Jimma University

College of Natural Sciences

Department of Chemistry

MSc Thesis on

Investigating of Physiochemical Properties and Volatile Organic Compounds of Honey Varity Produced in Bonga, Ethiopia

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Advisor: Abera Gure (PhD

Jimma, Ethiopia

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Advisor's Approval Sheet

We approve that **Haimanot Tesfaye Ewunetu** has carried out his MSc research work entitled as "***Investigation of Physiochemical Properties and Volatile Organic Compounds of Honey Produced in Bonga, Ethiopia***" under our supervision. In our judgment, the candidate has completed his research work, and therefore he is ready to proceed to the defense.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION

AC	Ash Content
AOAC	Association of Analytical Chemists
CACS	Codex Alimentarius Committee on Sugar
CSA	Central Statistical Agency
EC	Electrical Conductivity
EI	Electron Impact
EU	European Union Directive
EQSA	Ethiopia Quality and Standard Authority
FA	Free Acidity
FAO	Food and Agricultural Organization
GC-MS	Gas Chromatography Mass Spectroscopy
IHC	International Honey Commission
HMF	Hydroxymethylfurfural
LLE	Liquid Liquid Extraction
MC	Moisture Content
QSAC	Quality and Standard Authority Commission
VOCs	Volatile organic compounds
WHO	World Health Organization

ABSTRACT

In this thesis, physiochemical properties and volatile organic compounds (VOCs) of honey varieties from Bonga, Ethiopia were investigated. Three flora-origin honey sample including Schefflera abssinica, Croton macrostachyus and unknown flora honeys were collected from Bonga area. Different physiochemical parameters including moisture, ash contents, pH, free acidity, hydroxymethylfurfural (HMF), and honey colors were studied. The volatile organic compounds (VOC) were extracted by sequential liquid extraction using n-hexane and then, identified by gas chromatography-mass spectrometry (GC-MS). The obtained results showed that physicochemical parameters of all the three flora-origin honeys fall within the acceptable ranges set in the national and international guidelines. However, their statistical tests by ANOVA ($p<0.05$) demonstrated as the physicochemical parameters of the three studied samples were significantly different. Several VOCs were identified from each honey type. From C. macrostachyus, S. abssinica, and unknown flora honeys, 16, 17 and 18 VOCs were, identified, respectively. From these VOCs, six of them were detected in the three honey samples. Particularly, two VOCs, namely, (3-isopropyloxiran-2-yl) methanol, $C_6H_{12}O_2$ and 1, 2, 4-trimethyl-benzene, C_9H_{12} were take the share of 32.62, 29.88 and 29.98% as well as 24.06, 24.09 and 23.38%) in C. macrostachyus, S. abssinica and unknown flora honey samples, respectively. Some VOCs were detected in two of the honey samples but not in the third. Several VOCs, nearly 50% of the detected compounds from one flora honey were not detected in others. Generally, the obtained results showed that the VOCs of honeys are varied with their floral. However, further study is recommend to identify different flora origin honey markers of the Bonga area or other area honey as the method is promising to easily identify honey based on its flora-origin.

Keyword: Physiochemical properties, VOCs, Different flora-origin honeys, Bonga area, GC-MS.

1. INTRODUCTION

1.1. Background of the Study

Honey is a nutritious and indispensable product for human. It is produced from the nectar of plants (secretions of living parts of plants), in which honeybees collect, transform and combine with enzymes to breakdown complex sugars in the nectar to simple sugars (such as glucose and fructose). Then, they store in the honeycomb to ripen and mature [1]. Honey is a sweet, supersaturated mixture containing wide ranges of constituents such as simple sugars, organic acids, amino acids, proteins, minerals, vitamins, enzymes and volatile compounds that is responsible for the characteristic flavour of honey [1-3].

Honey contains more than 95% sugar. Honey also contains aromatic compounds at very low concentrations in the form of volatile mixtures. These, volatile organic compounds (VOCs) provide an aroma for the honey. Honey can be varied in its aroma and qualities because honeys produced from different nectar's have different chemical constituents. The quality of honey also dependent on microbes in honey, transformation of plant compounds by bees, honey processing, and condition of storage [3, 4]. Generally, honeys have different inherit properties from plants nectars, which directly and/or indirectly determine honeys color, aroma, flavor, density, physical and chemical properties [2, 3].

In worldwide about 1,850,868 tons of honey is produced per year. China, Turkey, and Argentina are the world leading honey producer, with average annual production of 457,203, 114,113, and 79,468 per year, respectively. Ethiopia takes the 10^{th} rank in the world with 50.000 tone production in a year. However, Ethiopia takes the first rank in honey production in Africa [5, 6].

Physicochemical study of honey is signicantly important in the global trade since honey is widely used as food and additive in food processing industries. The physicochemical parameters of honeys such as pH, free acidity, moisture content, ash content, electric conductivity, Hydroxymethylfurfural (HMF), apparent reducing sugar and sucrose contents as well as their Volatile organic compounds (VOCs)might be different for different honey varieties [7-9]. The composition and quality of honey also depend highly

on the types of flowers utilized by bees as well as regional and climatic condition [10, 13].

The main objective of this study was to investigate the physiochemical properties and VOCs of honey varieties produced in Bonga area, Kafa Zone, Ethiopia. The findings of the study were compared with the Ethiopian honey Quality Standard Authority [14] and International honey standards of the Codex Alimentarius [1].

1.2. Statement of the Problem

Kafa Zone is located in South West Region of Ethiopia. It is one of the high honey production areas. The area is known by its dense forest coverage and supplying quality honey to the local community, particularly Bonga Town and other parts of the country. In Kafa Zone, honey is produce from various floral sources such as *Schefflera abssinica* (*S. abssinica*) whose local name is Getama, *Croton macrostachyus* (*C. macrostachyus)* its local name is Bisana, and others. The composition and quality of honeys are greatly dependent on floral types, geographical and environmental factors [5].

The quality of honey is a key factor for both local and international markets for competitive premium prices and ensures human health [3]. Honey quality consideration is an aspect disregarded by producers and processors especially in developing countries [4]. Quality control of honey is significant to determine its suitability for processing and to meet the demand of the market or consumers. Due to increasing in international interests, studies in honey characterization have been carried out in relation to physicochemical parameters. Therefore, investigation of the physicochemical parameter and VOCs of honey at the production sites is so important to know whether the produced honeys meet the established quality standard at national and international levels. Therefore, in this study, the physiochemical properties and VOCs of honey produced in Bonga area, Kafa Zone, Ethiopia was investigated. The obtained findings were compared with the existing national and international guidelines.

1.3. Objectives of the Study

1.3.1. General objective

The general objective of this study was to investigate the physiochemical properties and volatile organic compounds of honey varity in Bonga, Kafa Zone, Ethiopia.

1.3.1. Specific objectives

- To determine the physiochemical parameters (such as colour, moisture content, ash content, electrical conductivity, pH, free acidity and HMF) of honey -in Bonga area.
- To identify main VOCs of the honey varieties produced in Bonga area by GC-MS.
- To compare the quality of honey produced in Bonga area with that of national and international quality standards.

1.4. Significance of the study

The finding of this study could have the following significances;

- It could give scientific information about the quality of honey varieties produced in Bonga area, Kafka Zone.
- It could be used as baseline information for honey producers and sellers in the study area.
- It could be used as a literature for other researchers on the physicochemical properties and VOCs of honey varieties of Bonga area, Kafka Zone.

2. LITERATURE REVIEW

2.1. Physicochemical properties of honey

Different physicochemical parameters such as color, ash, pH, free acidity, moisture content, electrical conductivity, refractive index, specific gravity, reducing sugar, sucrose, water insoluble solid, optical rotation specific rotation, diastase activity and HMF are used to determine the quality characteristics of honey varieties[5,15,16]. The physical properties of honey depend on it is the flora type from which it is produced, temperature of storage and the proportion of sugars it contains [16-19]. Analysis of these physicochemical properties of honey are used to verify the authenticity of the product and to reveal the presence and/or absence of an intentionally added adulterants [20-23]

2.1.1. Colour

Honeys have different colors, from nearly water white to dark amber [6]. The flavor and aroma of honey are dependent on its color. For instance, the light colored honey has typically a mild flavor [21], while the dark colored honey has usually stronger in flavor [2-5]. In general, the color of honey also indicates its mineral content and floral sources. Exceptionally, sometimes, light colored honey has very definite specific flavor. Of course, the flavor and aroma judgments are varied with individual preference.

2.1.2. Refractive Index

Refractive index of honey is used to provide a rapid, accurate and simple measure of moisture content. A study indicated the refractive index of honey from Argentina ranged from 1.4892 to 1.5043[21]. While, other study on showed that the refractive index of Algerian honeys ranged from 1.4889 to 1.4999 [23].

