



Original Research Article

Quantification of caffeine content in coffee bean, pulp and leaves from Wollega Zones of Ethiopia by high performance liquid chromatography

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ABSTRACT

Caffeine is a stimulant alkaloid found in aerial parts of many hot beverages, including coffee and tea. Due to its health impact, quantification of caffeine level in coffee is of paramount importance for consumers and traders, as well. Therefore, this study was designed to determine the caffeine content in coffee beverage prepared from coffee beans, pulp and leaves using high performance liquid chromatography coupled with a reverse phase C₈ column and UV-detector. In this study, caffeine sample was extracted from coffee beans, pulp and leaves with boiled distilled water followed by solvent partition with chloroform. The extracted caffeine samples were analyzed alongside caffeine standard solutions over the concentration range of 5-25 µg/mL. For quantitative purposes, the standard caffeine gave an equation of $Y=1270560x + 986903$ ($R^2 = 0.998$) and the retention time of 1.84 ± 0.01 min. In parallel to the standard caffeine solution, peak area of caffeine contents in 20 µL extracted caffeine samples of 2.5 g coffee powder in 100 mL of distilled water coffee beverage were registered. By using the peak area, caffeine concentration in injected sample and its concentration in the total sample solution was calculated. The percentage masses of caffeine (w/w%) in the original coffee samples were $1.30 \pm 0.11\%$ for beans, $0.90 \pm 0.11\%$ for pulp and $0.65 \pm 0.10\%$ for leaves. These results of caffeine contents in coffee beans in Wollega zone show high caffeine contents when compared with previously reported coffee beans (1.01 ± 0.04 - $1.19 \pm 0.02\%$) of other parts of Ethiopia using UV/Vis. spectrophotometric technique.

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1. Introduction

Coffee is derived from the name of the province Keffa where shepherds from Abyssinia/Ethiopia discovered the coffee plant in the 6th century (Ranheim and Halvorsen, 2005; Weldegebreal et al., 2017). It has become one of the most widely consumed beverages throughout the world due to its pleasant taste, aroma, stimulant effect and health benefits (Chen et al., 1998; Camargo, 1999; Schenker et al., 2002; Aresta et al., 2005; Perrone et al., 2008; Gebeyehu and Bikila, 2015). In Ethiopia, there are different coffee types recognized by their origin and quality and used as trade names including "Bebeka", "Harar", "Jimma", "Kaffa", "Wollega", "Limmu",

"Sidama", "Teppi" and "Yirgacheffe". Under each coffee type, 2-5 different local types are known. Such high level of diversity is partly attributed to the presence of indigenous traditional production systems of coffee in the country (Boot, 2011). Coffee beans are the seeds of a shrub belonging to the botanical family Rubiaceae and the genus *Coffea* (Clifford and Ramirez-Martinez, 1991). The generic name covers over sixty different species of which only three, namely *Coffea arabica*, *Coffea robusta* and *Coffea liberica* have commercial values (Boot, 2011). The two most important commercial species are *Coffea arabica* and *Coffea canephora*, usually known as *Arabica* and *Robusta* varieties, respectively (Martin et al., 1998; Boot, 2011). *Arabica* is considered to be a

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higher quality bean, prized for its complex aroma and flavors and is usually the most expensive one in the world market (Martin et al., 1998). Coffee has strong historical, cultural, social and economical importance. It is also the single most important tropical commodity traded worldwide, accounting for nearly half of total exports of tropical products (Fujioka and Shibamoto, 2008). The world's largest importer of coffee is the EU, accounting for 66% of worldwide imports ca 4.0 million tonnes, in 2008 followed by the United States (24%, 1.5 million tonnes) and Japan (7.0%, 423,602 tonnes) (Wondimkun et al., 2016). Most of the coffee in the world market is produced by developing countries (Yigzaw et al., 2007). Ethiopia is one of world top ten exporters of *Coffea arabica* and (first in Africa) leading to its domestic consumption in the continent. About 12 million Ethiopians make their living from coffee. While most of the coffee plants cultivated in Ethiopia are *Coffea arabica* (Fig. 1), there are, however, wide ranges of variability among coffee cultivars in the country. This variability in coffee beans has been attributed to variation in the soil, altitude and climate of the coffee growing areas (Yigzaw et al., 2007). These factors are believed to considerably influence coffees characteristics like chemical content, flavor or aroma (Gebeyehu and Bikila, 2015). Both species, *Arabica* and *Robusta*, are rich sources of biologically active compounds. The coffee beverage is rich in bioactive substances such as nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallol acid and especially caffeine (Minamisawa et al., 2004). Caffeine (1,3,7-trimethylxanthine) (Fig. 2) is an odorless and slightly bitter material that can be naturally found in the coffee and cocoa beans, tea leaves and other plant species (Castro et al., 2010; Amare and Admassie, 2012). It can be consumed in the form of coffee, tea, cocoa, chocolate and energy drinks where is intentionally added. Moreover, it is considered to be as one of the most commonly consumed behavioral active substances with more than 80 percent of the world's population daily consuming caffeine (Carrillo and Benitez, 2000; Norton et al., 2011). It is stimulant of central nervous system, cardiac muscle and respiratory system, diuretic delays and fatigue. The human salivary level, which indicates the extent of absorption, peaks around 40 minutes after caffeine consumption (Chou and Bell, 2007). Many studies have attracted considerable attention due to the antioxidant and anticancer properties as well as health benefits of caffeine containing plants. However, in excess amount, caffeine is known to cause a higher risk of developing bone problems, including osteoporosis problems in metal absorption and excretion, re absorption processes in intestines and kidney resulting in iron deficiency anemia (Shaker et al., 2010). A fatal dose of caffeine has been reported to be more than 10 g which is equivalent to about 170 mg/kg of body weight (Grujić-Letić et al., 2016).

The amount of caffeine in a cup of coffee can vary greatly, depending on both intrinsic factors involving the species and/or its origin along with extrinsic factors such as sampling localities and the method of brewing.

As caffeine contents of the coffee samples varied on the basis of geographical locations, there should be further studies on different geographical and environmental conditions that results these differences (Gebeyehu and Bikila, 2015). In the coffee plant, caffeine is present in all of its aerial parts. Caffeine biosynthesis takes place in the leaves and the outer part of the fruit. However, in aged leaves, the caffeine content is lower (Oestreich-Janzen, 2013). In the pulp tissues, light strongly stimulates the methylation step of caffeine synthesis. When the seed inside the fruit starts growing, caffeine is translocated through the membranes and accumulates in the endosperm. Then, the final value is reached 8 months after the flowering stage (Oestreich-Janzen, 2013). Researchers from England and France have discovered that a tea made from coffee leaves involves even more antioxidants and healthful compounds than either regular tea or coffee. Since geographical location is known to affect the caffeine content, coffee species belonging to the same variety can have different amounts of caffeine level (Gebeyehu and Bikila, 2015; Wondimkun et al., 2016). Due to the widespread consumption of caffeine and its potential physiological and pharmacological effects, it is important for both health professionals and consumers to know the exact caffeine content in food. It is therefore important to precisely determine the caffeine content in different coffee types, as a way to assess their caffeine contents in order to find a more precise relationship between the amounts of consumed caffeine and their physiological effects (Demissie et al., 2016). Despite the wide use of coffee in Ethiopia, there is the lack of extensive studies addressing the issues related to the caffeine content of coffee growing in Wollega, Ethiopia. Coffee pulps and leaves were also used sometimes as a beverage in different parts of Ethiopia after roasting, grinding and adding to the boiled water. For example, lactating mothers in some Ethiopian regions use these beverages of pulps and leaves with milk. Though there were reports of caffeine contents in coffee bean in literature, to the best of our knowledge, there were no reports on comparison of caffeine contents of coffee beans, pulps and leaves.

