PHYSICAL AND CHEMICAL CHARACTERIZATION OF FRESHLY HARVESTED AND FACTORY-PROCESSED MANGROVE HONEY FROM KILIFI COUNTY, KENYA

OMORI NANCY KWAMBOKA

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DECLARATION

I confirm that this thesis is my original work and has not been presented in any other university/institution for certification.

Signature_____ Date_____
Nancy Kwamboka Omori
Registration No. I56/CE/25151/2014
Department of Chemistry

We declare that the work reported in this thesis was carried out by the candidate under our supervision.

Signature_____ Date_____

Dr. Evans Changamu Ogwagwa
Department of Chemistry
Kenyatta University

Signature_____ Date_____

Dr. Robert M. Bichanga
Department of Chemistry
Kenyatta University

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ABBREVIATIONS AND ACRONYMS

AOAC	Association of Analytical Chemists
ASAL	Arid and Semi-arid Lands
ANOVA	Analysis Of Variance
DCM	Dichloromethane
DMRT	Duncan Multiple Range Test
DN	Diastase Number
EC	Electrical Current
EU	European Union
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GoK	Government of Kenya
HD	Hdrodistillation
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
HS-SPME	Head Space-Solid Phase Micro-Extraction
IHC	International Honey Commission
IN	Invertase Number
KShs	Kenya Shillings
LC-MS	Liquid Chromatography Mass Spectrometry
LLE	Liquid-Liquid Extraction
LRI	Linear Retention Indices
MS	Mass Spectrometry
NIST	National Institute of Standard and Technology
PAC	Polyacrylate
PDMS	Polydimethylsiloxane
pH	Potential of hydrogen
SD	Standard Deviation
SDE	Steam Distillation Extraction
SPME	Space-Solid Phase Micro-Extraction
SPSS	Statistical Packages for Social Sciences
TIC	Total Ion Current
USD	United States Dollar
USDA	United States Department of Agriculture
USE	Ultrasound-assisted Solvent Extraction
UV-Vis	Ultra-violet visible
VOC	Volatile Organic Compounds
WHO	World Health Organization

ABSTRACT

Mangrove honey from the mangrove forests at the Kenyan coastal region has been in production at the commercial level by beekeepers organized and registered in self-help groups for more than a decade now. The honey is said to have medicinal properties but it has not been fully characterized to determine whether it meets the Kenyan standards on Honey – Specification (KS EAS 36:2020), European Union (EU) Directive on Honey (2001/110/EC) and International Honey Commission (2009). The objective of this study was, therefore, to characterize Kenyan mangrove honey in terms of its physicochemical properties and volatile organic compounds present. It was also important to correlate the honey VOC profiles with the mangrove tree flowers extract profiles. Unprocessed honey sampling was done from selected beekeepers from the four mangrove forest regions of Kilifi County, namely, Mida, Kilifi, Watamu and Mtwapa creeks while processed honey samples were obtained from Kwetu Training Centre. Twenty (20) honey samples weighing approximately 250 g each were collected randomly from farmers and factory in different locations in Kilifi County, packed in 500 cm3 plastic bottles, ferried for refrigeration in the lab at a temperature of 4o C. Mangrove flower volatile organic compounds (VOCs) were collected by static headspace solid phase microextraction (HS-SPME) fiber while collection of VOCs in honey samples was done by ultrasound-assisted solvent extraction (USE) method. Mangrove honey was analyzed for moisture content (by refractometry), colour index, diastase, proline and hydroxymethylfurfural (by spectrophotometry), pH and electrical conductivity (by pH/conductivity meters), free acidity (by titrimetry), sugar content (high performance liquid chromatography) and volatile organic compounds (by GC-MS). Quantitative data was analyzed using the SPSS program version 21. The results showed that the mean moisture content ranged from 23.40±0.00 to 19.23±0.06%, mean pH from 4.45±0.05 to 4.00±0.00, mean electrical conductivity ranged from 0.54±0.00 to 0.32±0.00 MS/cm. The mean acidity ranged from 30.21±0.15 to 22.20±0.00 Meq/kg. The mean proline content ranged from 734.36±4.23 to 602.24±2.68 mg/kg. The mean HMF levels were in the range of 2.86±0.14 to 0 mg/kg. The mean invertase content ranged from 9.55±0.10 to 5.54±0.11 U/kg. The mean diastase content ranged from 11.48.47±0.06 to 8.11±0.64 S/units. In the VOCs profile of mangrove flower, factory and farm, 119, 90 and 70compounds were identified, respectively. Of the identified VOCs, 35 were found both in the honeys analyzed and in mangrove flower. Three compounds docosanoic acid, palmitoleic acid and n-hexadecanoic were found in large quantities in all samples analyzed and the mangrove flowers and can be considered to be chemical markers for mangrove honey. The HMF, diastase and invertase were found to be below the set limits suggesting that the mangrove honey studied was fresh and had not been heated by farmers during harvesting and extraction. The high proline content also revealed that nectar and pollen are produced in great quantities by mangrove plants. The rest of the physicochemical parameters were found to be within the limits set by the International Honey Commission, Codex Alimentarius and the Kenya bureau of standards (through the EAS).

CHAPTER ONE

INTRODUCTION

1.1 Background information

Mangrove forests are of great significance to the economic well-being of the regions where they occur since they provide a wide range of benefits including apiculture projects, marketable goods and eco-tourism sites (Kathiresan and Bingham, 2001). The Bangladesh Sundarbans mangrove forest for example, has created job opportunities for over 2,000 employees who are involved in the honey processing industry. Annually, the mangrove ecosystems of Bangladesh produce about 50% of the total honey production of the honey in their country (Gani, 2001). In Kenya the first mangroves-related apiculture project was introduced in 2010 in Kilifi County and it has become established and cherished by residents (Lang'at and Kairo, 2008). The honey produced in the established mangrove forests apiaries has not been systematically characterized even though it is marketed and consumed. It was also of interest to establish the volatile organic compounds profile of the honey from the unique mangrove habitat which is clearly different from the terrestrial habitats foraged by honeybees.

Generally, in the honey making process, honeybees collect nectar from flowers or plant secretions or plant sucking insects waste products obtained from plants living parts (Manyi-Loh *et al*., 2011a) which they then transform to honey through a combination of their own precise components. The nectar is then placed, dried and left to ripen and mature in honey cells, (White, 1962; White and Doner, 1980). Hence, honey gathered from various geographical sources is likely to differ in minor constituents only. The composition of honey is usually very closely associated with

the botanical nectar sources and the prevailing climatic conditions in the regions that bees forage (Castro-Vázquez *et al.*, 2010). Therefore, honey characterization from any given region in terms of important physicochemical characteristics like pH, moisture content, free acidity, levels of hydroxymethylfurfural, proline content, sugars (fructose, sucrose, maltose and glucose), enzymes and electrical conductivity (Bogdanov *et al.*, 1999), and in terms of the volatile organic compounds (VOCs) they contain is essential. Furthermore, characterization assures quality and authenticity when based on the quality criteria set out by the international honey standards (Alimentarius, 2001).

The honey quality criteria established by bodies like the Codex Alimentarius Commission, International Honey Commission (IHC) and the European Community Council (Alimentarius, 2001; IHC, 2002) prescribes the limits of the honey free acidity, moisture content, hydroxymethylfurfural (HMF), electrical conductivity and levels of sugars. Thus, all honeys are characterized against the said standards. Mangrove honey obtained from various parts of the world including Algeria (Ouchemoukh *et al.*, 2007), Portugal (Silva *et al.*, 2009), India (Saxena *et al.*, 2010) and Bangladesh (Islam *et al.*, 2017) have been conducted against the same standards.

This study sought to characterize Kenyan mangrove honey from Kilifi County in terms of its physicochemical parameters and the signature volatile organic compounds (VOCs) against the International Honey Commission quality criteria (Bogdanov *et al.*, 1999). It was necessary to systematically sample and analyze mangrove honey for physicochemical properties including hydroxymethylfurfural, acidity, diastase, invertase, moisture content, glucose, sucrose, fructose, pH, electrical conductivity and

color. It was also important to profile the VOCs found present in the honey and to compare the results with the profile obtained from the analysis of mangrove flower extracts to establish correlation.

The characterization of honey is important as producers strive to meet the ever-changing consumer needs, tastes and preferences. Therefore, it is of great significance to the honey industry to identify specific chemical markers for establishment of the botanical origin of honey. According to Persano Oddo and Piro (2004), the European legislation specification states that honey definition is complete when identified with its origin. Consequently therefore, physicochemical parameters alone are inadequate for honey quality authentication (Oddo *et al*., 2004). Defining the peculiarity and the specialty of honey helps to add value on functional uses and to attract customers. High quality and well researched honey attract a reasonable price in the world market, while lower quality and uninvestigated honey does not bring a good price. Most honeys in the market are priced lower than 4 USD per kg, whereas a well-defined and researched, Manuka honey from Australia, costs 52.97 US D per kg as reported in CIAFS (2012) and Tomblin *et al.*, (2014).

1.2 Statement of problem and justification

Kenyan mangrove honey has been produced in Kilifi County for a little over a decade now by beekeepers that organized in well-established self-help groups and a honey processing factory at the Kwetu training Centre within the region. The honey has, however, not been characterized against the Kenya Bureau standards Honey specifications (KS EAS 36:2020), the European Union Directive on Honey (2001/110/EC) and the Harmonized Methods of the International Honey Commission

(2009). Furthermore, the signature volatile organic compounds found in the Kenyan Mangrove honey have not been identified. Thus, the honey is regarded as poorly researched both in the local and international market. The objectives of this study were to characterize mangrove honey systematically sampled directly from the beehives in the mangrove forest in terms of its physicochemical properties, to identify the volatile organic compounds present in the honey and to compare the parameters found with those found in processed honey from the factory found in the region. The study also sought to correlate the volatile organic compound profiles of the honey with that of the mangrove tree flowers to help identify chemical markers for mangrove honey. Mangrove honey was analyzed for moisture content (by refractometry), colour index, diastase, proline and hydroxymethylfurfural (by spectrophotometry), pH and electrical conductivity (by pH/conductivity meters), free acidity (by titrimetry), sugar content (by high performance liquid chromatography) and volatile organic compounds (by GC-MS). The data collected is important both for the local market and for the international community as it will provide a means of distinguishing Kenyan mangrove honey form other honeys.

1.3 Hypotheses

i. The physicochemical properties of unprocessed and processed mangrove honey from Kilifi County are not significantly different and satisfy the European Union and IHC standards.

ii. The volatile organic compounds in mangrove honey are not different with those in mangrove flower and can be used as chemical markers for mangrove honey.

1.4 Objectives

1.4.1 General objective

To characterize Kenyan mangrove honey from Kilifi County in terms of its physicochemical characteristics and its signature volatile organic compounds relative to the volatile organic compounds profile of the mangrove flowers from the same region.

1.4.2 Specific objectives

The specific objectives of the study were to determine the:

i. pH, moisture content, free acidity, levels of hydroxymethylfurfural, proline content, sugars (fructose, sucrose, maltose and glucose), colour enzymes and electrical conductivity of raw (unprocessed) mangrove honey and compare with processed honey
ii. Volatile organic compounds in mangrove honey in comparison with those in mangrove flower.

1.5 Significance of the study

The physicochemical data generated and volatile organic compounds identified both in the Kenyan mangrove honey and mangrove flowers from the mangrove forests in Kilifi County will help to unequivocally identify the honey and its source. The signature VOCs will be used to identify future samples and help detect and probably deter mangrove honey adulteration.

1.6 Scope and limitations

Apiculture covers wide areas in the coastal region, but this study focused on mangrove honey from Kilifi County since it is adjacent to the natural mangrove plantations. Mangrove honey can be analyzed for a wide range of compounds and other properties but this study only focused on volatile organic compounds and physicochemical properties.

CHAPTER TWO

LITERATURE REVIEW

2.1 Apiculture

Kenyan apiculture projects produce honey approximated to be 100,000 metric tons per year, although it has been practiced over a long period of time due to challenges of natural resource degradation, climate change, lack of market information and farmers' difficulties in getting honey from remote rural areas to urban-based buyers (Lang'at and Kairo, 2008). The arid and semi-arid lands (ASAL) make up 80% of Kenyan land and are expected to be high in honey production (Morse-Jones *et al.,* 2011). Kenyan modernized apiculture projects began during the late 1960s and are among one of the innovative projects in the sector of livestock (Bosire *et al.,* 2003). The traditional log hives produce about 80% of the Kenyan honey and other hive products (Kinuthia *et al.,* 2009).

In Kenya the first mangroves-related apiculture project in Gazi Bay located at a longitude of 4°26'South and latitude of 39°30'East and also located south of the coastal city of Mombasa (50 km) was introduced in 2010 (Lang'at and Kairo, 2008). After problems arose from insufficient amount of flowers and freshwater, the first harvest was completed in early 2011. Apiculture was implemented by constructing twenty four (24) 9-column bee hives (Morse-Jones *et al.,* 2011). Each column produces 3 Kg of honey in 3 months. The 24 hives were able to produce 2,592 Kg of mangrove honey in one year, which is equivalent to KSh. 777,600 revenue per year (Bosire *et al.,* 2003).

Mangroves grow between the sea and land and are resistant to salinity and flooding hence can withstand salinity better than the terrestrial trees. Mangrove forests are a good source of food for bees because honey produced is far much better than that from any other apiary (Lang'at and Kairo, 2008). Kairo and co-workers (2009) notes that mangrove honey at the coastal region is priced higher than honey from other areas due to its associated medicinal properties than the ordinary honeys and also its salty, sweet taste. The characterization of honey generally involves determinations of its physical and chemical parameters including colour, pH, moisture content, electrical conductivity, free acidity, total sugars and HMF (Ouchemoukh *et al.,* 2007). Biochemical properties including enzymes like invertase and diastase are also usually measured (Crane, 1982).

2.2 Physicochemical characterization of honey

This refers the evaluation of the physical and chemical characteristics of honey relative the available honey quality standards to assure honey quality and authenticity which guarantee the consumer safety and protection against fraud (Shamsudin S., *et al,* 2019). The parameters evaluated include moisture content, 5-hydroxymethylfurfural (5-HMF) content, pH, free acidity, total soluble solids, sugar content, colour characteristics and intensity, and amino acid content. The quality and source or origin of honey influence its market price and consumer acceptance. This is particularly important for honey from new sources or honey from sources that have undergone floral changes. With increasing demand for bee honey unscrupulous dealers are tempted to adulterate honey by introducing other sweeteners such as high fructose or maltose syrup, beet sugar, cane sugar, corn syrup etc (Missio P., *et al,* 2016). Unscruplous beekeepers are also reported to feed honey bees on sucrose syrup

to boost production (Puscas A., *et al*, 2013). These practices rob the honey of its quality, alter its nutritional value as well as its medicinal benefits. This is why characterization of honey is very important.