2.1.3. pH value

Naturally, honey is acidic, due to the presence of organic acids that contribute to its flavor and its stability against microbial spoilage. The pH of a honey sample is not directly related to the free acidity because of the buffering action of various acids and minerals present. Most honeys are supersaturated solutions of fructose and glucose with pH values between 3.2 and 4.5. The acidic pH of honey is used to prevent the growth of many

bacteria. Honeys have low pH due to the presence of weak organic acids such as gluconic acids, ascorbic acid and acetic acid [18]. The natural acidity of the honey inhibits the growth of microorganism as the optimal pH for most organisms is between 7.2 and 7.4 [10].

Fredes *et al.*, [20] reported the honey pH values of Chilean honey, which were ranged from 3.75 to 4.61, with 4.1 mean values. Citrus and eucalyptus honeys from Andalusian had also pH values from 3.72 to 4.64 with the mean of 4.07 [24, 25]. Moreover, generally, honeys are acidic with a pH-value lying between 3.5 and 5.5[19]. The pH is indeed a useful index for identification of the possible microbial contamination and adulteration. Adulterated honey samples have higher pH than that of pure samples [3]. Honey pH has high relevance during the extraction and storage of honey since it affects the stability and shelf life of the product. Most bacteria and moulds grow in neutral and mildly alkaline environments. However, honey contains a number of acids: 0.05 to 0.1% amino acids, and 0.57% organic acids that are used to in controlling honey's pH at an average value, 3.9, with a typical variation from 3.4 to 6.1 [3, 19].

Free acidity is an important parameter related to the deterioration of honey and it is characterized by the presence of organic acids in equilibrium with lactones and internal esters and some inorganic ions like sulphates, phosphates, and chlorides. in general, the presence of different organic acids, geographical origins and harvest seasons can affect the honey‘s acidity [1, 19].

2.1.4. Electrical conductivity

Electrical conductivity (EC) is another good criterion for determination of honey quality and its botanical origin. The EC of honey lies between 0.06 and 2.17 mS/cm. Honeydew contains considerably higher amounts of minerals compared to blossom honeys. Blossom honeys, as well as the mixture of blossom and honeydew honeys should have EC less than 0.8 mS/cm. Honeydew and chestnut honeys should have more than 0.8 mS/cm EC [1, 21]. The maximum EC of pure floral honey is 0.8 mS/cm [1, 23].The EC of the honey is closely related to the concentration of mineral salts, organic acids and proteins. EC is useful for discriminating honeys of different floral origins [1]. Recently EC measurement has been included in the international standards instead of the ash content [14]. A reliable determination of the botanical origin, however, do not based on EC only [1, 23].

2.1.5. Optical density

The optical density of a medium is not the same as its physical density. It related to the honey sluggish or slow moving tendency to maintain the absorbed energy of an electromagnetic wave in the form of vibrating electrons before remitting it as a new electromagnetic disturbance. Honey density has higher than that of water by about 50% [5, 23]. Because of the variation in density, it is sometimes possible to observe distinct satisfaction of honey in large storage tanks. The higher water content or less denser honey settles above the denser honey. By thoroughly mixing of the honey such inconvenient separation can be avoided [5].

The optical density of honey measured with a colorimeter using the complete light spectrum offers a precise and reproducible method for classifying honey according to its color. This provides a practical systemic basis for blending honey to any required color and used for honeys from most floral sources [1, 5].

2.1.6. Viscosity

Like most viscose liquids, honey is thick and sluggish to flow at room temperature below20 °C [21]. The viscosity of honey is influenced by temperature, moisture content and flora source. If temperature increases viscosity decreases due to decrease, molecular friction and hydrodynamic forces reduced. Honey with higher water content has higher fluidity and lower viscosity [21]. The temperature effect on viscosity follow an Arrhenius type relationship and honey varieties exhibited Newtonian behavior [22].

2.1.7. Specific rotation

Honey has the property of rotating the polarization of the plane of polarized light. The major components of honey are sugars, which are chiral compounds like almost all naturally occurring carbohydrates (D-molecules). In a given concentration each sugar affects the rotation by an amount that characteristics the sugar. The relative proportions of the sugars present in a mixture can affect the optical rotation. To measure the optical rotation of honey, a supersaturated solution, mostly D-fructose and D- glucose with a small amount of sucrose is used [24].

The low content of sucrose and high content of glucose and fructose in honey are parameters of characterization of honey quality. Honeys and honeydews have the property of rotating the polarization plane of polarized light. This is largely depending on their type and relative proportion the sugars of honey [24, 25].

Normally, floral honeys have higher fructose content than whereas honeydew honeys. Each carbohydrate type has a specific angle of rotation of polarized light or specific rotation fructose $^{[\alpha]}D_{20}$ = -92.4° and Glucose $^{[\alpha]}D_{20}$=+52.7 [25].

Optical rotation is a parameter that to determination of botanical origin of honey groups blossom, honeydew and compound honeys. The specific rotation calculated from angular rotation, ray circuit length and mass of honey in gram taken [19]. Analysis of optical rotation is used to identify floral honeys from honeydew honeys [25]. The optical rotation for honeydew honeys is positive rotation, whereas nectar honeys have negative rotation [24, 26, 27].

2.2. Chemical composition of honey

2.2.1. Moisture content of honey

The moisture content of honey is a limiting factor that determines the honey quality, stability and resistance against spoilage yeast fermentation [9]. Honeys that have low moisture content, below 20%, have long shelf life. Such honeys have the ability to resist fermentation and granulation during storage [4, 10]. Honey may contain higher moisture content, up to 30%. Such high water content was observed due to premature harvesting, mixing with water or watery ingredients such as bee brood.

Moisture is largely influenced by geographical position from where the nectar and pollen producing plant and the bee colony, degree of maturity, botanical origin of honey and harvesting techniques [3, 4, 10]. For instance, honeydew honeys have lower water content than blossom honeys [5]. Honey moisture is the quality criterion that determines the capability of honey to remain stable and to resist spoilage. The permitted moisture content is from 13.6 - 19.4% [8]. The moisture content influences the preservation, flavor, specific weight, viscosity, crystallization and repeatability contributes to the development of fermenting microorganism [8, 9].

2.2.2. Ash content

Ash content is the residue, which obtain by heating ash dish in electrical furnace at ashing temperature [1]. The ash content in honey is low and is influenced by the nectar chemical composition of predominant on plants in their formation [1,7,8] and varies according to the different botanical sources involved in honey formation [26,27].

The variability of ash contents has been associated in a qualitative way with different botanical and geographical origins of honeys [1]. The highest allowable ash contents of honey according to the specifications of Ethiopian, European and Codex standards are < 0.6 [14], < 0.6 and < 0.8percentage, respectively [1].

Normally, nectar has low ash content. For floral honeys, the ash contents are expected between 0.1% and 0.3 %. Very high ash content about 1.0% is actually expected in honeydew honey [21, 25, 28].

Literature report showed that the ash content of different floral honeys fall in the range of 0.02 to 1.03 % [27]. Lazaridou *et al,* [29] reported that ash content of selected Greek honeys, which were ranged from 0.16 to 0.60 %, with an average of 0.32 % [29].

2.2.3. Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is an excellent indicator of honey freshness [30, 31]. It is a breakdown product of fructose or one of the main sugars in honey formed slowly and naturally, during the storage of honey, and much more quickly when honey is heated [30]. The presence of HMF is an aldehyde that is often use for the assessment of honey quality is considering an important physiochemical parameter to determine the status of honey samples. HMF is often use as an indicator for the quality of honey, is generate by the decomposition of fructose in acidic conditions, and occurs naturally over time in most honey. Additionally, high levels of HMF may be result due to inadequate storage and adulteration with sugar or severe heat treatment [20, 29].

The HMF amount present in honey an indicator which is used as a guide to the amount of heating that has taken place, i.e. the higher the HMF value considers, the lower the quality of honey [20,30]. In Some countries there is a set of limit for imported HMF value of honey (sometimes 40 milligrams per kilogram), and honey with an HMF value

higher than this limit will not be accepted. However, naturally some honeys have high HMF level. When the level of the 5-Hydroxymethylfurfural (HMF) increased and the activity of diastase is decline beyond the given limits. Therefore, it is consider that overheating of honey taken place or adulterated the honey sample present. The high level of HMF in honey was originally take to indicate adulteration with invert sugar but naturally, honey from tropical areas might contain over 40 mg/kg. If it is desired to show that a honey is adulterated rather than that it merely has a high HMF level, a more extensive analysis is necessary [27, 28, 31].

A maximum limit for HMF in honey after processing and pending is 80 ppm. Excessive heat use, liquefaction, or pasteurization of honey adverse effect on quality of honey, i.e. loss of volatile compounds, accumulation of HMF and reduction of diastase and invertase activities [1]. At pH 5 or lower HMF is produced and HMF occurs naturally in honey especially in warm climates [28, 32]. The useful tools in detecting induced heat defects in honey are quantification of HMF content and enzyme activities but cannot be used for the determination of botanical or geographical origin. HMF content varied between 0 and 4.12mg/kg, which can be considered fresh honey. The other factors that influence the level of HMF are the sugar profile presence of organic acid, pH, moisture content and floral source [32, 33]. The study indicated that the HMF value gives only an indication of overheating or inadequate storage conditions [26, 27, 31].As a result of bad storage and heating the HMF formation increases [28,34,35].

