ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DETERMINATION OF LEVEL OF SOME HEAVY METALS IN HONEY COLLECTED FROM DIFFERENT REGIONS OF ETHIOPIA

By

Esubalew Adugna

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List of Abbreviation

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CSA	Central Statistics Agency
DDA	Daily Dose allowance
DNA	Deoxyribonucleic Acid
FAAS	Flame Atomic Absorption spectrometer
GFAAS	Graphite Atomic Absorption spectrometer
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometer
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
IDL	Instrumental Detection Limit
MDL	Method Detection Limit
RSD	Relative Standard deviation

Abstract

The qualities of 12 Ethiopian multifloral honey samples were evaluated in terms of common physicochemical parameters namely: moisture content, pH, free acidity, lactonic acidity and total acidity. The values for studied quality parameters were moisture content, 14.23-19.2%; pH, 3.4-4.8; free acidity, 30.6-97.6 meq/Kg; lactonic acidity, 8.06-14.4meq/Kg and total acidity, 42.96-107.4meq/Kg. The analyzed samples showed proper maturity, absence of undesirable fermentation (<20% of moisture content), and were in agreement with standard values (Codex Alimentricus) and reported literatures.

The concentrations of trace heavy metals (Cd, Cr, Cu, Mn, Ni, Pb and Z n) in the 12 multifloral honey samples collected from different regions of Ethiopia were also evaluated using flame absorption spectrometry after wet digestion. The accuracy of the method was assessed by spiking honey samples with known amounts of standard metals, and examining recovery. Trace heavy metal contents in the honey samples were found to be in the range of ND-0.017 μg/g (Cd), ND-0.15 μg/g (Cr), 0.02-1.15 μg/g (Cu), ND-7.29 μg/g (Mn), ND (Ni), ND-2.53 μg/g (Pb) and 9.96-16.03 μg/g (Zn). Results obtained were in agreement with data reported elsewhere in literature.

Keywords: Honey, physicochemical parameters, Ethiopia, wet digestion, atomic absorption spectrometry, trace elements

1. Introduction

According to European Council Directive 2001/110/EC, honey is the natural sweet substance produced by bees (*Apis mellifera*) from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Codex Alimentricus, 2000). The difference between honeydew and nectar honey is that the former is a honey which comes mainly from the excretion of plant sucking insects (Hemiptera) on living parts of plants or secretions of living parts of plants, where as nectar honey is the honey which comes from nectars of plants (Kujawski & Namies´nik, 2008).

The earliest written records of honey used as medicine is in Egyptian papyri and Sumerian clay tablets dated from 1900 to 1250 BC and in one of these, honey was described to have been used in 30% of the prescriptions. Ancient Egyptians also used honey in embalming. They made salves with it for treating diseases of the eyes and skin. As well, the ancient Greeks are reported to have used honey to treat fatigue: athletes drank a mixture of honey and water before major athletic events (Chepulis, 2008). Hippocrates (460-357 BC) prescribed honey for various indications, including the management of wounds and gastritis (Kujawski & Namies´nik, 2008).

The Hindu people also had great faith in the medical virtues of honey. They use it mainly for coughs, pulmonary issues and gastric disorders. Similarly, populations in rural communities from almost all nations have documented the use of honey through time. German women, specifically, believed that a mixture of honey and crushed bees would have a beautifying and strengthening effect and that would regulate menstrual flow (Zareie, 2011).

Chemically speaking, honey is a highly hygroscopic and very concentrated aqueous solution of sugars. As an analytical sample, honey is a very complex matrix. Its composition depends strongly on the plant species from which nectar or honeydew was collected, and other factors, such as environmental conditions and climate (Kujawski & Namies´nik, 2008).

Africa is blessed with numerous types of wild honeybee. Ethiopia is one of the countries of the continent which own big honey production potential. Owing to its varied ecological and climatic conditions, Ethiopia is home to some of the most diverse flora and fauna in Africa. Its forests and woodlands contain diverse plant species that provide surplus nectar and pollen to foraging bees. Beekeeping is one of the oldest farming practices in the country. There is an ancient tradition for beekeeping in Ethiopia which stretches back into the millennia of the country's early history. However, the products obtained from this sub sector are still low as compared to the potential of the country. Although thousands of tones of honey are produced every year it is usually poorly managed and unattractive in appearance. The type of hives used the methods of removing and storage of honey play a vital role in the quality of honey (Deffar, 1998).

The total world production of honey is estimated at 1 170 000 tons and China, the largest producer, exports 70 000 tons of honey. Ethiopia, having the highest number of bee colonies and surplus honey sources of flora, is the leading producer of honey (41 million kilograms of honey/annum) and beeswax in Africa. According to the central statistics agency of Ethiopia 2002 E.C report, the total honey production is estimated at 41 million kilogram and from which Southern Nation Nationality and people Regions (SNNPR) (11,794,672 kg), Oromia (15,825,245 kg), Amhara (7,453,349 kg) and Tigray (3,203,088 kg) accounts the major portion of honey produced. The report accounts number of hive, frequency of honey production and honey production per harvest (CSA, 2002; Nandaa *et al.*, 2003).

1.1. Composition of Honey

The composition of honey is variable, owing to the differences in plant types, climate, environmental conditions, and contribution of the beekeeper (Kucuk *et al.*, 2007; De Rodriguez *et al.*, 2004). Honey contains fructose and glucose (60–85%) as the predominant monosaccharides, maltose and sucrose as the most important disaccharides, melezitose as the main trisaccharide and other low molecular weight oligosaccharides. The composition of honey (sugars and moisture content (< 20%)) is responsible for many of the physicochemical properties of honey, such as viscosity, hydroscopicity, and granulation etc (Lazaridou *et al.*, 2004; Wang & X. Li, 2011).

Other chemical groups present are organic acids (responsible for the taste of honey 0.05–1.2%), proteins (mainly albumin and globulin from bee pharyngeal glands, generally not above 0.5%), amino acids (0.03%), and variable amounts of sugar-tolerant yeasts.

It also constitute enzymes, coming from the glands of the bees, the most important being invertase, amylases and glucose oxidase. Honey also contains hormones (of plant and bee origin) (e.g., acetylcholine and its precursor, choline). Flavonoids, anthocyanins, a wide array of vitamins, essential oils, pigments, sterols and phospholipids are also present in honey (Kujawski & Namies´nik, 2008; Wang & Li, 2011).

In general over 300 components, belonging to a wide variety of groups of chemical compounds have been detected in different types of honey (Madejczy and Baralkiewicz, 2008). In addition to its complex matrix, honey has minerals and trace metals (which are important in the diet of human beings) as minority components ranging from 0.1%-1% (Lachman *et al.*, 2007), 0.02%-1.028% (Luque *et al.*, 2005), 0.1%-0.2% (Luque *et al.*, 2005). The main mineral elements present in honey includes: K, Ca, Na, P and the trace elements include Al, Ag, B, Ba, Cd, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Si, Sn, Pb, and Zn (Madejczy & Baralkiewicz, 2008). Honey contains approximately 1% of admixtures, such as pollen, dead bees and mineral substances (ashes) as well (Kujawski & Namies´nik, 2008).

1.2. Importance of Honey

The ancient Egyptians and Greeks used honey for wound care, and a broad spectrum of wounds are treated all over the world with natural unprocessed honey (Simon *et al.*, 2009). Research indicated that honey have functional properties in human health promotion which depend largely on the floral source of honey. These properties include antimicrobial activity (due to presence and formation of hydrogen peroxide and mild acidity, phenolic compounds), antioxidant (Estevinho *et al.*, 2007; Vilma *et al*, 2007), *in vivo* anti viral activity against herpes simplex virus, reduction of frequencies of diarrhea, maintenance of moist wound environment, prevention of spreading of infection (Medihoney^{TM} has been one of the first medically certified honeys) (Lusby *et al.*, 2005), protective action on neurotoxicity and Alzheimer's disease (Shati *et al.*, 2011), antimutagene, probiotic (Stankovska *et al.*, 2007), treatment of various gastrointestinal disorders, respiratory ailments, fatigue, vertigo, ophthalmic disorders, toothache, measles, burns, chest pains, postnatal disorders, male impotence and as a skin cleansing agent (Meda *et al.*, 2004).

Because of its sweetness, color and flavor, honey is often used as a sugar substitute, an ingredient or a natural preservative in many of manufactured foods. It can prevent oxidation reaction in foods (e.g., lipid oxidation in meat and enzymatic browning of fruits and vegetable) (Pyrzynska & Biesaga, 2009).

Honey besides its therapeutic importance, it is a useful bio-monitor for information related to the environment where the bees live. Since honey bees readily fly up to 4 km in all directions from their apiary and thus have access to an area of about 50 km^2 and the bees come in contact not only with air but also with soil and water, the concentration of heavy metals in honey reflects their amount in the whole environment. Therefore, honey has been recognized as a biological indicator of environmental pollution (Silici *et al.*, 2008).

1.3 Sources of Contamination

The contamination sources can be divided into environmental and apicultural ones. Contaminants can reach the raw materials of bee products (nectar, honey dew, pollen, plant exudates) by air, water, plants and soil and then be transported into the bee hive by the bees. Air and soil contain heavy metals, mainly from industry and traffic which can also contaminate the bee colony and its product (Bogdanov *et al.*, 2006).

