**ADDIS ABABA UNIVERSTIY**

**COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES**

**DEPARTMENT OF CHEMISTRY**


# DEVELOPMENT OF NEW ANALYTICAL METHODS FOR THE DETERMINATION OF CAFFEINE CONTENT IN AQUEOUS SOLUTION OF GREEN COFFEE BEANS

**BY: Blen Weldegebreal**

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**June, 2016**

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**A THESIS SUBMITTED TO THE OFFICE OF GRADUATE PROGRAM OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY.**

**BY**

**BLEN WELDEGEBREAL**

**JUNE, 2016**

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## DECLARATION

I the Undersigned, declare that this thesis is my original work, the results reported in this work were obtained by research carried out by me under the supervision of my Advisors in the College of Natural Sciences, Department of Chemistry, Addis Ababa University in the academic year 2015 - 2016. This thesis has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged. No part of this work shall be published in scientific journals or reported in the media or presented at a conference without the knowledge and consent of my advisors, who are the principal scientists responsible for any publication. Furthermore if the work is published the institutional address given should be that of the Chemistry Department, AAU.

**Name: Blen Weldegebreal** **Signature**_____ **Date** _____

This thesis has been submitted for examination with my approval as university advisors.

| | **Advisors** | **Signature** | **Date** |
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## TABLE OF CONTENTS

**Contents** **Page**

## LIST OF TABLES

**Tables** **Page**

## LIST OF FIGURES

**Figures** **Page**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| CF | Caffeine |
| Conc. | Concentration |
| FT-IR | Fourier Transform Infrared Spectrophotometer |
| ATR | Attenuated Total Reflectance |
| SD | Standard Deviation |
| UV-Vis | Ultraviolet and Visible |
| NIR | Near Infrared |
| ANOVA | Analysis of variance |
| DMF | N,N-dimethylformamide |
| DCM | Dichloromethane |

## ABSTRACT

In the present work direct determination of caffeine in aqueous solution of green coffee bean was performed by using FT-IR-ATR and fluorescence spectrophotometers. In all the experiments water which is environmentally friendly and easily available everywhere was used for the extraction of caffeine from coffee beans. Experiments were performed without suffering from expensive and toxic chemicals. Caffeine was also directly determined in DMF solution using NIR spectroscopy with univariate calibration technique. This method can be used as alternative with reduced amount of organic solvent. The percentage of caffeine for the same sample of green coffee bean was determined using the three methods. The caffeine content of the green coffee bean in % w/w has been found to be 1.52 ± 0.09 using FT-IR-ATR, 1.50 ± 0.14 using NIR and 1.497 ± 0.05 using fluorescence spectroscopy. The means of the three methods were also compared by applying one way analysis of variance (ANOVA) and based on the ANOVA result at 0.05 significance level the means were not significantly different. The percentage of caffeine in green coffee bean was also determined by using previously developed UV/Vis spectrophotometric method for comparison. The caffeine content of the green coffee bean in % w/w was found 1.397 ± 0.02 using UV/Vis spectrophotometer for the same coffee sample.



**Key words:** green coffee beans, caffeine, optical methods, FT-IR-ATR, NIR, fluorescence spectroscopy

## 1. BACKGROUND

### 1.1. Origin of coffee

The name coffee is derived from the name of the province Keffa where shepherds from Abyssinia/Ethiopia discovered the coffee plant in the 6$^{th}$ century. Then coffee has become one of the most widely consumed beverages throughout the world due to its pleasant taste, aroma, stimulant effect and health benefits (Gebeyehu and Bikila, 2015). The value of coffee as a human beverage was initially recognized from the invigorating effect of wild coffee berries on goats in Abyssinia, sometime around 850 AD (Thomas et al., 2011).

Coffee is a tropical plant that grows at 600–1800 m above sea level (Buffo and Freire, 2004). The plant produces red cherry-like fruits containing two seeds, which, after being separated from the fruit pulp, are known as green coffee. Coffee cherries are the raw fruit of the coffee plant, which are composed of two coffee beans covered by a thin parchment like hull and further surrounded by pulp (Figure 1). These cherries are usually harvested after 5 years of coffee trees plantation and when the bear fruit turns red.