2.2.4. Protein content

The interest of the protein content was provided to distinguish natural honey from artificial mixture and blend. Accordingly, the value of the protein content was found that 0.532 % [28, 29]. A study on four types of honey produced in the United State reported that an average protein content to be 0.46 % [28]. The level of protein is dependent on the type of flora and thus it is variable [3,35].The nitrogenous compounds amount of different honeys is at the range of 199-13100(ug/g) for protein content [14].Other report indicated that the nitrogen content ranged in American bee honeys was from 0-0.138% and averaged 0.041%. A reported showed in Sudanese bee honeys a range of 0.077-1.378% and average of 0.53% protein [14]. In honey of bee and plant origin, the presences of different amino acids were identified. The free amino acids proline, lysine,

histidine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, tyrosine, phenylalanine, and tryptophan were existed in honey [36, 37].

2.2.5. Carbohydrate content

In honey carbohydrates represented as the largest portion [5].The two main sugars present in honey are 38% w/w fructose and 31% glucose, lesser amounts of (1%) sucrose and, about 25 different oligosaccharides additionally have been detected [23]. The principal carbohydrates fructose and glucose are quickly transported into the blood in the process of digestion after honey intake and utilized for energy requirements by the human body [33, 38]. For a daily dose of 20 g, honey will cover about 3% of the required daily energy [19].

Much of the physical nature of honey like viscosity, hygroscopicity, granulation properties and energy values are responsible by sugars. According to the proposed Codex Standard for Honey requires a minimum reducing-sugar content of 65% for flower honeys; fructose and glucose both are clearly characterized as reducing sugars [1, 14]. Sucrose is a non-reducing, is hydrolyzed by either mineral acids or by enzyme invertase, each molecule combines with a molecule of water, and fructose and glucose are formed quantitatively in equal [29,39].

According to the study [27], fructose, glucose and sucrose were ranged between 27.2 and 44.3 %, 22.0 and 40.7% then 0.2 and 7.6% respectively. The total sugars, reducing sugar and sucrose reported values were 60.6-79.4%, 57.8 -75.7% and 0.6-10.5% respectively [28].In the other study found that the range of fructose and glucose were 22.1-41.3 % and 13.5 - 36.3% respectively [27]. Moreover, a report noted the range of glucose was 10.69-45.25%, fructose 13.55–45.0%, and sucrose 0.14–11.49% and the total sugars 80.0–83.5%[30].

2.3. Volatile compounds and Aroma compounds in honey

Volatile compounds are organic chemicals that have a high vapor pressure at standard room temperature due to low molecular weight. A volatile compound is the important components in honey. Volatile compounds are found low concentration and the main factors responsible for the aroma as it can evaluate the organoleptic and authenticity of foodstuffs [40].

A study conducted in honey showed that more than 600 volatile organic compounds (VOCs) have been identified. VOCs affect the sensory characteristic of honey; the type of plants affects all flavour, aroma, colour and texture and flowers bees visit [40].

Microbial and environmental contamination can also contribute to the number of VOCs [40]. Factors that affecting the honey aroma are a volatile element present in honey especially gluconic acid and proline [10]. Aroma is one of the most important features, since it also allows detection of adulteration of the product. Aroma in honey is attributed to different low-molecular weight chemical compounds. Some compounds, all of them volatile, are derived directly from the flowers visited by bees; therefore, the aroma has a floral origin.

Others, however, are generated during honey processing and storage. Because honey inherits plants properties, its color, aroma, flavor, density, and physical and chemical properties depend on the flowers used by bees, although weather conditions also influence honey composition and properties [40-42].

Volatile compounds in honey originate from plant components through the direct generation of aromatic compounds by bees, as well as thermo-generation of aromatic compounds and the action of microorganisms [40-43].

The main volatile compounds in honey have their origins, in general terms, in different chemical families, such as alcohols, ketones, aldehydes, acids, esters, terpenes. Honey volatiles are the substances responsible for the honey aroma [40, 42], it was found that most volatile compounds originate probably from the plant, but bees add some of them. Volatile compounds in honey have been reported to be derived from diverse sources such as plant constituents, transformation of plant constituents by honeybee, direct generation

of constituents by the honeybee, thermal processing of honey, and action of microorganisms or environmental contamination. They are grouped in different chemical families such as alcohols, ketones, aldehydes, hydrocarbons, acids, esters, terpenes and its derivatives, norisoprenoids, benzene derivatives and sulphur compounds [40, 43].

Accordingly, these compounds are extracted with low polarity solvents including hexane, diethyl ether, chloroform and dichloromethane. Nevertheless, their composition depends on the extraction methods. The main volatile compounds in honey have their origins, in general terms, in different chemical families, such as alcohols, ketones, aldehydes, acids, esters, terpenes. Formic acid is an organic compound found in nature. It appears in a diversity of organisms and is one of the natural compounds of honey [40].

Volatile compounds benzaldehyde and benzene acetaldehyde have been reported as common aromatic compounds in honey samples. Benzene acetaldehyde is considered one of the compounds that give honey its characteristic honey aroma [43-45].

Several techniques have been developed to identify and qualify volatile compounds that are responsible for aroma. However, the composition of volatile compounds depends on the extraction method. Not all compounds have an impact on honey aroma since the concentration of the volatile compound must exceed the perception threshold. However, a few compounds with own concentrations can still contribute significantly to honey aroma [42,45].Various methods have been employed in the extraction of volatile compounds in honey, which included solvent extraction, simultaneous distillation-extraction, headspace, ultrasound assisted extraction, hydro distillation and solid-phase micro extraction techniques[45-47].

2.4. Production of Honey and Bee Wax in Ethiopia

Ethiopia is a country with the highest honeybee population in Africa and a honey production estimated at 50,000 metric tons per annum, which constituted about 11% of the country's production potential. It is the 4th largest producer of beeswax and the 10th largest honey producer in the world. Ethiopia produces around 23.6% and 2.1% of the total African and world's honey, respectively [39]. According to CSA data, the total honey production in Ethiopia has been a small portion of the estimated potential, indicating the state of under utilization of the existing resources. The most important

honey producing regions in Ethiopia are Oromia, Amhara, SNNPR, Benishangul-Gumuz and Tigray. In Ethiopia, where more than 400 plant species are already identified as major honey plants [19], it is expected to have very diversified honey types. An investigation conducted in HBRC and Biochemical laboratory of the Ethiopian Authority of Standardization to characterize honey samples from all over the country indicated that about 63% of 542 honey samples had moisture content < 21%, those from highly humid areas having higher moisture content and those from low humid regions with lower moisture content[39]. More than 95% and 80% of the samples were found that the total reducing sugar and acidity in the range of standards, respectively, the mineral content was relatively lower than the standards, around 63% of the samples had HMF value below 40mg/kg and about 72% of the samples meet the diastase activity standards set by EU and FAO/WHO.

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted honey varieties produced around Bonga Town, which is located in the Southwest peoples' Regional State of Ethiopia. Bonga Town found at about 440 km from Addis Ababa. Currently, it is used as the capital of the Kafa Zone and the South West Peoples' Regional state. The town is found on a hill in the upper Barta valley at latitude and longitude of 7°16′N 36°14′E, coordinates: 7°16′N 36°14′E and an elevation of 1,714 m above sea level. Based on the 2007 Census conducted by the CSA [48], Bonga Town has a total population 20,858.

3.2. Chemicals and Reagents

All chemicals and reagents used were of analytical grade and distilled water used was for solution preparation. Different chemicals such as ethanol (87%), nitric acid (HNO_3, 65%) and sodium hydroxide (NaOH) from were from Blulux Laboratories PLtd. (Faridabad, Haryana, India). Potassium ferrocyanide ($K_4Fe(CN)_6{\cdot}3H_2O$) and Zinc acetate, $Zn(CH_3COO)_2{\cdot}2H_20$, 98%) were obtained from Merck Laboratory Chemicals (Nagpur, Maharashtra, India) to prepare Carrez I and II, respectively. Sodium bisulphite, ($Na_2S_2O_5$, 98%) was obtained from Riedei-deHaen (Rupert-Mayer-Str. Munich, Germany). Distilled water was used throughout the experiment.

3.3. Apparatus and Instruments

An analytical balance (Nimbus, Switzerland) with ± 0.0001 g precision, pH and conductivity meter (pH/Cond. Level-1, InloLab), digital refractometer (Reichert, AR200), Muffle furnace (Lenton Thermal, England), hot plate (SH3, Stuart Scientific) and fume cupboard (ENVAIR Ltd., England), Analytical Jena AG Specto-200 plus, double beam UV-Vis spectroscopy, Agilent Gas chromatography-mass spectrometry (GC-MSD), GC model 8890 and MSD model 5977B and other laboratory apparatus were used during the study.

