Determination of Caffeine Content of Bale Coffee Using HPLC Analysis

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Abstract

Ethiopia is the home land of biodiversity of *coffea arabica* seeds. The major and most known coffee producing regions in the country are Oromiya and Southern Nations Nationalities and Peoples Region. The objective of this study was to determine the caffeine content of coffee bean samples obtained from coffee growing areasof Bale zone, Oromia region. The samples were collected from farmers' union that supplies coffee to Ethiopian Commodity Exchange (ECX) or exporters. The collected samples were roasted and powdered to facilitate the extraction. The powders were then boiled with water, filtered and subjected to liquid-liquid extraction using dichloromethane. The extracts were subjected to High Performance Liquid Chromatographic analysis. The results revealed that caffeine levels to be $1.31 \pm 0.14\%$ for *Gololcha* coffee, $1.32 \pm 0.03\%$ for *Harena Buluk* forest coffee, $1.33 \pm 0.08\%$ for *Mena* forest coffee, $1.34 \pm 0.21\%$ for *Berbere* coffee, $1.36 \pm 0.02\%$ for *Harena Buluk* cultivated coffee and $1.36 \pm 0.16\%$ for Mena cultivated coffee (on dry matter basis). The finding also showed that there are no significant differences in the caffeine contents of the coffee samples used in the study. Moreover, the caffeine contents were in the range of export standards of Ethiopia.

Keywords: Caffeine content; High Performance Liquid Chromatography, *Coffea Arabica; Bale coffee*; Kaffa

1. Introduction

Coffee is the most widely consumed beverage in the world due to its pleasant taste, aroma, stimulant effect and health benefits [1-5]. The name coffee was originated from the name of the place 'Keffa' where shepherds from Ethiopia discovered the coffee beans in the 6th century [6.7]. Though there are over 80 species of coffee that grow in different parts of the world [8], two of them namely *Coffea arabica* and *Coffea robusta* type dominated the world coffee production or coffee market. Reports also showed that *Coffea arabica* and *Coffea robusta* constitute 66% and 34% of the world coffee production, respectively [9]. This facts revealed that *Coffee arabica* to be the most economical, and it controls three quarters of the world coffee productions [10].

Ethiopia is the home land of biodiversity of *Arabica coffee* seeds. Furthermore, genetically diverse varieties of *Coffea arabica* exist in Ethiopia than anywhere else in the world [6,9,11]. The recognition and worldwide demand of coffee, which stems from its distinctive flavor, made it currently one of the most desirable and commonly consumed beverages. Furthermore, it has a strong historical, cultural, social and economic value in each and every society in Ethiopia [11]. It also plays the leading role in the national economy being the primary source of foreign exchange earnings [12,13]. It is also an source of revenue of millions of Ethiopians [6,11]. The major and most known coffee producing regions in the country are Oromiya and Southern Nations Nationalities and Peoples Region [10,11,14]. The coffee beans produced in these regions have different qualities that can be attributed to several factors such as variation in genotype, climatic conditions, soil characteristics, agricultural practices, harvesting methods and post-harvest processing conditions (drying, storage, roasting and grinding) and chemical compositions such as caffeine contents [10,14,15].
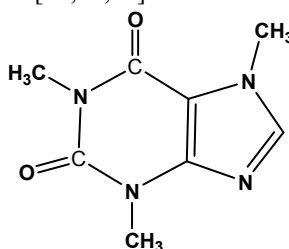
Figure 1. The chemical structure of caffeine.

Coffee quality refers a desirable appearance, attractive flavor, good cup taste and level of caffeine [6,14,16,17]. There are several factors that affect the quality of coffee beans. Among the factors, level of caffeine (caffeine content) [18,19] is an important factor. There are several reports on health benefits or desirable physiological effects of caffeine in humans [20-23] if it is taken in moderate amounts (130-300 mg/day) [24]. However, it has adverse effects if taken in higher amounts (>400 mg/day) [24]. These include increase in gastric secretion, severe restlessness, excitement, muscular tension, twitching and cardiovascular disturbance [25], enhance blood pressure, increase in blood sugar, increase in gastric acid and pepsin secretion [26] and cause significant sleep disturbance [27].