2.2.1 Moisture content

The moisture content of honey is key to its stability against fermentation and granulation in that when moisture content is low, honey can be stored for longer periods as it is protected from microbiological activity (El Sohaimy *et al.,* 2015) Honey moisture content depends on environmental conditions and the manipulation from beekeepers at the harvest period and can vary from season to season (Acquarone *et al*., 2007). It has been reported that moisture content of honey is greatly influenced by the location of bee colony, honey botanical origin, method of harvesting and honey ripeness (Belay *et al.,* 2017). Generally, honey moisture content ranges between 13 to 29% for honeys from various geographical and botanical origins (Lazaridou *et al.,* 2004). Moisture contents of honey samples from different parts of the mangrove agro-ecological zone ranged from 12.32 – 34.02 %. There was significant difference between the mean moisture content of 32.8 % obtained from Edo State honey and 12.32 % obtained from Delta State honey samples. The different moisture content of honey depends on harvest season, the degree of maturity reached in the hive and the moisture content of original plant (Finola *et al*., 2007).

Moisture content is a good parameter for determining if honey is stable and of good quality. According to Kamal *et al.,* (2002), monofloral honeys such as strawberry, sunflower and heather are prone to contain higher content of water than others. Honey stability and free fermentation can be determined by the amount of water content in it. Moisture is the condition that governs the ability of honey to stay stable and prevent

decomposition by yeast fermentation. The moisture content is the compositional standard, which needs to be satisfied in world honey trade. Honey with high water content is more likely to ferment. In practice, honey as high as 21g/100 g were very infrequently found (Bogdanov, *et al.,* 1999).

In routine honey control carried out by the IHA during the years 1989-97 on 30,000 honey samples, 91 - 95 % of all honeys had a water content of less than 20g/100g. In Switzerland, a standard of 20 g/100 g honey was effectively used, until the last revision of the Swiss Food Ordinance, where the European Union maximum value of 21g/100 g had accepted (2001/119/EC). Many domestic beekeeping institutions in Germany, Belgium, Austria, Italy, Switzerland, and Spain have set a moisture content of maximum values of 17.5-18.5 g/100g for special classes of quality honey (Bogdanov, *et al.,* 1999).

Codex Alimentarius (CA) and European Union (EU) have a moisture standard of ≤ 20g/100g. The Kenya Bureau Standards (KEBS) based on the East African Standards (EAS) for honey certifies moisture content of ≤ 22g/100g of honey (Table 2.2). Mangrove honey from Kilifi County may contain different moisture content that require investigations.

Table 2.1: Codex Alimentarius (CA), European Union (EU) and East African Standards (EAS) standards for honey quality

Quality Criteria	CA	EU	East African Standards (EAS)
Moisture content	≤20 g/100g	≤ 20 g/100g	22 g/100g
Sugar content			
Blossom honey	≥ 60g/100g	≥ 60g/100g	≥ 60g/100g
Honeydew honey	≥ 45g/100g	≥ 45g/100g	≥ 45g/100g
Sucrose	≤ 5 g/10g	≤ 5 g/100 g	≤ 5 g/100 g
Free Acidity	≤ 50 meq/kg	≤ 40 meq/kg	≤ 40 meq/kg
Electrical conductivity			
Blossom honey	≤ 0.8 mS/cm	≤ 0.8 mS/cm	np
Honeydew honey	> 0.8 mS/cm	> 0.8 mS/cm	np
Diastase activity (Schade units)			
General	≥ 8	≥ 8	≥ 8
Honey with low natural enzyme content	≥ 3	≥ 3	≥ 3
HMF			
General	≤ 60 mg/kg	≤ 40 mg/kg	≤ 80 mg/kg
Proline	<180 mg/kg	< 180 mg/kg	< 180 mg/kg

hydroxymethylfurfural; meq: milliequvalent; mS: milliSiemens;
Source: (Bogdanov, 2001; Bogdanov and Martin, 2002; EAS 36: 2000).

2.2.2 Free acidity and pH

Honey naturally contains organic acids which contribute to its acidic properties (Belay *et al.,* 2013). The acidic property contributes to the honey flavour and microbial stability (Bogdanov *et al.,* 2004). The low honey pH majorly makes honey to have a distinctive good taste. Gluconic acid is the predominant acid in honey and is obtained from the oxidation of glucose in presence of glucose oxidase enzyme. Honey acidity is determined by titration and expressed in meq kg^{-1}. Other minor acids present

in honey include: lactic, pyroglutamic, formic, citric, succinic maleic, acetic, and oxalic acids (MATO *et al.*, 2003).

The study by Kamal and coworkers on honey obtained from Pakistan flora revealed that there was a substantial distinction in pH for honey obtained from different botanical sources; this could be attributed to nectar producing source and climatic conditions of the area (Kamal *et al.*, 2002). The amount of acid in honey can be a good criterion in determining honey quality, because in honey which is fermented the honey acidity increases. Honey having a high acidic level has a lower demand in the market or by consumers. The findings of Bastos and coworkers described that honey from the Dry Forest of northern of Minas Gerais, Brazil had high acidity (50.11 meq.kg^{-1}) (Bastos *et al.*, 2016). Similarly, a range of 3.99 ± 0.12 - 3.86 ± 0.19 and 19.2 ± 4.14 - 16.8 ± 1.09 meq kg-^{1} were reported for pH and total acidity, respectively, of coffee honey samples (Kadri *et al.*,2016). The EU and EAS accept a maximum limit of 40 meq kg^{-1} and CA has 50 meq kg^{-1} for acidity (Table 2).

2.2.3 Electrical conductivity

Electrical conductivity (EC) of the honey is closely related to the concentration of mineral salts, organic acids and proteins; serving as electrolytes. These electrolytes conduct electrical current. Electrical conductivity is an important parameter that is used to determine honey quality and establishing honey floral source (Krauze and Zalewski, 1991). Honey conductivity is majorly related to the amount of amino acids, mineral salts, organic acids, complex sugars and ash content. Nectar and honeydew honey can be distinguished on the basis of electrical conductivity (Bertoncelj *et al*., 2011).

According to Szczęsna and Rybak-Chmielewska (2004), conductivity of an aqueous solution is the degree of its ability to carry an electrical current by means of ionic motion. It is affected by the type and number of ions in the solution. The finding of Truzzi *et al.* (2014) indicated that honey from Italian, Marche Region had an electrical conductivity range of 0.14 - 1.45 mS cm^{1}. Bogdanov, *et al.* (1999) reported extensive conductivity data of thousands commercial honeys. The quality criteria for blossom honeys, mixtures of blossom and honeydew honeys are set to be ≤ 0.8 mS/cm; and honeydew and chestnut honeys ≥ 0.8 mS/cm (Table 2). Exceptions to these criteria are *Arbutus, Banksia, Erica, Leptospermum, Melaleuca, Eucalyptus* and *Tilia* honeys as well as their blends, having an extremely high variation in their conductivity.

2.2.4 Proline content

Proline is an amino acid found in pollen, nectar and saliva of honey bees and is the principal amino acid in floral honey, followed by lysine, glutanic and asparitic acids (Hermosin *et al.*, 2003; Ouchemoukh *et al.*, 2007). The findings of Hermosín, *et al*., (2003) on 31 Spanish honeys of five different botanical origins indicated that proline, phenylalanine, tyrosine and lysine were found principally; followed by arginine, glutamic acid, histidine and valine. The findings of Chua *et al.,* (2014) indicated that proline content was abundantly found in all honey samples significantly reduced upon heat treatment.

A minimum limit of 180 mg proline/kg honey has been accepted as a quality standard set by CA, EU and EAS. Proline might originate from bees, and cover 50 - 85% of the amino acid fraction (Iglesias *et al*., 2004). The dominancy of proline is true only for

some type of monofloral honeys. The total amino acid contents ranged from 240.7 to 812.6 mg/kg (Dong *et al.,* 2013). This variation depends on the color of honey. Usually, light colored honey shows lower amino acid level than dark honey.

2.2.5 Colour

The color of honey is an optical property that varies from water white to dark amber, when it is determined in Pfund scale. Honey color can be identified instantly by consumers from its physical appearance. It is most likely linked to age, storage conditions, mineral content, handling and botanical origin (Terrab *et al.,* 2002). The variation in floral origin is related to the content of polyphenols, which have an effect on the color of honey (Olaitan *et al.,* 2007). Honey which is mildly flavored is known to be light in colour , unlike strong flavored honey which has a dark colour (Manyi-Loh *et al.,* 2011b). According to Saravana Kumar and Mandal, (2009), to gain insight into honey phenolic content, HMF, mineral and pollen content, colour is a vital characteristic. Seasonal and environmental conditions also contribute to the colour of honey (Terrab *et al.,* 2002).

The work of Tuberoso and workers showed that the honey color ranges from clear, to amber to almost black. Infrequently, bright yellow, reddish or greenish color are observed (Tuberoso *et al.,* 2014). Honey optical analysis can be used to determine honey floral origin, occurrence of artifacts and level of heat treatment. Colour of honey can be determined using the spectrophotometric method by measuring absorbance at 560 nm.

2.2.6 Diastase and invertase

Diastase is defined as the amount of enzyme which converts 0.01 gram of starch to the prescribed end-point in one hour at 40 ^{o}C under test conditions (Bogdanov, 2009d). Results of diastase are expressed in Gothe units (Schade units) per gram of honey. The minimum level of diastase activity used by IHC and EU directive is 8 Diastase number (DN) whereas the EAS has a minimum of 3 Diastase number (DN). Diastase activity is thermal sensitive and easily affected by improper storage conditions hence used to indicate honey freshness and storage time (Bogdanov, 2009d; Babacan *et al.,* 2002). Unprocessed fresh honey diastase levels vary according to the botanical source, for instance clover and citrus honeys comprise of low diastase quantities. Other aspects that have effect on the quantities of diastase are bees foraging patterns and nectar viscosity. Long storage at moderate temperatures and exposure to high temperatures inactivate diastase in honey (Babacan *et al.,* 2002). Diastase levels do not always correlate with honey quality. Therefore, an overwhelming consideration of the diastase level will not guarantee quality.

Glucose and fructose are formed from hydrolysis of sucrose by Invertase/Sucrase enzyme predominantly found in mature honey. During the stages of honey maturation, the invertase enzyme activity significantly increases. A number of chemical reactions involved in the transformation of nectar to honey are initiated by invertase enzyme. Generally in honey, invertase enzyme is found to in minute quantities and can be deactivated through thermal treatment (Babacan and Rand, 2005).

The source of invertase in honey is the honeybees themselves. The secretions of the hypopharyngeal and salivary glands of foraging bees are added to the nectar for honey ripening. A lot of secretions of the glands are introduced into honey cells to enable honey ripening especially when nectar is transferred to house bee from a forager bee ready for storage. The amount of invertase enzyme is a function of apiculture practices, colony stage, bee age, viscosity of nectar and environmental conditions (Karabournioti and Zervalaki, 2001).

Invertase value is calculated from the volume of a sucrose solution (ml) that reacts with honey invertase (1g) for one hour. Invertase Number (IN) shows sucrose quantity that undergoes enzymatic hydrolysis for one hour using the enzymes present in honey (10 g) during the experimental procedure (Balasubramanyam, 2011).

2.2.7 Hydroxymethylfurfural

Hydroxymethylfurfural is a heterocyclic water-soluble organic compound obtained from dehydration of sugars through the Maillard reaction (Bogdanov *et al.,* 2004). It is a good criterion of determining honey's freshness, because in fresh honey the amount of HMF is insignificant (Bogdanov *et al.*, 2004; Ajlouni and Sujirapinyokul, 2010). It accumulates in honey because of deterioration caused by heating, aging or storage (Gidamis *et al.,* 2004). Very low amounts of HMF are naturally found in fresh sugar-containing foods. Extra HMF is made during heating and accumulates due to prolonged storage of food especially in acidic conditions. It is also a possible indicator of decay, syrup or sugar adulteration and over-heating (Sanz *et al.,* 2003; Morales *et al.,* 2009)).

Determination of the HMF content aids in verifying the freshness and whether honey has been overheated during processing because in fresh honey the amount of HMF is insignificant (Bogdanov *et al.,* 2004; Ajlouni and Sujirapinyokul, 2010). Ultra-Violet Spectrophotometry at a wavelength of 240 nm absorbance is used to determine HMF levels. It has been reported that in considering the results of HMF evolution, 20 months could be proposed as the best-before date once opened for honeys. After this date, HMF formation is clearly speeded up (Mar Cavia *et al.,* 2008).

Markets in Germany, Belgium, Italy, Austria and Spain accept a maximum HMF limit of 15 mg kg^{-1}. In international trade, a maximum value of 40 mg kg^{-1} has proven satisfactory. The CA has maximum of 60 mg kg^{-1} standard (Table 2). The EU has maximum of 40 mg kg^{-1} HMF and the EAS has maximum of 80 mg kg^{-1}. Extremely high (> 500 mg/kg) HMF values demonstrate adulteration with invert syrup. Investigating the HMF content helps to verify the freshness of honey.

2.2.8 Sugars

Sugars accounts for 95% of the major solids in honey. Monosaccharaides (fructose, glucose, maltose and raffinose) are the predominant followed by disaccharides (sucrose) (Terrab *et al.,* 2001). Minor quantities of oligosaccharides and trisaccharides carbohydrates are also present in honey (Ouchemoukh *et al.,* 2010). The presence of oligosaccharides and trisaccharides in honey can be used to determine honey floral origin and adulteration (Persano Oddo *et al.,* 2004). The nutritional and physical characteristics of honey can be determined by the predominance of simple sugars like fructose levels (Persano Oddo *et al*. 2004).

The degree of honey sweetness depends on the honey type. Generally, in good quality honey, the amount of fructose is greater than that of glucose. After fructose, sucrose is the sweetest followed by maltose. It is, therefore, a honey sweetener and is the most predominant sugar in most types of honey. Honeys containing a high concentration of fructose are known to be sweeter than those containing high concentration of glucose (Kaakeh and Gadelhak, 2005).

