MODIFICATION OF THE WHITE'S METHOD FOR QUANTITATIVE EVALUATION OF 5-HYDROXYMETHYLFURFURAL IN HONEY

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DEPARTMENT OF CHEMISTRY,

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DECLARATION

I declare that this project tittle“Modification of The White’s Method for Quantitative Evaluation Of 5-Hydroxymethylfurfural In Honey” has been written and compiled by me under supervision of Dr. F.G Okibe, and Dr. G.A Shallangwa, Department of Chemistry, Faculty of Physical Science, Ahmadu Bello University Zaria, Kaduna. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution. Authors whose work (s) I have referred to, have been duly acknowledged in the reference section.

……..	……..	……
Name of Student	Signature	Date

CERTIFICATION

This projectentitled “MODIFICATION OF THE WHITE’S METHOD FOR QUANTITATIVE EVALUATION OF 5-HYDROXYMETHYLFURFURAL IN HONEY” by Ibrahim USMAN meets the regulation governing the award of the degree of Master Analytical Chemistry of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate Studies Signature Date

DEDICATION

This work is dedicated to the Almighty God, the giver of life, health and time for his divine protection over my life from the commencement of my Master's degree programme and the opportunity given to me to successfully complete the programme.

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ABSTRACT

The White's method for determination of 5-hydroxymethylfurfural (5-HMF) content in honey was successfully modified using perchloric acid ($HClO_4$) as replacement for zinc acetate ($Zn(CH_3CO_2)_2{\cdot}2H_2O$) and potassium ferrocyanide ($K_4Fe(CN)_6{\cdot}3H_2O$) to serve as deproteinizing agent, and sodium bisulphite ($NaHSO_3$) was replaced with sodium pyrosulphite ($Na_2S_2O_5$) for the chromophore removal of 5-HMF at 284 nm. The proposed method was validated by evaluation of parameters such as linearity, precisions (reproducibility and intermediate), accuracy, and Limit of Detection (LOD), Limit of Quantification (LOQ), ruggedness and robustness. The correlation coefficients for the calibration curves were 0.9994 and 0.9923. The method obeyed Beer-Lambert's law at the concentration range of 5, 10, 15, 20 and 25 mg/kg. The values of reproducibility and intermediate precision in honey samples were 2.65, 2.67, 3.03, 4.73, and 1.90 % respectively. The recoveries for the analyses were between 81.4 % and 104.6 %, LOD and LOQ were 0.12 and 0.36 mg/kg at 284 nm and 0.06 and 0.17 mg/kg at 336 nm respectively. The ruggedness of the method was 1.23 and 1.00 %, and the robustness were 0.64 and 0.42 %. The results obtained suggest that perchloric acid and sodium pyrosulphite can successfully replace zinc acetate, potassium ferrocyanide and sodium bisulphite which are scarce and expensive reagents. The modified method is suitable for routine determination of 5-HMF in honey samples.

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ABBREVIATIONS

A_{283} – Absorbance at 284 nanometer wavelengths

A_{366} –Absorbance at 336 nanometer wavelengths

AOAC - Association of Official Analytical Chemists

FDA – Food and Drug Administration

5-HMF – 5-Hydroxymethylfurfural

HPLC- High Performance Liquid Chromatography

ICH - International Conference on Harmonization

ICP – Increase Intracranial Pressure

IHC – International Honey Commission

LOD – Limit of Detection

LOQ – Limit of Quantification

RSD – Relative Standard Deviation

USP – United State Pharmacopeial

UV-Vis - Ultraviolet-Visible Spectrophotometer

CHAPTER ONE

1.0 INTRODUCTION

1.1Background of the Study

The Codex Alimentarius regulation for honey defined honey as the natural sweet substance produced by *Apis Mellifera* bees from the nectar of plants or extra floral secretion that bees transform and store (Velásquez *et al.*, 2013).

The use of honey and production have a long and varied history. In many cultures, honey has uses that go beyond its use as food. Honey is frequently used as a talisman and symbol of sweetness among Egyptians (Honey-Marie, 2011). Exactly how long honey has been in existence is hard to say because it has been around since as far back as we can record(Marlowe, 2005).Cave paintings in Spain dating back 7000 years ago show the earliest records of beekeeping, however, fossils of honey bees date back about 150 million years(Marlowe, 2005).Its ' magical' properties and versatility have given honey a significant part in history(Ghaderi *et al.*, 2015). The earliest record of keeping bees in hives was found in the sun temples erected in 2400BC CairoAfrica. The bee featured frequently in Egyptian hieroglyphs and being favored by the pharaohs, often symbolized royalty(Minhas and Dhaliwal, 2018).

The ancient Egyptians used honey as a sweetener, as a gift to their gods and even as an ingredient in embalming fluid. Honey cakes were baked by the Egyptians and used as an offering to placate their gods(Gene, 2015). The Greeks, too, baked honey cakes and offered them to their gods. The Greeks viewed honey as not only an important food, but also as a healing medicine. Greek recipes books were full of sweetmeats and cakes made from honey(Ståhlberg *et al.*, 2011). Cheeses were mixed with honey to make cheesecakes, described by Euripides in the fifth century

BC as being "steeped most thoroughly in the rich honey of the golden bee"(Civitello, 2011). The Romans also used honey as a gift to their gods and they used it extensively in cooking. Beekeeping flourished throughout the Roman Empire. Once Christianity was established, honey and beeswax production increased greatly to meet the demand for church candles(Sivakesava and Irudayaraj, 2001).

Honey starts out as nectar that bees collect from flowers. Basically, nectar is a reward that plants produce to attract pollinating insects and birds. It is a sugary fluid which includes the aromatic oils that give flowers their scent, as well as other trace substances(Winston, 2014). Bees collect this nectar by drawing it through their proboscis and storing it in their honey stomachs. Honeybees then carry it back to their hive(Pita-Calvo and Vázquez, 2017). The foraging bees regurgitate the nectar and pass it to worker bees in the hive. These bees then gradually transform the nectar into honey by evaporating most of the water from it(Minhas and Dhaliwal 2018).

Nectar is as much as 70 % water, while honey is only about 18 % water. According to Manyi-Loh*it al*(2011), “Only honeys with less than 18% water can be stored with little or no risk of fermentation”. Bees get rid of the extra water by swallowing and regurgitating the nectar over and over. They also fan their wings over the filled cells of the honeycomb(Pereira *et al*., 2011). This process retains lots of sugar and the plant's aromatic oils while adding enzymes from the bees' mouths. The finished honey is thick, sticky and very sweet. It contains several types of sugar such as glucose and fructose. Its flavor and colour depends on the flowers from which the bees harvested their nectar(Thompson, 2009).

Various honeys differ in physicochemical parameters such as moisture, pH, total acidity, density, dynamic viscosity, refractive index, electrical conductivity, and color, as well as relative amounts

of reducing and non-reducing sugars, total sugars, water insoluble solids, mineral composition, 5-hydroxymethylfurfural content and diastase value (Israili, 2014). The composition of honey is rather variable and primarily depends on the floral source; however, certain external factors also play a role, such as seasonal and environmental factors and processing condition (Alvarez-Suarez,2009). Honey contains at least 181 substances; it is a supersaturated solution of sugars, mainly composed of fructose (38%) and glucose (31%), containing also minerals (Calcium, Potassium, Copper, Iron, Magnesium, Manganese, Sodium, Phosphorous, Zinc), proteins, and vitamins (Ascorbic acid, Thiamine, Riboflavin, Niacin, Pantothenic acid and Pyridoxine) (Suarez and Battino, 2013). A wide range of minor constituents are also present in honey, many of which are known to have antioxidant properties(Murkovic *et al*., 2006). These include phenolic acids and flavonoids, certain enzymes (glucose oxidase and catalase) and amino acids (Proline, Glycine, Valine, Glutamic acid, Aspartic acid, Isoleucine, Leucine, Lysine, Alanine, Glutamine, Threonine, Arginine, Tryptophan etc, with Proline being the most abundant, contributing to about 50% of total amino acids in Honey (Gündoğdu*et al.*, 2019).

1.2 5-Hydroxymethylfurfural (5-HMF)

5-hydroxymethylfurfural is a six-carbon heterocyclic organic compound containing both aldehyde and alcohol (Hydroxymethyl) functional groups(Shapla¸*et al.,* 2018). The ring of the structure is centered on furan moieties, whereas the two functional groups, that is, formyl and hydroxy-methyl groups are linked at the second and fifth positions respectively showed in structure 1.1. 5-HMF is a solid, yellow substance that has a low melting point but is highly soluble in water and its chemical formula is $C_6H_6O_3$(Shapla¸*et al.,* 2018).5-HMF is considered the most important intermediate product formed during two reactions (i) acid-catalyzed degradation of hexose and (ii) decomposition of 3-deoxyosone in the Maillard reaction

(Lachman *et al*., 2007). 5-HMF formation is correlated with chemical characteristics such as pH, free acid content, total acidity, lactone content and mineral content, which in turn are related to the floral source of collected honey samples(Lin, e*t al.*,2003). The presence of simple sugars such as glucose and fructose and of many acids have been reported to be favourable for honey production (Shapla*et al*., 2018).

Structure 1.1 5 –(hydroxymethyl) furan – 2 – carbaldehyde, $C_6H_6O_3$

The presence of 5-HMF has been reported in several foods, including honey, grain products, biscuits, cereals, milk, tomato products, instant coffee, dried fruits, bread, pasta, citrus juices, beer, syrup, jams, canned peach, dried grape, alcohol, apple juice, milk and cereal-based infant formula(Suarez and Battino, 2013). The presence of 5-HMF in foods reflects a breakdown or change of substances containing sugar, which is why 5-HMF levels in food are generally analyzed for quality control purposes (Gökmen and Senyuva, 2006). Food processing conditions, such as temperature, time and water activity, affect 5-HMF content in foods. The daily intake of 5-HMF from foods may occasionally reached 150 mg/day and it may be present in foods at varying levels (Abraham *et al.*, 2011). Very high levels of 5-HMF can be found in such foods as dried fruits or caramelized products (> 1 g/kg) (Ünüvar,2018). In addition to caramelized foods, 5-HMF has also been identified in caramel-colored pharmaceutical syrups (Ünüvar, 2018). Although the concentrations reported in pharmaceutical syrups are very low, there are concerns

about the potential interactions between 5-HMF and functional amino groups of pharmaceutics (Douša, *et al*., 2012).

1.2.1 Synthesis of 5-Hydroxymethylfurfural in Honey

In principle, 5-HMF production seems easy; it is synthesized by the loss of three molecules of water from a hexose material (carbohydrate) in an acid catalyzed reaction. However, the synthesis of 5-HMF synthesis is quite complicated because, many other reactions occur (Román and Dumesic, 2009).

Several catalysts have been reported for the dehydration of carbohydrates.Rosatella*et a.* (2011) organized them into five groups: organic acids, inorganic acids, salts, Lewis acids, and others. In recent years, carbohydrate dehydration catalysts have undergone a remarkable process of evolution, and several new catalysts have been reported. Here, we group dehydration reactions by the catalysis type: acid catalysis (homogeneous liquid, heterogeneous liquid–liquid, solid-liquid and gas-liquid) and metal catalysis(Rosatella*et a.,* 2011).Carbohydrates can be converted to 5-HMF through a three-step reaction as showed in figure 1.1(Chen *et al.*, 2014);

Step1: Hydrolysis of glucan (a glucose-based polymer, e.g., cellulose and starch) to glucose catalyzed by a Brønsted acid;

Step2: Isomerization of glucose to fructose mediated by a Lewis acid;

Step3: Dehydration of fructose to 5-HMF facilitated by a Brønsted acid.

Figure1.1Reaction steps from glucan to 5-HMF

1.2.2Factors affecting 5-HMF formation in honey

Heating of honey during its processing isto reduce its viscosity which can prevent crystallization or fermentation leading to the formation of 5-HMF(Subramanian *et al.*, 2007).In addition to heating, several other factors influence the formation of 5-HMF in honey, such as honey's physicochemical properties (pH, free acid content, total acidity, lactone content and mineral content), water activity, the use of metallic containers (Shapla *et al.,* 2018), thermal and photochemical stress (Spano *et al.,* 2008).

5-HMF is easily formed at low temperatures in the presence of low-pH or acidic conditions while high temperature and long storage duration increase its concentration to a large extent(Shapla *et al.,* 2018). Nevertheless, a different pathway is proposed in dry and pyrolytic conditions under which 5-HMF is formed from fructose and sucrose(Shapla *et al.,* 2018). In addition to temperature and pH, the rate of 5-HMF formation in honey is also dependent on

honey's moisture content (Önür et al., 2018). Therefore, many steps are taken to maintain low moisture content in honey samples, including gamma irradiation and heat treatment to inhibit 5-HMF formation(Gökmen, 2006).

The rate of 5-HMF formation is also dependent on the fructose: glucose ratio and the type of sugars formed because it has been reported that at pH 4.6, fructose has five times more reactivity than glucose, and a high fructose: glucose ratio will accelerate the reaction (Shapla *et al.*, 2018).

1.2.3Uses of 5-hydroxymethylfurfural

5-HMF is very useful not only as intermediate for the production of the biofuel dimethylfuran (DMF) and other molecules, but also for important molecules (figure 1.2) such as levulinic acid, 2,5-furandicarboxylic acid (FDA), 2,5-diformylfuran (DFF), dihydroxymethylfuran and 5-hydroxy-4-keto-2-pentenoic acid ((Rosatella*et a.,* 2011).

figure1.2Products of 5-hydroxymethylfurfural

1.2.4Effects of 5-hydroxymethylfurfural on Human Health

In recent decades, HMF has drawn the attention of the scientific community for its carcinogenic potential to humans. Some studies have shown that this metabolite can be converted in vivo to 5-sulfooxymethylfurfural ($C_6H_6O_6S$), a genotoxic compound (Ribeiro, *et al.,* 2012). In addition, at high concentrations, HMF is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes (Janzowski *et al.,* 2000). For this reason, the Codex Alimentarius and the European Commission have set a maximum 5-HMF level for honey of 40 mg/kg(Kolodziejczyk *et al*., 2015).However, honeys coming from tropical countries and honeys with low enzyme levels, have their5-HMF limit set as 80 and 15 mg/kg respectively (Zappala *et al.,* 2005)

1.2.5Detection methods of 5-hydroxymethylfurfural

5-HMF content is internationally recognized for its ability to indicate the freshness or lack of freshness of honey and can be used to judge the processing and storage condition of honey. Honey is a natural product that must be delivered to the consumer with its essential composition and its quality minimally altered (Escriche *et al.,* 2008). However, Honeydecomposition starts on the fieldwhen honey is harvested and placed in barrels by the bee-keepers; it goes on with transport and storage previousto the industrial treatment and continues during theindustrial process and even after it(Escriche *et al.,* 2008). The loss of the natural quality is due to the decomposition of vitamins, destruction of the integrity of the enzymes and the development of 5-HMF content(Khalil *et al*., 2010).

