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M.Sc. Thesis

Influence of Altitude on Caffeine, Chlorogenic and Nicotinic Acid Contents of Arabica Coffee Varieties in Southwest Ethiopia

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INFLUENCE OF ALTITUDE ON CAFFEINE, CHLOROGENIC AND NICOTINIC ACID CONTENTS OF ARABICA COFFEE VARIETIES IN SOUTHWEST ETHIOPIA

M.Sc. THESIS

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Acronym and Abbreviations

ANOVA	Analysis of Variance
CGAs	Chlorogenic Acids
DAD	Diode Array Detector
ECX	Ethiopian Commodity Exchange
GARC	Gera Agricultural Research Sub-Center
HPLC	High-Performance Liquid Chromatography
ICO	International Coffee Organization
In Ga As	Indium Gallium Arsenic
ISO	International Standard Organization
JARC	Jimma Agricultural Research Center
JUCAVM	Jimma University College of Agriculture and Veterinary Medicine
LOD	Limit of Detection
LOQ	Limit of Quantification
M.a.l.s	Meters above level of sea
NIRS	Near-infrared spectroscopy
PLS	Partial Least Squares
Q1	Quality (specialty 1)
QSAE	Quality and Standards Authority of Ethiopia
RP/UV-HPLC	Reversed-Phase UV High-Performance Liquid Chromatography
SAH	S-adenosyl - L-homocysteine
SAM	S-adenosyl – L-methionine
SNNPRS	South Nation and Nationalities People of Regional Stat
TNSCC	Teppi National Specie Coordinating Center

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Abstract

In this study, the influence of altitude on caffeine, chlorogenic and nicotinic acid contents in coffee beans of some Arabica varieties grown in Southwest Ethiopia was investigated. High-performance liquid chromatography with diode array detector was used for the simultaneous determinations of the target analytes. Totally, 12 coffee samples were collected from 4 coffee varieties cultivated in high, mid and low altitudes. The moister content was ranged from (10.13 ± 0.04 to 12.49 ± 0.08) and the pH also ranges (5.36 ± 0.03 to 5.81 ± 0.04). The results of the chemical analysis showed that caffeine content in green coffee varieties was ranged from (12.34 ± 0.076 to 19.89 ± 0.288) mg/g, chlorogenic content was also recorded (27.17 ± 0.382 to 39.18 ± 0.24) mg/g and nicotinic acid was ranged from (7.13± 0.528 to 10.16 ± 0.75) mg/g. The caffeine content in roasted coffee varieties was ranged from (13.93 ± 0.299 to 20.88 ± 0.141) mg/g and chlorogenic content was recorded (8.56 ± 0.021 to 16.21 ± 0.109) mg/g and nicotinic acid ranged from (8.76 ± 0.114 to 6.34 ± 0.031) mg/g. A highly significant decrement was observed in chlorogenic acid content after roasting, as altitude increases the caffeine and chlorogenic acid decreased. In contrary, nicotinic acid increase as altitude increase in green coffee varieties. Two way ANOVA showed the presence of significant interaction between altitude and coffee varieties on chemical constituents of coffee. The caffeine content in green beans was lower as compared to roasted beans; significant degradation of chlorogenic acid was observed after roasting coffee of the same variety.

Keywords: Caffeine, Chlorogenic acid, Nicotinic acid, Coffee Varieties, Altitude,

1. Introduction

1.1. Background of the study

Coffee is the most popular beverage all over the globe its consumption is progressively increasing particularly in the western countries and the U.S.A. due to its distinct taste and aroma [1]. The world production of coffee is based on two commercial species, *Coffea arabica* (Arabica coffee) and *Coffea canephora* (Robusta coffee) which together account for about 98% of world supply [2].

Ethiopia is the single known center of origin and genetic diversity for Arabica coffee (*Coffea arabica* L.) [3]. Coffee grows widely in variable environments in Ethiopia has a variety of characteristics sought in the international market. The quality of coffee can be defined as its organoleptic cup-quality, physical appearances and inherent chemical constituents of a green bean produced [3]. Coffee quality encompasses beans flavor in fragrance, aroma, flavor, sweetness, acidity or overall taste felt by the consumer after drink as well as physical characteristics such as length, width, thickness or weights, shape and color of coffee beans [3].

Acidity is an important feature of coffee. The main acids in green coffee beans are citric, malic, chlorogenic and quinic. During roasting the first three acids decrease while quinic acid increases as a result of the degradation of chlorogenic acids [1].The acidity and sourness of coffee brews (together with aroma and bitterness) have always been recognized as an important attribute of their sensory quality [4].

Coffee quality results from interaction among many different factors including genotype and environment [5]. The quality of coffee is strongly influenced by an environmental factor including altitude, daily temperature fluctuations, amount and distribution of rainfall and the physical and chemical characteristics of the soil [4].

High altitude found to be an optimum growing environment for coffee, in particular for coffee Arabica where the bean quality observed to be enhanced as compared to another growing environment [5]. The diversity of coffee quality due to genotype and environment, result from influences on the biochemical components of the coffee bean accumulated during seed

development [5]. Consumers of high-quality coffee may exercise preference for genotype with the labeling of species (e.g. arabica) or environment of production (usually country) [6]. Numerous studies have been conducted in this field, especially in relation to biochemical constituents such as caffeine, trigonelline, chlorogenic acids (CGAs), sucrose and lipids, considered to influence commercially important sensory traits [5]. In this study, the influence of altitude on chlorogenic acid, nicotinic acid, and caffeine contents with Arabica coffee varieties in southwest Ethiopia was studied.

1.2. Statement of problem

Ethiopia is the origin coffee and produces only coffee Arabica varieties that have different cup qualities [3]. Nowadays, consumers are highly concerned about the quality of the coffee. Coffee quality is usually investigated in three ways chemical analysis, sensorial and physical methods [10]. Chemical analysis is better to get a common objective evaluation as compared to other cup quality evaluation methods. In addition to this, coffee quality is typically evaluated by professional cuppers trained to assess subjective contributions to flavor and aroma. Objective based coffee cup quality evaluation can also be done, by analyzing the levels of "Coffee Taste Descriptor", including caffeine, trigonelline, nicotinic acid, *N*-methyl pyridinium ion, 5-monocaffeoylquinic acid (5-CQA), and 5-hydroxymethylfurfural in the given coffee samples [9]. However, earlier reports demonstrated that the levels these coffee taste descriptors are highly influenced by altitude and coffee varieties [8,10]. Therefore, in this study, the effect of altitudes on the concentration levels of some of these chemical contents like caffeine chlorogenic acid, and nicotinic acid in *Coffea arabica* varieties of Southwest of Ethiopia was studied.

1.3. Objectives

1.3.1. General objective

- To investigate the influence of altitude on physical and main chemical constituents (caffeine, chlorogenic & nicotinic acid contents) of Arabica coffee varieties of Southwest Ethiopia

1.3.2. Specific objectives

- To assess the moisture and pH of coffee green beans *arabica coffee* varieties grown in high, mid and low altitudes of southwest Ethiopia
- To analyze the variation of caffeine, chlorogenic and nicotinic acid contents *of coffee arabica* varieties grown in high, mid and low altitudes in the southwest of Ethiopia,
- To investigate the effect of roasting on the caffeine, chlorogenic and nicotinic acid contents of coffee varieties

1.4. The significance of the study

- The significant of study gives information and knowledge for a researcher in the areas of coffee, commercial growers, exports, concerned body (Ethiopian Coffee and Tea Authority) and coffee quality control in ECX and to farmers.
- The chemical content analysis gives more objective evaluation method and it could use nearby sensorial or cup quality evaluation.
- Use more precise and accurate instrument like HPLC on the chemical analysis and separation technique could provide more appropriate results.