Honey sucrose content depends on the degree of its ripeness (Dimins *et al.,* 2008) and the invertase activity in honey (Dimins *et al.,* 2008). The contribution of sucrose to the total sugar in honeys can be increased, if honey is harvested before ripening. During the ripening process, in the cell combs, the sucrose level is reduced by the action of enzyme, invertase (Bogdanov *et al.,* 1999). Determination of the sucrose content from different sources of nectars collected is important in detection of adulteration. According to the International Honey Commission, some monofloral honeys for instance, Spanish rosemary honeys, are exempted on the amount of sucrose because they contain significantly higher levels compared to the acceptable limit of ≤ 5% (Bogdanov *et al.,* 1999).

Previous studies by De la Fuente *et al.,* (2011) on the carbohydrate composition of Spanish unifloral honeys identified about twenty six types of sugars, namely: fructose, glucose, sucrose, α,α-trehalose, α,β-trehalose, cellobiose, laminaribiose, maltulose, nigerose, turanose, maltose, kojibiose, trehalulose, palatinose, gentiobiose, isomaltose, raffinose+1-kestose, 6-kestose, neokestose, erlose, melezitose, planteose, theanderose, maltotriose, panose and isomaltotriose. Differences exist between the sugar of blossom and honeydew honeys. The honeydew honey contains higher

amount of oligosaccharides, mainly the trisaccharides melezitose and raffinose, which are absent in blossom honeys. In most blossom honeys, fructose and glucose represent the majority of sugars; but honeydew honey have high amounts of non-reducing oligosaccharides, such as maltotriose and raffinose. The CA, EU and EAS standards have a minimum limit of 60g/100g for reducing sugar, mainly fructose and glucose (Table 2).

2.2.9 Volatile organic compounds in honey

Volatile organic compounds (VOCs) in honey are derived from; flower nectaries, metabolic synthesis of plant substances by bees to form honey, bacterial or environmental adulteration or during honey processing (Cuevas-Glory *et al.,* 2007; Jerković I *et al.,* 2010). This means that honeys obtained from various floral origins can be distinguished by the volatile organic compounds (VOCs) present (Stanimirova *et al.,* 2010). Sensorial properties can be used to differentiate some monofloral honeys (Jerković and Marijanović, 2010). The aroma of honey can majorly be determined by the presence of certain VOCs that are also useful in linking honeys to their floral source. Product fraudulent labeling can be prevented through explication of the aroma compounds with floral origins of a specific honey type hence leading to standardization of honey quality (Jerković and Marijanović 2010). The concentration of VOCs in honey differ due to variation in temperature and duration of storage, since some VOCs are liable to heat and others arise from Maillard reaction (Castro-Várquez *et al.,* 2014).

The VOCs can be classified into various chemical families including aldehydes, terpenes, esters, hydrocarbons, carboxylic acids, alcohol, furans and pyrans (Baroni *et*

al., 2006). Verzer and co-workers affirmed the occurrence of 113 volatile organic compounds from the studied chestnut, eucalyptus and orange honeys (Verzera *et al.*, 2001). On the basis of the prior achievements on research work reported on Argentinean and European honeys, mangrove honey samples can be investigated using USE–GC–MS methods to come up with an appropriate method of classifying the VOCs (Alissandrakis *et al*., 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design

Descriptive quantitative research design was used in this study. It involved sampling and characterization of freshly harvested (unprocessed) and factory processed mangrove honey from four sampling regions of the mangrove forest in Kilifi County and from the honey processing factory found in the region. It also involved sampling of mangrove tree flowers, extracting, identifying and quantifying their volatile organic compounds. The honey samples were subjected to physical and chemical analyses for the important honey quality parameters as well as volatile organic compounds. The flower VOC profiles were compared with those of processed and freshly harvested (unprocessed) mangrove honey. The honey samples were analyzed for pH, moisture, specific sugars, acidity, HMF, electrical conductivity, proline content, enzymes (diastase and invertase) and volatile organic compounds present in honey by following Association of Analytical Chemists (AOAC) Method (AOAC 2000). One-way Analysis of Variance (ANOVA) was used to identify distinguishing physical and chemical features in the analysed honey samples. . The results of different parameters of all the analysed samples of honey were compared with the Codex Alimentarius and European Standards (Bogdanov *et al.*, 1999).

3.2 Study area

The study was conducted in mangrove forest area of Kilifi County (Figure 3-1). The County of Kilifi covers an area of approximately 12,609.7 km^2 in the Kenyan coastal region. The County is located within a longitude 39° 05’ and 40° 14’ East and latitude 2° 20’ and 4° 0’ South of the Greenwich Meridian (GoK, 2013). Prior to

carrying out the study, a reconnaissance survey was carried out in Kilifi County. Pre-requisite information was gathered from Kilifi County livestock and fishery resource and the ministry of public service, youth and gender affairs head office to identify the major apiculture areas and select registered self-help groups for beekeeping in the Kilifi County.

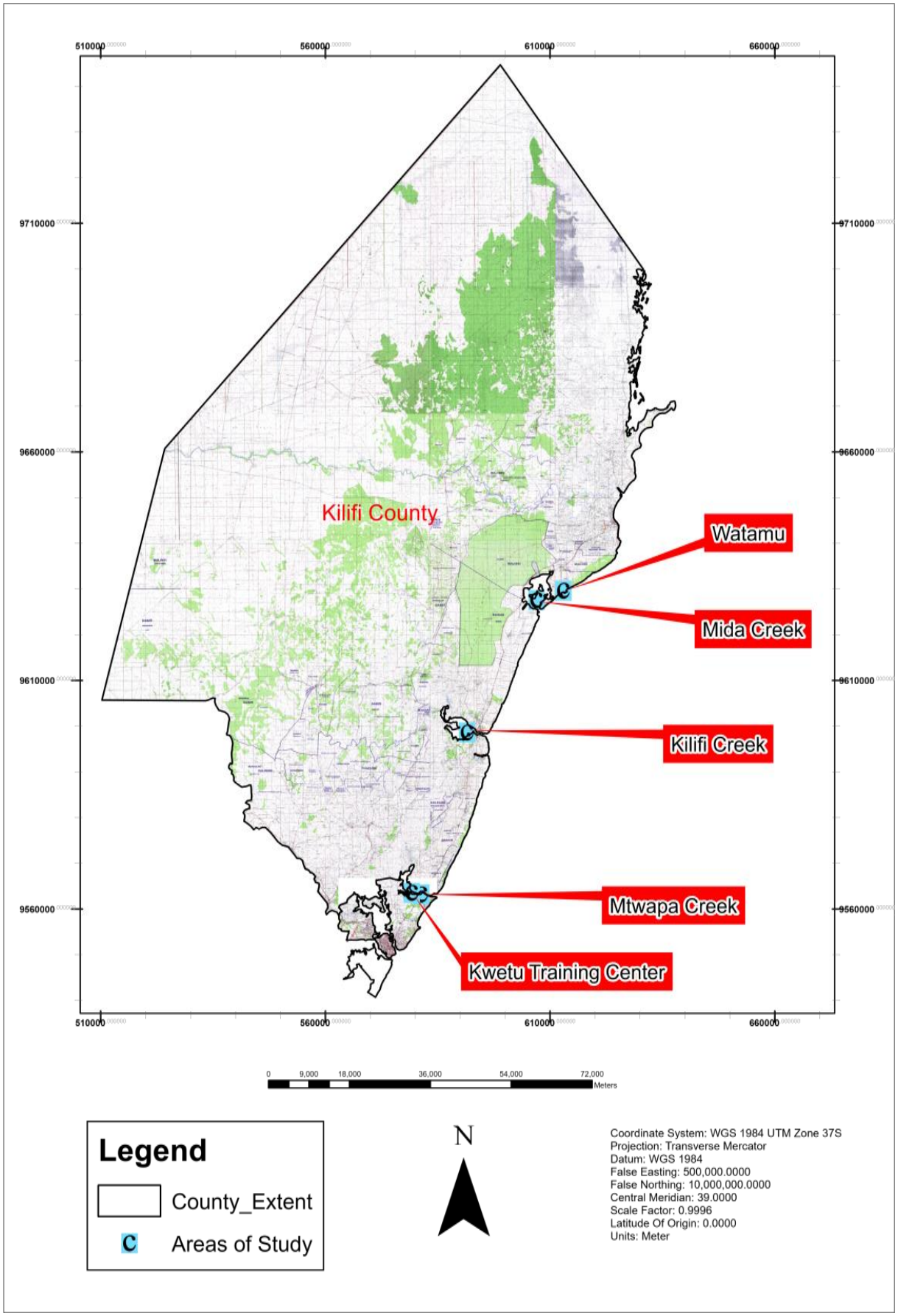

Figure 3.1: Sampling areas

A visit to the agricultural department in Kilifi County, revealed that beekeepers were organized into self-help groups through which they supply honey to a community-based training centre, Kwetu training centre, which also houses a honey processing plant. From the training centre it was established the major mangrove apiculture areas in the County were Mida creek, Kilifi creek, Watamu and Mtwapa creek and also Kwetu Training Centre which serves as a honey collection Centre for beekeepers from various community-based organizations in Kilifi County.

3.3 Samples and Sampling Procedures

3.3.1 Honey samples

Honey sampling was done from Kwetu training Centre (processed) and from selected beekeepers located in the four regions (Mida creek, Kilifi creek, Watamu and Mtwapa creek) and who are registered with various apiculture self-help groups located within the studied regions. The beekeepers who participated in the study were identified by the self-help groups and also through a questionnaire to identify the ones with well-established honeybee colonies and who supply honey to Kwetu training Centre between the months of November and March. Four mangrove honey beekeepers were selected from each of the sampling regions. Only beekeepers who collect large amounts of honey twice a year and sell to Kwetu Training Centre for processing were included.

Beekeepers who were still in their first and second year of beekeeping were omitted. This is because, their apiaries had new honeybee colonies and were not likely to harvest much honey since a new honeybee colony needs a full season to build up a large enough population to gather surplus honey. Similarly, beekeepers who produce

mangrove honey between April and October for were omitted to ensure the honey analyzed was within the same season of harvesting.

A total of four hives from each of the four beekeepers situated in the four regions were randomly selected from the mangrove farm apiaries between November 2016 and March 2017. The hives had established honeybee colonies and from every hive a freshly harvested honey sample weighing 250 g was obtained. The honey samples were stored in clean 500 cm^3 well stoppered plastic bottles capacity and transported to the laboratory within four days. At the laboratory, honey was separated from other debris according to a literature method (Belay *et al.,* 2017). The samples were kept in the laboratory at ambient temperature away from direct sunlight awaiting analysis.

3.3.2 Flower samples

Fresh mangrove flowers with nectar were collected from five regions of Kilifi County; namely, Mida creek, Kilifi creek, Watamu, Mtwapa creek and Kwetu Training Centre. Five flowering trees were randomly selected in each sampling regions for flower sampling. The mangrove trees flower inflorescences were covered with mosquito nets were used to cover for twenty-four hours before collection to keep off nectarivores. Four samples (well-developed flowers from the base of the shoots) were harvested at 8. 00 am the following day from each of the five different trees located in each of the five studied regions. Prior to extraction, flower samples were separated are subjected to freeze-drying, followed by grinding the fresh samples were placed into sampling bottles with a 50 ml-vial, refrigerated and ferried to the laboratory for VOC collection and analysis.

3.4 Reagents and equipment

Acetate buffer (0.1 M, pH 5.2), hydrochloric acid, glacial acetic acid, disodium hydrogen phosphate, sodium acetate trihydrate, Sodium hydroxide (0.5 M), tris-(hydroxymethyl) aminomethane, potassium hydrogen phosphate (KH_2PO_4), p-nitrophenyl-α-D-glucopyranoside, Fructose (Sigma Aldrich, Lot 092K0137, EC No: 200-333-3), pNPG (M Cat: 487510, Switzerland), Sucrose (Sigma Aldrich, S-8501 Lot 84H0557), D-(+)-Glucose (Sigma Aldrich, EC No: 20110425), (-), HPLC grade standards; water, acetonitrile and methanol (J.T. Baker, Avantor), formic acid 98 - 100% pure, ninhydrin, ethylene glycol monomethylether and 2-propanol, sodium chloride, iodine and soluble starch were all of analytical grade and were obtained from Sigma Aldrich and used as purchased.

Ultraviolet-visible spectra were recorded on a Jenway, 6715 UV/Vis spectrophotometer, refractive indeces were recorded on an Atago Refractometer, pH was recorded on a GS, TUV, Berlin digital pH-meter while electrical conductivities were recorded on a 3540, Jenway conductivity meter. VOCs were extracted using a Branso Ultrasonics 5510E-DTH, U.S.A. Sonicater, Whatman nylon 0.45 μm membrane filter, 7404-004, G3620153, U.K. membrane filter, HSW Henk Sass Wolf GmbH syringes and Agilent, 8010-0014, U.S.A. vials. VOCs were further identified by an Agilent 6890 GC-MS coupled to an Agilent 5975 quadrupole mass detector.

Hexamethylfurfural (HMF) and sugars were analysed on an Agilent Technologies HPLC equipped with a binary pump, an auto sampler, and a refractive index detector thermo-stated at 30 ^{o}C, column (ZORBAX Eclipse Plus, Rapid Resolution HD C18, 4.6x100 mm 3.5 Micron), 5 μm LC-NH_2 column (Supelco, Bellefonte, PA, USA) of

250 mm × 4.6 mm and sensitive balance. In analysis of HMF, an isocratic mobile phase of methanol mixed with water in the ratio (85:25 v/v) was used and separation was done at a 1.0 mL/min rate flow. Whereas in analysis of specific sugars, acetonitrile and water (75:25 v/v) were used as the gradient mobile phase and separation was done at flow rate 1 mL min^{-1} with a sample volume of 10 µml.

Samples were incubated in water at a constant temperature of 40ºC in a GFL Labortechnikmbh D3006 Burgwedel thermostated water bath. Small amounts of the sample were placed in vials, vortexed and further placed in cuvettes (1 cm) ready for spectroscopic analysis (Plastibrand, Cartilage. Number. 8009 20).

3.5 Ultra-Sound Extraction (USE) of volatiles in honey samples

Honey (5 g) was added to 5 ml distilled water in a 50 ml vial and comprehensively vortexed. The mixture was transferred into a tube followed by 5 ml of DCM and then the mixture was vortexed again at 1500 rpm for 3 min. USE was performed using indirect sonication mode in an ultrasound cleaning bath. Sonication was done at temperature of 25 ºC and 35 kHz frequency for 30 minutes. The top organic layer was removed through centrifugation at 2500 rpm for 5 min after sonication and filtration. To dry the residual 0.4 g of magnesium sulfate was added. The analyte solution was then transferred into a 2 ml auto sampler vial for GC-MS injection.