3.4. Sampling and Sample pre-treatment.

Three different honey samples were purposively collected in polyethylene bottles based on their flora varieties, in November 2021. Honey of three produced from *S. abssinica, C. macrostachyus and unknown flora* were considered in this study. For each variety, about 1 kg fresh honey sample was taken from beekeeper around Bonga town. Then, the samples were transported to Jimma University Analytical Chemistry Research Laboratory and kept in refrigerator until for analysis. Before analysis of physicochemical parameters of the samples, honey samples were warmed in water bath at approximately 40℃ for 30 min and shaken to homogenize and solubilize crystallized sugar [14, 38].

3.5. Analysis of physicochemical properties of honey

The physicochemical parameters of honey samples such as moisture and ash content, electrical conductivity, pH, free acidity and HMF content were of analyzed following the standard procedures: the European Union Directive (EU), international honey commission (IHC)[49], quality and standards authority of Ethiopia[14] and codex alimentarius commission methods [1,18]. All analysis was carried out in triplicate.

3.5.1. Colour analysis

To determine colours, the absorbance of 50% honey (w/v) in water were measured at 560 nm ageist glycerine as a blank sample. The honey colours were the classified according to the Pfund scale after conversion of the absorbance values y the following formula [14, 28].

mm Pfund (intensity of honey color) = -38.70 + 371.39 x A (absorption of honey solution).

Table 1*: Pfund scale for determining colour*

Colour	Pfund scale (mm)	Colour range (inc)
Water white	From 1 to 8	0.030 or less
Extra white	More than 8–17	More than 0.030–0.060
White	More than 17–34	More than 0.060–0.120
Extra light amber	More than 34–50	More than 0.120–0.188
Light amber	More than 50–85	More than 0.188–0.440
Amber	More than 85–114	More than 0.440–0.945
Dark amber	More than 114	More than 0.945

*Millimetre **Incidence absorbance at 560 nm [8]

3.5.2 Determination of moisture

To determine the moisture content of sample honey a standard refractometric method was used [49]. Accordingly, moisture content was determined using refractometer at 20 °C using refractive index of distilled water (1.333) as a reference. The method correlates the refractive index of the honey samples measured to its moisture content. The refractive index readings were converted to moisture content (g/100 g) of honey samples.

3.5.3. Determination of electrical conductivity

To measure the EC, 20 g honey sample was dissolved in 100 mL distilled water. Then, a portion of the resulting solution was transferred to a beaker and placed in thermostated water bath at 20 °C and the EC of the solution was measured using coductometry [14, 49].

3.5.4. Ash content

The ash content of honey samples was determined using the standard method used by the method reported in literature [49]. Accordingly, a crucible dish (quartz dish) was first heated in a furnace at 400 °C and then, after cooling in a desiccator to room temperature its weight (M_2) was measured. Following this, 10 g of honey sample was weighed (M_0) into the crucible dish. After addition of 2 drops of olive oil to prevent frothing, the

sample was placed in preheated furnace and heated for about 1 h at 350 °C, until constant weight (M_1) was obtained. Finally, the ash content (% by mass) was calculated:

$$\text{Ash(\% by mas)} = \frac{M_1 . M_2}{M_0}$$

Where, Mo, M_1 and M_2 are mass of the honey sample taken, weight of the ash and crucible, weight of empty crucible, respectively

3.5.5. pH and free acidity

The pH value was determined using pH meter [49]. To measure pH of the samples, 10 g of honey was accurately weighed and dissolved in 75 mL carbon dioxide free distilled water in a beaker. Then, after thoroughly stirring with magnetic stirrer the pH of the resulting solution was measured. The pH meter was calibrated using pH 4, and pH 7 prior to using for measurement.

The free acidity of honey samples was determined using the standard procedure [14, 49]. Accordingly, the earlier solution prepared for pH determination was titrated against 0.1 M NaOH until the pH 8.50 was attained, which indicated the end point of the titration. Eventually, the acidity (meq/kg) of the sample was calculated.

$$\text{Acidity} = 10V$$

Where V is the volume of 0.1 M NaOH consumed for titration and 10 is the dilution factor of honey sample.

3.5.6. Hydroxymethylfurfural (HMF)

The HMF contents of the samples were determined by AOAC method [49].Therefore, Carrez I and II solutions were separately prepared by dissolving 15 g $K_4Fe(CN)_6$, and 30 g $Zn(CH_3COO)_2.2H_2O$ in 100 mL distilled water, respectively. 0.2 % sodium bisulphite, ($NaHSO_3$) was also prepared by dissolving appropriate weight of the salt in distilled water. 5 g honey sample was taken in a beaker and dissolved in 25 mL distilled water. The resulting honey solution was transferred to 50 mL volumetric flask. After addition of each 0.5 mL Carrez I and II solutions, the remaining volume was adjusted by distilled water to the mark. The content was then filtered through the filter paper and the first 10 mL of filtrate was rejected. From the remaining filtrate, 5 mL was pipetted in two test tubes. 5 mL water was added to the first test tube and mixed well. To the second test

tube 5 mL 0.2 % $NaHSO_3$ was added, mixed well and used as a reference solution. The absorbance of the sample was determined against the reference solution at 284 and 336 nm wavelengths using 1 cm quartz cuvettes within 1 h [48, 49]. Finally, HMF expressed as mg/kg was computed by the following formula:

$$HMF = \frac{[A_{284}-A_{336}]\times 149.7\times 5\times D}{W}$$

W is weight in g of honey sample, D is dilution factor (in case dilution is necessary), A_{284} and A_{336} are absorbance at 284 nm and 336 respectively.

$$Factor = 149.7 = \left(\frac{126}{16830}\right)x\left(\frac{1000}{10}\right)x\left(\frac{1000}{5}\right)=constant$$

126 = molecular weight of HMF, 16830 = Molar absorptivity of HMF at 284 nm, 1000 = conversion of g into mg, 10 = conversion of 5 into 50 ml, and 1000 = conversion of gram of honey into kg

3.6 Extraction of Volatile Organic Compounds

To determine VOCs of honey samples, liquid-liquid extraction (LLE) earlier reported literature was used [42, 42]. Accordingly, 1g honey sample was first diluted with 2 mL distilled water. The mixture was shaken for 20-30 min to ensure mixing honey of the sample. Afterwards, LLE was performed sequentially three times using n-hexane. For each sequential step, 4 mL n-hexane was used and after addition of the extraction solvent, the content was shaken for 20 min the mixture. Then, after standing to allow separation of the organic layer from the aqueous sample phase. In each step, the floating top layer was transferred to same vial. Then, the obtained extract was passed through Na_2SO_4 to remove water residue ad finally, 1 µL was injected into GC-MS for VOCs identifications.

3.6.1 Chromatographic conditions

Chromatographic separation was performed using HP-5 capillary column (30 m × 0.25 mm inner diameter; 0.25-mm film thickness) coated with 5% phenyl methyl siloxane (model 19091J-433; Agilent). Separation was performed with oven temperature program indicated in Table 2. The total GC run time was 20 min. Helium (99.99% purity) was used as a carrier gas at a flow rate of 1 mL/min. An aliquot of 1 µL was injected in splitless mode and injection temperature of 280 ^{o}C. The separated samples were ionized

by electron impact ionization (EI) and the ionization temperature was 280 ^{o}C. Identification of VOCs preformed using NIST mass spectral library.

Table 2: The GC conditions

	Rate ℃/min	**Value** ℃	**Holding min**	**Total time**
Initial		40	0	0
Ramp1	15	150	2	9.333
Ramp2	15	250	4	20

3.7. Analysis of data

The obtained experimental data were reported as mean ± SD of triplicate measurements. The SPSS software version 22 was used for data analysis. Statistical analyses such as One-way ANOVA and Turkey's post hoc at $P < 0.05$ were used to compare the physicochemical parameters ad VOCs of the studied hooey varieties.

4. RESULT AND DISCUSSION

4.1. Result for the physicochemical Parameter of Honey Sample

The obtained results of the physicochemical parameters of the studied three honey varieties are presented in Table 3.

4.1.1. The colour of honey

Based Pfund scale, the colours of the investigated *C. macrostachyus*, *S. abssinica* and unknown flora honey samples were categorized as amber, white, and light amber, respectively. The colour of honey is related to its mineral contents. Awraris *et .al* [10] reported that dark colored (Amber) honey collected from Masha, Gesha, and Sheko Districts in Southwestern Ethiopia had higher mineral contents than light amber honey samples. On the other hand, the mineral contents of honey is highly dependent on the soil type where the nectar producing plant is located, and the type of flower used by bees for nectar [5,22]. Some studies also reported that the taste of honey varied with colours, i.e., lighter coloured honey has a mild taste, whereas dark honey has strong and has a slightly bitter taste [10].