2. Review of Literatures

Coffee (*Coffea arabica* L.) is the second most important agricultural commodity throughout the world and annually, worth up to the US $ 14 billion for producing country [5]. Ethiopia is among the top ten coffee-producing countries in the world and the largest exporter in Africa [9]. It is also the birthplace of Coffee Arabica and its varieties [3]. The country is naturally gifted with a suitable climate and has the potential to produce specialty Arabica coffee beans having a wide range of flavors [5].

Coffee grows in various altitudes ranging from 550 to 2750 meters above sea level. However, *Arabica* is best thrives and produced between altitudes of 1300 and 1800 meters above sea level with annual rainfall ranging from 1500 to 2500 mm, as well as minimum and maximum air temperatures of 15 and 25°C, respectively [3]. However, in some extreme cases, it grows up to 550 meters above sea level (like Bebeka) and in areas where annual rainfall ranges from 1000 to 2000 mm [4].

2.1. Coffee quality

According to the International Organization for Standardization (ISO) (2000), Quality is described as "the ability of a set of inherent characteristics of a product, system or process to fulfill the requirement of customers and other interested parties". These inherent characteristics can also be called "attributes"[11].

More specifically, ISO [10] defined a standard for green coffee quality (ISO 9116 standard) as, it requires several pieces of information, like the geographical and botanic origins of the coffee, the harvest year, the moisture content, the total defects, the proportion of insect-damaged beans and the bean size [11]. These ISO standards define methods of measurements for several of these qualities such as defects, moisture content, bean size, some chemical compounds and preparation of samples to perform cup tasting [12].

Assessment of coffee quality is usually focused on factors that influence utilization of the final product with consumer preferences being assessed in three primary ways: physical (e.g. bean size), sensorial (cup quality) and chemical analysis (key compounds attributed to quality) [5].

According to the definition of quality and standards authority of Ethiopia (QSAE) (2000) a quality is conformance with requirements or fitness for use in which the parties involved in the industry (customer, processor, supplier, etc.) should agree on the requirements and the requirements should be clear to all stakeholders involved in the process [11].

The quality of coffee can be defined as its organoleptic cup-quality, physical appearances and inherent chemical constituents of a green bean produced [4]. Coffee quality is of critical importance to the coffee industry. Production and supply of coffee with excellent quality appear more crucial than ever before for coffee exporting countries [3]. Quality coffee is a product that has desirable characteristics such as clean raw and roasted appearance, attractive aroma, and good cup taste. Coffee quality encompasses beans flavor in fragrance, aroma, flavor, sweetness, acidity or overall taste felt by the consumer after drink as well as physical characteristics such as length, width, thickness or weights, shape and color of coffee beans [3].

The quality of coffee is strongly influenced by an environmental factor. Altitude, daily temperature fluctuations, amount and distribution of rainfall and the physical and chemical characteristics of the coffee growing area soil [5]. The production of good quality coffee beans in specific areas characterized by their climatic conditions clearly showed that climate is one of the important factors in determining the quality of the coffee beverage. According to Sualeh [3], genetic origins greatly influenced coffee quality [3]. Coffee quality also depends upon the genetic make-up genotype/variety and the environmental conditions in which it is grown; this fevers genes of chemical compounds that behave as aroma precursors expressed during the coffee roasting process.

2.2. Physical quality

The International Coffee Organization (ICO, 2001) implemented a Coffee Quality Improvement Program (CQIP) with a recommendation to exporting countries. It is not recommended to export coffee with the characteristics having foreign material of non coffee origin; foreign materials of non bean origin, such as pieces of parchment or husk; abnormal beans for shape regularity or integrity; abnormal beans for visual appearance, such as black beans; abnormal beans for taste of the cup after proper roasting and brewing [10].

2.2.1. Moisture content

The moisture content of coffee bean is an important attribute and indicator of quality. The high moisture content of the beans is a loose sensorial defect. If coffee beans are too wet (above12.5 % moisture), can easily develop mold during storage [9]. In addition, if the beans are too dry (below 8 % moisture) they lose flavor. The moisture content can influence the way coffee roast and the loss of weight during roasting. Green coffee with low moisture contents tends to roast faster than those with high moisture content [10].

2.2.2. Bean size

Price is related to bean size and small beans of the same variety bring lower prices; however, larger beans do not necessarily taste better; ideally, roasting should be processed with uniform beans [10]. When uneven coffee beans are roasted, the smallest once tend to burn or over roasted while the largest is under roasted, which affects both the visual appearance of coffee beans and cup quality [11]. Arabica coffee beans have sizes ranging between 18-22 g and 12-15 g per 100 beans respectively. As a positive factor, shade increases and unifies bean size by reducing the solar radiance in the coffee canopy and results in lower air temperature and slowing down of coffee maturation [10].

2.3. Chemical attributes

The chemistry of coffee quality is highly complex with a wide range of compounds that change during fruit development. A few key components, such as caffeine, trigonelline, lipids, sucrose and chlorogenic acids (CGAs), are regarded as significant in influencing coffee quality. These components either stay stable and act as flavor attributes reaching the coffee brew or are degraded during roasting accounting for flavor precursors [11]. Table 1 presents the main components of coffee bean.

2.3.1. Caffeine

Caffeine, an alkaloid of the methylxanthine family, is a naturally occurring in the leaves, seeds or fruits of over 63 plants species [6]. The most commonly known sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves [6]. Pure caffeine occurs as odorless, white, fleecy masses,

glistening needles of powder. Its molecular weight is 194.19 g, melting point is 236°C, point at which caffeine sublimes is 178°C at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mmHg at 178°C, solubility in water is 2.17%, vapor density 6.7. [9, 13]. Nowadays, caffeine is the world's most famous behaviorally active drug and is consumed primarily from coffee [11]. Arabica coffee relatively contains lower caffeine content as compared to Robusta, with 0.6-1.8% and 1.2-4.0%, respectively [12].

Table 1: The main components in coffee beans, their flavor attribute and changes after roasting in cup test [11].

Component	**Flavor attribute**	**Influence of roasting**
Caffeine	Perceived strength, body and bitterness	Stable
Chlorogenic acids	Acidity, astringency & bitterness	59.7- 98% degraded
Trigonelline	Overall aromatic perception, bitterness	overall aromatic perception, bitterness 60-90% degraded
Sucrose	Flavor precursor	Disappear
Lipids	Flavor carriers, texture, and mouthfeel	Stable

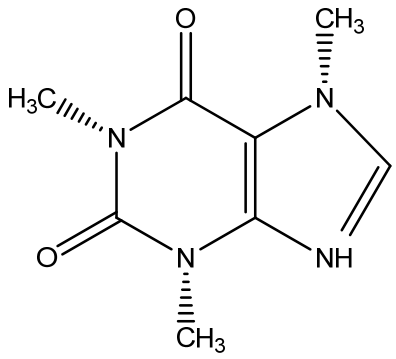

Figure 1: The chemical structure of caffeine (1, 3, 7-trimethylxanthine)[9].

