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Research Article

Physicochemical Analysis and Determination of the Levels of Some Heavy Metals in Honey Samples Collected from Three District Area of East Gojjam Zone of Amhara Region, Ethiopia

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ABSTRACT

This study analysis physicochemical property and some heavy metals levels of honey from three-district area of East Gojjam Zone, Ethiopia. The samples were collected purposively from the most potential beekeeping Woredas namely Debre Markos, Dejen, and Bichena. The results of pH, electric conductivity, moisture, total solid, ash content, free acidity, reducing sugar, total sugar, non- reducing sugar were found to be 3.98-4.12, 0.35-0.65 mS/cm, 17.5-18.19%, 81.8- 82.3%, 0.09-0.26%, 35.3-46.6 mg/kg, 45.1-63.8%, 61.4-68.1%,and 4.30-16.51%, respectively. Some of the levels of heavy metal contents were determined by using flame atomic absorption spectrometry (FAAS). The optimized wet digestion method for honey sample analysis was found to be efficient for the metals determined and it was validated through the recovery experiment and a good percentage recovery was obtained (84.45- 98.0%).Among the seven heavy metals analyzed for honey Cd and Pb were not detected, hence below the method detection limit. However, the concentration of Fe was found in highest amount with mean concentration ranging from (0.59 to 5.39 µg/g followed by Cr with mean concentration range of (0.22-0.46 µg/g, Cu (0.27-0.28 µg/g, Mn (0.09-0.33 µg/g and Ni (0.04-0.14 µg/g). The metals content and the physicochemical properties investigated in honey samples were found within the ranges established by national and international standards. except non-reducing sugar content from Bichena. The slight excess value of sucrose content of honey from Bichena may be due to adulteration of the honey by addition of commercial sugar to honey.

Keywords: Honey; Heavy metals; Physicochemical properties; Wet digestion; East gojjam zone

INTRODUCTION

Background of the Study

Ethiopia is known for its tremendous variation of agro-climatic conditions and biodiversity, which favored the existence of diversified honeybee flora and huge number of honeybee colonies. Already the hieroglyphs of the ancient Egyptians give a hint that this country has been a source for honey and beeswax ever since. Ethiopia is the largest honey producer in Africa and the ninth largest honey producer all over the world [1,2]. Honey is a natural sweet, viscous substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants, which honeybees collect, transform, and combine with specific substances of their own, store and leave in the honey comb to ripen and mature. It is the simplest and often the best way to soothe a sore throat and it can be taken at any time [3]. Freshly extracted honey is a viscous liquid, with a greater density (1.5 g/cm^3) than water (1 g/cm^3 at 4°C); having a strong hygroscopic character, relatively low heat of conductivity, low surface tension and various colors [4].

Honey is a concentrated aqueous solution of different carbohydrates, fructose, glucose, maltose, sucrose and other oligo- and polysaccharides. The major components of honey and the most dominant are the monosaccharide's fructose and glucose (accounting for 85 to 95%); the actual proportion of glucose to fructose in any particular honey depends largely on the source of the nectar. The average ratio of fructose to glucose is 1.2:1. The amount of glucose in honey is usually at a supersaturated level at normal temperatures [5]. It is the major product of honeybees, which has important nutritional value and provides significant economic contributions. Quality control of honey is important to determine its suitability for processing and to meet the demand of the market. Honey shall not have foreign taste, begun to ferment, heated to the extent of destroying its natural enzymes and a substance that endanger human health [1]. Honey is generally evaluated by a physicochemical analysis of its constituents. Several of these constituents are of great importance to the honey industry. These constituents influence the storage quality, granulation, texture, flavor and the nutritional quality of the honey. These are also responsible for the medicinal quality of honey. These

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constituents include: moisture content, electrical conductivity, pH, ash content as reported by [6]. Honey bees are a good biological indicators because they indicates the chemical impairment of the environment they live in through two signals: the high mortality rates in the presence of toxic molecules and the presence of residues in honey, pollen, and larvae due to the excessive existence of heavy metals, fungicides, and herbicides that are normally harmless to bees [7,8]. The bees are considered biological indicators due to their important morphological, ecological, and behavioral characteristics. Honey's composition, flavor, and aroma are derived from the plant utilized by the bees, as well as regional and climatic conditions. Therefore, the specific composition of honey and the possible presence of contaminants are also dependent on the crops surrounding the bee hives [9]. Elements such as Se, Cu, Mn, Fe, Ni, and Zn are essential for normal metabolism, but above tolerance limits they could be environmental pollutants that are hazardous for human health and trace elements such as Pb, Cd, and Al are considered as toxic and could damage the human metabolism. The levels of Pb, Cd, Ni, and Cr are unacceptable owing to their carcinogenic and cytotoxic influences. The mineral and toxic metal content of honey have been used as a quality indicator and toxic metal levels of honey depend on the biological and geographical origin [10]. However, there is no enough data available, which can give us information about the levels of these toxic metals in honey collected from three-district area of East Gojjam Zone. To our knowledge, no research has been carried out to the determination of physicochemical characteristics and the levels of heavy metals in honey consumed in East Gojjam Zone. Therefore, the present study was aimed to provide information on the physicochemical properties and level of heavy metal of honey samples obtained from three district Woredas of East Gojjam Zone.