4.1.2. The pH value and the free acidity

Generally, honeys are acidic and have low pH values. According to Bogdanov *et al.* [5, 49] honeys, have pH values between 3.2 and 4.5. As presented from above Table 3, the studied honey samples: *C. macrostachyus*, *S. abssinica* and unknown flora had pH value 3.59 ± 0.29, 3.61 ± 0.02 and 3.85 ± 0.01, respectively. The studied honey samples have pH values with in the acceptable range [2, 5, 19, 49]. The obtained pH values were relatively lower than the reported pH value of honey samples collected from Tigray, which was 4.1 ± 0.2[50].

The free acidity values of the three-honey samples were 18.68 ± 0.54, 22.16 ± 0.53 and 24.03 ± 2.38 meq/kg for *C. macrostachyus*, *S. abssinica* and unknown flora, respectively. The overall average free acidity of honey samples analyzed in the present study was 21.62 ± 2.38meq/kg. The free acidity values indicate the freshness and/or absence of unwanted fermentation of honeys [5, 12, 18].

The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or

Table 3 : The obtained physicochemical parameters of investigated honey samples

Parameter	*C. macrostachyus*	*S. abssinica*	*Unknown flora*	Average	QSAC [1,5]	CACS [1,5,9,10]	EU [5,6]	IHC [5,49]	EQSA [14,23]	WHO[[5,7]
Pfund scale (m)	88.32 ± 0.35	28.30 ± 0.38	74.23 ± 2.17							
pH	3.59 ± 0.29	3.61 ± 0.02	3.85 ± 0.01	3.68 ± 0.19						
FA	18.68 ± 0.54	22.16 ± 0.53	24.03 ±2.38	21.62 ± 2.38	≤ 40	≤ 40	≤ 40	≤ 40	≤ 40	40
AC	0.21 ± 0.01	0.19 ± 0.01	0.33 ± 0.01	0.24 ± 0.06	≤ 0.6	≤ 0.6	≤ 0.6	≤ 0.6	0.01-1.2	0.6-1
MC	22.86 ± 0.18	23.66 ± 0.63	21.82 ± 0.75	22.78 ± 0.94	< 21%	< 21%	< 21%	< 21%	< 23%	21-23%
EC	0.65 ± 0.01	0.183 ± 0.01	1.22 ± 0.01	0.69 ± 0.44	< 0.8		≤ 0.8	< 0.8		
HMF	30.21 ± 0.11	18.19 ± 0.38	23.13 ± 0.59	23.84 ± 5.24	≤ 40	≤ 60	≤ 40		≤ 60	≤ 80

FA = Free Acidity (meq/kg) , AC = Ash Content (% by mass), MC = Moisture Content (%),

EC = Electrical Conductivity (μmS/m) , HMF = Hydroxyl- Methyl-Furfural (mg/kg)

internal esters, and some inorganic ions, such as phosphate. High acidity can be indicative of the fermentation of sugars into organic acids. The main organic acid found in honey is gluconic acid. Honey also contains minor acidic components such as formic, acetic, citric, lactic, maleic, malic, oxalic, pyroglutamic and succinic acids [18]. The studied honey varieties exhibited lower free acidity values than the reported free acidity values from Masha, Gesha and Sheko district southwest Ethiopia, which had average free acidity value of 28.32 meq per kg [10]. The studied honeys have below the maximum free acidity limits of set in national (40 meq/kg) [14] and international guidelines (50 meq/kg) [49].

4.1.3. Moisture content

Water content is very important for the analysis of the shelf life of honey during storage [26]. It can lead to undesirable honey fermentation due to osmo-tolerant yeasts, which form ethyl alcohol and carbon dioxide. The moisture contents (%MC) of the analyzed honey samples were 22.86 ± 0.17 (for *C. macrostachyus*), 23.66 ± 0.63 (for *S. abssinica*) and 21.82 ± 0.75(for unknown flora). The highest %MC obtained from sample *S. abssinica* (23.66 ± 0.63) and the least was recorded in honey ample unknown flora (21.82 ± 0.75). The overall average %MC was 22.78 ± 0.94, which is it higher than the limit set in the national and international guidelines [1, 5, 10, 49]. The %MC is one of the most essential quality components of honey, because it determines the rate of fermentation, shelf life span and processing characteristics of honey [10].

The variation %MC of honey depends on harvesting season, the degree of maturity that honey reached in the hive, type of hive used, environmental temperature and the MC of original plant [9, 22]. Naturally, the MC of honey can be as low as 13 % or as high as 23 % depending on the source of the honey, climatic conditions and other factors [18]. In areas of high humidity, it can be difficult to produce honey of low MC [18]. On the other side, high MC could accelerate crystallization in certain types of honey and increases its water activity to ferment [2, 17], and thus, deteriorate its quality [22].

4.1.4 Ash content

The ash content of honey is also another parameter that is used to describe honey flora origin or sources. The obtained ash content (%w/w) were 0.21 ± 0.01 for *C. macrostachyus* , 0.19 ± 0.01 for *S. abssinica* and 0.33 ± 0.01g for unknown flora honey samples. The overall mean of %ash was 0.24 ± 0.06. The results of the study indicated that the ash content of the three honey samples were below the maximum limit set in different guidelines such as EQSA [14], QSAC [5], QACS [15], EU [1,5,10]and WHO [1,5]. The ash content of honey is directly related to the mineral contents of honey, which relies upon soil nature, origin of flora, a floral plant and ecological condition [2]. Literature affirmed that pollen gathered by the honeybees during scrounging on the plants is the principal factor that influences the ash (mineral) content of honey, which fluctuates from 0.02 to marginally more than 1% for with flora type [18].

Earlier reports showed the variations the %ash content of honey from different parts of Ethiopia. For instance, Gebru, *et al*., [50] reported the %ash content of honey samples collected from different regions of Ethiopia. Honey samples collected Gambella region exhibited %ash content of 0.34 ± 0.05, from Oromia region, Bale zone 0.39 ± 0.04, and from Amhara and Tigray Regions 0.21 ± 0.01. According to these authors, report the ash content of Ethiopian honey falls between 0.14 - 0.47%. The obtained ash content in the present study also fall in this rage.

4.1.5. Electrical conductivity

EC is one of the most important factors for determining the physical characteristics of honey. It is also an important physicochemical measurement for the authentication of unifloral honeys. The highest EC, 1.22 ± 0.01 mS/cm was recorded for honey sample labeled as unknown flora followed by honey *C. macrostachyus* that had 0.65 ± 0.01 mS/cm. The lowest EC, 0.183±0.01mS/cm, was recorded for *S. abssinica* honey sample. The obtained EC values have a linear relationship with their ash content of values. According to the revised codex, the maximum limit EC for pure flora honey is 0.8 mS/cm or 800 mS/m [1, 51].

The obtained EC of the studied honey varieties also fall within this range. EC indicates the levels of ionizable acids and compounds in aqueous solution and it is a good criterion

to predict the botanical origin of honey. The higher the content of the ionizable acids and compounds in aqueous solution higher is the EC of honey samples. The observed differences among the three honey samples, in EC confirmed that the honey samples were from different were produced flora species [18].

4.1.6. Hydroxymethylfurfural

The concentrations of HMF recorded 30.21 ± 0.11 mg/Kg, 18.19 ± 0.38 mg/Kg and 23.13 ± 0.59 mg/Kg for honey varieties *C. macrostachyus*, *S. abssinica* and unknown flora, respectively. The average HMF of the three investigated honey samples was 23.84 ± 5.24 mg/K. One way ANOVA at $P < 0.05$ revealed that honey sample from flora *C. macrostachyus* has significantly contains concentration of HMF than flora *S. abssinica* and unknown flora.

Broadly, HMF is recognized as a boundary of honey deterioration. It is produced from fructose, slowly and naturally during the storage of honey and much more quickly when honey is heated [1, 12]. Honeys that have higher concentration of HMF have lower quality. Its concentration arises with storage and prolonged heating of honey.

HMF is one of the most commonly monitored parameters for determining honey freshness and good practices by beekeepers are HMF [2, 3, 26]. Both the EU and the Codex Alimentarius set the maximum limit of HMF in Honey, i.e., not be higher than 40 mg/kg. If honey is stored, at a temperature of 20 °C, tits HMF content increases by 1-2 mg/kg per month. After two years of storage, the maximum value usually reached to 40 mg/kg or greater. In tropical conditions, it is probably reach even quicker. Honey from tropical area is therefore permitted by law to have a maximum HMF content up to 80 mg/kg as long as it is labeled as tropical honey [49, 52, 53].

4.2. Pearson correlation studies

Table 4 shows Pearson correlation ($P < 0.05$) among the investigated physicochemical parameters of C. macrostachyus, S. abssinica and unknown flora honey samples.