2.3.2. Caffeine biosynthesis pathway

Caffeine biosynthetic pathway involves four steps consisting of three methylations and one nucleoside reactions, Figure 2, [13]. The xanthine skeleton of caffeine is derived from purine nucleotides. The initial step in caffeine biosynthesis is the methylation of xanthosine by a SAM (S-adenosyl – L-methionine) dependent N-methyltransferase. Several N-methyltransferases with different substrate specificities contribute to the conversion of xanthosine to caffeine. Steps 1 & 2 are catalyzed by xanthosine N-methyltransferase.

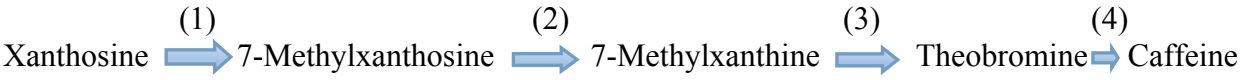

Figure 2: The major biosynthetic pathways of caffeine from xanthosine [13].

Where (1) 7-methylxanthosine synthase (xanthosine N-methyltransferase); (2) N methylnucleosidase; (3) Theobromine synthase (monomethylxanthine N-methyltransferase); (4) Caffeine synthase (dimethylxanthine N-methyltransferase); (3–4) dual-functional caffeine synthase.

2.3.3. Chlorogenic acids

A Chlorogenic acid (CGA) is another main component of Coffee [14]. CGAs are a group of phenolic compounds that show multiple attributes. During roasting, a large percentage of the CGAs degrade to form caffeic acid, lactones, and other phenol derivatives through Maillard and Strecker's reactions, which result in increased bitterness, astringency and aroma [15].

CGA is being an ester of trans-cinnamic acids, such as caffeic acid, ferulic and p-coumaric acids with (-) quinic acid [14]. CGAs are thermally unstable and in Arabica coffee the loss of CGAs after light roasting and after very dark roasting of beans corresponds to 60.9% and 96.5%, respectively, while in Robusta this loss corresponds to 59.7% to 98%, respectively [15].

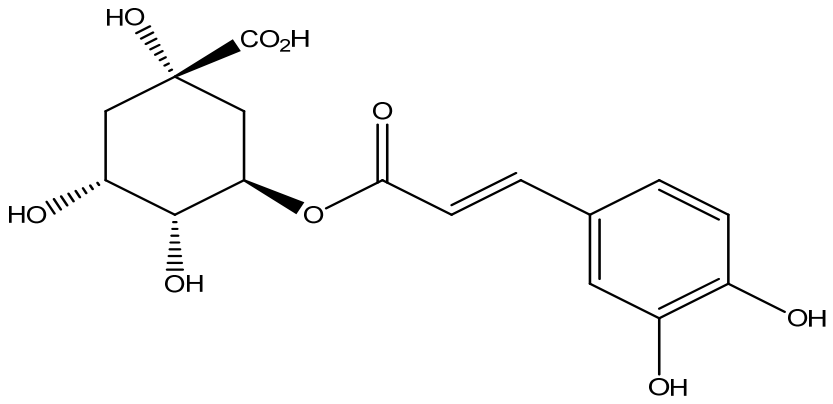

Figure 3: The chemical structure of chlorogenic acid [14].

In general, the percentage of CGA for regular green coffee beans on dry matter basis varies from 4 to 8.4% for Arabica and 7 to 14.4% for Robusta with some hybrids presenting intermediate levels [11].

They play a great role in the formation of pigments, taste, and flavor of coffee beans, which determine the quality and acceptance of the beverages [14]. The final acidity of the beverages and the formation of lactones and other phenol derivatives responsible for flavor and aroma are also contributed by CGA [15].

They are believed to have antioxidant properties which are suggested to play an important role in protecting food, cells and any organ from oxidative degenerative [10]. The report indicates that diet rich in CGA compounds play a great role in preventing various diseases associated with oxidative stress such as cancer, cardiovascular, aging and neurodegenerative disease[10]. Although most CGAs are lost by roasting, a sharp increase in total antioxidant activity was reported in the coffee beverage which may indicate that the breakdown products of CGAs are antioxidants [15].

2.3.4. Nicotinic acid

Trigonelline (N-methyl-nicotinate) was named after the leguminous plant *Trigonella foenumgraecum* L. (Fenugreek) from which the compound was first isolated and characterized [17]. The chemical formula for trigonelline is $C_7H_7NO_2$ and the chemical structure is as shown in Figure 4.

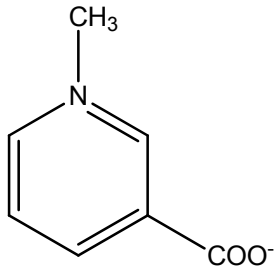

Figure 4: The chemical structure of Trigonelline acid [17].

It is a vitamin B6 derivative having a low, bitter taste in comparison to caffeine and probably the most substantial element which contributes to undue bitterness in coffee and is 100 percent water soluble. It is also known as Coffearin, Coffearine, and Gynesine [17].

Trigonelline comprises about 2% of the dry weight of green coffee but does not resist roasting temperature and thus breakdown to nicotinic acid, pyridine and other volatile compounds [16]. It is also found in various plants and in some animal species including sea urchins and jellyfish. It also appears in mammalian urine after administration of nicotinic acid. It is one of the responsible components for bitter tasting in the coffee brew. It is also thermally unstable and converted nicotinic acid and to certain flavor compounds during roasting [16].

2.4. Sensory evaluation

Flavor or cup quality is the primary standard in the worldwide coffee trade. Having an even bean size and good appearance without defective beans does not always result in good coffee flavor. For this reason, it is important to judge the flavor quality in relation to the final utilization, such as roasted, liquid canned coffee, etc [11].

Cup quality analysis aims to evaluate coffee flavor with a group of trained people in an objective and reproducible way to create a profile using established terminology, such as aroma, flavor, body, and acidity, which has been established by the International Coffee Organization (ICO) [11]. Coffee flavor is very sensitive to genetics and environment changes [11]. Acidity, for

example, ranges dramatically in different washed Arabica, while Robusta has been described as low or no acidity at all with coarse liquor, harsh and cereal notes and thick body.

Ultimately, Arabica coffee is sold as blends with varying proportions of Robusta coffee, but Robusta coffees are seldom used alone [17]. The same genotype planted in a different environment may vary greatly in quality [11]. For example, increasing positive attributes (appearance and preference) together with decreasing negative attributes (bitterness and astringency) was found in shade-grown coffee [17]. Coffee quality, especially liquor or cup quality, determine both the relative price and usefulness of a given quantity of coffee [10]. Cup quality often referred to as drinking quality or liquor quality, is an important attribute of coffee and acts as a yardstick for price determination [10].

2.5. Health benefit of coffee

Coffee has antioxidant properties, reduces the incidence of cancer, diabetes and liver disease, protects against Parkinson's disease and reduces mortality risk [17]. Green coffee bean extract shows a hypotensive effect in rats and reduces visceral fat and body weight. These properties are connected with bioactive compounds, not only chlorogenic acids and their derivatives but also caffeine, theophylline and theobromine, cafestol, kahweol, tocopherols and Trigonelline [18].

Coffee increases the level of alertness, improves short-term memory and permits better use of the prefrontal cerebral cortex. Coffee reduces the risk of hepatic cirrhosis and prevents the formation of gallstones [18]. Coffee provides protection against degenerative brain diseases like Alzheimer's and Parkinson's. Coffee combats caries and has anti-inflammatory properties. Coffee has a moderate slimming effect and improves performance in sports [19]. Coffee helps to alleviate asthma symptoms and helps to calm hyperactive children. Coffee has antioxidant and antitoxic properties at the cellular level [20].

