Level of selected physicochemical parameters and metals (Zn, Cu and Pb) of honeys from three districts of the Amhara Region, Ethiopia

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Abstract: As honey may be contaminated during its collection, processing, confection and storage, assessment of the level of metals and physicochemical parameters is very important to ensure its quality. This study describes the levels of Cu, Zn and Pb and some physicochemical parameters in honeys collected from three districts of Amhara region, Ethiopia.

The average values of the studied physicochemical parameters were in the range of ash 0.07–0.20%; EC 0.50–0.94 mS/cm; moisture 15.30–21.90%; pH 3.81–4.14 and free acidity 28.80–40.40 meq/kg. Except the moisture content of honeys from Wuchale (21.13%) and Yesmala (21.87%) districts which slightly exceeded the allowable limits, all the quality parameters were below acceptable standard limits by Codex Alimentarius, EU Council Directives and QSAE.

Linear dependence of inductively coupled plasma optical emission spectrometer emission intensity on the concentration of the studied metals with $R^2$ 0.99823–0.99975 supplemented by excellent recovery results in the range 94.92–97.29%, 87.05–93.23% and 79.83–83.26% for spiked copper, zinc and lead, respectively, in digested honey samples validated the method for determination of the metals in the honey samples. Although lead was not detected in all the analyzed samples, the mean concentrations in the range of 0.678–0.868 mg/kg Cu and 0.693–0.770 mg/kg

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PUBLIC INTEREST STATEMENT
Honey is one of the traditionally explored medicinal food types in the world. Honey, which is produced by honeybees from the plant nectars, possesses valuable nourishing, healing and prophylactic properties. As the quality of honey mainly depends on the soil composition and types of floral plants around the production area, it is imperative to assess the quality of the honey. Amhara Region being one of the most honey producing regions in Ethiopia, no work has been reported on the quality of honey produced in the region. Thus, this study can serve as benchmark for further researchers in the area and honey consumers to have information on the quality of the honey they consume.
Zn were all below the FAO/World Health Organization permitted levels and comparable with reported literature values.

ANOVA analysis results showed significant differences ($P < 0.05$) in ash content, moisture content, EC, free acidity and Cu and Zn content among almost all analyzed honey samples although no significant difference ($P > 0.05$) in pH was observed.

**Subjects:** Environmental Sciences; Agriculture and Food; Food Chemistry; Food Analysis; Analytical Chemistry

**Keywords:** Amhara Region; honey; ICP-OES; physicochemical parameters; quality; trace metals

### 1. Introduction

Honey is a natural sweet substance produced by honeybees from the nectar of blossoms or secretions of plant parts, which they collect, transform and combine with specific substances and store in honey combs (European Union Directive [EU], 2001). Honey possesses valuable nourishing, healing and prophylactic properties. It is often used as a sugar substitute, an ingredient or a natural preservative in hundreds of manufactured foods (Osman et al., 2007) which is attributed to its chemical composition. The various chemical components of honey include variety of macro and micronutrients in the form of supersaturated sugars, organic acids, proteins, aroma compounds, tannins, enzymes, vitamins and traces of minerals. The composition of honey depends highly on the type of flowers utilized by bees, climatic conditions in which the plants grow and maturation (Anklam, 1998).

Moreover, the type and amount of minerals and heavy metals in honey is largely dependent on the soil composition, as well as various types of floral plants, because minerals are transported into plants through the roots and are passed to the nectar and finally into the honey produced from it (Anklam, 1998). However, different anthropogenic activities such as industries nearby, waste dumps, traffic-related pollution and chemical-intensive agriculture, which contaminate the air, water, and soil, can also contribute to the levels of trace elements in honey. When forage areas are polluted, various pollutants can reach honey through nectar, pollen or honeydew of melliferous plants that grow on contaminated soils and absorb contaminated water. Bees themselves can transmit contaminants from the environment into hives and change quality of honey. Honey can also become contaminated with trace elements during its collection, processing, confection and storage (Pohl, 2009).

Trace metals are very important for life when present in optimum concentrations, but at higher concentrations contribute to toxicity in humans. Some metals such as iron, zinc, copper and manganese play important roles in biological systems and are therefore referred to as essential elements, while others like lead, arsenic, cadmium and mercury are nonessential elements which can be toxic even in trace amounts (Diyrikli, Mendil, Tuzen, Soylak, & Elci, 2006). The toxicity occurs due to the inability of the heavy metals to be metabolized by the body, leading to accumulation in human or animal soft tissues without being fully inactivated or destroyed. Accumulation of heavy metals at toxic level in the environment causes health problems such as headaches, metabolic abnormalities, respiratory disorders, nausea, vomiting, damage to the brain, damage to nervous system, vascular diseases, kidney or bone damage, irregular functioning of human and animal reproductive system (Ajibola et al., 2012).