Figure 1. Image of the coffee plant, coffee cherries and green coffee beans (Department of Agriculture, Forestry and Fisheries, 2012).

Coffee cherries are harvested each year when they are bright-red, glossy, and firm either by selective hand-picking or non-selective stripping of whole branches or mechanical harvesting (Wang, 2012). The hand-picking method is very time-consuming, but results in a superior product quality because only ripe cherries are selected. After harvesting, the coffee fruits are separated from the pulp, which is carried out by dry or wet processing. The dry process is simple and inexpensive. The whole cherries are dried under the sun in open air, followed by the separation of the hull (dried pulp and parchment) mechanically to yield the green beans.

On the other hand, the wet process requires greater investment and more care. In the wet process, the pulp of the coffee cherries, which is made up of exocarp and mesocarp, is removed mechanically, but the parchment remains attached to the beans. After drying either under the sun or in a dryer, the parchment is removed to produce the green coffee beans.

### 1.2. Coffee species

Coffee is a plant which is a member of *Rubiaceae* family, within which it constitutes the *Coffea genus* that was created by Linnaeus, in 1737. Coffee grows wild in Africa and Madagascar and the genus comprises more than 90 different numbers of species. However, only Coffee Arabica, Robusta, and Liberica are of commercial importance (Schenker, 2000). Coffee Arabica accounts for approximately 75% while Robusta accounts for about 25% and Liberica (< 1%) of the world's production, other species are of not much commercial value (Pohl et al., 2013).

*Coffea liberica* however, was devastated during the 1940s by epidemics of tracheomycosis, due to infection by *Fusarium xylaroides*, and commercial growth of this species has effectively ceased. *Coffea canephora* has a very wide geographic distribution, extending from the western to the central tropical and subtropical regions of the African continent, from Guinea and Liberia to Sudan and the Uganda forest, with a high concentration of types in the Democratic Republic of Congo (Carneiro, 1997). *Coffea canephora* or Robusta, as it is commonly called, grows at low altitudes (about 850 m), and accounts for 80% of African coffee production. However, Robusta has also been cultivated in American and Asian countries (Vinod et al., 2006).

Both *C. arabica* and *C. canephora* are available in a large number of varieties and cultivars. The former yields green coffee seeds bigger (longer) in size, better in aroma and generally fetches a higher price. The latter is smaller, round, and yields a thicker brew (Ramalakshmi and Raghavan, 1999). Due to its more pronounced and finer flavor qualities, Arabica is considered to be of better quality and accordingly is usually the most expensive one in the world market (Valdenebro et al., 1999).

### 1.3. Coffee in Ethiopia

It is believed that the Ethiopian province of Keffa is the fatherland of this beverage. Coffee is originated in the highlands of Ethiopia, and from there spread into the Arabic world and became known in Europe during the early 17th century, at first as a medicine and then as a social drink in the Arab tradition. Drinking coffee, which in the Amharic language is called "Bunna" is an important element of cultural beverage in Ethiopia. Enormous industrial plantations are situated in the South-West of the country, but it is possible to come across single small trees literally everywhere. The peoples of the South who cannot afford genuine coffee prepare a beverage with the use of the husks of coffee grains.

In Ethiopia, there are four types of coffee production system: forest coffee, semi-forest coffee, garden coffee, and plantation coffee. Forest coffee accounts for about 10%, semi forest coffee for about 35%, garden coffee for about 35%, and plantation for about 15% (5% government, 15% private) of total coffee production in Ethiopia. 95% of the coffee produced under these systems is organic. Coffee production using chemical fertilizers and herbicides account for about only 5% of total production (Ethiopian Commodity Exchange Authority, 2008).

For Ethiopians, coffee is the most important exported merchandise which accounts for half of the value of Ethiopian exports. It is a typical global commodity because it is usually produced in developing countries and consumed in developed countries. Therefore, marketing channels extend beyond borders, and the price of coffee is basically determined at international exchange markets in New York and London (Kodama, 2007). Most of Ethiopian coffee is practically

organic, and only a fee for the organic certificate is required. The export volume of Ethiopian organic coffee is the second largest in the world next to that of Peru (Kodama, 2007).

