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EFFECT OF ALTITUDE, SHADE AND PROCESSING METHODS ON ISOTOPES AND BIOCHEMICAL COMPOSITION OF GREEN COFFEE BEANS IN ETHIOPIA

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ABSTRACT: Though Arabica coffee has evolved in Ethiopian forest areas, it is being produced without shade in many countries at varying altitudes. Systematically establishing a relationship between geographic origin of coffee and its quality leading to the generation of reliable data is a priority area in Ethiopia. Thus, the objective of this study was to determine the composition, fingerprinting and association of isotopes and biochemical composition as a function of altitude, shade and processing methods. The study included washed and unwashed coffee samples from 1150, 1545 and 1802 m a.s.l. to determine carbon, nitrogen and oxygen isotopes and %N and %C using a continuous flow (CF) EA-IRMS; caffeine and chlorogenic acids using HPLC/THERMO; sucrose using Gas Chromatography. Univariate Analysis of Variance, Automatic regression modelling, and Stepwise canonical discriminant function were conducted. Shading disfavoured the composition of $\delta^{18}O$ as the altitude increased. The highest mean value of 5-caffeoylquinic acid (5-CQA) was obtained from unwashed-shaded coffee at 1545 m a.s.l. while unwashed-shaded coffee at 1802 was the lowest. The $\delta^{15}N$, higher $\delta^{13}C$ and $\delta^{18}O$ values may be useful indicators of altitudinal coffee. The association of isotopes and biochemical composition of green coffee beans was found to be weak although for caffeine percent N contributed significant positive weight ($b = 6.604$, $p \leq 0.002$) and %C significant negative ($b = -0.388$, $p \leq 0.004$) weight, respectively) for the model. Lowland coffees were well discriminated (94.4% of variation) by 4,5-DCQA from those at midland and highland grown coffees. In conclusion, isotopes, their respective elements, and biochemical composition of green coffee beans could be certainly exploited for discriminating growing environment and profiling of coffee quality.

Key words/phrases: Coffee quality profile, Coffee value chain, Fingerprinting, Growing environments.

INTRODUCTION

The importance and globalization of the coffee market increasingly raises concerns about labelling its origin. Signature (fingerprinting) of various nature could be used for sorting the products of unique nature (Meinzer *et al*., 1990). It is understood that the quality of coffee has a high variation

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according to its origin, and several attempts have been made to discriminate analytically the origin of green and roasted coffees. A review by Rodrigues *et al*. (2009) disclosed different approaches of origin identification of food stuffs for labelling. Analytical methods such as gas chromatography–mass spectrometry (Costa Freitas *et al*., 2001), near infrared spectroscopy (Bertrand *et al*., 2006), determination of organic compounds such as chlorogenic acids and fatty acid profiles (Martín *et al*., 2001), tocopherols and triglycerides (González-Rios *et al*., 2007) and stable isotope analysis of specific compounds extracted from the green coffee beans (Weckerle *et al*., 2002; Adugnaw Mintesnot, 2014) have been studied extensively with promising results.

Ethiopia, despite being the place of origin and diversification of Arabica coffee, has not yet fully exploited its genetic and environmental wealth discriminated in the world market (Petit, 2007). The country could play most roles as a supplier of some of the best coffees in the 'global coffee value chain', which could be accompanied by discrimination and promotional works that identifies it with unique inherent flavour. In this regard, the approach using stable isotope ratios and biochemical composition for origin discrimination seem quite promising (Serra *et al*., 2005). Serra *et al*. (2005) inferred the continental origin (Africa, Asia and America) of coffee using the combination of the isotopic fingerprints of carbon, nitrogen and boron, which are reportedly used as integrated proxies for environmental conditions and agricultural practices. They were successful in identifying 88% of the intercontinental origin of the samples but not an intracontinental discrimination by means of principal component analysis. Variation in stable carbon isotope discrimination among genotypes grown under identical conditions has been used for its potential as a means of selecting for improved water use efficiency (WUE) and yield in C_3 crop species (Meinzer *et al*., 1990).

Rodrigues *et al*. (2009) found some discrimination of the geographic origin of some of the coffees in Hawaii through multivariate analysis of isotopic composition of the bean ($\delta^{13}C$ VPDB, $\delta^{15}N$ VAIR, $\delta^{18}O$ VSMOW) and elemental composition (carbon and nitrogen percentage). They recognized that stable isotopes often integrate the isotopic signature of food provenance. The observed differences on stable isotopic and elemental composition were mainly explained by altitude and precipitation values associated with the different geographic locations. Technological advancement in the application of multi-element stable isotope analysis extends the scope for the detection of adulteration and for assignment of regional origin and authenticity of foods

(Rodrigues *et al*., 2009). For instance, Weckerle *et al.* (2002) demonstrated the high significance of $\delta^{18}O$ VSMOW values for assessment of the origin assessment of green coffee beans. Adugnaw Mintesnot (2014) reported instrumentality of isotopes and multi-elements and biochemical composition of green coffee beans for the successful discrimination of coffee origins.

The interpretation of isotopic results in terms of climatic parameters must generally take into account the numerous physiochemical and biochemical fractionation phenomena. Martin and Martin (2003) showed that physiochemical and biochemical fractionation phenomena occur in the course of water transport in the plant, photosynthesis, metabolism of sugars, and further biochemical transformations. Shibuya *et al*. (2006) demonstrated that the stable carbon and nitrogen isotopic ratios are related to the plants' climatic conditions during growth, mainly water and nutrient availability along with light intensity and temperature, and can be useful as indicatives of their origin, providing tools to delimit their potential cultivation areas if the conditions are significantly different. The isotopic composition of oxygen of plant leaf water, which is potentially derived not only from water but also from carbon dioxide, is a function of isotopic composition of source water and of atmospheric water vapour and also of the ratio of air and leaf vapour pressure (Flanagan *et al*., 1993). A study by Flanagan *et al*. (1993) showed that differences in altitude, annual precipitation, water stress, and processes like evaporation and transpiration and also the kinetics of the exchange of CO_2 with leaves will affect leaf-water isotopic composition.

The growing environment was also found to have an effect on biochemical composition (Gichimu *et al*., 2014). As the factors may differ from one region to the other, these chemical descriptors (concentrations of phenolic compounds and methylxanthines) are considered to be reliable geographical indicators, as well as chemo-taxonomical markers. Yigzaw Dessalegn *et al*. (2007) found significant variation for green bean caffeine, chlorogenic acid, sucrose, and trigonelline contents among 42 Ethiopian Arabica coffee accessions collected from different parts of Ethiopia and grown at Finoteselam, North-western Ethiopia. They observed respective values ranging from 0.91–1.32, 2.34–4.67, 5.30–8.98 and 1.04–1.71%. Ky *et al*. (2000) and Yigzaw Dessalegn (2006) reported direct relation of sucrose and coffee flavour.

Environmental factors, such as altitude, shade and rainfall may contribute to the quality of the coffee beverage (Bote and Struik, 2011; Likassa Ebisa, 2014). However, further studies are needed to investigate additional

environmental characteristics that affect coffee quality (Rodrigues *et al*., 2009; Decazy *et al*., 2003). Silvarolla *et al*. (2000) reported that an evaluation of the caffeine content of beans from 99 progenies revealed intra- and inter-progeny variability. In 68 progenies from the Kaffa region they found caffeine values in the range 0.46–2.82%, and in 22 progenies from Illubabor region these values ranged from 0.42 to 2.90%. Bertrand *et al*. (2006) reported a significant effect of elevation on bean biochemical composition of the cultivar 'Caturra' where they found increased concentration of chlorogenic acid and caffeine with increasing elevation above 1100 m. The reverse trend was observed for trigonelline concentration in one situation and had no effect on another. Caffeine and fat concentrations increased with increasing elevation up to a level where it decreased beyond that level.