Owing to its varied ecological and climatic conditions, Ethiopia is the leading producer of honey in Africa and stands 10th place in the world. While its potential is to produce 500,000 MT of crude honey per annum, Ethiopia’s current production is limited to 54,000 t of honey (MoARD, 2013). Although bee products are highly distributed across the different regions of Ethiopia, the most important honey producing regions are Oromia, Amhara, Southern Nation Nationalities and People’s, Benshangul-Gumuz and Tigray (Central Statistical Authority [CSA], 2012). Beekeeping is...
a very long-standing practice in the farming communities of Amhara region and it plays a significant role as source of additional incomes and nutrition for many subsistence farmers. Some recent studies indicated that the region has immense beekeeping resources attributed to existing bee floras, various cultivated oil crops, pulse and field flowers.

According to CSA (2012), The Amhara National Regional state has produced 86,843.93 q of honey from 965,293 numbers of hives which accounts about 25% of the total honey production of the country. Many districts both from the moisture stress and moist areas of the region including Gojjam and Gondar are famous for qualitative and quantitative honey production. Moreover, North Wollo and South Wollo zones are also known for their denser honey bee population density due to their high natural vegetation coverage (AWDARDO, 2014; MWDARDO, 2014). Among the districts in the stated zones of the region, Wuchale district (South Wollo), Filakit-Gereger district (North Wollo) and Yesmala district (West Gojjam) are potential honey producers per annum because the areas are endowed with different natural vegetation on which the bees forage to make honey.

Although large amount of honeys are produced per year in each of the district mentioned above, the level of trace metals in honeys and hence its quality have never been studied so far. Therefore, the present study is aimed to investigate the levels of selected trace metals (copper, zinc and lead) and some physicochemical properties (moisture content, ash content, electrical conductivity, pH and free acidity) of honeys obtained from the selected districts (Wuchale, Filakit-Geregera and Yesmala) of Amhara region, Ethiopia.

Analysis of minerals in honey is challenging due to the complex organic matter of the matrix. Several analytical methods have been reported for the determination of heavy metals in honey and other sweeteners. Recently, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectroscopy (Aghamirlou et al., 2015; Akbari et al., 2012; Altun, Dinc, Paksoy, Temamogullari, & Savrunlu, 2017) have been reported for determination of heavy metals in honey and other sweeteners at the same time. Of the reported methods, the ICP-AES is attractive for trace analysis, owing to the satisfactory sensitivity coupled with the advantage of simultaneous determinations of several metals at several spectral lines (Ioannidou, Zachariadis, Anthemidis, & Stratis, 2005). To the best of our knowledge, analysis results on metal content and physicochemical parameters of honey samples from the Amhara Region have not been reported. Thus, this study presents the level of selected metals and physicochemical parameters in honey samples collected from three districts (Wuchale, Filakit-Gereger and Yesmala) in the Amhara Region.

2. Materials and methods

2.1. Description of sampling areas

The study was conducted in three selected districts of Amhara National Regional State; Wuchale district from South Wollo zone, Filakit-Gereger from North Wollo zone and Yesmala from West Gojjam zone.

Wuchale is a town in Northern Ethiopia, which is located about 461 km north of Addis Ababa along the main Addis-Mekele road and 60 km north of Dessie in the South Wollo Zone of the Amhara Regional state. The town is the largest settlement in Ambassel woreda, having geographical coordinates 11° 30’ 0” north, 39° 36’ 0” east with an elevation of 1,711 m above sea level.

Filakit-Gereger is a town located about 204 km north eastern of the capital of Amhara regional state, Bahir Dar. The town is found in the North Wollo Zone of the Amhara Region, having geographical coordinates 11° 40’ 0” north, 38° 48’ 0” east and an elevation of 2,865 m above sea level.
Yesmala is a town located about 105 km north western of the capital of Amhara Regional state, Bahir Dar. It is located 11° 36′ 0″ N latitude and 36° 57′ 0″ E longitude with an elevation of 2,072 m above sea level.

2.2. Sample collection
Honey samples were collected from the three districts on 6 December 2015. Three different fresh honey samples (about 500 g in weight) were collected from each beehive which is found approximately 2 km apart from the nearest village in each district. The collected honey samples were stored in clean polyethylene bottles and sealed. The tightly sealed bottles containing the samples were delivered to Analytical Chemistry Laboratory at Bahir Dar University and kept at room temperature and away from direct sunlight till analysis.

2.3. Chemicals and reagents
Pentahydrated copper(II) sulfate, CuSO₄·5H₂O (98%, Blulux® Laboratories (P) Ltd.), hexahydrated zinc nitrate, Zn(NO₃)₂·6H₂O (98%, Blulux® Laboratories (P) Ltd.), and anhydrous lead(II) nitrate, Pb(NO₃)₂ (99%, UNI-CHEM® Laboratory Reagent) were used for the preparation of standard stock solutions Cu, Zn and Pb ions, respectively. Nitric acid (69%, Indenta Chemicals (Indio) Pvt. Ltd.) and perchloric acid ([60–62%], Blulux® Laboratories (P) Ltd.) were used for digestion of the honey samples. NaOH (98%, Blulux® Laboratories (P) Ltd.) was used for determination of free acidity of the honey samples by titration. Deionized distilled water was used throughout the experiment for preparing sample and standard stock solutions, dilution and rinsing apparatus prior to analysis. All chemicals and reagents used in the analysis were of analytical grade and were used without prior purification, unless otherwise stated.