3.6 Head Space-Solid Phase Micro-Extraction (HS-SPME) of flower volatiles.

Fresh inflorescences material were blended in an electric blender with stainless steel blades (Rigchina group company, Yongkang, China). A sample of the blended material (0.15 g) was placed in a 5 ml vial. The vial was sealed with a

polytetrafluoroethylene/silicone septum (and a black open-top polypropylene cap (Agilent Technologies, Santa Clara, CA). A pretreated polyacrylate (PA) fiber was inserted to the headspace of the vial which was then maintained at a temperature of 40 °C for 50 minutes. The temperature of 40 °C was used to ensure all the volatile organic compounds in the flower samples were extracted. The PA fiber was removed and placed in the injection port of the gas chromatography for 5 minutes to desorb the volatiles. The equilibrium time profile (solid matrix) was studied by the modified Da Porto and Decorti (2008) method.

3.7 Gas chromatography–mass spectrometry

Volatile organic compounds were identified using a mass spectrometer (Agilent 5973), fitted with an Agilent silica column (30 m x 250 μm) made of (5%-phenyl)-methylpolysiloxane. The GC–MS was programmed as follows: helium (99.99%) as the carrier gas, spilt ratio 50:1 and flow rate 1.5 ml/min. The temperature of the oven was initially set at 40 °C for 3 minutes before increasing it to 250 °C at 6 °C per minute, thereafter maintained constant for 5 minutes. Temperatures of 230 °C, 250 °C and 280 °C were set for the source, injection port and transfer line respectively. According to Adams (Essential Oil Components by Quadrupole GC/MS) the mass scan was set at a range of 41 to 415m/z. MSD ChemStation (E02.00.493) was used to acquire and process data (Agilent Technologies, Mulgrave, Australia).

3.8 Identification of the volatile organic compounds

Volatile organic compounds (VOCs) were identified by use of the reference library in the GC–MS (Adams, 2007, Wiley 7th, and NIST 2.0) based on a similarity match

(80%) cut off value. Electronic integration was used to determine the relative abundance of compounds and a mean of three replicates was obtained.

3.9 Retention indices

Retention times of n-alkane mixture series obtained under standard conditions was used to determine retention indices of Kovats standard (Adams, 2007).

3.10 Preparation of working solutions and standards (IHC, 2002)

Distilled water (40ml) was added to iodine (11.0 g) and potassium Iodide (22.0 g) and the solution comprehensively vortexed. The solution was further diluted to make 500 ml iodine stock solution and kept in a stoppered dark bottle. Dilute iodine solution was prepared by adding iodine stock solution (2 ml) to potassium iodine (20 g) and toped up with distilled water to 500 ml. Ethylene glycol monomethylether (50 ml) was added to ninhydrin (0.04 g) to make ninhydrin stock solution. A dilute solution of ninhydrin was prepared by adding 25 ml of 50 % propan-2-ol in water to 1 ml ninhydrin stock solution.

Standard sugar solutions were separately prepared by dissolving 2.0000 g fructose, 1.5000 g glucose, and 0.2500 g sucrose in 40 ml distilled water and quantitatively transferred to 25 ml methanol in a 100 ml conical flask and the solution diluted to the mark. Filtration of the solution into vials was done using a syringe fitted with 0.45 μm pore sized filter. The solutions were refrigerated at 4 °C to maintain stability for a longer time waiting for analysis.

3.11 Preparation of Stock Solutions

3.11.1 Preparation of buffer solution

Potassium hydrogen phosphate (11.66 g) mixed with disodium hydrogen phosphate (2.56 g) was dissolved in 1 L volumetric flask and the solution diluted to the mark using distilled water. Acetate buffer solution (pH 5.3) was prepared by dissolving sodium acetate (43.5 g) in water, standardized using glacial acetic (5 ml) to a pH of 5.3 and diluted to 250 ml. Reaction –terminating solution was prepared by dissolving tris- (hydroxymethyl) amino methane (363.42 g) in water and diluting to 1 L.

3.11.2 Substrate Solution

This was prepared by dissolving 6.0252 g of p-nitrophenly –α-D- glucopyranoside in buffer solution and making the solution up to 1 L in a volumetric flask.

3.12 Experimental procedures

Physiochemical parameters including: free acidity, pH, electrical conductivity, moisture content, sugars, diastase and invertase, proline, HMF, colour and VOCs were determined following the International Honey Commission procedures as described in sections.

3.12.1 Determination of the conductivity meter cell constant

A solution of KCl (0.1 M) in a beaker was allowed to equilibrate to 20 °C in a thermostatic water bath a (GFL Labortechnikmbh D3006 Burgwedel). A digital conductivity meter (3540, Jenway) was used to determine the electrical conductance of the solution. The value obtained was used to determine the cell constant for calculation of the electrical conductivity of honey samples.

3.12.2 Determination of Moisture Content

The moisture content of honey samples was measured on a refractometer (Atago PAL-1-model, Tokyo, Japan). The refractometer had a temperature compensation of 20^{o} C if the honey sample temperature was different from 20 ^{o}C. Honey (5.0 g) in tightly closed vials was homogenized and heated in a water bath at 50 °C to dissolve any sugar crystals present. The honey solution was cooled to room temperature before analysis. Three drops of the honey solution was placed on a clean and dry refractometer prism at 20 ^{o}C, spread evenly and refractive index determined after two minutes. A reference of 1.3330 obtained from distilled water was used to recheck the refractometer for any drift. All measurements were done in triplicate. Using the refractive index determined, moisture content of honey was read from Wedmore Table of water content based on the AOAC Table 969.38, (AOAC, 1990).

3.12.3 Determination of pH

A pH meter (3540, Jenway) was calibrated using buffer solutions with pH of 4.0, 7.0 and 9.0 obtained commercially and used to determine pH of the honey samples. A honey sample (10 g) was dissolved in carbon (IV) oxide-free distilled water (75 ml) and the solution vigorously stirred with a magnetic stirrer before pH electrodes were inserted to determine the pH at a temperature of 20 ^{o}C. Three readings were taken and averaged (AOAC, 1990).

3.12.4 Determination of Free Acidity

A honey sample (10 g) was dissolved in 25 ml carbon (IV) oxide-free distilled water. The solution was stirred using a magnetic stirrer before titration against NaOH (0.1 M) to a phenolphthalein indicator end point (pH of 8.3). A constant pH reading was

recorded in a duration of two minutes of starting the titration. These measurements were taken in triplicates.

3.12.5 Determination of Electrical Conductivity

The electrical conductivity of the honey samples was measured using digital conductivity meter (3540, Jenway). Honey (20 g) was dissolved in 120 ml distilled water, after which a 40 ml aliquot was pipetted into three different conical flasks and heated in a water bath at a temperature of 20 ^{o}C. The solution was allowed to equilibrate and its conductance measured. Three readings were taken for each aliquot to get an average value. Calculations of electrical conductance were given in the nearest 0.01 mS/ cm (Bogdanov, 2009d).

$$SH = K \times G \quad [3.1]$$

$$EC = K \times G \quad [3.2]$$

where,

G = conductance (mS).

K = cell constant (cm^{-1})

SH = honey solution electrical conductivity (mS/cm)

EC = Electrical conductivity

3.12.6 Determination of Diastase Activity

Diastase activity of the honey samples was determined according to a literature method (Schade *et al.,* 1958). Water quantity to be added to the mixture reacting was determined through calibrating of starch solution. This was done in order to ensure the solution of iodine and starch had an absorbance range of 0.745 to 0.770. Dilute iodine solution (5 ml) was pipetted into 6 test tubes containing 20, 21, 22, 23, 24 and 25 ml

of water, respectively. Water (10 ml) and starch solution (5 ml) mixture was added to every test tube. The solution was agitated before determination of its absorbance at a wavelength of 660 nm. The procedure was repeated for all the test tubes to obtain an absorbance range of 0.745 to 0.770. The amount of water required to dilute the starch solution determined in this manner was used for all determinations of diastase activity by UV/V is spectrophotometry at a wavelength of 660 nm. In a typical determination, 10 g of honey was dissolved in 15 ml distilled water and acetate buffer (5 ml). Sodium chloride solution (3 ml) was added to the honey solution in a volumetric flask (50 ml) and the analyte solution made up to the mark by addition of distilled water. The analyte and starch solutions (10 ml) in a boiling tube were placed in a water bath at temperature of 40 ^{o}C and allowed to equilibrate. Starch solution (5 ml) was pipetted into the analyte solution after 15 minutes. A 0.5 ml aliquot was pipetted into the dilute iodine solution (5 ml) and absorbance determined at a wavelength of 660 nm against distilled water blank in 4 cm cuvettes.

Graphs of the absorbance against time were plotted, and from the linear region of the slope, a regression equation was obtained. From this reaction, the reaction time (t_x) was determined in minutes at an absorbance 0.235. Diastase number (DN) was used to express diastase activity.

$$DN = 300/t_x.$$

3.12.7 Determination of Invertase Activity

Invertase was determined spectrophotometrically on the Jenway 6850 ultra-violet - visible spectrophotometer. Honey (5 g) was transferred to a buffer solution in a 25 ml volumetric flask, mixed well and refrigerated for one day. A 5 ml aliquot of the

solution was placed in two separate test tubes (sample and reference) and heated for 5 minutes at a temperature of 40 ^{o}C. To the sample and reference test tubes, 0.5 ml of the analyte and reaction-terminating solution were added, respectively. The two solutions were allowed to equilibrate for 15 minutes to room temperature before determining the reference and sample absorbance at 400 nm. Graphs of the absorbance against time were plotted, and from the linear region of the slope, a regression equation was obtained with a gradient of 21.64. Invertase in U/kg = 21.64 x A_{400}

where,

A_{400} = change in absorbance at 400 nm

3.12.8 Determination of proline

The determination of proline content in the honey samples was done according to a literature method (AOAC, 2000; Meda *et al.*, 2005). Honey (10 g) was dissolved in 100 ml distilled water in a beaker. A solution of ninhydrin (10 ml) to the solution in the beaker followed by formic acid. The mixture was heated to a temperature of 40 ^{o}C before addition of isopropanol. The absorbance of the solution was measured with distilled water as the blank at a wavelength of 520 nm on a Jenway 6850 UV- visible spectrophotometer. The proline content was read off the calibration curve.

3.12.9 Determination of HMF in honey samples

The amount of HMF in the honey samples was determined by HPLC system (Agilent Technologies). Honey samples (10 g each) were dissolved in distilled water (50 ml) and filtered through a filter membrane (0.45μm) before injecting 10 μL into HPLC system equipped with the Agilent ZORBAX Eclipse Plus column with Rapid

Resolution HD C18, 4.6x100 mm 3.5 Micron. HPLC grade Water (85%) and methanol (15%) at a flow rate of 1.0 mL/min were used as an isocratic mobile phase. Standard HMF solutions (Sigma-Aldrich) were used to determine the concentrations of HMF in the honey samples at a detection wavelength of 285 nm. The amount of HMF was expressed in mg/kg.

3.12.10 Determination of Specific sugars

Specific sugars in honey samples were determined by HPLC system (Agilent Technologies, USA) equipped with an auto sampler, binary pump, and an LC-NH_2 column with dimensions of 250 mm × 4.6 mm (Supelco, Bellefonte, PA, USA) for the separation of the sugars. Water (25%) and acetonitrile (75%) at a flow rate of 1 1ml/ min were used as the chromatographic mobile phase. The amount of sugar in the samples was quantified using calibration curves obtained from standard solutions and calculations were given in g/100 g.

3.12.11 Determination of colour

A Samples of honey (10 g) was dissolve in 10 ml distilled water and heated to a temperature of 40 ^{o}C for sugar crystals to dissolve. The solution was homogenized for five minutes and centrifuged at a speed of 3200 rpm. UV- visible spectrophotometer (Jenway 6850) was used to determine the absorbance at a wavelength of 635 nm. The absorbance obtained was used to calculate the Pfund value using the equation below;

$$\text{mmPfund} = -38.70 + 371.39 \times \text{Abs}$$

where,

mm Pfund = honey colour intensity in the Pfund scale

Abs = Honey solution absorbance.

Pfund scale was used to classify the honey color intensity (González-Miret et al., 2005)

3.12.12 Data Analysis

All analysis readings were taken in triplicates, averaged and reported as mean ± standard deviation. Statistical Analysis System (SAS, 2002) was used to subject the data to analysis of variance (ANOVA). Duncan Multiple Range Test (DMRT) was used to determine if the mean were significantly different. Associations between honey quality parameters were investigated using Pearson correlation analysis.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Physicochemical Parameters mangrove honey

The physicochemical characteristics measured include moisture content, pH, free acidity, electrical conductivity and proline content. Mean results of physicochemical parameters determined are summarized in Table 4.1.