Table 4 : Pearson correlation data of the physicochemical parameters

	pH	FA	mmpfund	AC	MC	EC	HMF
pH	1						
FA	0.599	1					
mmpfud	0.154	-0.385	1				
AC	0.654	0.674	0.400	1			
MC	-0.422	-0.338	-0.596	-0.763	1		
EC	0.565	0.383	0.696	0.934	-0.849	1	
HMF	-0.106	-0.710	0.919	0.015	-0.291	0.361	1

FA=Free Acidity (meq/kg) , AC=Ash Content (% by mass) ,
MC= Moisture Content (%), EC= Electrical Conductivity (μmS/m) ,
HMF= Hydroxyl- Methyl-Furfural(mg/kg)

The correlation study showed that the presence of very strong positive correlation between EC and ash content as well as their color and HMF. On the other hand, pH has moderate positive correlation with moisture content, ash content and EC. However, a strong negative correlation was observed between EC and moisture content of honey samples.

Moderate positive correlation was observed between free acidity and pH of the samples. However, free acidity exhibited a strong negative correlation with HMF. Colour showed a strong positive correlation with EC. On other hand, color had moderate correlation with ash content and HMF. However, it had moderate negative correlation with moisture content.

The findings showed a low negative correlation between pH and moisture content, free acidity and color, free acidity and moisture contents of honey samples. However, a low positive correlation was observed between free acidity and EC as well as color and ash contents of honey sample.

Although the finding showed a positive correlation between free acidity and pH ($r = 0.599$), but the pH value of the honey is not directly related to free acidity because of the buffer

properties of organic acids phosphate, carbonate and other mineral salts which are naturally present in honey [19].

One way ANOVA ($p < 0.05$) demonstrated that the presence of significance difference in the physicochemical properties among the studied honey samples: *C. macrostachyus*, *S. abssinica* and unknown flora.

4.3. VOCs of C. macrostachyus, S. abssinica and unknown flora honey samples

The identified VOCs of the three honey varieties are presented in Table 5 – 7. From each *C. macrostachyus*, *S. abssinica* ad unknown flora honey samples 16, 17 and 18 VOCs were identified. The three honey varieties contained (with their % in the parenthesis) an alcohol, (3-isopropyloxiran-2-yl) methanol, $C_6H_{12}O_2$ (32.62, 29.88 and 29.98%) and a benzene derivative compound, 1,2,4-trimethyl-benzene, C_9H_{12} (24.06, 24.09 and 23.38%) as the major components in *C. macrostachyus*, *S. abssinica* and unknown flora honey samples, respectively. In addition, cis/trans-5-Phenyl-2-tetrahydrofurylmethyl 2'-pyridyl sulphide, $C_{16}H_{17}NOS$, (furan derivative); 5-(1'-Ethylpropylidene)-1,3-cyclopentadiene, $C_{10}H_{14}$ (alkene), (+-)-trans-p-menthan-1,8-dien-5-acetate, $C_{12}H_{18}O_2$ (ester), and 3,7,7-trimethyl-, [1S-(1.alpha.,2.alpha.,6.alpha.)] -bicyclo [4.1.0] hept-3-ene-2-thiol ($C_{10}H_{16}S$) (Bicyclic mono terpenoid) are available in all the studied honey samples.

Table 5: VOCs identified in C. macrostachyus flora honey samples

Peak No	Identified VOCs	M. weight	t_R	%	Compound type
1	(3-isopropyloxiran-2-yl)methanol ($C_6H_{12}O_2$)	116	3.276	32.62	Alcohol
2	3,9-diethyl-6-Tridecanol ($C_{17}H_{36}O$)	256	3.52	0.93	Alcohol
3	2-(methylallyloxy) ethanol ($C_6H_{12}O_2$)	116	4.342	1.13	Alcohol
4	1,2,4-trimethyl-benzene (C_9H_{12})	120	5.153	24.06	Benzene derivative
5	1-Butoxyoctane ($C_{12}H_{26}O$)	186	5.398	3.82	Alkane
6	cis/trans-5-Phenyl-2-tetrahydrofurylmethyl 2'-pyridyl sulphide ($C_{16}H_{17}NOS$)	271	5.465	5.54	Furan derivative
7	1-Azido-1-ethenylcyclopropane ($C_5H_7N_3$)	109	5.576	1.89	Alkane
8	Dicinnamyl ether ($C_{18}H_{18}O$)	250	5.62	2.27	Ether
9	5-(1'-Ethylpropylidene)-1,3-cyclopentadiene ($C_{10}H_{14}$)	134	5.764	6.01	Alkenes
10	2-(1-phenylethyl)-Phenol ($C_{14}H_{14}O$)	198	5.831	4.845	Phenol derivative
11	3,7,7-trimethyl-, [1S-(1.alpha.,2.alpha.,6.alpha.)]-Bicyclo[4.1.0]hept-3-ene-2-thiol ($C_{10}H_{16}S$)	168	6.053	2.35	Bicyclic mono terpenoid
12	(+-)-trans-p-Menthan-1,8-dien-5-acetate ($C_{12}H_{18}O_2$)	194	6.453	1.301	Easter
13	(1R,2R)-2-(aminomethyl)-1-phenyl-1-butanol ($C_{11}H_{17}N O$)	179	6.709	1.699	Alcohol
14	(6R,7R)-7-(4-Methylphenyl)non-8-en-1,6-diol ($C_{16}H_{24}O_2$)	248	6.831	2.67	Alcohol
15	3-Hydroxy-2-methoxy-cyclopent-2-enone ($C_6H_8O_3$)	128	7.209	2.21	Ketone
16	(4aS,5R,10bS)-5-phenyl-3,4,4a,5,6,10b-hexahydro-2H-pyrano[3,2-c]quinolone,($C_{18}H_{19}NO$)	265	16.141	4.4794	Quinoline

Table 6: VOCs identified in* S. abssinica *flora honey sample

Peak No	Identified VOCs	M. weight	t_R	%	Compound type
1	(3-isopropyloxiran-2-yl)methanol ($C_6H_{12}O_2$)	116	3.276	29.88	Alcohol
2	Ethylbenzene (C_8H_{10})	106	3.776	12.19	Benzene derivative
3	meso-2,5-Dimethyl-3,4-hexanediol ($C_8H_{18}O_2$)	146	4.343	0.77	Alcohol
4	1-Dotriacontanol ($C_{32}H_{66}O$)	466	4.476	2.79	Alcohol
5	1,2,4-trimethyl-benzene (C_9H_{12})	120	5.154	24.09	Benzene derivative
6	(2R)-2-(2-iodanylethyl)oxirane (C_4H_7IO)	198	5.398	1.79	Alkane
7	cis/trans-5-Phenyl-2-tetrahydrofurylmethyl 2'-pyridyl sulphide ($C_{16}H_{17}NOS$)	271	5.465	4.72	Fural derivative
8	cis-1,2-diamine cyclohex-4-ene ($C_6H_{12}N_2$)	112	5.576	1.43	Alkenes
9	(+-)-6-phenyl-3-hydroxy-1,5-hexadiene ($C_{12}H_{14}O$)	174	5.62	1.88	Alkenes
10	5-(1'-Ethylpropylidene)-1,3-cyclopentadiene ($C_{10}H_{14}$)	134	5.754	3.62	Alkenes
11	2-tert-butyl-2-chloro-3,3-dimethylbutanal ($C_{10}H_{19}ClO$)	190	5.831	4.48	Aldehyde
12	1,6,6-trimethyl-3-methylene-1,4-cyclohexadiene ($C_{10}H_{14}$)	134	6.042	1.845	Alkenes
13	(+-)-trans-p-Menthan-1,8-dien-5-acetate ($C_{12}H_{18}O_2$)	194	6.12	3.59	Easter
14	n-Cetyl thiocyanate ($C_{17}H_{33}NS$)	283	6.198	2.036	Thiocyanate
15	3, 7, 7-trimethyl-, [1S-(1.alpha.,2.alpha.,6.alpha.)] - bicyclo[4.1.0]hept-3-ene-2-thiol ($C_{10}H_{16}S$)	168	6.498	1.158	Bicyclic mono terpenoid
16	Benzo[c]thiophene, 1-ethyl-1,3-dihydro-, 2,2-dioxide ($C_{10}H_{12}O_2S$)	196	6.709	1.45	Thiophene
17	(1R,2R)-2-(aminomethyl)-1-phenyl-1-butanol ($C_{11}H_{17}NO$)	179	6.831	2.24	Alcohol