The floral type of honey plants, floral density, and the chemical composition of nectar, pollen, and other forage sources extensively differ with the location of the apiary, regional conditions, as well as season and type of vegetation. As a result, the botanical origin of honey has a remarkable effect on the content of certain metals in honey (Pohl, 2009).

Beside the geographical and botanical origins of metals in honey, the content of some heavy metals can result from different anthropogenic sources. Usually, the neighborhood of mines and steelworks, industrialized and urban areas, or highways in the forage area can result in the contamination of the apiary locality. Honey can also be polluted with certain metals during its harvesting and processing and this is primarily related to the apiculture technology used by the beekeepers. Metals can be released from materials (stainless steel, galvanized steel, and aluminum) of tools and equipment used for honey crop extraction, centrifugation, or ripening. The materials of the containers applied for honey processing, shipping, and storage of the extracted and ripened honey or lids may also contribute to the contamination of honey (Pohl *et al.*, 2009).

1.4 Essentiality and Toxicity of Heavy Metals in Human Health

1.4.1 Essential Metals

Chromium

Cr III, the most abundant environmental form, is an essential element that plays a role in glucose metabolism. Chromium deficiency causes changes in the metabolism of glucose and lipids and may be associated with maturity-onset diabetes, cardiovascular diseases, and nervous system disorders (emeA, 2007).

Copper

Copper (Cu) is an essential yet potentially toxic element to all organisms. Cu serves as a catalytic and structural cofactor for enzymes that function in energy generation, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting, and signal transduction. It is also used as a co-factor in several enzymes and several proteins have evolved to tightly regulate the distribution of copper in the cell. In humans, unbalanced concentration of copper has been implicated in Wilson's disease (excess), Menke's syndrome (deficiency), and neurodegenerative diseases (Alzheimer's disease, amylotrophic lateral sclerosis and prion disease). According to Codex Alimentarius Commission, the maximum allowable limit for Cu in multifloral nectar honey is 5 μg/g (Blanchard, 2009; Valentine *et al.*, 2006; Kim *et al.*, 2008).

Manganese

Manganese (Mn), an essential element, is crucial for a number of biological and physiological processes in the body, including immune function, regulation of cellular energy, reproduction, digestion, bone and connective tissue growth, and blood clotting. Mn also plays an important role as a cofactor for many enzymic reactions including amino acid, lipid, protein, and carbohydrate metabolism (Yoon *et al.*, 2011).

Signs of manganese deficiency include impaired growth, skeletal abnormalities, disturbed or depressed reproductive function, ataxia of the newborn, neurotoxic effects, defects in lipid and carbohydrate metabolism (Thompson *et al.*, 2011).

Nickel

The essentiality of nickel in mammals is questionable. It is generally present in the environment and appears to be an essential element for some plant life and bacteria. For the general population, the primary health concern is an allergic response from skin contact. Nickel is one of the few proven human carcinogens. Contact dermatitis is also a common workplace hazard (Gilbert, 2005).

Zinc

Zinc is an essential trace element that plays important role in a wide range of Zn – containing proteins necessary for growth, development, and DNA replication. Zn also has a variety of critical functions including maintaining appetite, wound healing, immune competence, cofactor of the superoxide dismutase enzymes, and stabilization of phosphate groups and coordination with organic bases in DNA. However, it also has the potential to interact with many biological functions to induce adverse effects such as nausea, vomiting, diarrhea, fever and lethargy. According to Codex Alimentarius Commission, the maximum allowable limit of Zn in multifloral nectar honey is 0.5 ppm (Yamada *et al.*, 2007; emeA, 2007).

1.4.2 Toxic Metals

Cadmium

Cadmium, a ubiquitous environmental contaminant, damages several major organs in humans and other mammals (Martin *et al.*, 2007). It can induce various toxic effects such as hepatotoxicity, nephrotoxicity (Tokumoto *et al.*, 2011), osteotoxicity, immunotoxicity, cytotoxicity (Honda *et al.*, 2010; Yang *et al.*, 2007) autophagic cell death (Wang S *et al.*, 2009).

So far, there are no studies available demonstrating that cadmium is a human teratogen, but is a potent teratogen in laboratory animals, causing exencephaly when administered at early stages of development According to Codex Alimentarius Commission, the maximum allowable limit of Cd in multifloral nectar honey is 0.05 ppm (Ferna´ndez *et al.*, 2003).

Lead

Lead is one of the most ubiquitous toxic metals known to man. Lead occurs naturally in plants and in soils throughout the world. Chronic lead exposure has been linked with cardiac arrhythmia, renal insufficiency, hypertension, and osteoporosis. Studies indicated that apoptosis may be associated with the lead-induced oxidative stress and DNA damage (Jamieson *et al.*, 2006; Seth *et al.*, 2004).

1.5 Determination of Metals in Honey

The most common methods used nowadays for the determination of heavy metals in different samples involve highly sensitive spectroscopic techniques, such as atomic absorption spectroscopy (FAAS, GFAAS) and inductively coupled plasma-optical emission and mass spectrometry (ICP-AES and ICP-MS). Selection of the most appropriate analytical method to solve a specific problem depends on the content and range of the study elements in the samples, determination limits as well as acceptable level of accuracy and precision and economic criteria. Studies revealed that GFAAS is more suitable for determination of elements in environmental samples (Especially for Cd, Cr, Ni, Pb and V), primarily with respect to convenient determination limits than FAAS and ICP-AES (Sastre *et al.*, 2002)

In all these techniques, the sample is required to be transformed into solution where the metal content is determined. Although honey is soluble in water and metals can directly be determined in solutions prepared by dissolving honey samples in water or acidic solutions, samples are usually decomposed before measurements. The resulting residues (ashes and digests) are re-dissolved in water or acid solutions, and the mineral components are transferred into solution (Pohl, 2009).

The use of other methods including capillary zone electrophoresis (Luque *et al.*, 2005), Potentiometric stripping analysis (Mun˜oz and Palmero, 2006), particle-induced X-ray emission (PIXE) (Braziewicz *et al.*, 2002), total reflection X-rayfluorescence (TXRF) (Khude *et al.,* 2010), ion chromatography (Buldini *et al.*, 2001), isotope dilution inductively coupled plasma mass spectrometry with direct injection nebulization (ID-ICP-MS) (Packera & Gineb, 2001) have been implicated in determination of trace metals in honey.

Interest in the analysis of honey due to its metal content has significantly increased over the past 15 years. The aim of this study is therefore, to assess the levels of different metals, especially heavy metals, as a general objective aimed at characterizing various honeys in view of the implications for their safety. Such information is important to beekeepers, as it helps them to avoid possible contamination during honey processing, and to consumers, as it assures them that the product is of good quality.

2. Objectives

2.1. General Objective

To determine the amount of some heavy metals (Cd, Cr, Cu, Mn, Ni, Pb and Zn) in different honey samples collected from top honey producing regions of Ethiopia.

2.2. Specific Objectives

- To collect honey samples from different regions of Ethiopia.
- To measure some physicochemical properties (pH, free acidity, total acidity, and moisture content) of honey samples.
- To determine the level of some selected heavy metals in collected samples if any.
- To compare the content of heavy metals in honey from each sampling region.
- To compare the level of heavy metals in honey with that of different countries.
- To compare the values obtained with standards

3. Methods and Materials

3.1. Reagents and Chemicals

Nitric acid (69%), hydrogen peroxide (30%), sodium hydroxide, potassium hydrogen phthalate (KHP), (all from BDH, England), and stock solutions with 1000ppm concentration of Cd, Cr, Cu, Mn, Ni, Pb, and Zn, all in nitrate form (Inorganic Ventures, USA) were used. All standards were of analytical grade.

3.2. Instruments and Equipment

Atomic absorption spectrophotometer: Flame & graphite furnace system (PG990, United Kingdom) fitted with appropriate hollow cathode lamps, analytical balance (Mettler Toledo, Switzerland), volumetric flasks, measuring cylinder, conical flasks, burette, porcelain dish, water bath, Abbe refractometer (ATAGO, UK), magnetic stirrer, pH meter (JENWAY 3510, UK), Whatman No.42 filter paper, plastic containers, hotplate and pipettes.

3.3. Cleaning Apparatus

Apparatus such as volumetric flasks, measuring cylinders and digestion flasks, and all the necessary materials used for the experiment were washed with detergents and tap water, rinsed with deionized water, soaked in 10% (v/v) HNO_3 solution for 24 hrs, rinsed with de-ionized water and kept in dust free cabinet until used, and digested solutions were kept in refrigerator until analysis.

3.4. Sampling Techniques

The samples of honey were collected by referring the report of CSA of Ethiopia for the major honey producing regions of Ethiopia. The top most honey producing regions (SNNPR > Oromia > Amhara > Tigray regions) were selected. From each region, the major three honey producing districts and/or zones were selected. Then from each zone and/or district, four commercial honey traders were randomly selected and about 500g of typical multi floral *Apis mellifera* honey samples were purchased. Then, this four samples (for a given district and or zone) were mixed, homogenized and preserved in plastic containers and kept at 4~5°C in a refrigerator until analysis.

3.5. Study Area

Bure is a town in western Ethiopia, located in the West Gojjam Zone of the Amhara Region. It has a longitude and latitude of 37°4′E, and 10°42′N respectively with an elevation of 2091 meters above sea level.

Debre Marqos is a town in east-central Ethiopia, located in the Eastern Gojjam Zone of the Amhara Region. It has a latitude and longitude of 10°20′N, and 37°43′E respectively and with an elevation of 2446 meters above sea level.