MATERIALS AND METHODS

Reagents, chemicals and equipment(s)

Electronic analytical balance with ± 0.0001 g precision (AA-200DS, Deriver Instrument Company, Germany) was used to weigh honey samples. pH meter (Model: Elmetron CPI-501, Poland) was used for determination of pH of the honey. Electrical conductivity meter (Model: SCHOTT handlab LF11, Germany) was used for determination of EC of the honey. Digestive furnace (Model: KDN-20C, China), Kjeldahl tubes fitted with reflux condenser were used in Kjeldahl digestion block apparatus to digest honey samples, spiked honey samples and blank solutions. A refrigerator (Beko RDP 6900, Japan) was used to keep the collected samples and digested samples until analysis. Flame atomic absorption spectrometer (Model: AA-500AFG, UK) equipped with deuterium ark background correctors and hollow cathode lamps with air-acetylene flame was used for analysis of the digested honey samples for the metals Pb, Cu, Cr,Ni, Fe, Mn and Cd. All the reagents used were of analytical grade. 69-72% nitric acid (HNO_3) and 70% perchloric acid ($HClO_4$) (Fine Chemical, Mumbai, India) were used for the digestion of honey samples. Stock standard solutions containing 1000 mg/L, in 2% HNO_3, of the metals Pb, Cu, Cr, Ni, Fe, Mn and Cd (Buck Scientific PuroGraphictm, USA) were used for preparation of calibration standards and in the spiking experiments. Deionized water (chemically pure <1.5 µscm-1) was used throughout the experiment for sample preparation and dilution.

Sample collection

The honey samples were collected from three district Woredas of East Gojjam Zone of Amhara Region, Ethiopia. The samples were collected purposively from the most potential beekeeping Woredas namely Debre Markos, Dejen, and Bichena (Figure 1). Nine honey samples (three from each Woredas market)) were collected randomly from the market directly the beekeepers during December 2019 and the three sub-samples from each Woredas was mixed to make a composite sample that represents each sampling area. Finally, three honey samples (one from each stated areas) were collected, put in clean cooled glass jars with proper labelling and stored in glass jars. The honey samples were transported to Ambo University Laboratory and kept there in refrigerator at 4^0C for further analysis.

Sample preparation

In accordance with AOAC 920.180, honey samples were heated in a water bath at 65°C to dissolve any fine crystals. The samples were filtered to remove any coarse particles, which may affect the analysis and indeed to decrease viscosity for more uniform distribution. The samples were then cooled and weighed for subsequent analysis [11].

Determination of pH value

The pH of the sample was measured by pH meter (Model: Elmetron CPI-501, Poland), which was calibrated with standard buffer solutions of pH 4.0 and 7.0 [12]. Ten grams of honey were added to 75 mL of distilled water in a 250 mL beaker. The electrodes of the pH meter were immersed in the solution and the pH was measured directly.

Determination of electrical conductivity

The electrical conductivity was determined by conductivity meter

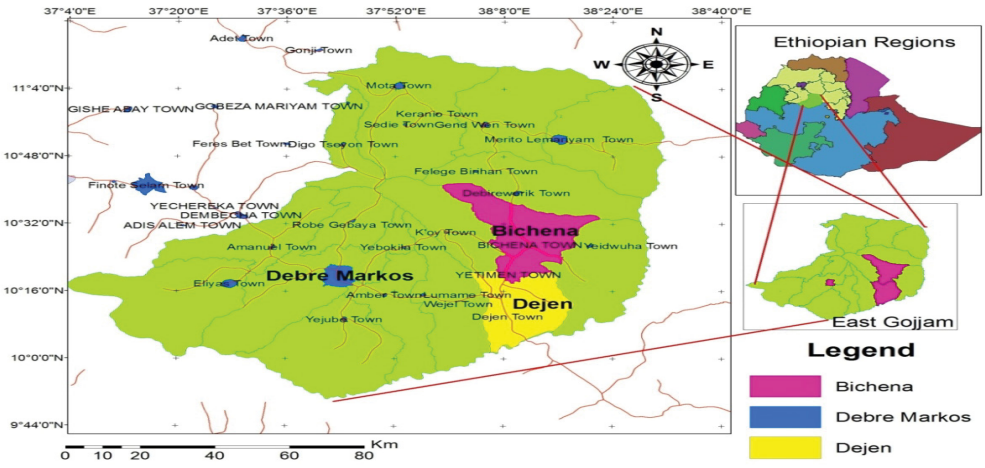

Figure 1: Map of the study area.

(Model:SCHOTT handlab LF11, Germany). About 10 g of sample was mixed with 75 mL of distilled water in a 200 mL beaker and the mixture was stirred for about 30 min [3]. The instrument was calibrated using 2 M potassium chloride (KCl), which has an electrical conductivity of 1413 mS/cm at 25°C.

Determination of moisture content

Five grams of each sample was weighed and placed into a pre-weighed aluminum drying dish. The samples were dried to constant weight in an oven at 105°C for overnight under [12]. Percent moisture was calculated as follows:

$$\textbf{Moisture}(\%) = \frac{M_1 - M_2 \times 100}{M_1 - M_0} \quad (1)$$

Where: M_o = Weight of empty aluminum dish ,M_1 = Weight of the fresh sample + dish, and M_2 = Weight of the dried sample + dish

Determination of total solids

The percentage total solid of each sample were determined by using the following formula:

Total solids (%) =100 – Moisture content (2)

Determination of ash content

Samples were prepared according to 920,181 method of the A.O.A.C. Honey sample of Two grams was weighed accurately into a pre-weighed porcelain crucible and gently heated on a hot plate until the sample was turned in to black and dry and hence there was no danger of loss by foaming and overflowing. The sample was then ignited at 550°C in a furnace (overnight) to constant weight. Then the samples were cooled in a desiccator and weighed [13,14].