A study by Bertrand *et al*. (2006) showed no clear trend of sucrose with elevation. Furthermore, there was no significant relationship between sucrose concentration and elevation for the TC varieties or the hybrids. Elevation explained little of the variation in chlorogenic acid concentration for the F1-A and the F1-B hybrids (R^2 values of 0.15 and 0.30, respectively). For caffeine, a significant linear regression was found for the TC in Experiment 1 (R^2 = 0.61) and for the F1-A hybrids (R^2 = 0.25). This relationship was not found for the TC and F1-B varieties. Their coefficients of regression were almost identical (0.65 and 0.64, respectively) and both had R^2 values of 0.39. Fat concentration increased with increasing elevation for the traditional varieties but not for the hybrids. Post-harvest processing was also reported to influence the final quality and characteristics of the product (Silvarolla *et al*., 2000; Bertrand *et al*., 2006).

Natural diversity in Ethiopian specialty coffees may represent an important opportunity to add value to the economic and social development of the country as well as to the advancement of coffee science. Systematically establishing a relationship between geographic origin of coffee and its quality leading to the generation of reliable data is a priority area in Ethiopia (Adugnaw Mintesnot, 2014). However, studies of objective specialty coffee discrimination and authentication methods have been commenced very recently in Ethiopia. Such methods are believed to complement the existing subjective organoleptic classification methods. Thus, the objective of this study was to describe the composition, fingerprinting and association of isotopes and biochemical compounds as a function of altitude, shade and processing methods.

MATERIALS AND METHODS

Site selection and experimental design

The study included washed coffee and unwashed coffee samples where possible from south-western Ethiopia from three farms located at different altitudinal ranges (1150, 1545 and 1802 m a.s.l.) designated as lowland, midland and highland coffee growing areas, respectively (Table 1). According to the data obtained from National Meteorological Services Agency, the study region is characterized by mono-modal rainfall of about 1565 mm, maximum and minimum temperature of 26.1 and 13.2°C, respectively, relative humidity of 73.3%, sunshine hours of 5.4 and altitudinal range between 1150 and 1820 m a.s.l. The farms were purposely selected considering the altitudinal ranges and uniformity to management methods. The selected farms belong to the then Coffee Plantation Development Enterprise. Nine sub coffee farms were selected from the three farms. From the three coffee farms shaded and unshaded plots were identified and two types of processing methods were applied in three replications. Ripe coffee cherries were handpicked at their peak ripening phase during the 2010/11 crop season out of which washed coffee and unwashed coffee green coffee beans were carefully prepared without contamination.

Table 1. Description of study sites.

Farm	Region	Latitude	Longitude	Altitude (m a.s.l.)
Bebeka (N=3)	Benchmaji/ SNNP	6°56.580′ N	35°30.607′ E	1150
Kossa (N=3)	Jimma/ Oromia	7°57.223′ N	36°52.664′ E	1802
Goma (N=3)	Jimma/ Oromia	7°55.253′ N	36°37.069′ E	1545

Laboratory analysis

Green coffee beans were subjected to freeze drying just before grinding to fine powder using a hand-held electrical blade coffee grinder (Bosch MKM 6003 UC, Bean Container Capacity: 75 g, Power: 180 Watt). Grinding was assumed to be sufficient when the powder escaped to the ceiling of the cap of the grinder. The powder was immediately packed in a plastic cup with a tight stopper, and kept in a deep freezer until laboratory analysis.

Carbon, Nitrogen isotopes and elements measurement

Carbon and Nitrogen isotopes and %N and %C were measured using a continuous flow (CF) EA-IRMS, Sercon stable isotope mass spectrometers (UK) (Otsuki, 1983). Finely ground green coffee bean powder (0.95–1.4 mg each) rolled in small tin capsules were loaded onto an auto sampler in a duplicate. The samples were purged by a helium (He) flow into a combustion tube and completely oxidized at a temperature of 1000°C. A packed GC

column removes impurities and separates N_2 and CO_2. A mass spectrometer ionizes gaseous molecules and separates the ions into a spectrum according to their mass-to-charge ratio (m/z), using electric and magnetic fields. The relative abundances of the molecules of different m/z were then found by measuring the currents generated by these separated ion beams. A high vacuum keeps the analyzer pressure low enough (10–5 mbar) to reduce collisions between ions and background gas to an acceptable level. A permanent magnetic field was used (fixed B) and masses were selected by varying the tensions of the electric field V. A universal triple collector was used and B and V were kept constant for each element that has to be measured (Otsuki, 1983).

Oxygen ($\delta^{18}O$) isotope measurement (VSMOW)

It was done via a TC/EA (thermal conversion/elemental analyzer) coupled to an isotope ratio mass spectrometer (IRMS) (20-20, SerCon Ltd, Crewe, UK) (SerCon, 1983). The green coffee beans powder was further dried overnight at 60–80°C and stored in desiccators after which samples of 1 mg each rolled in a silver cup in duplicate were loaded onto an auto sampler. The samples were pyrolyzed at 1400°C in a molybdenum lined, aluminium oxide reduction tube filled with glassy carbon and topped with a glassy carbon crucible. The produced N_2 and CO gases were separated via a 1 m gas chromatography (GC) column (E3030, Elemental Microanalysis Ltd., Okehampton, UK) at a temperature of 50°C, Helium (He) carrier gas pressure of 1.6 bar, Helium (He) flow retention time of about 250 s, and sample analysis time of 1000 s, and analyzed via isotope ratio mass spectrometry (IRMS) for $\delta^{18}O$. The internationally accepted reference values of $\delta^{18}O$-KNO_3 for USGS32 (25.7±0.4 and USGS34 (-27.8±0.4 (Brand *et al*., 2009) and $\delta^{18}O$-$NaNO_3$ for USGS35 (56.8±0.3 (IAEA, 2004) were used to correct raw $\delta^{18}O$ values to $\delta^{18}O$ (‰) (Otsuki, 1983).

Caffeine, trigonelline and chlorogenic acids

The caffeine and chlorogenic acid contents were determined using HPLC/THERMO following the method of Alonso-Salces *et al*. (2009): 0.1 g freeze-dried green coffee powder was weighed in an Erlenmeyer flask of 50 ml. Ten ml of MeOH/Acetic Acid (30:7.5:2.5) containing 2 mg/ml ascorbic acid was added and then placed in an ultrasonic bath for 15 minutes. The extract was filtered using Whatman filter paper No. 2, and subsequently over a 0.45 micrometer PTFE filter after which 1 ml of the filtrate was taken in a vial and injected on HPLC/THERMO. The standard solutions of chlorogenic acid, caffeine and caffeic acid were mixed each at 0.5/1/1.5 mg/ml in one

mixture in methanol and each solution was injected twice for calibration. A calibration curve was made using the standard concentration and area of sample and subsequently used to calculate the composition of the respective biochemical component using the area generated after the retention time. The detection was carried out at 278 nm (caffeine and trigonelline), and 324 nm (CGA). For the identification and quantitative analysis, a standard curve was prepared using standards of caffeine, trigonelline and chlorogenic acids.