Tilli is a town in western Ethiopia, located in the west Gojjam zone of Amhara region. It has a longitude and latitude of 37^0 01' 15'' E, and 10^0 51' 05'' N respectively with an elevation of 2445 meters above sea level.

Bench Maji is one of the 13 Zones of Southern Nations, Nationalities and people's Region (SNNPR). It has a latitude and longitude of 6°20′30"N, and 35° 37′36"E respectively and with an elevation of 1035 meters above sea level.

Bonga is a town in southwestern Ethiopia. It has a latitude and longitude of 7°16′N, and 36° 14′ respectively and with an elevation of 1714 meters above sea level.

Dawero is a town in southwestern Ethiopia. It has a latitude and longitude of 6°59′44"N, and 35° 36′22"E respectively and with an elevation of 1424 meters above sea level.

Adigrat is situated in Eastern Tigray, Tigray region. It is located at a latitude and longitude of 14^0 16' 37'' N, and 39^0 27' 39'' E respectively and with an elevation of 2457 meters above sea level.

Erob is situated on Eastern Tigray, Tigray region. It's located at a latitude and longitude of 14^0 24' 58'' N, and 39^0 28' 43'' E respectively and with an elevation of 2239 meters above sea level.

Hawzene is situated on Eastern Tigray, Tigray region. It's located on a latitude and longitude of 13^0 58' 39'' N, and 39^0 25' 45'' E respectively.

Jimma is the largest city in southwestern Ethiopia. Located in the Jimma Zone of the Oromia Region, it has a latitude and longitude of 7°40′N 36°50′E and with an elevation of 1730 meters above sea level.

Ginchi is situated in Oromia region. It's located at a latitude and longitude of 9^0 03' 37'' N, and 38^0 07' 11'' E respectively and with an elevation of 2276 meters above sea level

Arsi Robe is a town in south-central Ethiopia, located in the Arsi Zone of the Oromia Region. This town has a latitude and longitude of 7°7′N 40°0′E with an elevation of 2492 meters above sea level.

3.6. Sample Preparation

The procedure described by (AOAC, 2000b) was used for the determination of physicochemical (pH, total acidity, free acidity and moisture content) properties of honey.

Liquid or strained honey: Samples which were free from granulation were mixed thoroughly by stirring and/or shaking before weighing portions for determination and samples which are granulated were placed in water bath without submerging and heated for 30 minutes at 60^0C with occasional shaking. Then, the samples were cooled, and a portion liquefied honey was weighed for determination.

The procedure described by (Tuzen *et al.*, 2007) was followed for the digestion of honey samples. One gram of honey sample, mixed and homogenized, was weighed and placed in a conical flask. 8 ml of concentrated nitric acid (69%) and 4 ml of hydrogen peroxide (30%) was added to the flask containing the sample. Then, the sample mixture was placed on the water bath and heated for 4 hrs to dryness.

The flask was removed from the water bath and cooled to room temperature. To the cooled sample, deionized water was added to dissolve the dried mass, and the content was filtered in 10 ml volumetric flask with Whatman No 42 filter paper. Subsequently, the solution was made up to volume using deionized water.

Similarly, reagent blank was prepared by taking mixture of the reagents (concentrated nitric acid and hydrogen peroxide) and treating in the same manner as the sample. The content of the flask was then analyzed for the level of the selected heavy metals (Cd, Cr, Cu, Mn, Ni, Pb, and Zn) if any. The whole procedure was done in triplicate.

3.7. Determination of Moisture Content

After homogenization of the sample, samples were placed in flask. The flask was closed using a watch glass and placed in water bath at about 50^0C. Then, the sample was cooled at room temperature and stirred well. Then, the surface of the prism of Abbe Refractometer was covered evenly and refractive index was read after 2 minutes in triplicate and the average value was considered.

3.8. Determination of pH and Total Acidity

10 g of honey was dissolved in 75 ml of carbon dioxide- free water in a 250 ml beaker, and stirred with a magnetic stirrer. After homogenization was achieved, the pH electrode was immersed and the pH was measured. Starting from this pH value, the sample solution was titrated against a standardized 0.05 M NaOH to pH 8.5, and addition of 0.05 M NaOH was stopped at pH 8.50 (free acidity), immediately a volume of 10 ml 0.05 M NaOH was added and, without delay, back-titrated with 0.05 M HCl to pH 8.30 (lactonic acidity). Total acidity results were obtained by adding free and lactone acidities (AOAC, 2000b). Free acidity is calculated as milliliters of 0.05M NaOH times ten, where as lactonic acidity was calculated as $(10\text{ml} - \text{ml of HCl from burret})\ x\ \frac{50}{\text{g}}\text{sample portion}$

3.9. Determination of Heavy Metals

A stock standard solution of 1000ppm was used for each metal and an intermediate solution was prepared and used immediately for the determination of metals. Five different working standard solutions were prepared for each metal from the respective intermediate standards in a blank treated in the same way as a sample to minimize matrix effects. Cd, Cr, Cu, Mn, Ni, Pb and Zn and were determined with FAAS equipped with deuterium arc background corrector and standard air-acetylene flame system using external calibration curve after the parameters (burner and lamp alignment, slit width and wavelength adjustment) were optimized for maximum signal intensity of the instrument. The acetylene and air flow rates were managed to ensure suitable flame conditions. Then their absorbance measured and the calibration curves were obtained by plotting absorbance versus concentration. Table 1 depicts the operating parameters of the instrument for each metal.

Table 1: Parameters of FAAS determination

Parameters	Cd	Cr	Cu	Mn	Ni	Pb	Zn
Wavelength (nm)	228.8	357.9	324.7	279.5	232.0	283.3	213.9
Spectral interval width (nm)	0.4	0.4	0.4	0.2	0.2	0.4	0.4
Supply current of discharge lamp (mA)	2.0	4.0	3.0	2.0	2.0	2.0	3.0
Background correction	D_2	No	No	D_2	D_2	D_2	D_2

3.10. Statistical Analysis

The statistical analysis was conducted using statistical package of SPSS version 19. One way ANOVA coupled with post hoc Tukey HSD test was performed in order to compare the mean values of each element in honey samples collected from different regions of Ethiopia. The level of significance was compared at $P = 0.05$, $P < 0.05$ was used as a cutoff point to show significant difference. Microsoft Office Excel 2007 was used to calculate mean, standard deviation and to draw calibration graphs and bar graphs.

4. Result and Discussion

4.1. Physicochemical Parameters

Results for the studied physico-chemical parameters of honey from different regions of Ethiopia are summarized in Table 2. Moisture level in honey is a quality criterion that determines the capability of honey to remain stable and to resist spoilage by yeast fermentation (Bogdanov *et al.*, 2002). Moisture content depends on the botanical origin of the sample, harvest season, proper time of extraction, and degree of ripening of honey in the hive (O¨zcan *et al.*, 2006). It is also important for the shelf life of honey during storage (Nandaa *et al.*, 2003).

Table 2: Statistical analysis of some studied physico-chemical parameters of Ethiopian multifloral honey

Sample	*pH*	*Moisture content (%)*	*Free acidity (meq/Kg)*	*Lactonic acidity (meq/Kg)*	*Total acidity (meq/Kg)*
Adigrat	3.8 ±0.05a	17.16±0.15f	33.3 ± 2.88dg	12.76 ± 0.60ab	46.06 ± 2.35b
Arsi	3.6 ± 0.10a	18.82±0.03e	63.3 ± 2.31ade	9.33 ± 1.21a	72.33 ± 3.44d
Benchi Majji	3.4 ± 0.10a	14.23 ± 0.05a	97.6 ± 6.80a	9.8 ± 0.6a	107.4 ± 6.23a
Bure	4.5 ±0.05d	14.56±0.05a	42.6 ± 2.51ec	8.43 ± 1.88a	51.03 ± 3.12bc
Bonga	4.1 ± 0.05b	15.11±0.10b	36.0 ± 1.00b	9.9 ± 0.1a	45.9 ± 0.90b
Dawero	4.7 ± 0.11c	16.57±0.39c	40.6 ± 3.05c	8.06 ± 0.11a	48.66 ± 3.03b
D/Markos	4.8 ±0.00c	19.2±0.01e	41.33 ± 2.31c	9.73 ± 1.60a	51.06 ± 2.71bc
Erob	3.8 ±0.05a	16.39±0.03c	31.0 ± 3.46dg	14.4 ± 0.75b	45.4 ± 4.09b
Ginchi	4.2 ± 0.05b	17.64±0.07d	44.6 ±0.57bd	11 ± 0.3a	55.6 ± 0.85cb
Jimma	4.1 ±0.00b	14.47±0.03a	55.0 ± 5.00bf	10.26 ± 0.68a	65.26 ± 4.52cd
Hawzene	3.8 ±0.00a	18.38±0.02g	30.6 ± 1.52dbg	12.36 ± 0.57ab	42.96 ± 1.10b
Tilli	4.5 ± 0.10d	17.32±0.03df	33.33 ± 1.52eg	10.86 ± 2.11a	44.19 ± 2.72b
Codex[a]	3.5-5.5	<20	<50	-	-

NB: Mean in a column with different letters (a-g) are significantly different ($P<0.05$). Codex Alimentricus, 2002 [a]

Among the analyzed samples, honey samples collected from Debre Markos had the highest moisture content (19.2%), where as honey samples collected from Benchi Maji showed the lowest moisture content (14.23%). The moisture content of Adigrat and Hawzene honeys were significantly different from the rest of analyzed samples ($P<0.05$). There was no significant difference between moisture content of honey samples collected from Benchi Maji, Jimma and Bure ($P>0.05$). None of the honey samples exceeded the permitted limit established by the European Community Directive which is moisture content not more than 20%. (Codex Alimentricus, 2002). The value found corresponds to mature honey, and indicates that the beekeepers had followed the proper time of extraction. In general, high moisture content causes the honey to ferment, spoil and lose flavor, causing honey-quality loss (De Rodriguez *et al.*, 2004).