Table 4.1: Moisture content, pH, acidity, electrical conductivity, proline value (mean ± sd) of mangrove honey

Region	Moisture (%)	pH	Free acidity (meq/kg)	Electrical conductivity (mS/cm)	Proline (mg/kg)
Mida creek (unprocessed) (n=4)	19.8± 0.00	4.04± 0.05	26.19± 0.27	0.43± 0.00	619.25± 16.92
	19.50±0.01	4.10± 0.03	26.12± 0.60	0.41± 0.00	613.09± 12.04
	19.40± 0.10	4.21± 0.01	26.33± 0.50	0.42± 0.10	606.54± 3.94
	19.33± 0.06	4.03± 0.00	26.03± 0.82	0.44± 0.01	613.37± 2.88
Kilifi creek (unprocessed) (n=4)	22.23± 0.06	4.06± 0.04	30.21± 0.15	0.32± 0.01	616.16± 4.55
	23.17± 0.10	4.01± 0.00	30.18± 0.64	0.35± 0.00	609.19± 3.29
	23.40± 0.00	4.07± 0.01	30.13± 0.53	0.32± 0.00	617.08± 1.47
	22.27± 0.06	4.00± 0.00	30.11± 0.62	0.34± 0.01	604.24± 2.68
Watamu (unprocessed) (n=4)	21.37± 0.06	4.18± 0.01	25.18± 0.61	0.44± 0.00	606.37± 3.56
	21.20± 0.10	4.05± 0.02	25.21± 0.60	0.42± 0.01	630.95± 2.29
	21.10± 0.10	4.13± 0.03	25.13± 0.99	0.41 ± 0.00	616.59± 1.89
	21.27± 0.06	4.16± 0.03	25.17± 0.60	0.43± 0.00	632.28± 2.88
Mtwapa creek (unprocessed) (n=4)	22.40± 0.00	4.05± 0.04	27.11± 0.66	0.40± 0.00	661.43± 2.07
	22.47 ± 0.58	4.17± 0.02	27.21± 0.66	0.46± 0.01	604.82± 3.41
	22.21± 0.06	4.34± 0.01	27.16± 0.43	0.44± 0.01	617.77± 3.33
	22.23± 0.06	4.42± 0.02	27.23± 0.85	0.41± 0.00	611.27± 1.92
Grand mean (Unprocessed) (n=16)	**21.45± 0.09**	**4.13± 0.03**	**27.17± 0.60**	**1.40± 0.04**	**617.53± 4.32**
Kwetu training Centre(processed) (n=4)	20.20± 0.10	4.33± 0.03	22.34± 0.96	0.52± 0.01	631.31± 2.76
	20.19± 0.06	4.37± 0.01	22.23± 1.10	0.53± 0.00	621.37± 4.65
	20.17 ± 0.15	4.45± 0.05	22.20± 0.00	0.51± 0.00	734.36 ± 4.23
	20.17 ± 0.06	4.30 ±0.05	22.29 ± 0.05	0.54± 0.00	611.28 ± 3.82
Grand mean (processed) (n=4)	**20.18± 0.09**	**4.36± 0.04**	**22.27± 0.53**	**0.53± 0.01**	**645.08± 3.87**

4.1.1 Moisture content

The moisture content values of mangrove honey are presented in Table 4.1. The highest moisture content (23.40 ± 0.00%) and was observed in Kilifi creek and the lowest moisture content (19.33 ± 0.06%) in Mida creek. 23.40% moisture content

value was above the permitted limits set by Codex Alimentarius Commission (Alimentarius, 2001), EU and the KEBS based on the EAS. The findings were in accord with Adenekan *et al.,* (2012), who reported about Nigerian mangrove honey from the Agro-Ecological mangrove zones had moisture content in the range of 12.32 to 34.02%. The high moisture content can be attributed to the humid conditions in the region and therefore, nectaries foraged there have higher level of water content, resulting in high moisture content in the honey. Mangrove honey moisture content for the five regions lies within this range and compares well with the high moisture content (19.8 to 29.0%) of Chinese honeys (Junzheng and Changying, 1998).

It is clear from figure 4.1 that there was no significant difference ($p < 0.05$) in moisture content observed between unprocessed and processed mangrove honey.

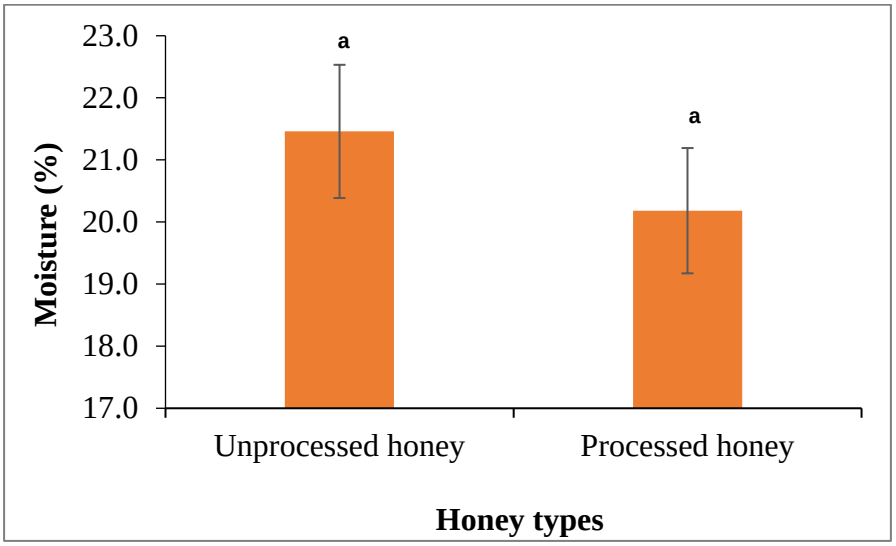

Figure 4.1: Comparison of mean of moisture content (%) in processed and unprocessed honeys in Kilifi County

4.1.2 pH

It can be seen from Table 4 that the pH values for mangrove honey samples ranged from 4.45± 0.05 (Kwetu Training Centre) to 4.00± 0.00 (Kilifi creek). These are within the accepted limits set by Codex Alimentarius, EU and KEBS. The findings of this study indicated that mangrove honey was acidic. The acidic nature of honey has also been reported by different authors (Belay *et al.*, 2013; Scandurra *et al.*, 2013). Similarly Bangladeshian mangrove honeys have been reported to have pH value in the range of 3.9 to 4.4 (Islam *et al.*, 2017), Brazilian honey (da C Azeredo *et al.*, 2003) and Indian honey (Saxena *et al.*, 2010).

Figure 4.2 shows that the pH values of unprocessed and processed honey were significantly different ($p < 0.05$). The pH values alone are not enough for the discrimination of honey from different geographical regions. The organic acids such as oxalic acid, lactic acid gluconic acid and glucono lactone present in honey and their variation have a direct effect on honey pH values (Nanda *et al.*, 2003).

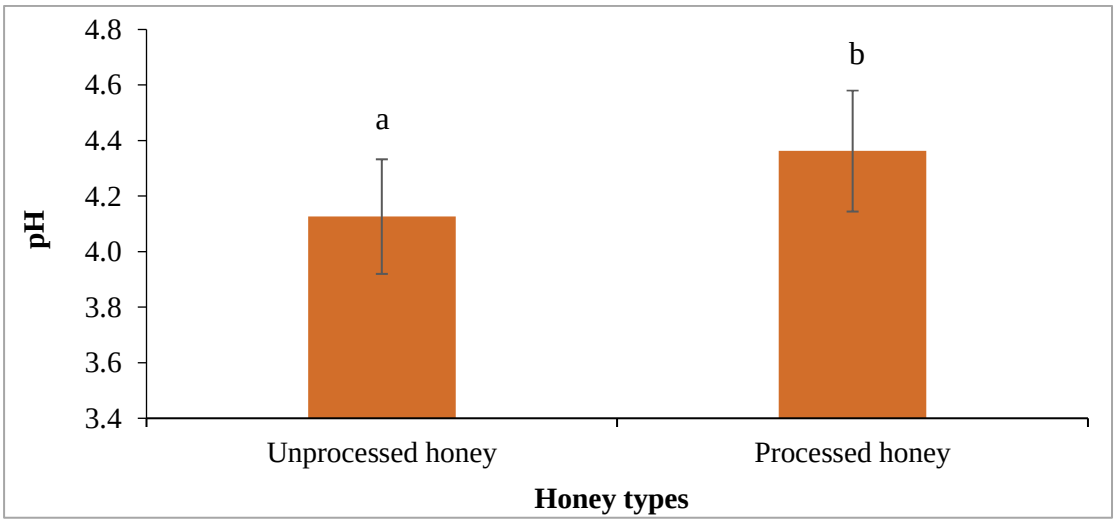

Figure 4.2: Comparison of mean pH values of processed and unprocessed honeys from Kilifi County

4.1.3 Free acidity

The free acidity values for mangrove honey are presented in Table 4.1. The highest free acidity of 30.21 ± 0.15 meq/kg was recorded for Kilifi region honey while the lowest of 22.20 ± 0.00 meq/kg was recorded for Kwetu Training Centre region honey. The free acidity of mangrove honey was within the permitted limit of 40 meq/kg set by the EU (Bogdanov *et al.*, 2002), KEBS based on the EAS and 50 meq/kg set by Codex Alimentarius Commission (Alimentarius, 2001). The findings are in accord to those reported by Islam *et al.*, (2017) for mangrove honey from sundarbans mangrove forest of Bangladesh.

The Figure 4.3 shows that the free acidity values of unprocessed and processed honey were significantly different ($p < 0.05$). This is attributed to the fact that different regional plants have varying amounts of organic acids in their nectaries which include gluconic acid. Hence blending of the collected mangrove honeys can lead to the variation in honey free acidity.

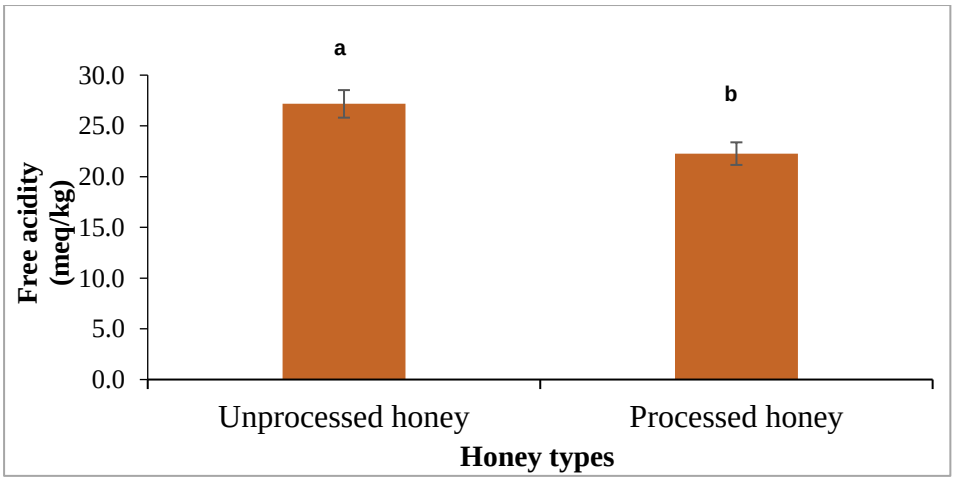

Figure 4.3: Comparison of Mean free acidity levels of processed and unprocessed honeys from Kilifi County

4.1.4 Electrical conductivity (EC)

The results of the electrical conductivity of mangrove honey are presented in Table 4.1. The highest electrical conductivity value (0.54 ± 0.00 mS/cm) was observed in Kwetu Training Centre and the lowest value (0. 32 ± 0.00 mS/cm) in Kilifi creek. All the analysed mangrove honey samples showed electrical conductivity values within the permitted limits of not more than 0.8 mS/cm for floral honeys as set by Codex Alimentarius Commission (2001), EU and KEBS based on the EAS (2018).

According to the EU, (Bogdanov *et al.,* 2002) and Codex Alimentarius (Alimentarius, 2001), honeydew honeys have more than 0.8 mS/cm. Thus, the electrical conductivity values obtained of less than 0.8 mS/cm reveals that mangrove honey is floral honey. The electrical conductivity values were similar to those previously reported for Bangladeshi sundarbans mangrove forest honeys (Rabiul *et al.,* 2017). The electrical conductivity data values were less than the permitted 0.8 mS/cm set by Codex Alimentarius (2001).

The Figure 4.4 shows that the electrical conductivity of unprocessed honey was significantly different ($p < 0.05$) from processed honey. The high acidity in processed honey may be associated with increase in temperature during heating which leads to an increase in ionazability of ionic compounds present honey.

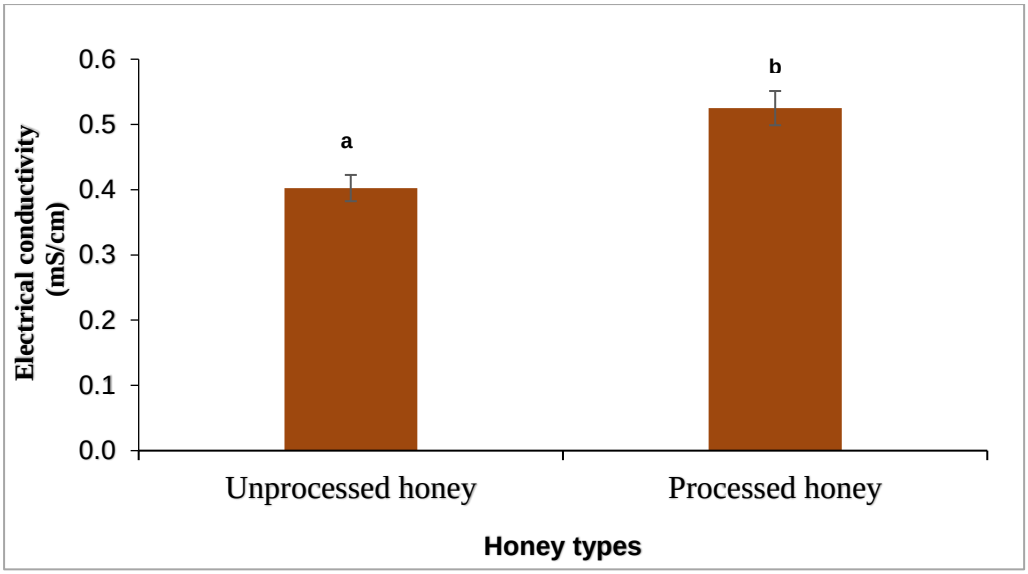

Figure 4.4: Comparison of Mean Electrical conductivity of processed and unprocessed honeys from Kilifi County

4.1.5 Proline content

From the Table 4.1 the highest proline content (734.36± 4.23 mg/kg) was observed in Kwetu Training Centre and the lowest proline content (604.24± 2.68 mS/cm) in Kilifi creek. Proline content is used as indicator for honey ripeness. A minimum limit of 180 mg proline/kg honey has been accepted by Codex Alimentarius Commission (2001), EU and KEBS based on the EAS. Based on this criterion, all the mangrove honey samples had a value above the minimum acceptable limit. A number of researchers Iglesias *et al.* (2004) and Truzzi *et al.* (2014) also indicated that proline is the predominant amino acid fraction in honey. This is in line with the results of mangrove honey. Proline content varies according to the honey floral origin (Cotte *et al.*, 2004). Mangrove honey proline content was higher than that of honeys from other Kenyan regions (Bichanga 2010), Algeria (Ouchemoukh *et al.*, 2007), India (Saxena *et al.*, 2010), and Cuba (Alvarez-Suarez *et al.*, 2010).

As can be noted from the Figure 4.5, the mean of proline values of processed and unprocessed honey was significantly different at $p < 0.05$. This variation depends on the color of honey. Usually, light colored honey shows lower amino acid level than dark honey (Dong *et al.*, 2013).