Weldegebreal et al. (2017) reported a direct method of determination of caffeine in aqueous solution of green coffee bean using FT-IR-ATR and fluorescence spectrometry. In this regard, among the several analytical techniques which have been developed for the determination of caffeine and the quality control of products containing caffeine, high performance liquid chromatography (HPLC) is the method of choice (Grujić-Letić et al, 2016; Abbood and Aldiab, 2017) whenever the sample cannot easily be converted to the gas phase. Therefore, in this context, this article will highlight the concentration of caffeine in coffee beans, pulps and leaves in some areas of Wollega zone, Ethiopia and compare their contents using HPLC technique. Additionally, in this study, we have focused on the determination and comparison of caffeine contents of coffee beans growing in Wollega zones.



Fig. 1. Picture of the aerial parts of coffee plant.

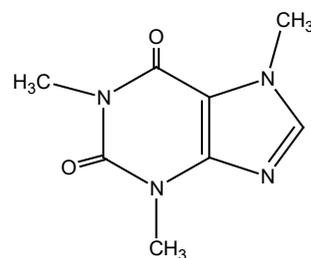


Fig. 2. The chemical structure of caffeine.

2. Experimental

2.1. Sample collection

2.1.1. Description of the study areas

The study was conducted in three zones of Oromia region Kelem Wollega, Western Wollega and East Wollega which are located in the west of the country. Dembi Dollo town is the capital and administrative centre of Kelem Wollega zone and 652 km far from Addis Ababa. For West Wollega, Gimbi town is the capital and administrative centre of zone and 441 km from Addis Ababa. Nekemte town is the capital and administrative centre of East Wollega zone and found at 322 km from Addis Ababa. The study area is located between latitude 8°12'-10°03' N and longitude 34°08'-36°10' E. The zones are classified into three agro-climatic zones locally known as Kola, Woinadega and Dega. These selected study areas have suitable afro-montane rain forest, altitudes ranging from 1,300 to 1,800 m above sea level which contain density of coffee, annual rainfall varies from 1000 to 2400 mm and a wide range of soil types (from acidic to slightly acidic with low availability of phosphorous) where its fertility is maintained by organic recycling and suitable temperature of 15-25 °C, cool, shady environment of the forest of south western highlands of Ethiopia (Beyene et al., 2015). Wollega zone is one of most coffee cultivating areas of the country.

2.1.2. Study design and selection of coffee sample site

Data were collected in February 2016. All samples of beans, pulp and leaves were collected from the similar areas at an altitude range of 1300-1800 m above sea level.

Coffee pulp and beans were collected and removing of pulp from this bean was made at laboratory.

In order to prepare representative data for Wollega zone, three districts were selected purposely based on their selection by dweller (consumers, traders and agricultural office of zones) of the areas from high to low selective features. Based on the above selection, coffee samples were collected from Anfilo, Sayyo and Gidami for Kelem Wollega, Nole, Gimbi (Gambela) and Begi for West Wollega and Sasiga, Harolimu and finally Nunukumba for East Wollega. 10-g portions of coffee samples were measured from each district. The measured 10-g portions of Anfilo, Sayyo and Gidami coffee samples were added together and roasted as Kelem Wollega coffee sample representative. Again Nole, Gimbi (Gambela) and Begi were added together for West Wollega and Sasiga along with Harolimu and Nunukumba for East Wollega coffee sample representatives. The coffee sample from these three zones may be representative of Wollega brand coffee sample. In support to this data, some papers dealing with the Ethiopian coffee, have stated that there is only 2-5 local variability for one brand of coffee (Boot, 2011).

2.2 Chemicals

The main chemicals and reagents used in this experiment were distilled water, anhydrous sodium carbonate (Na₂CO₃), HPLC grade chloroform (CHCl₃), diluted NaOH (10%), anhydrous sodium sulphate (Na₂SO₄), acetone, anhydrous caffeine and HPLC grade methanol. Among various solvents used to extract caffeine from raw coffee solution, chloroform was selected due to its best caffeine extracting ability (Islam et al., 2002).

2.3. Caffeine extraction

Coffee beans, pulp and leaves were extracted from commercial *Arabica* variety. 30-g portions of each coffee beans, pulp and leaves were roasted using a local coffee roasting machine. Each of the roasted samples were ground and screened through 250 μm sieves to get a uniform texture. In the next step, 2.5 g of sample powder was measured and added into 100 mL beaker. Then, 50 mL of the boiled water was added over the coffee bean powder. The beaker containing solution of coffee was put on hot plate and occasionally stirred for 15 min. The coffee solution was then poured through cheesecloth into another beaker and pressed out the solution. Thereafter, the extracted solution was put aside. The coffee bean powder residue on the cheesecloth was washed twice with 25 mL of hot water for 10 min. Then, the obtained extracts were combined. The final coffee beverage extracts was filtered by vacuum suction filtration method to remove any insoluble solids and cooled at room temperature. 1.0 g of sodium carbonate (Na_2CO_3) was added to this solution in order to remove some inorganic compounds that can react with Na_2CO_3 (Alliance and Chan, 2013) and then 25 mL of HPLC grade chloroform (CHCl_3) was added to the sample solution and the mixture was vigorously swirled for 10 min and allowed to stand and being separated into two layers; a dark aqueous top layer and a clear chloroform bottom layer. The organic and aqueous layers were separated using a separatory funnel. The above procedure was repeated three times by adding 25 mL of chloroform to the aqueous layer. 5.0 mL of an aqueous NaOH (10%) solution was added into the combined organic extracts to remove inorganic impurity. 1.0 g of Na_2SO_4 , as a dehydrating agent, was added to remove any trace water molecule and the resulting extract was then filtered. The organic layer was concentrated using rotary evaporator and yielded caffeine. The entire above step were repeated for both coffee pulps and leaves. Qualitatively, the extracted sample was checked by TLC, HPLC and UV/Vis.-based spectroscopic methods.

2.4. Quantitative determination of caffeine in extracted samples

2.4.1. Preparation of stock standard and working solutions

Caffeine stock standard solution (1000 ppm) was prepared by dissolving 100 mg of caffeine standard in 80 mL of distilled water and sonicated for 10 min. Then, the obtained solution was transferred to 100 mL volumetric flask and filled to the mark with distilled water. This stock solution was stored in a dark place at +4 $^\circ\text{C}$ for two days. From the prepared stock solution, 10 mL was transferred to 100 mL volumetric flask and volume was made up to the mark with distilled water to make the working solution (100 $\mu\text{g}/\text{mL}$).