Besides, its health problems higher caffeine content is associated with less quality coffee beans [28]. Thus,

high caffeine content adversely affects the price of coffee and consumer preferences to coffee products (beans). This fact made it mandatory/necessary to determine caffeine contents of coffee products before they got marketed or exported to other countries. There are several analytical methods that have been used or reported to be used in caffeine level determination coffee (and coffee products) and other local and industrial beverages [29-39]. Accuracies of the methods may vary from method to method indicating that selection of appropriate analytical method is necessary for the accurate estimation of caffeine in test samples. UV/VIS and HPLC methods are two of the most commonly and widely used (reported) chromatographic techniques in the determination of caffeine contents of coffee products. HPLC is a more recent and reliable method of caffeine determination combined with several detectors such as UV/VIS, MS and IR spectrometry, because of its rapidity, high accuracy and reproducibility [37, 40-45].

The two methods namely UV/VIS and HPLC have widely been used in caffeine content determination of coffee beans from different parts of Ethiopia. For instance, caffeine contents of coffee samples of five districts of *Wolaita zone* (Ethiopia) were determined using UV/VIS spectrophotometric method. According to the report, the finding of the research showed caffeine contents of the five districts namely *Bolso sore* coffee, *Boloso bomba*, *Kindo koisha* coffee, *Sodo zuria* coffee and *Humbo* coffee to be 507.15 mg/L, 564.07 mg/L, 501.97 mg/L, 553.66 mg/L and 527.85 mg/L, respectively [46]. A report from North western part of the country such as *Wembera, Goncha, Zegie* and *Burie* areas also revealed caffeine content of $1.53 \pm 0.003\%$, $1.41 \pm 0.040\%$, $1.29 \pm 0.033\%$ and $0.97 \pm 0.049\%$, respectively [47]. The use of UV/VIS to determine caffeine contents of Ethiopian *Arabica coffee* from different localities were also reported. These reports showed that the caffeine contents to be in the range of 0.601- 0.903 % for *Hararghe* coffee beans [48], 0.46 - 2.82% (or average 1.18%) for *Kaffa* coffee, 0.42 - 2.90% (or average 1.10%) for *Illubabor* coffee [49], $1.1 \pm 0.01\%$ for *Bench Maji* coffee, $1.01 \pm 0.04\%$ for *Gediyo Yirgachefe*, $1.07 \pm 0.02\%$), *Tepi* coffee and $1.19 \pm 0.02\%$ for *Godere* coffee [32]. There is also a report that showed the use of HPLC technique to determine caffeine contents of medium roasted coffee beans of different coffee varieties [45]. Use of HPLC method some Ethiopian coffee varies such as *Kaffa* coffee and *Illubabor* showed caffeine contents of 0.46-2.82% (average value of 1.18%) and 0.42-2.90% (average value of 1.10%), respectively [49]. Similar analyses employed by other researcher on 42 Ethiopian coffee samples showed the amount of caffeine content of the coffee samples used in the study to be 1.10 % (on average) and in the range of 0.96 ± 0.01 % to 1.23 ± 0.06 % [50]. The present study aims on the determination of caffeine content of coffee bean samples collected from four main coffee producing districts of Bale zone (*Gololcha* district, *Berbere* district, *Mena* district and *Harena Buluk* district) using HPLC method. The results of the study would serve as baseline information for other researchers who want to conduct similar studies in other districts of the zone and also provide information to consumers and concerned authorities about caffeine levels of coffee beans form Bale area.