Sucrose measurement

Sucrose of the coffee beans was determined using GC VARIAN 3800 following the standard method. A sample of green coffee powder was freeze-dried and weighed (0.5–1 g) in 50 ml volumetric flask to which 30 ml distilled water plus 5 ml frozen Internal Standard Solution (ISS) (Phenyl-B-D-pyranoside) was added. It was placed at 60°C for 30 minutes after which it was cooled. Next, 0.5 ml each of Carre I (15 g $ZnSO_4$ and 7.5 g Carre II (K_4FClCN)6 was added to de-protein the sample. The distilled water was then filled to label the mark on the 250 volumetric flasks and shaken well to homogenize the mixture. The solution was immediately filtered with Whatman filter papers, and subsequently 1 ml filtrate was taken in small bottles using glass Pasteur pipette and dried under nitrogen-drier using hollow needles to let nitrogen in to the bottle. To this dry extract was added 1 ml STOX (2.5 g hydroxylamine hydrochloride diluted with dry pyridine to 100 ml) under hood and kept at 60°C for 30 minutes and then cooled down. Then, 1 ml of HMDS (hexamethyldisilazan) was added and subsequently 0.1 ml TFA (trifluor acetic acid) was added before sedimentation for 60 minutes to get clear extract solution. From the clear extract solution, 1 ml was taken in a vial with rubber stopper to inject to GC VARIAN 3800.

Statistical analysis

Univariate Analysis of Variance and Tukey HSD method of mean separation were applied to determine significant difference between samples. Moreover, multiple regression analyses were conducted to examine the relationship between coffee quality attributes and various potential predictors using SPSS 16 v2 software. Stepwise canonical discriminant function and Stepwise Regression between isotopes and biochemical composition of green coffee beans were conducted to identify the best predictor for discriminating coffees of different altitudinal gradients.

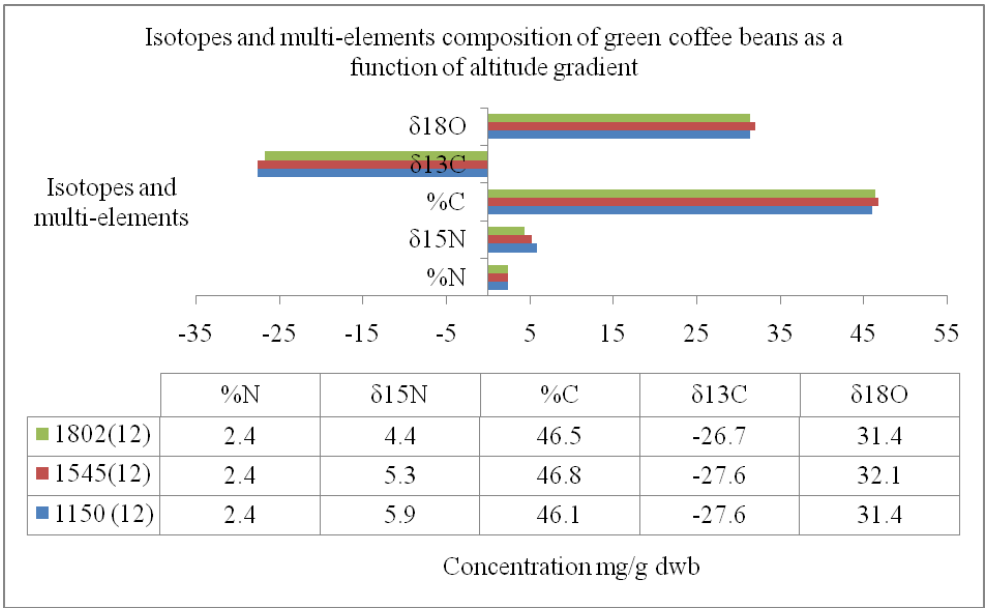
RESULTS

Isotopic and percent N and C composition of green coffee beans as a function of altitude, shade and processing methods

There were significant effects ($p \leq 0.05$) of altitude on $\delta^{15}N$ and $\delta^{13}C$; shade on $\delta^{13}C$ and non-significant, otherwise ($p > 0.05$) (Tables 2-4 and Fig. 1). With regards to $\delta^{15}N$ the mean value at 1802 m a.s.l. was significantly lower as compared to the mean value at both 1150 and 1545 m a.s.l. The trend showed that $\delta^{15}N$ concentration increased in the order of lowland, midland and highland samples (Fig. 1). For $\delta^{13}C$ mean value at 1150 m a.s.l. was significantly lower as compared to the value at 1545 and 1802 m a.s.l. There was significant effect of shading on $\delta^{13}C$ and $\delta^{18}O$ levels of green coffee beans. With regards to $\delta^{18}O$ unshaded coffee showed higher mean value as compared to shaded counterpart whereas for $\delta^{13}C$ the shaded coffee showed higher mean value. Processing methods did not show any significant effect on any of the studied variables.

Table 2. Effect of altitude, shade and processing methods on stable isotopes and %N and %C.

Factor	Level	%N	$\delta^{15}N$	%C	$\delta^{13}C$	$\delta^{18}O$
Altitude	1150 (12)	2.4±0.1 a	5.9±0.3 a	46.1±0.8 a	-27.6±0.2 b	31.4±0.3 a
	1545(12)	2.4±0.1 a	5.3±0.3 a	46.8±0.8 a	-27.6±0.2 b	32.1±0.3 a
	1802(12)	2.4±0.1 a	4.4±0.3 b	46.5±0.8 a	-26.7±0.2 a	31.4±0.3 a
	p-value	0.770	0.006	0.861	<0.001	0.184
Shade	Shaded	2.4±0.0	5.0±0.3	46.3±0.7	-27.6±0.1	31.1±0.2
	Unshaded	2.4±0.0	5.3±0.3	46.7±0.7	-27.0±0.1	32.1±0.2
	p-value	0.904	0.419	0.712	0.005	0.005
GM		2.4±0.0	5.2±0.2	46.5±0.5	-27.3±0.1	31.±0.2
SD		0.2	1.2	2.5	0.7	1.7
CV%		6.5	23.3	5.4	-2.7	5.4



	%N	δ15N	%C	δ13C	δ18O
1802(12)	2.4	4.4	46.5	-26.7	31.4
1545(12)	2.4	5.3	46.8	-27.6	32.1
1150 (12)	2.4	5.9	46.1	-27.6	31.4

Fig. 1. Isotopes and multi-element composition of green coffee beans as a function of altitude gradient.

The bi-factor interaction effect of altitude * shade, and altitude * processing methods were significant for $\delta^{18}O$ (Tables 3 and 4), and non-significant, otherwise. Shading disfavoured the composition of $\delta^{18}O$ in green coffee beans as the altitude increases while favouring at lower altitude. Unwashed coffee showed higher $\delta^{18}O$ composition of green coffee beans at higher altitudes but lower at low altitude. Both shading and washing could discriminate coffees of higher altitudes from that of lowlands. The tri-factor interactions resulted in significant effect of none of the response variables (Table 3).

Table 3. Mean effect of bi-factor interactions on the composition of stable isotopes and %N and %C of green coffee beans.

Altitude * Shade		**%N**	**$\delta^{15}N$**	**%C**	**$\delta^{13}C$**	**$\delta^{18}O$**
1150	Shaded	2.3±0.1	5.4±0.4	45.9±1.2	-27.9±0.2	32.2±0.4
	Unshaded	2.4±0.1	6.4±0.4	46.4±1.2	-27.4±0.2	30.6±0.4
1545	Shaded	2.4±0.1	5.4±0.4	46.5±1.2	-27.8±0.2	31.6±0.4
	Unshaded	2.4±0.1	5.2±0.4	47.1±1.2	-27.3±0.2	32.5±0.4
1802	Shaded	2.4±0.1	4.3±0.4	46.5±1.2	-27.1±0.2	29.4±0.4
	Unshaded	2.4±0.1	4.4±0.4	46.6±1.2	-26.3±0.2	33.3±0.4
p-value		0.89	0.442	0.967	0.676	<0.001

Table 4. Mean effect of bi-factor interaction on the composition of stable isotopes and %N and %C of green coffee beans.