2.5. Preparation of extracted sample solution

The caffeine extracted from each coffee sample was placed into 100 mL volumetric flask and diluted up to the mark with HPLC grade distilled water. The resultant solution was sonicated for 5 min. The solution was then filtered using Whatman No. 1 filter paper. From the filtrate solution, 15 mL was diluted to 100 mL using HPLC grade distilled water. Then, its peak was analyzed alongside with standard caffeine using HPLC.

2.6. HPLC analysis of caffeine

Caffeine in the filtered beverages of coffee samples was analyzed using column German (Zobax-SB-C8) HPLC and detected by UV/Vis detector at the wavelength of 272 nm and quantified using a calibration graph. The extracted caffeine was identified by comparing the retention times and spectral data with those of authentic standards. All analyses were repeated three times. The relative peak areas were determined for three replicates of each dilute sample including standard caffeine. In chromatographic analysis, sample was purified before being injected into HPLC. Reverse phase HPLC column (Zobax-SB-C8) was used to determine the concentration of caffeine in coffee beverage drinks. Using the high performance liquid chromatography system made a fast and easy separation of caffeine from any other substances in the extracted caffeine sample. Standard solution of caffeine was prepared and injected into the HPLC. From the resulting chromatograms, measurements of retention time (t_r) and peak areas were performed. In this chromatographic determination, retention time (t_r) was used as a qualitative measure, whereas the peak area was used as quantitative measure. A calibration curve for peak area against the concentration of the caffeine standards was employed to determine the concentration of caffeine in the coffee beverages.

2.7. HPLC conditions

HPLC-UV analysis was performed on an Agilent liquid chromatograph system (HP 1220, Agilent, USA). A Zobax-SB-C₈ reversed-phase packed column, German, Agilent Technology (4.6 mm x 150 nm: 5 μm) column was used throughout this study. The concentration of caffeine was determined by using high performance liquid chromatography equipped with UV detector (HPLC-UV) set at 272 nm and the run time of 6 min at a flow rate of 1 mL/min at room temperature. An isocratic elution was used using HPLC grade methanol (100%) with a total run time of 6 min.

2.8. Statistical analysis

All measurements and analyses were carried out in triplicates. The results were expressed as mean \pm standard error of three parallel replicates. Analysis of variance was performed by using one way ANOVA. The results with $p < 0.05$ were regarded to be statistically significant. Data were statistically analyzed using SPSS multiple comparison Tukey HSD programs.

2.9. Preparation of calibration curve

Chromatogram of standard solutions (5, 10, 15, 20, 25 $\mu\text{g/mL}$) being prepared from the working standard solution (100 $\mu\text{g/mL}$) was monitored. The concentration versus peak area response was also registered (Table 1). The external standard calibration method was used. Alongside this standard solution, chromatogram of the unknown concentration chloroformic extracted

caffeine sample was drawn and registered (Table 1). Construction of calibration curve was done by taking standard caffeine solutions (5, 10, 15, 20, 25 $\mu\text{g/mL}$) from the corresponding chromatogram. Finally, caffeine content of coffee beverage under test was calculated from the extracted chloroformic sample solutions of coffee beans, pulp and leaves using linear regression equation obtained from drawn calibration curve (Eqn. 1).

Table 1

Peak area of caffeine standard and extracted samples.

Conc. of standard caffeine ($\mu\text{g/mL}$)	Peak area of standard (units) ²	Part of coffee	Sample area	Amount injected (μL)	Peak area (units) ² (Mean \pm SD) (n = 3)	Accepted precision
						RSD%
5	7,853,096	Beans	Kelem Wollega	20	14579975.7 \pm 249671.1	1.71
10	13,224,913		West Wollega	20	13114625.7 \pm 113786.7	0.87
			East Wollega	20	12516925.4 \pm 18969	1.5
15	19,877,730	Coffee pulp	Kelem Wollega	20	10226526.67 \pm 204407.1	1.99
			West Wollega	20	10226986.67 \pm 170008.8	1.66
			East Wollega	20	8453430.7 \pm 144988.7	1.73
20	26,082,152	Coffee leaves	Kalem Wollega	20	8170453 \pm 123643.1	1.5
25	33,188,404		West Wollega	20	7044954.3 \pm 14620.9	0.23
			East Wollega	20	6426735.3 \pm 39894.4	0.67

(Average \pm Standard deviation (n = 3).

2.10. Method validation

Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Due to this reason, precision, linearity, accuracy, sensitivity, limit of detection (LOD) and limit of quantification (LOQ) of the technique used were checked on the sight of ICH guidelines (Gowrisankar et al., 2010).

2.10.1. Detector linearity

Five aliquots were prepared from the secondary stock solution 100 $\mu\text{g/mL}$ ranging from 5-25 $\mu\text{g/mL}$ and analyzed according to the experimental conditions. The caffeine solution was scanned using HPLC against mobile phase as blank. It was found that the linearity of sample between the ranges of 5-25 $\mu\text{g/mL}$ showed acceptable linear regression coefficient ($R^2 = 0.9983$). The calibration graph was generated using 20 μL injection loops. Then, the calibration curve was established according to the obtained response (peak area) and the concentrations of caffeine in standard solutions. The results show a good linear relationship.

2.10.2. Precision

According to the ICH guidelines (Association; Gowrisankar et al., 2010), precision should be performed at two different levels; repeatability and reproducibility. Repeatability is an indication of how easy it is for an operator in a laboratory to obtain the same result for the same batch of material using the same method at different times using the same equipment and reagents in the same day with minimum of determinations covering the specified range of the procedure. Reproducible precision results from variations such as different days, analysts and equipment. Precision criteria for an assay method are that the instrument precision and the intra-assay precision (RSD) will be $\leq 2\%$ (Gowrisankar et al., 2010).

2.10.2.1. Intra-day (repeatability) precision study

In intra-day variation studies, solutions of the same concentration (5 $\mu\text{g/mL}$) were analyzed six times using the same method at the same time using the same equipment and reagents in the same day. Then, sample peak area was measured by HPLC and the mean standard deviation and RSD of sample were calculated (Table 2).

2.10.2.2. Inter-day (reproducibility) precision study

In the inter-day variation studies, solutions of the same concentration (5 µg/mL) were analyzed three times for the two consecutive days by the same method at the same time using the same equipment and reagents and the sample peak area and the mean retention time, standard deviation and RSD(%) were calculated (Table 2).

2.10.3. Accuracy study

The accuracy of the study was determined by percent of recovery. For percent of recovery study, one sample of known caffeine concentration from different types of coffee beverages was spiked with 5 mg/L of caffeine standard and recovery was calculated as summarized in (Table 1). All analyses were carried out in triplicate. General equation used to calculate the recovery (%) is given in equation below (APHA, 1999).

$$\text{Percent of recovery} = \frac{\text{Spiked sample value} - \text{Sample value}}{\text{Spiked standard value}} \times 100 \text{ (Association) (Eqn. 1)}$$

2.10.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. It is expressed as a concentration at a specified signal:noise ratio usually as 3:1. The LOQ signal:noise ratio of 10:1 is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. As ICH has recommended, LOD and LOQ may also be calculated based on the standard deviation (SD) of the response and the slope of the calibration curve(s) at levels approximating the LOD according to the formula given below (Gowrisankar et al., 2010).

$$\text{LOD} = \frac{3s_a}{b} \text{ and } \text{LOQ} = \frac{10s_a}{b} \text{ (Eqn. 2)}$$

Where S_a is the standard deviation of residue and b the slope of the calibration line, respectively. However, in order to calculate LOD and LOQ, the value of standard deviation of residue and intercept were calculated by microsoft office excel windows 10 program from calibration curve of the standard caffeine $S_a = 245549.8374$ and $S_b = 1288737.953$.

