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DETERMINATION OF CAFFEINE LEVEL IN COFFEE AND ARTEMISININ IN *ARTEMISIA ANNUA*

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Organic Stream

Determination of Caffeine level in Coffee and Artemisinin in *Artemisia annua*

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Minbale Gashu Tadesse

Advisor: Ermias Dagne (Professor)

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ABSTRACT

Determination of Caffeine level in Coffee and Artemisinin in *Artemisia annua*

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Coffee is the second important raw material within the international trade, the most important foreign exchange supplier for many agricultural oriented countries, and the most popular drink. Assessment of the caffeine level is crucial to coffee quality.

An HPLC-UV method with microwave assisted extraction was developed, which allowed the determination of caffeine in green, roasted, home-and coffee-house brewed coffee samples. The chromatographic separation was achieved on reversed-phase C-18 pre-packed analytical column (RP-18 Lichrospher®, Waters, 5μm, 4.6 X 150 mm i.d.) using water: Acetonitrile (70:30, v/v) as isocratic mobile phase at 0.8 mL/min flow rate. The method has been applied to samples to afford average caffeine contents (0.8-1.0%) green and roasted, (48-102 mg) home- brewed, (24-56%) coffee-house brewed. The overall procedure had percent recoveries of 93%. The within day and between day percent RSD in coffee samples ranged from 2.4-6 and 0.8-14%, respectively. HPLC-UV is a method used for caffeine routine analysis.

Artemisia annua is the only commercial source of artemisinin. Due to the absence of appropriate UV absorption, artemisinin should be converted into UV active compound before HPLC analysis. It can be analyzed using Quantitative TLC method with anisaldehyde visualization reagent

1. Coffee (C*offea arabica*)

1.1. Coffee and its Importance

Ethiopia is full of several unique natural products such as coffee, civet, Khat, etc some of which are well known worldwide [1].

Coffee belongs to the botanic family Rubiaceae and the genus C*offea* [2, 3]. *Coffea* is a large genus of flowering plants containing 25-100 species [4]. However, only two coffee tree species are used in the coffee bean production namely C*offea arabica* [5, 6, 7] and *Coffea canephora* [5, 6, 7], commonly known as `Arabica` and `Robusta` coffees, respectively.

Ethiopia is considered as primary center of genetic diversity of Arabica coffee [8, 9, 10,11]. The botanist Linnaeus named it as *Coffea arabica* [12] after viewing Coffee trees cultivated by the Arabs. Although the Coffee fruit is edible to some extent, the beverage obtained by brewing roasted Arabica Coffee is what made Coffee known in the areas of its origin and beyond [4].

Coffee is one of the most popular and widely consumed beverages throughout the world due to its characteristic bitter taste and aroma, and stimulant effect [13,14,15]. A number of recent studies have found beneficial health properties attributed to moderate consumption of coffee [14,15,16,17], even though, there are reports that it is not necessarily good for human health [18,19, 20].

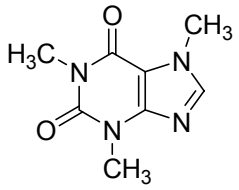
Coffee ranks as one of the worlds major commodity crops and is the major export product of some countries [11]. It is produced commercially in 82 countries worldwide on over 100 billion square meters. Average yields are 77.6 g/m^2 [4]. Four countries (USA, Germany, France, and Japan) consume over half of the world's coffee [4]. Coffee now traded in the international market is usually a blend of beans grown in different parts of the world [9, 21].

Table 1. Top 10 countries that produce 70% of world production

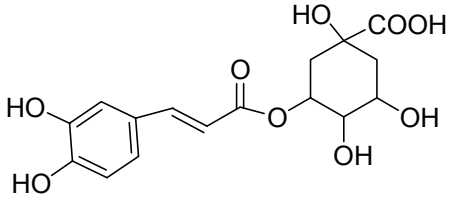
1.Brazil	25%	6.Mexico	4%
2. Colombia	9%	7. Ethiopia	3%
3. Vietnam	9%	8.Uganda	3%
4. Indonesia	8%	9. Cote d`Ivoire	3%
5. India	4%	10. Honduras	2%

1.2. Caffeine and the Other Main Constituents of Coffee

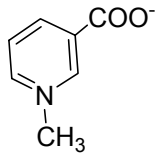
Coffee contains a multitude of substances [10]. The principal secondary metabolites present in coffee bean include: caffeine (**1**), chlorogenic acid (ester of quinic and caffeic acid) (**2**), trigonelline (N-methyl nicotinic acid) (**3**), sucrose (**4**). These are in addition to other constituents such as theobromine, theophylline tannins, fats, proteins, cellulose**,** carbohydrates, and minerals [12, 16, 22, 23].



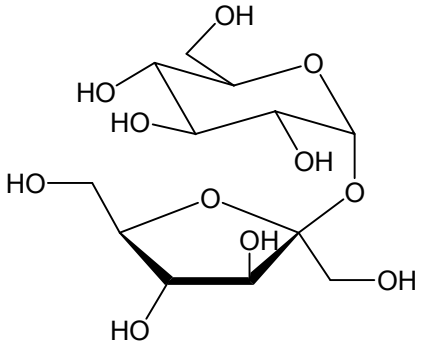
1 Caffeine



2 Chlorogenic acid



3 Trigonelline



4 Sucrose

Fig.1. Principal secondary metabolites in coffee

In the roasting process, characteristic odor and flavor are developed together with the formation of dark brown color. Aroma, sourness and bitterness of coffee brews have been recognized as important attributes of their sensory quality [7, 9]. Most of the aroma is due to the volatile coffee oil fraction, which comprises about 10% of the roasted beans [9, 12, 22]. The volatile oil contains mainly ethyl acetate and furfural with traces of several other compounds [12, 13]. The fixed oil fraction of coffee oil is non-volatile. The most important compounds in coffee that are relevant for flavor and aroma are caffeine (**1**), chlorogenic acid (**2**), trigoneline (**3**), and sucrose (**4**) [6, 11, 24]. Some aroma precursors such as sucrose and trigonellne, give rise to appreciated flavor products including furans, pyrazins, alkyl pyridines and pyrroles. Carboxylic acids are responsible for sourness while chlorogenic acid and caffeine increase bitterness [6, 9].

The contents of caffeine in coffee may be influenced by coffee bean species, geographical origin and roasting conditions which in turn affect the quality of the different coffee beverages [6, 13, 23]. Arabica coffee with its lower sourness, bitterness and better flavor is the most cultivated and more liked by consumers [14, 25, 26]. Robusta coffee beans contained the higher concentration of caffeine and chlorogenic acid compared with Arabica, while Arabica is richer in trigonelline [27]. Beans of Arabica and Robusta contain about 1% and 2% caffeine, respectively. The caffeine content of seeds of different coffee species varies from 0.4-2.8% [8, 26, 28].

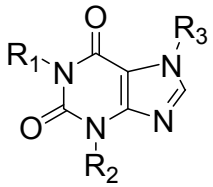
A wide range of reported values for caffeine content in coffee and other caffeine containing beverages arise from differences in reference volumes, analytical methods, product sources and methods of preparation [23, 29]. In order to put the values on a comparable basis, the caffeine content values were analyzed to a common reference volume of 150 mL [23]. In the US, the caffeine content of coffee ranged from 47 to 140 mg per cup, depending on brewing method, cup size, amounts of ground coffee per cup and extraction efficiency. Based on the same factors, caffeine content ranged from 23 to 150 mg per cup in the UK and

from 32 to 201 mg in Denmark [23]. The resulting mean caffeine contents per 150 mL were approximately 55 mg caffeine (range 24 to 88 mg) for percolated coffee, 48 mg (range 13 to 72 mg) for instant and 56 mg (range 26 to 111 mg) for filtered [22, 23, 29].

1.3. Other Minor Purine Alkaloids in Coffee

Alkaloids are classes of naturally occurring compounds containing nitrogen and having the property of an organic amine base (alkaline, hence alkaloid) [12]. They are also defined as cyclic nitrogen containing molecules which are true secondary metabolites (of limited occurrence and produced by living organisms) [30]. They are structurally the most diverse class of secondary metabolites [22].

The purine derivatives caffeine (**1**), theobromine (**6**) and theophylline (**7**) are usually referred to as purine alkaloids [22, 28, 31]. They are all methyl derivatives of xanthine [22, 25]. Methylxanthines are important naturally occurring alkaloids [31].