2.0 Materials and Methods
2.1 Plant materials collection
Coffea arabica from *Gololcha* (cultivated), *Berbere* (cultivated), *Mena* (cultivated and forest) and *Harena Buluk* (cultivated and forest) coffee bean samples were obtained from farmers unions and coffee suppliers in the month of August 2017. The areas (Figure 2) were selected purposely as they are major coffee producing areas of the zone. Coffee production area of *Gololcha* district covers 4,000 hectares and *Berbere* district has 14,000 hectares cultivated coffee. *Mena* district has a total area of 16,000 hectares (9,000 hectares forest coffee and 7,000 hectares cultivated coffee), and *Harena Buluk* district has 11,500 hectares (6,500 hectares forest coffee and 5,000 hectares cultivated coffee) [personal communication with office of coffee and tea development authority office].
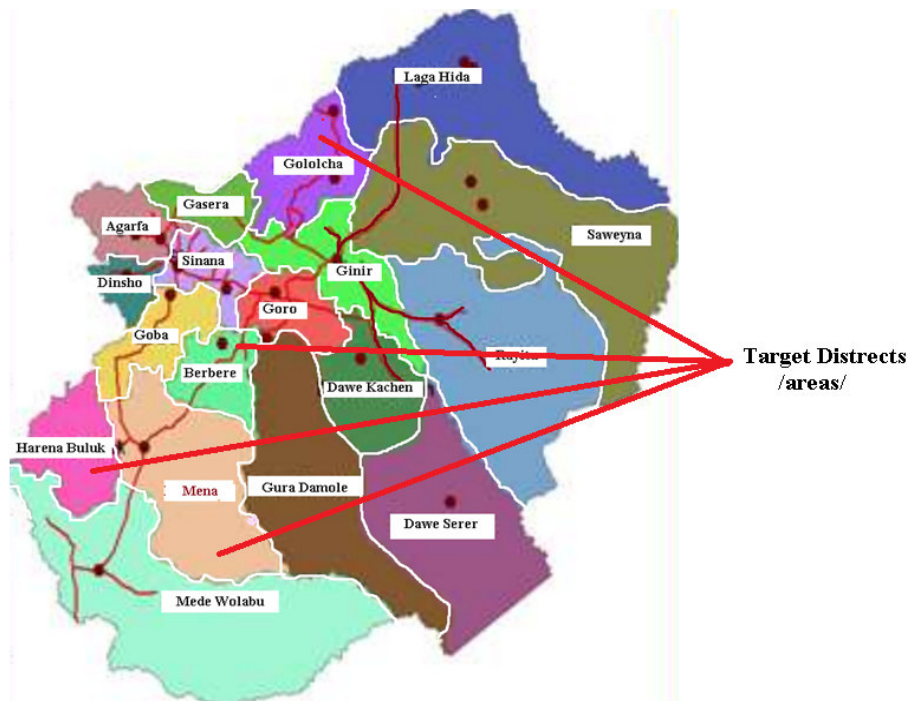
Figure 2. Map of Bale zone and geographical location of target districts [53].

3.2 Sample preparation and extractions

30g of each of the six raw coffee bean samples (Figure 3) which were obtained from farmers union and coffee supplier were roasted using conventional coffee roasting method.

Figure 3. The physical appearance of coffee samples from the four districts (A-raw B- roasted).

Each of the roasted coffee bean samples were ground and screened through 250 μm sieve to get a uniform texture. Exactly 0.50 g of each ground and sieved coffee samples (Figure 4) were put into 250 ml beakers, and were boiled in 100 ml of distilled water in a temperature range of 80-90 ^{o}C with continuous stirring using

magnetic stirrer (for 30 minutes). The coffee solution samples were cooled to room temperature, and then filtered into conical flasks using 0.45 μm filter paper using suction filtrations. Small amount of hot water was added on the scum left over the filter paper to wash caffeine remains (if there were any).

Figure 4. The ground coffee samples prepared for liquid-liquid extraction

To each of the filtrate, 3 g of Na_2CO_3 was added to remove fats, and also to facilitate formation clear of solution. The filtrates were subjected to liquid-liquid extraction by adding 100 ml dichloromethane to each of the filtrates in a separatory funnel. The organic phases were separated from aqueous phase and collected in clean and dry flasks. The aqueous phases were extracted 3 times with 30 ml of dichloromethane. The fraction of organic phases each coffee samples were mixed together and were dried over anhydrous Na_2SO_4. The solutions were filtered using suction filtration followed by solvent evaporation under reduced pressure (or using rotary evaporator). The masses of crude caffeine extracts were collected in dry and weighed test tubes, and kept in refrigerator until used for HPLC analysis.

2.3. Preparation of standard solutions

Caffeine stock solution of 1000 ppm was prepared by dissolving 100 mg of standard caffeine powder (obtained from JIJE Analytical *Testing* Service Laboratory) with 50 ml of warm ultra-pure water in a 100 ml volumetric flask and filled to the final volume with distilled water after cooling down to room temperature. 100 ppm of intermediate standard solution was prepared by pipetting 10 ml of stock solution in to a 100 ml volumetric flask and brought up to volume with ultra-pure water. The caffeine working standard solutions of different concentrations 0, 5, 10 and 25 ppm were prepared from the intermediate solution in a 10 ml volumetric flasks with ultrapure water.