Determination of free acidity

Ten grams of honey were weighed in a glass beaker and then 75 mL of distilled water were added. The solution was titrated with 0.1 N NaOH solution until a pH of 8.3 was attained [15]. The results were expressed as meq/kg honey.

Free acidity = V × 10 (3)

Where: V = Titer value " the volume of mL (0.1 N) NaOH used in the neutralization of 10 g honey" and 10 indicate the dilution factor of honey sample during analysis.

Determination of sugar

Five grams of the homogeneous sample honey were transferred to a flask and diluted with distilled water to 100 mL. 2-3 drops of phenopthelene was added, and then 20 mL of NaOH solution was added till the solution turns to pink color. Ten mL of HCl acid was added to the solution to turn to its original color and then added distilled water to 200 ml total volume (honey solution). Five mL of honey solution was taken in the burette. Five mL of Fehling's solution A and five mL of Fehling's solution B were transferred to 250 mL Erlenmeyer flask and approximately five to ten mL of distilled water was added and heated until it starts boiling. Two drops of 0.2 % of Methylene blue indicator was added and titrate with honey solution till brick red colored end point.

$$\text{Reducing sugar}(\%) = \frac{\text{Fehling solution constant}(0.052) \times \text{total volume of solution} \times 100}{\text{Weight of sample solutio} \times \text{volume of titrate}} \quad (4)$$

Fifty milliliter of honey solution, from the solution prepared for reducing sugar in above solution was placed in a graduated flask, together with 15 mL distilled water, and boils for 15 minutes and cools it. Then neutralize it as in reducing sugars and then the total volume 200 mL (V_2) distilled water was added (diluted honey solution). Then 5 mL of honey solution, 5 mL of Fehling A, 5 mL of Fehling B and 5 to 10 mL of distilled water were taken in a 250 mL conical flask and heated till it starts boiling. During boiling, 2-4 drops of 0.2 % of methylene blue indicator were added to the flask and titrate with honey solution till it turns in to brick red color. The percentage of total sugar was calculated using equation[3].

$$\text{Total sugars} = \frac{\text{Fehlingsolutionconstant}(0.051 \times 200 \times 200 \times 100}{\text{Weightofthesample} \times 50 \times \text{volumeofhoneysolutionusedfortitration}} \quad (5)$$

Non-reducing sugars = total sugars – reducing sugars (6)

Optimization of digestion procedures for honey samples

0.5 g of honey sample was accurately weighed and transferred quantitatively in to a 250 Kjeldahl tubes digestion flask. 3.5 mL of a mixture of HNO_3 (65-68%) and $HClO_4$ (70%) were added with volume ratio of 2:1.5 mL. The sample was swirled gently to homogenize the mixture then it was fitted to a reflux condenser and digested continuously for 2:40 hours on a Kjeldahl digestion block by setting the temperature dial at 6 (240°C) until clear solution was obtained. Each honey sample was digested in triplicate and hence a total of nine digests were made for the three types of honey samples. The digest was allowed to cool for 10 minutes without dismantling the condenser and further 10 minutes after removing the condenser. To the cool solution, deionized water was added to dissolve the precipitate formed on cooling and to minimize dissolution of the filter paper by the digest residue while filtering with whatman (No 41) filter paper. The digestive flasks further were rinsed subsequently with deionized water in to 100 mL volumetric flasks and finally the volumetric flasks were made up to the mark with deionized water. Digestion of a reagent blank was also performed for correcting the effect of the blank in parallel with the honey samples keeping all digestion parameters the same. For the analysis of the honey samples, three reagent blank samples were prepared. All the digested samples were stored in a refrigerator until analysis using FAAS.

Method detection limit

Method detection limit (MDL) is defined as the minimum concentration of analyte that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, but it may not necessarily be quantified as an exact value. It is the amount of analyte that gives a signal equal to T-test times the standard deviation of the blank [16]. In the present study, seven reagent blank solution were digested and each of the blank samples were analyzed for metal concentrations of Pb, Cu, Cr, Ni, Fe, Mn and Cd by FAAS. The detection limits were obtained by multiplying the standard deviation of the reagent blank by three. The detection limits were found in the range 0.0011-0.0099 µg/g that clearly showed that the method developed is applicable to determine the metal concentration in the honey samples at trace levels (µg/g). Limit of quantification (LOQ) were found obtained by multiplying the standard deviation of the reagent blank by ten times.