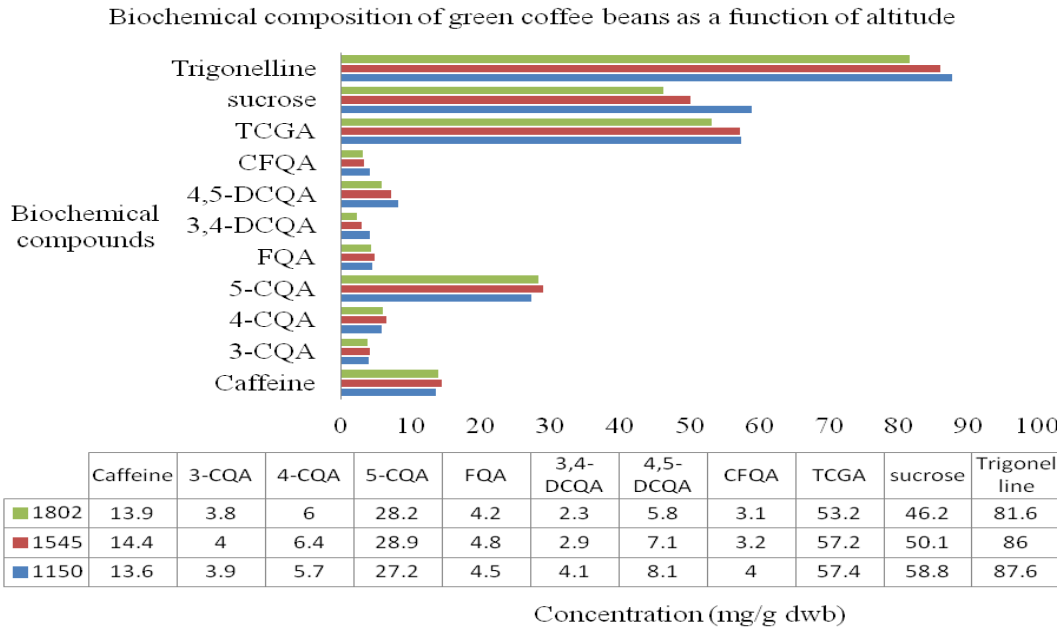
Altitude * Processing		%N	$\delta^{15}N$	%C	$\delta^{13}C$	$\delta^{18}O$
1150	Washed	2.3±0.1	5.9±0.4	45.7±1.2	-27.4±0.2	31.7±0.4
	Unwashed	2.4±0.1	6.0±0.4	46.5±1.2	-27.9±0.2	31.0±0.4
1545	Washed	2.3±0.1	5.7±0.4	46. 2±1.2	-27.6±0.2	31.0±0.4
	Unwashed	2.4±0.1	4.9±0.4	47. 4±1.2	-27.5±0.2	33.1±0.4
1802	Washed	2.4±0.1	4.7±0.4	45.9±1.2	-26.5±0.2	31.2±0.4
	Unwashed	2.4±0.1	4.0±0.4	47.2±1.2	-26.8±0.2	31.5±0.4
p-value		0.875	0.552	0.977	0.365	0.01

Biochemical composition of green coffee beans as a function of altitude, shade and processing methods

The results of the biochemical composition of green beans are presented on Tables 5-7 and Fig. 2. Altitude had significant effect ($p \leq 0.05$) on 4-CQA, 5-CQA, FQA, 3,4-DCQA, 4,5-DCQA, CFQA, TCGA, sucrose, trigonelline composition of green coffee beans. However, altitude did not have significant effect ($p > 0.05$) on caffeine and 3-CQA compositions. For 4-CQA and 5-CQA lowland samples showed the least mean value as compared to both midland and highland samples. On the other hand, for FQA, 4,5-DCQA, and TCGA, the highland sample showed the least mean value as compared to both lowland and midland samples. The composition of 3,4-DCQA, CFQA, and sucrose was significantly the lowest at lowland compared to both midland and highland samples. The effect of shade was significant for CFQA, and non-significant, otherwise. Unshaded coffee gave higher mean value of CFQA composition of green coffee beans as compared to shaded counterpart. Processing methods had significant effect on 3-CQA, 5-CQA, 4,5-DCQA, CFQA, TCGA and trigonelline composition, and non-significant, otherwise. Washed coffee showed higher mean value for 5-CQA, 4,5-DCQA, TCGA, and trigonelline composition of green coffee beans while unwashed coffee resulted in higher mean value for 3-CQA and CFQA composition of green coffee beans. As seen in Fig. 2 the mean value for 3,4-DCQA, 4,5-DCQA, CFQA, TCGA, sucrose, and trigonelline decreased in the order of lowland, midland and highland samples. However, for caffeine, 4-CQA, and 5-CQA the value increased in the order of midland, highland and lowland whereas for 3-CQA and FQA in the order of midland, lowland and highland samples.

The effect of altitude by shade interaction was significant for sucrose and trigonelline composition of green coffee beans, and non-significant, otherwise (Table 5). In this regard, shaded coffee at 1150 m a.s.l. showed the highest mean value while at 1802 m a.s.l. the lowest for both sucrose and trigonelline composition of green coffee beans. The effect of altitude *

processing was significant for 5-CQA, CFQA, TCGA, sucrose and trigonelline of composition of green coffee beans (Table 6). Washed coffee at 1802 m a.s.l. for 5-CQA, unwashed coffee at 1150 m a.s.l. for CFQA, unwashed coffee at 1545 m a.s.l. for TCGA, unwashed coffee at 1150 m a.s.l. for both sucrose and trigonelline showed the highest mean value while unwashed coffee at 1802 m a.s.l. for 5-CQA, TCGA, sucrose and trigonelline, and washed coffee at 1545 m a.s.l. for CFQA showed the least value. The study did not observe any significant effect of shade * processing methods. The tri-factor interaction effect of altitude, shade and processing methods showed significant effect on 5-CQA composition of green coffee beans, and non-significant, otherwise (Table 7). In this regard, the highest mean value was obtained from unwashed coffee and shaded coffee at 1545 m a.s.l. while unwashed coffee and shaded coffee at 1802 was the lowest.



	Caffeine	3-CQA	4-CQA	5-CQA	FQA	3,4-DCQA	4,5-DCQA	CFQA	TCGA	sucrose	Trigonelline
1802	13.9	3.8	6	28.2	4.2	2.3	5.8	3.1	53.2	46.2	81.6
1545	14.4	4	6.4	28.9	4.8	2.9	7.1	3.2	57.2	50.1	86
1150	13.6	3.9	5.7	27.2	4.5	4.1	8.1	4	57.4	58.8	87.6

Fig. 2. Biochemical composition of green coffee beans as a function of altitude gradient.

Table 5. Effect of altitude, shade and processing methods on biochemical composition of green coffee beans.