5-HMF content and enzymatic activities are the recognized honey quality parameters in the international market(Janzowski *et al*., 2000). Heating is of great importanceto commercial honey

processing industry; hence it needs to have standard guidelines for the use of optimum heating temperature and time duration (Yeshitila and Tekeba, 2019).Accurate quantitative analysis of HMF is of great importance because HMF is a marker of quality deterioration, thermal processing and other adulteration practices(Hegazi, 2011). HMF is also important in clinical research and therapeutics. 5-HMF is beneficial to human health by proving antioxidant, antiallergic, anti-inflammation, anti-hypoxic, anti-sickling effect (Gidamis *et al.,* 2004).

The International Honey Commission (IHC) has recommended three main methods for HMF determination: two spectrophotometric methods by White's and Winkler and a reversed-phase high-performance liquid chromatography (HPLC) method (*Önür et al.,* 2018). Prior to the availability of the spectrophotometric methods, both optical and chemical methods were used. The White's Methodinvolves measurement of UV- absorbance of aqueous honey solutions with and without bisulfite(White, 1992). On the other hand, theWinkler Method involves measurement of UV- absorbance of aqueous honey solutions with p-toluidine and Barbituric acid (Zappala *et al.,* 2005). HPLC method according to Jeuring and Kuppers, honey is first dissolved in water and 5-HMF content is determined on a reversed phase HPLC column with water and methanol as isocratic mobile phase (Ünüvar, 2018).

These methods are fast, they lack good sensitivity and specificity. Moreover, the method described by Winkler requires the use of *p*-toluidine, which is carcinogenic (Veríssimo *et al.,* 2017). Although the HPLC method is relatively more expensive, it is advantageous with respect to both labour and time. In addition, the method is deemed as an automated and sensitive method that can exclude much interference from other related compounds (Pascual-Mate *et al.,* 2018). Nevertheless, although HPLC is a sophisticated technique, the method is still not satisfactory to

some who have recommended further development and modification of the method (Oleszek, 2002).

1.3Perchloric Acid ($HClO_4$)

Perchloric acid is one of the strongest mineral acids and presents a high oxidizing power at high temperature, making it very suitable for the digestion of matrices such as fat, proteins, and lipids as the resulting perchlorates are readily soluble in water(Singh, 2020). Perchloric acidshould be handled with extreme care due to its rapid reactivity with organic matrices and sometimes it can become explosive(Gradvol *et al*., 2015).

Perchloric acid can be decomposed at 245 °C in a closed vessel to produce dangerous amounts of gaseous by-products and excess of pressure (Müller*et al.,* 2014). For this reason, perchloric acid should not be used in closed vessels. The high boiling temperature of $HClO_4$(203 °C) in comparison with HNO_3 and HCl (83 ^{o}C and 85.1^{o}C)allows the evaporation of these acids which present relatively low-boiling-temperature (Alfassi, 2012).

In some cases, it may be possible to test samples (food and blood samples) directly with appropriate dilution in distilled water. However, if this is not adequate then deproteinization with either perchloric acid ($HClO_4$) or trichloroacetic acid or Carrez reagents is required (Sharma*et al.,* 2013).Among the deproteinization protocols developed over the last half century, perchloric acid ($HClO_4$) precipitation has been extensively used in many different sample preparation procedures, since not only does it remove most of the protein present but it also functions to stabilize many of the small analytes (Hu, & Qi, 2014).). Ketones react with formaldehyde and perchloric acid to produce 2-isoxazolines(C_9H_9NO), and with urea in α – methylnaphthalene at 200 ^{o} Cto produce 2- carbamoylisoxazolidines as shown in Figure 1.3 below(Sharma*et al.,* 2013).

Figure1.3Reaction ofKetones with formaldehyde and perchloric acid to produce 2-isoxazolines

1.4Sodium Bisulfite($NaHSO_3$)

Sodium bisulfite (or sodium hydrogen sulfite) is a chemical mixture with the approximate chemical formula $NaHSO_3$. Sodium bisulfite in fact is not a real compound (Tudela, *et al.,* 2003) but a mixture of salts that dissolve in water to give solutions composed of sodium ions and bisulfite ions.

In organic chemistry "sodium bisulfite" has several applications. It forms a bisulfite adduct with aldehyde groups and with certain cyclic ketones to give a sulfonic acidas shown in Figure 1.4(Chauhan*et al.*, 2014).

Figure1.4Reaction of sodium bisulfite with aldehydes to give a sulfonic acid

The reverse reaction takes place in presence of a base such as sodium bicarbonate or sodium hydroxide and the bisulfite is liberated as sulfur dioxide as shown in Figure 1.5 (Furigay*et al*., 2018).

Figure1.5Reverse reaction with sodium bicarbonate to liberate bisulfite as sulfur dioxide.

1.4.1Addition of sodium bisulfite to aldehydes and ketones

This reaction only works well for aldehydes as shown in Figure 1.6. In the case of ketones, one of the hydrocarbon groups attached to the carbonyl group needs to be a methyl group as shown in figure 1.7 (Pessah et al., 2006). Bulky groups attached to the carbonyl group get in the way of the reaction happening. The aldehyde or ketone is shaken with a saturated solution of sodium hydrogensulphite in water. Where the product is formed, it separates as white crystals(Furigay*et al*., 2018).

Figure1.6Reaction of ethanal with sodium bisulfite to give1-hydroxyethane-1-sulfonate

Figure1.7Reaction of propanone with sodium bisulfite to give1-hydroxypropane-1-sulfonate.

The reaction is usually used during the purification of aldehydes (and any ketones that it works for). The addition compound can be split easily to regenerate the aldehyde or ketone by treating it with either dilute acid or dilute alkali. If you have an impure aldehyde, for example, you could shake it with a saturated solution of sodium hydrogen sulfite to produce addition aldehydes and ketones. These crystals could easily be filtered and washed to remove any other impurities. Addition of dilute acid, for example, would then regenerate the original aldehyde. It would, of course, still need to be separated from the excess acid and assorted inorganic product (Corbett*et al.*, 2006).

1.5Sodium Metabisulfite ($Na_2S_2O_5$)

Sodium metabisulfite is a chemical compound with the formula$Na_2S_2O_5$. It is also known as sodium pyrosulphite and sodium disulfite shown in Figure 1.8 below. $Na_2S_2O_5$ is an ionic compound containing the sodium cation (Na^+) and the metabisulfite anion ($Na_2S_2^-$) In its standard state, sodium metabisulfite exists as a white or yellowish-white powder (Tudela, *et al.*, 2003). As mentioned earlier, sodium metabisulfite is made up of two sodium ions and one metabisulfite ion.Sodium bisulfite is not sufficiently stable in the solid state to be marketed for

commercial use. The sodium bisulfite of commerce consists chiefly of sodium pyrosulphite. The sodium pyrosulphite and its reactions are nontoxic. Sodium pyrosulphite does not generate poisonous gasses, in contrast to sodium bisulfite which give sulfur dioxide when it is contact with an acidic environment(Madan*et al.,* 2007).

Figure 1.8 Structureof Sodiummetabisulfite

When a solution of $NaHSO_3$ (sodium bisulfite) is saturated with SO_2 (sulfur dioxide) and then allowed to evaporate, a residue of sodium pyrosulphite is obtained as shown in equation (1)(ANS, 2016).

…… (1)

1.6Statement of the Problem

The three methods for determination of 5-HMF described and validated by the IHC, only two of them are recommended for use: the HPLC and the White's method. The Winkler method should not be used for determination of 5-HMF because one of the reagents (p-toluidine) is carcinogenic (Dzugan*et al.,* 2021)". The Carrez reagents used inThe White's and HPLC methods (Zinc acetate and potassium ferrocyanide) are expensive and not readily available.

Sodium bisulfite is not sufficiently stable in the solid state to be marketed for commercial use, the sodium bisulfite of commerce consists chiefly of sodium pyrosulphite (Alfassi,

2012).Anklam (1998) states in his recentreview, “The suitability of the analytical methods for 5-HMF is unsatisfactory and requires further investigation”.

1.7Justification

In some cases, it may be possible to test samples directly with appropriate dilution in distilled water. However, if this is not adequate then deproteinization with either perchloric acid ($HClO_4$), trichloroacetic acid ($C_2HCl_3O_2$) orCarrez reagents is required (Sharma *et al.*,201).

Among the deproteinization protocols developed over the last half century, perchloric acid ($HClO_4$) precipitation has been extensively used in many different sample preparation procedures, since not only does it remove most of the protein present but it also functions to stabilize many of the small analytes by reducing matrix effects (HuandQi, 2014).

Sodium pyrosulphite and its reactions are nontoxic. Sodium pyrosulphite does not generate poisonous gases, in contrast to sodium bisulfite which gives sulfur dioxide when it is in contact with an acidic environment (ANS, 2016).

Using perchloric acid and sodium pyrosulphite;the method is easy, convenient, and can be used for the preparation of a large number of samples (Brayshe*et al.*, 2019).Therefore, it is very essential to modify a new rapid and simple validated spectrophotometric method for determination of 5-hydroxymethylfurfural in honey.

1.8Aim and Objectives

1.8.1 Aim

This study is aimed at modifying the White’s method for determinationof 5-hydroxymethylfurfural in honeyusing perchloric acid and sodium pyrosulphite as replacement for the scarce and expensive carrez reagents and sodium bisulphite.

1.8.2 Objectives

i.To determine the physicochemical parameters of the honey such as pH, conductivity, Specific gravity and moisture content.

ii. To determine of 5-HMF content in honey using The White's method and a modifiedmethod

iii. To compare the results of the two separate methods with linear regression analysis, ANOVA (single factor) and student's t-test (paired two samples for mean).

iv. Tovalidate the proposed modified method with the determination of Linearity, precision (repeatability and intermediate precision), accuracy, robustness, ruggedness, Limit of Detection (LOD) and Limit of Quantification (LOQ).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Properties of Honey

The properties of honey vary depending on water content, the type of flora used to produce it, temperature and the proportion of the specific sugars it contains. Fresh honey is a supersaturated liquid, containing more sugar than the water can typically dissolve at ambient temperatures. At room temperature, honey is a super cooled liquid, in which the glucoseprecipitates into solid granules (Eshlak, 2019).

2.1.1 Viscosity

Since honey normally exists below its melting point, it is a supercooled liquid. The viscosity of honey is affected greatly by both temperature and water content. The higher the water percentage, the easier honey flows(Ghaderi *et al.*, 2015). Aside water content, the composition of honey also has little effect on viscosity, with the exception of a few types. At 25 °C (77 °F), honey with 14% water content generally has a viscosity around 400 poise, while a honey containing 20% water has a viscosity around 20 poise. At very low temperatures, honey will not freeze solid (Temaru *et al.*, 2007).

Instead, as the temperatures become lower, the viscosity of honey increases. Viscosity increasesat low temperature and decreases at high temperature. Honey containing 16% water, at 70 °C (158 °F), will have a viscosity around 2 poise, while at 30 °C (86 °F), the viscosity is around 70 poise.

As cooling progresses, honey becomes more viscous at an increasingly rapid rate; reaching 600 poise around 14 °C (57 °F). However, while honey is very viscous, it has rather low surface tension (Temaru *et al.*, 2007).

2.1.2Electrical and Optical Properties of Honey

Because honey containselectrolytes, in the form of acids and minerals, it exhibits varying degrees of electrical conductivity. Measurements of the electrical conductivity are used to determine the quality of honey in terms of ash content. Honey with a low percentage of glucose (such as chestnut or tupelo honey) do not crystallize (Kayode and Oyeyemi, 2014).

The effect of light on honey is useful for determining the type and quality of honey. Variations in the water content alter the refractive index of honey. Water content can easily be measured with a refractometer. Typically, the refractive index for honey will range from 1.504 at 13% water content to 1.474 at 25%. Honey also has an effect on polarized light, in that it will rotate the polarization plane. The fructose will give a negative rotation, while the glucose will give a positive one. The overall rotation can be used to measure the ratio of the mixture (Nicola, 2009).

2.1.3Hygroscopic and Fermentation of Honey

Honey has the ability to absorb moisture directly from the air, a phenomenon called hygroscopy. The amount of water the honey will absorb is dependent on the relative humidity of the air. Because honey contains yeast, this hygroscopic nature requires that honey be stored in sealed containers to prevent fermentation, which usually begins if the honey's water content raises much above 25%. Honey will tend to absorb more water in this manner than the individualsugars would allow on their own, which may be due to other ingredients it contains(Temaru *et al.*, 2007).

Fermentation of honey will usually occur after crystallization because, without the glucose, the liquid portion of the honeyprimarily consists of a concentrated mixture of the fructose, acids, and water, providing the yeast with enough of an increase in the water percentage for growth. Honey

that is to be stored at room temperature for long periods of time is often pasteurized, to kill any yeast, by heating it above 70 °C (158 °F) (Sreejaand Gawai,2019).

2.1.4 Thermal characteristics

Like all sugar, honey will caramelize if heated sufficiently, becoming darker in colour, and eventually burn. However, honey contains fructose, which caramelizes at lower temperatures than the glucose. The temperature at which caramelization begins varies, depending on the composition, but is typically between 70 and 110 °C (158 and 230 °F). Honey also contains acids, which act as catalysts, decreasing the caramelization temperature even more(Sreeja and Gawai, 2019). The amino acids form darkened compounds called melanoidins, during a Maillard reaction. The Maillard reaction will occur slowly at room temperature, taking from a few to several months to show visible darkening, but will speed-up dramatically with increasing temperatures. However, the reaction can also be slowed by storing the honey at colder temperatures(Temaru *et al.*, 2007).