Xanthine (**5**), $R_1 = R_2 = R_3 = H$
Caffeine (**1**), $R_1 = R_2 = R_3 = CH_3$
Theobromine (**6**), $R_1 = H$, $R_2 = R_3 = CH_3$
Theophylline (**7**), $R_1 = R_2 = CH_3$, $R_3 = H$

Fig. 2. Xanthine and its naturally occurring derivatives

Different amounts of methylxanthines are present in coffee, tea leaves (*Camellia sinensis*), cocoa beans (*Theobroma cacao*), cola nuts (*Cola nitida*), guarana seeds (*Paullinia cupana*) and mate leaves (*Ilex paraguensis*), etc [22, 32]. Caffeine (**1**) is a socially accepted natural stimulant [17, 29, 31].

Table 2. Purine Alkaloids (% dwb)

Purine alkaloids	**Coffee**	***Camellia sinensis***	***Cola nitida***	***Theobroma cacao***	***Ilex paraguensis***	***Paullinia cupana***
Caffeine	1-2	1-4	3	0.2-0.5	0.8-1.7	3-5
Theophylline	trace	0.05	-	-	trace	0-0.25
Theobromine	trace	0.05	0.1	1-4	0.3-0.9	0.02-0.06

Methylxanthines cause stimulation of the central nervous system and the skeletal muscles resulting in an increased alertness [12, 25]. Caffeine is powerful in this respect. Caffeine can also cause mild addiction. There are publications that indicate that caffeine has an effect on the cardiovascular system with a slight increase in blood pressure and a modest decrease in heart rate. Some of the effects of caffeine, such as those on the heart and blood vessels, are inconsistent, and may only be noticeable when regular consumers suddenly cut out caffeine. Theobromine and theophylline are analgesic to cardiac muscles and play a role in relaxing the muscles and expansion of coronary artery [12, 22]. Theophylline relaxes smooth muscles of the bronchi and has been used as a treatment of asthma [3, 10]. Theobromine increases blood vessel dilation and urine volume [10, 12].

1.4. Methods of Determination of Caffeine Content in Coffee

Several analytical techniques have been developed for the determination of caffeine in coffee and other beverages [5]. The most widely used methods for the individual as well as simultaneous quantitation of caffeine in coffee and its derived products include spectrophotometric [5, 17], electrochemical [33], near infrared spectroscopy (NIRS)[25], ion-chromatography [15, 32], solid phase extraction and High Performance Liquid Chromatography (SPE-HPLC) [34], LC-MS [13], HPLC-MS [25], HPLC-UV [14, 27, 35, 36], Quantitative proton NMR (Q^1HNMR) and Quantitative TLC (QTLC) [21]. Spectrophotometric method is fast and simple way of caffeine determination directly in coffee beans, but limited to

use due to spectral overlap. The derivative spectrophotometer requires larger concentration of caffeine in samples [5]. GC is applied to the caffeine analysis but derivatization is needed [37]. HPLC is especially suited to the analysis of compounds not readily assayed by GC such as thermally labile compounds at ambient temperature and polar non-volatile compounds [38]. Since caffeine is a polar and water-soluble organic molecule, HPLC is the most frequently used and nondestructive method for routine qualitative and quantitative determination, and separation based on reverse phase chromatographic separation with UV-diode array absorbance detection [35] and with mass spectroscopy [25]. They provide fast and reliable data [32, 34, 36]. Q^1HNMR and QTLC are other alternatives.

1.5. HPLC: Backgrounds and Basic Principles

HPLC is a form of liquid chromatography with the main difference being that much smaller and uniform stationary phase particles are used to separate compounds that are dissolved in solution [38, 39, 40].

The usual mode of HPLC called reverse phase LC chromatography is a type of partition chromatography where columns are packed with a chemically bonded octadecylsilyl (ODS) coated silica (C-18) and are very non polar. The eluent used with reversed phase columns is relatively polar liquid, such as mixtures of water and methanol or acetonitrile. Here, the more non-polar the material is, the longer it will be retained where as the more polar components of the mixture elute first [38, 40].

HPLC instruments consist of a reservoir of mobile phase, a pump, a degasser to eliminate bubbles, an injector, a separation column (10-25 cm long and 2.1- 4.6 mm i.d.), and a detector [38, 40].

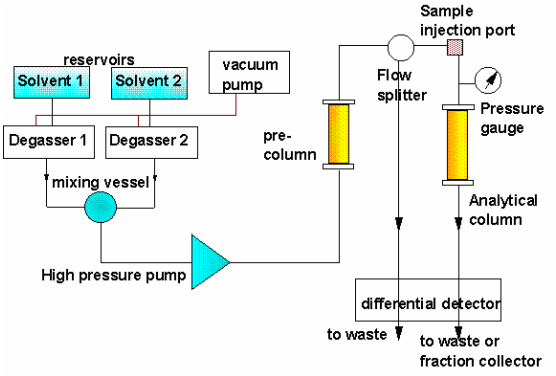


Fig. 3. Schematic of HPLC instrument

The detector for an HPLC is the component that emits a response due to the eluting sample compound and signals a peak on the chromatogram. The output of the detector is an electrical signal that is proportional to some property of the solutes and /or the mobile phase. Some of the more common detectors used with HPLC include: Ultraviolet (UV) detectors, Refractive Index (RI), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS). Most of the popular detectors in HPLC are selective [38, 40].

Ultraviolet (UV) detectors detect only those solutes that absorb UV (or visible) radiation such as alkenes, aromatics and compounds having multiple bonds between C & O, N or S. The mobile phase on the other hand should absorb little or no radiation [38, 41],

1.6. Quantitative Proton NMR Spectroscopy

Nuclear magnetic Resonance (NMR) spectroscopy is one of the most versatile analytical tools available for qualitative and quantitative analysis of all NMR-sensitive nuclei [21]. It is simple and nondestructive.

Quantitative proton NMR is the most commonly used NMR technique in the analysis of foods, pharmaceuticals, natural products etc [21]. The intensity (or area) of a sample peak is directly proportional to the number of nuclei producing

the signal, and the integration ratio of selected sample peaks with respect to that of an internal standard give the mole ratio for quantification [21, 42].

1.7. Quantitative Thin-layer Chromatography

Thin-layer Chromatography (TLC) can be applied in different areas of analysis [21, 42]. Accurate identification of the spot position and quantitative estimation of its content has been developed by *in situ* scanning of TLC plates employing optical instrumentation. In most instruments, the plate surface can be examined for the light absorbed, reflected, scattered or fluorescent light from the spot. Densitometric evaluation using Camag TLC scanner 3 is the common technique used in quantitative analysis [21, 42].

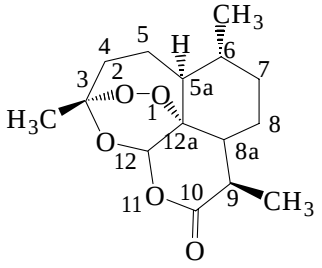
1.8. Objectives of the Coffee Project

The main goal of this project is to develop and validate high performance liquid chromatography (HPLC) system to enable the quantification of caffeine routinely in different types of green, roasted, home- and coffee-house brewed coffee samples.

2. Chinese wormwood (*Artemisia annua)*

Backgrounds

Artemisia annua L. (sweet or annual wormwood, family Asteraceae) grows wild in China. Artemisinin (**8**) is a sesquiterpene lactone with an endoperoxide oxygen bridge across the seven member rings which is the main active constituent of the plant [42, 43]. It is a promising and potent antimalarial drug. It is now being used as a new type of antimalarial drug against chloroquine-resistant malaria [42].



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Artemisinin is usually isolated from the aerial parts of *Artemisia annua* L. The natural Artemisinin content found in the leaves and flowers of *A. annua* is approximately in 0.01-1.1% dry weight, with the highest content just before flowering [42].

The techniques that are reported to determine Artemisinin in *Artemisia annua* include quantitative HPTLC, quantitative TLC, HPLC with UV, ELSD, MS, chemiluminescence and electrochemical detection, GC and GC-MS and QHNMR [42, 43].

The compound is present in low concentrations in the plant and it is thermolabile (>150^{0}C), unstable and acid sensitive that lacks chromophore. This makes its determination difficult [42].