### 1.4. Importance of coffee

Coffee is the second important raw material within the international trade, the most important foreign exchange supplier for many agricultural oriented countries, an attractive source for tax yield, and the most popular drink (Huck et al., 2005). Coffee is consumed by around 40% of the world's population, for many people, especially in western countries coffee drinking is a part of their lifestyle and an everyday habit (Pohl et al., 2013).

Due to the economic importance of coffee there is an increasing demand for proper quality control for certification of contents and substandard products. Therefore, sensitive and accurate analytical methods for both qualitative and quantitative determinations and characterization of chemical substances in coffee are required. Coffee is nowadays produced in a large number of countries worldwide.

In addition to the economic importance green coffee contains antioxidants which are good for the heart and arteries. Studies suggest that moderate coffee-drinkers have an 80% lower risk of Parkinson's disease, a 25% lower risk of colon cancer, a 50% lower risk of kidney stones and an 80% lower risk of cirrhosis of the liver. In addition, it has recently been shown that coffee may cut the risk of type II diabetes and that a decaffeinated extract of green coffee may be effective in controlling weight. Green coffee can minimize liver glucose production and enhance reverse cholesterol of high-density lipoprotein (Hala and Safaa, 2014).

Important health benefits of coffee according to the Centre of Scientific Information on Coffee (COSIC), based in Oxford, England (Illy and Pizano, 2003) are: (1) Coffee increases the level of alertness, improves short-term memory and permits better use of the prefrontal cerebral cortex. (2) Coffee has antioxidant and antitoxic properties at cellular level. (3) Coffee reduces the risk of hepatic cirrhosis and prevents the formation of gallstones. (4) Coffee provides protection against degenerative brain diseases like Alzheimer and Parkinson. (5) Coffee provides protection against

colon and skin cancers. (6) Coffee combats caries and has anti-inflammatory properties. (7) Coffee has a moderate slimming effect and improves performance in sports. (8) Coffee helps to alleviate asthma symptoms and helps to calm hyperactive children.

### 1.5. Constituents of green coffee beans

The chemical composition of green coffee mainly depends on the variety of the coffee, although slight variations are possible due to agro-climatic conditions, agricultural practices, and processing and storage. The composition of green coffee beans is given in Table 1.

**Table 1.** Chemical composition of green coffee beans, mass percent in dry matter (Solange et. al, 2011).

| Constituents | Arabica green<br>% DW | Robusta green<br>% DW |
|---|---|---|
| Caffeine | 0.8 - 1.4 | 1.7 - 4.0 |
| Trigonelline | 0.6 -1.2 | 0.3 - 0.9 |
| Soluble carbohydrates | 9 - 12.5 | 6 - 11.5 |
| Insoluble polysaccharides | 46 - 53 | 34 - 44 |
| Chlorogenic acids | 6.7 - 9.2 | 7.1 - 12.1 |
| Lipids | 15 - 18 | 8 - 12 |
| Proteins | 8.5 - 12 | 8.5 - 12 |
| Amino acids | 7.9 - 18.5 | 7.9 - 18.5 |
| Organic acids | 2.0 - 2.9 | 1.3 - 2.2 |
| Volatile aroma | Trace | Trace |
| Water | 8 - 12 | 8 - 12 |
| Ash (minerals) | 3 - 5.4 | 3.0 - 5.4 |

Coffee has many volatile and non-volatile components. In addition to caffeine, coffee contains substantial amounts of bioactive components which are a family of conjugated hydroxycinnamates, collectively referred to as chlorogenic acids, diterpenes and trigonelline (Figure 2) (Nuhu, 2013). The main chlorogenic acids are 5-O-caffeoylquinic acid (5-CQA) and its isomers 3-O-caffeoylquinic acid (3-CQA) and 4-O-caffeoylquinic acid (4-CQA) and these account for 80% of the total chlorogenic acids (Thomas, 2011). Chemical structures of bioactive components of coffee are shown in Figure 2.