Unlike many other liquids, honey has very poor thermal conductivity, taking a long time to reach thermal equilibrium. Melting crystallized honey can easily result in localized caramelization if the heat source is too hot or if it is not evenly distributed. However, honey will take substantially longer to liquefy when just above the melting point than it will at elevated temperatures. Melting 20 kilograms of crystallized honey at 40 °C (104 °F) can take up to 24 hours, while 50 kilograms may take twice as long(Abeshu and Geleta, 2016).

2.1.5 Therapeutic Properties of Honey

Meda *et al.* (2004) reported that honey is becoming acceptable as a reputable and effective therapeutic agent by practitioners of conventional medicine and by the general public. Its

therapeutic properties have been ascribed to its antimicrobial, anti-inflammatory and antioxidant activities.

2.1.6 Antimicrobial activities

Antimicrobial activity of honeyis very important therapeutically, especially in situations where the body's immune response is insufficient to clear infection. In other words, it has shownpowerful antimicrobial effects against pathogenicand non-pathogenic microorganisms (yeasts and fungi) and even against those that developed resistance to many antibiotics.

Furthermore, honey is a supersaturated sugar solution; these sugars have high affinity for water molecules leaving little or no water to support the growth of microorganisms (bacteria and yeast). Consequently, the microorganisms become dehydrated and eventually die. However, the major antimicrobial activity has been found to be due to hydrogen peroxide (Temaru,*et al.*, 2007), produced by the oxidation of glucose by the enzyme glucose-oxidase.When honey is dilutedas Hydrogen peroxide decomposes as honey is diluted, it generates highly reactive free radicals that react and kill the bacteria. In most cases, the peroxide activity in honey can be destroyed easily by heat or the presence of catalase (Mandal and Mandal, 2011).

2.1.7 Anti-inflammatory activities

It has also been shown that honey reduces skin inflammation, promotes wound healing, diminishes scar size and stimulates tissue regeneration (Molan, 2001). Although inflammation is a vital part of the normal response to infection or injury, when it is excessive or prolonged it can prevent healing or even cause further damage. The most serious consequence of excessive inflammation is the production of free radicals in the tissue. These free radicals are initiated by

certain leucocytes that are stimulated as part of the inflammatory process (Van den Berg *et al.*, 2008), as inflammation is what triggers the cascade of cellular events that give rise to the production of growth factors which control angiogenesis and proliferation of fibroblasts andepithelial cells (Reuter*et al.,* 2010). They can be extremely damaging and break down lipid, proteins and nucleic acids that are the essential components of the functioning of all cells. However, the anti-inflammatory properties of honey have been well established in a clinical setting and its action is free from adverse side effects(Escuredo *et al*., 2014).

2.1.8 Antioxidant activities

Antioxidant capacity is theability of honey to reduce oxidative reactions within the human body. It has been found to have a significant antioxidant content measured as its capacity to scavenge free radicals. This anti-oxidant activity may be at least part of what is responsible for its anti-inflammatory action because oxygen free radicals are involved in various aspects of inflammation (Manyi-Loh*et al.,* 2011). Even when the antioxidants in honey do not directly suppress the inflammatory process, they can be expected to scavenge free radicals in order to reduce the amount of damage that would otherwise have resulted(Escriche *et al*., 2009).

Honey exerts its anti-oxidant action by inhibiting the formation of free radicals, catalyzed by metal ions such as iron and copper. Flavonoids, other polyphenols and common constituents of honey have the potential to impound these metal ions in complexes, preventing the formation of free radicals in the first place. The main antioxidants in honey are the phenols, such as quercetin, hesperetin and chrysin and the Maillard products called melanoidins (Abeshu and Geleta, 2016).

2.1.9pH and acidity

All the various varieties of honey are acidic with a pH-value generally lying between 3.5 and 5.5, due to the presence of organic acids that contribute to honey flavour and stability against

microbial spoilage. In honey the main acid is gluconicacid, which is found together with the respective glucono-lactone in a variable equilibrium (Bogdanov*et al,* 2004). Free acidity, total acidity and pH have some classification power for the discrimination between unifloral honeys, while lactones, due to their strong variability, do not provide useful information (Bogdanov*et al*, 2004).

A highly acidic honey sample indicates the possible fermentation of sugars into organic acids. None of the investigated samples exceeded the allowed limit (3.7-4.5), which may be considered as an index of freshness of all honey samples. Furthermore, the maximum pH value of 3.4 was found in Acacia variety whereas 3.5 and 3.2 were determined in Berry and Herbal varieties respectively. These observations are in accordance with those made by Codex Almentariou Commission (2001) where acceptable ranges of pH of honey were predetermined between 3.2 and 4.5. The results of this study are also in agreement with those of Avila*et al*. (2018), who reported the pH of 3.0 to 5.0 in pure honey. These pH ranges are mainly due to the variation of different acid and minerals present in the honey. Likewise, the floral difference may also cause the ranges of pH(Delgado-Andrade *et al*., 2007.

All honey samples analyzed were acidic in nature, with pH values varying from 3.7 to 5.0. The pH values of honeys were in accordance with AOAC (Tornuk*et al.,* 2013). These results are comparable to 3.42 to 4.68 for Brazilian and 3.2 to 4.5 for Bangladeshi honeys (Wakil*et al.,* 2021). This parameter is of great importance during the extraction and storage of honey as it influences the texture, stability and shelf life of honey (Terrab *et al.,* 2004). Floral and geographic origins can cause great variations in the pH values honey, as the nectar pH and soil conditions can greatly influence honey physicochemical characteristics (Wang and Qianxi, 2011). Habib *et al.* (2014) reported pH range of 3.99 – 6.33 in honey samples of honeys from

arid regions. Fahim*etal.* (2014) showed that in Algeria, honey SamplespH ranged from 3.7 to 5.Kazi *et al.*,(2019) reported pH in the range of 3.20 to 4 in local and imported honey samples from Pakistan. Rebiai and Lanez (2014) reported 3.80 to 5.24 pH value in different flora honey samples of Algeria. Yohannes*et al.* (2018)reported pH values of 4.75 and 4.25 in natural and industrial honeys respectively.

2.2.0Moisture content

The water content is a quality parameter, important above all for honey shelf life. It has a minor importance for the characterization of unifloral honeys(Chua 2014). However, depending on the production season and the climate, unifloral honeys show some typical differences in water content, which affect the physical properties of honey (viscosity, crystallization) and also influence the value of the glucose/water ratio (Oddo *et al.*, 2004). However, water content can be artificially altered during honey processing.

Khan (2000) reported 18.7 % as compared with 19.5 % for summer honeys. Mairaj*et al.* (2008) showed that in Pakistani honeys, water content ranged from 14.3 to 18.6%. Vijayakumar (2020) reported 20.90 per cent average moisture contents in 80 honey samples from India.

Farkas & Zajácz (2007) reported 19.5% average moisture content for 5 types of Korean honey (acacia, bush clover, chestnut, rape and multiflora). Disayathanoowat et al.(2020) reported four groups of honey: 27 from *Apis Mellifera*, 5 from *A. dorsata*, 9 from *A. cerana* and 14 of unknown origin (described as commercial). Values for moisture content of honey from different *Apis species* differed significantly; mean values were 19.5, 23.1, 22.00 and 20.00, respectively. Ghoshdastidar and Chakrabarti (1992) recorded 16.0-23.4% moisture of the samples received

from the Central Bee Research Institute, Pune. Finola *et al.,* 2007; Cantarelli *et al.,* 2008; Omafuvbe *et al.,* 2009 and Buba *et al.,* 2013 showed a wide range of the moisture content in honey samples.

Habib *et al.* (2014) reported moisture content in arid regions honey sample range from 13.63 to 20.60%. Fahim *et al.* (2014) reported of moisture content in Pakistan honey sample range from 13.80 to 16.60%. Ali*et al.* (2014) reported of moisture content in various flora honey of Tunisia range from 17.27 to 19.73%. Fahim*et al.* (2014) showed that in Algeria honeys, water content ranged from 15.87 to 18.05%. Sabir*et al.* (2014), Kayode and Oyeyemi(2014), Rebiai and Lanez (2014), Krishna *et al.* (2015), Watson*et al.* (2015), Prica *et al.*, 2015, Prodhan*et al.* (2015), Ambily*et al.* (2015) &Sansano*et al.* (2015) also reported moisture content in apiary honey in the range of 13.9 to24.1%.

2.2.1 Specific gravity

Specific gravity is an important physical characteristic of honey. According to USA standards, Grade "A" honey must have specific gravity not less than 1.415 (g/cm^3), Standard honey should have specific gravity more than 1.406 (g/cm^3). The British National Mark scheme has covered honey under the best quality if Specific gravity of honey is at least 1.315 (all at 60^o F). Karunarathna (2013) reported specific gravity (at 60^o F) between 1.411 and 1.420, the mean was given as 1.415. Alqarni*et al.* (2014) reported that clover honey had the lowest specific gravity among the major Egyptian honeys. Hussain (1989) reported that specific gravity on 153 Oman honey samples in the range of 1.357 to 1.446. Johnson*et al.* (2015) reported specific gravity in Natural honey as 1.433 and in Industrial honey, it was found to be 1.427.

2.2.2Electrical Conductivity

The measurement of electrical conductivity (EC) was introduced a long time ago (Corwin & Lesch,2005). At present it is the most useful quality parameter for the classification of unifloral honeys, which can be determined by relatively inexpensive instrumentation. This has been confirmed by the data reportedby Oddo *et al*.,(2004). On the basis of an extensive survey of EC values on honeys originating from different parts of the world (Bogdanov*et al.,* 2004), this parameter was included recently in the new international standards for honey (Bogdanov*et al.,* 2004), replacing the determination of ash content. Indeed, EC correlates well with the mineral content of honey(Bogdanov*et al.,* 2004). In these standards maximal EC values for blossom honeys (except for chestnut honey) are introduced for differentiation between honeydew and blossom honey.

According to the European Legislation, blossom honeys have electrical conductivity values below 0.80 ms/cm, while the honeydew honeys exceeded this value (Manzanares*et al.,* 2011). However, there are many exceptions to this rule. Some mono-floral honeys such as those from chestnuts, strawberry plants, heather, eucalyptus, lime, manuka tea tree or jelly bush honeys, regarded as blossom honeys, often have electrical conductivity values above 0.8 ms/cm (Oddo *et al.,*2004). Habib *et al.* (2014) reported of electrical conductivity in Arid regions honey sample range from 0.25 to 0.69mS/cm. Ali*et al.* (2014) reported electrical conductivities in various flora honey of Tunisia with range from 0.39 to 0.89mS/cm. Andualem (2014) reported of electrical conductivity in honey 0.488 to 3.27mS/cm. Ahmed *et al.* (2014) showed that in Algeria honeys electrical conductivity ranged from 0.23 to 1.52mS/cm. Oshomah *etal.* (2015) got electrical conductivity mean value of 0.61 mS/cm in commercial honeys from EDO state, Nigeria. Johnson*et al.* (2015) reported of Electrical conductivity in natural honey to be 0.35 and Industrial

honey 0.27mS/cm.Somanathan*et al.* (2015) reported of optical density in natural honey as 1.061 and Industrial honey as 1.056.

2.2.3Sugar content

Honey is mainly a solution of glucose and fructose with other minor constituents. There are great variations in the sugar composition of honey. Alghamdi*et al.* (2020) analyzed several hundred honey samples for sucrose content. It was found that the majority of samples contained less than 5 per cent sucrose. Dong*et al.*(2021) reported the average sugar composition to be glucose (32.29%), fructose (39.28%), sucrose (1.62%) and higher sugars (1.03%). Mairaj*et al.* (2008) reported that sample of Pakistan honey had reducing sugars 71.1-76.9 per cent (fructose 39.01-53.8%, glucose 27.7-34.2%) and sucrose 1.9-2.75%. Honey contain a number of components that act as preservatives; these include ascorbic acid, flavonoids, and other phenolics and enzymes such as glucose oxidase, catalase, and peroxidase (Nagai*et al.,* 2001). The monosaccharides, fructose and glucose, are the main sugars found in honey (Ouchemoukh et al., 2007).

The composition of honey has a role on the HMF formation kinetics (Turhan*et al.,* 2008). Glucose and fructose together correspond for 85–95% of honey carbohydrates and their amounts depend on the honey source (Cavia *et al.,* 2002) subsequently, composition of honey as well as storage conditions affects both crystallization and HMF formation. Analysis of 175 Bulgarian honeys by Ivanov and Khan (2000) showed that amount of invert sugar varied from 62.20 to 77.76 % and total sugar content ranged from 68.98 to 79.80 %. Dumté (2010). reported that amount of glucose plus fructose as 72.3 %, sucrose as 5.55 % in Italian honeys. Mitchell,

(2004)reported reducing sugars values ranged from 0 to 4.81 %. There values were within the limits of the Japanese standard: not less than 65 per cent and not more than 5 %, respectively.

White (1975) recorded 79.59, 69.47 and 10.12 % total sugars, reducing sugars and other sugars such as sucrose, maltose and other disaccharides respectively. Thirteen honey samples produced in Taiwan analyzed by Chen*et al.* (2019) showed that invert sugar ranged from 66 to 77, glucose 30 to 36, fructose 34 to 40 and sucrose 0.1 to 5.7 %. The invert sugar content ranged from 65 to 74 %. The ratio of glucose to fructose was 1.3:1.1 in liquid honeys, 1.1:1.0 in soft honeys and 1.0:1.1 in solid honeys.

2.2.4 5-hydroxymethylfurfural

Fresh honey does not contain hydroxymethylfurfural (HMF). Thus, HMF is not a criterion for the botanical classification of honey. However, before determining storage-dependent parameters like enzyme activity and colour, one should ensure that honeys are fresh and unheated. Before testing these parameters, it should be checked that the HMF content is below 15 mg/kg. Three methods for the determination of HMF are described and validated by the IHC (Bogdanov et al., 2004). Only two of them are recommended for use: the HPLC and the White method. The Winkler method should not be used because one of the reagents (p-toluidine) is carcinogenic. Since the publication of the IHC methods there is a change in the procedure of the HMF determination by HPLC: a Carrez treatment of the honey solution is necessary in order to prevent 5-HMF break-down (Kgozeimeh*et al*.,2014).