	Caffeine	**3-CQA**	**4-CQA**	**5-CQA**	**FQA**	**3,4-DCQA**	**4,5-DCQA**	**CFQA**	**TCGA**	**Sucrose**	**Trigonelline**
Altitude											
1150 (120	13.6±0.2a	3.9±0.2a	5.7±0.1b	27.2±0.3b	4.5±0.2a	4.1±0.2a	8.1±0.3a	4.0±0.1a	57.4±0.6a	58.8±2.2a	87.6±0.3a
1545 (12)	14.4±0.2a	4.0±0.2a	6.4±0.1°	28.9±0.3a	4.8±0.2a	2.9±0.2b	7.1±0.3a	3.2±0.1b	57.2±0.6a	50.1±2.2b	86.0±0.3b
1802 (12)	13.9±0.2a	3.8±0.2a	6.0±0.1°	28.2±0.3a	4.2±0.2b	2.3±0.2b	5.8±0.3b	3.1±0.1b	53.2±0.6b	46.2±2.2b	81.6±0.3c
p-value	0.08	0.585	0.013	0.002	0.014	<0.001	<0.001	<0.001	<0.001	0.001	<0.001
Shade											
Shaded	14.2±0.2	3.8±0.1	5.9±0.1	27.9±0.2	4.6±0.1	2.9±0.2	7.2±0.2	3.2±0.1	55.4±0.5	52.9±1.8	84.8±0.3
Unshaded	13.7±0.2	4.1±0.1	6.1±0.1	28.2±0.2	4.4±0.1	3.2±0.2	6.8±0.2	3.7±0.1	56.5±0.5	50.5±1.8	85.3±0.3
p-value	0.111	0.123	0.201	0.531	0.259	0.215	0.354	0.002	0.12	0.344	0.274
Processing methods											
Washed	14.2±0.2	3.7±0.1	6.0±0.1	28.5±0.2	4.6±0.1	3.1±0.2	8.1±0.2	2.8±0.1	56.7±0.5	49.2±1.8	85.9±0.3
Unwashed	13.7±0.2	4.1±0.1	6.0±0.1	27.6±0.2	4.3±0.1	3.1±0.2	5.9±0.2	4.1±0.1	55.2±0.5	54.2±1.8	84.2±0.3
p-value	0.111	0.029	0.867	0.025	0.085	0.945	<0.001	<0.001	0.029	0.064	<0.001

Table 6. The bi-factor interaction effect of altitude, shade and processing methods on biochemical composition of green coffee beans.

		Caffeine	**3-CQA**	**4-CQA**	**5-CQA**	**FQA**	**3,4-DCQA**	**4,5-DCQA**	**CFQA**	**TCGA**	**Sucrose**	**Trigonelline**
Altitude*Shade												
1150	Shaded	14.1±0.3	4.0±0.2	5.7±0.2	26.9±0.4	4.7±0.2	4.0±0.3	7.9±0.4	3.8±0.2	57.1±0.8	64.7±3.1	88.0±0.5
	Unshaded	13.1±0.3	3.9±0.2	5.7±0.2	27.4±0.4	4.2±0.2	4.2±0.3	8.2±0.4	4.2±0.2	57.7±0.8	53.0±3.1	87.3±0.5
1545	Shaded	14.6±0.3	3.8±0.2	6.3±0.2	28.8±0.4	4.9±0.2	2.7±0.3	7.4±0.4	2.9±0.2	56.7±0.8	48.4±3.1	85.0±0.5
	Unshaded	14.1±0.3	4.2±0.2	6.5±0.2	29.0±0.4	4.8±0.2	3.0±0.3	6.8±0.4	3.5±0.2	57.8±0.8	51.8±3.1	87.0±0.5
1802	Shaded	13.8±0.3	3.5±0.2	5.7±0.2	28.2±0.4	4.1±0.2	2.1±0.3	6.1±0.4	2.9±0.2	52.5±0.8	45.7±3.1	81.5±0.5
	Unshaded	13.9±0.3	4.1±0.2	6.2±0.2	28.2±0.4	4.2±0.2	2.5±0.3	5.5±0.4	3.3±0.2	53.9±0.8	46.7±3.1	81.6±0.5
	p-value	0.295	0.480	0.366	0.883	0.354	0.878	0.428	0.618	0.901	0.049	0.019
1150	Washed coffee	13.8±0.3	3.8±0.2	5.7±0.2	27.3±0.4	4.7±0.2	4.1±0.3	9.3±0.4	3.1±0.2	57.8±0.8	49.4±3.1	87.6±0.5

		Caffeine	3-CQA	4-CQA	5-CQA	FQA	3,4-DCQA	4,5-DCQA	CFQA	TCGA	Sucrose	Trigonelline
Altitude*Shade												
Altitude*Processing												
	Unwashed coffee	13.4±0.3	4.1±0.2	5.8±0.2	27.0±0.4	4.2±0.2	4.1±0.3	6.8±0.4	5.0±0.2	57.0±0.8	68.2±3.1	87.7±0.5
1545	Washed coffee	14.4±0.3	3.8±0.2	6.3±0.2	28.6±0.4	4.8±0.2	2.7±0.3	7.8±0.4	2.4±0.2	56.4±0.8	50.3±3.1	84.3±0.5
	Unwashed coffee	14.3±0.3	4.3±0.2	6.4±0.2	29.1±0.4	4.9±0.2	3.0±0.3	6.5±0.4	4.0±0.2	58.1±0.8	49.9±3.1	87.6±0.5
1802	Washed coffee	14.3±0.3	3.6±0.2	6.0±0.2	29.5±0.4	4.5±0.2	2.5±0.3	7.2±0.4	2.8±0.2	55.9±0.8	48.0±3.1	85.8±0.5
	Unwashed coffee	13.4±0.3	4.0±0.2	5.9±0.2	26.8±0.4	3.9±0.2	2.2±0.3	4.5±0.4	3.3±0.2	50.5±0.8	44.4±3.1	77.3±0.5
	p-value	0.444	0.933	0.933	0.003	0.199	0.6	0.165	0.002	0.001	0.003	<0.001
Shade* Processing												

Table 7. The tri-factor interaction effect of altitude, shade and processing on biochemical composition of green coffee beans (mg/g dwb).

Altitude	Shade	Processing methods	Caffeine	3-CQA	4-CQA	5-CQA	FQA	3,4-DCQA	4,5-DCQA	CFQA	TCGA	Sucrose	Trigonelline
1150	Shaded	Washed coffee	14.2±0.5	3.8±0.3	5.7±0.3	26.6±0.6	5.0±0.3	4.1±0.4	9.2±0.6	3.0±0.2	57.2±1.1	50.8±4.4	87.8±0.7
		Unwashed coffee	13.9±0.5	4.1±0.3	5.8±0.3	27.2±0.6	4.5±0.3	3.9±0.4	6.6±0.6	4.7±0.2	56.9±1.1	78.5±4.4	88.2±0.7
	Unshaded	Washed coffee	13.3±0.5	3.7±0.3	5.6±0.3	27.9±0.6	4.4±0.3	4.1±0.4	9.4±0.6	3.2±0.2	58.3±1.1	48.0±4.4	87.3±0.7
	Shaded	Unwashed coffee	12.9±0.5	4.1±0.3	5.7±0.3	26.8±0.6	4.0±0.3	4.2±0.4	7.0±0.6	5.2±0.2	57.1±1.1	57.9±4.4	87.2±0.7
1545		Washed coffee	14.6±0.5	3.5±0.3	6.2±0.3	29.5±0.6	4.5±0.3	2.2±0.4	7.8±0.6	1.9±0.2	55.6±1.1	46.5±4.4	83.1±0.7
	Unshaded	Unwashed coffee	14.6±0.5	4.2±0.3	6.3±0.3	28.0±0.6	5.3±0.3	3.2±0.4	7.1±0.6	3.9±0.2	57.8±1.1	50.4±4.4	86.8±0.7
	Shaded	Washed coffee	14.2±0.5	4.0±0.3	6.4±0.3	27.7±0.6	5.0±0.3	3.2±0.4	7.7±0.6	3.0±0.2	57.2±1.1	54.1±4.4	85.5±0.7
		Unwashed coffee	14.0±0.5	4.4±0.3	6.5±0.3	30.2±0.6	4.5±0.3	2.9±0.4	5.9±0.6	4.1±0.2	58.3±1.1	49.4±4.4	88.5±0.7
1802	Unshaded	Washed coffee	14.5±0.5	3.2±0.3	5.6±0.3	29.8±0.6	4.4±0.3	2.1±0.4	7.7±0.6	2.4±0.2	55.2±1.1	48.0±4.4	85. 9±0.7
	Shaded	Unwashed coffee	13.1±0.5	3.9±0.3	5.8±0.3	26.5±0.6	3.9±0.3	2.1±0.4	4.5±0.6	3.3±0.2	49.9±1.1	43. 3±4.4	77.2±0.7
		Washed coffee	14.2±0.5	4.0±0.3	6.4±0.3	29.1±0.6	4.6±0.3	2.8±0.4	6.7±0.6	3.2±0.2	56.7±1.1	48.1±4.4	85.8±0.7
	Unshaded	Unwashed coffee	13.7±0.5	4.1±0.3	6.1±0.3	27.2±0.6	3.8±0.3	2.3±0.4	4.4±0.6	3.3±0.2	51.2±1.1	45.4±4.4	77.4±0.7
p-value			0.714	0.721	0.799	0.007	0.299	0.41	0.397	0.108	0.96	0.293	0.881