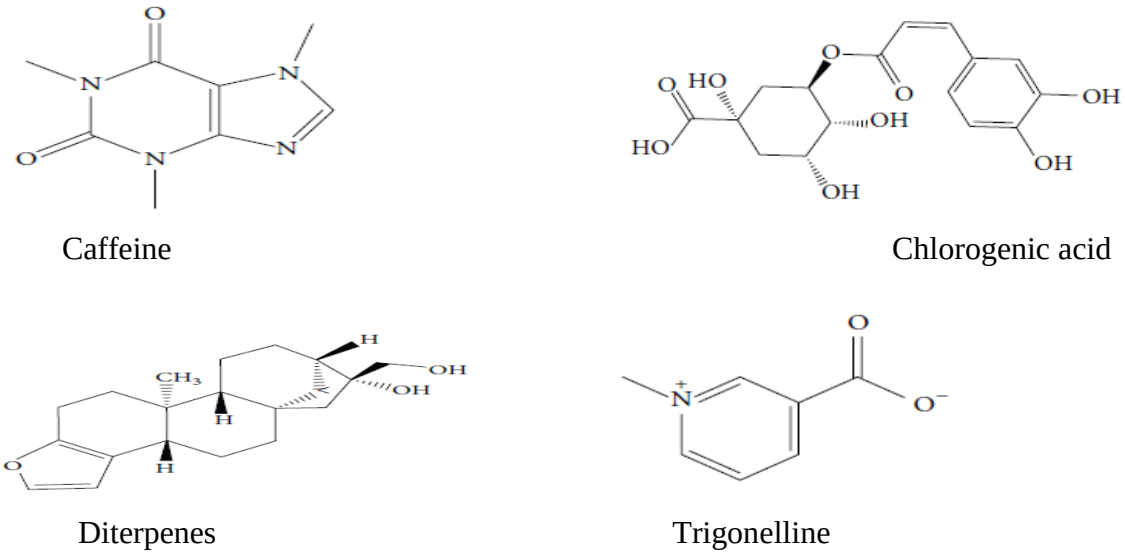



Figure 2. Chemical structures of bioactive components of coffee (Nuhu, 2014).

Among the substances present in the chemical composition of coffee, only caffeine is thermo stable, i.e., it is not destroyed by excessive roasting. Other substances such as proteins, sugars, chlorogenic acid, trigonelline, and fat may be preserved or even destroyed and transformed into reactive products during the coffee roasting process (Solange et al., 2011).

## 1.6. Caffeine

### 1.6.1. Chemical structure and sources of caffeine

Caffeine is an alkaloid, a class of naturally occurring compounds containing nitrogen and having the properties of an organic amine base (alkaline, hence, alkaloids). Caffeine have a chemical formula of ($C_8H_{10}N_4O_2$), which is chemically known as 1,3,7-trimethylxanthine (or 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione) (Nuhu, 2014). Caffeine is an alkaloid of the

methylxanthine family which is a naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide (Wanyika et al., 2010).

Pure caffeine occurs as odorless, white fleecy masses, glistening needles of powder. Its molecular weight is 194.19 g/mole, melting point 236 $^{o}$C, point at which caffeine sublimes is 178 $^{o}$C at atmospheric pressure, specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mmHg at 178 $^{o}$C; solubility in water is 2.17%, vapor density 6.7 and pH values in the range of 6 to 9 (Belay, 2011).

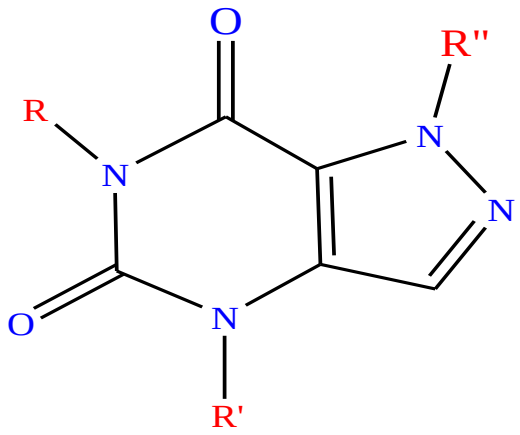
The world's primary source of caffeine is the coffee bean which is actually the seed of the coffee plant, from which coffee is brewed (Wanyika et al., 2010). The highest concentrations are found in the leaves and beans of the coffee plant, in tea, yerba mate, guarana berries, the kola nut and cocoa (Meltzer et al., 2008). In the literature the amount of caffeine found in these products have been reported as the highest amounts are found in guarana (4–7%), followed by tea leaves (3.5%), mate tea leaves (0.89–1.73%), coffee beans (1.1–2.2%), cola nuts (1.5%), and cocoa beans (0.03%) (Clifford et al., 1990).