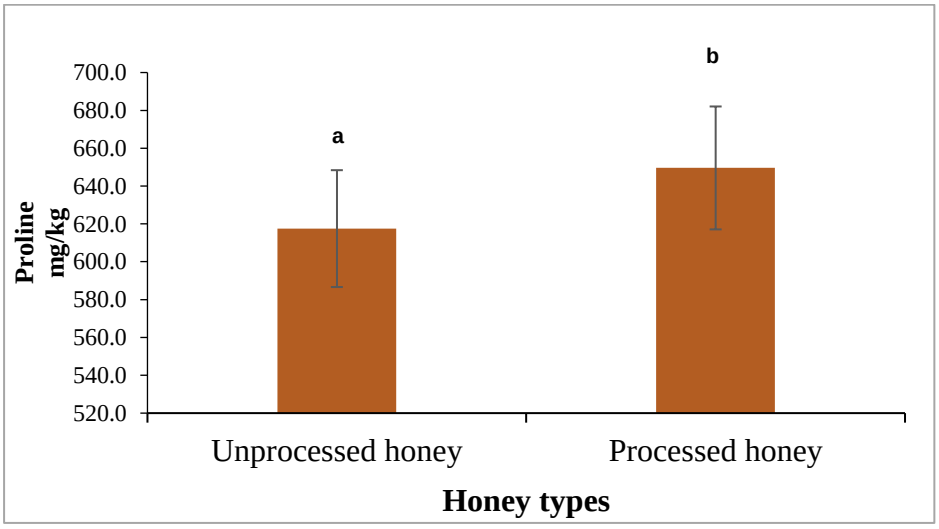

Figure 4. 5: Comparison of mean proline of processed and unprocessed honeys in Kilifi County.

4.1.6 Colour

The color intensities of mangrove honey samples analysed and classified according to the United States Department of Agriculture (USDA), approved colour standards 1985 are summarized in Table 4.2 and figure 4.6. In the studied mangrove samples, the color ranged from extra light for mida creek honey amber to dark amber for Kwetu Training Centre. The highest Pfund value (117.28 mmPfund) for honey from Kwetu Training Centre and lowest Pfund value was recorded in Mida creek honey (46.35). Changes in colour may be attributed to beekeeper's interventions and different ways of handling the combs such as use of old wax combs for producing honey and exposure to heat during processing (Moniruzzaman *et al.*, 2013). Honey samples obtained from Kwetu Training Center had the highest colour intensity suggesting that it probably

possessed high antioxidant properties since is a blend obtained from various regions. According to Moniruzzaman *et al.*, (2013), honey color intensity is a good parameter that shows availability of antioxidant activities like flavonoids and carotenoids.

Table 4.2: Colour of Mangrove

Region	Pfund Scale, millimeters	Color name
Kwetu Training Centre	117.28	Dark amber
Mtwapa creek	111.71	Amber
Kilifi creek	105.40	Amber
Watamu	50.80	Light amber
Mida creek	46.35	Extra light amber

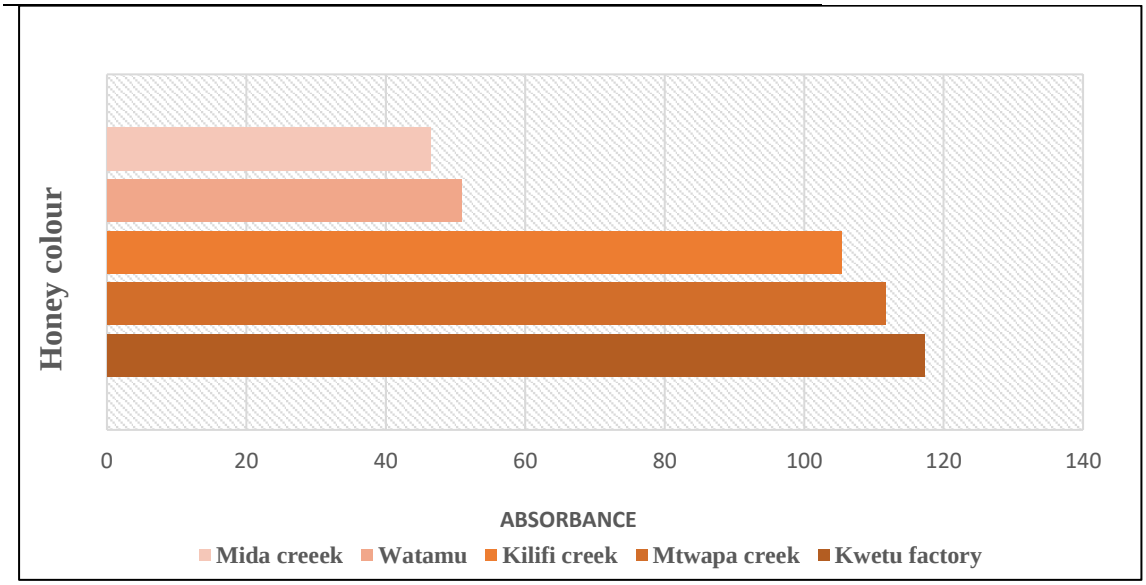

Figure 4.6: Comparison of colour of mangrove honeys from Kilifi County

4.1.7 Enzymes

The Table 4.3 represents the mean values of diastase and invertase enzyme in mangrove honey samples.

Table 4.3: Diastase, invertase and HMF mean ± sd values of mangrove honey (n =20)

Region	HMF mg/kg	Diastase Schade Units	Invertase U/kg
Mida creek (unprocessed) (n=4)	2.54± 0.05	8.11± 0.64	7.24 ± 0.20
	2.84± 0.05	8.54± 0.22	7.19 ± 0.01
	2.64± 0.02	8.17± 0.09	7.76± 0.79
	2.65± 0.09	8.59± 0.30	7.74± 0.09
Kilifi creek (unprocessed) (n=4)	2.65± 0.20	11.46± 0.22	6.86± 0.12
	2.71± 0.15	11.42± 0.21	6.74± 0.10
	2.85± 0.09	11.34± 0.01	6.77± 0.12
	2.72± 0.16	11.31± 0.14	6.77± 0.08
Watamu (unprocessed) (n=4)	0	9.13± 0.25	9.55± 0.10
	0	9.18± 0.13	7.12± 0.06
	0	9.39± 0.15	7.65± 0.10
	0	9.83± 0.63	7.28± 0.16
Mtwapa creek (unprocessed) (n=4)	2.74± 0.10	10.16± 0.14	5.61± 0.07
	2.77± 0.11	10.63± 0.17	5.54± 0.11
	2.61±0.01	10.31± 0.33	6.84±0.15
	2.55±0.02	10.05 ± 0.04	6.77± 0.08
Grand mean	**2.02± 0.16**	**9.85± 0.34**	**7.09± 0.15**
Kwetu Training Center (processed) (n=4)	2.81± 0.16	11.48± 0.06	6.67± 0.26
	2.86± 0.14	11.30± 0.15	6.68± 0.15
	2.80± 0.14	11.47 ± 0.05	6.41± 0.81
	2.69± 0.17	11.34± 0.11	6.76± 0.09
Grand mean	**2.79± 0.15**	**11.40± 0.10**	**6.63± 0.33**

4.1.7.1 Diastase

Results of diastase activity in the honey analyzed from different regions in Kilifi are tabulated in Table 4.3. The diastase activity was in a range of 8.11 to 11.48 DN. Mangrove honey from Mida creek had the lowest while Kwetu Training Center had the highest diastase activity. The permitted mean minimum diastase activity limit for honey is 8 DN as set by Codex Alimentarius Commission (2001), EU and KEBS based on the EAS. All the mean diastase activity values shown in the table for all the regions investigated are above this limit, suggesting that most of the honey was fresh. Natural fresh honey should have diastase activity above8 S/units but for honeys where

the enzyme content is low it should be not less than 3 S/units (Bogdanov *et al.*, 2002). The activity of diastase mangrove honey from Mida creek was found to be low indicating early maturity of the honey. Generally, low diastase activity shown by mangrove honey, may be indicative of the contribution of unifloral honeys in which diastase activity is usually low.

Figure 4.7 shows that the mean diastase activities in processed and unprocessed honey were significantly different at $p < 0.05$ ($p = 0.023$). The lower diastase activity in unprocessed honey can also be indicative of early maturity of the honey in which diastase enzyme had catalyzed all the enzymatic reactions fast leading to its low concentration.

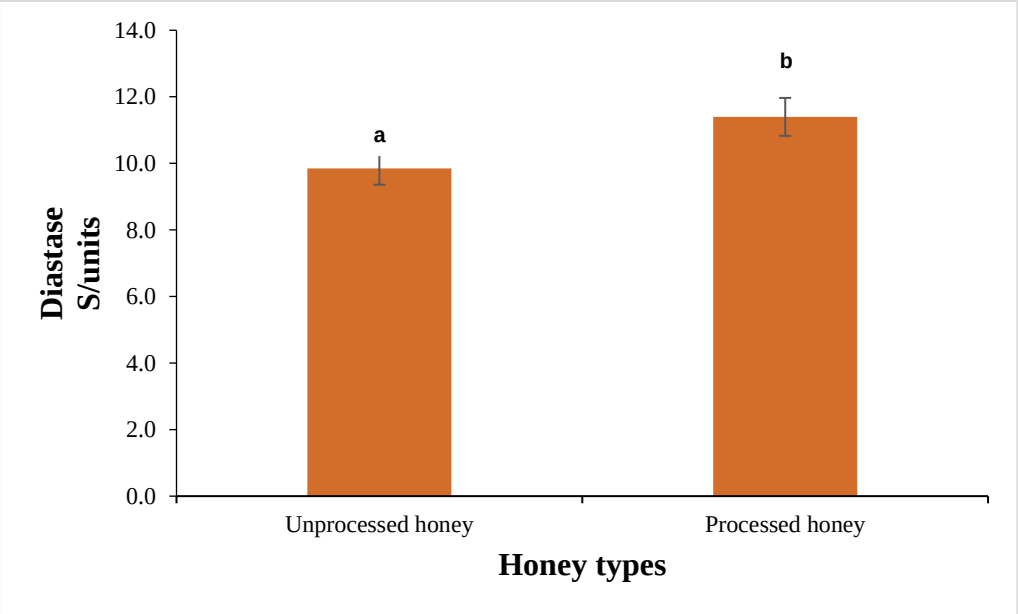

Figure 4.7: Comparison of the Mean diastase activity of in processed and unprocessed honeys from Kilifi County

4.1.7.2 Invertase

The mean invertase activity of mangrove honey samples are tabulated in Table 4.4. The mean invertase (IN) activity was in a range of 5.54 U/kg to 9.55 U/kg. Invertase

activity was highest in mangrove honey from Watamu and lowest in Mangrove honey from Mtwapa creek.

It is clear from figure 4.8 that there was no significant difference between the mean invertase of the processed and that of the unprocessed honey at 95% confidence limit ($p = 0.36$). Processed honey had a relatively lower invertase activity value probably because invertase has higher thermal sensitivity than diastase (White *et al.*, 1964). Invertase activity has no fixed limits and is commonly used in countries like Switzerland, Italy and Germany as an indicator of freshness, due to its sensitivity to heat and storage damage (Bogdanov, 2009).

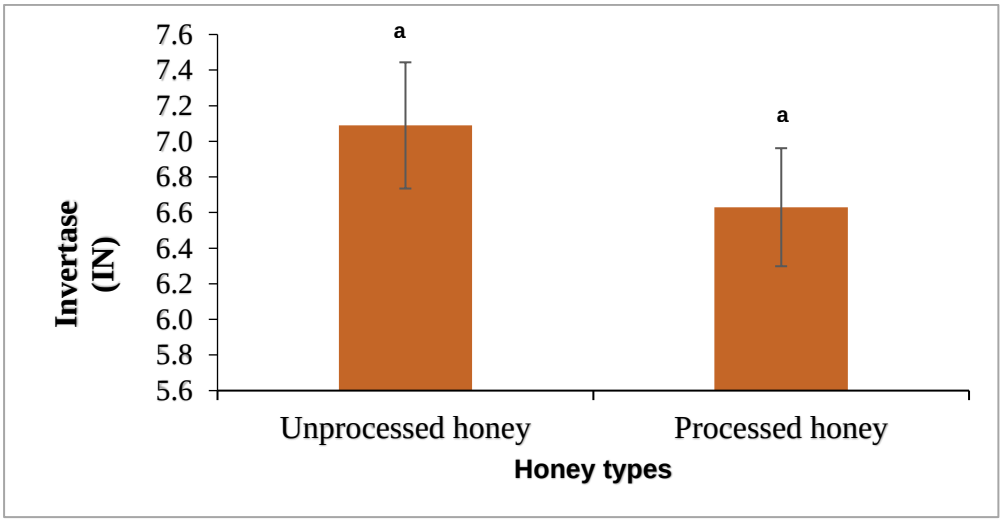

Figure 4. 8: Comparison of mean invertase activities of processed and unprocessed honeys from Kilifi County

4.1.8 Hexamethylfurfural (HMF)

The results obtained from the analysis of mangrove honey samples for HMF are summarized in Table 4.4. The mean HMF content was found to be 1.9135 ± 0.08 mg/kg. This is lower than the set accepted limits of 40 mg/kg, 60 mg/kg, 40 mg/kg and 80 mg/kg by the EU, KEBS based on the EAS and Codex Alimentarius

Commission (2001) respectively. Mangrove honey samples from the four regions showed negligible variations in their HMF values. HMF content was absent in samples from Watamu but was highest in honey samples from Kwetu Training center.

It can be seen from figure 4.9 that there was no significant difference in the mean HMF content in the processed and unprocessed honey at 95% confidence limit ($p = 0.23$). In processed honey the HMF content was high and this can be attributed to heating during processing. HMF is absent in fresh honey but is present in stored honey due to ageing, handling during processing, thermal treatment and floral sources influence their levels (Islam *et al.*, 2017). Artifacts that lower honey quality may be introduced into honey especially due to poor conditions of storage and over-heating during processing (Fallico *et al.*, 2008).

HMF is a good criterion for determination of honey quality. It is an indicator of the extent of honey deterioration due to over-heating and/or storage of the honey (Sancho *et al.*, 1992). It is also a measure of honey adulteration by invert syrups (Ramirez *et al*., 2000).