The pH values of all honey samples collected from the different regions are in the acidic range (3.4-4.8). The lowest pH was found for honey samples collected from Benchi Maji, and the highest pH was recorded for honey sample from Debere Markos. Except the pH of honey collected from Benchi Maji, all the mean pH values were within permitted limit established by the European Community Directive (3.5-5.5). As it can be inferred from Table 2, pH values of honey collected from Debere Markos has no significant difference with honey samples collected from Dawero ($P>0.05$), whereas there was a significant difference on the pH value of honey collected from Tilli, Bure and the rest of honey samples collected from different regions of Ethiopia ($P<0.05$). Similarly, there was a significant difference between Benchi Maji, Bonga, Dawero, Bure and Tilli ($P<0.05$). These differences may be due to the geographical locations from which samples are collected as composition of organic acids and ions contribute to the acidity of honey (Terrab *et al.*, 2004). pH is of great importance during honey extraction and storage, due to influence on texture, stability and shelf life. It is also a useful index of possible microbial growth. Most bacteria grow in a neutral and mildly alkaline environment, while yeasts and moulds are capable of growth in acidic medium (4.0-4.5) and do not grow well in alkaline media (Conti, 2000).

The acidity of honey is due to the presence of organic acids, particularly the gluconic acid, in equilibrium with their lactones or esters and inorganic ions such as phosphate and chloride (Ouchemoukh *et al.*, 2007). The mean values of free acidity in studied sample showed the minimum value as 30.6 meq/Kg for Hawzene and the maximum free acidity value for Benchi Maji (97.6 meq/Kg). Lactonic acidity, considered as the acidity reserve when the honeys become alkaline, ranges from 8.06 meq/Kg (Dawero) to 14.4 meq/Kg (Erob), while the total acidity was between 42.96 meq/Kg (Hawzene) and 107.4 meq/Kg (Benchi Maji). 25% (3/12) of honey samples exceeded the maximum limit of free acidity established by the European Community Regulation which is not more than 50 meq/Kg (Codex Alimentricus, 2000).

As briefly described in table 2, there was no significant difference on the level of free acidity between honey samples collected from Dawero, Debre Markos, and Bure, as well as samples collected from Adigrat, Hawzene, Erob and Tilli ($P>0.05$). Statistically significant difference was observed on the level of free acidity between honey samples collected from Benchi Maji, Bonga, Arsi, Jimma, and Ginchi ($P<0.05$). Similarly, there was a significant difference on the mean total acidity of honey samples collected from Benchi Maji and the rest of honey samples ($P<0.05$), but there was no significant difference between samples collected from Tigray region ($P>0.05$). The variation in acidity among different honey samples may be attributed to variation in the constituents of organic acids, due to extraction season, and floral types (Nandaa *et al.*, 2003).

As shown in figure 1, honey samples collected from Tigray region had the highest level of moisture content and Lactonic acidity where as honeys collected from SNNPR showed the highest amount of free and total acidity. The highest pH value was found for honey samples collected from Amhra region, and the lowest pH was recorded in honey samples collected from Tigray region.

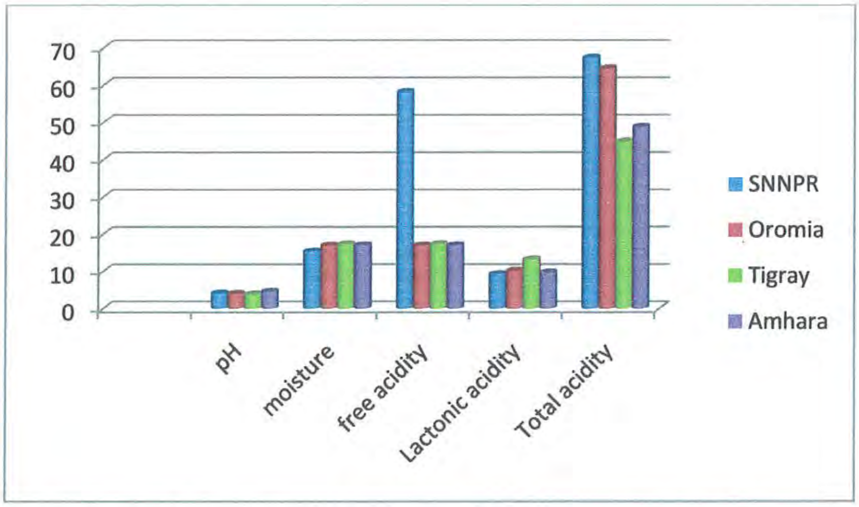

Figure 1: Physicochemical properties of honey samples collected from different regions of Ethiopia

One way ANOVA (Table 3) confirmed that there was no significant difference between the four regions in terms of pH and moisture content (P>0.05). There was a significant difference between the mean lactonic acidity of honey samples collected from Tigray region and Amhara, SNNPR, Oromia (P<0.05), but there was no statistical significance on the mean lactonic acidity of honey samples collected from SNNPR, Oromia, and Amhara (P>0.05). Except honey sample collected from SNNPR, there was no significant difference between the mean free acidity of the three regions (P>0.05). The mean total acidity of honey samples collected from SNNPR and Oromia showed significant difference (P<0.05) unlike the mean total acidity of honey samples collected from Tigray and Amhara region (P>0.05).

Table 3: Data for statistical analysis of the physicochemical parameters for honey from different regions of Ethiopia

Regions	*pH*	*Moisture content (%)*	*Free acidity (meq/Kg)*	*Lactonic acidity (meq/Kg)*	*Total acidity (meq/Kg)*
Amhara	4.6 ± 0.17a	17.02±2.33a	17.02±5.02a	9.67± 1.21a	48.76±3.95a
Oromia	3.8 ± 2.24a	16.97±2.24a	16.39±9.36a	10.19±.0.83a	64.39±8.39b
SNNPR	4.06±0.65a	15.33±1.22a	58.06±3.4b	9.25±1.03a	67.32±3.4b
Tigray	3.80 ±0.0a	17.31±1.00a	17.31± 1.47a	13.17±1.08b	44.80±1.63a

NB: Mean in a column with different letters (a-b) are significantly different (P<0.05)

4.2. Comparison of Physicochemical Parameters with Reported Data

There are reports from different countries on the physicochemical parameters of different honey types (honeydews, and or blossom honey). It's important to compare the results obtained from the analysis of Ethiopian multifloral honey samples with other countries to know the quality of honey in terms of pH, moisture content, free acidity, lactonic acidity, and total acidity. In addition, comparison may provide information on any deviations (if any) from the international guideline for honey. Summary of this comparison is given in Table 4.

The mean pH value of Ethiopian honey (4.1) was very close to pH values reported from Turkey (O¨zcan *et al.*, 2006), Spain (Terrab *et al.*, 2004), Saudi Arabia (Osman *et al.*, 2004), Chili (Fredes *et al.*, 2004), and Algeria (Ouchemoukh *et al.*, 2007). The pH of honey from Slovenia (Kropf *et al.*, 2010), Nigeria (Achudume *et al.*, 2010), Portugal (Almeida-Silva *et al.*, 2011), New Zealand (Vanhanen *et al.*, 2011), and Italy (Conti, 2000) was slightly higher than the mean pH value for Ethiopian honey. In addition, the mean pH value of Ethiopian honey was slightly higher than the mean pH values reported from Venezuela (De Rodriguez et al., 2004). From the reported pH values, the lowest value was obtained from Venezuela and the highest being from Slovenia. From the results obtained it was observed that Ethiopian honey was having good quality in terms of pH.

The mean moisture content (16.56 %) of Ethiopian honey was comparable with results reported for honey from Turkey (O¨zcan *et al.*, 2006), Syria (Khuder *et al.*, 2011), New Zealand (Vanhanen *et al.*, 2011), Italy (Conti, 2000), India (Nandaa *et al.*, 2003), and Algeria (Ouchemoukh *et al.*, 2007). On the other hand, the moisture contents reported from Nigeria (Achudume *et al.*, 2010), and Spain (Terrab *et al.*, 2004) were higher than, the mean moisture content of Ethiopian honey while moisture content reported from Turkey (Kahraman *et al.*, 2010), Saudi Arabia (Osman *et al.*, 2004) and Greek (Lazaridou *et al.*, 2004) was slightly lower than Ethiopian honey. Ethiopian honey was found to be of a good quality in terms of moisture content since none of the samples exceeded the maximum permitted the amount stipulated by Codex Alimentricus (<20%)

The mean free acidity of Ethiopian honey (45.81meq/Kg) was found to be the highest compared to the reported values from countries such as Turkey (Kahraman *et al.*, 2010), Spain (Terrab *et al.*, 2004), Slovenia (Kropf *et al.*, 2010), and India (Nandaa *et al.*, 2003). Comparison of level of Lactonic acidity revealed that, Ethiopian honey (10.57meq/Kg) had a lower value than Indian honey (Nandaa *et al.*, 2003)) but it was slightly higher than Spanish (Terrab *et al.*, 2004), and Slovenian honey (Kropf *et al.*, 2010). Table 4 shows that the total acidity of honey samples collected from Ethiopia was higher than Indian (Nandaa *et al.*, 2003), Saudi Arabian (Osman *et al.*, 2004), Slovenian (Kropf *et al.*, 2010), Spanish (Terrab *et al.*, 2004), Turkish (O¨zcan *et al.*, 2006), and Venezuelans (De Rodriguez *et al.*, 2004) honey samples. With some reservation, it was found that Ethiopian honey had met the criterion set by international organizations and is of good quality in terms of free acidity.