Turhan*et al.* (2018) showed that HMF content can serve as an indicator showing the influence of heat on honey during bottling and storage. Out of the 1724 samples of honey from Germany

analyzed by him it was found that samples lying in range between 0 to 15 ppm and 5.4 per cent had greater than 40 ppm HMF. They found that light honeys had less HMF than dark honeys. Mohammed(2015) determined HMF content in 400 samples of honey obtained direct from beekeepers (group A) and in 400 honeys commercially available in Italy group B, samples (average 13.4 mg/kg), all contained less than the maximum of 40 mg/kg allowed by the European Codex, but in group B (average 59.6 mg/kg), only 292 samples confirmed to the Cordex, and 24 samples contained more than 100 mg/kg.

White (1980) reported that HMF in honey samples from U.S.A. resulted from heating which could affect the validity of tests for HMF as an indicator of adulteration with invert syrup. It was suggested that a level of 20 mg HMF/100g honey would allow differentiation of honeys possibly adulterated with invert syrup (HMF content 170-650 mg/100g from normally stored and processed (4 mg/100g).

Bricage (1989) reported that 16 out of the 88 honey samples exceeded the acceptable limit of 40 mg/kg from French honey while 35 out of 38 honeys sold directly by local beekeepers had a 5-HMF content below 15 mg/kg. Imported honeys were mainly of quality equivalent to that of locally sold French honeys; 18 of 32 samples had an HMF content of below 15 mg/kg. Balenovic *et al.* (1988) determined HMF content in 22 samples of honey obtained directly from apiaries and 22 samples of industrial honey of the 44 honeys, 54.5 percent met the standard requirements (HMF should not be > 40 mg/kg). In general, apiary honey was of a higher quality than industrial honey.

Vázquez*et al.* (2017) reported the absence of HMF in the fresh Himachal honeys. Thompson *et al.* (2003) investigated the effect of the treatment (in the “Easy Bee” method of extracting

granulated honey, the combs are put in a stainless-steel melting trey which is heated by hot water) on the HMF level. Torre (1991) reported results for 38 samples; mean HMF content was 16.9 gm/kg; all except 8 samples contained less than 40 mg/kg. Ghoshdastidar and Chakrabarti (1992) reported nil – 12.0 ppm HMF in the honey samples received from the Central Bee Research Institute, Pune. Gomez *et al.* (1993) carried out physico-chemical analysis of 25 Spanish Commercial Eucalyptus honey and reported an average value of 3.63 ppm for HMF. Several techniques were proposed to determine metallic impurities in honey, but in most cases, a matrix mineralization is required. Habib *et al.* (2014) reported of HMF in Arid regions honey samples to range from 0.17 to 79.26mg/kg. Ali*et al.* (2014) reported of HMF in various flora honey of Tunisia range from 12.07 to 27.43mg/kg. Ambily*et al.* (2015) reported HMF value in unbranded & branded honey in the range of 2.41 to 2.86% & 9.73 to 22.88% respectively. Ahmed *et al.* (2015) reported HMF in honey in the range of 3.8 to 21.4 mg/kg.

2.2.5 Transition metals

The largely preferred analytical approaches to the determination of transition metals in honey are spectroscopy techniques (Muñoz& Palmero, 2006). For metal ion analysis, electrochemical techniques are potentially the cheapest and quickest method of carrying out a determination when compared with instrument technique such as atomic absorption spectroscopy and inductively coupled plasma (Muñoz& Palmero, 2006). Potassium is the major metal, followed by calcium, magnesium, sodium, sulphur and phosphorus. Trace elements include iron, copper, zinc and manganese. Many trace elements in different types of bee honeys, have been analyzed (Rashed & Soltan, 2004) and new methods for their determination have been developed(Rashed & Soltan, 2004). Gonzalez-Miret *et al.* (2005) by multiple linear regression analysis found and

established significant correlations and equations relating honey lightness and colour to the mineral content.

The application of multivariate analysis to the general physicochemical parameters, minerals, trace elements, and sugars has been used to differentiate types of monofloral honeys, honeydew and blossom honeys over the last few decades (Terrab *et al.*, 2002; Terrab *et al.*, 2003; Marini *et al.*, 2004).

Anklam (1998) published a review of the analytical methods used to determine the geographical and botanical origins of honey. Such methods include the determination of amino acids and proteins, aroma compounds, sugars, enzyme activity, fermentation products, flavonoids, organic acids, phenolic compounds, pollen analysis, minerals and trace elements, and specific stable isotopic ratios. Habib *et al.* (2014) reported of total protein in arid regions honey samples to range from 0.2 to 0.57%.

2.3Analytical Methods Validation

Validation is the process of establishing documentary evidence demonstrating that a procedure, process, or activity carried out in testing maintains the desired level of compliance at all stages. In the pharmaceutical industry, it is very important that in addition to final testing and compliance of products, it is also expected that the process will consistently produce the expected results (Kakad*et al.*, 2020).

The process of validation of analytical method is adopted to confirm that the employed analytical procedure for a specific test meet the intended requirements. Guidelines from the USP, ICH, FDA etc., can provide a framework for validations of pharmaceutical methods. Results from the method validation can be considered to judge its quality, reliability as well consistency

pertaining to analytical results (Krull, 2003). In pharmaceutical industry, the reasons for validating assay are the integral part of the quality-control and regulatory requirement (ICH, 2005). Analytical methods have been validated in pursuance of ICH guidelines (2005), these are Validation parameters which include linearity, range, precision, accuracy, robustness, ruggedness, limit of detection and limit of quantification:

2.3.1 Linearity and Range

The linearity of a method is a measure of how well a calibration plot of response against concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity (ICH, 2005).

2.3.2Precision

The precision of an analytical procedure represents the nearness of agreement between a series of measurements got from multiple sampling of the same homogenous sample under the similar analytical conditions and it is divided into 3 categories:

i. Repeatability: precision under same operating conditions, same analyst over a short period of time.
ii. Intermediate precision: method is tested on multiple days, instruments, analysts etc.
iii. Reproducibility: inter-laboratory studies.

The ICH (2005) guidelines suggest that repeatability should be conformed duly utilizing at least 9 determinations with specified range for the procedure (e.g., three concentrations / three

replicates each) or a minimum of 6 determinations at 100 % of the test concentration (ICH, 2005).

2.3.3Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies (ICH, 2005). There are three ways to determine accuracy:

i. Comparison to a reference standard.
ii. Recovery of the analyte spiked into blank matrix.
iii. Standard addition of the analyte. It should be clear how the individual or total impurities are to be determined.

2.3.4Limit of Detection

Limit of detection is the minimum level at which the analyte can reliably be detected, but not necessarily quantitated as precise value, under the stated experimental conditions (ICH, 2005). The detection limit is generally expressed in the concentration of analyte (ppm) in the sample. A number of approaches are recommended by the ICH for determining the detection limit of sample, depending on instrument used for analysis, nature of analyte and suitability of the method (ICH, 2005). The acceptable approaches are;

i. Visual evaluation.
ii. Signal-to-noise ratio.
iii. Standard deviation of the response.

iv. Standard deviation of the slope of linearity plot.

2.3.5Limit of Quantitation

Limit of quantitation is the least concentration of drug in a sample which is estimated with appropriate precision and accuracy under the affirmed experimental conditions (ICH, 2005).

i. Visual evaluation.

ii. Signal-to-noise ratio.

iii. Standard deviation of the response.

iv. Standard deviation of the slope of linearity plot.

2.3.6Robustness

Robustness is defined by the measure of the capability of an analytical method to stay unchanged by small deliberate changes in method parameters. The variable method parameters in HPLC technique may involve flow rate, column temperature, sample temperature, pH and mobile phase composition (ICH, 2005).

Joseph *et al.* (2019) developed a simple, rapid, accurate, and less expensive spectrophotometric method for the quantitation of 5-hydroxymethylfurfural (5-HMF) levels in canned malt drinks and fruit juice drinks sampled in the Kumasi Metropolis, Ghana. The quantitation is based on the selective maximum absorption of ultraviolet radiation by 5-HMF at the wavelength (λ_{max}) of 284 nm using acetonitrile: water (50: 50 v/v) as the solvent system. The method was established to be specific, precise, and accurate over a concentration range of 0.001 mg/ml to 0.02 mg/ml. 5-HMF levels in fruit juice samples (A1 to A10) were between 0.132 mg/ml and 0.438 mg/ml, and

these levels were shown to be comparable (t = 2.200; p = 0.0553) to the contents in the canned malt samples (M1 to M10) which were between 0.3140 mg/ml and 0.7170 mg/ml. The study failed to show any dependence of 5-HMF levels on the composition of the product as well as the manufacturing process adopted. The length of storage did also not significantly affect the 5-HMF levels in the products.

Jalili and Farzaneh (2015) reported the mean recovery values ranged from 84.4 to 105.8%. Varying amounts (11.42-929 mg kg-1) of HMF were found in 48 out of the 70 (87%) samples analyzed. Thepercent Relative Standard Deviation (% RSD) was found to be 3.3 % and 7.5 % for spiking of milk and canned fruit at 100 and 50 mg/kg respectively. The LOD and LOQ were 0.02 and 0.06 mg/kg, respectively.

Hameed *et al* (2019) validated the HPLC method for Hydroxymethylfurfural (HMF) and furosine in milks, the intra-day and inter-day relative standard deviation (RSD %) were 5.9% and 4.1% for 5-HMF and furosine, respectively. The recovery rates were 85% and 91% and the LOD values for 5-HMF and furosine were 0.05 and 0.02 ppm, respectively.

Ibrahim *et al* (2011) developed an accurate, simple, sensitive and selective reversed phase liquid chromatographic method for the determination of ebastine in pharmaceutical preparations. The proposed method depends on the complexation ability of the studied drug with Zn^{2+} ions. Reversed phase chromatography was conducted using an ODS C18 (150 × 4.6 mm id) stainless steel column at ambient temperature with UV-detection at 260 nm. A mobile phase containing 0.025% w/v Zn^{2+}in a mixture of (acetonitrile/methanol; 1/4) and Britton Robinson buffer (65:35, v/v) adjusted to pH 4.2, has been used for the determination of ebastine at a flow rate of 1

mL/min. The calibration curve was linear over the concentration range of 0.3 - 6.0 µg/ml with a detection limit (LOD) of 0.13 µg/ml, and quantification limit (LOQ) of 0.26 µg/ml.

Ivana *et al*(2008) developed and validated asimple and accurate UV-spectrophotometric method for the estimation of phenylephrine hydrochloride inpharmaceutical nasaldrops formulations. Phenylephrine hydrochloride was estimated at 291 nm in 1 mol/dm^3 sodium hydroxide (pH 13.5). Beer's law was obeyed in the concentration range of 10–100 µg/cm^3 (R^2 = 0.9990) in the sodium hydroxide medium. The apparent molar absorptivity was found to be 1.63×103 $dm^3mol^{-1}cm^{-1}$. Themethod was tested and validated for various parameters according to the ICH (International Conference on Harmonization) guidelines. The detection and quantitation limits were found to be 0.892 and 2.969 µg/cm^2, respectively. The proposed method was successfully applied for the determination of phenylephrine hydrochloride in pharmaceutical nasal drops formulations. The results demonstrated that the procedure is accurate, precise and reproducible (relative standard deviation < 1 %), while being simple, cheap and less time consuming, and hence can be suitably applied for the estimation of phenylephrine hydrochloride in different dosage forms.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Area

Kachia is one of the LGAs in the southern part of Kaduna state. It is bounded in the north by Chikun and Kajuru LGAs, in the east by Zango Kataf LGA and the south by Kagarko and Jaba LGAs. Kachia LGA is located between latitude 7 °32' and 10 °10′N and longitude 7°00′E and 8°05′E.The LGA is divided into three main relief features: the western zone comprised mainly of plains and lowland areas with 602 m above the sea level as the highest peak, while the central part comprised of upland areas with elevation ranging between 602 m and 676 m above the sea level. The eastern margins of the LGA are more strongly dissected with elevation ranging from 671 m to 926 m. There are three major rivers in Kachia LGA. The largest of these rivers is the River Bishimi which is located in the southern extreme of the LGA. At the middle of the LGA is River Amful, which drains the central as well as the western parts of the LGA. River Sarkin Pawa forms a natural boundary between Kachia LGA and Chikun and Kajuru LGAs in the north.

Figure 3.1 Map of Kaduna State showing Kachia Local Government Area

3.2 Sample Collection

For the purpose of this study, five honey samples (n=5) were collected across the Local Government Area of Kachia, Kaduna State. The honey samples that were collected are the ones harvested between January and September 2019, and werestored at ambient temperature, in the dark, until the experiment. The Samples were labels S1, S2, S3, S4 and S5.

3.3 Equipment and glassware

i. Cary 300 UV-Vis Spectrophotometer (Agilent Technologies)

ii. Sartorius ED224 Digital Analytical Balance

iv. Bench-top pH meter – pH 2700 (Turbo Technology)

v. Beakers

vi. Glass funnel

vii. Pipettes

viii. Volumetric flasks

ix. Whatman filter paper no. 41

x. Spatula

xi. Hand gloves

3.4 Chemicals and Reagents

5-hydroxymethylfurfural ($C_6H_6O_3$) and Ethanolwere purchased from SigmaAldrich(Santa Ana, CA, USA).

Sodium Pyrosulphite ($Na_2S_2O_5$), Carrez solution II: 30 g zinc acetate (Zn $(CH_3COO)_2 \cdot 2H_2O$), Carrez solution I: 15 g potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$), Sodium bisulphite ($NaHSO_3$)and Perchloric acid ($HClO_4$) were purchased from were of analytical reagent grade.

3.5 Preparation of Solutions and Reagents

3.5.0 Preparation of Standard Stock Solution of 5-HMF

Stock solutions of 5-HMF were prepared in MeOH: water (50:50, v/v) at a 1000 mg/L concentration and stored at 4^0C until analysis.

3.5.1Carrez solution I

15 g potassium ferrocyanide (K_4Fe $(CN)_6 \cdot 3H_2O$) was dissolved in distilled water and diluted to 100 mL.

3.5.2 Carrez solution II

30 g zinc acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$) was dissolved in distilled water and diluted to 100 mL.

3.5.3 Sodium bisulfite ($NaHSO_3$) solution

0.20% in 100 mL distilled water.

3.5.4Perchloric acid ($HClO_4$)

8.6 mL perchloric acid was added to 92.4 mL of distilled water and mixed thoroughly.

3.5.5Sodium Pyrosulphite ($Na_2S_2O_5$) solution

0.20% of Sodium pyrosulphite was prepared using 100 mL distilled water.