2.4. Instruments and apparatus
Electronic balance (Nimbus, Switzerland) with ±0.0001 g precision, pH and conductivity meter (pH/Cond. Level-1, InloLab), digital refractometer (Reichert, AR200), muffle furnace (Lenton Thermal, England), hot plate (SH3, Stuart Scientific) and fume cupboard (ENVAIR Ltd., England) were among the apparatus used. Inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer® Optima™ 8000) instrument was used for determination of the concentration of selected trace metals (Cu, Zn and Pb) in the digested honey samples.

2.5. Procedures

2.5.1. Cleaning apparatus
All apparatus (plastics and glass wares) such as polyethylene bottles, hand-held polyethylene spoons, measuring cylinders, beakers, Erlenmeyer flasks, burettes, volumetric flasks, funnels and pipettes were washed with detergents and tap water, rinsed with deionized water, soaked in 10% (v/v) HNO₃ for 24 h before rinsing with deionized distilled water, dried at room temperature and kept in dust free place until analysis begins.

2.5.2. Sample handling and pre-treatment
A pooled honey sample representing each district was obtained by mixing 100 g from each sampling area. Prior to analysis, honey samples were warmed with rotation in a water bath (in their original containers) at approximately 60°C for 30 min and occasionally shaken to guarantee the homogenization and solubilization of sugar crystal in accordance to AOAC (1995). This would also help to liquefy the honey for easy handling and analysis. The samples were later sieved through a mesh sieve of 0.50 mm, diam. so as to remove any foreign matter such as wax, sticks, bees and particles of comb in the honey sample (Bogdanov, 2009). The liquefied honey samples were kept in dark place at 3–5°C in a refrigerator and subsequently digestion was performed prior to trace element analysis.

2.5.3. Analysis of selected physicochemical parameters
Five physicochemical parameters were analyzed using the European Union Directive (EU, 2002), IHC (Bogdanov, 2009), Quality and Standards Authority of Ethiopia [QSAE] (2005) and Codex
Alimentarius Commission (2001) methods. Analysis of each honey samples was conducted in three replicate for each test and during the same period of time to ensure uniform conditions.

2.5.3.1. Determination of moisture content. Refractometric method was used to determine the moisture content in honey as described by the IHC (Bogdanov, 2009). The homogenized sample was put in a 50-mL flask and placed in a water bath at 50°C (±0.2) until all the sugar crystals were dissolved and make sure that the flask is air tighten. The resulting solution was allowed to cool to room temperature, stirred and immediately covered. The refractive index was measured after 2 min of application of few drops of the filtrate on the surface of the refractometer prism. The moisture content of honey was then determined using the measured refractive index of the honey by making reference to a standard table.

2.5.3.2. Determination of ash content. Ash content was determined after the sample was burnt in an electric muffle furnace (Bogdanov, 2009; QSAE, 2005). The ash dish was cleaned, then heated in the electrical furnace to ashing temperature and subsequently cooled in a desiccator to room temperature and immediately weighed (m₂). Then, 5 g of filtered honey sample weighed to the nearest 0.001 g (m₀) was put in the prepared ash dish. After ashing the sample with hot plate, the dish was placed in the preheated muffle furnace (at 600°C) and heated for 6 h. The ash dish was cooled in the desiccator and weighted. The ashing procedure was continued until constant weight was reached (m₁). Each sample measurements were carried out in triplicate and the ash content was expressed in percentage (%). The proportion of ash Wₐ in g/100 g honey is calculated using the formula below

\[ Wₐ = \left( \frac{m₁ - m₂}{m₀} \right) 100 \]

where \( m₀ \) = weight of honey taken, \( m₁ \) = weight of dish + ash and \( m₂ \) = weight of dish.

2.5.3.3. Determination of pH and free acidity. Free acidity of honey defined as the content of all free acids expressed in meq./kg of honey was determined based on IHC (Bogdanov, 2009). Ten gram of the honey samples was dissolved in 75 mL of carbon dioxide-free water (distilled water) in 250 mL beaker and stirred with the magnetic stirrer. Then the pH was measured with digital pH meter (pH/Cond. Level-1, InloLab). The solution was further titrated against 0.1 M NaOH solutions in burette using phenolphthalein (4–5 drops) as indicator. The titration was carried out till the solution turns to pink from colorless, which persists for 10 s. Free acidity expressed as milliequivalents of acid/kg honey (meq./kg) = mL of 0.1 M NaOH × 10, and the result was expressed to one place of decimals.