3. Results and Discussion

3.1. Ethnomedicinal values of some plants in the study area

The study areas in Wollega region have several traditional medicinal plants which belong to families including Asteraceae, Solanaceae, Zygophyllaceae, Fabaceae, Rosaceae, Musaceae, Euphorbiaceae, Rutaceae, Malvaceae, Brassicaceae and Cucurbitaceae (Feyissa et al., 2017). (Sarker and Nahar 2018) reviewed phytochemicals which are natural antioxidants such as curcumin, resveratrol, epicatechin, ellagic acid, flavone and apigenin (Ng et al., 2000; Reuter et al., 2010; Ribeiro et al., 2015) and are used in cosmetic industries to prepare various anti-ageing products. Additionally,

Table 2 A. intra-day precision study of 5 µg/mL standard caffeine peak area and retention time; B. inter-day precision of 5 µg/mL caffeine peak area and retention time at different day.

Parameter	No of replicates						Precision measuring parameter		
	1	2	3	4	5	6	Mean	SD	RSD%
Peak area	782259	776897	767542	762318	782745	783576	775889	8961.4	1.15
Retention time	1.847	1.843	1.857	1.837	1.847	1.843	1.84	0.0066	0.36
Parameter	05/07/2016						Precision measure parameter		
	No of replicates						Mean	SD	RSD%
Peak area	782259	776897	767542	754231	776897	774231	772009.5	9937.76	1.28
Retention time	1.837	1.843	1.843	1.847	1.843	1.857	1.84	0.0067	0.36

many phytochemicals such as phenolic compounds and mono- and sesqui-terpenes or phyto-extracts including *Castanea sativa* are also added to skin-care cosmetic products because of their antimicrobial properties (Ribeiro et al., 2015). Wansi et al. (2018 and 2019) have reviewed several essential oils extracted from various plant parts, such as leaves, bark, fruit, roots and rhizomes which exhibited bioactivities against *Plasmodium falciparum*, food borne microbes, dermatophytes, the malaria vector *Anopheles gambiae*, cancer cell lines, river blindness as well as plant pathogen weevils and fungi. The *Achillea* species were reported to have tonic, sedative, diuretic, carminative remedies which promote breast-feedings and regulate women menstruation and are extensively prescribed for the treatment of stomachache, inflammation, gastrointestinal, hemorrhoid, hay fever and wound healing in indigenous medicines (Mohammadhosseini et al., 2017).

(Abera 2014) reviewed the majority of medicinal plant parts in Gimbi, Wollega zone, Ethiopia which are prepared either in combination with other medicinal plant parts or with other additives such as boiled coffee, honey and local beverages (*tella*) for different purposes either to increase the healing potential or to improve the flavour and taste or to avoid abdominal discomfort (Tamene, 2000; Balemie et al., 2004). For instance, a traditional medicine applied to treat tape worm infection is prepared by the combination of several medicinal plant parts, i.e. *Hagenia abyssinica*, *Glinus lotoides* with other additives such as local beverages and salt (Balemie et al., 2004). There were also different studies that showed the presence of a wide range of herbal medicines in the Wollega zone being used for treating various ailments due to the presence of

various medicinal plants fairly distributed throughout the region (Feyissa et al., 2017). The identified plants in the region have a broad spectrum of activities and are used for the treatment of multiple ailments and have medical value against many diseases. (Feyissa et al. 2017) reported that *Citrus aurantifolia* is among the traditional medicinal plants available in this study area having insecticidal property against lice infestation. Additionally, *C. aurantifolia* is considered as tonic for libido and as antidote for poison. The diluted form of the *C. aurantifolia* fruit juice is used for mouth wash to treat sore mouth, sore throat and is useful to treat irritation, diarrhea and swelling due to mosquito bites (Aibinu et al., 2007; Khare, 2007; Akhtar, 2013).

3.2. Detection of caffeine from coffee bean, pulp and leaves

Quantification of caffeine content in the test samples was performed by an HPLC instrument coupled with C8 column and UV-detector at the wavelength of 272 nm. Fig.s 3-4 show general features of standard and extracted caffeine chromatograms. The relative peak areas of standard caffeine and three replicates of each diluted chloroformic extraction of coffee bean, pulp and leaf samples were shown in (Table 1). From standard caffeine concentration prepared over the range 5-25 µg/mL, linear regression calibration curves were made (Fig. 5). Before HPLC analysis of the standard samples, we checked the volume of the sample which gives the best peak. Accordingly, 20 µL injection volume was found to be the best for the analysis. Regarding the similar studies of contents of caffeine analysis, 20 µL was selected as an optimal injection volume in such sorts of HPLC-based determinations (Ali et al. 2012).

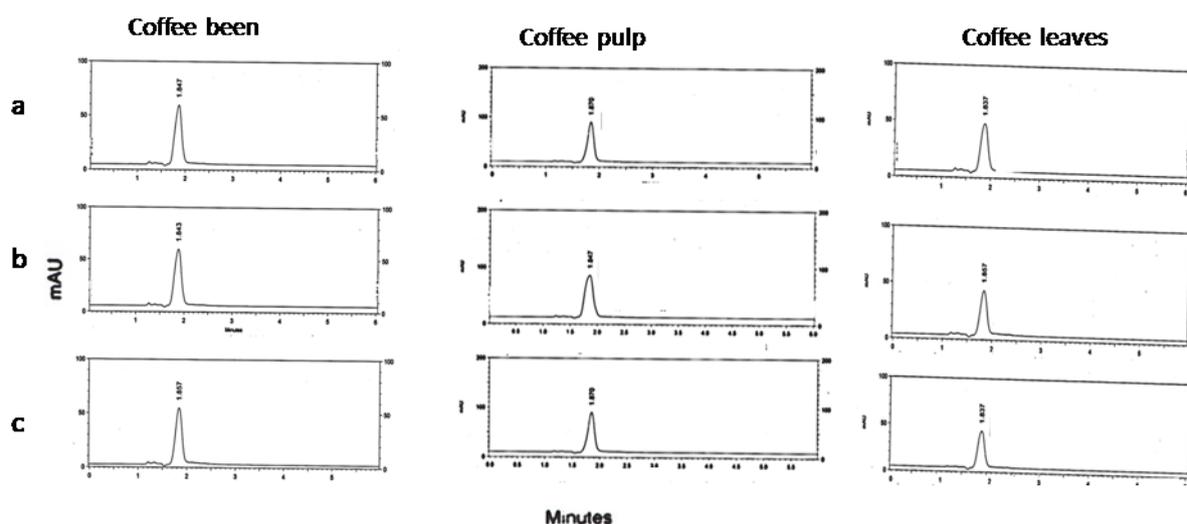


Fig. 3. HPLC chromatogram of caffeine extracted from coffee beans, pulp and leaves(a: Kelllem Wollega; b: West Wollega; c: East Wollega).