Objectives of the Chinese wormwood Project

The task of this analysis is to develop a quick method of determining artemisinin levels in *Artemisia annua* leaves introduced recently in Ethiopia.

3. MATERIALS AND METHODS

3.1. Caffeine Content Determination

3.1.1. Coffee Samples, Chemicals and Standards

Ten samples were used in this work. Three green coffee bean samples were obtained from private shops at different times. Some amount from the two green coffee bean samples was roasted. All samples were milled to powder. Abyssinia Coffee (a commercially roasted, ground coffee sample) was bought from a super market. Two home brewed coffee samples (*Abol* and *Bereka*) were obtained privately, and two coffee-house brewed samples were taken from common room of Chemistry Department and Cordial Cafeteria, in front of Science Faculty.

Caffeine (99%), Theophylline (99%) and deuterated water (99.8%) were brought from Sigma-Aldrich (St. Louis, Germany) while magnesium oxide was purchased from British Drug House (BDH, England), analytical grade chloroform (99.98%) and Acetone (99.99%) (Fisher chemicals, UK) and, methanol (99.5%) and HPLC grade acetonitrile (99.9%) (Riedel-de Haën, Germany), distilled and deionized water (AAU, Professor Wendimagegn Mammo Laboratory).

3.1.2. Preparation of Standards and Samples

Two stock solutions were prepared for HPLC by dissolving appropriate amount of the compounds in MeOH:H_2O (1:1) for the preparation of standard solutions and spike analysis. Stock solution **1**: 10 mg of pure caffeine was dissolved in 10 mL of MeOH:H_2O (1:1) to give 1 mg/mL solution. Stock solution **1** was diluted to obtain 10, 20, 40, 45, 80,100 µg/mL caffeine standard solutions. Stock solution **2**: 10 mg of theophylline in 10 mL of MeOH:H_2O (1:1)(1 mg/mL) was prepared. This was diluted into 15 mL volume with the same solvent system as internal standard. For TLC analysis, two standards were prepared. Stock solution **3**: 10

mg of caffeine was dissolved in 10 mL of chloroform (1 µg/µL) and used as a stock solution. Stock solution **4**: stock solution **3** was diluted with chloroform 1:10 (100 ng/µL). For QHNMR analysis, one standard solution was prepared. Stock solution **5**: 10 mg of theophylline was dissolved in 25 mL acetone (0.4 mg/mL). All solutions were stored at 24^0C.

Green, roasted and brewed coffee samples were extracted according to the method cited elsewhere [21] as follows: 100 mg of dried and ground green or roasted coffee sample was mixed with 100 mg of MgO and 1 mL of distilled water in a round bottom flask. The mixture was heated on steam bath for 20 min. To the residue, 5 mL chloroform was added and extracted by sonication for 15 min. The mixture was filtered through Whatman No.1 filter paper and washed with chloroform twice. Some anhydrous Na_2SO_4 was added to the filtrate as a dehydrating agent. After some time the solution was filtered and the final volume was adjusted to 15 mL with chloroform. Brewed coffee samples (5 mL each) were taken and 100 mg of MgO was added to each. The mixture was freeze dried by Christ Alpha 1-2 for 6 h. To each residue, 5 mL chloroform was added, and then follows the same procedure as above.

For HPLC-UV determination, the chloroform extract was dried on rotary evaporator and weighed. It was dissolved in 10 mL volumetric flask using MeOH:H_2O (1:1). To 1 mL of the extract solution, an equal volume of theophylline (66 µg) as internal reference was added to the HPLC vial. 10µL solutions of each sample were injected into the chromatograph. For QHNMR analysis, the filtrate was dried in a rotary evaporator; a known amount of theophylline reference solution (0.4 mg/mL in acetone) was added to it and dried. The mixture was then dissolved in deuterated water to 0.6 mL volume and transferred to NMR tube. For TLC analysis, the filtrate was directly used as a test solution.

3.1.3. Apparatuses and Experimental Conditions

Analytical High Performance Liquid Chromatographic Analysis. The chromatographic analysis were carried out on the LC system consisting of a high pressure gradient control HPLC pump (Model 600, Waters), a degassing system, an Auto sampler (Model 717plus, Waters), and a photodiode array detector (PDA, Model 996, Waters) with a 10 mm path length flow cell. Separations were performed on a reversed-phase C-18 pre-packed analytical column (RP-18 Lichrospher®, Waters, 5 μm, 4.6 X 150 mm i.d.). Data were collected, stored and analyzed on a computer-based data system (Millennium ^{32} soft ware version 3.20, Waters) managed by Workstation 4.0.

A solution of two solvents acetonitrile-water (ACN: H_2O, 30:70, v/v) were used as mobile phase at 0.8 mL/min flow rate for 35 min elution time. And 10 µL of sample was injected. The elution condition was isocratic. All standards and samples were injected three times. The optimum temperature and pressure for separation were 25^0C and 900-1030 psi, respectively. The instrument was monitored for about 50 min using distilled and deionized water and 20 min with the mobile phase before starting running any analyte. The UV detection of chromatograms was carried out at maximum wave length of 268.9 and 271.3 nm corresponding to theophylline and caffeine, respectively.

Quantitative Proton NMR (QHNMR) Analysis. The proton NMR experiments were performed on a Bruker ACQ 400 AVANCE spectrometer operating at 400 MHz equipped with a 5 mm multinuclear probe and running Topspin 1.3 software at 298 K. The spectra of samples were recorded in neutral D_2O media. Typical acquisition parameters for ^{1}H NMR experiments were as follows: acquisition time 4.0 s, spectral width 8278.146 Hz, relaxation delay 1 s and number of scans 4.

The spectral data processing included Fourier transformation (FT) of the free induction decay (FID) data using MestRe-C software, phase correction and

baseline correction for the entire spectral range. Areas of peaks were determined by electronic integration of expanded regions of selected resonances. The procedures and conditions were as referenced elsewhere [21].

Quantitative Thin-layer Chromatographic (QTLC) Analysis. Samples and standards were applied on pre-coated TLC plates (aluminium sheets, silica gel 60 F_{254}, 10x10 cm, 0.25 mm layer thickness, Merck) as 8 mm bands, 14 mm distance between tracks using the 'spray on' technique. A Camag Linomat 5 (Switzerland) was used for sample application. TLC plates were developed with chloroform: methanol (10:1, v/v) over a distance of 50 mm from the lower edge of the plate using Camag twin trough chamber (Switzerland), saturated for 10 min with the mobile phase. The developed plates were then dried in air for 5 min.

The quantification of caffeine was performed by densitometric evaluation in absorption mode at 276 nm using deuterium and tungsten lamps using Camag TLC scanner 3. The size of the scanning slit was adjusted to 6.0 x 0.45 mm and the scanning speed to 20 mm/s at a data resolution of 100 µm/step. The peak heights and areas of chromatograms were determined using winCATS Software. Separation conditions and methods of analysis were as written elsewhere [21].

3.1.4. Calibration Curve Preparation in HPLC Analysis

The calibration curve was determined by using four different concentrations of standard solutions of caffeine. To a known amount of theophylline internal reference solution (66 µg/mL): 20, 45, 80, 100 µg/mL of caffeine standard solutions were added in to a vial that fit to the Auto sampler and the mixtures were analyzed by the proposed method. Each calibration standard solution was injected three times. The calibration curve was generated by plotting the peak area ratio of the analyte relative to the internal standard as a function of the concentration of the standard caffeine.

The concentration of caffeine in samples was calculated using the regression equation of the calibration curve in the linear range within 20-100 µg/mL caffeine. In this project the peak area measurements were used for all calculations.

3.1.5. Precision, Accuracy and Specificity in HPLC Analysis

The intra-day and inter-day variability of the method for the determination of caffeine was analyzed at three concentration levels of 20, 40 and 100 µg/mL and it was expressed in terms of percent relative standard deviation (%RSD).

The accuracy of the procedure was evaluated by means of recovery experiments carried out from spiked samples at different analyte concentration levels. Three pre-analyzed samples were spiked with extra 10, 20, 40 µg/mL of the standard caffeine and the mixture was re-analyzed.

The peak for caffeine in the sample was identified by comparing the retention time values and the ultraviolet spectra (at cut-off filter wave length, 271 nm) of the sample with that of authentic standards. No other peaks were observed.