3. Materials and Methods

3.1. Description of the Study Areas

The study was carried out from November 2017 to August 2018. Coffee bean samples were collected from November to December 2017 from three different altitudes: Gera Agricultural Research Sub-Center (altitude 1940-1960 m) and Jimma Agricultural Research Center (altitude 1750-1760 m) in Jimma Zone, Oromiya Regional State as well as from Teppi National Spies Coordination Center (altitude:-1100-1200 m) which is located in south nations, nationality people (SNNPRS) Regional State, all from Southwest of Ethiopia. Geographic location and biophysical data of the study areas are presented in Table 2.

Table 2: Geographic location and biophysical data of the study areas in southwest Ethiopia

Biophysical data	Highland (Gera)	Midland (Jimma)	Lowland (Teppi)
Altitude (a.s.l)	1940 – 1960 m	1750 - 1775 m	1100 - 1200 m
Mean temperature	19°C - 25°C	20.5°- 27°C	25°C - 30°C
Rain fall	1880 - 2080 mm	1525 - 2000 mm	800 - 2000 mm
Latitude/Longitude	6^{o}30'N and 32 ^{o}15'E	7^{o}40'N and 36^{o} 50' E	9^{o}08'N and 37^{o}13'E

m.a.s.l is meters above sea level

3.2. Chemicals and reagents

Chemicals and reagents used were analytical grades and solvents were HPLC grades. Analytical grade standards of chlorogenic acid (99.9%), caffeine (99.9%), and nicotinic acid (99.9%) were obtained from Sigma Aldrich ((St. Louis, MO, USA). HPLC grades of acetonitrile (99.9%) and Glacial acetic acid (99.9%) were obtained from Carlo Erba reagents S.A.S (Mumbai, India). Ultra-pure water was also used throughout the work dilation and other purposes.

3.3. Instrumentation and apparatus

Instruments such as Agilent HPLC quaternary solvent system equipped with the Flexar Solvent manager, DAD, with LC Chem. Station), Digital analytical balance (Mettler Toledo -AL204-IC,

America), Refrigerator, Roasted coffee machine (Jos. Hansen & Soehne, BR2-6, Germany), grinding machine (Stawar-Hamburg-70, Germany), Raw coffee grinding machine (M 20 S000, Germany) and Gallonkamp water bath (DQ.O59). Apparatus like volumetric flasks, measuring cylinders and pipettes were used.

3.4. Standard solutions

An individual stock solution containing 1000 mg/L of nicotinic acid, caffeine and CGA was prepared acetonitrile. Then, an intermediate solution containing 100 mg/L of each standard solution was also prepared in acetonitrile. Working solutions were then prepared by diluting the intermediate solution. Serial dilution was used for the preparation of concentrations of standards for constructing the external calibration curves, at five concentration points, 1, 2.5, 5, 7.5, 10 mg/L for the three analytes; nicotinic acid, caffeine, and chlorogenic acid. Stock and intermediate standard solutions were kept in the refrigerator below 4 °C when not used.

3.5. Sampling and sample collection

Jimma Agricultural Research Center (JARC) has given different codes and/or names for different coffee varieties grown in southwest Ethiopia. These codes and/or names include 741, 744, 7440, 7454, 74112, 74110, 74140, 74148, 74165, 8136, 7416, 7514, Geisha, Catimor -19, and Catimor-21, which are naturally (pure line) grown in semi-forest of Southwest Ethiopia [23, 24]. From these varieties, 4 coffee varieties including 74110, 7454, 7440 and 74112 were purposively sampled from three farmlands of the agriculture research center and which are located in different altitudes. The three sampling sites with their altitudes in parenthesis are Gera research sub-center (Highland), Jimma agricultural research center (Midland) and Teppi national spices coordinating center (lowland). Coffee samples were collected based on the reported by Sultana and coworkers [3]. Accordingly, 6 kg red ripe coffee cherries (beans) were collected (handpicked) from 30 randomly selected coffee trees in each farmland. Before pulping, immature cherries and other unnecessary materials were removed. The selected coffee varieties are a pure line (not hybrids) and grow in three different altitudes (Highland, Midland, and lowland). They are also characterized by wide adaptation (covers large areas) and have a different flavor; tastes and aroma [23].

3.5.1. Roasting and grinding

Roasting and grinding of coffee samples were adopted from Sultan and coworkers [3]. Accordingly, the roasting machine was heated first at about 200°C. Then, 100 g green coffee bean sample was added and roasted for 8 min on average. Afterward, the roasted sample was transferred to the cooling tray and cold air was blown for rapid cooling. Subsequently, after the loose silver skins were removed the sample was ground to medium seized ground.

3.6. Method of physical and chemical analysis

3.6.1. Moisture content

Moisture content was determined by AOAC procedure [30]. Accordingly, first fully matured coffee samples were pulped using single disc manual pulpier of beans from the skin and pulp. Then, 100 beans were taken and their weight was recorded. The green coffee samples were dried in an oven at 105 ºC for 24 h until constant weight was obtained, and then, the weight of the dried beans was measured to calculate the percentage of moisture content.

3.6.2. pH

The pH was measured using the procedure reported by Natalina and co-workers [24] at 25°C, after calibrating the pH meter at pH 4.0 and 7.0. To measure the pH, 10 g of ground green coffee was mixed with 200 mL water. Then, after boiling for 5 min and then cooling at room temperature, the content was filtered using Whatman No. 1 filter paper. , Finally, the pH of the filtrate was measured.

3.7. Sample preparation for HPLC analysis

For HPLC analysis coffee samples were prepared based on the method reported by Alves, et al [7]. Accordingly, 0.5 g of roasted or raw ground coffee sample was mixed 30 mL of a solution of water: Acetonitrile (95:5, *v/v*) and then, allowed to boil in a water bath in 80 $^{\circ}C$ for 10 min. Then, after filtering with Whitman No.1 filter paper, 5 mL was taken into 25 mL volumetric flask and filled to the mark with the water: acetonitrile (95:5 *v/v*) solution. Finally, about 2 mL of the

extract was filtered through syringe filter into 2 mL autosampler vial to inject 20 µL into the HPLC-DAD system.

3.7.1. Chromatographic conditions

Spheris orb ODS-1 column (150 mm × 4.6 mm; 5 µm) was used for chromatographic separations of the analytes. Other chromatographic conditions were adopted from Alves and co-workers report [7]. Accordingly, separation was done using binary solvents, containing acetic acid:H_2O (5:95 *v/v*) (solvent A) and acetonitrile (Solvent B) in the ration of (95:5 *v/v)* in isocratic elution mode, 30 ºC column temperature and.1.0 mL/min elution flow rate. The target analytes were analyzed at various wavelengths, nicotinic acid (260 nm), caffeine (272 nm) and chlorogenic acid (320 nm).

3.7.2. Method validation

The method was validated by determining, the Accuracy and Precision, Recovery, limits of detection and limits of quantification [5]. In this study, the precision of results was evaluated by the standard deviation of replicates analysis. Preparing duplicate of samples & each sample was injected in duplicates. Total of four replicate (n = 4) measurements. Similarly, the accuracy of the method was assessed percentage recovery study. LOD and LOQ were determined as 3 and 10 times signal-to-noise ratio (S/N) of the chromatogram [29].