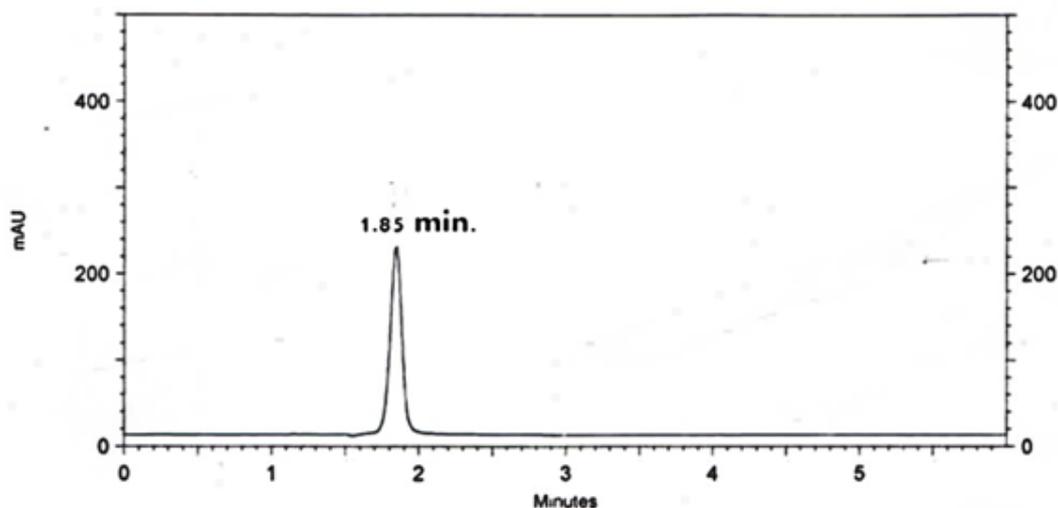


Fig. 4. HPLC Chromatogram of caffeine standard sample.

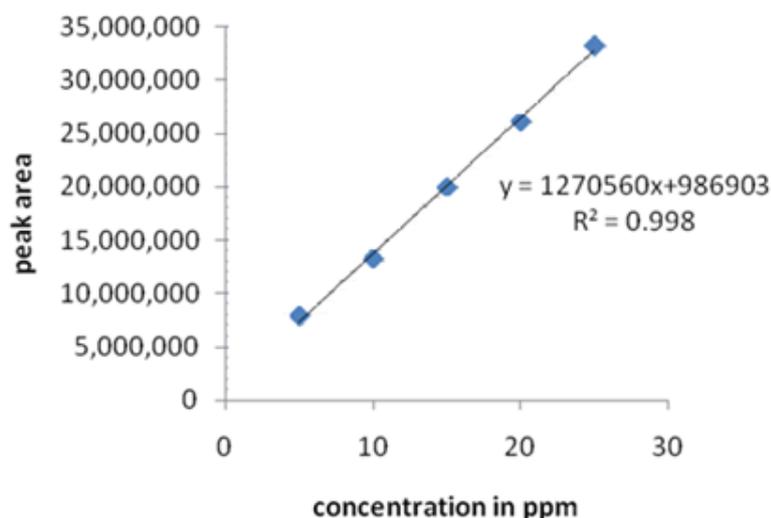


Fig. 5. Calibration curve of standard caffeine.

3.3. Method validation

The method was validated in terms of linearity range, intra-day precision (Table 2), inter-day precision (Table 2) and analytical recovery and accuracy (Table 3), limit of detection and limit of quantification (Table 3). Generally, the obtained calibration curve was found to be linear over the concentration range of 5-25 $\mu\text{g}/\text{mL}$ with an acceptable correlation coefficient (R^2) and a linear regression equation used to calculate concentration of caffeine in the extracted sample. From the quantitative analysis, acceptable relative standard deviation of 1.15% and 1.28% with stable retention time 1.84 ± 0.0066 min were resulted. Therefore, Table 2 represents the intra- and inter-day precision of the new method, confirming adequate sample stability and method reliability over a 24 h period.

The mean recoveries of the obtained results were found

to be not significantly different from the value of added caffeine concentration. The result of accuracy (Table 3) was within the range of ICH guideline. The accuracy(%) indicated non-interference from the component of solution. The results of analysis of beans were good and shown in Table 3.

3.4. Detection and quantification limits

In Table 3, the calculated LOD and LOQ have been shown using microsoft office excel windows 10. Hence, the lowest concentration that can be quantified with an acceptable accuracy and precision (LOQ) of extracted caffeine samples and lowest concentration that can be detected but cannot be quantified (LOD) were given in Table 3. The chromatograms of extracted caffeine samples obtained from coffee beans, pulp and leaves were shown in Fig. 3.

Table 3

Method detection and quantification limits of measured caffeine.

Standard deviation residue	slope of the calibration line	Calculated LOD	Calculated LOQ
$S_a=245549.8374$	$b=1288737.953$	0.63 µg/mL	1.90 µg/mL

 General formulae used to calculate $LOD = \frac{3s_a}{b}$ and $LOQ = \frac{10s_a}{b}$

3.5. Linearity

A linear regression of peak area versus standard caffeine concentration gave Eqn. 3 which is used to determine the concentration of unknown chloroformic extracted caffeine sample.

$$y = 1270560x + 986903 \quad (\text{Eqn. 3})$$

Using the regression equation (Eqn. 3) of standard caffeine, the caffeine concentration of the extracted sample solutions were calculated as shown in Table 4. A linear regression concentration calculated from peak area of injected chloroformic caffeine sample allowed to calculate the total concentration of caffeine (Eqn. 4) in the extracted sample solution (Table 4).

Table 4

Caffeine concentration of beans, pulp and leaves of extracted samples.

Part of coffee plant	Sample area	Caffeine Conc. in diluted Sample (µg/mL)	Caffeine Conc. in concentrated sample (µg/mL)	Caffeine Content (mg)	Caffeine content (mg/100 mL)	Caffeine to coffee Ratio. (Wt/Wt%)	Total average (Wt/Wt%)
Beans	Kelem Wollega	10.69 ± 0.19	71.34 ± 1.30	35.67 ± 1.30	35.67 ± 1.30	1.43 ± 0.19	1.30 ± 0.11
	West Wollega	9.54 ± 0.085	63.55 ± 0.43	31.81 ± 0.43	31.81 ± 0.43	1.27 ± 0.08	
	East Wollega	9.08 ± 0.14	60.58±0.97	30.29±0.97	30.29±0.97	1.21 ± 0.14	
Coffee pulp	Kelem Wollega	7.24 ± 0.13	48.28 ± 0.92	24.14 ± 0.81	24.14 ± 0.81	0.96 ± 0.13	0.90 ± 0.11
	West Wollega	7.25 ± 0.16	48.33 ± 0.93	24.17 ± 0.87	24.17 ± 0.87	0.97 ± 0.16	
	East Wollega	5.88 ± 0.11	39.15 ± 0.76	19.57 ± 0.76	19.57 ± 0.76	0.78 ± 0.11	
Coffee leaves	Kelem Wollega	5.68 ± 0.05	37.80 ± 0.34	18.90 ± 0.34	18.90 ± 0.34	0.76 ± 0.05	0.65 ± 0.10
	West Wollega	4.76 ± 0.02	31.71 ± 0.17	15.85 ± 0.17	15.85 ± 0.17	0.63 ± 0.02	
	East Wollega	4.29 ± 0.03	28.53 ± 0.20	14.26 ± 0.20	14.26 ± 0.20	0.57 ± 0.03	

