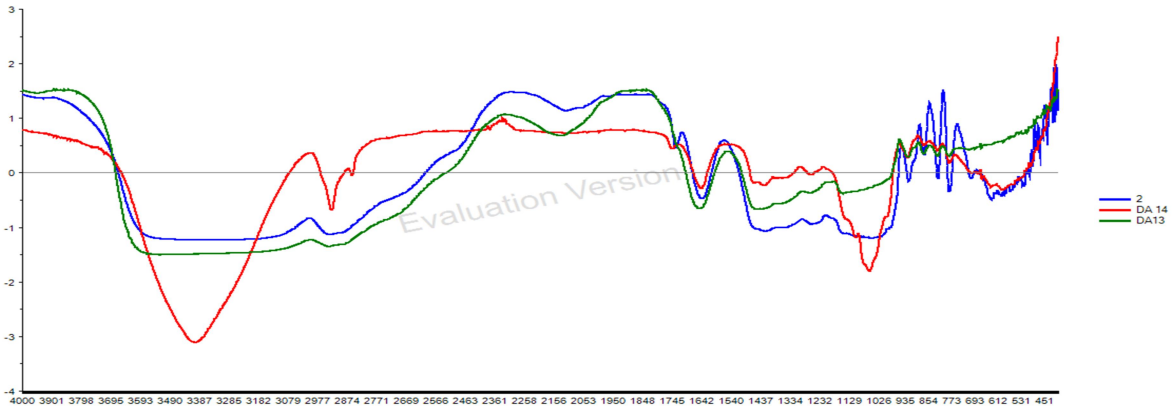
Annex 14: FTIR spectrum for Sugar adulterated honey



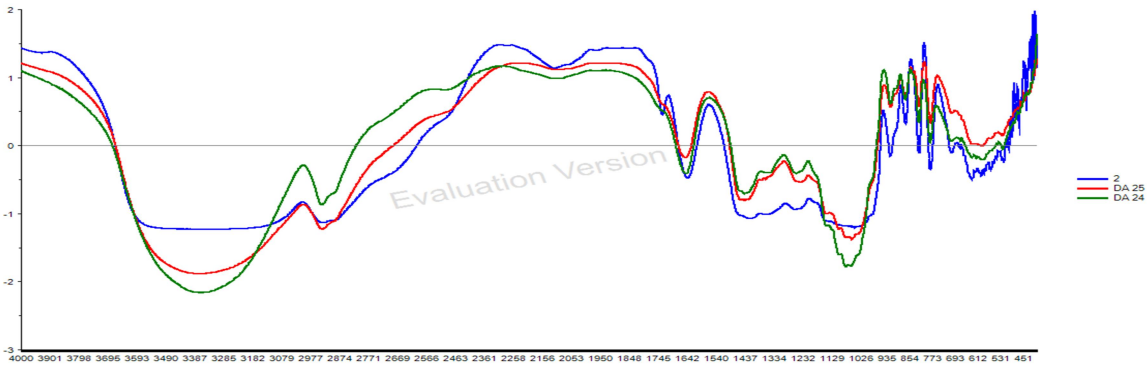
Annex 15. FTIR spectrum for banana adulterated honey