In the meanwhile, the percentage of caffeine in green and roasted coffee samples and its amount (in mg) per cup (150 mL) of the brewed coffee samples were determined.

3.2. Artemisinin Level Determination

3.2.1. Chinese wormwood Samples, Chemicals and Standards

Fresh, green-yellowish leaves of *Artemisia annua* were collected from NAPRECA garden, Addis Ababa University, Science Faculty and allowed to dry in an oven at 35^0C overnight.

Artemisinin, anisaldehyde, sulfuric acid, toluene, acetic acid, ethyl acetate, hexane, ethanol, acetone (99.99%) (Fisher) and chloroform (99.98%) (Fisher), 4-(Dimethylamino) benzaldehyde, MeOH, acetonitrile were used.

3.2.2. Preparation of Standards and Samples

Stock solution **1**: 10 mg of pure artemisinin in 100 mL toluene (100 ng/µL) for TLC analysis. Stock solution **2**: 20 mg pure artemisinin was reconstituted in 5 mL acetonitrile (4 mg/mL). Stock solution **3**: Stock solution **2** was diluted eight times to obtain 500 µg/mL artemisinin for HPLC analysis.

Artemisinin was extracted based on the method cited [42] as follows: the dried leaves of the plant were milled to powder. 200 mg of the powder (30 g) was extracted with 10 mL toluene by sonication for 10 min followed by centrifugation. For TLC analysis, the supernatant is directly used as a test solution.

3.2.3. Apparatuses and Experimental Conditions

Analytical High Performance Liquid Chromatographic Analysis. The chromatographic analysis was carried out on the LC system described above (3.1.3.). The mobile phase was isocratic and composed of 60% acetonitrile and 40% of a 0.1% aqueous acetic acid (pH 3.2) with a flow-rate of 1.0 mL/min and a data collection window of 15 min. And 10 µL of sample was injected at 25^0C. The

instrument was monitored for about 20 min using the mobile phase before starting running the analyte. The UV detection of chromatograms was carried out (three times) using photodiode array detector (PDA) at 192 nm without derivatization. The chromatographic conditions were as cited elsewhere [43].

Quantitative Thin-layer Chromatographic (QTLC) Analysis. Since this analysis was a continuation [42], the instrumentation and chromatographic conditions were the same. Samples and standards were applied on pre-coated TLC plates (aluminium sheets, silica gel 60 F_{254}, 10x10 cm, 0.25 mm layer thickness, Merck) as 8 mm bands, 14 mm distance between tracks using the 'spray on' technique. A Camag Linomat 5 (Switzerland) was used for sample application. TLC plates were developed with cyclohexane, ethyl acetate and acetic acid (20:10:1 v/v/v) over a distance of 70 mm from the lower edge of the plate using Camag twin trough chamber, saturated for 20 minutes with the mobile phase. The developed plates were then dried in a stream of cold air for 5 minutes. The dried Plates were dipped into freshly prepared anisaldehyde reagent for two seconds. After waiting for 1 min to allow complete absorption of the reagent, the plates were heated using Camag-TLC plate heater 3 at 100^0C to visualize the bands of standard and sample artemisinin. Evaluation of the chromatogram was performed by UV at 366 nm.

The quantification of artemisinin was performed by densitometric evaluation in fluorescence mode at 520 nm using deuterium and tungsten lamps. A Camag TLC scanner 3 was used for scanning the plates. The size of the scanning slit was adjusted to 4.00 x 0.20 mm and the scanning speed to 20 mm/s at a data resolution of 100 µm/step. The peak areas of chromatograms were determined using winCATS software.

3.2.4. Derivatizing Reagents

The following reagents were used to derivatize artemisinin prior to TLC analysis.

i. Modified anisaldehyde reagent: a mixture of acetic acid (10 mL) and sulfuric acid (2 mL) was added into a mixture of ethanol (50 mL) and distilled water (40 mL) followed by addition of 1 mL anisaldehyde.

ii. A solution of 4-(Dimethylamino) benzaldehyde (0.5 g), conc.H_2SO_4 (0.5 mL), glacial AcOH (3 drops) and 95% EtOH (18 mL), and the plate heated slowly to 70 ^{0}C after spraying.

iii. A solution of 10% sulfuric acid in methanol by dipping

Finally, the amount of artemisinin in the plant was quantified with preferred derivatizing reagent.

4. RESULTS AND DISCUSSION

Ethiopia produces 260,239,200 Kg Coffee on 3,912,960,000 square meter land annually [44]. Coffee is important raw material within the international trade and the first export product in Ethiopia in which 30% of the total export derived income was generated by coffee [45]. Caffeine is one of the responsible components for coffee flavor, its excessive intake can cause health problems, and thus it affects the quality of coffee. Analysis of caffeine in Coffee is used as a tool for evaluating its quality. Therefore, establishing an analytical method for the determination of caffeine in coffee is of interest.

4.1. Analysis of Caffeine Levels Using Analytical HPLC

Establishment of the Optimum Conditions

The HPLC method was established to separate the caffeine peak from the rest of the chromatogram, and to quantify it in the coffee samples by considering pressure, mobile phase type and composition, flow rate, column type, detection wavelength and temperature.

The LC-UV chromatograms obtained by direct injection of a sample and a standard (66 µg/mL) (Fig. 4) using the optimized mobile phase composition showed its capability to resolve the target compound. Good resolution with well-defined peaks was obtained for caffeine in the extracts and standards at the optimized conditions.

The proposed method allowed the identification of caffeine in coffee samples. It was performed by comparison of the retention time of peaks in the standard and sample, and confirmed by the addition of the standard caffeine to the sample. Caffeine elutes at about 5 min in 35 min running time. The UV spectra of caffeine in the standard and sample were also used for qualitative analysis of the peaks in the chromatogram. The chromatograms were obtained with almost the same

retention time and λ_{max} as their standard reference that show the peaks identity. This condition was applied to the determination of caffeine in coffee samples.

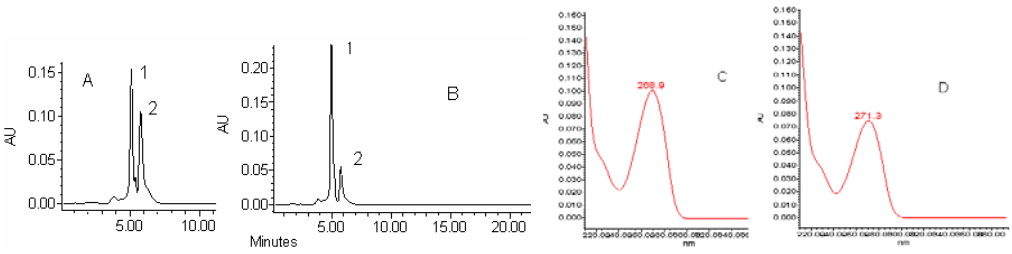


Fig. 4.Typical HPLC Chromatograms of A: Caffeine in coffee sample to which theophylline reference has been added B: Of reference samples of caffeine and theophylline at optimum conditions. Peaks 1: Theophylline 2: Caffeine C: The characteristic UV spectra of theophylline and D: Caffeine in the reference and extract generated online at the experimental conditions

Determination of Caffeine Levels in Samples

Quantitation was performed by using theophylline as internal standard. The area of the peaks was measured. The relative peak area ratio of the analyte to the internal standard was used for quantitative analysis, which is calculated as:

$$\text{Relative peak area ratio} = \frac{\text{Peak area of caffeine}}{\text{Peak area of theophylline}} X \text{ Concentration of theophylline}$$

Since the concentration of theophylline in the HPLC vial was known (66 µg/mL), the concentration of caffeine (in µg/mL) in each sample vial was determined from the calibration curve by the software using the relative peak area ratio obtained.

The caffeine content of the raw/roasted sample was calculated by

$$\% \text{ caffeine} = \frac{C}{M} 100\%$$

Where C is Amount of caffeine obtained in mg (Conc. from calibration curve (µg/mL) times 10 mL extract volume times 10^{-3}). M is mass of weighed ground coffee (in mg)

The amount of caffeine in brewed samples was calculated per cup (150mL) of brewed coffee.

In order to evaluate the applicability of the method: green, roasted and brewed coffee samples were analyzed. A resolved peak was observed in their chromatograms along with the internal standard. By the optimized HPLC method, the retention time and the relative peak area of the extracted caffeine and that of the standard caffeine were almost similar, which confirms the identity of caffeine peak.