2.5.3.4. Determination of electrical conductivity. The electrical conductivity of honey is defined as that of a 20% w/v solution in water at 20°C, where 20% refers to honey dry matter (Bogdanov, 2009). Twenty gram of dry matter of honey was weighed and dissolved in 100 mL distilled water. Electrical conductance of the specified solution was measured using previously calibrated conductivity meter (pH/Cond. Level-1, InloLab). All honey samples were measured in triplicate and the results were expressed in mS/cm.

2.5.4. Analysis of selected trace metals in honey samples
2.5.4.1. Digestion of honey samples. Sample pretreatment is usually required to destroy the organic matrix and to extract the metal ions bound in organic complexes (Bakircioglu, Kurtulus, & Ucar, 2011). The honey samples were digested following the optimized procedure after 11 repeated trials. Five gram of homogenized honey samples was accurately weighed and transferred to a 250-mL Erlenmeyer flask. To this, 35 mL of digesting acid mixtures (25 mL conc. HNO₃ and 10 mL conc. HClO₄) was added and digested gently on an electrically heated metal plate by setting
the temperature first to 60°C for 30 min and then increased to 130°C for 2:10 h in a fume cupboard. After cooling the flask at room temperature, 25 mL of deionized water was added to dissolve the precipitate formed on cooling and minimize dissolution of filter paper by the digest residue during filtration. The content in the flask was then boiled to expel any chlorine or oxides of nitrogen until the volume is reduced to about 3–5 mL but not to dryness. This confirmed completion of digestion due to formation of colorless solution. Then after cooling the flask for the second time, the digested solution was filtered using Whatmann® No.42 (110 mm, diam.) filter paper and the filtrate was transferred to a 50-mL volumetric flask and diluted to the mark with deionized water. Digestions of each honey sample were carried out in triplicate. The blank solutions were prepared by digesting the mixture of reagents following the same digestion procedure. All the digested samples and blank solutions were kept at 4°C in a refrigerator until analysis (Cantarelli, Pellerano, Marchevsky, & Camiña, 2008; Corbella and Cozzilino, 2006).

2.5.4.2. Preparation of standard solutions of metals. Hundred milliliter of 1,000 ppm standard stock solution of each analyzed trace metal (copper, zinc and lead) was prepared by dissolving appropriate amount of the corresponding nitrate salt in deionized distilled water, while 50 mL of 10 ppm intermediate standard solution was prepared from the respective stock solution. Calibration standard solutions of 0.50, 1.00, 2.00, 4.00, 8.00 mg/L for those relatively abundant elements copper and zinc and 0.20, 0.40, 0.80, 1.60, 3.20 mg/L for the rarely abundant lead were prepared from their respective intermediate standard solutions.

2.5.4.3. Determination of trace elements in honey using ICP-OES. ICP-OES technique was used for quantitative determination of the studied metals (copper, zinc and lead) in honey samples after calibration of the instrument using working standards and blank solutions. Following its calibration, triplicate measurements were recorded for each sample and digested blank solution. Table 1 describes the operating conditions for the determination of selected trace metals by ICP-OES. The level of each analyzed metal in each honey sample was thus calculated using the regression equation drawn for the dependence of the emission intensity on concentration for each metal.

2.5.5. Method verification
As there were no readily available certified reference material candidates for trace elements in honey, the recovery results for spiked metals in honey samples were used as estimates of the accuracy of the method (Ioannidou et al., 2005). Each digested honey sample was divided into two equal portions and placed in 25 mL volumetric flasks. While one portion was spiked with known amounts of the

| Table 1. Optimized operating conditions for the determination of selected metals by ICP-OES |
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| **Parameters** | **Values** |
| RF power | 1,500 W |
| Plasma gas flow rate | 8 L/min |
| Auxiliary gas flow rate | 0.4 L/min |
| Nebulizer gas flow rate | 0.6 L/min |
| Viewing height | 15 mm |
| Purge flow | High |
| Plasma view | Axial |
| Read parameters(s) | Auto (1–5(min–max)) |
| Peristaltic pump flow rate | 1.5 mL/min |
| Replicates | 3 |
| Element wavelength (nm) | Cu (324.754) |
| | Zn (213.856) |
| | Pb (220.353) |
studied metals, the other portion was left as unspiked to serve as a control. The “spiked” honey sample was spiked with 457 μL of 100 mg/L of Cu, 343 μL of 100 mg/L of Zn and 1,144 μL of 10 mg/L of Pb which were added at once and then filled with deionized distilled water up to the mark. The “unspiked” honey sample was just filled with the deionized distilled water up to the mark. Both the spiked and unspiked samples were analyzed in similar experimental conditions.

2.5.6. Method detection limits
Mostly used and accepted definition of LOD is the concentration that gives a signal three times the standard deviation of the blank or background signal. It can also be estimated by the relation:

\[ \text{LOD} = \frac{3 \times SD}{m} \]

where \( m \) is the slope of the regression equation for the calibration curve and SD is the standard deviation of nine measurements for a blank solution (UNODC, 2009). Similarly in this study, three reagent blank solutions were digested and triplicate readings were recorded for each of the sample at the respective wavelength.