Table 4: Comparison of Physico-chemical parameters of Ethiopian honey with results from other countries

Country	Physicochemical Parameters					References
	pH	*Moisture content (%)*	*Free acidity (meq/Kg)*	*Lactonic acidity (meq/Kg)*	*Total acidity (meq/Kg)*	
Algeria	3.49-4.43	14.64-19.04	NR	NR	NR	Ouchemoukh *et al.*, 2007
Chili	4.23	NR	NR	NR	NR	Fredes *et al.*, 2004
Ethiopia	3.4-4.8	14.23-19.2	30.6-97.6	8.06-14.4	42.96-107.4	Present study
Greek	NR	13.0-18.9	NR	NR	NR	Lazaridou *et al.*, 2004
India	NR	13.97-18.65	14.57-32.65	14.64-18.61	30.03-47.37	Nandaa *et al.*, 2003
Italy	4.32	16.36	NR	NR	NR	Conti , 2000
New Zealand	3.57-5.04	15.7-18.0	NR	NR	NR	Vanhanen *et al.*, 2011
Nigeria	4.017-5.05	26.39-31.34	NR	NR	NR	Achudume *et al.*, 2010
Portugal	4.5	NR	NR	NR	NR	Almeida-Silva *et al.*, 2011
Saudi Arabia	3.88-4.58	14.40-15.95	NR	NR	21.84-10.90	Osman *et al.*, 2004
Slovenia	4.09-5.61	NR	11.67-17.11	1.62-2.88	14.55-18.73	Kropf *et al.*, 2010
Spain	3.56-4.79	14.2-19.8	17.59-39.81	4.3-11.3	25.6-48.6	Terrab *et al.*, 2004
Syria	NR	13.2-19.4	NR	NR	NR	Khuder *et al.*, 2011
Turkey	NR	15.3-16.9	NR	NR	23.9-24.4	O¨zcan *et al.*, 2006
Turkey	3.94	15.36	22.8	NR	NR	Kahraman *et al.*, 2010
Venezuela	3.3-4.3	17.8-20.40	NR	NR	24.4-53.3	De Rodriguez *et al.*, 2004

4.3. Determination of Metals

4.3.1 Calibration Curve

The instrument was calibrated using five series of working standards. The working standard solutions of each metal were prepared using their respective reagent blank as a solvent so as to minimize matrix interference. The concentration of the working standards and value of correlation coefficient for each metal is shown in Table 5.

Table 5: Concentrations of working standard solutions and correlation coefficients of the calibration curves

Metals	Working Standard Solution (mg/L)	Regression Equations	Correlation Coefficient (r)
Cd	0.25, 0.5, 1, 1.5, 2	$A = 0.188C + 0.025$	0.99849
Cr	0.5, 1, 2, 4, 8	$A = 0.013C + 0.002$	0.99949
Cu	0.25, 0.5, 1, 1.5, 2	$A = 0.123C + 0.001$	0.99949
Mn	0.5,1, 2, 3, 4	$A = 0.143C + 0.018$	0.99949
Ni	0.5, 1, 2, 4, 8	$A = 0.083C + 0.05$	0.99949
Pb	0.25, 0.5, 1, 1.5, 2	$A = 0.127C + 0.005$	0.99849
Zn	0.25, 0.5, 1, 1.5, 2	$A = 0.121C + 0.015$	0.99949

NB: A = Absorbance, C= Concentration

4.3.2 Evaluation of Analytical Method

The accuracy of the method was assessed by spiking honey samples with known amounts of standard metals, and examining recovery. Recovery was calculated using the equation below

$$\% \, Recovery = \frac{(S) - (NS)}{The \ spiked \ metal \ content} x100$$

Where: S is metal content of spiked sample, and NS is metal content of non spiked sample

The recovery values for honey samples are given in Table 6. The table shows that the recovery results for the metals lie in the range of 89.6 – 109.5 % with a RSD value ranging from 2.65-9.76%. The results show the validity of the proposed methods and a good repeatability for analysis of honey samples. Reading of control solution between three consecutive samples showed the instrument's precision (97.5%-102.1%).

Table 6: Recovery test results for honey samples.

Metals	*Sample conc. (μg/g)*	*Amount spiked(μg/g)*	*Conc. In spiked sample(μg/g)*	*Amount recovered (μg/g)*	*Recovery (%)*	*RSD (%)*
Cd	0.030	0.015	0.044	0.014	93.3	3.2
Cr	6.66	3.33	10.04	3.38	101.5	9.76
Cu	1.15	0.575	1.71	0.56	97.4	2.3
Mn	7.29	3.64	11.16	3.87	106.3	5.62
Ni	ND	4	4.12	4.11	102.7	2.65
Pb	2.53	1.26	3.66	1.13	89.6	4.65
Zn	14.62	7.31	22.63	8.01	109.5	7.31

4.3.3 Analytical Method Detection Limit

The limit of detection is the smallest mass of analyte that can be distinguished from statistical fluctuations in a blank, which usually correspond to the standard deviation of the blank absorbance times a constant. Usually it is defined as the amount of analyte that gives a signal equal to three times the standard deviation on the blank (Woldegebriel, 2007). To evaluate the method detection limit (MDL), six blank solutions (all treatment procedures for the analysis of sample were done except inclusion of the sample itself) were digested in triplicate and the absorbance was recorded. Then, MDL was calculated as three time the standard deviation of the blank (3σ blank). Table 7: summarizes the instrumental and method detection limits. As it has been shown in Table 7, all the MDL values were found to be greater than the instrumental detection limit.

Table 7: Method and Instrumental detection limits for honey samples

Metals	IDL (mg/L)	MDL (mg/L)
Cd	0.0028	0.0159
Cr	0.005	0.0718
Cu	0.004	0.0041
Ni	0.008	0.0375
Mn	0.002	0.0169
Pb	0.03	0.0258
Zn	0.003	0.0243

IDL: Instrumental Detection Limit, MDL: Method Detection Limit

4.4. Levels of Metals in Honey Samples

In the analysis of honey obtained from different regions of Ethiopia, it was observed that Zn was present in all honey samples collected from the different regions. Cu was also detected in all honey samples except honey sample collected from Hawzene. 25% of the samples showed the presence of Cd, and Mn while 33.3% of the samples showed the presence of Pb, and Cr. Ni was found to be below the method detection limit for all analyzed samples. Table 8 summarizes the concentrations of metals in the analyzed honey samples. The concentration of metals in honey samples varied in the order of Zn > Mn > Cr > Pb > Cu > Cd > Ni. The variations in metal levels in different honey samples may be attributed to different factors including the botanical origin, soil composition, agricultural practices involving the use of pesticides and fertilizers around the forge area, instruments used for extraction of honey from hive, containers employed for honey processing, paints for modern hive, shipping, and storage, high traffic and industrialization etc. As it has been depicted in Table 8 there was a significant difference in the heavy metal content between the analyzed samples originating from different regions of Ethiopia.

Table 8: Trace metal contents in wet digested honey samples from different regions of Ethiopia (mean ± SD, n=3) wet mass

Sample	*Cd(µg/g)*	*Pb (µg/g)*	*Zn (µg/g)*	*Cu (µg/g)*	*Cr (µg/g)*	*Mn (µg/g)*	*Ni (µg/g)*
Adigrat	ND	0.23 ± 0.12c	12.77 ± 0.40c	0.10 ± 0.09d	5.89 ± 0.44a	ND	ND
Arsi	0.017 ± 0.0025a	ND	13.63 ± 1.09c	0.21 ± 0.01c	ND	ND	ND
B/Maji	ND	ND	16.03 ± 0.29a	0.45 ± 0.04a	ND	ND	ND
Bonga	ND	ND	11.62 ± 0.63b	0.026 ±0.04b	ND	ND	ND
Bure	0.035 ± 0.005b	ND	14.62 ± 0.13c	0.02 ± 0.004b	5.89 ± 0.00a	0.7 ± 0.07c	ND
Dawero	ND	ND	14.3 ± 0.16c	0.21 ±0.04c	ND	ND	ND
D/Markos	ND	2.53 ± 0.03d	9.96± 0.4b	1.15 ± 0.04e	0.15 ±0.00b	0.36 ± 0.00b	ND
Erob	ND	ND	12.58 ± 0.39c	0.16 ± 0.00d	0.16 ± 0.00b	ND	ND
Ginchi	ND	1.64 ± 0.08a	10.43 ± 0.64b	0.24 ±0.00c	ND	ND	ND
Hawzene	ND	ND	10.65 ± 0.38b	ND	ND	ND	ND
Jimma	0.03 ± 0.0015b	1.23 ± 0.07b	15.17 ± 0.33a	0.08 ± 0.00b	ND	ND	ND
Tilli	ND	ND	14.02 ±0.31c	0.32 ± 0.00bc	6.66 ±0.44a	7.29 ± 0.04a	ND
Australia[a]	0.05	1.5	-	10	-	-	-
Codex[b]	0.05	0.3	5	5	-	-	-
India[c]	1.5	2.5	-	30	-	-	50