Stepwise regression between isotopes and biochemical composition of green coffee beans

A stepwise multiple regressions were conducted to evaluate whether stable isotopes and multi-elements were necessary to predict biochemical composition of green coffee beans. The regression analysis between stable isotopes ($\delta^{18}O$, $\delta^{15}N$, and $\delta^{13}C$), %N and %C, and biochemical compounds of green coffee beans showed significant contribution of the model for caffeine, 4,5-DCQA, TCGA, and sucrose composition of green coffee beans (Table 8) indicating significant positive regression weights. All the requested variables entered into the regression equation for all dependent variables at step 1 of the analysis. Thus, only one model was employed. The model was significantly related to dependent variable F (5, 30) = 3.147, p = 0.021 for caffeine; = 5.007, p = 0.002 for 4,5-DCQA; = 3.292, p = 0.017 for TCGA; = 2.672, p = 0.041 for sucrose (Tables 9 and 10), and non-significant, otherwise. For caffeine, 4,5-DCQA, TCGA and sucrose about 34%, 46% 35% and 31% of the variation was accounted for by the model, respectively. For caffeine, percent N contributed significant positive weight (b = 6.604, p = 0.002) and %C contributed significant negative (b = -0.388, p = 0.004), respectively) for the model. The study showed that all the studied stable isotopes did not contribute significant weight to the model. Moreover, a t-test showed that biochemical compounds in the list 3-CQA, 4-CQA, 5-CQA, FQA, and CFQA were not significantly influenced (p>0.05) by any of the predictors (Tables 9 and 10). The study indicated that there was significant contribution of $\delta^{15}N$ for variation of 4,5-DCQA (p = 0.002), 3,4-DCQA (p = 0.043), and TCGA (p = 0.003).

Table 8. The model for regressing biochemical compounds on stable isotopes and %N and %C content of coffee beans.

Dependent variable	Model	R	R^2	Adjusted R^2	SE of the Estimate	Model p-value (Regression)
Caffeine	1	0.587	0.344	0.235	0.7926	0.021
Trigonelline	1	0.474	0.225	0.096	3.7218	0.155
Sucrose	1	0.555	0.308	0.193	9.8744	0.041
3-CQA	1	0.467	0.218	0.088	0.5349	0.171
4-CQA	1	0.361	0.130	-0.015	0.5272	0.494
5-CQA	1	0.305	0.093	-0.058	1.5686	0.689
FQA	1	0.454	0.206	0.074	0.5917	0.202
4,5-DCQA	1	0.674	0.455	0.364	0.7975	0.002
3,4-DCQA	1	0.448	0.201	0.067	1.6497	0.218
CFQA	1	0.428	0.183	0.047	0.9391	0.272
TCGA	1	0.595	0.354	0.247	2.6674	0.017

Table 9. Stepwise regression of caffeine, trigonelline, sucrose with stable isotopes and %N and %C content of coffee beans.

Dependent variable	**Independent variables in the model**	**Unstandardized Coefficients B**	**Std. Error**	**Standardized Coefficients Beta**	**T**	**Sig.**
Caffeine	(Constant)	4.272	7.336		0.582	0.565
	%N	6.604	1.958	1.134	3.373	0.002
	$\delta^{15}N$	-0.044	0.124	-0.058	-0.352	0.728
	%C	- 0.388	0.122	-1.071	-3.166	0.004
	$\delta^{13}C$	-.280	0.204	-0.231	-1.373	0.180
	$\delta^{18}O$	0.144	0.083	0.270	1.736	0.093
Trigonelline	(Constant)	43.020	34.445		1.249	0.221
	%N	-6.669	9.194	-0.265	-0.725	0.474
	$\delta^{15}N$	1.247	0.583	0.386	2.139	0.041
	%C	.278	0.575	0.177	0.483	0.633
	$\delta^{13}C$	-0.975	0.958	-0.186	-1.018	0.317
	$\delta^{18}O$	0.378	0.389	0.165	0.972	0.339
Sucrose	(Constant)	-252.614	91.388		-2.764	0.010
	%N	-9.396	24.394	-0.133	-0.385	0.703
	$\delta^{15}N$	0.840	1.547	0.093	0.543	0.591
	%C	1.576	1.526	0.359	1.033	0.310
	$\delta^{13}C$	-7.836	2.541	-.533	-3.084	0.004
	$\delta^{18}O$	1.117	1.032	0.173	1.082	0.288

Table 10. Stepwise regression of chlorogenic acid families and stable isotopes and %N and %C content of coffee beans.

Dependent variable	**Variables in the model**	**Unstandardized Coefficients B**	**Std. Error**	**Standardized Coefficients Beta**	**T**	**Sig.**
3-CQA	%N	-1.007	1.321	-.280	-.762	0.452
	$\delta^{15}N$	0.136	0.084	.294	1.621	0.116
	%C	0.113	0.083	.503	1.364	0.183
	$\delta^{13}C$	-0.063	0.138	-.084	-.456	0.652
	$\delta^{18}O$	0.091	0.056	.278	1.636	0.112
4-CQA	%N	-0.785	1.302	-.233	-.602	0.551
	$\delta^{15}N$	0.105	0.083	.244	1.274	0.212
	%C	0.066	0.081	.317	.813	0.422
	$\delta^{13}C$	0.045	0.136	.064	.329	0.745
	$\delta^{18}O$	0.077	0.055	.250	1.394	0.173
5-CQA	%N	-1.457	3.875	-.149	-.376	0.710
	$\delta^{15}N$	-0.079	0.246	-.063	-.320	0.751
	%C	-0.029	0.242	-.048	-.121	0.905
	$\delta^{13}C$	0.463	.404	.227	1.147	0.260
	$\delta^{18}O$	0.005	.164	.005	.028	0.978
FQA	%N	0.810	1.462	.205	.554	0.584
	$\delta^{15}N$	0.079	.093	.156	.851	0.401
	%C	-0.032	0.091	-.132	-.355	0.725
	$\delta^{13}C$	-0.267	0.152	-.325	-1.757	0.089
	$\delta^{18}O$	0.109	0.062	.302	1.761	0.088
4,5-DCQA	%N	-1.833	1.970	-.285	-.931	0.359