Precision and accuracy

Precision and accuracy of the analytical method was assessed by

repeatability and recovery studies of matrix spike and laboratory control samples. Recovery study was performed by spiking three replicate Honey samples with a known concentration of metal standard solution (mid-range calibration concentration). The spiked samples were then subjected to the same digestion procedure like the actual sample. Precision was expressed as relative standard deviation (RSD) of the three replicate results. The relative standard deviations of the sample were obtained as % RSD = (standard deviation/mean value) x 100. Accuracy is expressed as matrix spike recovery and the percent recovery results were calculated by the following equation [17].

$$\%\text{Recovery} = \frac{C(spiked) - C(non-spiked) \times 100}{C(added\ metal\ concentration)} \quad (7)$$

Laboratory control samples (LCS)

For the honey sample ,three replicates of reagent blank($HClO_4$ and HNO_3) spiked with a mixture of standard and digested like the sample including exposure to all glassware, digestion media, apparatus, solvents and reagents that used with honey samples. The value was found under the recommended control limits 80-120% for LCS recovery [17]. The percent LCS recoveries for each metal of interest were calculated using the following equation [18].

$$\%R = \frac{LCS \times 100}{S} \quad (8)$$

Where: %R=Percent recovery, LCS=Laboratory control sample results and S=amount of spike added

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the significant differences in the mean values of physicochemical parameters and heavy metal levels among groups of Honey. A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were done by SPSS version 20.0 software for windows. Data were expressed as mean ± standard deviation (SD) of three replicate experiments

RESULTS AND DISCUSSIONS

pH

The pH of the honey samples were in the range 3.98–4.12 in Table 1, which were in the standard range of 3.3–4.6 specified by the Codex Alimentarius Commission [19-21]. Therefore, it was found that all the studied honey samples were acidic in nature. Among all the honey samples, Bichena honey was the most acidic (pH 3.98 ± 0.01) followed by Dejen honey (4.05 ± 0.02). The lowest acidity was detected in Deber Markos honey (4.12 ± 0.01). There was significant difference recorded between the three studied types of honey concerning pH values ($P<0.05$). The results obtained were in the range indicated by [6] who reported that the pH of honey was between 3.73 and 4.60 in Nigeria honey. Similarly, these results were in agreement with the finding of [22], who reported that the pH of honey was between 3.40 and 4.60 in India honey. However, the value was very low as compared to the previous work of [23] who reported a range of 4.56-5.87 in Ethiopia honey. The low pH of honey has an advantage to prevent the presence and growth of microorganisms. In addition, the pH of honey mainly indicates the buffering action of the inorganic cation constituents of the acids present. The pH values have great importance during the extraction and storage of honey as it influences the texture, stability and shelf life of honey [22,24].

Electrical conductivity

EC values ranged from 0.35 to 0.65 mS/cm in (Table 1). Statistical test of significance using ANOVA revealed significant difference ($P<0.05$) between the value of electrical conductivity in the honey samples obtained from the three sites. The present study indicated that the electrical conductivity value of the honey samples were in agreement with those reported values of honey samples from Nigeria by [25] which ranges from 0.25–0.64 mS/cm, and also similarly, these results were in agreement with the findings of [24] who reported 0.384 to 0.646 mS/cm in West Shewa honey. However, the values were very low compared to the previous works of [26] who reported a range of 0.44 to 1.14 mS/cm in Nigeria honey. The electrical conductivity of the honey is closely related to the concentration of mineral salts, organic acids, and proteins; it is a parameter that shows great variability according to the floral origin and is considered one of the best parameters for differentiating between honeys with different floral origins [27]. Analyses of results for electrical conductivity of all samples were within the acceptable limit (i.e. <0.8 mS/cm) [21].

Moisture content

The moisture content in the investigated honey samples were found to be in the range of 17.5–18.2% (Table 1), which are within the limit (≤ 20%) recommended by the international quality regulations Codex Alimentarius Commission [21] and European Union [28]. There was significant difference in the moisture content among the three study areas in honey samples ($P<0.05$). The results of these study on moisture content were also in agreement with the findings of [29] who reported (14.5–19.0%) in

Table 1: Results of physicochemical parameters of the Honey sample (mean ± SD, n=3) and comparison with national and international standards.

	Location of Honey Sample			Standards	
Parameters	**Debre Markos**	**Bichena**	**Dejen**	**National**	**International**
pH	4.12 ± 0.01[a]	3.98 ± 0.01[b]	4.05 ± 0.02[c]	-	3.20 - 4.50
EC	0.65 ± 0.01[a]	0.35 ± 0.00[b]	0.46 ± 0.01[c]	-	0.22 - 1.52
Moisture Content (%)	17.6 ± 0.96[a]	17.5 ± 0.29[b]	18.2 ± 0.25[c]	17.5 – 21.0	18.0 – 23.0
Total solid (%)	82.4 ± 0.96	82.4 ± 0.13	81.8 ± 0.25		
Ash content (%)	0.26 ± 0.00[a]	0.09 ± 0.00[b]	0.17 ± 0.0012[c]	<0.6	0.25-1.00
Free Acid (meq/kg)	39.0 ± 1.00[a]	35.3 ± 0.58[b]	46.7 ± 2.08[c]	<40	<50
Reducing Sugar (%)	63.8 ± 1.20[a]	45.1 ± 2.48[b]	51.7 ± 11.2[c]	>65	60.0-70.0
Total sugars (%)	68.1 ± 7.00	61.4 ± 10.5	61.6 ± 13.7		
Non-Reducing sugars(%)	4.30 ± 8.30	16.5 ± 8.81	9.89 ± 24.7	<5	<10

Source: Quality and Standards Authority of Ethiopia [19,20] and so on

Mean values in the same row with different alphabets are significantly different ($P<0.05$).