3.6 Physicochemical Analysis

Physicochemical properties of honey were analyzed in order to verify honey quality. The following techniques were used: determination ofpH, Moisture content, specific gravity and electrical conductivity.

3.6.1 Determination of pH

This was carried out using a pH meter. The honey samples were heated in a water-bath at 27^0C and stirred until it become homogenous. Four grams of honey was accurately weighted into a beaker and 20 mL of deionized water was added. The samples werestirred using magnetic stirrer and the exact temperature was recorded. All samples were measured in triplicate and the average pH value was calculated(Bogdanov*et al.,* 2002).

3.6.2Determination of conductivity

The electrode was rinsed with deionized water, wiped and dipped into each of the honey sample and left for some time for reading to stabilize. The reading displayed on the screen was then recorded in micro Siemens per centimeter (*u*S/cm) (Bogdanov*et al.,* 2002).

3.6.3 Determination of specific gravity (Sp.gr)

The specific gravity of honey was determined as the ratio of the mass of honey to the mass of equal volume of water. The density of the honey was determined using a measuring cylinder. A 10 mL measuring cylinder was cleaned, dried and weighed (W_0) and then filled with the honey

sample and reweighed to give (W_1). The measuring cylinder was empty, washed, dried and was substituted with distilled water and reweighed to give (W_2)(Bogdanov*et al.,* 2002).

The specific gravity (Sp.gr) wasthen calculated from the expression:

$$\text{Sp.gr} = \frac{mass\ of\ the\ substance}{mass\ of\ an\ equal\ volume\ of\ water} = \frac{W_1 - W_0}{W_2 - W_0}$$

3.6.4Determination of moisture content

The moisture content of the honey samples was determined by hot oven method. The crucibles were washed, dried and their weighted were obtained. 10 g of each of the honey samples was measured. Each sample in a crucible was dried in an oven at 80^0 C for 2 hours and at 100^0C for the next 4 hours until the weighted was constant. The samples were cooled in the desiccators and the dried weighted of the sample plus crucible was taken(Bogdanov*et al.,* 2002).

The percentage moisture content was calculated as:

Percentage (%) moisture content = B-C/A x 100

Where A = Sample weight before drying,

B = Weight of crucible + sample prior to drying

C = Weight of crucible + sample after drying

B-C = Loss in weight of sample after drying.

3.7 Determination of 5-hydroxymethylfurfural

The spectrophotometric method that was used for 5-hydroxymethylfurfural determination was described by White and it is called “White Method”. It involves measurement of UV absorbance of aqueous honey solutions with and without bisulphite (Bogdanov*et al.,* 2005).

3.7.1 Determination of 5-Hydroxymethylfurfural usingThe White’smethod.

Five gramsof honey samples were weighed into a 50 mL beaker. The sample was dissolved in approximately 25 mL of distilled water and transferred quantitatively into a 50 mL volumetric flask. 0.5 mL of Carrez solution I was added and mixed. 0.5 mL of Carrez solution II was added and mixed. The solution was diluted to volume with distilled water (a drop of ethanol was added to suppress foaming) and followed by filtration. 5.0 mL of the solution was pipetted into each of the two test tubes. 5.0 mL of water was added to one of the test tubes and mixed well. Again, 5.0 mL of sodium bisulphite solution was added to the second test tube and mixed well (the reference solution). The absorbance of the sample solution against the reference solution at 284 and 336 nm in 10 cm quartz cells were determined (Bogdanov*et al.*,2005).

3.7.2 Determination of 5-Hydroxymethylfurfural using themodified method

Five grams of honey sample were weighed into a 50 mL beaker. The sample was dissolved in approximately 25 mL of water and transferred quantitatively into a 50 mL volumetric flask. 2 mL of ice-cold Perchloric acid was added and mixed. The sample solution was placed on ice for 5 minutes and followed by filtration. 0.5 mL of neutralization solution was added to the sample solution and mixed to neutralize the sample and precipitate excess Perchloric acid.

5.0 mL of the solution was pipetted into each of the two test tubes. 5.0 mL of water was added to one of the test tubes and mixed well. Again, 5.0 mL of sodium pyrosulphite solution was added to the second test tube and mixed well (the reference solution) in which the 284 nm chromophore of HMF was destroyed. The absorbance of the sample solution against the reference solution at 284 and 336nm in 10 cm quartz cells was determined(Bogdanov*et al.*, 2002).

The Hydroxymethylfurfural content of honey was calculated using the following equation:

5-HMF (mg/kg of honey) $= \frac{(A284 - A336)x\ 74.87}{W}$

Where; W = weight of Sample (g)

A284, A336 = Absorbance reading at 284 and 336 nm

Factor = $\frac{126 \text{ x } 100 \text{ x } 1000 \text{ x } 100}{16830 \text{ x } 1000} = 74.87$

Where;126 = Molecular Weight of HMF

16830 = Molar absorptivity of HMF at 284 nm

The results were expressed in mg/kg (IHC, 2005)

3.8Validation of the Analytical Method

The Modified method was validated according to International Conference on Harmonization (ICH)2005 guide lines for validation of analytical procedures in order to determine the linearity, precision, and accuracy, limit of detection, limit of quantification, ruggedness and robustness.

3.8.1 Linearity and range

Standard solution was prepared at five concentrations, typically 5, 10, 15, 20 and 25 mg/kg. The absorbance of the 5-Hydroxymethylfurfural were measured at 284 nm and 336 nm and the concentration of 5-HMF was calculated. Thedata were used for calibration plot. Limit of detection (LOD) and limit of quantification (LOQ) for the assay were calculated from standard deviation of y-intercept (ICH, 2005).

The LOD was calculated using the formula;

LOD = 3.3 δ/S

Where δ = standard deviation of intercepts of calibration curves.

S = the slope of linearity plot

The LOQ was calculated using the formula

LOQ = 10 δ/S

Where δ = standard deviation of response.

S = Mean of slopes of the calibration curves.

3.8.2 Precision-repeatability

The precision of the procedures was determined by repeatability (intra-day) and intermediate precision (inter-day).One sample solution containing the target level of analytes was prepared. Eight replicates were made from sample solution and analyzed according to the final method procedure. The absorbance of reference and sample solutions were recorded; the concentration of 5-hydroxymethylfurfural, mean, standard deviation, and relative standard deviation were also calculated (ICH, 2005)

The relative standard deviation (RSD %) was calculated using the formula;

RSD % = SD/X × 100

Where S is the standard deviation and X is the mean.

3.8.3 Intermediate precision

Intermediate precision (within-laboratory variation) was demonstrated by two analysts, using two Spectrophotometric systems on two different days. The absorbance of reference and sample solutions were recorded; the concentration of 5-hydroxymethylfurfural, mean, standard deviation, and relative standard deviation were also calculated (ICH, 2005).

The relative standard deviations were calculated using the formula;

RSD % = SD/X × 100

Where S is the standard deviation and X is the mean.

3.8.4 Accuracy

The accuracy of the method was evaluated with the recovery of the standards from excipients. Spike sample was prepared at three difference concentrations over the range of 50 to 150 % of the target concentration. The mean concentration, standard deviation, Relative standard deviation and percent recovery were calculated (ICH, 2005).

The percentage recovery was computed using the formula:

$$\% \text{ Recovery} = \frac{(\text{spike sample result} - \text{unspiked sample result}) \times 100\%}{\text{known spike added concentration}}$$

The average of these determinations was taken as the percentage recovery.

3.8.6 Ruggedness

Ruggedness analysis was carried out by two different analysts. One sample containing the target level of analytes was prepared andfive replicates were made from sample solution and analyzed according to the final method procedure. The respective absorbancewas recorded and 5-HMF concentrations was calculated(ICH, 2005).

3.8.7 Robustness

Robustness analysis was carried out at four difference wavelengths (284, 283. 336 and 335 nm).Five replicates were made from sample solution and analyzed according to the final method procedure. The respective absorbance and 5-HMF concentration were calculated (ICH, 2005).

3.9 Statistical analysis

All analyses werecarried out in triplicates and the data were presented as means ± standard deviations.Analysis of variance (ANOVA) and student's t-test were used to compare the two method.

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Analysis

The results of pH,electrical conductivity, moisture content and specific gravity are shown inTable 4.1.The pH values for this study are between 3.22 and 4.88 and the rangedis within standard limit (3.5 to 5.5). Sample S4 (3.22) is lower than the pHvalues of sample S1, S2, S3 and S5 (3.76, 3.88, 3.46 and 3.65) which means that it is more acidic than honeys from sample S1¸S2, S3, and S5.The electrical conductivity is within the standard limit (<1.40). There is no significance different in the electrical conductivity of samples S1 and S2.Sample S5 recorded the highest moisture content (9.2) followed by sample S1. All samples are within the Standard limit of ≤20% for moisture content. There was no statisticallysignificant difference in the specific gravity of honey from all the samples.

Table 4.1 Results of pH, Moisture content, Electrical conductivity and Specific gravity of Samples.

Sample	pH	Electrical conductivity (µS/cm)	Moisture (%)	Specific gravity (g/cm^3)
S1	3.76±0.006	0.64±0.014	8.5±0.141	1.35±0.045
S2	3.88±0.010	0.22±0.000	7.8±0.141	1.49±0.014
S3	3.46±0.007	0.39±0.006	5.4±0.283	1.51±0.014
S4	3.22±0.010	0.54±0.006	4.4±0.071	1.44±0.014
S5	3.65±0.007	0.93±0.007	9.2±0.071	1.51±0.014

4.2 Calibration Curves

The calibration curves for determination of 5-HFM at 284 nm and 336 nm obeyed the Beer-Lambert's law as shown in Figure 4.1 and 4.2. The calibration parameters, absorbances and the concentrations for determination of 5-HMF content in honey are listed in Table 4.2, 4.3 and 4.4.The linear relationship between absorbance (y) and concentration (x)in mg/kg gives the regression line. The limit of detection and limit of quantification were calculated by using standard deviation of y-intercept from the two calibration curves recommended by ICH (2005). The LOD and LOQ values for absorbance at 284 nm were 0.12 and 0.36 mg/kg and for the absorbance at 336 nm were 0.06 and 0.17 mg/kg.

Table 4.2 CalibrationParameters

Wavelength of maximum absorption(nm)	284	336
Regression equation	y = 0.1534x - 0.0345	y =0.002x +0.303
Coefficient of determination (R^2)	0.999	0.992
Intercept (a)	- 0.035	0.303
Slope (b)	0.153	0.002
LOD (mg/kg)	0.120	0.060
LOQ (mg/kg)	0.360	0.170

Table 4.3 Results of the calibration curve of 5-hydroxymethylfurfural at 284 nm

Standard	Concentration (mg/kg)	Absorbance (284 nm)
Std. 1	5	0.7005
Std. 2	10	1.5164
Std. 3	15	2.3087
Std. 4	20	3.0253
Std. 5	25	3.7808

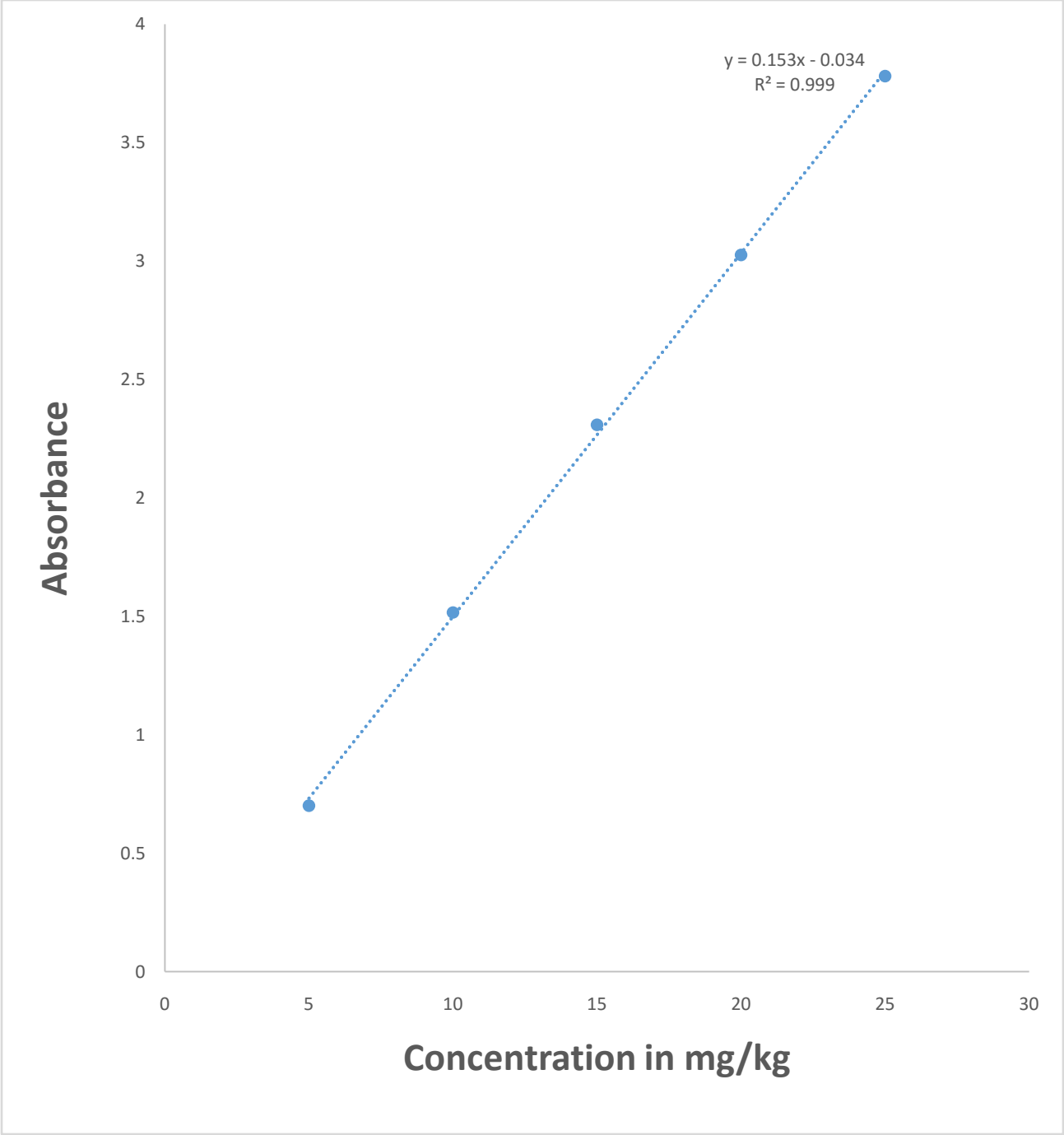

Figure 4.1 Calibration curve of 5–Hydroxymethylfurfural at 284 nm

Table 4.4 Results of the calibration curve of 5-hydroxymethylfurfural at 336 nm

Standard	Concentration (mg/kg)	Absorbance (336 nm)
Std. 1	5.0	0.3142
Std. 2	10	0.3223
Std. 3	15	0.3324
Std. 4	20	0.3413
Std. 5	25	0.3546