3.7.3. Accuracy and precision

In this study, the precision of the results was evaluated by the standard deviation of replicate analysis. The replicate measurements were done by preparing duplicate samples and injecting each sample in duplicates to have the total of four replicate (n = 4) measurements [27, 28]. Similarly, the accuracy of the method was assessed percentage recovery study. Recovery study was conducted by spiking known concentrations of the target analytes onto the coffee sample. Percentage recovery was then determined as the ratio of the obtained increase concentrations of the analytes after spiking coffee samples to the known concentrations of the standard solution added to the sample multiplied by 100 [30].

$$\% \text{ Recovery} = (Cs – C)/S * 100$$

Where S is concentration the analyte added to the sample, Cs is a concentration in the spiked sample, C is a concentration in the unspiked sample

3.7.4. Determination of detection and quantification limits

Limit of detection (LOD) is the lowest concentration of the analyte that can be detected by a given method. Likewise, limit of quantification (determination), LOQ, is the lowest concentration of the analyte that can be measured in the sample matrix at an acceptable level of precision and accuracy [28-29]. In this study, LOD and LOQ were determined as 3 and 10 times signal-to-noise ratio (S/N) of the chromatogram [29].

3. 8. Statistical analysis

Various descriptive statistical procedures (mean, SD, as well as % RSD), and two-way ANOVA were utilized for reporting and comparing the obtained results. Statistical analyses were done by SAS 9.0.Software. In this study, all measurements were reported as the mean ± SD of a replicate of measurements.

4. Results and Discussion

4.1. Validation of the method

The HPLC method was validated by constructing the external calibration curves, at five concentration points, 1, 2.5, 5, 7.5, 10 mg/L for the three analytes; nicotinic acid, caffeine, and chlorogenic acid. Each concentration level was prepared in duplicates, and each solution was also injected duplicates. The calibration curves which were established by plotting peak areas as a function of concentrations of the standards showed good linearity in the employed concentrations range (1.0 – 10 mg/L) with a coefficient of determination (r^2) 0.996, 0.997 and 0.998 for nicotinic acid, chlorogenic acid, and caffeine, respectively.

LOD and LOQ of the method were determined as the lowest concentration of the analytes that can give signal equals to signal 3 and 10 times S/N, respectively. The observed LOD and LOQ were 0.08 and 0.23; 0.09 and 0.26; as well as 0.07 and 0.20 for nicotinic acid, chlorogenic acid, and caffeine, respectively.

The accuracy of the method was also evaluated through a recovery study, by spiking raw (green) and roasted coffee samples with two concentration levels of the analytes. The observed results are displayed in Table 3.

Table 3: Recovery values (Mean % R ± SD, n = 4) of caffeine, chlorogenic acid, and nicotinic acid.

		Recovery, %		
Sample	**Spiked in mg/l**	**Nicotinic acid**	**Chlorogenic acid**	**Caffeine**
Green coffee	5	100 ± 3.40	101 ± 0.30	95 ± 0.80
Roasted coffee	5	99 ± 0.12	95 ± 0.60	88 ± 2.10

As can be seen, the obtained recoveries at the two concentration levels in both sample types from 88 – 102%, indicating the accuracy of the method for the analysis of the target analytes from green and roasted coffee samples.

4.2. Moisture content and pH of coffee varieties

4.2. 1. Moisture content

Moisture content is one of the most important quality parameters of green coffee beans. Most coffee importing and exporting countries consider moisture content of green coffee beans as one of the quality parameters. The safety range for moisture content of coffee is 8.0–12.5%, based on the fresh matter [31]. In this study, the moisture contents of coffee varieties, which were cultivated in different altitudes, were evaluated (Table 4).

Table 4 : Moisture contents of coffee varieties with altitudes

Coffee varieties	**Altitude**			
	Highland	**Midland**	**Lowland**	**Mean**
74110	12.49 ± 0.08	12.34 ± 0.10	11.37 ± 0.02	12.08 ± 0.07
7454	12.17 ± 0.13	12.42 ± 0.08	11.26 ± 0.09	11.97 ± 0.08
7440	12.47 ± 0.38	12.40 ± 0.05	10.56 ± 0.01	11.89 ± 0.15
74112	12.25 ± 0.08	12.15 ± 0.09	10.13 ± 0.05	11.53 ± 0.04
Mean	12.32 ± 0.19	12.40 ± 0.04	10.83 ± 0.09	11.87 ± 0.09
CV	2.89			

It was observed moisture contents of green coffee varieties were varied from 12.17 ± 0.127 to 12.49 ± 0.08; 12.15 ± 0.09 to 12.42 ± 0.08 and 10.13 ± 0.045 to 11.37 ± 0.017 for Highland, Midland, and lowland, respectively. A statistical test, two ways ANOVA ($P < 0.05$) showed the presence of significant interaction between coffee varieties and altitudes in terms of their moister contents (Appendix Table 1). The highest moisture content was observed in 74110 coffee varieties which were collected from Highland. The lowest moisture content was recorded for 74112 coffee variety collected from lowland. Generally, the moisture contents of coffee varieties exhibited decrements with altitudes. Highland coffee varieties have exhibited the higher moisture contents, than the similar coffee varieties from Midland and lowland, this could be attributed to the presence of relatively high temperate and wind flow rate at Midland and lowland. The moisture contents of all the studied coffee varieties were in the recommended safe range, i.e., below 12.50%, for importing or exporting coffee beans [31].

Moisture content outside the safety range impairs the bean quality and safety. Beans with a moisture content above 12.5% are not allowed to be shipped and traded since it facilitates fungal growth and mycotoxin production (e.g., ochratoxin A) that are risks to human health [31]. On the other hand, moisture content below 8% also causes shrunken beans and an unwanted appearance.

4.2.2. pH of coffee varieties

Table 5 shows pH of green coffee varieties. The pH of green bean coffee varieties was varied from 5.36 ± 0.04 - 5.81 ± 0.04. A statistical test, two ways ANOVA ($P < 0.05$) showed that there the existence of significant interaction between coffee varieties and altitude in pH contents of green beans (Appendix Table 2). The highest pH was observed green beans of variety 7440, which was collected from the highland. Generally for all coffee varieties the pH of highland > Midland > lowland coffee.

Table 5: pH of green coffee varieties

Coffee	**Altitude**			
varieties	**Highland**	**Midland**	**Lowland**	**Mean**
74110	5.80 ± 0.04	5.78 ± 0.03	5.69 ± 0.01	5.75 ± 0.09
7454	5.74 ± 0.09	5.68 ± 0.09	5.36 ± 0.04	5.59 ± 0.09
7440	5.81 ± 0.04	5.58 ± 0.14	5.56 ± 0.13	5.65 ± 0.06
74112	5.66 ± 0.13	5.63 ± 0.05	5.62 ± 0.05	5.63 ± 0.19
Mean	5.73 ± 0.06	5.67 ± 0.06	5.57 ± 0.06	5.66 ± 0.14
CV	0.85			

4.3. Caffeine, chlorogenic acid and nicotinic acid contents of green coffee varieties

Separation and determination of caffeine, chlorogenic acid and nicotinic acid were performed using HPLC-DAD by monitoring at various wavelengths including 260 nm (for nicotinic acid), 272 nm (for caffeine) and 320 nm (for chlorogenic acid). Representative chromatograms of the target analytes, obtained from one representative green coffee bean sample are presented in Figure 5.