2.4. HPLC analyses of caffeine

All HPLC analyses were carried out at JIJE Analytical *Testing* Service Laboratory, Addis Ababa. The standards and the samples were run in the HPLC system Agilent 1260 equipped with G1310B pump, G1316A column comportment, G1329B auto sampler and G4286Bdetector (VWD). The HPLC conditions followed during the experiment were: column size: Agilent Zorbax Eclipse XDB–C_{18}, 4.6 x 75 mm and 5 μm particle diameters. The flow rate was set at 1.0 ml/min, detector (VWD/DAD) at 272 nm, and the sample injection volume at 20 μL, column temperature at 25 ^{O}C and data rate at 10 Hz (Table 1). The mobile phase used in the experiment was solution of water: methanol (75:25, v/v). A calibration curve of peak areas versus concentration of the standards was plotted using standard solutions of different concentrations (0, 5, 10 and 25 ppm).

Table 1. The condition for running the HPLC experiments

Parameter	Value
Mobile phase	Water 75%, Methanol 25%
Flow rate	1.0 mL/min
Elution conditions	Isocratic, 25 % methanol
separation Column	Agilent Zorbax Eclipse XDB–C18, 4.6 x 75mm, 5μm
Injection volume	20 μL with Column 1
Column temperature	25 °C
Detector (VWD/DAD)	272 nm, Data rate 10 Hz
limit of detection (LOD)	0.0018 mg/L
limit of quantification (LOQ)	0.0055 mg/L
Recovery	101.45 %

2.5. Method validation

To determine the caffeine content of the coffee samples, the method was validated using the standard solution prepared for the experiment. The standard solutions used in experiment were 0, 5, 10 and 25 ppm. The solutions were then injected into the HPLC machine following the chosen chromatographic conditions (Section 2.4). The solution with 0 ppm (blank solution) did not give any peak (Appendix 1). On the other hand, the rest standard solutions (5, 10 and 25 ppm) gave single peak at retention time of 1.305, 1.299 and 1.304 minutes, respectively (Appendix 2 A-C). Moreover, the peak areas were found to increase with concentration. The peak areas were 288.518, 547.941 and 1342.375 for 5, 10 and 25 ppm solutions, respectively. A calibration curve for peak area against concentration of working caffeine standards was constructed to validate the HPLC quantification of caffeine in terms of linearity, sensitivity, precision and for calibration purpose. The curve showed good linear relationship between the peak area and concentrations of the standard solutions. Its equation was derived as $Y = 52.8x + 22.9$ and calibration curve of standard ($R^2 = 1.0000$) (Figure 5) where Y is peak area, X is concentration of caffeine (mg/L) and R is the linear correlation factor. Hence, the chosen method was taken as suitable and reproducible for the quantitative determination of caffeine extracted in coffee samples [44].
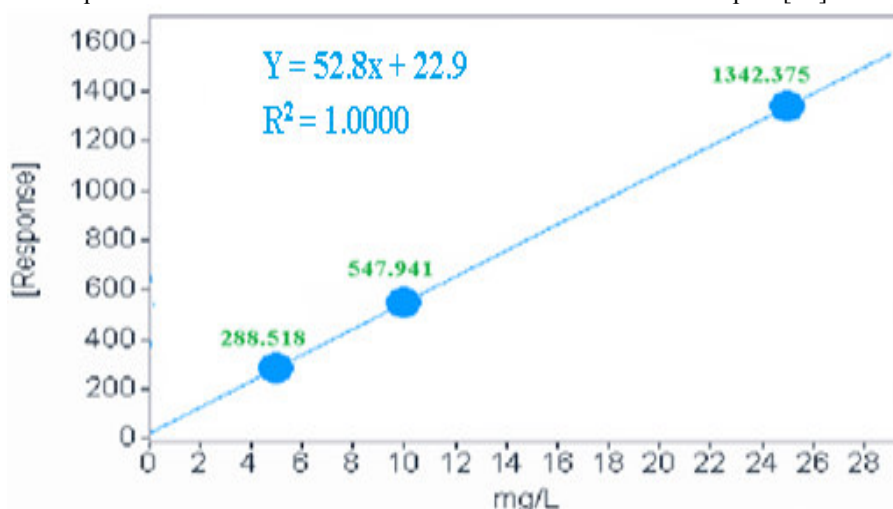

Figure 5. The calibration curve that was used in the HPLC analysis

3.0 Results and Discussion

In this project, it was intended to determine caffeine levels of coffee samples obtained from selected (major coffee producing) areas of Bale zone, and also to see differences (if any) between caffeine contents of forest and cultivated coffee beans. To do so, HPLC method was employed [51].