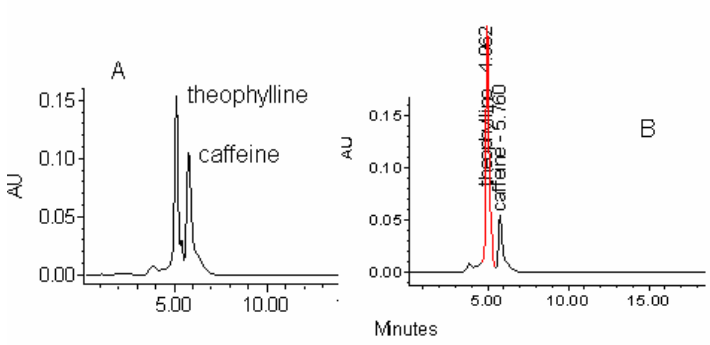
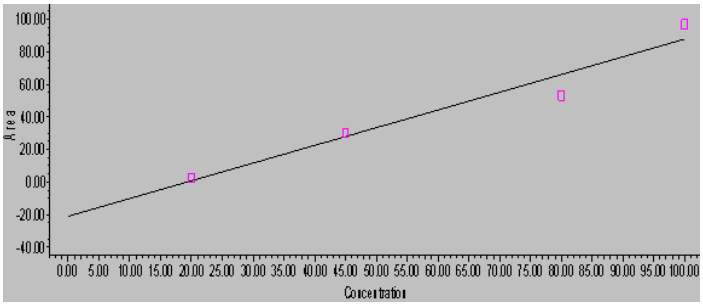


Fig. 5. HPLC-UV Chromatograms of caffeine when the proposed method applied to samples (A) and reference (B) at 271 nm to which theophylline was added as internal reference.

The calibration curve is used to determine the concentration of the extracted caffeine in samples from their relative peak area (Tables 5 & 6).

Calibration Curve

The peak area ratios of the caffeine standards (ranged from 20-100 µg/mL) relative to the internal standard versus caffeine standard concentrations allowed the preparation of the calibration plot and develop regression equation. The peak area increases from the lowest standard to the highest. The peaks were identified at retention time of around 5 min. The slope of the curve was linear which was used to determine caffeine in the coffee extract at 271 nm. The developed method for estimation of caffeine showed good correlation coefficient (r = 0.903470) in the concentration range with respect to the peak area. The mean value (± S.D) of slope and intercept were 1.12 ± 0.09 and -22 ± 8, respectively.



Linear Regression Equation:

P= A + B * C P= peak area ratio (caffeine: theophylline X 66 µg/mL)

A = Intercept

B = Slope

C = Concentration of caffeine (µg/mL)

parameter	value	error
A	-22	1.7
B	1.1	0.01

R	SD	N
0.90347	2.2	4

Fig. 6. Calibration curve for 20 - 100 µg/mL caffeine standard concentration in 70:30 H_2O:ACN.

4.2. Validation of the HPLC Method

Repeatability

In order to express the precision under the same operating conditions over a short interval of time, repeatability of caffeine concentration was determined by intra-and inter-day assay and expressed as %RSD. Three different concentration of caffeine were analyzed three times for the intra- and inter-day assay. The %R. S. D. for repeatability of measurement of peak areas were ranged from 2.4-6 for intra-day and 0.8-14 for inter-day variation. The results of intra-day assay showed lower values of %R. S. D than that of inter-day.

Table 3. Intra- and Inter-day precision of HPLC method (n=3)

Concentration analyzed (µg/mL)	Intra-day (n=3)		Inter-day (n=3)	
	Amount found (µg/mL) Mean± SD	%RSD	Amount found (µg/mL) Mean ± SD	%RSD
20	18 ± 1	5.5	16.8 ± 2.5	14
40	37.5 ± 2.25	6	35 ± 3	9
100	90.5 ± 2.25	2.4	90 ± 0.8	0.8

Recovery Study

To check the method performance for caffeine determination, the accuracy of the quantification was analyzed by calculating the percentage recovery of the known amounts in a pre-analyzed sample. Three samples with pre-determined caffeine content were individually spiked with three different amounts of caffeine. The proposed method, when used for estimation of caffeine, afforded recovery of 93 %.

Table 4. Results of Recoveries (n = 3)

Predetermined concentration in sample (µg/mL)	Concentration added (µg/mL)	Theoretical concentration (µg/mL)	Concentration found (mean ± SD) (µg/mL)	Recovery %
80	10	90	81 ± 0 .3	90
71	20	91	84 ± 2	92
45	40	85	82 ± 0.3	96

4.3. Quantitative ^{1}HNMR Analysis of Caffeine in Samples

The samples were analyzed by Q^{1}HNMR for comparison by the procedure and conditions cited. The spectrum of theophylline exhibited two characteristic sharp singlet peaks at δ 3.24 and 3.43 that belong to the methyl groups linked to the nitrogen atoms as well as a singlet peak at δ 7.89 corresponding to the =C-H proton. The spectrum of caffeine consisted of three sharp singlets at δ 3.22, 3.40 and 3.83 due to the three methyl groups connected to the nitrogen atoms as well as a singlet peak at δ 7.78 corresponding to the =C-H proton. In all spectra, a sharp singlet peak was observed at δ 4.70 corresponding to water. The methyl signals of theophylline at δ 3.24 and 3.43 overlap with that of caffeine.

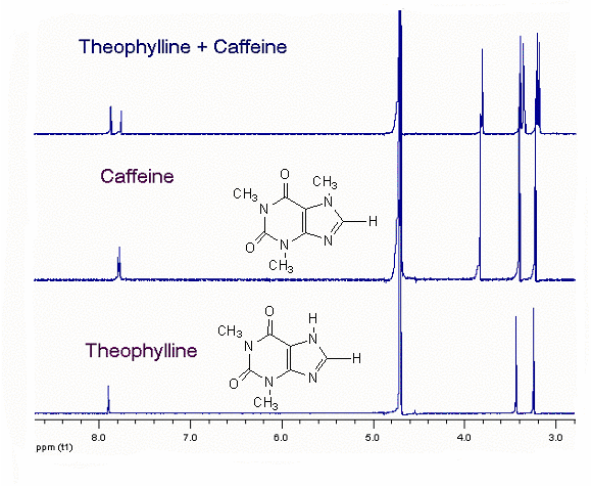


Fig. 7. Proton NMR spectra of theophylline, caffeine and sample containing theophylline and caffeine.

For quantification purpose, the spectral region showing well-resolved NMR peaks displaying one-to-one correspondence between caffeine and theophylline (7.72 - 7.78 and 7.82 - 7.88 ppm, respectively) was chosen and integrated.

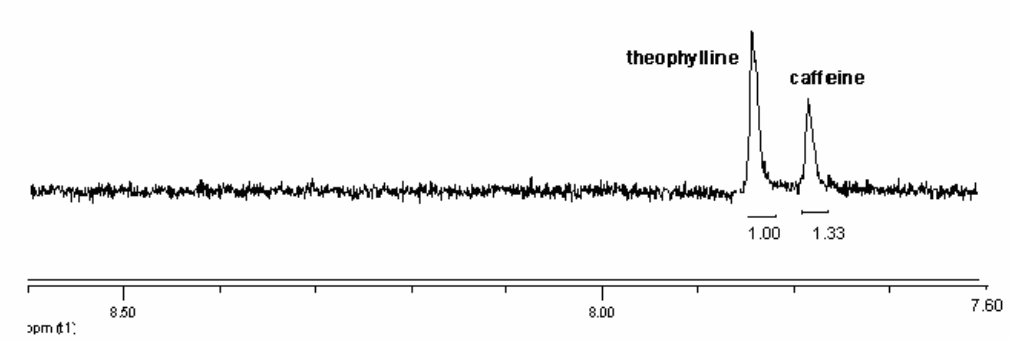


Fig. 8. Selected and integrated olefinic ^{1}HNMR spectrum of 0.4 mg/mL of theophylline and coffee extract

The integration ratio of signals belonging to different compounds with respect to those of an internal standard gives the mole ratio of caffeine and theophylline in the NMR tube. The mass of theophylline is known, so the mass of caffeine in the NMR tube can be calculated (Tables 5 & 6).