Palestine honey. Similarly, these results were in agreement with the finding of [30] who reported (5.4–18.4%) in Argentinian honey. According to the Ethiopian Standard, ES [19], honey is grouped into three grades based on moisture content: Grade A: 17.5–19.0%; Grade B: 19.1–20.0%; and Grade C: 20.1–21.0%. The East Gojjam Zone honey could be grouped as 'Grade A' honey based on the Ethiopian standard. Honey moisture is one of the quality criteria that determine the capability of honey to remain stable and to resist spoilage by yeast fermentation. The higher the moisture content is the higher probability of honey fermentation during storage [31]. Lower moisture content (<20%) elongates honey shelf life during storage [24]. Honey is an excellent hygroscopic product and has tendency to absorb atmospheric moisture and thus readily increase its moisture levels. Further, the moisture levels may also largely depend on methods of harvesting and extraction of honey, which may differ from location, species, and practices [32]. Overall, the low moisture content in honey samples of this investigation indicates that all the samples have good storage ability and quality.

Total solids

The total values in honeys from all studied areas were very high. They ranged between (81.8–82.4%) indicating that they were within the acceptable total solids range. There was no significant difference ($p>0.05$) in the amount of total solid among honey samples analyzed from all the three study areas. The results of this study on total solids were also in agreement with the previous work of [24] who reported 81.36-83.39% in West Shewa honey. Similarly, these results were in agreement with the findings of [6] who reported the values of total solid from Nigeria honey that ranged between 76.6 - 90.73%.

Ash content

Ash content was considered to be an indicator of the cleanliness of honey samples . The ash content in honey is generally small and depends on nectar composition of predominant plants in their formation [24]. The ash content in the investigated honey samples were varied between 0.09 % to 0.26 % (Table 2). The ash content of all the analyzed honey samples lied within the acceptable range 0.01-1.2% set by the Ethiopian standard [33] and below 0.6% maximum limit set by Codex Alimentarius Commission [21]. There was significant difference between samples in ash content ($P<0.05$). The results of present study were in agreement with those reported 0.09 to 0.54% in West Gojjam in Dangilla Woredas by [2]. The results of present study on Ash content were also in the range with the finding of [29] who reported 0.034 to 0.214% in Palestine honeys. However, the values were very low compared to the previous works of [13] who reported a range of 0.17–0.46 % and [20] who reported a ranged of 0.14 to 0.3% in Ethiopia honey. Variation in the ash content of the honey samples might be due to differences in the floral origin of the honeys. The ash content is a measure of mineral content of honey. Though the quantities of minerals are less, they play a vital role in determining the color and nutritional value of honey [20].

Free acidity

The free acidity as recorded in this study ranged from 35.33to 46.7 meq/kg (Table 1), which was less than 50 meq/kg established by [28]. One-way ANOVA test showed that there was a significant difference in the free acidity between examined honey samples obtained from the three sites ($P<0.05$). The results indicated that the free acidity values of the honey samples were in agreement with those reported values of honey samples from India by [34] which range from 9.20–41.4 meq/kg in and also similarly, these results were in agreement with the findings of [35] who reported 18.9–32.3 meq/kg free acidity of honey produced by different plant species in Ethiopia. However, the present study higher than the values reported by [26] who reported a range of 5.60 to 24.77 mg/kg in Nigeria honeys. This result revealed that the freshness of honey samples and the absence of unwanted fermentation [22,24]. Low acidity value is indicative of the freshness of honey sample while high acidity indicates fermentation of sugars into organic acids [27]. Values obtained for the all honey samples except Dejen honey sample were within the required limits (below 40 meq/kg) [19]. Variation in free acidity among different honeys could be due to floral origin or variation in harvest season.

Reducing sugars

The reducing sugars values of honey analyzed in the present study were ranged between 45.1–63.8%, which fulfills the requirements of [19,21]. Therefore, all honey samples were qualified an international standard for content of reducing sugars in honey. There was significant difference between samples in reducing sugar ($P<0.05$).The results of present study on reducing sugar were in agreement with the findings of [36] who reported 62.0 to 71.0% in honey samples analyzed from Homesha District of Western Ethiopia by [19] who reported 63.4 to 71.7% in honey samples analyzed from Sekota District, Northern Ethiopia, and also similarly, these results were in agreement with the finding of [24] who reported (61.38–72.87%) in West Shewa honey. The high sugar content of the analyzed honey samples could be attributed to its high acidity and low moisture content.

Total sugar

The percentage total sugar content of the various honey samples was shown in Table 1. Total sugar concentration is the other parameter to assess honey quality. The total sugar content of the samples considered in the present study showed in the range of (61.40% to 68.11%). The highest sugar content was observed in Deber Markos (68.1 ± 7.00%), whereas the samples from Bichena and Dejen honey have comparable total sugar content. One-way

Table 2: Instrument detection limit (IDL), method detection limit (MDL) and limit of quantification (LOQ) for the determination of metals in honey samples.

Metals	IDL(mg/L)	MDL(µg/g)	LOQ (µg/g)
Cu	0.0018	0.0020	0.0055
Cr	0.0018	0.0019	0.0050
Pb	0.0011	0.0015	0.0040
Cd	0.0072	0.0074	0.0200
Fe	0.0015	0.0040	0.0110
Ni	0.0099	0.0100	0.0270
Mn	0.0033	0.0037	0.0100

ANOVA test showed that there was no significant difference ($p>0.05$) among the percentage mean of the three sampling sites. The results of present study on total sugar were in agreement with the findings of [16] who reported 61.7–72.4% in Pakistan honey and were lower than the values obtained by [37] who reported (69.1–82.1%) in Algerian honey and [26] who reported a range of 71.48% to 83.18% in Nigeria honey.