Average ± Standard deviation (n = 3)

$$\text{Caffeine in total sample (ppm)} = \text{Conc. in injected (ppm)} \times \text{dilution factor} \quad (\text{Eqn. 4})$$

The final caffeine content in mass unit (mg) of the beverage under test was then calculated from the extracted sample solution concentration using Eqn. 5 and Eqn. 6.

$$\text{Caffeine content in injected sample (mg)} = \frac{\text{Conc.} \left(\frac{\text{µg}}{\text{mL}} \right) (\text{Total sample (mL)})^2}{\text{Used sample (mL)} \times 1000} \quad (\text{Eqn. 5})$$

(Wondimkun et al., 2016)

$$\text{Caffeine content in total sample (mg)} = \text{Caffeine content in injected sample (mg)} \times \text{dilution factor} \quad (\text{Eqn. 6})$$

The final result of the study was reported in terms of percentage mass of calculated caffeine to total mass of original sample used (W/W%). The yield of isolated caffeine from the collected samples of coffee is presented in Table 4. The results indicate that coffee beans contain higher amounts of caffeine than coffee

pulps and leaves. Caffeine content of coffee beans ranged from 1.21 ± 0.14% to 1.43 ± 0.19%, caffeine content of coffee pulp was within range of 0.78 ± 0.11%-0.97 ± 0.16% and that of coffee leaves was within range of 0.57 ± 0.03%-0.76 ± 0.05%. This result of the study can be graphically shown in Fig. 6.

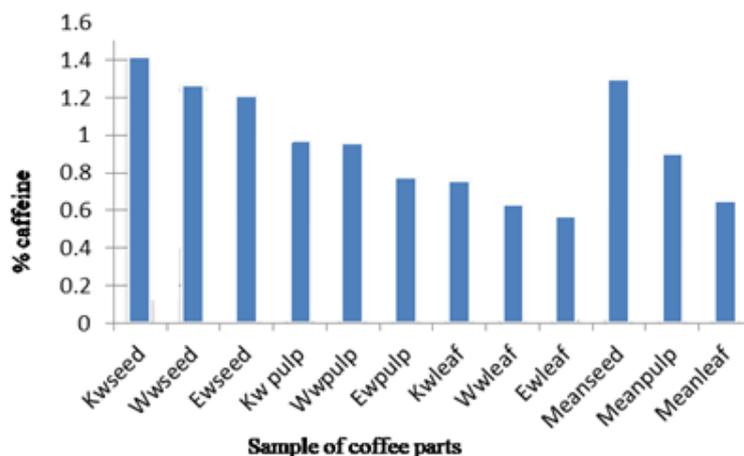


Fig. 6. Graphical representation of determined caffeine %Wt/Wt of coffee beans, pulp and leaves collected from study area (0.5-1.5%). KWseed: Kelem Wollega coffee bean; Wwseed: West Wollega bean, EWseed: East Wollega bean; Kw pulp: Kelem Wollega pulp; Wwpulp: West Wollega pulp; Ewpulp: East Wollega pulp; Kwleaf: Kelem Wollega leaf; Wwleaf: West Wollega leaf; Ewleaf: East Wollega leaf. Meanseed: mean of bean; Meanpulp: mean of pulp and Meanleaf: mean of leaf.

3.6. Comparison of caffeine content in different parts of the plants and among the regions

As shown in Fig. 3, the caffeine level of coffee beans, pulp and leaves determined in the study areas were decreased in the order of coffee beans, coffee pulp and coffee leaves. Within each coffee bean, the caffeine content decreases in order of Kelem Wollega ($1.43 \pm 0.19\%$), West Wollega ($1.27 \pm 0.08\%$) and East Wollega ($1.21 \pm 0.14\%$). When caffeine content of coffee pulp is considered, it decreases in order of West Wollega ($0.97 \pm 0.16\%$), Kelem Wollega ($0.96 \pm 0.13\%$) and East Wollega ($0.78 \pm 0.11\%$). For coffee leaves, order of their caffeine content decreases from Kelem Wollega ($0.76 \pm 0.05\%$), West Wollega ($0.63 \pm 0.02\%$) and East Wollega ($0.57 \pm 0.03\%$) (Table 4).

The result of our analysis on caffeine content in the studied area is in a good agreement with previously reported *Coffea arabica* beans caffeine content (Illy, 2013) which state less than 1.5% for *Coffea arabica* variety. In case of coffee leaves, caffeine content of the studied area was within a range of reported literature for *Coffea arabica* variety range between 0.62 to 0.98% (Oestreich-Janzen, 2013). For coffee pulps, caffeine content obtained was between ranges of *Coffea* species caffeine content (0.54-1.67%) stated in literature (Clifford and Ramirez-Martinez, 1991). According to this study, quantity of caffeine present in all aerial parts of coffee plant (beans, pulp and leaves) have good agreement with previously reported papers in literature that state in coffee plant, caffeine is present in all parts of the plants (Oestreich-Janzen, 2013). This fact consistent with the literature report which shows caffeine biosynthesis takes place in the leaves and the outer part of the fruit but when the seed inside the fruit starts growing, caffeine is translocated through the membranes and accumulates in the endosperm

(Oestreich-Janzen, 2013).

In support to this study, different caffeine contents in coffee beans have been reported by the previous researchers. For instance, average caffeine content of coffee beans (1-2.2%) (Komes et al., 2009; Ali et al., 2012) were found to be over the range of $0.96 \pm 0.01\%$ - $1.23 \pm 0.06\%$ for Arabic green coffee beans (Gebeyehu and Bikila, 2015) together with 0.8-1.4% for *Coffea arabica* (Belitz et al., 2009) and less than 1.5% for *Coffea arabica* (Illy, 2013). Moreover, the caffeine content in Ethiopian *Coffea arabica* grown in Bench Maji, Gediyo Yirgachefe, Tepi and Godere which has been determined by UV/Vis spectrophotometry to be $1.1 \pm 0.01\%$, $1.01 \pm 0.04\%$, $1.07 \pm 0.02\%$ and $1.19 \pm 0.02\%$, respectively (Belay et al., 2008) accounting for less caffeine content than the coffee beans of studied area. On the other hand, caffeine content in Ethiopia and Eritrea *Coffea arabica* grown in Gojjam area, Geisha, Harar and Eritrea has been determined by HPLC technique to be 0.89-1.04%, 0.93%, 1.18% and 0.95%, respectively (Gebeyehu and Bikila, 2015). In general, comparison of previous literature reports with this study for *Coffea arabica* indicates Wollega *Coffea arabica* has greater caffeine content than other parts of Ethiopian *Coffea arabica*.