4.4. Quantitative TLC Analysis of Caffeine in Samples

The samples were analyzed by QTLC for comparison. The optimized thin layer chromatography procedure gave well-defined spots for caffeine in the standard as well as in the extracts at average retention time of 0.62. The peak identity of caffeine was assessed by comparing their UV spectra. This enables to detect and quantify caffeine in the samples (Tables 5 & 6).

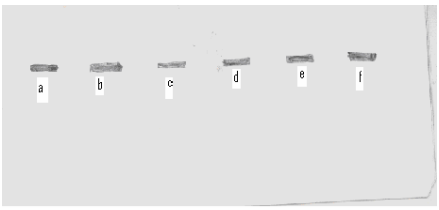


Fig. 9. TLC of caffeine standards (200 ng, **a**; 600 ng, **c**; 1000 ng, **e**) and extracts (543 ng, **b**; 373 ng, **d**; 426 ng, **f**) developed with $CHCl_3$: MeOH (10:1)

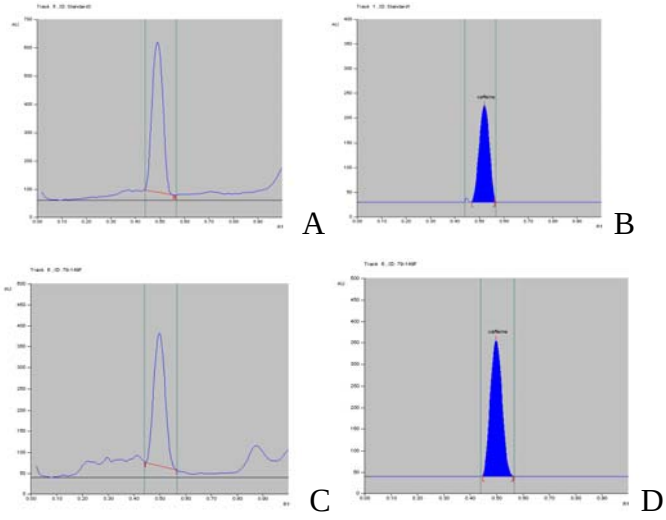


Fig. 10. Densitogram of caffeine in coffee camples (A, B) and reference (C, D) at 276 nm through baseline and peak area display.

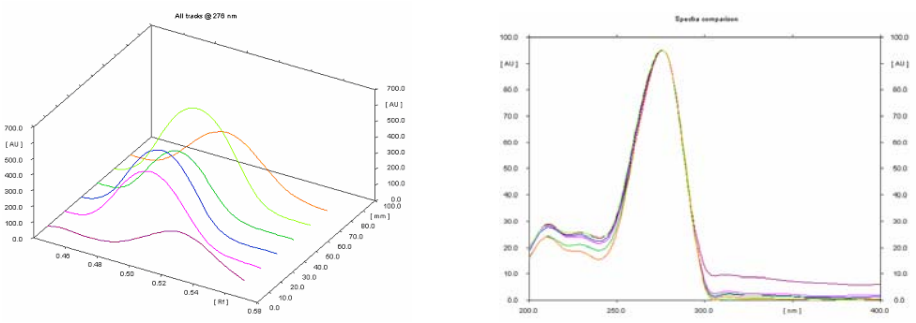
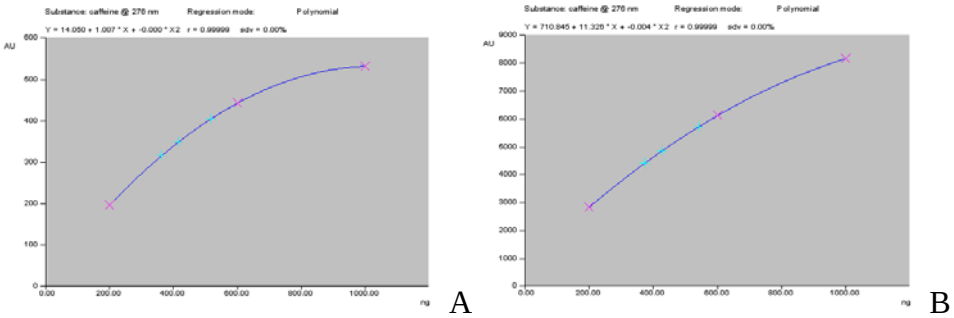


Fig. 11. The UV spectra comparison of caffeine in the standard and coffee extracts



A B

Fig.12. Quantification of caffeine in samples on TLC plate using polynomial regression mode through Height (A) and Area (B)

4.5. Summary of Caffeine Levels Determinations

In order to ensure high quality for both the coffee processor and the consumer, determination of caffeine levels is used as a clue. The contents of caffeine obtained by HPLC-UV method is ranged from 0.8-1.0% (dwb) in green (A, B, C) and roasted (D, E, F) coffee samples, and that of brewed coffee samples in mg/cup were 24-56 (coffee-house brewed) and 48-102 (home-brewed).

In general, the HPLC-UV analysis results of green and roasted coffee samples were similar in Q ^{1}HNMR and QTLC analysis, but that of brewed coffee samples were not. This variation was assumed to be due to the variation of the length of brewing time and analytical method.

Table 5. Percent caffeine contents of green and roasted coffee samples

Sample	Percentage of caffeine (dwb)		
	HPLC-UV	Q^1HNMR	QTLC
Green coffee			
A	0.9	0.7	0.6
B	0.7	0.6	0.7
C	0.8	0.6	0.6
Ground roasted coffee			
D[a]	1	0.8	0.9
E	0.9	0.8	0.8
F	0.9	0.7	0.8

[a] Commercial ground, roasted coffee sample named 'Abyssinia Coffee`

Table 6. Caffeine content of brewed coffees

Sample	Content of caffeine (mg/150 mL)		
	HPLC-UV	Q^1HNMR	QTLC
Coffee-house brewed	24	9	12
Coffee-house brewed[b]	56	10	16
Home- brewed Abol	60-102	12	17
Home- brewed Bereka	48-80	7	12

[b] Samples taken from different cafeteria at different times.

4.6. Determination of Artemisinin level in *Artemisia annua*

In the course of this project we undertook another analytical problem using similar methods as described above. Our aim was to attempt if HPLC method could be used to determine artemisinin levels in the antimalarial plant *Artemisia annua*. To determine the amount of artemisinin in the source plant there is a need for rapid analytical technique. The starting point for this work was the recent report by Ferreira, *et al*. (2009) where artemisinin in the plant can be quantified by HPLC-UV without derivatization.

The analysis of underivatized artemisinin by HPLC-UV was tested at conditions cited by Ferreira. The paper reported that the UV absorption of artemisinin (Rt= 5.05 min) and its related compounds is sufficiently high to allow their quantification from crude plant samples and artemisinin-based pharmaceutical preparations by UV detection without derivatization at 192 nm.

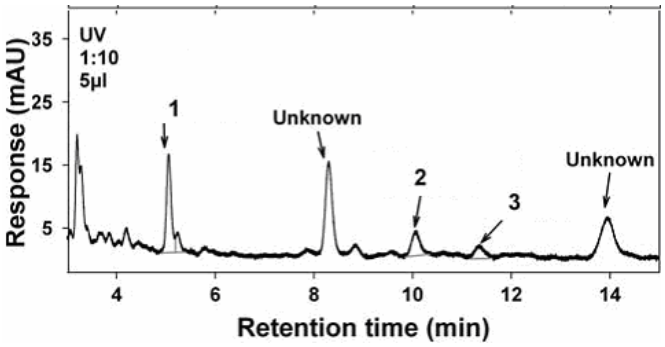


Fig.13. HPLC chromatogram of a greenhouse-grown, oven-dried, *Artemisia annua* analyzed by HPLC-PDA at 192 nm. In a 10.0 µL injection of 1:10 dilution of the sample, artemisinin (**1**) was present at 12 µg/mL

However, 500 µg/mL of artemisinin was injected into the Waters HPLC-UV under the same conditions to afford a noised signal using isocratic mobile phase composed of acetonitrile: 0.1% aq. AcOH, P^H 3.2 (60:40) at 192 nm. This showed the difficulty of analysis of underivatized artemisinin by using UV detection.