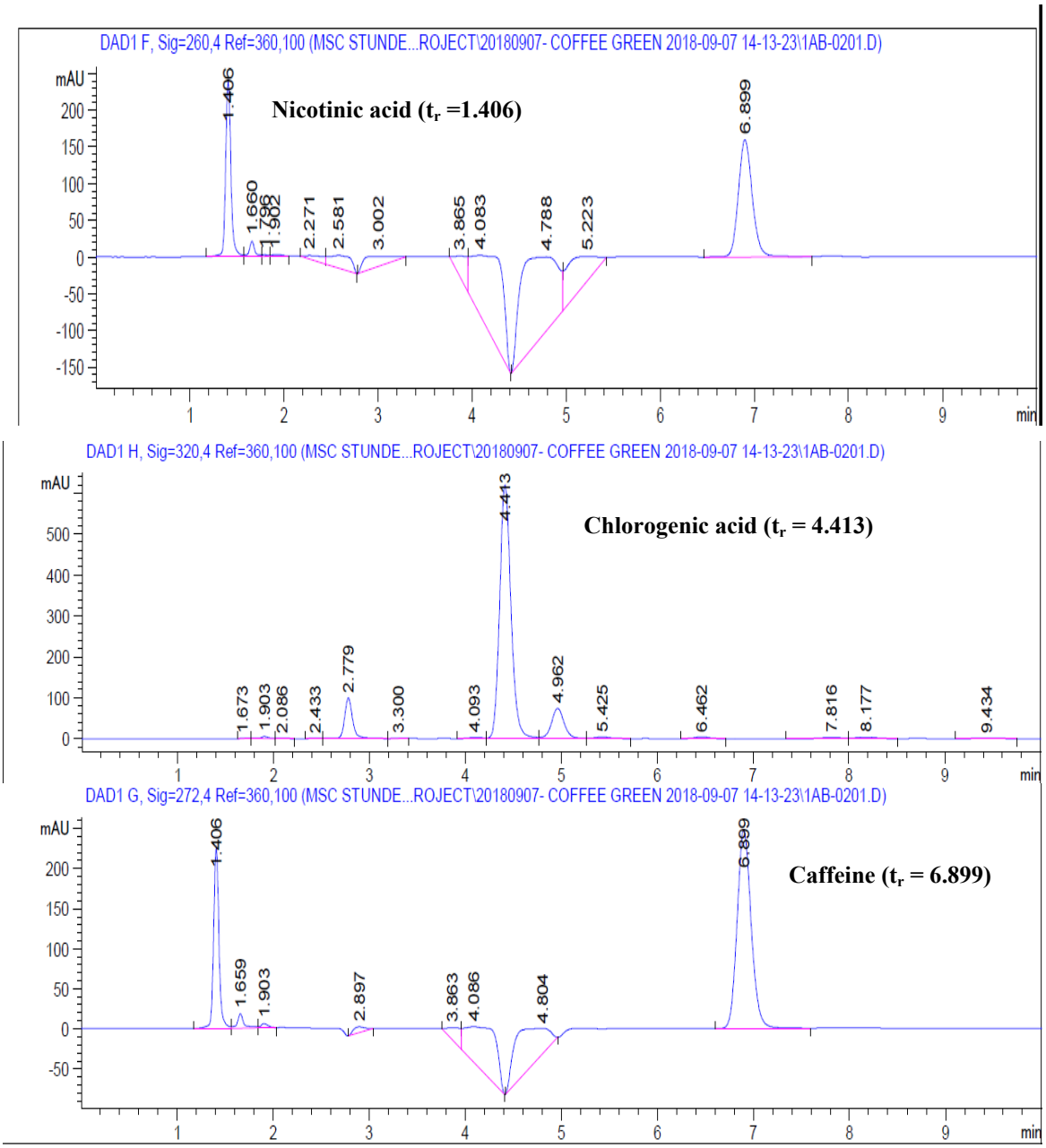

Figure 5: Representative chromatograms of nicotinic acid, caffeine and chlorogenic acid obtained from green coffee variety 74110.

4.3.1. Caffeine

Table 6 shows concentrations of caffeine in green coffee beans varieties collected from different altitudes. The caffeine content of green beans varieties were varied from 15.14 – 19.89 mg/g (lowland); 13.83 - 16.07 mg/g (Midland) and 12.34 – 13.58 mg/g (Highland).

The lowest (12.34 ± 0.076) mg/g and highest (19.89 ± 0.288) mg/g caffeine concentrations were observed in coffee varieties 47110 (at Highland) and 7454 (at lowland), respectively.

Table 6 : Concentrations (mg/g) caffeine in green coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4).

Coffee varieties	**Altitude**			
	Highland	**Midland**	**Lowland**	**Mean**
74110	12.34 ± 0.076	14.51 ± 0.082	15.14 ± 0.392	14.00 ± 1.271
7454	13.58 ± 0.146	16.07 ± 0.575	19.89 ± 0.288	16.5 ± 2.732
7440	13.10 ± 0.445	13.83 ± 0.537	15.60 ± 0.955	13.84 ± 1.441
74112	13.30 ± 0.770	13.87 ± 0.532	15.67 ± 0.331	14.28 ± 1.175
Mean	13.08 ± 0.625	14.48 ± 1.340	16.42 ± 2.185	14.66 ± 2.033
CV	3.53			

Two way ANOVA results indicated caffeine content of green coffee beans is significantly affected by coffee varieties and altitude ($P < 0.05$) (Appendix Table 3). For all coffee varieties, the caffeine content of the lowland > Midland > highland coffees. However, the obtained caffeine contents ranged from 12.34 to 19.89 mg/g. The results are in agreement with previous studies by Kassaye et al [36] and Ky et al [37] and Bekele [38].

4.3.2. Chlorogenic acid

Chlorogenic acid is another important chemical for verification of coffee varieties. The influence of altitude on its concentrations in green coffee beans was investigated (Table 7). The concentrations of chlorogenic acid were ranged from 27.17 ± 0.382 - 34.56 ± 2.087 mg/g (in highland); 30.72 ± 0.643 –34.62 ± 0.065 mg/g (in Midland); and 32.06 ± 1.755 - 39.18 ± 0.240 mg/g (in lowland) coffee varieties. The lowest and highest concentrations of the compound were both observed in the same variety (7454) collected from highland and lowland, respectively. Coffee variety, 7454 demonstrated variations of the concentrations of chlorogenic acid with altitude, whereas the remaining three varieties exhibited almost similar concentrations in all altitudes.

Table 7: Concentrations (mg/g) chlorogenic acid in green coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4).

Coffee varieties	Altitude			
	Highland	**Midland**	**Lowland**	**Mean**
74110	29.72 ± 0.711	31.34 ±1.666	32.16 ± 2.111	32.12 ± 0.112
7454	27.17 ± 0.382	33.85 ± 2.258	39.18 ± 0.240	35.21 ± 1.214
7440	30.40 ± 1.439	30.72 ± 0.643	32.06 ± 1.755	31.06 ± 1.441
74112	34.56 ± 2.087	34.62 ± 0.065	33.62 ± 0.876	34.27 ± 1.275
Mean	31.50 ± 3.052	33.15 ± 2.182	33.23 ± 3.946	32.63 ± 3.179
CV	3.57			

The obtained results were similar to the studies reported by Kassaye and co-workers [36] and Ky and co-workers [37] and Bekele [38]. However, these values showed the effects of altitude and coffee varieties on the concentration of chlorogenic acids. That means the concentration of chlorogenic is influenced by both variations of the altitudes and coffee variety. Generally, according to the finding of this study, chlorogenic contents of green coffee beans follow similar trends as that of caffeine contents.