2.6. Data analysis
All the determinations of trace metals and physicochemical parameters were conducted in triplicate and results were expressed as mean ± standard deviation. Calibration graphs for trace metal determination were plotted using Origin software (Version 8). The data for the determination of pH, electrical conductivity, free acidity, ash content, moisture content, and selected trace metals were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference post hoc test \( (P < 0.05) \). ANOVA analysis was carried out by statistical software (SPSS version 20.0).

3. Results and discussion

3.1. Physicochemical parameters of honey samples
The mean results for the analyzed selected physicochemical quality parameters (ash content, EC, moisture content, pH and free acidity) in honey samples collected from the three selected districts of Amhara region are summarized in Table 2.

3.1.1. Ash content
The ash (mineral) content of the analyzed honey samples ranged between 0.07% and 0.20% all of which are within the acceptable standard limit (less than or equals to 0.60%) prescribed by Codex Alimentarius (EU, 2002), the Quality and Standards Authority of Ethiopia (QSAE, 2005) and EU Council Directives (Codex, 2001). The highest ash content obtained for honey samples from Wuchale district (0.20%) was almost three times the ash content of honeys from Yesmala district (0.07%). The variation in the ash contents between honeys from different districts might be due to many factors including the botanical origin, the materials gathered by the bees during foraging, the difference in soil composition and atmospheric conditions/environmental pollution as well as the type and physiology of each plant visited by the honey bee (Anklam, 1998).

3.1.2. Electrical conductivity
As can be seen from Table 2, the highest electrical conductivity was recorded for honey samples from Wuchale district (0.94 ± 0.06 mS/cm) followed by Filakit-Geregera district (0.88 ± 0.05 mS/cm) and Yesmala district (0.50 ± 0.03 mS/cm) which was the same trend as the ash content. Although the honey from Yesmala which showed EC value under the permissible limits can be characterized as nectar/multi-floral blossom honeys, the honeys from Filakit-Geregera and Wuchale districts which showed EC values beyond the permissible limits were classified as honeydew honeys (Codex Alimentarius Commission, 2001; EU, 2002). The variation of EC values among honey samples could probably be due to the difference in the botanical origin of the samples. Furthermore, high values of EC might be due to storage time, temperature, water content and concentration of ions and minerals (Guo, Liu, Zhu, & Zhuang, 2011).
3.1.3. Moisture content

The moisture content of the studied honey samples (Table 2) was all under the national and international permissible level (Codex Alimentarius Commission, 2001; EU, 2002; (QSAE), 2005), although honeys from Yesmala district had the highest moisture content while honeys from Filakit-Geregera district the least. The least moisture content of honey from Filakit-Geregera might be due to harvesting ripened honey, the dry harvest season with low humidity and the honey was stored under suitable conditions; and this indicated that honey was protected from attack by microorganisms. On the other hand, the high moisture contents of the rest honey samples might be due to wet season with high humidity of the studied areas, inappropriate honey harvesting time before ripening and storage conditions; and this showed that the honeys were easily exposed to attack by microorganisms (Cantarelli et al., 2008).

3.1.4. pH value

The pH values of investigated honeys from Filakit-Geregera, Wuchale and Yesmala districts were 4.14 ± 0.07, 3.81 ± 0.20 and 3.90 ± 0.13, respectively (Table 2), all of which being within the permissible limit (Codex Alimentarius Commission, 2001; EU, 2002). The low pH values of the studied honey samples indicated that the honeys were not suitable for the growth of microorganisms and hence improved stability and durability of the honey samples. The variations in pH values could be accounted for difference in acidity, minerals present in the honey and the floral/nectar source (Muli, Munguti, & Raina, 2007).

3.1.5. Free acidity

In the present study, the free acidity of the studied honeys varied from 28.80 to 40.40 meq/kg (Table 2) (Bogdanov, 2009; Codex Alimentarius Commission, 2001; EU, 2002; QSAE, 2005). As can be referred from the table, except the honeys from Filakit-Geregera which showed a free acidity value a little higher than the national allowed limit (QSAE, 2005) but still under the international limit (Codex