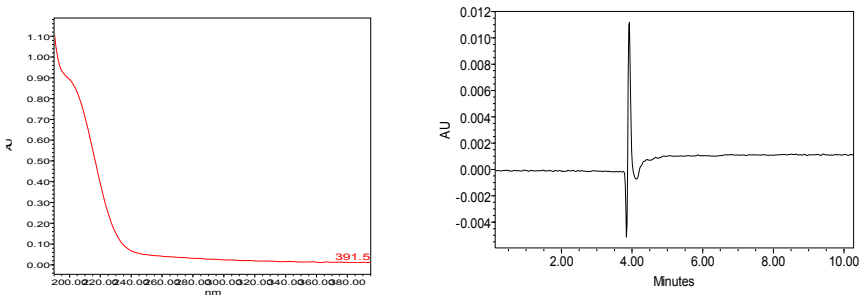


Fig.14. The UV spectra and chromatogram of pure artemisinin when HPLC-PDA method applied at 192 nm.

The TLC method reported recently by Tsegaye (2008) was reinvestigated. TLC method to be used for quantification must be able to resolve the principal compound to avoid interference during detection. The solvent showing good resolution of artemisinin was found to be cyclohexane, ethyl acetate, acetic acid (20:10:1 v/v/v). Since artemisinin lacks an appropriate chromophoric group responsible for UV absorption, derivatization must be required to change it into UV-active compound so that detection is possible.

The quantitative TLC procedure developed was tried using different derivatizing reagents. These are anisaldehyde, 4-(dimethylamino)benzaldehyde and sulfuric acid reagents. However, visualization reagent with anisaldehyde gave better spot for quantification where artemisinin was detected at R_f value of 0.67. An ordinary TLC was employed as stationary phase in this study. The densitogram of artemisinin in the crude extract is shown as a baseline and peak area display in Figure 15.

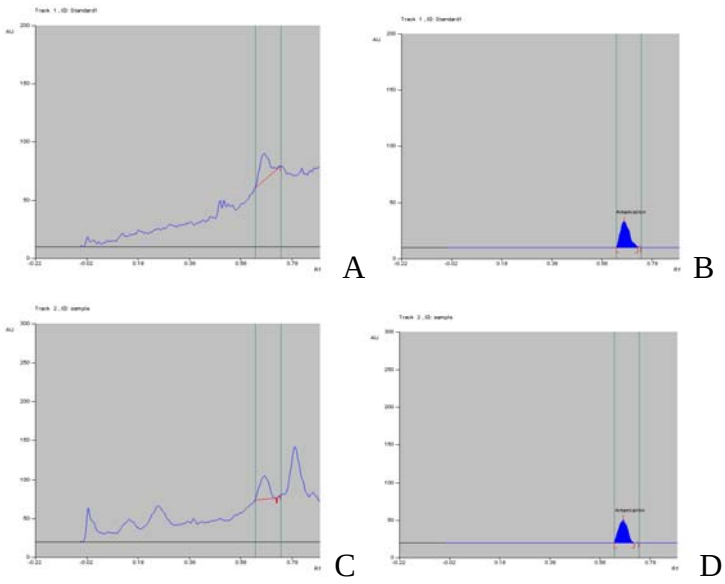


Fig.15. Densitogram of standard artemisinin (A, B) and *A. annua* extract (C, D) through baseline and peak area display.

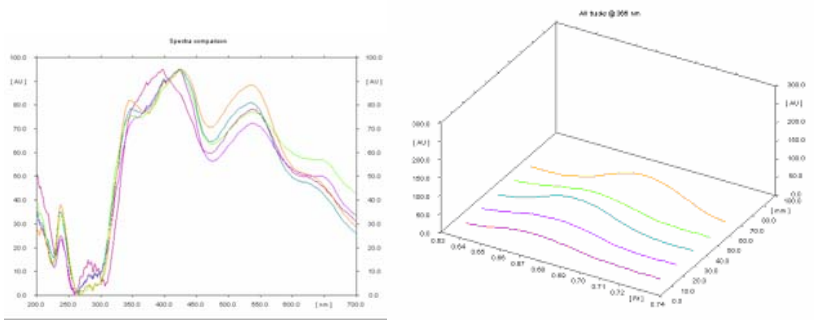


Fig.16. The UV spectra comparison of artemisinin in the standard and extract

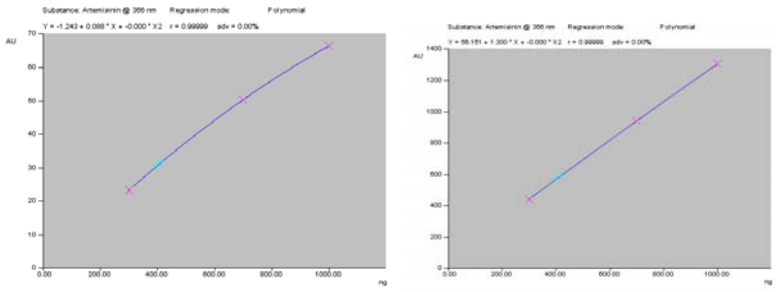


Fig.17. Quantification of artemisinin in samples on TLC plate using polynomial regression mode through Height and Area

The mass of artemisinin in the applied volume was determined as 399 ng via area. The total mass of artemisinin in the total volume of the extract solution (10 mL) was 2 mg. The percentage of artemisinin in the plant was determined to be 1% by the Camag QTLC method of analysis.

Table 7. Substance summary for artemisinin quantification using Camag-TLC

Substance: Artemisinin at 366 nm Regression mode: Polynomial

Regression via area $Y= 56.15 + 1.3X + 5.18 X 10^5x^2$ r=0.99999 sdv = 0.0%

Track	vial	Rf	Amount of fraction	Area (AU)	X(calc)	Remark
1	1	0.67	300 ng	441		Std level1
2	1	0.68	700 ng	940		Std level2
3	2	0.67		567	399 ng	sample
4	1	0.69	1000 ng	1304		Std level3

5. CONCLUSION AND RECOMMENDATION

HPLC coupled to PDA with microwave assisted extraction as efficient sample preparation technique was optimized. The results indicate that the technique proposed herein allows determination of the caffeine level in coffee samples. An easily adaptable method for both qualitative and quantitative determination of caffeine from coffee was developed.

The closeness of the values between HPLC and other techniques such as Q ^{1}HNMR and QTLC can be used to check the method sensitivity for the sample. Further experiments and more data are necessary to ensure the reproducibility of HPLC results of samples with that of Q ^{1}HNMR and QTLC.

Determination of other components of coffee such as chlorogenic acid, trigonelline and sucrose gives clue about its quality.

The absence of chromophore in artemisinin makes its detection challenging. So artemisinin should be converted into UV active compound before HPLC analysis. Artemisinin can be quantified better using the camag QTLC with anisaldehyde visualizing reagent.

6. REFERENCES

[1] Ermias, D. **2008**. Ten outstanding natural products of Ethiopia. Conference proceeding, ALNAP, AAU, pp15-16 Id 25327.[e]

[2] Clifford, M. N.; Gibson, C. L.; Rakotomalala, J. J. R.; Crost, E.; Charrier, A. **1991**. Caffeine from green beans of *Mascarocoffea*. *Phytochemistry* **30**, 4039-4040.[b]

[3] Martin, M. J.; Pablos, F.; Gonzalez, A. G. **1998**. Characterization of Green coffee varieties according to their metal content. *Analytica Chimica Acta* **358**, 177-183.[a]

[4] http;//www.uga.edu/fruit/coffee.html.[a]

[5] Abebe, B.; Kassahun, T.; Mesfin, R.; Araya, A. **2008**. Measurement of Caffeine in coffee beans with UV/vis spectrometer. *Food Chemistry* **108**, 310-315.[b]

[6] Ky, C. L.; Louarn, J.; Dussert, S.; Guyot, B.; Hamon, S.; Noirot, M. **2001**. Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild Coffea arabica L. and C. canephora P. accessions. *Food Chemistry* **75**, 223-230.[b]

[7] Rodrigues, C. I; Marta, L.; Maia, R.; Miranda,M.; Ribeirinho, M; Maguas, C. **2007**. Application of solid phase extraction to brewed coffee caffeine and organic acid determination by UV/HPLC. *Journal of food composition and analyis* **20**, 440-448.[a]

[8] Silvarola, M. B.; Mazzafera, P.; Alves, de Lima, M. N. **2000**. Caffeine content of Ethiopia *coffea arabica* beans. *Genetics and molecular biology* **23**, 213-215 Id 25474.[e]