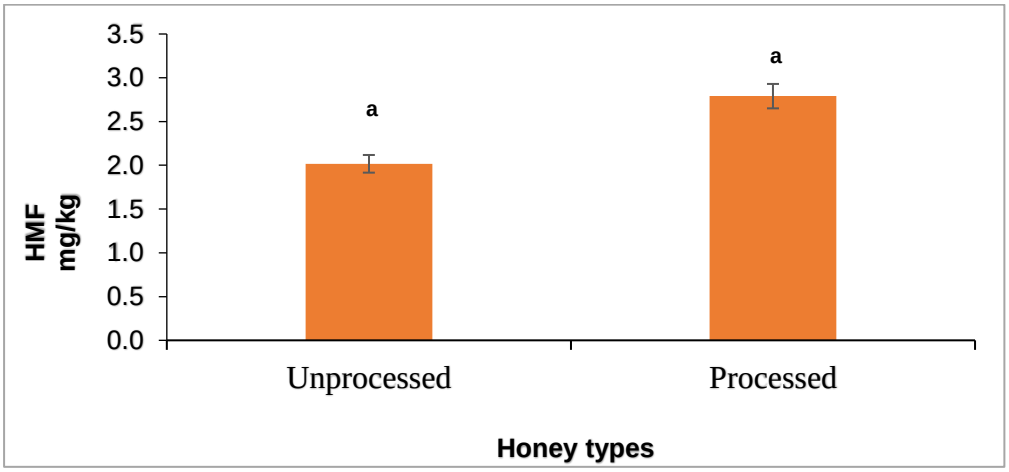

Figure 4.9: Comparison of mean HMF of processed and unprocessed honeys from Kilifi County

4.1.9 Specific sugars

The mean values of glucose, fructose and sucrose content in mangrove honey samples are summarized in Table 4.4. The highest concentration of fructose (37.66± 0.05 g/100 g) was found in Watamu honey and the lowest (34.48± 0.04 g/100 g) in Kwetu Training Center. From figure 4.10 there was no significant difference ($p < 0.05$) observed between processed and unprocessed mangrove honey in fructose content. The highest glucose content was found in Mida creek (30.45± 0.09 g/100 g) and the lowest in Kilifi creek (28.33± 0.01 g/100 g). From figure 4.10 there was no significant difference ($p < 0.05$) observed between processed and unprocessed mangrove honey in glucose content. The of fructose and glucose amount in the analyzed mangrove honey samples was in accordance with the legislations of the Codex Alimentarius Commission (2001), EU and KEBS based on the EAS (which sets a minimum of 60%). Among the monosaccharide sugars, the fructose content was found to be higher than that of glucose. This is in agreement with the findings of other researchers on monofloral honeys (De La Fuente *et al*., 2011; Ouchemoukh *et al*., 2010).

The highest concentration of sucrose (0.19± 0.00 g/100 g) was found in Kwetu Training Center and the lowest (0.08± 0.00 g/100g) in Mida creek. In the figure 4.11 a significant difference ($p < 0.05$) was observed between processed and unprocessed mangrove honey in sucrose content. Sucrose was found to be the 3rd dominant sugar in mangrove honey. In this study, lower level of sucrose was observed compared to the maximum limit (5 g/100 g honey) of Codex Alimentarius (CA), European Union (EU) and East African Standards.

The ratios of fructose to glucose (F/G) and glucose to water (G/W) were in the range of 1.17 to 1.31 and 1.38 to 1.55, respectively. The value of F:G ratio is used as a flavour indicator since fructose is much sweeter than sucrose and glucose (Kamal and Klein, 2011). Hence, mangrove honey is sweet. Both F:G and G:W ratios can be used to assess the likelihood of the honey to crystallizing upon storage, because glucose is less water soluble (de Rodríguez *et al.*, 2004). The results of this study, show that mangrove honey is more likely to crystallize fast.

Table 4. 4: Glucose, Fructose and Sucrose content in different honey samples

Region	Glucose g/100g	Fructose g/100g	Sucrose g/100g	Estimated fructose/ glucose ratio	Estimated glucose/water ratio	Estimated fructose / glucose ratio
Mida creek (unprocessed) (n=4)	29.30± 0.87	35.17±0.44	0.11±0.00	1.20±0.03	1.44±0.03	32.24±0.04
	29.34±0.27	36.01±0.31	0.08±0.00	1.22±0.02	1.43±0.01	32.68±0.05
	30.45±0.09	36.84±1.02	0.13±0.02	1.21±0.04	1.49±0.05	33.65±0.01
	29.35±0.01	34.76±0.14	0.11±0.00	1.18±0.01	1.44±0.00	32.06±0.03
Kilifi creek (unprocessed) (n=4)	28.35±0.01	35.30±0.48	0.11±0.00	1.25±0.02	1.47±0.00	31.83±0.00
	30.05±0.00	35.75±0.12	0.15±0.00	1.19±0.00	1.55±0.00	32.90±0.02
	30.23±0.01	36.07±0.06	0.14±0.00	1.19±0.00	1.53±0.00	33.15±0.00
	28.33±0.01	35.68±0.10	0.12±0.00	1.26±0.00	1.40±0.00	32.01±0.01
Watamu (unprocessed) (n=4)	29.42±0.01	37.13±0.07	0.09±0.00	1.26±0.02	1.45±0.00	33.28±0.02
	28.36±0.01	37.66±0.05	0.14±0.00	1.30±0.04	1.40±0.01	33.01±0.00
	29.53±0.01	35.89±0.07	0.09±0.00	1.22±0.04	1.47±0.01	32.71±0.00
	30.04±0.01	35.24±0.09	0.10±0.00	1.17±0.00	1.48±0.00	32.71±0.00
Mtwapa creek(unprocessed) (n=4)	29.43±0.01	36.08±0.08	0.14±0.00	1.23±0.00	1.44±0.00	32.75±0.01
	28.36±0.01	35.65±0.10	0.16±0.00	1.26±0.00	1.40±0.00	32.00±0.04
	29.43±0.01	34.57±0.04	0.11±0.00	1.18±0.00	1.45±0.00	32.00±0.00
	29.04±0.01	36.08±0.04	0.08±0.00	1.24±0.00	1.44±0.00	32.56±0.02
Grand mean	**29.31± 0.08**	**35.87± 0.02**	**0.47± 0.01**	**1.22± 0.03**	**1.46± 0.02**	**30.56± 0.01**
Kwetu Training Center (processed) (n=4)	30.12±0.00	34.48±0.04	0.12±0.00	1.14±0.00	1.50±0.01	32.30±0.00
	28.35±0.01	37.11±0.04	0.12±0.00	1.31±0.02	1.40±0.00	32.73±0.01
	29.77±0.01	36.17±0.06	0.17±0.00	1.22±0.03	1.38±0.01	32.97±0.02
	28.95±0.01	35.29±0.09	0.19±0.00	1.28±0.04	1.54±0.00	32.12±0.03
Grand mean	**29.30± 0.01**	**35.76± 0.06**	**0.15± 0.00**	**1.24± 0.03**	**1.46± 0.01**	**32.53± 0.02**

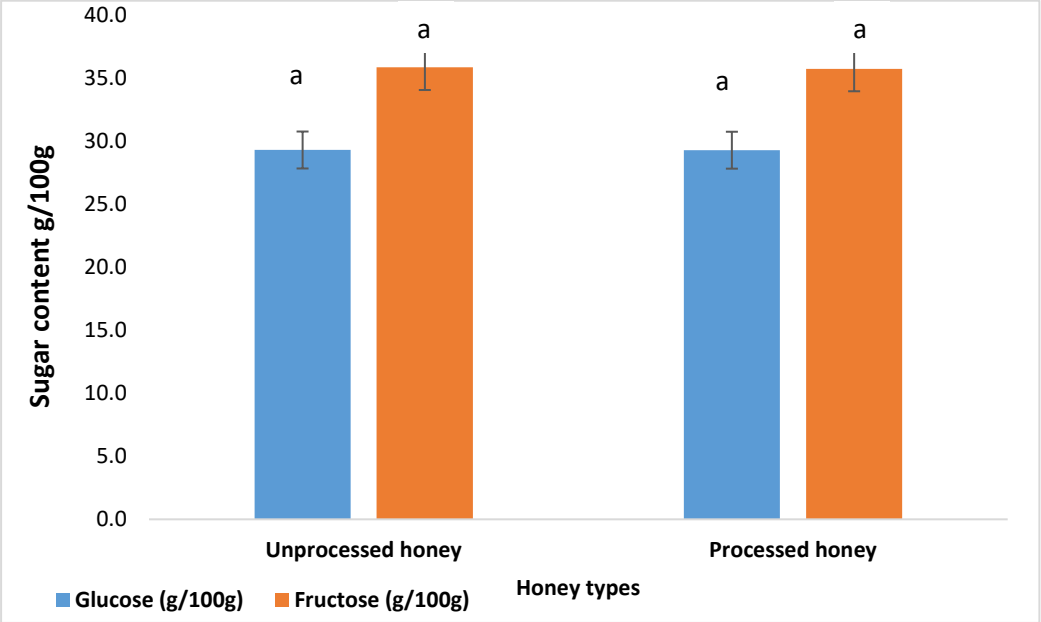

Figure 4.10: Comparison of mean glucose and fructose of processed and unprocessed honeys in Kilifi County

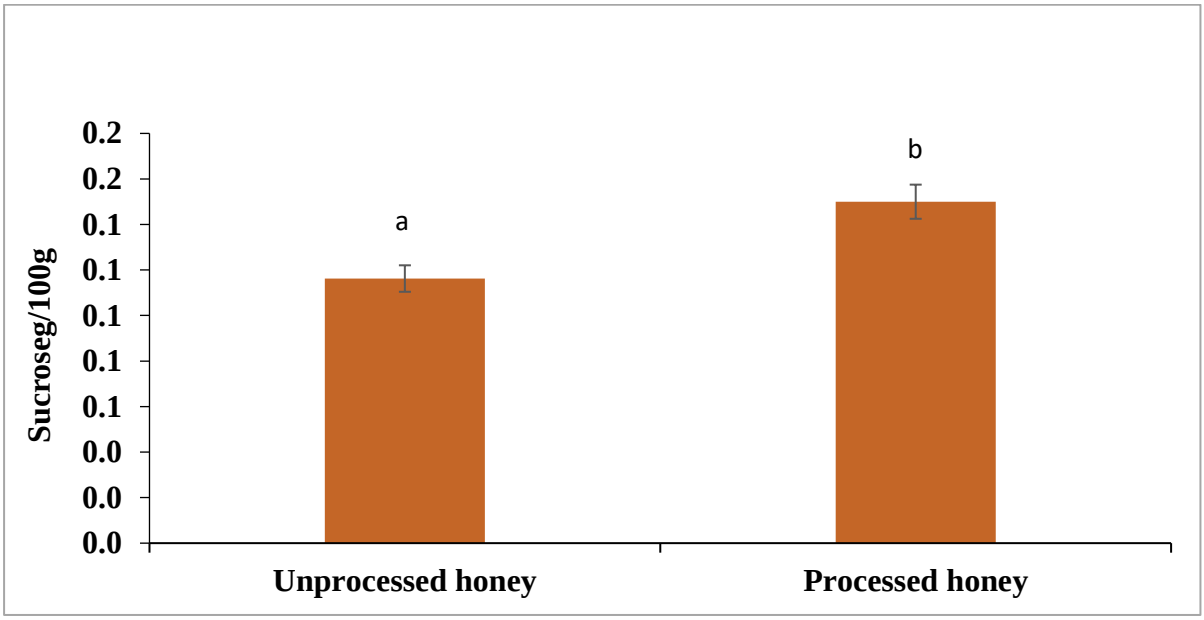

Figure 4.11: Comparison of mean sucrose of processed and unprocessed honeys in Kilifi County

4.1.10 Volatile organic compounds

The volatile organic compounds in mangrove honey and mangrove flowers were identified GC-MS and the results are summarized in Table 4.5. In the volatile organic

compounds profiles, 119 compounds were identified in mangrove flower extracts, 90 compounds in mangrove honey from the factory (processed) and 70 compounds in mangrove honey from the farms (unprocessed). Thirty-five (35) of the 119 compounds found in mangrove tree flower extracts, were also found in the factory mangrove honey as well as in the unprocessed honey. A typical flower and honey VOC chromatogram are given in Figures 4.12, 4.13 and 4.14).

Table 4.5: VOCs in mangrove flower and honey samples, given as % of total chromatogram area

No.	Compounds	Retention Time	Peak Area in flower samples	Peak Area in processed honey samples	Peak area in unprocessed honey samples
1	Ethyl acetate	3.2332	0.4792	0.7596	0.3952
2	Amylene hydrate	3.4379	0.3624	0.3768	0.458
3	2-Pentanone	3.6661	3.0719	3.4184	4.4385
4	2-Chloro-2-methyl-butane,	3.7246	5.6981	5.3722	7.6165
5	3-Penten-2-ol	4.0229	0.1624	0.2183	0.2698
6	1-Chloro-3-methyl-2-Butene,	4.1575	0.1408	-	0.2219
7	3-Hydroxy-3-methyl-2-butanone	4.9707	0.0991	0.1094	0.113
8	4-Methyl-Heptane,	5.813	0.1793	0.0617	-
9	1-chloro-2-methyl2-butene,	5.8716	0.1592	0.1983	-
10	N-Ethyl-butanamide,	6.1465	0.1953	0.2725	0.3274
11	4-Methyl,2-pentanol	6.8251	1.151	1.3699	-
12	2,4-Dimethyl-1-heptene	7.7026	0.8208	0.2813	0.0901
13	3,6,6-Trimethyl-cyclohex-2-enol	9.4167	0.2338	0.2872	-
14	3,3-dimethyl-hexane	11.353	0.5637	0.1665	-
15	Dodecane	14.4711	0.4108	0.1579	-
16	Tridecane	15.2433	0.2875	0.0694	-
17	6-Demethoxy ageratochromene	15.3311	0.6993	0.1935	-
18	3,3-Dimethyl-1-hexene	16.0272	0.7595	0.2617	-
19	4-Isopropyl-1,3-cyclohexanedione	16.2671	0.6587	0.2352	-
20	Tetradecane	17.2791	0.8155	0.1815	0.1757
21	Hexadecane	18.0981	0.3662	-	0.1407
22	Pentadecane	18.2034	0.4741	0.095	-
23	2,4-Bis-1,1-dimethylethylphenol	18.7299	1.1754	1.3099	1.2502
24	2-Methylbutyl-cyclopentane,	19.1979	0.3395	0.0921	-
25	Hexadecane	19.7478	0.5467	0.1057	-
26	7,9-Ditertbutyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.2988	0.3475	0.1124	0.1233
27	Hexadecanoic acid	23.6498	7.033	1.7735	2.6305
28	Palmitoleic acid	23.8019	1.4746	1.5084	1.6112
29	Heneicosane	24.9134	0.0796	0.6395	0.5785
30	Octadecanoic acid	25.4926	2.0069	0.5385	0.313
31	Docosane	25.8085	0.5576	0.163	
32	Tricosane	26.6801	0.2995	0.319	0.2081
33	Tetracosane	27.505	0.6165	0.2612	0.166
34	Docosanoic acid	28.8212	1.742	3.5027	1.6687
35	1-Docosene	33.864	0.3885	0.2503	0.1453