Table 2. Summary results of analyzed physicochemical parameters of the honey samples collected from the three districts versus international and national permitted values

| Parameters (unit) | Present study | Standards |
|-------------------|---------------|-----------|
|                   | District       | Mean ± SD* | International (Codex and EU) (range) | National (QSAE) (max. limit) |
| Ash content (% mass) | FG            | 0.13 ± 0.03a | 0.6–1.2 | 0.60 |
|                   | WC            | 0.20 ± 0.05b |          |          |
|                   | YS            | 0.07 ± 0.01a |          |          |
| EC (mS/cm)        | FG            | 0.88 ± 0.05a | ≤0.8    | –        |
|                   | WC            | 0.94 ± 0.06b |          |          |
|                   | YS            | 0.50 ± 0.03ab|          |          |
| Moisture content (% mass) | FG          | 15.27 ± 0.61ab | ≤20.0 | 21.0 |
|                   | WC            | 21.13 ± 0.90a |          |          |
|                   | YS            | 21.87 ± 0.46a |          |          |
| pH                | FG            | 4.14 ± 0.07a | 3.40–6.10 | –        |
|                   | WC            | 3.81 ± 0.20a |          |          |
|                   | YS            | 3.90 ± 0.13a |          |          |
| Free acidity (meq/kg) | FG          | 40.37 ± 0.74a | ≤50.0 | 40.0 |
|                   | WC            | 28.80 ± 0.80a |          |          |
|                   | YS            | 34.70 ± 0.46a |          |          |

*Mean of triplicate measurements; SD at 95% confidence limit; means down a column for the same parameter with same superscript letter were significantly different (P < 0.05); FG: Filakit-Geregera; WC: Wuchale district; YS: Yismala district.
Alimentarius Commission, (2001; EU, 2002), the free acidity values for all the studied honeys were under the national and international permitted levels. This confirmed that the studied honey samples were newly harvested/fresh and will not be fermented (Muli et al., 2007).

3.2. Analysis of trace elements in honey samples

3.2.1. Instrument calibration
Calibration curves are plotted to determine the concentration of metals in the sample solution. The quality of the results emanated from the analysis of metals in a given sample matrix using ICP-OES is highly affected by calibration and solution preparation procedures. Concentrations of working standard solutions, linear regression equations and correlation coefficients of the calibration curve for the determinations of each trace element by ICP-OES method are described in Table 3.

Determination coefficient value greater than 0.998 for each trace metal confirmed good linearity of the signal with the concentration within the selected analytical range.

3.2.2. Recovery analysis
To ensure the reliability of the result obtained for the analysis of the studied trace elements in honey samples, a recovery test was conducted. The percentage recoveries (%R) of the detected trace metals in the spiked honey samples were calculated to be in the range 94.92–97.29% for Cu, 87.05–93.23% for Zn and 79.83–83.26% for Pb. All percentage recovery values were within the acceptable range for analysis of sugar rich food stuff such as honey. Therefore, excellent recovery results confirmed the suitability of the analytical method for the determination of trace metals in honey samples.

3.2.3. Concentration of trace elements in honey samples
The average concentration of the analyzed trace elements in all honey samples collected from three different districts with their corresponding % RSD is summarized in Table 4. The results indicated that with the exception of Pb, which was found below the method detection limit and not detected in all honey samples, the rest two trace elements were detected in all honey samples.

As designated in the table, the concentrations of trace elements differed significantly between the honey samples from all the districts under study. Among the honey samples analyzed, higher concentration of trace metals (Cu and Zn) was obtained in honey samples from Filakit-Geregera and Wuchale districts, and the lowest concentration for honey samples was collected from Yesmala district. However, lead concentrations were found to be below the method detection limit in all the studied honey samples.

The concentration of copper in the current study ranged from 0.678 to 0.865 mg/kg with the overall mean value of 0.794 ± 0.101 mg/kg (Table 4). The determined level of copper in all analyzed honey samples was below the permissible value set by Codex Alimentarius (2001). The mean value of copper intake from honey consumption in the studied districts was 0.794 mg/kg, which is less than the amount recommended by PTDI as 3 mg based on the body weight of an average 60 kg adult (WHO, 1999). The result indicated that significant differences were obtained (P < 0.05) between the amounts of copper in all analyzed honey samples (Table 5). Relatively higher amounts of copper in honey samples from Filakit-Geregera and Wuchale districts could be highly associated with the diversity in practice of growing plants which were utilized by the bee for nectar preparation and might also be the natural weathering of soils or the use of different fertilizers.

The mean amounts of zinc obtained under this investigation were 0.711 ± 0.006, 0.770 ± 0.007 and 0.693 ± 0.008 mg/kg for honey samples from Filakit-Geregera, Wuchale and Yesmala districts, respectively. The minimum and maximum zinc contents observed were 0.693 mg/kg in honey sample from Yesmala and 0.770 mg/kg in Wuchale districts, respectively (Table 4). All the resulted amounts of zinc were below the permissible limit established by East Africa Community (Codex
| Metal | Wavelength (nm) | Working standards (mg/L) | Linear regression equation | $R^2$ | LoD (µg/kg) |
|-------|-----------------|--------------------------|---------------------------|------|-------------|
| Cu    | 324.754         | 0.50, 1.00, 2.00, 4.00, 8.00 | $I = -81,820.08286 + 2,295,730C$ | 0.99954 | 0.035       |
| Zn    | 213.856         | 0.50, 1.00, 2.00, 4.00, 8.00 | $I = 3,728.48571 + 337,951.8765C$ | 0.99975 | 0.089       |
| Pb    | 220.353         | 0.20, 0.40, 0.80, 1.60, 3.20  | $I = 2,679.64286 + 35,957.10369C$ | 0.99823 | 2.060       |
Moreover, the overall mean concentration of zinc (0.725 ± 0.040 mg/kg) in honeys from the studied districts was within the acceptable limits recommended as daily consumption in food for adults by World Health Organization (WHO) (World Health Organization (WHO), 1999). Statistically significant differences (P < 0.05) were existed between the contents of zinc among honey samples collected from different districts under study (Table 4). The elevated concentration of zinc in honey samples collected from Wuchale district might be attributed to its natural abundance in the soil, intensive use of agrochemicals such as fertilizers and pesticides. It might also be due to use of honey storage materials, especially zinc-coated equipment by beekeepers.