3.1 Amount and percent yield of crude caffeine extract

The data obtained from the experiment showed that caffeine contents of the coffee samples collected from different areas were comparable to each other. Moreover, there is only slight difference among the caffeine contents of coffee samples obtained from cultivated and forest (Table 2). The percent yield was calculated using the following formula;

$$\% \text{ yiled of crude caffeine} = \frac{\text{mass of the crude caffiene}}{\text{Mass of coffee powder used for extraction}} \times 100\%$$

Table 2. The masses and percentages of the caffeine extracts

Type of sample	Mass of crude caffeine (g)	% by mass of crude caffeine sample
Gololcha coffee (cultivated)	0.010 ± 0.002	2.0 ± 0.001
Berbere coffee (cultivated)	0.009 ± 0.004	1.8 ± 0.014
Delo Mena cultivated coffee	0.009 ± 0.002	1.8 ± 0.018
Delo Mena forest coffee	0.010 ± 0.001	2.0 ± 0.014
Harena Buluk cultivated coffee	0.010 ± 0.004	2.0 ± 0.012
Harena Buluk forest coffee	0.009 ± 0.001	1.8 ± 0.002

### 3.2. Quantitative determination of caffeine using HPLC method

The validated method (Section 2.4), was used to determine the concentration of caffeine in the coffee samples were determined quantiatively by injecting the prepared solutions of crude caffeine extracts of the coffee

samples used in the study. From each sample of cultivated and forest coffee beans, 20 µl of solution were injected to the HPLC. The result showed that the elution or the retention time for caffeine for *Berbere* district cultivated coffee sample to be 1.296 min (Figure 6). At this retention time, the concentration of caffeine was determined to be 31.78 mg/L or 1.34 ± 0.21% w/w in dry basis (Table 3).
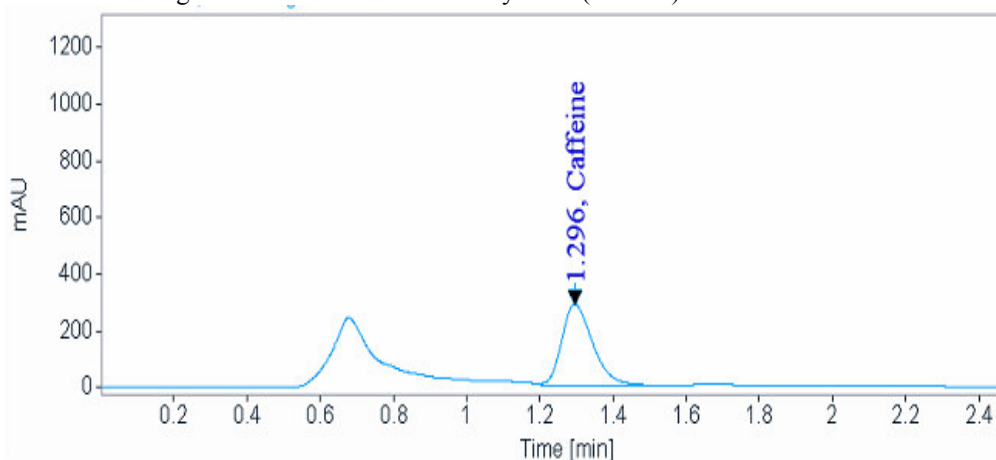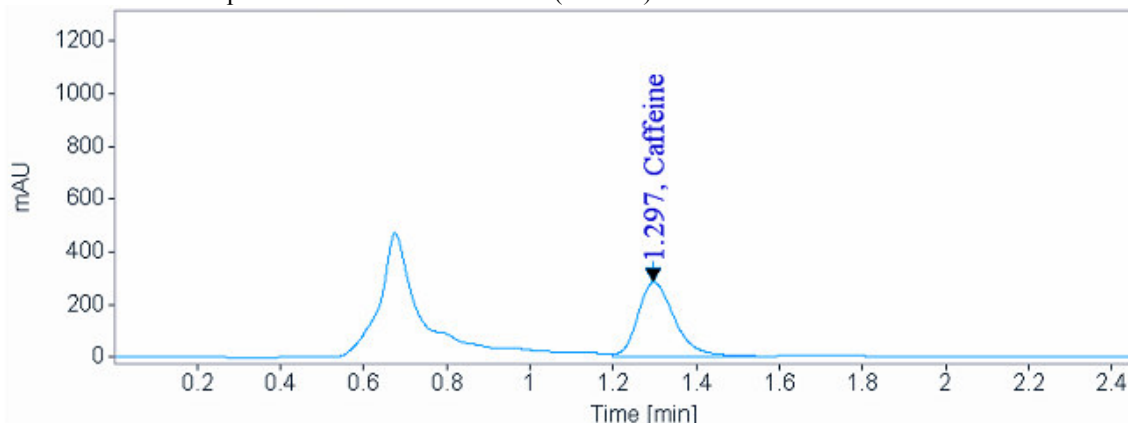