4.3.3 Nicotinic Acid

The observed concentrations of nicotinic acid in green coffee varieties are presented in Table 8. The nicotinic acid contents of green coffee varieties were varied from 7.13 ± 0.528 - 8.25 ± 0.271 mg/g; 7.38 ± 0.963 - 8.34 ± 1.572 mg/g; and 8.08 ± 0.162 - 10.16 ± 0.752 mg/g in lowland, Midland and highland coffee varieties. The smallest and highest nicotinic acid contents were observed in the same coffee variety, 7454, collected from lowland and highland, respectively. Generally, in contrast to caffeine and chlorogenic acid, lowland coffee varieties contained the lowest concentrations of nicotinic acid than coffee varieties collected from Midland and lowland. Statistical evaluation using two way ANOVA ($p < 0.05$) revealed the presence of interaction between coffee varieties and altitude on nicotinic acid of contents green coffee beans (Appendix Table 5).

Generally, in contrast to caffeine and chlorogenic acid, lowland coffee varieties contained the highest concentrations of nicotinic acid than coffee varieties collected from Midland and lowland.

Table 8 : Concentrations (mg/g) nicotinic acid in green coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4).

Coffee	**Altitude**			
Varieties	**Highland**	**Midland**	**Lowland**	**Mean**
74110	8.16 ± 0.041	8.15 ± 0.194	7.59 ± 0.683	7.96 ± 0.684
7454	10.16 ± 0.752	8.34 ± 1.572	8.25 ± 0.271	8.91 ± 0.051
7440	8.53 ± 0.454	7.38 ± 0.963	7.13 ± 0.528	7.68 ± 0.127
74112	8.08 ± 0.162	7.87 ± 0.494	7.54 ± 0.713	7.83 ± 0.577
Mean	8.73 ± 1.118	7.91 ± 0.457	7.63 ± 0.051	8.09 ± 0.044
CV	5.17			

4.4. Caffeine, chlorogenic acid and nicotinic acid contents of roasted coffee varieties

4.4.1 Caffeine

The observed concentrations of caffeine in roasted coffee varieties collected from different altitudes are presented in Table 9. The caffeine content of roasted beans varieties were varied from 19.21 ± 0.662 - 20.88 ± 0.141; 14.21 ± 0.714 - 18.62 ± 0.478; and 13.93 ± 0.299 -17.22 ± 0.762 mg/g in coffee verities of lowland; Midland and Highland, respectively. The lowest (13.93 ± 0.299) and highest (20.88 ± 0.141) mg/g caffeine concentrations were observed in coffee varieties 74112 (from the highland) and 7454 (from the lowland), respectively. For all coffee varieties, the concentrations of caffeine were reduced when the altitude increases and thus, the highest caffeine concentration was observed for each variety of coffee collected from lowland. Two way ANOVA results ($P < 0.05$) also confirmed the presence of interaction between coffee varieties and altitude on caffeine content in roasted beans (Appendix Table 6).

Table 9: Concentrations (mg/g) caffeine in roasted coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4).

Coffee varieties	Altitude			
	Highland	**Midland**	**Lowland**	**Mean**
74110	17.22 ± 0.762	18.62 ± 0.478	20.28 ± 0.637	18.71 ± 1.388
7454	16.92 ± 1.378	17.72 ± 1.462	20.88 ± 0.141	18.51 ± 2.071
7440	14.49 ± 1.697	16.55 ± 0.398	20.54 ± 0.091	17.19 ± 2.776
74112	13.93 ± 0.299	14.21 ± 0.714	19.21 ± 0.662	15.78 ± 2.561
Mean	15.99 ± 2.196	16.43 ± 1.582	20.23 ± 0.044	17.55 ± 2.489
CV	4.23			

Generally, the caffeine contents of the studied roasted coffee varieties ranged from 13.93 - 20.88 mg/g. For each variety, the observed concentration of caffeine in the roasted coffee was higher than the corresponding green coffee beans. Literature also indicated that roasted coffee has higher caffeine content than green coffee beans [41, 42].

4.4.2. Chlorogenic acid

The recorded chlorogenic acid concentrations in roasted coffee varieties are given in Table 10. The concentrations of chlorogenic acid were ranged from 8.56 ± 0.021 -11.48 ± 0.462 mg/g; 12.51 ± 0.211 - 15.25 ± 1.198 mg/g; and 14.19 ± 0.321 - 16.21 ± 0.109 mg/g for coffee varieties collected from Highland, Midland and lowland, respectively. For every coffee variety, the lowest and highest concentrations of the analytes were observed in highland and lowland, respectively. Two way ANOVA ($P < 0.05$) indicated the interaction between roasted coffee varieties and altitude on chlorogenic acid contents (Appendix Table 7). The chlorogenic acid content of roasted beans of varieties 7440 higher ($P < 0.05$) (16.21 ± 0.109) compared mid-altitude (12.51 ± 0.211) mg/g and high altitude (8.77 ± 0.385) mg/g ,(Table 11). The coffee variety 7454 (8.56 ± 0.021) mg/g at high altitude has the lowest chlorogenic acid content than other varieties.

Generally, the chlorogenic acid contents of the studied coffee samples ranged from 8.56 to 16.21 mg/g. As can be seen, irrespective of the variations of the altitude, roasted coffee varieties have

lower chlorogenic contents than green coffee beans, indicating the possibility of transformation of the compound into other compounds [14].

Table 10: Concentrations (mg/g) chlorogenic acid in roasted coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4).

Coffee	**Altitude**			
varieties	**Highland**	**Midland**	**Lowland**	**Mean**
74110	11.48 ± 0.462	13.16 ± 0.438	14.82 ± 1.506	13.15 ± 1.668
7454	8.56 ± 0.021	15.25 ± 1.198	15.27 ± 0.017	13.03 ± 2.913
7440	8.77± 0.385	12.51 ± 0.211	16.21 ± 0.109	12.50 ± 3.183
74112	12.59 ± 0.211	13.61 ± 0.363	14.19 ± 0.321	13.46 ± 0.804
Mean	13.03 ± 3.362	12.02 ± 2.492	14.05 ± 1.319	13.03 ± 3.362
CV	4.83			

It was also reported that chlorogenic acid is thermally unstable can be lost 60.9% and 96.5% after light and dark roasting Arabica coffee respectively [15]. The presence of variation of concentrations of chlorogenic acid with the variation of altitudes as well as coffee varieties was also reported in the literature [5, 36].

4.4.3. Nicotinic Acid

The observed concentrations of nicotinic acid in roasted coffee varieties are given in Table 11. The nicotinic acid contents of green coffee varieties were ranged from 6.55 ± 0.332 - 7.55 ± 0.345, 6.66 ± 0.035 - 7.98 ± 0.696 mg/g; and 7.92 ± 0.187 - 8.76 ± 0.114 mg/g in lowland, Midland and highland coffee varieties. The smallest and highest nicotinic acid contents were observed in the coffee varieties, 7454, and same variety collected from lowland and highland, respectively. Unlike caffeine and chlorogenic acid, lowland coffee varieties had the lowest concentrations of nicotinic acid than coffee verities collected from Midland and lowland.