Dependent variable	Variables in the model	Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	T	Sig.
	$\delta^{15}N$	0.420	0.125	.509	3.358	0.002
	%C	0.211	0.123	.529	1.715	0.097
	$\delta^{13}C$	-0.418	0.205	-.312	-2.037	0.051
	$\delta^{18}O$	0.075	0.083	.127	.895	0.378
3,4-DCQA	%N	-0.434	4.075	-.040	-.106	0.916
	$\delta^{15}N$	0.547	0.259	.388	2.114	0.043
	%C	-0.084	0.255	-.122	-.328	0.745
	$\delta^{13}C$	-0.095	0.424	-.041	-.223	0.825
	$\delta^{18}O$	-0.033	0.172	-.033	-.191	0.850
CFQA	%N	1.294	2.320	.209	.558	0.581
	$\delta^{15}N$	0.104	0.147	.131	.705	0.486
	%C	0.019	0.145	.050	.133	0.895
	$\delta^{13}C$	-0.365	0.242	-.283	-1.510	0.141
	$\delta^{18}O$	0.124	0.098	.220	1.265	0.216

Canonical discriminant functions

A stepwise canonical discriminant function analysis of green coffee bean biochemical and stable isotopes and their respective element composition showed that 4,5-DCQA, TCGA, trigonelline, $\delta^{13}C$, 3,4-DCQA, $\delta^{15}N$, 4-CQA, sucrose, 5-CQA, FQA, CFQA in descending order of importance significantly ($p \leq 0.05$) contributed for discrimination of altitudinal ranges (Table 11). At each step, the variable that minimizes the overall Wilks' Lambda was entered. About 83.3% of original grouped cases were correctly classified. Two out of four functions were sufficient to accommodate the variation up to 100%. In fact, the first function accommodated 94.4% of the variation which is contributed by 4,5-DCQA composition of green coffee beans (Fig. 3). The first function completely separated coffees grown at 1150 m a.s.l. from those grown both at 1545 and 1802 m a.s.l. About 5.6% of the variation was accounted for by function 2 which separated 75% each of coffees grown at 1802 and 1545 m a.s.l. on one hand and about 70% of coffees grown at 1150 from that of 1802 m a.s.l., on the other (Table 12). Both 4,5-DCQA and 4-CQA composition of green coffee beans contributed for by the variations at function 2.

Table 11. Tests of equality of group means.

Predictor	Wilks' Lambda	F	df1	df2	Sig.
4,5-DCQA	0.442	20.824	2	33	0.000
TCGA	0.596	11.18	2	33	0.000
Trigonelline	0.558	13.069	2	33	0.000
$\delta^{13}C$	0.637	9.415	2	33	0.001
3,4-DCQA	0.707	6.85	2	33	0.003
$\delta^{15}N$	0.717	6.506	2	33	0.004
4-CQA	0.732	6.043	2	33	0.006
Sucrose	0.763	5.117	2	33	0.012
5-CQA	0.784	4.533	2	33	0.018
FQA	0.793	4.32	2	33	0.022
CFQA	0.8	4.137	2	33	0.025
Caffeine	0.861	2.674	2	33	0.084
$\delta^{18}O$	0.962	0.654	2	33	0.526
3-CQA	0.969	0.52	2	33	0.599
%N	0.98	0.332	2	33	0.72
%C	0.988	0.195	2	33	0.824

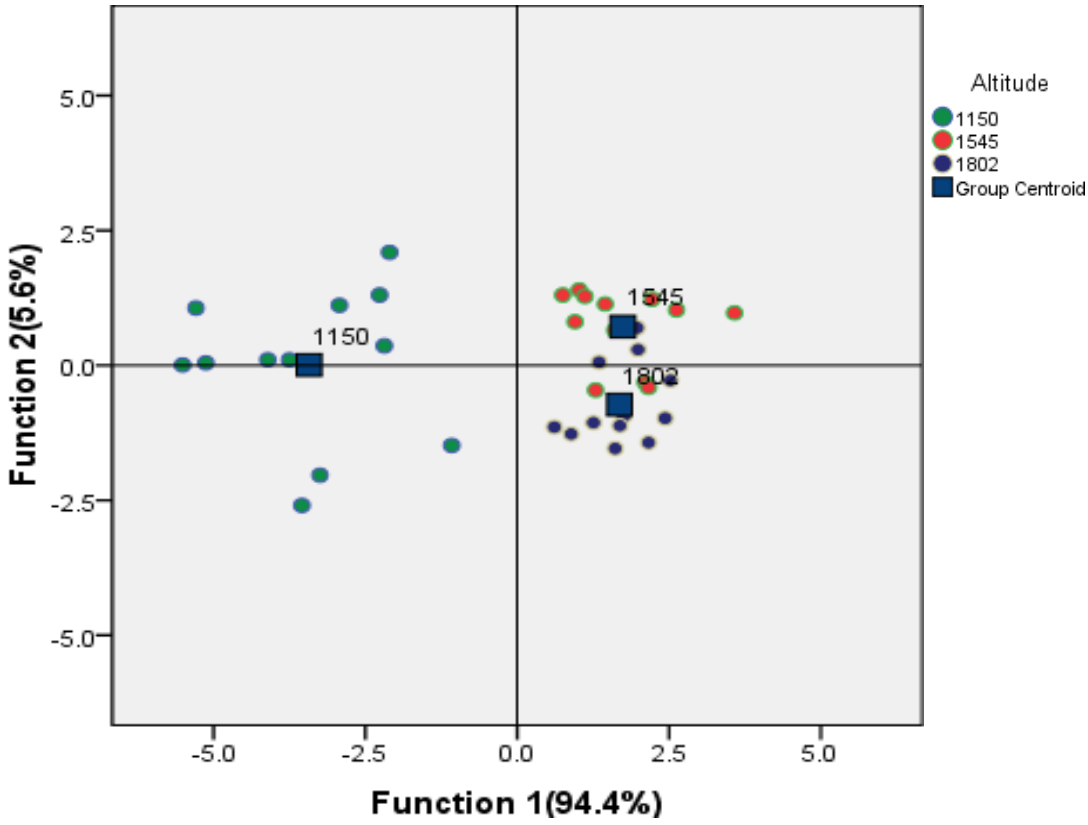


Fig. 3. Canonical discriminant plot of altitudinal coffee groups.

Table 12. Eigenvalue and Wilks' Lambda.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation	Wilks' Lambda	Chi-square	Df	Sig.
1	6.410a	94.4	94.4	0.930	0.098	73.227	8	0.000
2	0.380a	5.6	100.0	0.525	0.725	10.138	3	0.017

a-First 2 canonical discriminant functions were used in the analysis

DISCUSSION

As noted by Ehleringer and Rundel (1989) most elements of biological interest have two or more stable isotopes, although one isotope is usually present in far greater abundance. While there is no evidence for biological fractionation of the isotopes, they may be potentially useful markers of ecosystem process studies. The mean value of $\delta^{15}N$ at 1802 m a.s.l. was significantly lower as compared to the mean value at both 1150 and 1545 m a.s.l. likely due to lower microbial deposition and hence discrimination during the decomposition process of N at higher altitudes. One of the factors controlling the mineral content of plant material is the availability of plant nutrients in the nutrient medium. Moreover, the work of several researchers has revealed that natural abundance $\delta^{15}N$ of plants reflects the net effect of a range of processes (Shibuya *et al*., 2006). In this regard, the presence of multiple N-sources with distinct isotopic values, mycorrhizal associations, temporal and spatial variation in N availability, and changes in plant demand can all influence plant $\delta^{15}N$. In addition, the farm at higher altitude may be planted with legume shade trees where it appears and may supply N by atmospheric nitrogen fixation processes instead of discriminating against $\delta^{15}N$. Consequently, the $\delta^{15}N$ values of leguminous plants are often close to 0‰ (Ehleringer and Rundel, 1989). Ehleringer and Rundel (1989) showed that roughly 30% of the nitrogen fixed by a legume was transferred to the associated coffee trees. Snoeck *et al*. (2000) also proposed certain amount of N transferred by legume to sole coffee trees via litter fall.