ND: Concentration of metals below method detection limit (< MDL). Mean in a column with different letters (a-e) are significantly different ($P<0.05$)

NB: Codex Alimentarius Commission, 2002[b], India regulation, Nandaa *et al*., 2003 [c], Australia Food standard code[a]

Cadmium

Cadmium was detected in three honey samples ranging from 0.017μg/g-0.035 μg/g, the highest being from Bure and the lowest concentration from Arsi. The high concentration of Cd in Bure may be attributed to the high traffic, poor sewage system, use of fertilizers, and use of animal waste product for collection of honey from hives. The mean level of Cd in present studied honey samples (0.027 μg/g) did not exceed the limit established by codex alimentricus commission, 0.05μg/g (Codex Alimentarius Commission, 2002), Turkish codex 0.03 μg/g, (Leblebici *et al.*, 2008), and Indian regulations, 1.5 μg/g, (Nandaa *et al.*, 2003), and Australia Food standard code (0.05 μg/g), Macedonia legislation, 0.03 μg/g, (Stankovska *et al.*, 2007). The mean level of Cd in honey samples collected from Arsi showed significance difference with honey sample collected from Jimma and Bure ($p<0.05$), but there was no significant difference in mean level of Cd in honey samples collected from Jimma and Bure ($P>0.05$).

Chromium

Chromium level was the highest in honey samples obtained from Tilli (6.66 μg/g) followed by Bure (5.89 μg/g), Adigrat (5.89 μg/g), Erob (0.16 μg/g) and Debre Markos (0.15 μg/g). The mean Cr level in Erob honey was not significantly different from honey samples originated from Bure and Tilli, as well as Erob and Debre Markos ($P>0.05$) but there was a significant difference between honey samples collected from Debre Markos and Tilli, as well as Bure and Adigrat ($P<0.05$).

Copper

Copper was one of the heavy metals detected in all honey samples analyzed except honey samples collected from Hawzene. The lowest and the highest copper concentrations were 0.02 μg/g, in the honey sample from Bure and 1.15 μg/g in honey sample from Debre Markos.

One way ANOVA revealed that there was a significant difference between honey samples collected from Bench Maji and the remaining samples ($P<0.05$), but there was no significant difference on honey samples collected from Erob, Dawero, Ginchi, Arsi, and Tilli ($P>0.05$) but the mean Cu level of honey sample from Debre Markos was significantly different from the rest of the samples ($P<0.05$). The mean Cu levels in studied samples did not exceed the limit established by codex alimentricus commission, 5μg/g (Codex Alimentarius Commission, 2002), and Indian regulations (30 μg/g), and Australia Food Standard Code (10 μg/g).

Manganese

Only samples originated from Amhara regions (Bure, Debre Markos and Tilli) showed the presence of manganese. It was found that the maximum concentration of Mn was 7.29 μg/g from Tilli, followed by Bure (0.7μg/g) and Debre Markos (0.36 μg/g). One way ANOVA showed there was significant difference between honey samples collected from Amhara region ($P<0.05$).

Lead

Lead was detected in 33.3% (4/12) of the analyzed samples ranging from 0.23 μg/g to 2.53 μg/g. The lowest and the highest lead concentrations were 0.23 μg/g, in the honey sample from Adigrat and 2.53 μg/g in the honey sample from Debre Markos. There was a significant difference between honey samples which showed the presence of lead ($P<0.05$). The variation of the mean level of lead may be attributed to the presence of highways, presence of metal workshops, house construction tools, and use of extensive fertilizers for production of crops (e.g. Debre Markos). The mean lead value of honey samples collected from Adigrat (0.23 μg/g) was in agreement with maximum permitted level set by Codex Alimentricus Commission (0.3 μg/g), and Indian regulations (2.5 μg/g), and Australia Food Standard Code (1.5 μg/g). The mean lead level of honey samples collected from Ginchi, and Debre Markos exceeded the maximum permitted amount set by Australia Food Standard Code (1.5 μg/g) and Codex Alimentricus Commission.

Honey samples collected from Jimma showed mean lead value within stated amount by Indian regulations and Australia Food Standard Code.

Zinc

In this study, Zn was the only metal detected in all analyzed honey samples. The maximum concentration of Zn was found in honey sample collected from Benchi Maji (16.03 μg/g), and the lowest concentration of Zn was observed in honey sample collected from Debre Markos (9.96 μg/g). Analysis for difference between honey samples using one way ANOVA showed that honey samples collected from SNNPR (Benchi Maji, Bonga and Dawero) and Amhara (Bure and Debre Markos) were significant ($P<0.05$), while there was no significant difference on the mean level of Zn in honey samples collected from Arsi, Dawero, Ginchi, and Tilli ($P>0.05$), as well as honey samples collected from Bench Maji, and Bure ($P>0.05$). Usually, the use of galvanized containers is the most prominent source of contamination of honey besides the soil and flora differences of forge area (Tuzen &Soylak, 2007). All the honey samples analyzed for presence of Zn exceeded the maximum permitted level established by Codex Alimentricus Commission, 5 μg/g (Codex Alimentarius Commission, 2002), but were within the limit established by Indian regulation (50 μg/g).

As shown in figure 2, honey samples collected from SNNPR had the highest Zn level, followed by Oromia, Amhara, and Tigray. Cu was found in the highest amount in samples collected from SNNPR followed by Amhara, Oromia, and Tigray. Honey collected from SNNPR and Oromia did not contain Cr while it was available in samples from Amhara and Tigray regions. Amount of Cr in samples from Amhara region was observed to be higher than that of the Tigray region. Mn was found in honey samples collected from Amhara region only. Pb was detected in samples from the studied regions except SNNPR. In addition, it has been shown that samples collected from Oromia and Amhara regions show presence of Cd but it was not detected in honey samples originating from Tigray and SNNPR regions.

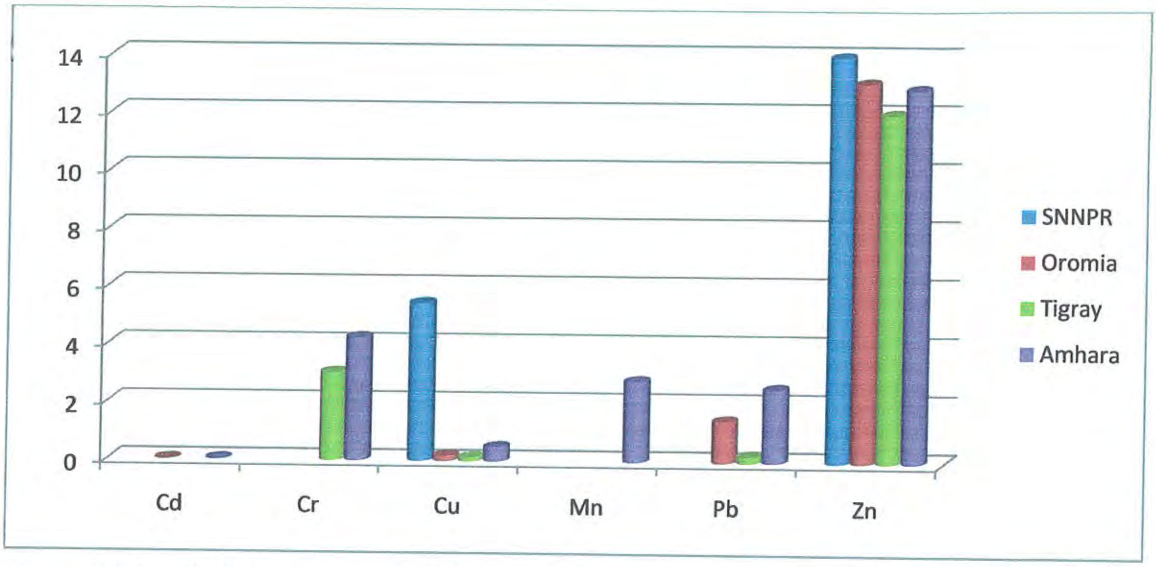

Figure 2: Distribution of trace metals in honey from different regions of Ethiopia

4.5. Comparison of Trace Metals with Reported Data

There are reports from different countries on the levels of trace metals of different honey types. It's important to compare the results obtained from the analysis of Ethiopian multifloral honey samples with that of other countries to know the quality of honey in terms of residue of trace metals and also to obtain any information on any deviations (if any) from the international guideline of honey. Summary of this comparison is given in Table 9.

The mean value of cadmium from the present study (ND-0.017 μg/g) was comparable with reports from Egypt, 0.01-0.5 μg/g, (Rashed & Soltan, 2004), New Zealand, 0.01-0.45 μg/g, (Vanhanen *et al.*, 2011) and Turkey Central Anatolia, 0.09-0.24 μg/g, (Leblebici *et al.*, 2008) but it was much lesser than the value for the honey samples collected from Turkey, 0.9 μg/g, (Tuzen &Soylak, 2007), Nigeria 25.49 μg/g, (Achudume *et al.*, 2010) and Lithuania, 4.1 μg/g, (Staniškienė *et al.*, 2006).