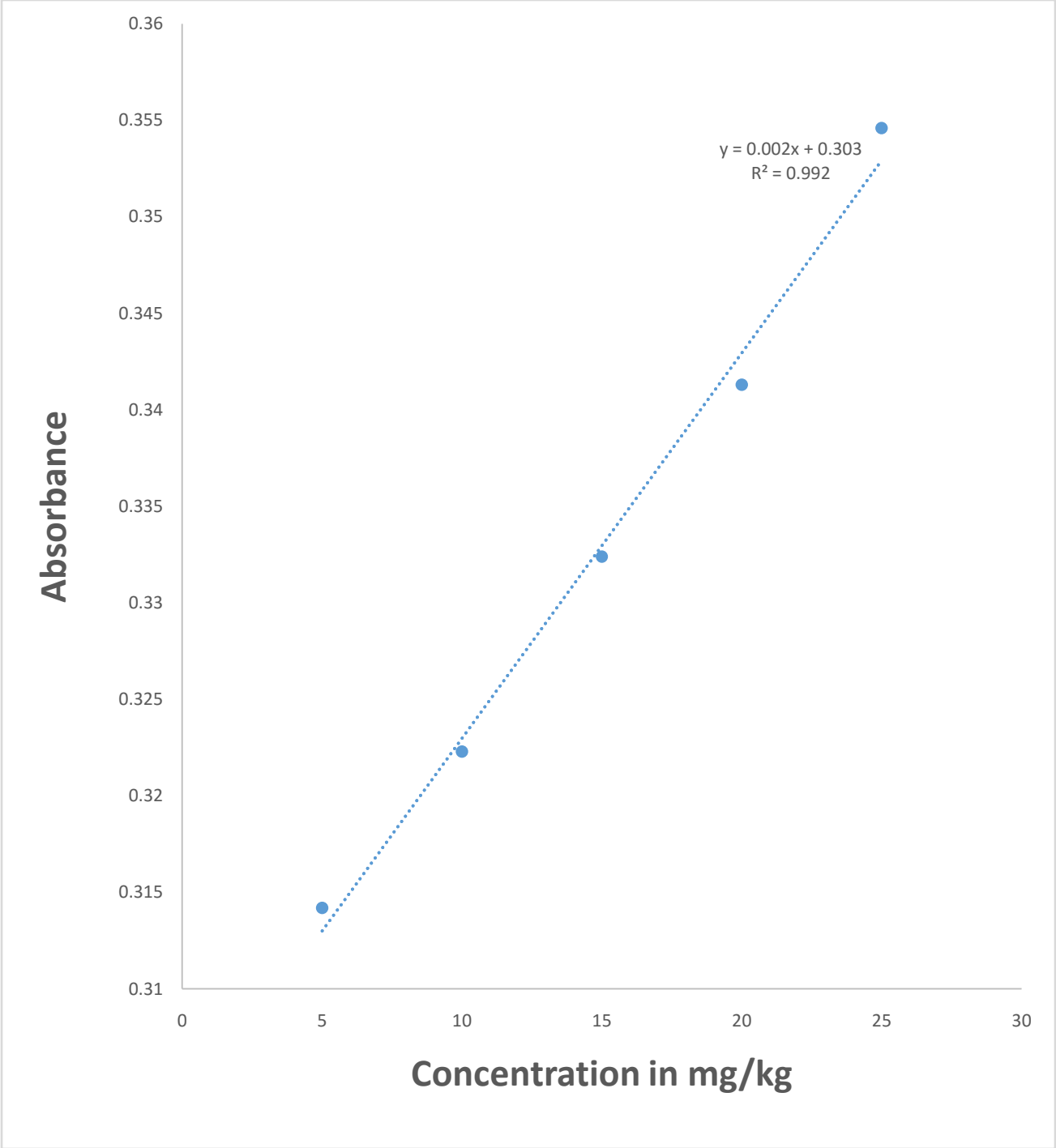

Figure 4.2Calibration curve of 5-hydroxymethylfurfural at 336 nm

4.3 Comparison of results between White Method and Modified Method.

The comparison of results of two separate methods for determining HMF was performed with Analysis of Variance (single factor) and student's t-test (pair two sample for mean) as shown in Table 4.5 and 4.6.

Table 4.5 Comparison betweenthe White's and the ModifiedMethods

Sample	The White's method (mg/kg)	The Modified method (mg/kg)
S1	43.26±0.01	45.93±1.06
S2	52.64±0.14	56.00±0.01
S3	28.13±0.01	32.63±0.01
S4	26.51±0.01	24.17±4.14
S5	54.02±0.00	39.94±0.06
F-statistic	0.89	
F-critical	5.31	
P-value	0.37	
Alpha level	0.05	

Table 4.6Comparison of the reagents used between Modified method and some of the reported methods

Methods	Reagents	Comments
Modified	•Sodium Pyrosulphite ($Na_2S_2O_5$)	Stable, sensitive and not expensive.
	•Perchloric acid ($HClO_4$)	
White	• Sodium bisulphite ($NaHSO_3$)	
	•Carrez solution I: potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$)	Expensive and not readily available
	•Carrez solution II: zinc acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$)	
Winkler	• p-toluidine (C_7H_9N)	Carcinogenic, less sensitive and not readily available
	• Barbituric acid ($C_4H_4N_2O_3$)	
HPLC	• Carrez solution I: 15 g potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$)	Expensive and not readily available
	• Carrez solution II: 30 g zinc acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$)	

4.4 5-hydroxymethylfurfural Determination using The White'sMethod

Sample S5 recorded the highest 5-HMF content (54.02 mg/kg) followed by sample S2 (52.64 mg/kg) and sample S1 (43.26mg/kg) which exceeded the maximum 5-HMF value of ≤40 mg/Kg shown in Table 4.7.5-HMF content for sample S3 (28.13 mg/kg)) and S4 (26.51 mg/kg) were very low. Honey from these two samples met Codex Alimentarius and the European Commission maximum (Codex, 1987; European Commission, 2001).

Table 4.7 Results of 5-hydroxymethylfurfural using White method

Sample	A_{336}(nm)	A_{284}(nm)	HMF (mg/kg)	RSD %
S1	0.0822±0.0001	2.9709±0.0007	43.26±0.01	0.02
S2	0.6033±0.0006	4.2188±0.1414	52.64±0.14	0.27
S3	0.0928±0.0007	1.9720±0.0001	28.13±0.01	0.04
S4	0.3437±0.0001	2.1143±0.0007	26.51±0.01	0.04
S5	0.3981±0.0001	4.0055±0.0001	54.02±0.00	0.00

4.5 Hydroxymethylfurfural determination using The Modified method

Sample S2 recorded the highest HMF content (56.00 mg/kg) followed by sample S1 (45.93 mg/kg) which exceeded the maximum HMF value of ≤40 mg/Kg shown in Table 4.8. 5-HMF content for sample S3 (32.63 mg/kg)), S4 (24.17 mg/kg) and S5 (39.94 mg/kg) were very low. Honey from these three samples met Codex Alimentarius and the European Commission maximum HMF level for honey of ≤40 mg/Kg. (Codex, 1987; European Commission, 2001).

Table 4.8 Results of 5-hydroxymethylfurfural usingModified Method

Sample	A_{336} (nm)	A_{284} (nm)	HMF (mg/kg)	RSD %
S1	0.3949±0.0007	3.4133±0.0006	45.93±1.06	2.31
S2	0.3829±0.0003	4.1225±0.0007	56.00±0.01	0.02
S3	0.2216±0.0001	2.4014±0.0007	32.63±0.01	0.03
S4	0.7155±0.0073	2.3292±0.2836	24.17±4.14	17.13
S5	0.7105±0.0175	3.3629±0.0001	39.94±0.06	2.65

4.6 Validation of the Modified method

The accuracy, precisions, percentage recoveries, ruggedness and robustness for the method are shown in table 4.9. The relative standard deviation (RSD) which are within the acceptable limit of <5 RSD show the precision of the methods (Bhinge *et al.*, 2014). Satisfactory percentage recoveries, ruggedness and robustness were achieved for 5-hydroxymethylfurfural in the method.

Table 4.9Results of validation parameters

Parameter	HMF (mg/kg)	RSD %
Precision-Repeatability	56.12	2.65
Precision-intermediate: Operator 1, Instrument 1, Day 1.	55.76	2.67
Operator 2, Instrument 2, Day 2.	54.82	3.03
Operator 1, Instrument 1, Day 2.	56.85	4.73
Operator 2, Instrument 2, Day 2.	56.36	1.90
Ruggedness: Analyst 1	57.29	1.23
Analyst 2	57.03	1.00
Robustness: At 284 and 336 nm	57.32	0.64
At 283 and 335 nm	57.33	0.42
Recovery (%): At 1 mg/kg	81.4 %	2.65
At 2 mg/kg	84.6 %	0.48
At 3 mg/kg	104.6 %	0.22

4.6.1Precision - repeatability

Repeatability (intra-day precision) was carried out by eight consecutive analyses of samples. One sample solution containing the target level of analytes was prepared. Eight replicates were made from sample solution and analyzed according to the final method procedure.The mean concentration, standard deviation and relative standard deviation were calculated to be 56.72 mg/kg, 1.49 and 2.65 %.

Table 4.10 Results obtained from repeatability studied for evaluation of HMF

Analysis No.	A_{336}	A_{284}	HMF (mg/kg)
Replicate 1	0.3178	3.9720	54.72
Replicate 2	0.3470	4.1383	56.77
Replicate 3	0.3780	4.1939	57.14
Replicate 4	0.5011	4.3955	58.31
Replicate 5	0.3039	4.0798	56.54
Replicate 6	0.3193	3.9776	54.78
Replicate 7	0.3282	3.9276	53.90
Replicate 8	0.3577	4.1511	56.80
Mean	0.36	4.10	56.12
SD	0.063	0.15	1.49
RSD	1.70	3.70	2.65

4.6.2Intermediate Precision

Intermediate precision (within-laboratory variation) was demonstrated by two analysts, using two Spectrophotometric systems on two different days. The calculated mean concentration, standard deviation and relative standard deviation were recorded in Table 4.11, 4.12, 4.13 and 4.14.

Table 4.11 Results obtained from intermediate precide studied for evaluation of 5- HMF from Operator1, Instrument 1 and Day 1.

Analysis No.	A_{336}	A_{284}	HMF (mg/kg)
Replicate 1	0.3341	4.0131	54.45
Replicate 2	0.3034	4.1120	57.03
Replicate 3	0.3218	3.9114	53.75
Replicate 4	0.2998	4.0230	55.75
Replicate 5	0.3184	4.1281	57.05
Replicate 6	0.3210	3.9801	54.79
Replicate 7	0.3301	4.2034	58.09
Replicate 8	0.3204	4.0181	55.37
Mean	0.32	4.049	55.76
SD	0.012	0.093	1.49
RSD %	3.70	2.30	2.67

Table 4.12 Results obtained from intermediate precide studied for evaluation of 5- HMF from Operator2, Instrument 2 and Day 1.

Analysis No.	A_{336}	A_{284}	HMF (mg/kg)
Replicate 1	0.3214	3.9114	53.76
Replicate 2	0.3102	4.0121	55.43
Replicate 3	0.3181	4.0213	55.45
Replicate 4	0.3411	4.2201	58.08
Replicate 5	0.3481	4.0139	54.89
Replicate 6	0.3212	3.8223	52.43
Replicate 7	0.3013	3.8935	53.79
Replicate 8	0.3311	3.9873	54.75
Mean	0.32	3.98	54.82
SD	0.055	0.12	1.66
RSD %	2.97	2.98	3.030

Table 4.13 Results obtained from intermediate precide studied for evaluation of 5- HMF from Operator1, Instrument 1 and Day 2.

Analysis No.	A_{336}	A_{284}	HMF (mg/kg)
Replicate 1	0.3210	4.1231	56.93
Replicate 2	0.3133	4.0116	55.38
Replicate 3	0.2991	4.2512	59.18
Replicate 4	0.3039	3.8912	53.71
Replicate 5	0.3113	4.0017	55.26
Replicate 6	0.2898	4.3145	60.27
Replicate 7	0.3033	4.3181	60.12
Replicate 8	0.3312	3.9349	53.96
Mean	0.31	4.11	56.85
SD	0.013	0.17	2.69
RSD %	4.21	4.17	4.73

Table 4.14 Results obtained from intermediate precide studied for evaluation of 5- HMF from Operator2, Instrument 2 and Day 2.

Analysis No.	A_{336}	A_{284}	HMF (mg/kg)
Replicate 1	0.3119	3.9910	55.09
Replicate 2	0.3013	4.1019	56.91
Replicate 3	0.3314	4.0171	55.19
Replicate 4	0.2998	4.0315	55.88
Replicate 5	0.3370	4.2109	58.01
Replicate 6	0.3040	4.0935	56.72
Replicate 7	0.2809	3.9963	55.63
Replicate 8	0.3297	4.1643	57.42
Mean	0.31	4.075	56.36
SD	0.019	0.081	1.07
RSD %	1.18	1.99	1.90

4.6.3Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients. The concentration of unspiked was 54.67 mg/kg. The mean concentrations of the spiked (58.74 mg/kg, 58.91 mg/kg and 59.90 mg/kg), standard deviation (1.15, 0.28 and 0.13), relative standard deviation (2.65, 0.48 and 0.22 %) and the percent recoveries (81.4, 84.6 and 104.6 %) for the spiked samples were recorded as shown in Table 4.15.

Table 4.15 Results obtained from accuracy studied for evaluation of 5-HMF in honey

Levels %	Concentration added(mg/kg)	Concentration found(mg/kg)	Recovery %	RSD %
50 %	1	58.74±1.15	81.4	2.65
100 %	2	58.91±0.0.28	84.6	0.48
150 %	3	59.90±0.13	104.6	0.22

Mean recovery = 90.2 %.