3.2.4. Comparison of level of trace metals in the present study against reported values in the literature

Even if there might be a difference in sampling technique, sample digestion technique and analysis method, the concentrations of trace metals obtained in the present study could be compared with the results that have been reported by different authors. The comparison of trace metals concentrations in honey samples of the present study with those reported values in the literature was summarized in Table 5.

| Metal | LOD (µg/kg) | FG | WC | YS | Over all mean |
|-------|-------------|----|----|----|--------------|
| Cu    | 0.035       | 0.865 ± 0.008<sup>a</sup> | 0.838 ± 0.015<sup>a</sup> | 0.678 ± 0.024<sup>a</sup> | 0.794 ± 0.101 |
| Zn    | 0.089       | 0.711 ± 0.006<sup>b</sup> | 0.770 ± 0.007<sup>b</sup> | 0.693 ± 0.008<sup>b</sup> | 0.725 ± 0.040 |
| Pb    | 2.060       | ND | ND | ND | –            |

Mean ± SD in the same row with the same superscript letter were significantly different (P < 0.05); ND: not detected.

Alimentarius Commission (2001). Moreover, the overall mean concentration of zinc (0.725 ± 0.040 mg/kg) in honeys from the studied districts was within the acceptable limits recommended as daily consumption in food for adults by World Health Organization (WHO) (World Health Organization (WHO), 1999). Statistically significant differences (P < 0.05) were existed between the contents of zinc among honey samples collected from different districts under study (Table 4). The elevated concentration of zinc in honey samples collected from Wuchale district might be attributed to its natural abundance in the soil, intensive use of agrochemicals such as fertilizers and pesticides. It might also be due to use of honey storage materials, especially zinc-coated equipment by beekeepers.

### Table 4. Concentration (mean ± SD) and relative standard deviation (%RSD) of trace elements (wet weight) in honey samples collected from three selected districts of Amhara Region (n = 3 at 95% confidence level)

| Metal | LOD (µg/kg) | Concentration of trace elements (mg/kg) in honey samples |
|-------|-------------|--------------------------------------------------------|
|       |             | FG          | WC          | YS          | Over all mean |
| Cu    | 0.035       | 0.865 ± 0.008<sup>a</sup> | 0.838 ± 0.015<sup>a</sup> | 0.678 ± 0.024<sup>a</sup> | 0.794 ± 0.101 |
| Zn    | 0.089       | 0.711 ± 0.006<sup>b</sup> | 0.770 ± 0.007<sup>b</sup> | 0.693 ± 0.008<sup>b</sup> | 0.725 ± 0.040 |
| Pb    | 2.060       | ND          | ND          | ND          | –            |

<sup>a</sup>Mean (range) in mg/kg; <sup>b</sup>mean (range) in µg/kg; <sup>###</sup>not reported.

### Table 5. Comparison of trace metal concentrations in honey samples of the present study against reported values in literature

| Country     | Concentration of trace elements Mean (range) | Reference |
|-------------|---------------------------------------------|-----------|
|             | Cu (mg/kg) | Zn (mg/kg) | Pb (mg/kg) |
| Ethiopia    | 0.37–13.99 | 0.370–1.124 | 36.00–1880.00 | Nigussie, Subramanian and Mebrahtu (2012) |
| Kenya       | 0.16 (0.07–0.24) | 1.67 (1.01–2.10) | 30.0 (10.00–50.00) | Irene (2012) |
| Morocco     | 0.51–4.75 | 0.04–2.74 | 36.00–1880.00 | Hasna, Mohamed and Abdelkader (2008) |
| Saudi Arabia | 0.29 (0.24–0.39) | 0.53 (0.21–0.75) | 61.0 (38.00–80.00) | Eusman et al. (2007) |
| Turkey      | 0.64 (0.23–2.41) | 3.59 (1.1–12.7) | 26.5 (8.4–106.0) | Tuzen, Silici, Mendil and Soyak (2007) |
| France      | 0.06–1.71 | 0.17–6.42 | 280.00–1,080.00 | Devillers et al. (2002) |
| Argentina   | 0.05–0.68 | 0.14–3.87 | 380.00–1,080.00 | Fernandez-Torrez et al. (2005) |
| Uruguay     | 0.91 | 1.82 | 80.0 | Corbella and Cazzolina (2006) |
| Spain       | 0.85 (0.53–2.12) | 3.96 (1.33–7.83) | 380.00–1,080.00 | Fernandez-Torrez et al. (2005) |
| Ethiopia    | 0.794 (0.678–0.865) | 0.725 (0.693–0.770) | ND | Present study |