Based on the previous literature reports, there may be slightly variation of roasting condition (Illy, 2013) since roasting does not significantly affect the content of caffeine. Other factors like brewing strength, grinding degree, brewing methods and serving size were controlled under the experimental conditions. Therefore, the variation in caffeine level of coffee samples may be due to geographical origins which might have different altitude, soil type, rain fall and other agricultural conditions, e.g. harvesting time, exposed to sun etc. as well as environmental conditions. In supports to this reason, other researchers reported that suitable environmental condition improves photosynthesis

and increases leaves area index, resulting in better producing of larger and heavier fruits with better beans quality (Gole, 2003; Bote and Struik, 2011; Gebeyehu and Bikila, 2015; Wondimkun et al., 2016). Therefore, greater caffeine content of *Coffea arabica* grown in Wollega zones with suitable afro-montane rain forest, altitudes range, annual rainfall and a wide range of soil types (Gole, 2003) may be due to this suitable environmental condition.

On the other hand, as ANOVA result indicates caffeine content of coffee grown in Wollega zones determined from beans in Kelem Wollega, West Wollega and East Wollega shows variation. The level of statistical significance association between the level of caffeine content in beans, pulp and leaves were statistically significant ($p < 0.05$) except for Kelem Wollega and West Wollega coffee pulps which are insignificant. The major source of this variation may be due to quality of coffee seed originated from different agricultural system and harvested time.

In case of caffeine content, difference among Kelem Wollega, West Wollega and East Wollega coffee leaves the main source of difference may be originated from total caffeine content of original coffee from where they are collected. In support to this idea, experimental results of this study show greater caffeine contents of Kelem Wollega coffee beans which have in turn greater caffeine content in case of their leaves, as well. Coffee leaves of East Wollega have lower caffeine content which corresponds to less caffeine containing coffee beans according to this report. On other hand, even though all this coffee leaves are collected at similar season, agro-climatic difference may cause different maturation stages that causes variation of caffeine in leaves. This fact is briefly stated in literature (Illy, 2013; Oestreich-Janzen, 2013). Additionally, for caffeine content variation of Kelem Wollega, west Wollega and East Wollega coffee pulp, the main reasonable origin of the variation may be beans quality of caffeine content.

In summary, in spite of the fact that the samples were analyzed under similar experimental condition and relatively similar geographical location, variation of caffeine content within each coffee beans from the three study areas could be due to harvesting time, agricultural system as well as environmental conditions. For instance, as stated in literature (Bote and Struik, 2011), coffee plants grown under shade trees produce larger and heavier fruits with better beans quality than those grown in direct sunlight. Accordingly, the coffee sample growing around Kelem Wollega under shaded area compared to those located in West Wollega and East Wollega has different quality even though Wollega zone belongs to rain forest part of Ethiopia. This fact is supported by experimental result which shows Kelem Wollega coffee has high caffeine content in all parts of coffee plant except caffeine content of coffee pulp which is less than west Wollega coffee pulp. Taking into account the above explanation, caffeine content variation between *Coffea arabica* samples in different parts of Ethiopia is due to geographical origins which might have different altitude, soil type, rain fall and

other agricultural as well as environmental conditions.

On the other hand, when brewing strength of this study, 25 g/L coffee beverage was explained in terms of 70 g/L ISO common brewing strength (Oestreich-Janzen, 2013) of coffee beverage and the caffeine contents of Kelem Wollega, West Wollega and East Wollega coffee beans were respectively 99.88, 89.26, 84.81 mg and in good agreement with previously reported data in literature which state that on the average, a cup of coffee contains 80 mg to 175 mg of caffeine depending on what "bean" (seed) is used and how it is prepared (Juliano and Griffiths, 2004).

4. Concluding remarks

Following extraction of caffeine from coffee and quantification by chromatographic HPLC analysis using peak area of extracted sample alongside standard caffeine calibration curve, the following results were reported in % mass of determined caffeine to its original coffee sample. The obtained results of this study are in good agreement with previously reported caffeine contents of *Coffea arabica* even though it has a greater value than those reported for the same coffee beans in some parts of Ethiopia. Although the number of coffee samples analyzed here is still small, the data presented in this study gave a representative data about the caffeine content level of coffee plant that grows in Wollega zone. Even though coffee pulps and leaves are used rarely as beverage substituting coffee beans in different parts of Ethiopia, most of the time, these parts of the plants are considered as by-products of coffee plants. Our results of caffeine contents of these parts of the plant may be of interest for researchers interested to work further in the preparation of beverages of coffee pulps and leaves with lower costs compared with those of coffee beans.

Conflict of interest

The authors declare that there is no conflict of interest.

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References

Abera, B., 2014. Medicinal plants used in traditional medicine by Oromo people, Ghimbi District, Southwest Ethiopia. J.



Ethnobiol Ethnomed. 10(1), 40. <http://www.ethnobiomed.com/content/10/1/40>.

Abbood, A., Aldiab, D., 2017. HPLC determination of caffeine in some beverages and pharmaceutical dosage forms available in Syrian market. J. Chem. Pharm. Sci. 10(3), 1174-1179.

Aibinu, I., Adenipekun, T., Adelowotan, T., Ogunsanya, T., Odugbemi, T., 2007. Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. Afr. J. Tradit. Complement. Altern. Med. 4(2), 185.

Akhtar, S., 2013. Evaluation of cardiovascular effects of *Citrus aurantifolia* (Linn.) fruit. Available at SSRN: <https://ssrn.com/abstract=2279447>.

Ali, M.M., Eisa, M., Taha, M.I., Zakaria, B.A., Elbashir, A.A., 2012. Determination of caffeine in some Sudanese beverages by high performance liquid chromatography. Pak. J. Nutr. 11(4), 336.

Alliance, C., Chan, S.C., 2013. Chemical Test for Caffeine. Hong Kong Chemistry Olympiad for Secondary Schools (2013-14), Memorial College.

Amare, M., Admassie, S., 2012. Polymer modified glassy carbon electrode for the electrochemical determination of caffeine in coffee. Talanta 93, 122-128.

American Public Health Association (APHA), 1999. Standard Methods for the Examination of Water and waste Water. Washington DC. 20th Ed.

Aresta, A., Palmisano, F., Zambonin, C.G., 2005. Simultaneous determination of caffeine, theobromine, theophylline, paraxanthine and nicotine in human milk by liquid chromatography with diode array UV detection. Food Chem. 93(1), 177-181.

Anonymous, 1985. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, Water Environment Federation, Washington.

Belay, A., Ture, K., Redi, M., Asfaw, A., 2008. Measurement of caffeine in coffee beans with UV/Vis spectrometer. Food Chem. 108(1), 310-315.

Balemie, K., Kelbessa, E., Asfaw, Z., 2004. Indigenous medicinal plant utilization, management and threats in Fentalle area, Eastern Shewa, Ethiopia. E. J. Biol. Sci. 3(1), 37-58.

Belitz, H.-D., Grosch, W., Schieberle, P., 2009.

Coffee, tea, cocoa. Food Chem. 938-970.

Beyene, B., Tolosa, T., Rufael, T., Hailu, B., Teklue, T., 2015. Foot and mouth disease in selected districts of western Ethiopia: seroprevalence and associated risk factors. Rev. Sci. Tech. Off. Int. Epiz 34(3), 2.

Bote, A.D., Struik, P.C., 2011. Effects of shade on growth, production and quality of coffee (*Coffea arabica*) in Ethiopia. J. Hortic. For. 3(11), 336-341.