Figure 6. The HPLC chromatogram of *Berbere* district cultivated coffee sample.

The chromatogram showed that for *Mena* district cultivated coffee sample the elution or the retention time for caffeine was at the time 1.299 min (Figure 7). At this retention time, the concentration of caffeine was determined to be 31.98 mg/L or 1.36 ± 0.16**%** w/w in dry basis (Table 3).
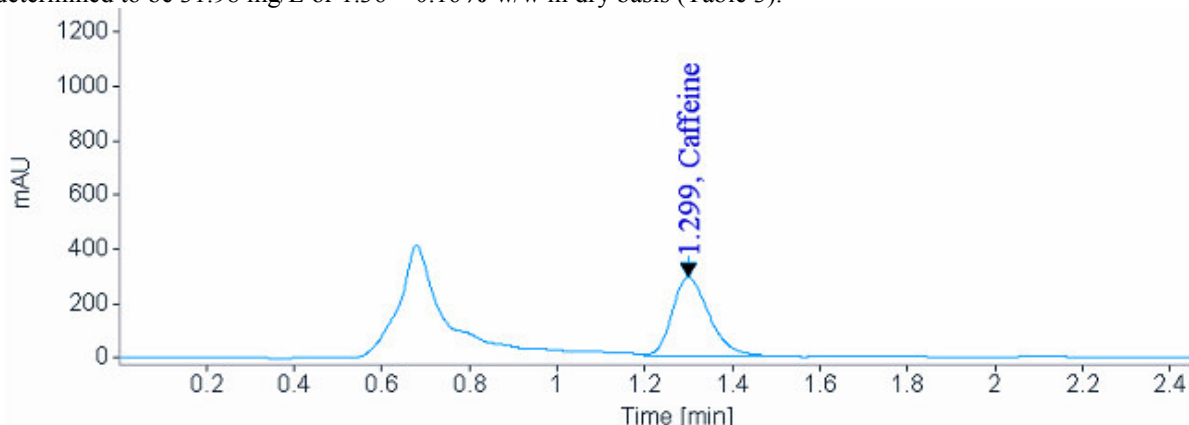
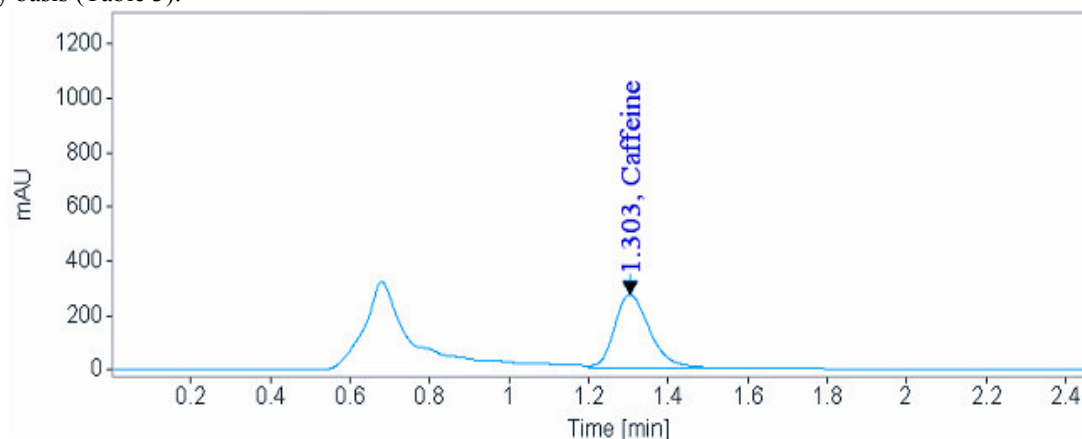**Figure 7.** The Chromatogram of *Mena* district cultivated coffee sample.