Table 11: Concentrations (mg/g) nicotinic acid in roasted coffee varieties grown in different altitudes of southwest Ethiopia (Mean ± SD, n = 4)

Coffee	**Altitude**			
varieties	**Highland**	**Midland**	**Lowland**	**Mean**
74110	7.92 ± 0.187	7.74 ± 0.455	7.55 ± 0.345	7.74 ± 0.352
7454	8.76 ± 0.114	7.98 ± 0.696	6.34 ± 0.031	7.69 ± 0.467
7440	8.44 ± 0.089	7.23 ± 0.332	7.36 ± 0.886	7.67 ± 0.051
74112	7.98 ± 0.151	6.66 ± 0.035	6.55 ± 0.332	7.07 ± 0.704
Mean	7.84 ± 0.696	7.33 ± 0.674	6.23 ± 0.834	7.13 ± 0.663
CV	3.32			

Statistical analysis using two way ANOVA ($p < 0.05$) revealed the presence of interaction between coffee varieties and altitude on nicotinic acid contents of roasted coffee beans (Appendix Table 8).

Irrespective of their varieties and altitudes, roasted samples showed relatively lower concentrations of nicotinic acid than green coffee samples. But, the roasted 7440, which was collected from lowland, exhibited higher concentration nicotinic acid than its corresponding green coffee. The obtained results were agreed with previous literature findings [5, 40].

5. Conclusions and Recommendations

5.1. Conclusions

In the present study, the influence of altitude on caffeine, chlorogenic acid and nicotinic acid contents on four Arabica coffee varieties grown in high, mid and low lands in the southwest of Ethiopia were investigated. For the study, totally 12 samples, (i.e., 4 samples from each altitude) were collected from purposively selected three JARC farmlands, located in low, mid and high altitudes.

The moisture contents and pH of the green coffee bean varieties were also evaluated after drying in open air. Although variations were observed in terms of the moisture contents among varieties and altitudes, the values samples were in the acceptable ranges. pH of the green beans samples was also shown slight variations with coffee varieties and altitudes.

The observed results demonstrated that concentrations of caffeine and chlorogenic acid decrease against the altitude in both green and roasted coffee varieties, whereas, the concentrations nicotinic acid increase with altitudes. On the other hand, irrespective of their varieties and altitude differences, concentrations of the analytes were varied after roasting of coffee beans.

Generally, coffee varieties grown in higher altitude areas have in higher quality than mid and low altitude. Chemical constituent coffee varieties in higher altitude showed higher cup taste followed by mid- and low altitude due to the reduced caffeine and chlorogenic acid and increased nicotinic acid. However, coffee varieties in low altitude showed lower cup taste relatively with higher altitude due to complete (higher) caffeine and chlorogenic acid and decreased nicotinic acid content.

5.2. Recommendation

Based on the observed findings,the researched would like to forward the following recommendations:

- Caffeine, chlorogenic and nicotinic acid contents were chemical discriminators and also trigonelline, kahweol and cafestol would be added and performed in the future, it will make the work more meaningful and complete.
- This research work was carried out in four varieties and three altitudes in raw and roasted coffee, it could be better more varieties would be added and, studied at a different altitude of another part of the country
- Even though HPLC-DAD was accurate and precise, HPLC –MS or HPLC MS-MS could be helpful in confirmation of the chemical structure of these chemical constituents.

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7. Appendixes

Appendix table 1: ANOVA table of showing moisture content of green coffee beans varieties

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	336.988	24.0706	250.53	<0.0001
rep	3	286.032	95.338	992.28	<0.0001
alt	2	16.147	8.274	86.16	<0.0001
var	3	1.519	0.507	5.27	0.005
alt*var	6	2.239	0.373	3.88	0.0063
Error	27	2.594	0.0961		
Corrected total	41	339.583			

R-Square	Coeff Var	Root MSE	Moisture Mean
0.992	2.888	0.309	0.730

Appendix table 2: ANOVA showing pH content of green coffee varieties

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	0.577	0.042	10.56	<0.0001
Rep	3	0.003	0.001	0.03	0.9930
Alt	2	0.186	0.093	23.90	<0.0001
Var	3	0.132	0.044	11.32	<0.0001
alt*var	6	0.243	0.0404	10.37	<0.0001
Error	27	0.577	0.004		
Corrected total	41	0.105	0.682		

R-Square	Coeff Var	Root MSE	PH Mean
1.102	0.845	0.0624	5.665

Appendix table 3: ANOVA table showing caffeine content in green coffee varieties and Altitudes (P < 0.05)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	185.434	13.245	49.52	<.0001
rep	3	0.122	0.0405	0.15	0.928
alt	2	89.857	44.928	167.98	<0.0001
var	3	56.390	18.796	70.28	<.0001
alt*var	6	39.066	6.5111	24.34	<0.0001
Error	33	8.826	0.267		
Corrected total	47	194.261			

R-Square	Coeff Var	Root MSE	Caffeine Mean
0.955	3.528	0.517	14.656

Appendix table 4: ANOVA table of showing chlorogenic acid on green coffee beans and altitude.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	430.529	30.752	10.56	<0.0001
rep	3	7.314	2.438	1.80	0.1666
alt	2	30.503	15.251	11.25	0.0002
var	3	99.786	33.262	24.54	<0.0001
alt*var	6	292.926	48.821	36.02	<0.0001
Error	33	44.725	1.355	22.69	<0.0001
Corrected total	47	475.255			

R-Square	Coeff Var	Root MSE	chlorogenic acid Mean
0.906	3.568	1.1642	32.629

Appendix table 5: ANOVA table of showing nicotinic acid content in green beans varieties and altitude.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	46.581	3.327	20.41	<.0001
rep	3	0.378	0.126	0.77	0.5176
alt	2	21.202	10.601	65.04	<0.0001
var	3	6.705	2.235	13.71	<0.0001
alt*var	6	18.297	3.049	18.71	<0.0001
Error	33	5.3791	0.1631		
Corrected total	47	51.961			

R-Square	Coeff Var	Root MSE	Nicotinic acid Mean
0.896	5.175	0.408	7.801

Appendix table 6: ANOVA table of showing caffeine in roasted coffee content in varieties and altitude

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	273.162	19.51155	35.46	<.0001
rep	3	7.183	2.395	4.35	0.0109
alt	2	173.726	86.863	157.87	<.0001
var	3	66.018	22.006	40.00	<.0001
alt*var	6	26.234	4.372	7.95	<.0001
Error	33	18.157	0.551		
Corrected total	47	291.319			

R-Square	Coeff Var	Root MSE	Caffeine Mean
0.937	4.227	0.742	17.548

Appendix table 6: ANOVA table of showing chlorogenic acid contents in roasted coffee beans varieties and altitude

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	266.328	19.0235	48.10	<.0001
Rep	3	2.203	0.734	1.86	0.1561
Alt	2	32.895	16.447	41.59	<.0001
Var	3	5.828	1.943	4.91	<.0001
alt*var	6	225.403	37.567	94.99	<.0001
Error	33	13.051	0.395		
Corrected total	47	279.379			

R-Square	Coeff Var	Root MSE	chlorogenic acid Mean
0.954	4.824	0.629	13.034

Appendix table 7: ANOVA shows nicotinic acid in roasted coffee content in varieties

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Model	14	30.695	2.193	35.75	<.0001
Rep	3	1.321	0.441	7.18	0.0008
Alt	2	16.064	8.032	130.95	<.0001
Var	3	3.557	1.186	19.33	<.0001
alt*var	6	9.753	1.626	26.50	<.0001
Error	33	2.0241	0.0614		
Corrected total	47	32.719			

R-Square	Co-eff Var	Root MSE	Nicotinic acid Mean
0.938	3.318	0.248	7.464