*Table 7: **VOC***

Table 7: Identified in* unknown *flora honey samples

Peak No	identified VOCs	M. weight	t_R	%	Compound type
1	(3-isopropyloxiran-2-yl)methanol ($C_6H_{12}O_2$)	116	3.287	29.98	Alcohol
2	3,9-diethyl-6-tridecanol ($C_{17}H_{36}O$)	256	3.509	2.48	Alcohol
3	Ethylbenzene (C_8H_{10})	106	3.776	12.98	Benzene derivative
4	4-(tetrahydrofuranyl-2-oxy)-4-methyl-2-pentanone ($C_{10}H_{18}O_3$)	186	4.476	4.13	Furan derivative ketone
5	1,2,4-trimethyl-benzene (C_9H_{12})	120	5.153	23.38	Benzene derivative
6	(2S)-2-(2-iodanylethyl)oxirane (C_4H_7IO)	198	5.309	0.87	Alkane
7	(5E)-8-bromanylocta-1,5-dien-3-ol ($C_8H_{13}BrO$)	204	5.398	1.69	Alcohol
8	cis/trans-5-Phenyl-2-tetrahydrofurylmethyl 2'-pyridyl sulphide ($C_{16}H_{17}NOS$)	271	5.465	4.31	Furan derivative
9	1-methyl-3-(phenylsulfonyl)pyrrolidine ($C_{11}H_{15}NO_2S$)	225	5.576	1.45	Payroll
10	1-ethyl-2,3-dihydro-1H-indene ($C_{11}H_{14}$)	146	5.62	1.71	Alkenes
11	5-(1'-Ethylpropylidene)-1,3-cyclopentadiene ($C_{10}H_{14}$)	134	5.753	3.13	Alkenes
12	2-tert-butyl-2-chloro-3,3-dimethylbutanal ($C_{10}H_{19}ClO$)	190	5.831	3.491	Aldehyyde
13	(+-)-trans-p-menthan-1,8-dien-5-acetate ($C_{12}H_{18}O_2$)	194	6.053	1.765	Easter
14	3, 7, 7-trimethyl-, [1S-(1.alpha.,2.alpha.,6.alpha.)] - bicyclo[4.1.0]hept-3-ene-2-thiol ($C_{10}H_{16}S$)	168	6.12	2.16	Bicyclic mono terpenoid
15	(1-methylenepropyl)-benzene ($C_{10}H_{12}$)	132	6.198	2.973	Benzene derivatives
16	1,6,6-trimethyl-3-methylene-1,4-cyclohexadiene ($C_{10}H_{14}$)	134	6.498	1.16	Alkenes
17	1-ethenyl-3,5-dimethyl-benzene ($C_{10}H_{12}$)	132	6.709	1.22	Benzene derivative
18	(6R,7R)-7-(4-Methylphenyl)non-8-en-1,6-diol ($C_{16}H_{24}O_2$)	248	6.831	2.17	Alcohol

Two VOCs, namely, 9-diethyl-6-Tridecanol, $C_{17}H_{36}O$ (0.93 and 2.49%); and (6R, 7R)-7-(4 Methyl phenyl) non-8-en-1, 6-diol, $C_{16}H_{24}O_2$ (2.67 and 2.17%) were identified with their % compositions in parenthesis from *C. macrostachyus* and unknown flora honey samples, respectively. However, these compounds were not detected in *S. abssinica* honey samples, respectively. Likewise, VOCs such as Ethylbenzene, C_8H_{10} (12.98 and 12.19%); 2-tert-butyl-2-chloro-3,3-dimethylbutanal, $C_{10}H_{19}ClO$ (4.48 and 3.49%); and 1,6,6-trimethyl-3-methylene-1,4-cyclohexadiene, $C_{10}H_{14}$ (1.845 and 1.16%) were identified from *S. abssinica* and unknown flora honey samples, with their % compositions in parenthesis, respectively. Nevertheless, these VOCs were not available in the honey sample of *C. macrostachyus* origin. One VOC, which is named as 1R,2R)-2-(aminomethyl)-1-phenyl-1-butanol, $C_{11}H_{17}NO$, was identified from *C. macrostachyus* (1.699%) and *S. abssinica* (2.24%) honey samples but it was not detected from the unknown flora honey origin. This VOC might be used for confirmation of honey from flora of C. *macrostachyus* and *S. abssinica.*

From 16 identified VOCs in *C. macrostachyus* flora honey, 8 of them are available only in this honey sample. These VOCs include 2-(methylallyloxy) ethanol, $C_6H_{12}O_2$ (1.13%); 1-Butoxyoctane, $C_{12}H_{26}O$ (3.82%), 1-Azido-1-ethenylcyclopropane, $C_5H_7N_3$(1.89%); Dicinnamyl ether, $C_{18}H_{18}O$ (2.27%); 2-(1-phenylethyl)-Phenol, $C_{14}H_{14}O$ (4.84%); (6R,7R)-7 (4-Methylphenyl)non-8-en-1,6-diol, $C_{16}H_{24}O_2$ (2.67%); ad 3-Hydroxy-2-methoxy-cyclopent-2-enone, $C_6H_8O_3$ (2.21%). These compounds could be used for identification of *C. macrostachyus* honey type.

Similarly, from 17 VOCs identified in the *S. abssinica* flora honey sample, 7 VOCs such as 4-(tetrahydrofuranyl-2-oxy)-4-methyl-2-pentanone, $C_{10}H_{18}O_3$ (4.13%); (2S)-2-(2-iodanylethyl) oxirane, C_4H_7IO (0.87%); (5E) -8-bromanylocta-1,5-dien-3-ol, $C_8H_{13}BrO$ (1.69%), 1-methyl-3-(phenylsulfonyl)pyrrolidine, $C_{11}H_{15}NO_2S$ (1,45%); 1-ethyl-2,3-dihydro-1H-indene. $C_{11}H_{14}$ (1.71%); (1-methylenepropyl)-benzene, $C_{10}H_{12}$ (2.97%); and 1-ethenyl-3, 5-dimethyl-benzene, $C_{10}H_{12}$ (1.22) were recorded only this flora honey sample. These compounds could also be used for identification of *S. abssinica* flora honey from others.

Likewise, from 18 VOCs identified in the unknown flora honey sample, 7 compounds including meso-2,5-Dimethyl-3,4-hexanediol, $C_8H_{18}O_2$ (0.77%); 1-Dotriacontanol, $C_{32}H_{66}O$ (2.79%); (2R)-2-(2-iodanylethyl)oxirane, C_4H_7IO (1.79%); cis-1,2-diamine cyclohex-4-ene, $C_6H_{12}N_2$ (1.43%); (+-)-6-phenyl-3-hydroxy-1,5-hexadiene, $C_{12}H_{14}O$(1.88%);n-Cetyl thiocyanate, $C_{17}H_{33}NS$ (2.04%); and Benzo[c]thiophene,1-ethyl-1,3-dihydro-2,2-dioxide, $C_{10}H_{12}O_2S$ (1.45%) were detected only in this flora honey sample.

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

In this study, the physicochemical characteristics and VOCs in the three honey varieties: *C. macrostachyus*, *S. abssinica* and unknown flora honeys were investigated. The studied physicochemical characteristics such as pH, free acidity, HMF, Ash content ,moisture content and EC of the 3 honey varieties agreed with the set national and international quality guidelines. However, statistical they exhibited significant differences in terms of their physicochemical properties. Besides, several VOCs were identified using GC-MS. From identified VOCS, (3-isopropyloxiran-2-yl) methanol, $C_6H_{12}O_2$ and 1,2,4-trimethyl-benzene, C_9H_{12} were the observed major components the three floral honey samples. The obtained findings also showed that each honey flora contain several unique VOCs, which could be used for the identification of the honey flora type. Generally, we can conclude the type and number of VOCs of a honey is dependent on its floral origin. Because, honeys from different can contain different VOCs.

5.2. Recommendation

Based on the obtained results of the study the following recommendation could be drawn.

- To draw general conclusion on the physicochemical characteristics and VOCs of Kafa Zone, specifically, Bonga area further study is recommended by considering additional flora origin honeys,
- The composition of VOCs of honey is promising for identification of its flora origin. Thus, identification of VOCs as markers for different flora origin honey needs further studied.
- Additional study is needed to characterize the aroma constituents as well as to determine the botanical and geographical origins of honeys to standardize its quality.