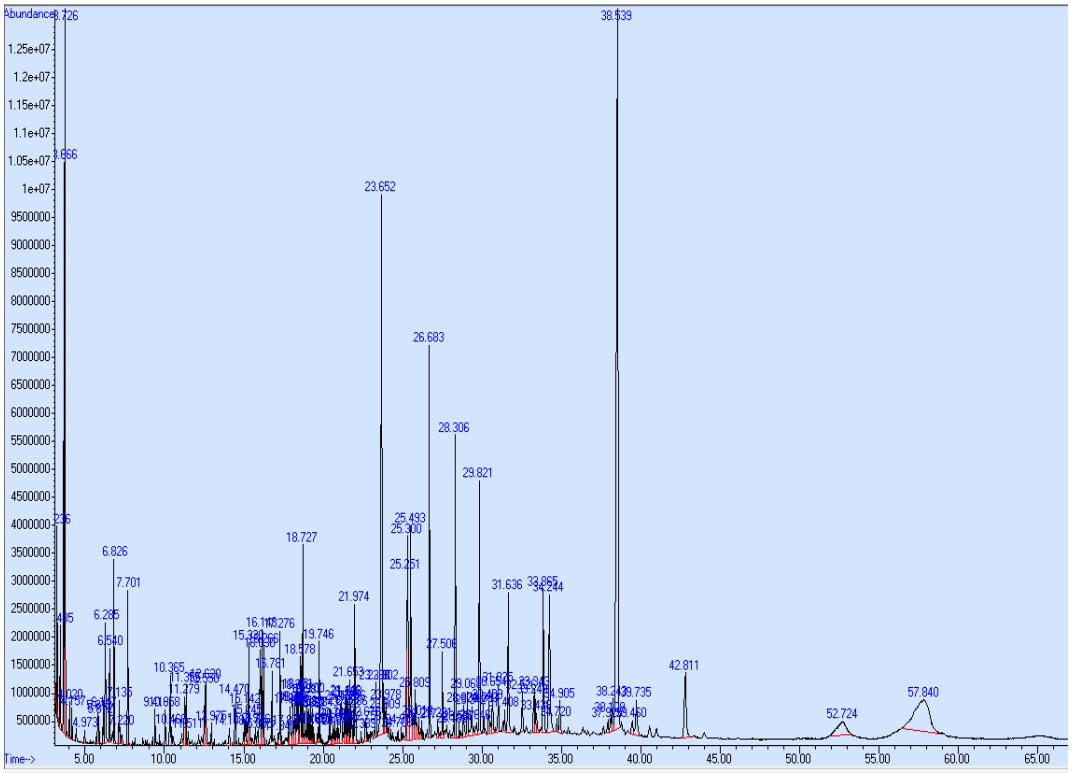

Figure 4.1: Chromatogram of mangrove flower volatile organic compounds showing 119 VOCs

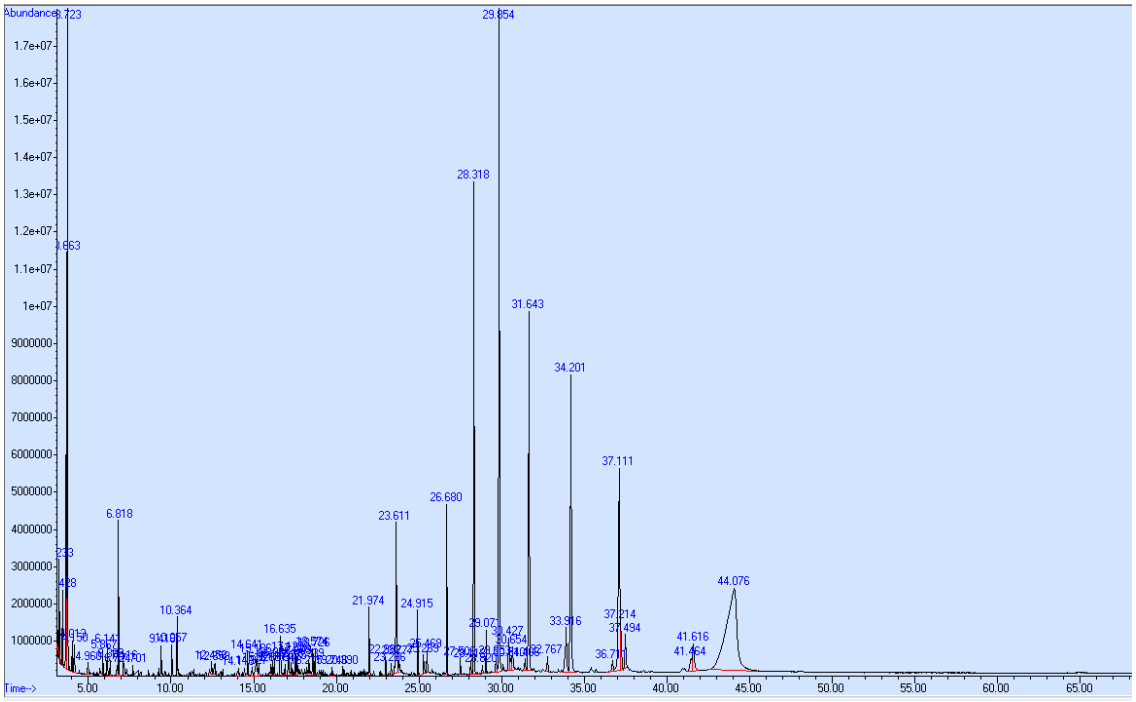

Figure 4.12: Chromatogram of mangrove honey (processed) volatile organic compounds showing 70 VOCs

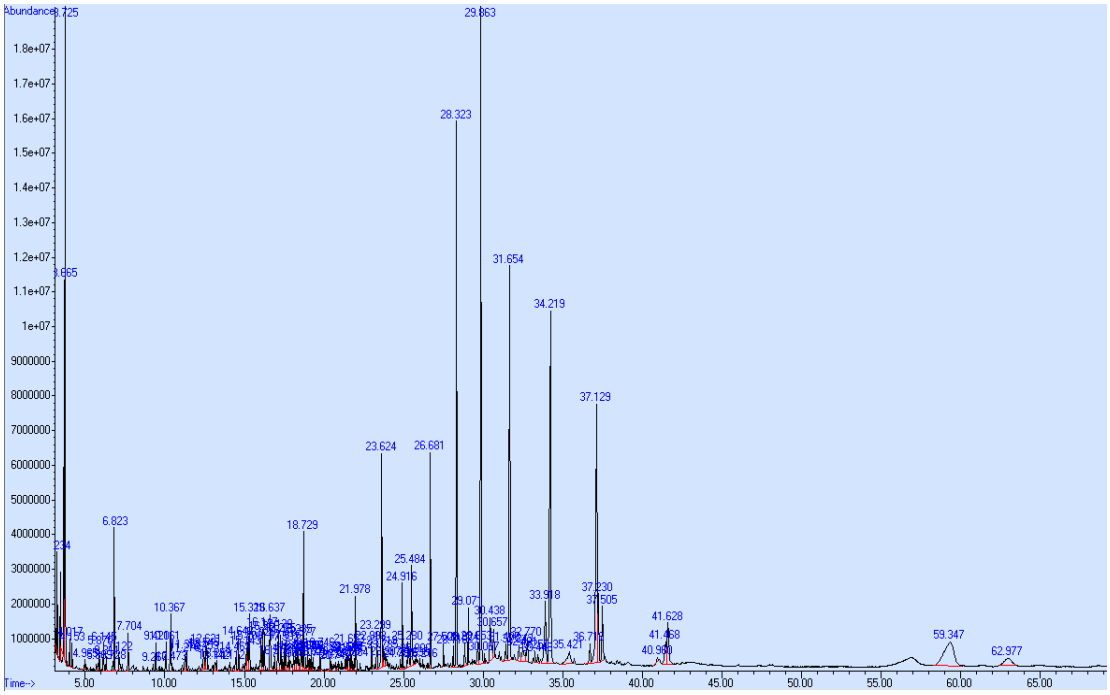

Figure 4.13: Chromatogram of mangrove honey (unprocessed) volatile organic compounds showing 107 VOCs

The volatile organic compounds identified were classified into 5 groups namely, esters, hydrocarbons, ketones and aldehydes, carboxylic acids and alcohols (Table 4.6). In all the analysed samples esters like ethyl acetate and carboxylic acids like n-hexadecanoic acid, palmitoleic acid, octadecanoic acid and docosanoic acid were present. There are many reports of VOCs profiling in honey from various floral sources (Soria *et al.*, 2004; Baroni *et al.*, 2006 and Wolski *et al.*, 2006).

Table 4.6: Classification of VOCs found in mangrove flower, factory and farm honey

Class	Compound
Hydrocarbons	Tridecane , Cyclopentane, 2-Methylbutyl-heneicosane, Docosane, Tricosane, Tetracosane, Docosane 1-Docosene, 2-Chloro-2-methyl-butane, Tetradecane, 4-Methyl-heptane, 3,3-dimethyl-hexane, Dodecane, 1-Hexene, 3,3-dimethyl-hexadecane, Pentadecane, Hexadecane, 2,4-Dimethyl-1-heptene, 2-Butene, 1-chloro-3-methyl-2-butene,
Ketones and Aldehydes	Pentanone, 3-Hydroxy-3-methyl-2-butanone, Isopropyl-1,3-cyclohexanedione, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione.
Esters	Ethyl acetate,
Carboxylic acids	Hexadecanoic acid, Palmitoleic acid, Octadecanoic acid, Docosanoic acid,
Alcohols	Amylene hydrate, 3-Penten-2-ol, Pentanol<4-methyl-2>, 3,6,6-Trimethyl-cyclohex-2-enol
Others	N-ethyl-butanamide, 6-Demethoxy-ageratochromene

A number of volatile organic compounds like nonanoic tricosane, tetracosane and octadecanoic acid identified in mangrove honey have also been identified in other honeys (Overton and Manura, 1995; Radovic *et al.*, 2001; Verzera *et al.*, 2001; Piasenzotto *et al.*, 2003; Soria *et al.*, 2004; De la Fuente *et al.*, 2006).

In the analyzed mangrove honey hexadecanoic acid, docosanoic and palmitoleic acid were found to be the major components present in high concentrations with peak areas of 2.6305, 1.6687 and 1.6112 respectively. These have not been reported in other honeys and can be used as chemical markers of mangrove honey.

Nonanoic acid has been reported as a marker present in Eucalyptus honey extracted by SPME (Piasenzotto *et al*, 2003). It has also been found to be present in heather, lime-honeydew honey, and buckwheat honey from Poland (Wolski *et al.*, 2006). Compounds such as tricosane, tetracosane and octadecanoic acid which were present in mangrove honey have been previously identified as characteristic markers of *Prunus mahaleb* honey in Croatia (Jerković *et al.*, 2009; Jerković and Marijanović, 2010).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The objective of this study was to characterize mangrove honey from the mangrove forests in Kilifi County in terms to its physical and chemical characteristics and also volatile organic compounds found therein. A total sixteen unprocessed honey samples and four processed honey samples were sampled from beekeepers' apiaries in the four sampled regions and Kwetu factory, respectively. The honey samples were subjected to physical and chemical analyses a well as chemical profiling to identify the volatile organic compounds found therein by GC-MS. To confirm that volatile organic compounds present in the honey samples came from the mangrove flowers, the flowers were also sampled and their volatile organic compounds collected by solid phase microextraction fiber and also profiled. Based on the findings, the following conclusions can be made:

i. All the mangrove honey samples collected were successfully analyzed and the physicochemical parameters were within the recommended range of values by the International Honey Commission, Codex Alimentarius and the KEBS honey specifications. Moisture content was relatively high, probably influenced by the humid conditions in the coastal region. The low HMF, diastase and invertase observed revealed that mangrove honey studied was fresh and had not been heated by farmers during harvesting and extraction. The high proline content also revealed that nectar and pollen are produced in great quantities by mangrove plants.

ii. The volatile organic compounds present in the honey samples and mangrove tree flowers were identified GC-MS. Thirty five of the 119 VOC identified in flower

extrats were also present in the honey samples analysed indicating a clear correlation between the flower VOC profiles and the honey VOC profiles. Three compounds docosanoic acid, palmitoleic acid and hexadecanoic and can be considered to be chemical markers for mangrove honey as they were found in all samples analysed and the mangrove flowers.

5.2 Recommendations

5.2.1 Recommendations from the study

On the basis of the findings of this study, the following recommendations can be made:

i. Kenyan mangrove honey is suitable for consumption locally and for exportation as it meets all the international standards.

ii. The identified VOCs (docosanoic acid, palmitoleic acid and hexadecanoic) in mangrove honeys should be used as markers to help distinguish mangrove honey from other honeys.

5.2.2 Recommendations for further work

i. Determination of total phenolic and flavonoids in relation to their radical scavenging activity.

ii. Determination of water activity and as a possible alternative indicator of honey quality by international and national standard agencies.

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Appendix 1: Questionnaire for Key informants

Purpose of the survey: the survey questionnaire is designed to select pertinent honey potential study areas and suitable beekeepers who will represent and enable to characterize Mangrove honey.

1. Respondent's name …….....
2. Occupation ……
3. Work experience (duration)……..
4. Have you ever involved/know about mangrove beekeeping

   Yes…….. No……
5. If yes; which of the mangrove area are customary for your activity?

   ……
6. How old is your bee colony…….
7. How often do you harvest honey…….
8. Where do you take your honey after harvesting……

   ……
9. Have you ever come across the research findings on the quality factors of mangrove honey? Yes……. No……

If yes, please mark on the following

I. Physicochemical properties Yes.....No……

II. Volatile organic compounds Yes…… No…….

III. What other quality of forest honey do you want to know?

…… …….

8. Which potential area do you recommend to study, and possibly produce mangrove honey?

Region	Recommended for the study	Honey harvesting time(months)
	1	
	2	

9. Do you have any suggestion please?

……..

THANK YOU FOR YOUR RESPONSE