Data in Table 9 showed that the mean value of Cd in honey samples collected from Ethiopia is higher than mean values reported from Turkey middle Anatolia, 10.9-21.2μg/Kg, (Tuzen & Soyla, 2005), Macedonia, ND-11.28 μg/Kg, (Stankovska *et al.*, 2007), Italy Siena, 1.0-15.3 μg/Kg, (Pisani *et al.*, 2008) and Croatia, 1.00-24.0 μg/Kg, (Bilandzˇic´ *et al.*, 2011).

The level of copper observed in the present study (ND-1.15 μg/g) was in agreement with results reported for samples from Chile,0.06-2.00 μg/g, (Fredes *et al.*, 2006), Czech, 0.11-0.88 μg/g, (Lachman *et al.*, 2007), Turkey black sea region, 9.75-35.8 μg/Kg, (Silici *et al.*, 2008), and Turkey middle Anatolia, 0.25-1.10 μg/g, (Tuzen & Soyla, 2005) but it was lower than the value for honey samples from Canary Island,0.1-1.73 μg/g, (Herna´ndez *et al.*, 2005), Croatia, 36-41.217 μg/g, (Bilandzˇic´ *et al.*, 2011), Egypt 1.00-1.70 μg/g, (Rashed & Soltan, 2004), Italy (Siena), 172-5900 μg/Kg, (Pisani *et al.*, 2008), Italy(Lazio), 0.31 μg/g, (Conti, 2000), Lithuania, 119.6-342.9 μg/Kg, (Staniškienė *et al.*, 2006), Nigeria, 25.49 μg/g, (Achudume *et al.*, 2010), Poland, 0.26-1.82 μg/g, (Madejczyk & Baralkiewicz, 2008), Poland, <LD-2.63 μg/g, (Chudzinska *et al.*, 2010), Portugal, 0.405 μg/g, (Almeida-Silva *et al.*, 2011), Slovenia, 0.37-15.5 μg/g (Golob *et al.*, 2005"), Spain, 0.89 μg/g, (Latorre *et al.*, 1999), Turkey Central Anatolia, 0.01-0.8 μg/g, (Leblebici *et al.*, 2008), Turkey, 0.23-2.41 μg/g, (Tuzen & Soylak, 2007). Higher concentration of copper was reported in honey samples collected from Croatia, 36-41.217 μg/g, (Bilandzˇic´ *et al.*, 2011) and the lowest level was observed from honey samples collected from Turkey Central Anatolia, (0.01-0.8 μg/g) (Leblebici *et al.*, 2008).

The mean concentration of Cr in Ethiopian honey ranged from (ND- 6.66 μg/g) which was lower than values obtained for honey samples collected from Turkey, 2.4-37.9 μg/g, (Tuzen & Soylak, 2007) and Slovenia, 0.11-33.8 μg/g, (Golob *et al.*, 2005) but was much higher than values reported in Chile, 0.03-1.92 μg/g, (Fredes *et al.*, 2006), Italy (Siena), <2.0-54 μg/Kg, (Pisani *et al.*, 2008), Nigeria, 0.32 μg/g, (Achudume *et al.*, 2010), Poland, 0.01-0.093 μg/g, (Madejczyk & Baralkiewicz, 2008), Turkey, 3.12 μg/g, (O¨zcan *et al.*, 2006), Turkey black sea, 1.57-12.9 μg/Kg, (Silici *et al.*, 2008), and Turkey Central Anatolia, 0.09-1.89 μg/g, (Leblebici *et al.*, 2008).

From the reported literature values, the highest mean concentration of Mn was observed in honey sample collected from Egypt, 100-300 μg/g, (Rashed & Soltan, 2004) where as the lowest was observed in honey samples collected from Chile, 0.01-3.14 μg/g, (Fredes *et al.*, 2006). Ethiopian honey contained lower Mn level (ND-7.29 μg/g) than samples from Slovenia, 0.12-66.4 μg/g, (Golob *et al.*, 2005), Poland, 0.125-13.2 μg/g, (Chudzinska *et al.*, 2010), Italy, 0.13-16.9 μg/g, (Pisani *et al.*, 2008), and Egypt 100-300 μg/g (Rashed & Soltan, 2004) but it was observed that the value obtained was comparable with results reported by Madejczyk & Baralkiewicz, 2008 from Poland (0.02-7.37 μg/g), Almeida-Silva *et al.*, 2011, from Portugal (0.83 μg/g), Luque *et al.*, 2005, from Spain (2.3-5.0 μg/g,), and Tuzen & Soylak, 2007, and from Turkey (0.32-4.56 μg/g). Data presented in Table 9 further showed that the mean concentration of Mn in Ethiopian Honey was slightly higher than values reported for samples from Turkey central Anatolia, 0.02-1.56 μg/g, (Leblebici *et al.*, 2008), Turkey black sea, 3.22-74.2μg/g, (Silici et al., 2008), Turkey Anatolia, 0.18-1.2 μg/g, (Tuzen & Soyla, 2005), Turkey (O¨zcan *et al.*, 2006), Italy Lazio (Conti, 2000), Czech Republic (Lachman *et al.*, 2007) and Chile (Fredes *et al.*, 2006).

The results from the present study showed that, the mean concentration of Pb in Ethiopian honey (ND-2.53 μg/g) was lower than values reported for honey samples collected from Turkey (Tuzen &Soylak, 2007) and Slovenia (Golob et al., 2005). The lead level in Ethiopian honey was higher than the mean concentration reported by Chudzinska *et al.*, 2010 from Poland, Turkey (O¨zcan *et al.*, 2006), Turkey central Anatolia (Leblebici *et al.*, 2008), Turkey Black sea region (Silici *et al.*, 2008), Nigeria (Achudume *et al.*, 2010), New Zealand (Vanhanen *et al.*, 2011), Lithuania (Staniškienė *et al.*, 2006), and Italy sienna (Pisani *et al.*, 2008).

The mean concentration of Zn reported from Poland (Chudzinska *et al.*, 2010), India (Nandaa *et al.*, 2003), Egypt (Rashed & Soltan, 2004), Canary Island (Herna´ndez *et al.*, 2005), Poland (Madejczyk & Baralkiewicz, 2008), and Slovenia (Golob *et al.*, 2005) was comparable with the mean concentration of Zn in samples of honey collected from Ethiopia (9.96-16.03 μg/g).

It was found that, the Zn level of Ethiopian honey samples were higher than the values reported from Chile (Fredes *et al*., 2006), Czech (Lachman *et al*., 2007), Italy (Siena) (Pisani *et al*., 2008), Italy(Lazio) (Conti, 2000), Lithuania (Staniškienė *et al*., 2006), New Zealand (Vanhanen *et al*., 2011), Nigeria (Achudume *et al*., 2010), Poland (Pohl *et al*., 2011), Portugal (Almeida-Silva *et al*., 2011), Spain (Latorre *et al*., 1999), Turkey (O¨zcan *et al*.,2006), Turkey black sea (Silici *et al*., 2008),Turkey Central Anatolia (Leblebici *et al*., 2008), and Turkey (Tuzen & Soylak, 2007). From the reported values it was observed that the highest mean concentration of Zn was observed in honey samples collected from Poland (Chudzinska *et al*., 2010), where as the minimum mean Zn concentration was resulted from honey originating from Chile (Fredes *et al*., 2006).

Table 9: Comparison of studied heavy metals with reported values

Country	Trace metal content in honey samples (µg/g)							Ref
	Cd	***Cr***	***Cu***	***Mn***	***Ni***	***Pb***	***Zn***	
Canary Island	NR	NR	0.1-1.73	NR	NR	NR	0.18-19.1	Herna´ndez *et al.*, 2005
Chile	NR	0.03-1.92	0.06-2.00	0.01-3.14	0.01-1.04	0.01-0.11	0.01-4.73	Fredes *et al.*, 2006
Croatia	1.00-24.0*	NR	36-41.217	NR	NR	10-841*	NR	Bilandzˇic´ *et al.*, 2011
Czech	NR	NR	0.11-0.88	0.06-0.40	0.06-0.40	NR	0.4-2.42	Lachman *et al.*, 2007
Egypt	0.01-0.5	NR	1.00-1.70	100-300	1.25-4.1	4.2-6.3	5.00-9.3	Rashed & Soltan, 2004
Ethiopia	***0.017-0.035***	***0.15-6.66***	***0.02-1.15***	***0.36-7.29***	***<MDL***	***0.23-2.53***	***9.96-16.03***	Present study
India	NR	NR	1.74-2.9	NR	NR	NR	2.55-16.77	Nandaa *et al.*, 2003
Italy (Siena)	1.0-15.3*	<2.0-54*	172-5900*	0.13-16.9	77-2760*	28.2-304*	0.72-3.66	Pisani *et al.*, 2008.
Italy (Siena)	NR	NR	0.31	3.0	NR	NR	3.14	Conti, 2000
Lithuania	4.1-14.6*	NR	119.6-342.9*	NR	NR	2.9-22.1*	514-563*	Staniškienė *et al.*, 2006