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7. **ANNEXES**

Annex 1: Refractive Index of the Honey Reference to the Standard Moisture Content

Refractive idea	**Moisture (%)**	**Refractive index**	**Moisture (%)**
1.4740	25.0	1.4865	20.0
1.4745	24.8	1.4870	19.8
1.4750	24.6	1.4875	19.6
1.4755	24.4	1.4880	19.4
1.4760	24.2	1.4885	19.2
1.4765	24.0	1.4890	19.0
1.4770	23.8	1.4895	18.8
1.4775	23.6	1.4900	18.6
1.4780	23.4	1.4905	18.4
1.4785	23.2	1.4910	18.2
1.4790	23.0	1.4915	18.0
1.4795	22.8	1.4920	17.8
1.4800	22.6	1.4925	17.6
1.4805	22.4	1.4930	17.4
1.4810	22.2	1.4935	17.2
1.4815	22.0	1.4940	17.0
1.4820	21.8	1.4946	16.8
1.4825	21.6	1.4951	16.6
1.4830	21.4	1.4956	16.4
1.4835	21.2	1.4961	16.2
1.4840	21.0	1.4966	16.0
1.4845	20.8	1.4971	15.8
1.4850	20.6	1.4976	15.6
1.4855	20.4	1.4982	15.4
1.4860	20.2	1.4987	15.2

Source: Bogdanov, S. [55]

Temperature corrections “Refractive index”

- Temperature above 20 ºC → 0.00023/ ºC was added.
- Temperature below 20º C→ 0.00023 per℃ was subtracted

Annex 2: ANOVA analysis of the physicochemical properties of honey

		Sum of Squares	df	Mean Square	F	Sig.
Ph	B/n Groups	0.132	2	0.066	2.279	0.184
	Within Groups	0.173	6	0.029		
	Total	0.305	8			
FA	B/n Groups	44.223	2	22.111	112.736	0.000
	Within Groups	1.177	6	0.196		
	Total	45.399	8			
Mmpfund	B/n Groups	5911.412	2	2955.706	1772.691	0.000
	Within Groups	10.004	6	1.667		
	Total	5921.416	8			
AC	B/n Groups	0.035	2	0.017	316.770	0.000
	Within Groups	0.000	6	0.000		
	Total	0.035	8			
MC	B/n Groups	5.104	2	2.552	7.732	0.022
	Within Groups	1.980	6	0.330		
	Total	7.084	8			
EC	B/n Groups	1.615	2	0.808	7698.624	0.000
	Within Groups	0.001	6	0.000		
	Total	1.616	8			
HMF	B/n Groups	219.080	2	109.540	636.689	0.000
	Within Groups	1.032	6	0.172		
	Total	220.112	8			

FA =Free Acidity (meq/kg), AC=Ash Content (% by mass),

MC = Moisture Content (%), EC= Electrical Conductivity (μmS/m),

HMF = Hydroxyl- Methyl-Furfural (mg/kg)

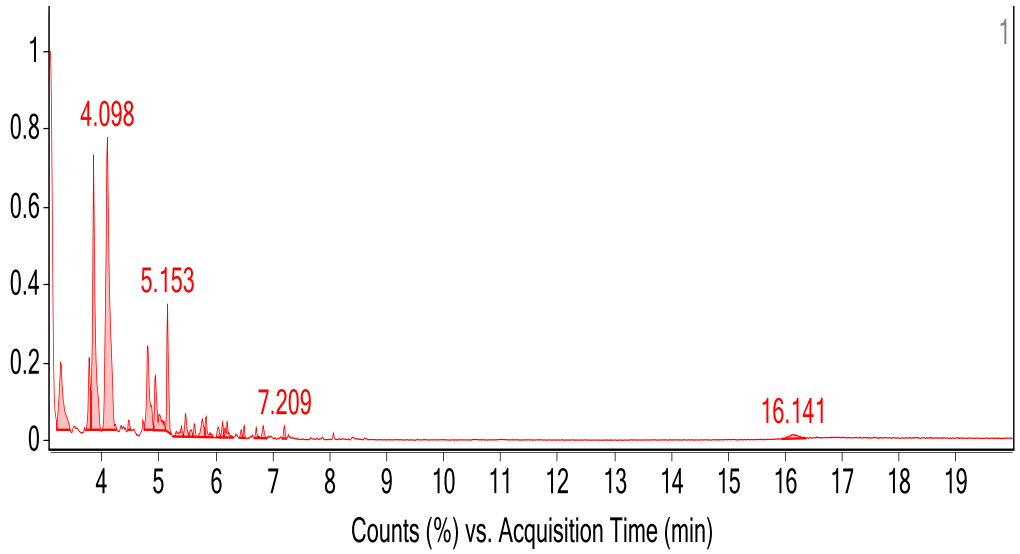

Figure 1: Total ion chromatogram of C. macrostachyus (Bissana) flora sample honey

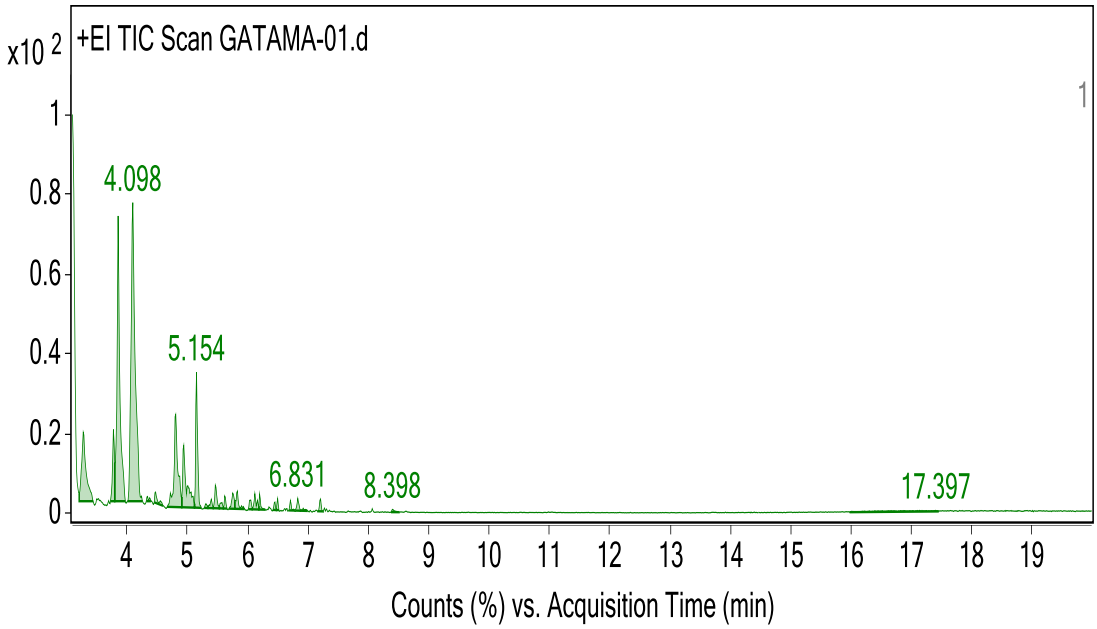

Figure 2: Total ion chromatogram S. abssinica (Gittema) flora sample honey

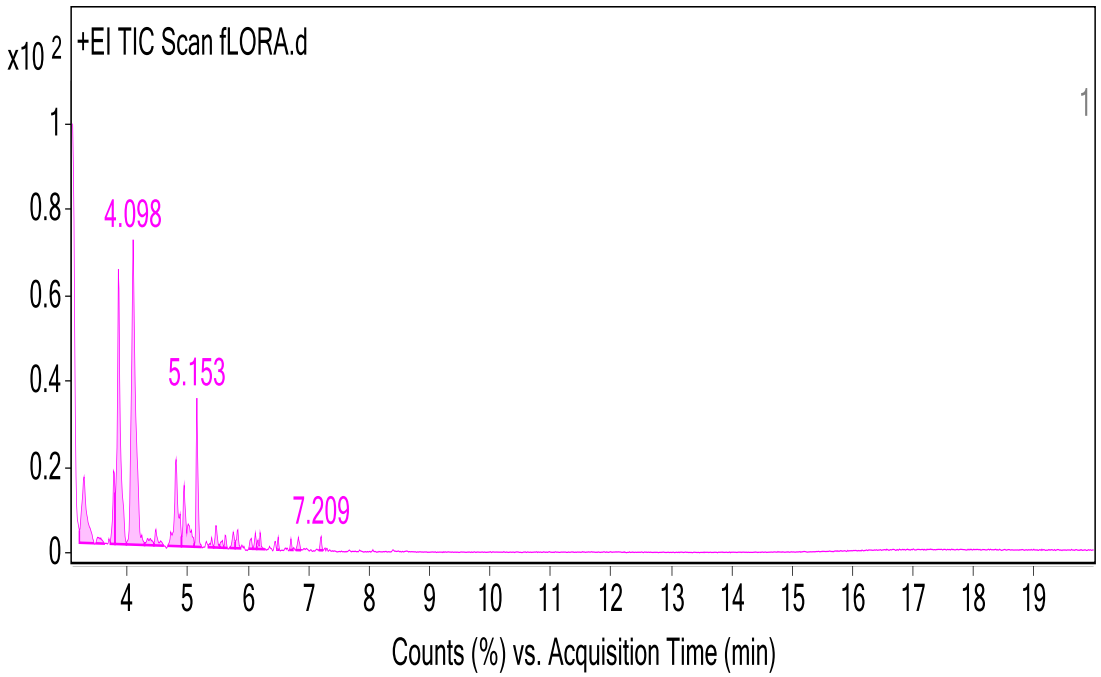

Figure 3: Total ion chromatogram Unknown flora sample honey