<sup>###</sup>Mean (range) in mg/kg; <sup>##</sup>mean (range) in µg/kg; <sup>##</sup>not reported.
As indicated in the table, most of the reported results in literature are in parallel with the findings of this study. The concentration of lead was below the method detection limit in all analyzed honey samples. This undetected level of lead in the studied honey samples was in line with values reported for honey samples from Argentina (Cantarelli et al., 2008), Ethiopia (Nigussie et al., 2012) and Spain (Fernandez-Torrez et al., 2005).

As compared to the present study, higher mean concentrations of copper were reported as 0.85 mg/kg for honeys from Spain (Fernandez-Torrez et al., 2005) and 0.91 mg/kg from Uruguay (Corbella & Cozzolina, 2006) while lower values were quoted in the literature as 0.29 mg/kg (Osman, Al-Doghairi, Al-Rehiayani, & Helal, 2007), 0.64 mg/kg (Hasna et al., 2008) and 0.16 mg/kg (Irene, 2012) for honeys from different regions of Saudi Arabia, Turkey and Kenya, respectively. However, the range of the results obtained in this study is in agreement with those reported values (Devillers et al., 2002; Fernandez-Torrez et al., 2005; Hasna et al., 2008; Nigussie et al., 2012). Hence, on average, the mean concentration of copper in the current study was within the ranges of previously reported values. The differences in the contents of copper in honeys of different region/country might be due to the floral type, the botanical origin, environment contamination, soil composition, production and storage conditions (Pohl, 2009).

The investigated mean concentration of zinc was below all the reported results cited in Table 5 (Corbella & Cozzolina, 2006; Fernandez-Torrez et al., 2005; Irene, 2012; Tuzen et al., 2007) except in case of honeys from Saudi Arabia (0.53 mg/kg) (Osman et al., 2007). The contents of zinc reported as 0.370–1.124 mg/kg (Nigussie et al., 2012) for honeys within the studied country of Tigray region are in line with the results obtained in this study. In addition to this, the levels of zinc in this study are similar to that in honeys from Argentina (0.14–3.87 mg/kg) (Cantarelli et al., 2008), Morocco (0.04–2.74 mg/kg) (Hasna et al., 2008) and France (0.17–6.42 mg/kg) (Devillers et al., 2002). Therefore, mean concentration of zinc in the present study was in good agreement with the reported values in the literature. The variation in the amounts of zinc between the different localities is not only due to varying amounts of minerals in the soil but also because of the different types of plants visited by the bees (Afzal et al., 2014).

4. Conclusion
This study in-depth focused on quantitative determination of selected trace elements (copper, zinc and lead) using ICP-OES and some physicochemical parameters of honeys produced in three selected districts of Amhara region, Ethiopia.

The analysis results revealed that the average ash content, EC value, free acidity and pH value in all the analyzed honey samples were in agreement with permitted limits proposed at national and international level by Codex Alimentarius, EU Council Directives and QSAE. Except honey samples from Filakit-Geregera, the moisture contents of honey samples collected from Wuchale (21.13%) and Yesmala (21.87%) districts slightly exceeded the allowable limits. This indicates that inappropriate handling during harvesting and storage was practiced for honeys from Wuchale and Yesmala districts. Low free acidity, acidic range of pH and low ash content values confirmed that the collected honey samples were fresh, clean and not exposed to fermentation. Even though the moisture contents of honeys from Wuchale and Yesmala districts indicate a slight deviation from the allowable limits, the overall physicochemical analysis results of honeys from different districts showed good quality.

An ICP-OES method verified for metal analysis by its reasonably excellent correlation coefficients (≥0.998), and recovery results in the range 79.83–97.29% was used for determination of selected metals copper, zinc, and lead.

While the elements copper and zinc were detected in all the analyzed honey samples, lead was not detected in any of the collected honey sample. The average concentrations of copper and zinc obtained in all analyzed honey samples were below the permitted values prescribed by FAO/WHO. The ANOVA showed that there was a significant difference (P < 0.05) in the concentrations of
copper and zinc between each of honey samples collected from different districts. The differences may be due to the floral type, the botanical origin, equipment used for honey storage and processing, natural composition of soils and intensive use of agrochemicals such as fertilizers and pesticides.

Though the findings of this study are limited in describing the level of various trace metals in the collected honey samples, combining with the results obtained from physicochemical analysis provides a clue about their safe level for human consumption. Moreover, the absence of toxic trace metal (Pb) in this investigation revealed that the collected honey samples are free from toxic contaminants and have good quality level.

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