4.6.4 Ruggedness

The ruggedness was determined by performing the assay with the same condition by two different analyst using different instrument. The mean concentration of 5-HMF and relative standard deviation determined by Analyst 1 are 57.29 mg/kg and 1.23 % are shown in Table 4.16. The mean concentration of 5- HMF and the relative standard deviation (RSD %) determined by Analyst 2 are 57.03 mg/kg and 1.00 %are shown in Table 4.17.

Table 4.16 Result of Ruggedness by Analyst 1.

Concentration (mg/kg)	Absorbance (336)	Absorbance (284)	Statistical analysis
56.82	0.3162	4.2358	
57.77	0.3168	4.1761	Mean = 57.29 mg/kg
57.39	0.3169	4.1511	SD = 0.71
56.35	0.3170	4.0819	RSD = 1.23 %
58.10	0.3165	4.1981	

Table 4.17 Result of Ruggedness by Analyst 2.

Concentration (mg/kg)	Absorbance (336)	Absorbance (284)	Statistical analysis
56.66	0.3150	4.0991	
57.51	0.3150	4.1558	Mean = 57.03 mg/kg
56.21	0.3159	4.0698	SD = 0.57
57.43	0.3170	4.1522	RSD = 1.00 %
57.33	0.3144	4.1434	

4.6.5 Robustness

The robustness analysis was carried out aftera change in wavelength. The mean concentrationof 5-HMF at 336 and 284 nmis 57.32 mg/kg and relative standard deviation is 0.64 % while the mean concentration of 5-HMF and the relative standard deviation (RSD) at 337 nm and 283 nm are 57.33 mg/kg and 0.42 %are shown in Table 4.18 and 4.19.

Table 4.18Result showing Robustness at 336 nm and 284 nm

Concentration (mg/kg)	A_{336} nm	A_{284} nm	Statistical analysis
57.62	0.3044	4.1538	
57.61	0.3038	4.1510	Mean = 57.32 mg/kg
56.91	0.3039	4.1042	SD = 0.37
56.94	0.3031	4.1056	RSD = 0.64 %
57.52	0.3038	4.1452	

Table 4.19Result showing Robustnessat 337 nm and 283 nm

Concentration (mg/kg)	A_{337} nm	A_{283} nm	Statistical analysis
57.50	0.3018	4.1416	
57.18	0.3005	4.1190	Mean = 57.33 mg/kg
57.59	0.3021	4.1483	SD = 0.24
57.40	0.3051	4.1382	RSD 0.42 %
57.00	0.3072	4.1138	

CHAPTER FIVE

5.0 DISCUSSION

5.1Physical Parameters of Honey

The pH values of five honey samples were measured and the results obtained results confirmed that all tested samples were acidic (pH 3.22–3.88) (Table 4.1.) andwithin the standard limit (pH 3.40–6.10) (Codex Alimentations, 2001) that insures honey samples' freshness. The values of pH gotten from this study were more acidic than that the values recorded by Ahmed *etal.* (2014) for Algeria honeys, pH value ranged from 3.7 to 5.1, Sharif*et al.* (2081) reported pH in the range of 3.20 to 4.0 in local and imported honey samples in Pakistan. Rebiai and Lanez (2014) reported 3.80 to 5.24 pH value in different flora honey samples of Algeria, Johnson*et al.* (2015) reported pH value in Natural honey 4.75 and Industrial honey 4.25 and Ambily *et al.* (2015) reported pH value in unbranded honey of 4.13 to 4.56 and branded honey 3.14 to 4.09. The reason might be that the honey samples are from different geographic regions, also, the major botanical origin of the honey was different.

Baroni (2009) added that the pH is a measure that allows the determination of floral or botanical origin of the honey. Published reports indicated that the pH should be between 3.2 and 4.5 (Bogdanov *et al*, 2002). The honey from nectar has a pH between 3.5 and 4.5 and those from honeydew are between 5.0 and 5.5. The pH of honey samples is important during the extraction process because it affects the texture of honey as well as its stability and shelf life (Terrab, *et al.*, 2004). Indirect relationship may exist between pH and electrical conductivity of honey. We have found that honey which has a high electrical conductivity, recording a high pH in some analyzed types of honey, this relationship can give an idea about the origin of honey.

The electrical conductivity is a good criterionof the botanical origin of honey and it is determined in routine honey control instead of the ash content (Adenekan *et al.,* 2010).This measurement depends on the ash and acid content of honey; the higher ash and acid content, the higher the resulting conductivity(Adenekan *et al.,* 2010).The obtained values of electrical conductivity were between 0.22 and 0.93 mS/cm (Table 4.1). The lowest value of the electrical conductivitywas obtained in S2(0.22 mS /cm) and the highest value has been found in honey sample S5 (0.93 mS /cm)There is a linear relationship between the ash content and the EC but there was no significant differencebetween examined samples. According to the European Legislation (European Economic Community, 2002), blossom honeys have electrical conductivity values below 0.80 mS/cm, while the honeydew exceeded this value. However, there are many exceptions to this rule. Some mono-floral honeys such as those from chestnuts, strawberry plants, heather, eucalyptus, lime, manuka tea tree or jelly bush honeys, regarded as blossom honeys, often have electrical conductivity values above 0.8 mS/cm (Oddo *et al*., 2004).

Moisture content of honey is a limiting factor in determination of its quality, stability and spoilage resistance against yeast fermentation. The higher the moisture content is the higher probability of honey fermentation during storage. Lower moisture limits (<20%), elongates honey shelf life which would be met by a large majority of the commercial honeys, have been proposed by some countries for the revision (Codex Alimentations, 2001).The moisture contents obtained ranged from 9.2 % (S5) to 4.4 % (S4) with an average of 6.8 % and are below the maximum limit which is recommended by European standards (Directive Council of the European Union, 2002). Only sample S5 showed slightly higher level of moisture but not exceeding the permitted limit of 20% (Directive Council of the European Union, 2002). This confirms that the fermentation rate is very low in the analyzed samples. Whereas, the lowest

value was 4.4 % in the sample S4. This means that the area is characterized by a warm and dry climate. The moisture content of honey samples in this study was lower than that of Habib *et al.* (2014) reported moisture content in Arid regions honey sample range from 13.63 to 20.60%, Fahim *et al.* (2014) reported of moisture content in Pakistan honey sample range from 13.80 to 16.60%, Ali*et al.* (2014) reported of moisture content in various flora honey of Tunisia range from 17.27 to 19.73%, Ahmed *et al.* (2014) showed that in Algeria honeys, water content ranged from 15.87 to 18.05%.

The specific gravity of five analyzed honey samples range between 1.51 and 1.35. Therefore, all honey samples meet the standards as recommended by U.S.A standards, "A" Grade honey must have specific gravity not less than 1.4155 g/cm^3, Standard honey should have specific gravity more than 1.406 g/cm^3. Sample S1 with specific gravity of 1.35 was below the U.S standard. The specific gravity of sample S2 and S4 agrees with that of Johnson*et al.* (2015), which reported specific gravity in natural honey as 1.433 and in industrial honey 1.427. Sample S1 was below that of Krishna *et al*while sample S3 and sample S5 were higher than that reported byJohnson*et al*. (2015). The variations in the density of honey come mainly from changes in the water content of the honey, chemical composition (dry matter) and temperature. Sample S3 and S5 represents the densest honey with specific gravity of 1.51 g/cm^3 each and Sample S1 represents the less dense Honey with a specific gravity of 1.35 g/cm^3.

5.2 Calibration curves

Linearity for determination of 5-hydroxymethylfurfural in honey obeyed Beer-Lambert's law within the range of 5-25 mg/kg. Correlation coefficient is a statistical measure that calculates the strength of the relationship between the relative movements of two variables (ICH, 2005). The

values of correlation coefficients for 5-HMF were found to be 0.9994 for absorbance at 284 nm and 0.9923 for the absorbance at 336 nm shown in Table 4.2,Figure 4.1 and Figure 4.2. It was observed that the absorbance at 336 nm revealed the smallest correlation coefficient. There was direct relationship and positive correlation between the absorbance and the concentrations used for this evaluation using Modified method. Joseph *et al.* (2019) development and Validated a UV-Visible Spectrophotometricmethod for the determination of 5-hydroxymethylfurfuralcontent in canned malt drinks and fruit juices in the Kubama metropolis Ghana,and reported a coefficient of determination(R^2) to be0.9997. However,this value is higher in comparison to proposed values of 0.9994and 0.9923. The proposed method has a better calibration curves compared to Hameed *et al*. (2019) reported the $R^2 = 0.988$.

5.3 5-hydroxymethylfurfural (5-HMF)

5-Hydroxymethylfurfural is often used as indicator for the quality of honey. 5-HMF is practically absent in fresh and untreated honey and foods, but its concentration tends to rise as a result of heating processes or long-term storage (Fallico *et al.,* 2004).Codex Alimentarius and the European Commission have set a maximum HMF level for honey of 40 mg/kg, except for honeys coming from tropical countries, the HMF limit of which was set at 80 mg/kg(Codex, 1987; European Commission, 2001).

The analysis of the samples revealed that 5-HMF contents in honey are between 26.51 and 54.02 mg / kg using the White's method and 24.17and 56.00 mg/kg using modified method. Samples S1, S2 and S5 in White method and samples S1 and S2 in modified method are above the maximum limit of 40 mg/kg.This means that the warm climate of Kachia may be the cause of this phenomenon or long-time storage of honey but agrees with the standard set for tropical

honey types for which limit is 80 mg / kg (Codex Alimentarius). Samples S3 and S4 in White method and samples S3, S4 and S5 in Modified method agrees with the standard set by Codex Alimentarius and the European Commission that the maximum HMF level for honey is 40 mg/kg(Codex, 1987; European Commission, 2001). The values of 5-HMF gotten from this study agree with the values recorded by Habib*et al.* (2014) for the 5-HMF in Arid regions honey sample range from 0.17 to 79.26mg/kg but disagree with the values reported by Ali*et al.* (2014) for the HMF values in various flora honey of Tunisia range from 12.07 to 27.43mg/kg. The 5-HMF value depends on many factors: pollinated species, soil, race of bees, physiological state of the colony, the type of sugar, its concentration, and duration of storage, temperature and acidity or pH which are not related to the origin of the samples (Anklam, 1998).

5.3 The comparison of results obtained by the two methods

The comparison of both methods was carried out on different honey samples (sample S1, S2, S3, S4 and S5) (Table 4.1.5).As far as White method gave result in the ranged of 28.13 to 54.02 mg/kg, Modified method gave result in the ranged of 24.17 to 56.00 mg/kg. Big difference was found in the sample S5 where the concentration was 54.02 mg/kg by White method and 39.94 mg/kg by modified method, but the difference was very small between the two methods in sample S1, S2, S3 and S4. The p-value (0.374) calculated for the reference and proposed method was greater than the alpha level chosen (0.05). The t-test indicating that there was no statisticallysignificant difference between the mean concentration of 5-HMF determined by Modified method and reference method. Also, the calculated F-test value (0.022) was lower thanthe critical one indicating that there was no significant difference between the two methods.

5.4 Validation parameters

The validation parameters of the method were checked using the International Conference onHarmonization(2005). The precision (repeatability) for the method was 2.65 % while intermediate precision was found to be 2.67, 3.03, 4.73 and 1.90 % respectively for Operator 1-Day 1, Operator 2-Day1, Operator 1-Day 2 and Operator 2-Day 2 respectively. The relative standard deviation is within the acceptable limit andthe low values of the % RSD indicate the repeatability of the modified method (Bhinge *et al*., 2014).Viviane (2012)and Hameed et al. (2019) reported the precisions of 5.41 and 12.5 % respectively. In comparison, these values are higher than the one of the modifiedmethods, which shows that the modified method ismore precise.

Percentage recovery values ranged from 81.4 to 104.6 % for the spiking solution at three different concentrations(1, 2 and 3 mg/kg). The relative standard deviations were found to be 2.65, 0.48and 0.22 % respectively. Jalili and Ansari (2015) reported % RSD of 6.1 and 7.4 % which were relatively higher than the ones reported by this method. The mean concentration of the recovery is 90.2 % and it is within the acceptable ranged (80 to 120 %)which shows that the proposed methodis accurate (Santos-Fandila *eta al*., 2013).

Limit of detection and limit of quantification are the functions of sensitivity of the analytical method. LOD is the lowest quantity of analytes an analytical method can detect but not necessary quantify (Aneta, 2008). The LOD's values were 0.12 and 0.06 mg/kg whichshowed good sensitivity of the method compared to 0.02 and 0.0.15 mg/kg reported by Jalili and Ansari (2015) and Hameed*et al.*, (2019). LOQ is the small quantity of analyte an analytical method can detect

and quantify. The LOQ's values are 0.36 and 0.17 mg/kg, which showed good sensitivity of the method compared to 0.06 and 0.07 mg/kg reported byJalili and Ansari (2015) and Hameed*et al.*,(2019).The LOD and LOQ were found to have small values indicating the sensitivity of the method (ICH, 2005).

The ruggedness of the method was carried out by two different analysts and the respective absorbance and concentrations of 5-HMF were recorded.The relative standard deviation determined by Analysts 1 and 2 were 1.23 and 1.00 % indicating that the method is rugged compared to ICH (2005) guideline. The robustness analysis was carried out to determine the effluence of small but deliberate variation in the wavelength. The relative standard deviationat wavelength 284 nm and 336 nm were0.37%, and 0.24 %respectively. The % RSD of ruggedness and robustness were found to be not more than 2 % which are within the acceptable limit (Table 4.16, 4.17, 4.18 and 4.19) indicating that the method is rugged and robust (ICH, 2005).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The White's method was successfully modified for determination of 5-HMF in honey using perchloric acid and sodium pyrosulphite as replacement for the scarce and expensive Carrez reagents and sodium bisulphite. Satisfactory results were obtained in relation to linearity, precision (repeatability and intermediate precision), accuracy, ruggedness, and robustness, limit of detection (0.12 and 0.06) and limit of quantification (0.36 and 0.17) which showed that the proposed method is suitable for 5-HMF evaluation in honey. From the results obtained, it can be concluded that there was no statistically significant difference between the mean concentrations of 5-HMF of the Modified method and that of the Reference method.