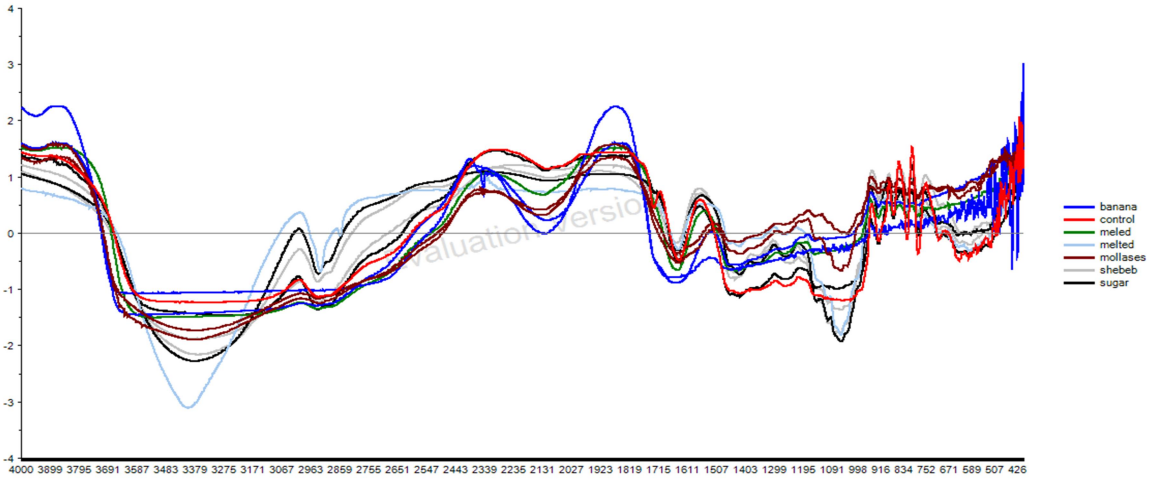
Annex 16: FTIR spectrum for molasses adulterated honey



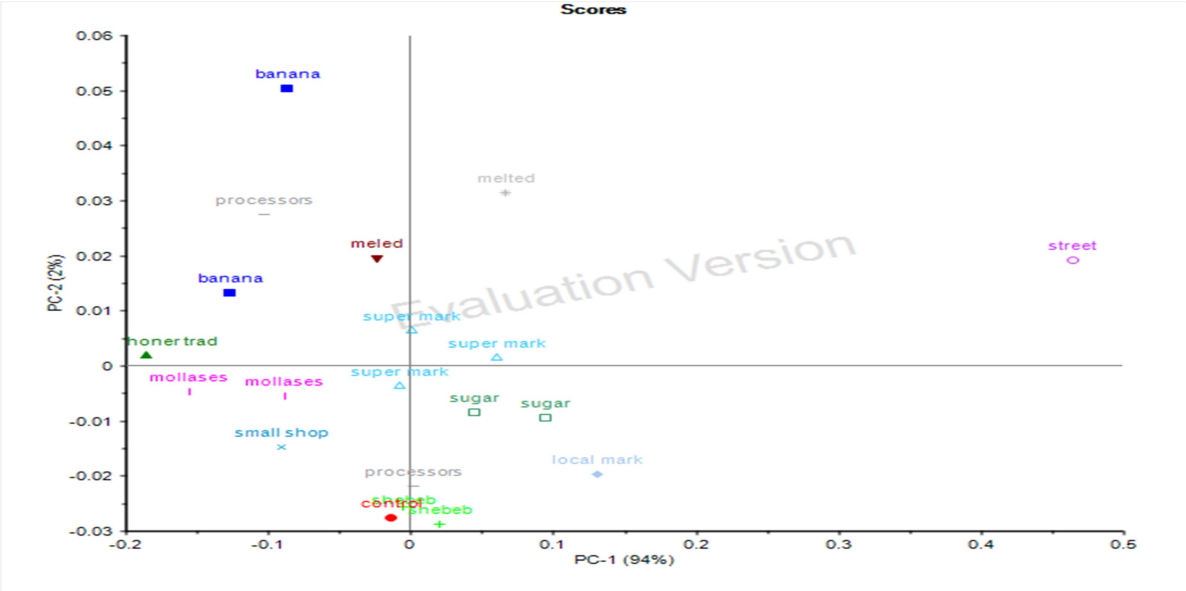
Annex 17: FTIR spectrum for melted candy adulterated honey



Annex 18: FTIR spectrum for Shebeb adulterated honey



Annex 19: FTIR spectrum for adulterants materials



Annex 20: Loading of a sample from PCA