The chromatography result showed that in the injection volume of 20 µL crud extract of *Mena* district forest coffee sample. The elution or the retention time for caffeine was at the time 1.297 min (Figure 8). At this retention time the concentration of caffeine determined was 31.72 mg/L or 1.33 ± 0.08**%** w/w in dry basis. When compared *Mena* district forest coffee sample with *Mena* district cultivated coffee sample, the *Mena* district forest coffee sample has lower caffeine content (Table 3).

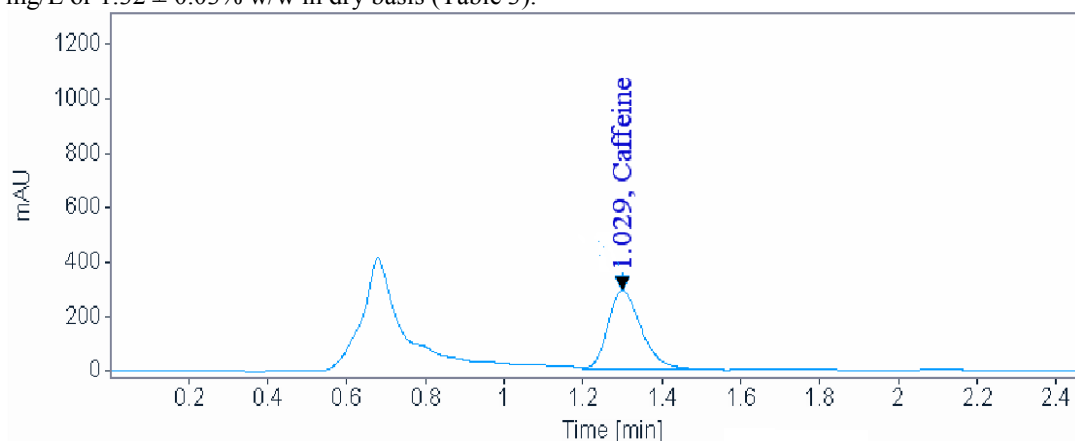**Figure 8.** Chromatogram of *Mena* district forest coffee sample.

The HPLC analysis of cultivated coffee sample of *Gololcha* district showed retention time of 1.303 min

(Figure 9). At this retention time the concentration of caffeine determined was 31.42 mg/L or 1.31 ± 0.14**%** w/w in dry basis (Table 3).
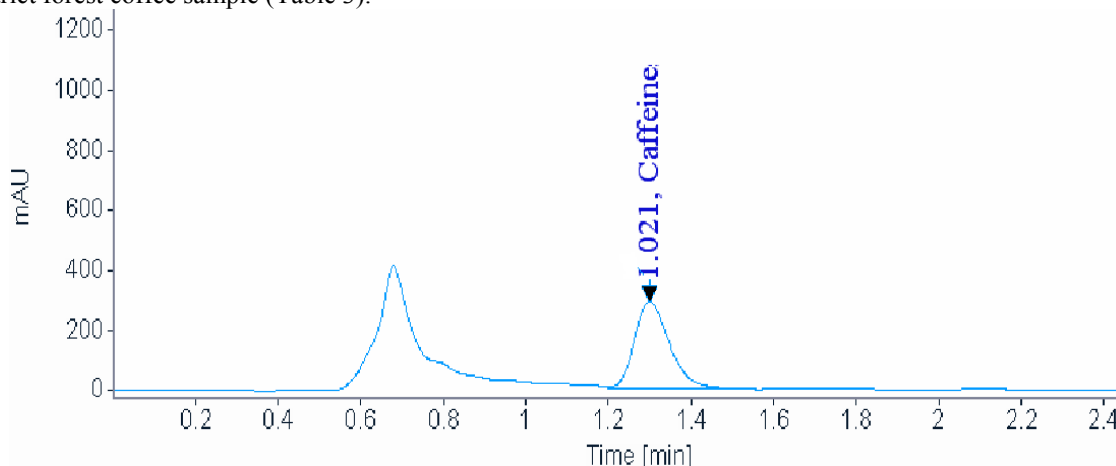

Figure 9. Chromatogram of *Gololcha* district cultivated coffee sample.

The chromatogram of *Harena Buluk* district cultivated coffee sample gave the retention time of 1.029 min (Figure 10). At this retention time, the concentration of caffeine determined was 31.87 mg/L or 1.36 ± 0.02**%** w/w in dry basis (Table 3). Similar procedure on *Harena Buluk* district forest coffee sample revealed that retention time of 1.021 min (Figure 11). At this retention time the concentration of caffeine determined was 31.52 mg/L or 1.32 ± 0.03% w/w in dry basis (Table 3).