6.2 Recommendations

The modified method gavesatisfactory results for 5-HMF determination in honey and it is recommended as a simple, cheap, precise, accurate, rugged, robust and easily applicable method for determination of 5-HMF content in honey.The overall results demonstrated that the modified method can be applied as an alternative (or complementary) analytical technique to the recommended White's method for total estimation of 5-HMF content in honey.

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Appendix I

Calculations of 5-Hydroxymethylfurfural Content

5-hydroxymethylfurfural content of honey was calculated using the following formula:

$$\text{HMF (mg/kg of honey)} = \frac{(A284 - A336) \times 74.87}{W}$$

Where W = weight of Sample (g)

A284, A336 = Absorbance reading

$$\text{Factor } 74.87 = \frac{126 \times 100 \times 1000 \times 100}{16830 \times 1000}$$

Where126 = Molecular Weight of HMF

16830 = Molar absorptivity of HMF at 284 nm

100 and 1000 are dilution factors

The result is express in mg/kg (IHC, 2002)

Reference method (White method)

Sample one (S1)

i. $\text{HMF} = \frac{(2.9704 - 0.0822) \times 74.87}{5}$

$= 43.25$ mg/kg

ii. $\text{HMF} = \frac{(2.9714 - 0.0823) \times 74.87}{5}$

$= 43.26$ mg/kg

Mean concentration = 43.27 mg/kg

Sample two (S2)

i. $\text{HMF} = \frac{(4.3187 - 0.6037) \times 74.87}{5}$

$= 52.63$ mg/kg

ii. $\text{HMF} = \frac{(4.1188-0.6029)\text{x } 74.87}{5}$

$= 52.65$ mg/kg

Mean concentration = 52.64

Sample three (S3)

i. $\text{HMF} = \frac{(1.9719-0.0923)\text{x } 74.87}{5}$

$= 28.14$ mg/kg

ii. $\text{HMF} = \frac{(1.9721-0.0933)\text{x } 74.87}{5}$

$= 28.12$ mg/kg

Mean concentration = 28.13 mg/kg

Sample four (S4)

i. $\text{HMF} = \frac{(2.1142-0.3436)\text{x } 74.87}{5}$

$= 26.51$ mg/kg

ii. $\text{HMF} = \frac{(2.1143-0.3980)\text{x } 74.87}{5}$

$= 26.50$ mg/kg

Mean concentration = 26.51 mg/kg

Sample five (S5)

i. $\text{HMF} = \frac{(4.0054-0.3980)\text{x } 74.87}{5}$

$= 54.02$ mg/kg

ii. $\text{HMF} = \frac{(4.0055-0.3981)\text{x } 74.87}{5}$

$= 54.02$ mg/kg

Mean concentration = 54.02 mg/kg

Proposed method (Modified method)

Sample one (S1)

i. HMF = $\frac{(3.4127-0.3954)\text{x } 74.87}{5}$

= 45.18 mg/kg

ii. HMF = $\frac{(3.4119-0.2944)\text{x } 74.87}{5}$

= 46.68 mg/kg

Mean concentration = 45.93 mg/kg

Sample two (S2)

i. HMF = $\frac{(4.1230-0.3827)\text{x } 74.87}{5}$

= 56.01 mg/kg

ii. HMF = $\frac{(4.1220-0.3831)\text{x } 74.87}{5}$

= 55.99 mg/kg

Mean concentration = 56.00 mg/kg

Sample three (S3)

i. HMF = $\frac{(2.4009-0.2215)\text{x } 74.87}{5}$

= 32.63 mg/kg

ii. HMF = $\frac{(2,4019-0.2216)\text{x } 74.87}{5}$

= 32.65 mg/kg

Mean concentration = 32.64 mg/kg

Sample four (S4)

i. HMF = $\frac{(2.5297-0.7206)\text{x } 74.87}{5}$

= 27.09 mg/kg

ii. HMF = $\frac{(2.1287-0.7103)\text{x } 74.87}{5}$

= 21.24 mg/kg

Mean concentration = 24.17 mg/kg

Sample five (S5)

i. HMF = $\frac{(3.3629-0.7228)\text{x } 74.87}{5}$

= 39.98 mg/kg

ii. HMF = $\frac{(3.3628-0.6981)\text{x } 74.87}{5}$

= 39.90

Mean concentration = 39.94 mg/kg

Precision – RepeatabilityCalculations

HMF (mg/kg of honey) =$\frac{(\text{A}284-\text{A}336)\text{x } 74.87}{5}$

Replicate 1. = $\frac{(3.9720-0.3178)\text{x } 74.87}{5}$

=54.72 mg/kg

Replicate 2 = $\frac{(4.1383-0.3470)\text{x } 74.87}{5}$

= 56.77 mg/kg

Replicate 3 = $\frac{(4.1939-0.3780)\text{x } 74.87}{5}$

= 57.14 mg/kg

Replicate 4 = $\frac{(4.3955-0.5011)\text{x } 74.87}{5}$

= 58.31 mg/kg

Replicate 5 = $\frac{(4.0798-0.3039)\text{x } 74.87}{5}$

= 56.54 mg/kg

Replicate 6 = $\frac{(3.9776-0.3193)\text{x } 74.87}{5}$

= 54.78 mg/kg

Replicate 7 = $\frac{(3.9276-0.3282)\text{x } 74.87}{5}$

= 53.90 mg/kg

Replicate 8 = $\frac{(4.1511-0.3577)\text{x } 74.87}{5}$

= 56.80 mg/kg

Mean concentrations = 56.12 mg/kg

I**ntermediate Precision calculations**

Operator 1, Instrument 1, Day 1.

Replicate 1 = $\frac{(4.0131-0.3341)\text{x } 74.87}{5}$

= 54.45 mg/kg

Replicate 2 = $\frac{(4.1120-0.3034)\text{x } 74.87}{5}$

= 57.03 mg/kg

Replicate 3 = $\frac{(3.9114-0.3218)\text{x } 74.87}{5}$

= 53.75 mg/kg

Replicate 4 = $\frac{(4.0230-0.2998)\text{x } 74.87}{5}$

= 55.75 mg/kg

Replicate 5 = $\frac{(4.1281-0.3184)\text{x } 74.87}{5}$

= 57.05 mg/kg

Replicate 6 = $\frac{(3.9801-0.3210)\text{x } 74.87}{5}$

= 54.79 mg/kg

Replicate 7 = $\frac{(4.0181-0.3204)\text{x } 74.87}{5}$

= 58.09 mg/kg

Replicate 8 = $\frac{(4.0181-0.3204)\text{x } 74.87}{5}$

= 55.37 mg/kg

Mean concentrations = 55.67 mg/kg

Operator 2, Instrument 2, Day 1.

Replicate 1 = $\frac{(3.9114-0.3214)\text{x } 74.87}{5}$

= 53.76 mg/kg

Replicate 2 = $\frac{(4.0121-0.3102)\text{x } 74.87}{5}$

= 55.43mg/kg

Replicate 3 = $\frac{(4.0213-0.3181)\text{x } 74.87}{5}$

= 55.45 mg/kg

Replicate 4 = $\frac{(4.2201-0.3411)\text{x } 74.87}{5}$

= 58.08 mg/kg

Replicate 5 = $\frac{(4.0139-0.3481)\text{x } 74.87}{5}$

= 54.54.89 mg/kg

Replicate 6 = $\frac{(3.8223-0.3212)\text{x } 74.87}{5}$

= 52.43 mg/kg

Replicate 7 = $\frac{(3..8935-0.3013)\text{x } 74.87}{5}$

= 53.79 mg/kg

Replicate 8 = $\frac{(3.9873-0.3311)\text{x } 74.87}{5}$

= 54.75 mg/kg

Mean concentrations = 54.82 mg/kg

Operator 1, Instrument 1, Day 2.

Replicate 1 = $\frac{(4.1231-0.3210)\text{x } 74.87}{5}$

= 56.93 mg/kg

Replicate 2 = $\frac{(4.0116-0.3133)\text{x } 74.87}{5}$

= 55.38 mg/kg

Replicate 3 = $\frac{(4.2512-0.2991)\text{x } 74.87}{5}$

= 59.18 mg/kg

Replicate 4 = $\frac{(3.8912-0.3039)\text{x } 74.87}{5}$

$$= 53.71 \text{ mg/kg}$$

$$\text{Replicate 5} = \frac{(4.0017 - 0.3113)\text{x } 74.87}{5}$$

$$= 55.26 \text{ mg/kg}$$

$$\text{Replicate 6} = \frac{(4.3145 - 0.2898)\text{x } 74.87}{5}$$

$$= 60.12 \text{ mg/kg}$$

$$\text{Replicate 7} = \frac{(4.3181 - 0.3033)\text{x } 74.87}{5}$$

$$= 60.12 \text{ mg/kg}$$

$$\text{Replicate 8} = \frac{(3.9349 - 0.3312)\text{x } 74.87}{5}$$

$$= 53.96 \text{ mg/kg}$$

Mean concentrations = 56.83 mg/kg

Operator 2, Instrument 2, Day 2.

$$\text{Replicate 1} = \frac{(3.991 - 0.3119)\text{x } 74.87}{5}$$

$$= 55.09 \text{ mg/kg}$$

$$\text{Replicate 2} = \frac{(4.1019 - 0.3013)\text{x } 74.87}{5}$$

$$= 56.91 \text{ mg/kg}$$

$$\text{Replicate 3} = \frac{(4.0171 - 0.3314)\text{x } 74.87}{5}$$

$$= 55.19 \text{ mg/kg}$$

$$\text{Replicate 4} = \frac{(4.0315 - 0.2998)\text{x } 74.87}{5}$$

$$= 55.88 \text{ mg/kg}$$

Replicate 5 = $\frac{(4.2109-0.3370)\text{x } 74.87}{5}$

= 58.01 mg/kg

Replicate 6 = $\frac{(4.0935-0.3040)\text{x } 74.87}{5}$

= 56.72 mg/kg

Replicate 7 = $\frac{(3.9963-0.2809)\text{x } 74.87}{5}$

= 55.63 mg/kg

Replicate 8 = $\frac{(4.1643-0.3297)\text{x } 74.87}{5}$

= 57.42 mg/kg

Mean concentrations = 56.36 mg/kg

Percentage Recovery calculations (% R)

$$\% \text{ Recovery} = \frac{(\text{spike sample result} - \text{unspiked sample result})\text{x } 100\%}{\text{known spike added concentration}}$$

Level 1

$$\% \text{ Recovery} = \frac{(58.74-54.67)\text{x } 100\%}{5}$$

% R = 81.4 %

Level 2

$$\% \text{ Recovery} = \frac{(58.91-54.67)\text{x } 100\%}{5}$$

% R = 84.6 %

Level 3.

$$\% \text{ Recovery} = \frac{(59.90-54.67)\text{x } 100\%}{5}$$

% R = 104.6 %

Mean recovery = 90.2 %

Ruggedness analysis calculation (Analysis 1)

1. $$\text{HMF} = \frac{(4.2358-0.3162)\text{x } 74.87}{5}$$

   = 56.82 mg/kg

2. $$\text{HMF} = \frac{(4.1761-0.3168)\text{x } 74.87}{5}$$

   = 57.77 mg/kg

3. $$\text{HMF} = \frac{(4.1511-0.3169)\text{x } 74.87}{5}$$

   = 57.39 mg/kg

4. $$\text{HMF} = \frac{(4.0819-0.3170)\text{x } 74.87}{5}$$

   = 56.35 mg/kg

5. $$\text{HMF} = \frac{(4.1981)\text{x } 74.87}{5}$$

   = 58.10 mg/kg

Mean concentrations = 57.29 mg/kg

Ruggedness analysis calculation (Analysis 2)

1. $$\text{HMF} = \frac{(4.0991-0.3150)\text{x } 74.87}{5}$$

   = 56.66 mg/kg

2. HMF = $\frac{(4.1558-0.3150)\text{x } 74.87}{5}$

= 57.51 mg/kg

3. HMF = $\frac{(4.0698)\text{x } 74.87}{5}$

= 56.21 mg/kg

4. HMF = $\frac{(4.1522-0.3170)\text{x } 74.87}{5}$

= 57.43 mg/kg

5. HMF = $\frac{(4.1434-0.3144)\text{x } 74.87}{5}$

= 57.33 mg/kg

Mean concentrations = 57.04 mg/kg

Robustness analysis calculations at 284 and 366 nm

1. HMF = $\frac{(4.1538-0.3044)\text{x } 74.87}{5}$

= 57.62 mg/kg

2. HMF = $\frac{(4.1510-0.3038)\text{x } 74.87}{5}$

= 56.91 mg/kg

3. HMF = $\frac{(4.1042-0.3039)\text{x } 74.87}{5}$

= 56.91 mg/kg

4. HMF = $\frac{(4.1056-0.3031)\text{x } 74.87}{5}$

= 56.94 mg/kg

5. HMF = $\frac{(4.1452-0.3038)\text{x } 74.87}{5}$

= 57.52 mg/kg

Mean concentrations= 57.32 mg/kg

Robustness analysis calculations at 283 and 335 nm

1. HMF = $\frac{(4.1416-0.3018)\text{x } 74.87}{5}$

   = 57.50 mg/kg

2. HMF = $\frac{(4.1190-0.3005)\text{x } 74.87}{5}$

   = 57.18 mg/kg

3. HMF = $\frac{(4.1483-0.3021)\text{x } 74.87}{5}$

   = 57.59 mg/kg

4. HMF = $\frac{(4.1382-0.3051)\text{x } 74.87}{5}$

   = 57.40 mg/kg

5. HMF = $\frac{(4.1138-0.3072)\text{x } 74.87}{5}$

   = 57.00 mg/kg

Mean concentrations = 57.33 mg/kg

Appendix II.

t-test: Paired Two Sample for Means

	WHITE (HMF mg/kg)	*Modified (HMF mg/kg)*
Mean	40.912	39.734
Variance	171.419	148.9301
Observations	5	5
Pearson Correlation	0.818272	
Hypothesized Mean Difference	0	
Df	4	
t Stat	0.343328	
P(T<=t) one-tail	0.374319	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.748638	
t Critical two-tail	2.776445	

Appendix III

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
W	5	204.56	40.912	171.419

M	5	198.67	39.734	148.9301

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.46921	1	3.46921	0.021659	0.886639	5.317655
Within Groups	1281.396	8	160.1746			
Total	1284.866	9				