For $\delta^{13}C$ mean value at 1150 m a.s.l. was significantly lower as compared to the value at 1545 and 1802 m a.s.l. and the shaded coffee showed higher mean value most probably due to better temperature regulation for photosynthetic process of higher altitudes and shade. In C_3 plants, discrimination against ^{13}C by the carboxylating enzyme, Rubisco (»27‰), is linked to photosynthesis via the ratio of intercellular to atmospheric CO_2 concentrations (ci/ca) (Dawson *et al*., 2002). This ratio reflects the relative magnitudes of net assimilation and stomatal conductance that relate to demand and supply of CO_2, respectively. Carbon-13 data are thus a useful index for assessing intrinsic water use efficiency (*A*/*g*; the ratio of carbon

acquired to water vapor losses via stomatal conductance, g) and may even provide information on actual water use efficiency (the ratio of assimilation to transpiration) when the leaf-to-air vapor pressure difference is known (Ehleringer and Vogel, 1993). A few recent studies are now using C isotope data to investigate competition. The $\delta^{13}C$ values were useful in showing how the efficiency of resource use varied in the presence or absence of different neighbours (Williams *et al*., 1991).

With regard to $\delta^{18}O$ unshaded coffee showed higher mean value as compared to shaded counterpart likely due to better accumulation of the heavy isotope at unshaded condition because of less humidity as compared to humid shaded condition. Oxygen isotope ratios have been most useful in tracing and describing water movement and as indicators of humidity regimes. Oxygen derived from CO_2 undergoes a complete exchange with the oxygen of the water in the plant during the synthesis of cellulose, and thus $\delta^{18}O$ of tissue water is the primary influence on the $\delta^{18}O$ of fixed oxygen in cellulose (Ehleringer and Rundel, 1989). The $\delta^{18}O$ generally varies with ambient humidity, which in turn reflects changes in water use (Meinzer *et al*., 1990). The $\delta^{18}O$ of leaf and tree ring cellulose are largely determined by the integrated leaf-to-air vapor pressure gradient during photosynthetic gas exchange (Ehleringer and Vogel, 1993). This leaf-air vapour pressure gradient changes with environmental conditions (atmospheric humidity, soil moisture, air temperature) and plant response to these environmental changes (e.g., changes in water use, leaf temperature, and net assimilation). Measurement of the $\delta^{18}O$ composition of plant tissues may thus aid for the interpretation of differences in $\delta^{13}C$ among individual plants growing in the same location and among species in different environments. Moreover, the determination of WUE is greatly improved by the simultaneous use of $\delta^{13}C$ and $\delta^{18}O$ in plant tissues (Saurer *et al*., 1997). By considering concurrent variations $\delta^{13}C$ and $\delta^{18}O$, one can distinguish between biochemical and stomatal limitations to photosynthesis in response to a change in environmental conditions.

Shading disfavoured the composition of $\delta^{18}O$ in green coffee beans as the altitude increases while favouring at lower altitude. This indicates shading requirement reduces as altitude increases because of lower amplitude of temperature and higher relative humidity. Bote and Struik (2011) noted that shade triggers differences in physiological behaviour of the coffee plants, such as improved photosynthesis and increased leaf area index, resulting in better performance than possible in direct sun light. Shaded plants had greater biochemical and physiological potential for high dry matter production which

would help them to maintain high coffee yields in the long term.

Rodrigues *et al*. (2009) observed differences in stable isotopic and elemental composition and associated them to altitude and precipitation values associated with the different geographic locations. The isotopic compositions of carbon and nitrogen, which are the main elements of living organisms, are used as proxies for environmental parameters that characterize a certain crop or area (Meinzer *et al*., 1990). Since different elements represent different characteristics of the environment (water cycle, plant physiology, agricultural practices, hydric stress, soil geology, etc.), the combination of these indicators may be used to unequivocally identify a certain ecosystem of origin. A noticeable feature of using stable isotope abundance data, in a multivariate approach to discriminate the origin of a product, is that each single isotopic signature is in fact the result of more than one unique phenomenon, and is thus very difficult to counterfeit for fraud purposes.

A study by Sera *et al*. (2005) showed that the small climatic differences among regions which do not have very marked differences may show little isotopic variation. Stable isotope ratios of carbon, nitrogen and boron have been shown to be good indicators of geographical-dependent parameters, and therefore to be useful tools to infer the region of production of green coffee (Rodrigues *et al*., 2009; Adugnaw Mintesnot, 2014). An inherent limitation of isotope ratio techniques is due to the mismatching between national borders and climatic borders, delimiting different geographical regions; for this reason, coffees produced in small adjacent countries with similar climatic conditions cannot be distinguished from one another on the basis of different isotope ratio values, while large countries with a large variety of climatic areas may show samples with a range of isotope ratio values, displaying wide dispersion.

Processing methods did not show any significant effect on any of the studied variables. Unwashed coffee showed higher $\delta^{18}O$ composition of green coffee beans at higher altitudes but lower at low altitude. Both shading and washing could be used to distinguish coffees of higher altitudes from that of lowlands.

Most of the time, elevation and the variety of Arabica coffee (*Coffea arabica* L.) cultivated are important indicators of quality in world market. For 4-CQA and 5-CQA lowland samples showed the least mean value as compared to both midland and highland samples. The composition of 3,4-DCQA, CFQA, and sucrose was significantly the lowest at lowland compared to both midland and highland samples. On the other hand, for FQA, 4,5-DCQA, and TCGA, the highland sample showed the least mean value as compared to

both lowland and midland samples. A study by Bertrand *et al*. (2006) complements this result and showed that elevation had a significant effect on bean biochemical composition, with chlorogenic acid and fat concentrations increasing with increasing elevation for some varieties and little effect on the variation of chlorogenic acid concentration but not on fat concentration. The stepwise regression analysis showed significant positive influence of percent N and significant negative effect of percent C on green coffee bean caffeine composition but all the studied stable isotopes did not contribute significant weight to the model. Therefore, the weak association of stable isotopes and biochemical composition may require further investigation to be used as a tool for distinguishing green coffee origins. The first function of the canonical discriminant function completely separated coffees grown at 1150 m a.s.l. from those grown both at 1545 and 1802 m a.s.l. An experiment conducted in Central America by Bertrand *et al*. (2006) gave complementary result which distinguished the majority of the samples grown at high elevations from the samples grown at the lowest elevations.

CONCLUSION

Higher $\delta^{15}N$ and higher $\delta^{13}C$ values may be useful indicators of lowland and highland coffee samples, respectively. $\delta^{15}N$ was found to be a significant indicator of variation of 4,5-DCQA; 3,4-DCQA and TCGA. Similarly, higher $\delta^{18}O$ could discriminate highland unshaded coffees. The association of isotopes and biochemical composition of green coffee beans was found to be weak. In fact, for caffeine percent N contributed significant positive weight and percent C significant negative weight, respectively, for the model. This implies the study revealed independency of the regressed variables to each other. With the exception of caffeine $\delta^{18}O$, 3-CQA, %N, and %C most variables were useful predictors of altitudinal ranges of coffee growing sites. In fact, 4,5-DCQA accounted for 94.4% of the variation in composition of green coffee beans to classify lowland coffees from those at midland and highland grown coffees.

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