Non-reducing sugar

The non-reducing sugar content of the honey samples analyzed in this study varied between 4.30 - 16.5% (Table 1). There was no significant difference ($p>0.05$) in the amount of non-reducing sugar among honey samples analyzed from all the three area. According to [38] reported the range of non-reducing sugar (1.18-14.9%) in Algerian honey and similarly values were observed by [34] that ranged between (15-27%)in India. The two honey samples fulfilled the requirements of non-reducing content set by [19] in which is <10%, while the Bichena honey has slight excess value than the Ethiopian standard [19]. The slight excess value of non-reducing sugar of honey from Bichena may be due to adulteration of the honey by addition of commercial sugar to honey.

Optimization of the sample digestion procedure for metal analysis

Generally, the extraction should be performed in such a way that the analyte is separated from the interfering matrix without loss, contamination, or change of speciation and with minimum interference [13]. Accordingly, nine digestion procedures were tested for the digestion of honey sample. The optimum procedure of honey digestion was selected depending on on clarity of digest, minimum reagent consumption, minimum digestion time, and minimum temperature applied for complete digestion of the sample. Finally the optimal procedure was chosen on the basis of these criteria requiring 2:40 hours for complete digestion of 0.5 g honey sample with 2.0 mL of 69-72% nitric acid (HNO_3) and 1.5mL70% perchloric acid ($HClO_4$).

Recovery test results and laboratory control samples results

This showed that the analytical method provided results in the required level of accuracy. The precision of the method was expressed as relative standard deviation (RSD) of three replicate readings. The RSD value obtained for honey sample (Table 3) ranged between (0.58-7.68%) for all metals. The percent recovery values of LCS results lied in the range of 83.13% to 106.42% and the RSD values ranged from 0.59 to 8.21% (Table 4). All the values were found under the recommended control limits 80–120% for LCS recovery and Recovery test Results [39] and ≤ 10% RSD. These results showed that the analytical method possesses the required precision and accuracy.

Copper

In the present study, copper level in honey sample was in the range of 0.268 to 0.279 µg/g (Table 5). However, there was no significant difference ($p>0.05$) in the content of Cu between the three sampling sites. Cu contents of honey samples in our study were determined below the limit standards. The average recommended daily intake in foods is reported to be 30 mg/day for copper [40]. The concentrations of Cu in all tested samples were below the guideline value of 5 µg/g [21]. Copper is a vital element to the health of all living things and in humans. However, too much ingestion of copper can lead to adverse health effects in the body. So, it is necessary to consider the daily intake of copper from different sources like food.

Chromium

Based on our findings, chromium concentrations ranged from 0.22-0.46 µg/g. The highest mean chromium concentrations were 0.46 µg/g in the honey samples from the Debre Markos. However, the concentration of chromium both in Bichena and Dejen were comparable range. One-way ANOVA test showed that there was significant difference ($p<0.05$) variation in the content of Cr between the three sampling sites. Trivalent chromium is the most common natural state of chromium and an essential nutrient. It's recommended Daily Intake is 30 to 100 µg/day for adults. However, the primary route of non-occupational exposure to

Table 3: Recovery tests for the optimized procedure for the honey samples (mean ± SD n=3).

Metals	[a]Conc.in sample(µg/g)	Amount added (µg/g)	[b]Conc.in spiked sample (µg/g)	[c]Recovery (%)	[d]RSD (%)
Cu	0.27 ± 0.01	0.16	0.42 ± 0.00	93.8 ± 3.73	3.98
`Cr	0.46 ± 0.00	0.36	0.81 ± 0.00	98.00 ± 2.00	2.04
Pb	ND	0.72	0.67 ± 0.51	92.8 ± 7.13	7.68
Cd	ND	0.18	0.17 ± 0.00	96.1 ± 0.56	0.58
Fe	0.59 ±0.08	0.27	0.81 ± 0.09	84.5 ± 5.14	6.09
Ni	0.14 ±0.01	0.36	0.46 ± 0.00	90.1 ± 0.80	0.89
Mn	0.01 ± 0.01	0.16	0.16 ± 0.01	91.6 ± 4.14	4..52

[a]Concentration value are average of the three replicate measurements the analyzed samples ± standard deviation (n=3).
[b]Concentration of in spike value are average of the three replicate measurements the analyzed samples ± standard deviation.
[c]Recovery values are mean ± standard deviation, [d]RSD relative standard deviation.