Macedonia	ND-11.28*	NR	NR	NR	NR	NR	NR	Stankovska *et al.*, 2007
New Zealand	0.01-0.45	NR	NR	NR	NR	ND-0.0170	0.2-2.46	Vanhanen *et al.*, 2011
Nigeria	25.49	0.32	25.49	NR	1.41	0.13	0.14	Achudume *et al.*, 2010
Poland	NR	0.01-0.093	0.26-1.82	0.02-7.37	0.03-1.33	NR	0.13-9.93	Madejczyk & Baralkiewicz, 2008
Poland	NR	NR	NR	NR	NR	NR	0.26-2.61	Pohl *et al.*, 2011
Poland	<LD-0.103	NR	<LD-2.63	0.125-13.2	0.010-1.87*	<LD-9.20	<LD-39.7	Chudzinska *et al.*, 2010
Portugal	NR	NR	0.405	0.83	NR	NR	0.41	Almeida-Silva *et al.*, 2011
Slovenia	NR	0.11-33.8	0.37-15.5	0.12-66.4	0.00-12.7	0.21-79.1	0.55-11.2	Golob *et al.*, 2005
Spain	NR	NR	NR	2.3-5.0	0.25-4.7	NR	NR	Luque *et al.*, 2005
Spain	NR	NR	0.89	5.2	<0.02	NR	2.00	Latorre *et al.*, 1999
Turkey	NR	3.12	NR	0.848	1.93	0.451	2.94	O¨zcan *et al.*, 2006

Turkey Anatolia	10.9-21.2*	NR	0.25-1.10	0.18-1.2	NR	17.6-32.1*	NR	Tuzen & Soyla, 2005
Turkey black sea	1.57-12.9*	1.57-12.9*	9.75-35.8*	3.22-74.2*	1.31-1.35*	1.54-36.7*	0.55-6.27*	Silici *et al.*, 2008
Turkey Central Anatolia	0.09-0.24	0.09-1.89	0.01-0.8	0.02-1.56	0.03-1.44	0.02-1.50	0.15-5.39	Leblebici *et al.*, 2008
Turkey	0.9-17.9	2.4-37.9	0.23-2.41	0.32-4.56	2.6-29.9	8.4-106	1.1-12.7	Tuzen & Soylak, 2007

* Concentrations are expressed in terms of μg/Kg, NR: Not Reported

4.6. Evaluation of Nutritional Value of Ethiopian honey

To evaluate the nutritional value, one and two table spoons of each honey were weighed. Considering the concentration of the elements in the honey samples, the contents of those elements in one and two spoons were calculated and compared to their daily dose allowance (D.D.A.) in the human body (Almeida-Silva *et al.*, 2011). Because of lack of information on metals such Cr, this study tries to compare three studied metals (Cu, Mn, and Zn). Table 10: summarizes the contribution of two table spoon of honey on daily dose allowance. Since there is no stated daily consumption of honey in Ethiopia it's difficult to compare the toxic effect of heavy metals studied with the continuous and immediate ingestion of honey. The ingestion of two table spoons of Ethiopian honey represent between 0.0358% - 7.29% of the D.D.A.

Table 9: Trace metal Concentrations, amount in one and two table spoons of honey, and the contribution of two spoons of ingested honey to (Daily Dose Allowance) D.D.A.

Metal	*Sample*	*Concentration*	*Amount in one table spoon (μg/g)*	*Amount in two table spoon (μg/g)*	*DDA*	*Contribution to DDA (%)*
Cu	Benchi Majji	0.45 ± 0.04	9	18	3 mg	0.6
	Bonga	0.026 ±0.04	0.52	1.04		0.035
	Dawero	0.21 ±0.04	4.2	8.4		0.28
	Ginchi	0.24 ±0.00	4.8	9.6		0.32
	Arsi	0.21 ± 0.01	4.2	8.4		0.28
	Jimma	0.08 ± 0.00	1.6	3.2		0.11
	Adigrat	0.10 ± 0.09	2	4		0.13
	Erob	0.16 ± 0.00	3.2	6.4		0.21
	Tilli	0.32 ± 0.00	6.4	12.8		0.43
	D/Markos	1.15 ± 0.04	23	46		1.53
	Bure	0.02 ± 0.004	0.4	0.8		0.03
Mn	Tilli	7.29 ± 0.04	145.8	291.6	4 mg	7.29
	D/Markos	0.36 ± 0.00	7.2	14.4		0.36
	Bure	0.7 ± 0.07	14	28		0.7
Zn	Benchi Majji	16.03 ± 0.29	320.6	641.2	15 mg	4.27
	Bonga	11.62 ± 0.63	232.4	464.8		3.10
	Dawero	14.3 ± 0.16	286	572		3.81
	Ginchi	10.43 ± 0.64	208.6	417.2		2.78
	Arsi	13.63 ± 1.09	272.6	545.2		3.63
	Jimma	15.17 ± 0.33	303.4	606.8		4.05
	Adigrat	12.77 ± 0.40	255.4	510.8		3.41
	Erob	12.58 ± 0.39	251.6	503.2		3.35
	Hawzene	10.65 ± 0.38	213	426		2.84
	Tilli	14.02 ±0.31	280.4	560.8		3.74
	D/Markos	9.96± 0.4	199.2	398.4		2.66
	Bure	14.62 ± 0.13	292.4	584.8		3.90

As shown in figure 3, honey samples collected from Debre Markos, Tilli and Benchi Maji may contribute the highest amount of D.D.A. of Cu, Mn, and Zn if two table spoons of honey ingested on a daily bases respectively. On the other hand, honey samples collected from Bonga, Debre Markos provide the lowest amount of D.D.A of metals such as Cu, and Zn respectively. Honey samples collected from Benchi Majji and Jimma contribute a comparable amount to daily dose allowance (4.27%, 4.05% respectively) of Zn.

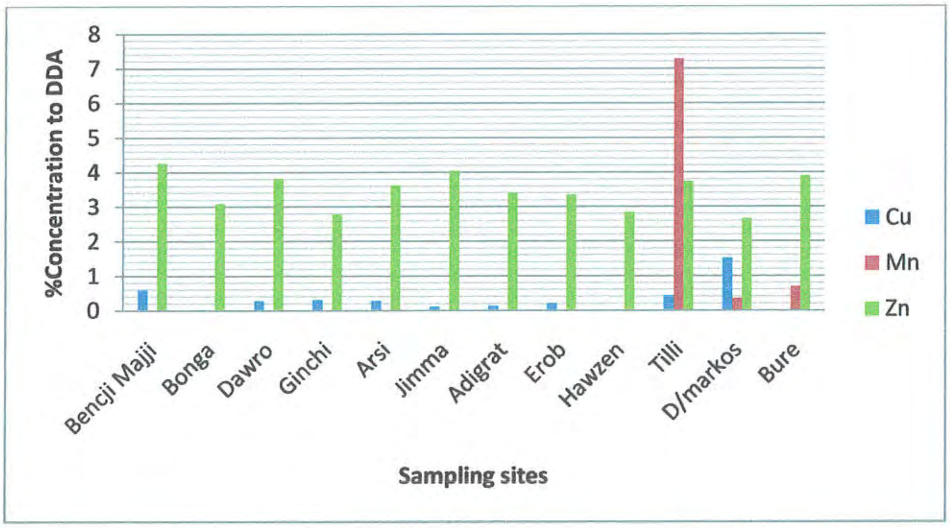

Figure 3: Percentage contribution of trace metals in honey from different regions of Ethiopia to daily dose allowance

5. Conclusion and Recommendations

Honey is a very complex matrix; its composition depends strongly on the plant species from which nectar or honeydew was collected and other factors, such as environmental conditions and climate. In the present study, the physicochemical parameters in terms of moisture content, pH, free acidity, Lactonic acidity and total acidity were evaluated and observed that none of the honey samples exceeded the permitted limit established by the European Community Directive in terms of moisture content (Moisture content not more than 20%), and all studied samples except samples collected from Benchi Majii were in agreement with permitted limit in terms of pH and 75% (9/12) of the studied samples were in agreement with permitted limit in terms of free acidity. There was a significant difference between samples collected from different regions in terms of studied physicochemical parameters. In addition some of the values obtained from this study were comparable with other data reported in various literatures.

The study confirmed the presence of Cd, Cr, Cu, Mn, Pb and Zn but not Ni in the honey samples studied. Among the trace metals analyzed, Zn was detected in all honey samples collected from different regions of Ethiopia. Honey samples collected from Bure, and Benchi Maji showed the highest concentration of Cd and Zn respectively. Cr and Mn were highest in Honey sample collected from D/Markos, whereas, honey samples collected from Tilli showed the highest concentration of Cu and Pb. Honey sample collected from Arsi, Bure and Adigrat showed the lowest concentration of Cd, Cu and Pb respectively. Honey samples collected from D/Markos showed the lowest concentration of Mn, Zn and Cr. In addition the level of contamination of honey varied as Amhara >Tigray>Oromia> SNNPR. As mentioned above the variation in trace metals may be attributed greatly to geographical factors, distance of bee forage area from road sides, soil composition and anthropogenic sources, etc.

In general the present study tried to address some physicochemical parameters and trace metals to assess the quality of honey. But as every region in Ethiopia produces honey, full picture about quality of honey may be obtained if the number of sample collection site increases, and considers more quality parameters including ash content determination, hydromethylfurfural, diastase activity, electric conductivity tests, sugars, insoluble matter, proline determination, specific rotation, and other toxic metals such as mercury and arsenic.

In addition to the above mentioned quality assessments, further investigation on plants, soil where plants grow and used as apiary, and bee themselves could provide valuable information on the level of contamination of honey and environment.

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Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

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