Figure 10. Chromatogram of *Harena Buluk* district cultivated coffee sample.

When compared *Harena Buluk* district forest coffee sample with *Harena Buluk* district cultivated coffee sample, the forest coffee sample showed lower caffeine content (Table 3). On the other hand, the comparison of the data also indicated that cultivated coffee sample of *Harena Buluk* and *Mena* districts to have almost similar caffeine contents. Whereas the comparisons of caffeine contents of forest coffee samples of *Harena Buluk* and *Mena* districts showed that the *Harena Buluk* district forest coffee sample has less caffeine content than *Mena* district forest coffee sample (Table 3).

Figure 11. Chromatogram of *Harena Buluk* district forest coffee sample.

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The data showed that the caffeine contents of the coffee samples used in the study were found to be in the range of 31.42 mg/L to 31.98 mg/L (or $1.31 \pm 0.14\%$ to $1.36 \pm 0.16\%$) (Table 3). The data also showed that the amount of caffeine in cultivated coffee sample from *Mena* was maximum (31.98 mg/L or $1.36 \pm 0.16\%$) whereas that of *Gololcha* coffee was the minimum (31.42 mg/L or $1.31 \pm 0.14\%$). The data (Table 3) also showed that the amounts of caffeine in *Mena* district cultivated coffee sample and *Harena Buluk* district cultivated coffee sample have the maximum of caffeine content, 1.36 ± 0.16 % and 1.36 ± 0.02 %**,** respectively, while that of *Gololcha* district coffee have the minimum value of $1.31 \pm 0.14\%$ in the zone.

When compared to coffee grown in other areas of Ethiopia (e.g., *Kaffa* and *Illubabor*), the caffeine contents of the coffee samples obtained from the study areas (of Bale zone) were slightly higher than caffeine contents of *Kaffa* (0.46-2.82 % or average value of 1.18%) and *Illubabor* (0.42-2.90% or average value of 1.10%) (Table 3) [49]. However, the data is in the range of caffeine contents of export standard Ethiopian coffee (i.e., 0.46 to 2.82 % as determined by HPLC) [49,45,52]. Moreover, no significant differences were observed between cultivated and forest coffee samples used in the study.

Table 3. The caffeine concentrations (%) of coffee samples

Coffee sample	Caffeine concentration w/w%	References
Gololcha coffee	1.31 ± 0.14**%**	
Berbere cultivated coffee	1.34 ± 0.21**%**	
Mena cultivated coffee	1.36 ± 0.16**%**	
Mena forest coffee	1.33 ± 0.08**%**	
Harena Buluk cultivated coffee	1.36 ± 0.02**%**	
Harena Buluk forest coffee	1.32 ± 0.03**%**	
Kaffa	Average value 1.18%)	[49]
Illubabor	Average value 1.10%)	[49]

4. Conclusions

Quantification of caffeine contents of coffee beans using HPLC analyses showed that there is no much difference in the caffeine contents of the coffee varieties obtained from districts of Bale zone used in the study. The HPLC analysis was obtained the following results: the sample obtained after the extraction with liquid-liquid extraction had a caffeine content of $1.34 \pm 0.21\%$ for *Berbere* coffee, $1.36 \pm 0.16\%$ for *Mena* cultivated coffee, $1.33 \pm 0.08\%$ for *Mena* forest coffee, $1.31 \pm 0.14\%$ for Gololcha coffee, $1.36 \pm 0.02\%$ for *Harena Buluk* cultivated coffee and $1.32 \pm 0.03\%$ for *Harena Buluk* forest coffee.. The study also showed absence of significant differences in the caffeine levels when forest coffee and cultivated coffee samples are compared to each other. But the caffeine content of Bale coffee is a little bit higher than the caffeine content reported for *Keffa coffee* (1.18%) determined by the same method. Generally, the data showed that the caffeine contents of the coffee samples used in the study are within the limits of reported standards for export Ethiopian *Arabica* coffee that is in the range of 0.46 - 2.82% (as determined by HPLC).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Author contributions

Legesse A. designed and supervised the research and prepared the manuscript; **Mesfine S** conducted the research/experiment, generated and interpreted data**; Israel A**. co-supervised the research work.

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