Table 4: Recovery and precision test results for the laboratory control samples (mean ± SD, n=3)

Element	Amount added (c)	Conc. in Spiked Sample (µg/g)	Recovery (%)	RSD (%)
Cu	0.16	0.15 ± 0.01	91.2 ± 5.25	5.76
Cr	0.36	0.32 ± 0.03	89.3 ± 7.33	8.21
Pb	0.72	0.68 ± 0.01	93.7 ± 0.70	0.75
Cd	0.18	0.16 ± 0.00	89.6 ± 0.53	0.59
Fe	0.27	0.29 ± 0.01	106.4 ± 2.38	2.24
Ni	0.36	0.38 ± 0.02	105.0 ± 4.41	4.20
Mn	0.16	0.13 ± 0.00	83.1 ± 0.63	0.76

chromium is food ingestion. Chromium in foodstuffs is considered to be in the trivalent form [41]. According to the Expert Group on Vitamins and Minerals (EVM) a total daily intake of about 0.15 mg chromium (III)/kg b.w/day (10 mg/person/day) would be expected to be without adverse health effects, whereas the WHO considered that supplementation of chromium should not exceed 250 µg/day [42]. The maximum permissible limit (MPL) of chromium was 1.5 µg/g established by [43]. According to these regulations and data, chromium concentrations in studied honey samples can be considered in an acceptable range. The chromium concentrations found in this study were lower than the value reported by [13] in earlier studies of Ethiopian honey which was between 1.20 to 4.33 µg/g.Variations in Cr concentrations may be due to a combination of certain factors like botanical, geochemical and anthropogenic activities. In order for the quality of honey to be maintained, each of these factors must be taken into consideration. The lower Cr concentrations may indicate better quality honey in our samples.

As it can be seen in Table 5, both Pb and Cd were not detected in the three study areas. Lead and Cadmium serves no useful purpose in the human body and its presence in the body can lead to toxic effects. Lead and cadmium toxicity can result in severe damage to organs including the liver, kidneys, heart, and male gonads. In present study, the concentrations of Pb and Cd were not found in traceable amounts in the honey samples from the three study areas. Hence, these metals may be below the detection limit (<0.0074 µg/g) and (<0.0015 µg/g) of the instrument. Therefore the three study area honey samples are free from the non essential toxic metal both Pb and Cd.

Iron

Based on our findings, iron concentrations ranged from 0.59 to 5.39 µg/g. One-way ANOVA test showed that there was significant difference ($p<0.05$) among the mean concentration of iron in the honey samples. Fe overload as a result of dietary intake is unusual in the normal population [44]. According to the standard values determined by Codex Alimentarius Commission; the maximum Fe value that must be found in sweet nutrients such as sugar and honey is reported as 15 µg/g. Therefore, the recorded Fe levels do not pose a health risk to consumers because the concentrations of Fe in all tested samples were below the guideline value [21]. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have set a limit for heavy metal intake based on body weight (b.w.). For an average adult (60 kg b.w.), the provisional tolerable daily intake for Fe was 48 mg (Joint FAO/WHO Expert Committee on Food Additives [45]. Our results indicating that the iron value falls within the range reported by [46] within the range of (0.40-52.51 µg/g). Iron (Fe) is one of the essential trace minerals that is vital for life and has unique role in the body. Fe is the center of the protein tetramer hemoglobin, which is essential for the transport of oxygen and carbon dioxide through the blood stream [47]. Therefore, its suitable amount must be present in all food ingredients. The differing concentrations of iron may be attributed to the climatic conditions of the locality under observation.

Nickel

The mean concentration of nickel in honey samples were 0.14 µg/g in Debre Markos, 0.12 µg/g in Bichena and 0.04 µg/gin Dejen. One-way ANOVA test showed that there was no significant difference ($p>0.05$) among the mean concentration of nickel in honey samples. The intake of nickel via food is related to several factors such as the source of nickel and distance from the contamination source. Nickel is present in the air, water, and soil and is generally distributed uniformly through the soil profile. The level of 5 mg/kg body weight/day was determined for nickel by joint FAO/WHO Expert Committee on Food Additives. The results of present study indicated that the Nickel concentration of the honey samples were in agreement with those reported values of honey samples [48,49] within the range of 0.004–3.23 µg/g. No significant variations in the concentration of Ni could be attributed to the similarly in the chemical composition of the soil at each location, as well as the existence of the same types of plants.

Manganese

The levels of manganese in honey samples ranged from 0.008 to 0.33 µg/g. Debre Markos and Dejen honey have comparable concentration; While Bichena honey has the highest concentration in the three areas. One-way ANOVA test showed that there was significant difference at ($p<0.05$) in the content of Mn between the sampling sites. Manganese values found in the present study are in agreement with the manganese levels of honey samples from Chile [50].

Comparison of metal levels in honey sample with literature values

Although various chemical investigations target similar objectives there may be differences in the sampling, sample preparation and other analytical techniques they followed.

The concentrations of heavy metals in honey sample found in this study were compared with some other related published reports conducted in some parts of the world (Table 6).

In the present study Cu, Cr and Ni was slightly higher concentration than those found in Black Sea (Turkey) honeys. However, Fe was within the concentration range found in Chile and Switzerland

Table 5: Concentration (µg/g) of heavy metals in three types of honey samples

Concentration of metal (mean ± SD) (µg/g) (n = 3)			
Metal	**Deber Markos**	**Bichena**	**Dejen**
Cu	0.27 ± 0.01	0.27 ± 0.01	0.28 ± 0.01
Cr	0.46 ± 0.00[a]	0.22 ± 0.01[b]	0.29 ± 0.00 [c]
Pb	ND	ND	ND
Cd	ND	ND	ND
Fe	0.59 ± 0.08 [a]	1.56 ± 0.02 [b]	5.39 ± 0.54 [c]
Ni	0.14 ± 0.01	0.12 ± 0.09	0.04 ± 0.02
Mn	0.01 ±0.01 [a]	0.33± 0.12 [b]	0.02 ± 0.00 [c]

*ND.=not detected (less than the instrument sensitivity).
Mean values in the same row with different alphabets are significantly different ($p<0.05$)

Table 6: Comparison of the levels of heavy metals (µg/g) in honey of the present study with literature values.