[9] Buffo, R. A.; Freire, C. C. **2004**. Coffee flavor: An overview. *Flavour and fragrance journal* **19**, 99-104.[b]

[10] Ranheim, T.; Halvorsen, B. **2005**. Coffee consumption and human health-beneficial or detrimental? - Mechanisms for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 Diabetes mellitus. *Molecular Nutrition & Food Research* **49**, 274-284.[b]

[11] Yigzaw, D.; Labuschagne, M. T.; Osthoff, G.; Herselman, L. **2007**. Variation for green bean caffeine, chlorogenic acids, sucrose and trigonelline contents among Ethiopian arabica coffee accessions. *SINET: Ethiopian Journal of Science* **30**, 77-82.[b]

[12] Pavia, D. L.; Lampman, G. M.; Kriz, G. J. **1976**. Introduction to organic Laboratory techniques: a contemporary approach. W. B. Saunders Company, Philadelphia, USA, pp. 54-65.[b]

[13] Perrone, D.; Donangelo, C. M.; Farah, A. **2008**. Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. *Food Chemistry* **110**, 1030-1035.[b]

[14] Fujioka, K.; Shibamoto, T. **2008**. Chlorogenic acid and caffeine contents In various commercial brewed coffees. *Food Chemistry* **106**, 217-221.[b]

[15] Silveira,T. M. L.; Tavares, E.; Gloria, M. B. A. **2007**. Profile and levels of bioactive amines in instant coffee. *Journal of Food Composition and Analysis* **20**, 451-457.[a]

[16] Dupas, C. J.; Marsset-Baglieri, A. C.; Ordonaud, C. S.; Ducept, F. M. G.; Maillard, M.-N. **2006**. Coffee antioxidant properties: effects of milk addition and processing conditions. *Journal of Food Science* **71**, S252-S258.[b]

[17] Singh, D. K.; Sahu, A. **2006**. Spectrophotometric determination of caffeine and theophylline in pure alkaloids and its application in pharmaceutical formulations. *Analytical Biochemistry* **349**, 176-180.[b]

[18] Heaney, R. P. **2002**. Effects of caffeine on bone and the calcium economy. *Food and Chemical Toxicology* **40**, 1263-1270.[b]

[19] Nawrot, P.; Jordan, S.; Eastwood, J.; Rotstein, J.; Hugenholtz, A.; Feeley, M. **2003**. Effects of caffeine on human health. *Food Additives and Contaminants* **20**, 1-30.[b]

[20] Smith, A. **2002**. Effects of caffeine on human behavior. *Food and Chemical Toxicology* **40**, 1243-1255.[b]

[21] Belete, A. **2008**. Detrmination of caffeine level in coffee. M.Sc.Thesis,

AAU.[e]

[22] Dewick, P. M. **2001**. Medicinal natural products: a biosynthetic approach. John Willey & Sons, West Sussex, UK, pp. 392-396.[c]

[23] Barone, J. J.; Roberts, H. R. **1996**. Caffeine consumption. *Food and Chemical Toxicology* **34**, 119-129.[b]

[24] Farah, A.; Monterio, M. C.; Calado, V.; Franca, A. S.; Trogo, L. C.**2006**. Correlation between cup quality and chemical attributes of Brazilian coffee. *Food Chemistry* **98**, 373-380.[b]

[25] Huck, C. W.; Guggenbichler, W.; Bonn, G. K. **2005**. Analysis of caffeine, theobromine and theophylline in coffee by near infrared spectroscopy (NIRS) compared to high-performance liquid chromatography (HPLC) coupled to mass spectrometry. *Analytica Chimica Acta* **538**, 195-203.[b]

[26] Silvarolla, M. B.; Mazzafera, P.; Fazouli, L. C. **2004**. A naturally decaffeinated arabica coffee. *Nature* **429**, 826.[b]

[27] Casal, S.; Oliveira, M. B.;Ferreira, M. A. **2000**. HPLC/diode array applied to the thermal degradation of trigonelline,nicotinic acid and caffeine in coffee. *Food Chemistry* **68**, 481-485.[a]

[28] Ashihara, H; Sano, H: Crozier, A.**2008**. Caffeine and related purine alkaloids; biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* **69**, 841-856.[a]

[29] Khan, K.; Naeem, M.; Arshad, M. J.; Asif, M. **2006**. Extraction and chromatographic determination of caffeine contents in commercial beverages. *Journal of Applied Sciences* **6**, 831-834.[a]

[30] Mann, J.; Davidson, R. S.;Hubbs, J. B.; Banthorp, D. V.; Harborne, J. B. **1994**. Natural products; their chemistry and biological significance. Long man scientific and technical, Hong Kong, pp.389-445.[d]

[31] Aresta, A.; Palmisano, F.; Zambonin, C. G. **2005**. Simultaneous determination of caffeine, theobromine, theophylline, paraxanthine and nicotine in human milk by liquid chromatography with diode array UV detection. *Food Chemistry* **93**, 177-181.[b]

[32] Chen, Q.-C. ; Mou, S.-F. ; Hou, X.-P. ; Ni, Z.-M. **1998**. Simultaneous determination of caffeine, theobromine and theophylline in foods and pharmaceutical preparations by using ion chromatography. *Analytica Chimica Acta* **371**, 287-296.[b]

[33] Bewketu, M**. 2007**. Mechanism of the Electrochemical Oxidation of Caffeine: further improvement of the voltammetric method of determination of caffeine by using 1,4-benzoquinone modified carbon paste electrode. M.Sc Thesis, AAU.[c]

[34] Mumin, M. A.; Akhter, K. F.; Abedin, M. Z.; Hossain, M. Z. **2006**. Determination and characterization of caffeine in tea, coffee, and soft drinks by SPE-HPLC. *Malaysian journal of chemistry* **8**,45-51.[a]

[35] Brunetto, M. R. ; Gutierrez, L; Delgado,Y. Gallignani, M.; Zambrano, A.; Gomez, A.; Ramos, G.; Romero, C. **2007**. Detrmination of theobromine, theophylline and caffeine in cocoa samples by a HPLC method with on-line sample clean up in a switching-column system. *Food chemistry* **10**, 459-467.[a]

[36] Baumann,T. W.; Sondahl, M. R.; Waldhauser, S. M.; Kretschmar, J. A. **1998**. Non-destructive Analysis of natural variability in bean caffeine content of Laurina coffee. *phytochemistry* **49**, 1569-1573.[a]

[37] Carlos, A. B.; De Maria ;Ricardo, F. A. Moreira. **2007**. Analytical methods for caffeine. Química Nova 30. [a]

[38] Lindsay, S. **1992**. Analytical chemistry by open learning HPLC, 2nd ed, John Wiley and Sons, London, pp.1-12, 69-145,207-307.[d]

[39] Skoog, D. A.;West, D. M.; Holler, F. J. **1997**. Fundamentals of Analytical chemistry, 7th ed , Harcourt college publishers, pp.701-724.[c]

[40] http;//elchem.kaist.ac.kr/vt/chem-ed/sep/lc/lc.html.[a]

[41] Purdie, N.; Brittain, H.G. **1994**.Analytical application of circulardichroism 14: Elsevier; Amsterdam, pp. 1-13,131-154, 242-275.[d]

[42] Tsegaye, D. **2008**. Quantitative determination of artemisinin in *A.annua* L. and Arthemeter-Lumefantrin Tablet, M.Sc. Thesis, AAU.[e]

[43] Ferreira, J.F.S.; Gonzalez, J.M. **2009**. Analysis of underivatized Artemisinin and related sesquiterpene lactones by HPLC-UV. *Phytochemical Analysis*, **20**, 91-97.[a]

[44] FDRE: Central Statistical Agency. **2009**. Agricultural Sample Survey Report on Area and Production of Crops. *Statistical Bulletin* (A.A), **1**, 17[f]

[45] FDRE: Central Statistical Agency. **2004**. External Merchandize Trade Statistics. *Statistical Bulletin* (A.A), **310**, 5[f]

Availability of the above cited references

[a] References available on the web.

[b] Books and Journals found in our laboratory,217

[c] References found in Chemical Information Center, AAU.

[d] References found in science main library, AAU

[e] References found in ALNAP library, Urael, AA

[f] References found in CSA library, AA