Carrillo, J.A., Benitez, J., 2000. Clinically significant pharmacokinetic interactions between dietary caffeine and medications. Clin. Pharmacokinet. 39(2), 127-153.

Castro, J., Pregibon, T., Chumanov, K., Marcus, R.K., 2010. Determination of catechins and caffeine in proposed green tea standard reference materials by liquid chromatography-particle beam/electron ionization mass spectrometry (LC-PB/EIMS). Talanta 82(5), 1687-1695.

Chen, Q.-c., Mou, S.-f., Hou, X.-p., Ni, Z.-m., 1998. Simultaneous determination of caffeine, theobromine and theophylline in foods and pharmaceutical preparations by using ion chromatography. Anal. Chim. Acta 371(2-3), 287-296.

Chou, K.H., Bell, L., 2007. Caffeine content of prepackaged national-brand and private-label carbonated beverages. J. Food Sci. 72(6), C337-C342.

Clifford, M., Ramirez-Martinez, J., 1991. Tannins in wet-processed coffee beans and coffee pulp. Food Chem. 40(2), 191-200.

Demissie, E.G., Woyessa, G.W., Abebe, A., 2016. UV/Vis spectrometer determination of caffeine in green coffee beans from Hararghe, Ethiopia, using beer-lambert's law and integrated absorption coefficient techniques. Sci. Study Res. Chem. Chem. Eng. Biotechnol. Food Ind. 17(2), 109.

Feyissa, T.F., Hailemariam, T.B., Regassa, T., Kergano, N.K., 2017. Ethnobotanical study of ethnoveterinary plants in Kelem Wollega Zone, Oromia Region, Ethiopia. J. Med. Plants Res. 11(16), 307-317.

Fujioka, K., Shibamoto, T., 2008. Chlorogenic acid and caffeine contents in various commercial brewed coffees. Food Chem. 106(1), 217-221.

Grujić-Letić, N., Rakić, B., Šefer, E., Milanović, M., Nikšić, M., Vujić, I., Milić, N., 2016. Quantitative determination of caffeine in different matrices.

Maced. Pharm. Bull. 62(1) 77-84.

Khare, C., 2007. Indian Medicinal Plants: An Illustrated Dictionary Springer-Verlag. Berlin pg, 699-700.

Gebeyehu, B.T., Bikila, S.L., 2015. Determination of caffeine content and antioxidant activity of coffee. Am. J. Appl. Chem. 3, 69-76.

Gole, T.W., 2003. Vegetation of the Yayu forest in SW Ethiopia: impacts of human use and implications for *in situ conservation* of wild *Coffea arabica* L. populations. Cuvillier.

Gowrisankar, D., Abbulu, K., Bala Souri, O., Sujana, K., 2010. Validation and calibration of analytical instruments. J. Biomed. Sci. Res. 2(2), 89-99.

Ily, E., 2013. The complexity of coffee. Sci. Am. 86-91.

Islam, M., Rahman, M., Abedin, M., 2002. Isolation of caffeine from commercially available tea and tea waste. Jahangirnagar Uni. J. Sci. 25, 9.

Juliano, L.M., Griffiths, R.R., 2004. A critical review of caffeine withdrawal: empirical validation of symptoms and signs, incidence, severity and associated features. Psychopharmacology 176(1), 1-29.

Komes, D., Horzic, D., Belscak, A., Kovacevic Ganic, K., Bljak, A., 2009. Determination of caffeine content in tea and maté tea by using different methods. Czech J. Food Sci. 27, 213-216.

Martin, M.J., Pablos, F., González, A., 1998. Characterization of green coffee varieties according to their metal content. Anal. Chim. Acta. 358(2), 177-183.

Minamisawa, M., Yoshida, S., Takai, N., 2004. Determination of biologically active substances in roasted coffees using a diode-array HPLC system. Anal. Sci. 20(2), 325-328.

Mohammadhosseini, M., Sarker, S.D., Akbarzadeh, A., 2017. Chemical composition of the essential oils and extracts of *Achillea* species and their biological activities: A review. J. Ethnopharmacol. 199, 257-315.

Ng, T., Liu, F., Wang, Z., 2000. Antioxidative activity of natural products from plants. Life Sci. 66(8), 709-723.

Norton, T.R., Lazev, A.B., Sullivan, M.J., 2011. The "buzz" on caffeine: Patterns of caffeine use in a convenience sample of college students. J. Caffeine Res. 1(1), 35-40.

Oestreich-Janzen, S., 2013. Chemistry of

coffee. CAFEA GmbH, Hamburg, Germany.

Perrone, D., Donangelo, C.M., Farah, A., 2008. Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. Food Chem. 110(4), 1030-1035.

Ranheim, T., Halvorsen, B., 2005. Coffee consumption and human health-beneficial or detrimental?-Mechanisms for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 diabetes mellitus. Mol. Nutr. Food Res. 49(3), 274-284.

Reuter, J., Merfort, I., Schempp, C.M., 2010. Botanicals in dermatology. Am. J. Clin. Dermatol. 11(4), 247-267.

Ribeiro, A., Estanqueiro, M., Oliveira, M., Sousa Lobo, J., 2015. Main benefits and applicability of plant extracts in skin care products. Cosmetics 2(2), 48-65.

Sarker, S.D., Nahar, L., 2018. Phytochemicals and phyto-extracts in cosmetics. Trends Phytochem. Res. 2(4) 185-186.

Schenker, S., Heinemann, C., Huber, M., Pompizzi, R., Perren, R., Escher, R., 2002. Impact of roasting conditions on the formation of aroma compounds in coffee beans. J. Food Sci. 67(1), 60-66.

Shaker, S.A., Farina, Y., Mahmmod, S., 2010. Synthesis and characterization of mixed ligand complexes of caffeine, adenine and thiocyanate with some transition metal ions. Sains. Malays. 39(6), 957-962.

Tamene, B., 2000. A floristic analysis and ethnobotanical study of the semi-wetland of Cheffa area South Wello. Unpublished MSc. Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Wansi, J.D., Sewald, N., Nahar, L., Martin, C., Sarker, S.D., 2018. Bioactive essential oils from the Cameroonian rain forest: A review - Part I. Trends Phytochem. Res. 2(4), 187-234.

Wansi, J.D., Sewald, N., Nahar, L., Martin, C., Sarker, S.D., 2019. Bioactive essential oils from the Cameroonian rain forest: A review - Part II. Trends Phytochem. Res. 3(1), 3-52.

Weldegebreal, B., Redi-Abshiro, M., Chandravanshi, B.S., 2017. Development of new analytical methods for the determination of caffeine content in aqueous solution of green coffee beans. Chem. Cent. J. 11(1), 126.

Wondimkun, Z.T., Jebessa, A.G., Molloro, L.H., Haile, T., 2016. The determination of caffeine



level of wolaita zone, Ethiopia coffee using UV-visible spectrophotometer. *Am. J. Appl. Chem.* 4(2), 59-63.

Yigzaw, D., Labuschagne, M., Osthoff, G., Herselman, L., 2007. Variation for green

bean caffeine, chlorogenic acids, sucrose and trigonelline contents among Ethiopian Arabica coffee accessions. *SINET: Ethiop. J. Sci.* 30, 77-82.