Country	Concentration of Metals(µg/g)							Reference
	Cu	Cr	Cd	Pb	Fe	Ni	Mn	
Saudi Arabia	0.206-0.389	NR	0.038-0.08	0.002-0.037	0.310-3.195	NR	0.188-0.373	[51]
Black Sea Region (Turkey)	0.009-0.035	0.00124-0.013	0.00028-0.0023	0.00151-0.0553	1.21-12.9	0.00121-0.0023	1.11-61	[40]
Chile	0.06-2.00	0.03-1.92	0.01-0.05	0.01-0.11	0.1-6.36	0.01-1.04	0.01-3.14	[50]
Ethiopia	ND-0.4676	1.20-4.33	ND-0.69	ND	5.37-12.43	0.8-4.46	ND-0.885	[13]
Switzerland	0.051-3.317	0.001-0.037	0.001-0.026	0.003-0.329	0.136-9.85	0.001-1.966	0.125-12.354	[52]
Ethiopia (Present study)	0.271-0.278	0.22-0.46	ND	ND	0.59-5.39	0.04-0.14	0.008-0.33	Present study

*ND=Not detected<MDL)
*NR=Not reported

honeys. The levels of Cu, Cr, Fe, and Ni were in a very good agreement, i.e. within the values found in the country: Chile, whereas Fe and Mn are comparable with the reports from Chile honey and also the levels of Cu, Fe, and Mn were in a very good agreement, i.e. within the values found in the countries: Saudi Arabia and Switzerland honeys. In the present study Fe was higher concentration than those found in Ethiopia honeys by [13] and also Ni was not found in the range reported by [13], but Fe was found in somewhat higher concentration those that reports in Saudi Arabia honey. The heavy metals such as Pb and Cd in the present study was not detected because both metals below the detection limit, i.e. < 0.0074 and <0.0015 µg/g and thus were in a very good agreement with most of the results reported from different literature values by [13] who reported in Ethiopia honeys and similarly, these results were in agreement with the findings of [51-53] who reported both Pb and Cd not detected in Libya honey. In general, the results obtained in this study were remarkably in a good agreement with those reported from other parts of the world implying acceptability and validity of this work regardless of some factors contributing deviation in some ways.

STATISTICAL ANALYSIS

Variations in the mean levels of metals between the samples were tested whether it was from just random error or treatment. The result indicated that significant difference were obtained (P<0.05) at 95% confidence levels for Cr, Fe and Mn in honey samples collected from all the three sites. The concentration of Cu and Ni for the honey samples were not significant (P>0.05) in the three sites. The analysis of variance showed that there was significant variation in levels of elements between each brand of honey. The mean levels of physicochemical parameter between the samples were tested. The result indicated that significant difference were obtained (p<0.05) at 95% confidence levels for pH, Free acidity, electrical conductivity, ash contents, moisture content, reducing sugar in honey samples collected from all the three sites. However, the variation of, total solid, non-reducing sugar, total sugar for honey samples were not significant (P>0.05).The analysis of variance showed that there was significant variation in levels of physicochemical parameters between each types of honey. The difference may be due to the floral type, the botanical origin, storage conditions anthropogenic factor, season of the year, rainfall.

CONCLUSIONS

The physicochemical properties and levels of some heavy metals in honey samples collected from three district Woredas of East Gojjam Zone were analyzed. Physicochemical properties data have shown that almost all the samples of honey analyzed were within the acceptable range of Ethiopian standard, international standard and Codex Alimentarius except non-reducing sugar content from Bichena:- From statistical analysis, there were significant differences (p<0.05) in the quantity of free acidity, electrical conductivity, ash content, pH, moisture content of the honey samples, except for total solid, total sugar , non-reducing sugar analyzed from the three different areas, However, one of Bichena honey samples, the non-reducing values slightly higher than the set standards. Slight excess value may be due to adulteration of the honey by addition of commercial sugar to honey. The quality parameters included in this study fits with most of the country; this makes the honey samples of the areas to be good for consumption, except non-reducing sugar in Bichena. However, further studies are required to evaluate the quality of the studied honeys based on nutritional, medicinal, and antioxidant properties. In the present study, honey samples were analyzed for the concentration of heavy metal (Cu, Cr, Pb, Cd, Fe, Ni and Mn).

The pattern concentration of metal in honey samples at Deber Markos was in the order of (Fe>Cr>Cu> Ni >Mn), Bichena (Fe>Mn>Cu>Cr>Ni) and Dejen (Fe>Cr ≥Cu>Ni>Mn). Cd and Pb were also below the detection limit in all honey samples at the three sites. Statistical test significant using one way ANOVA revealed that there were significant difference (p<0.05, at 95% confidence levels) in honey sample in the concentration of Cr, Fe and Mn in Deber Markos, Bichena and Dejen site, except Cu and Ni. Generally, heavy metal results indicated that East Gojjam Zone (Debre Markos, Bichena, and Dejen) honey is rich in nutritive elements that are important for human health and clean of toxic metals, like Cd and Pb. This indicates safe and high quality honey. Moreover, low levels of heavy metals in honey indicates clean environment.

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