Other naturally occurring methylxanthines include theobromine and theophyline. Coffee also contains trace amounts of theophyline, but no theobromine (Wanyika et al., 2010). Theophyline and theobromine are related compounds, characterized by only having two methyl groups each attached to environmentally different nitrogen groups. When metabolized, methylxanthines are de-methylated and excreted, the end product mainly being xanthine.

In coffee plants caffeine is synthesized from xanthosine via 7-methyl xanthosine, 7-methyl xanthine, and theobromine. *S*-adenosyl methionine (SAM) is the actual source of the methyl groups. The caffeine is degraded relatively slowly and involves demethylation steps to yield theobromine and theophyline. Theophyline is catabolized to xanthine via 3-methyl xanthine. However, it is unclear either 3-methyl xanthine or 7-methyl xanthine are intermediates in the conversion of theobromine to xanthine (Kumar et al., 2006). The chemical structure of xanthine

and its naturally occurring N-methyl derivatives (theobromine, theophyline and caffeine) are given in Table 2.

**Table 2**. The chemical structure of xanthine and its naturally occurring N-methyl derivatives (theobromine, theophyline and caffeine).

| Structure of the molecule | R | R’ | R” | Compound Name | Chemical formula |
|---|---|---|---|---|---|
|  | H | H | H | Xanthine | $C_5H_4N_4O_2$ |
| | H | $CH_3$ | $CH_3$ | Theobromine | $C_7H_8O_2N_4$ |
| | $CH_3$ | $CH_3$ | H | Theophyline | $C_7H_8O_2N_4$ |
| | $CH_3$ | $CH_3$ | $CH_3$ | Caffeine | $C_8H_{10}O_2N_4$ |



### 1.6.2. Caffeine consumption

Currently, regular daily consumption of caffeine-containing beverages is widespread throughout the world. Caffeine is provided through a number of different sources, most commonly through coffee, tea and soft drinks. It was consumed daily in coffee, tea, cocoa, chocolate, some soft drinks, energy drinks and some drugs. It is metabolized in the liver into three primary metabolites, paraxanthine (84%), theobromine (12%) and theophyline (4%) (Amos, 2014). Caffeine and its main metabolic products are shown in Figure 3.

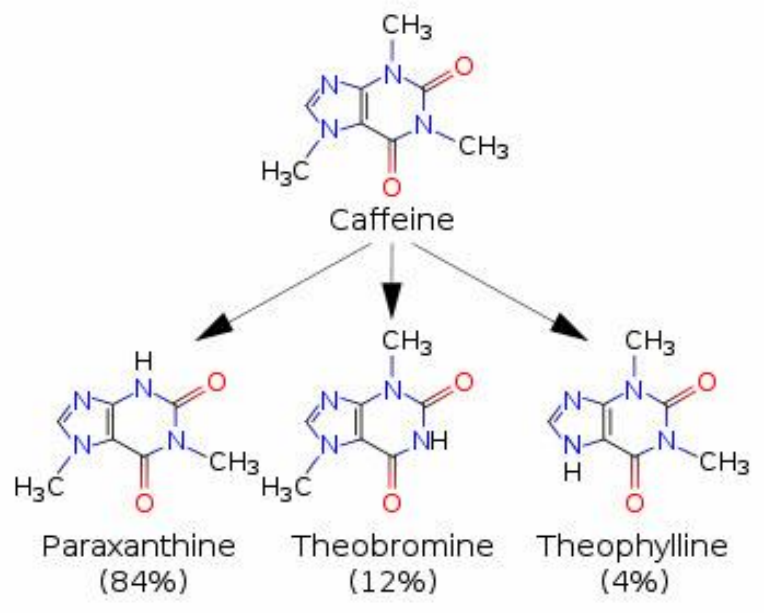



Figure 3. Caffeine and its main metabolic products (Wanyika et al., 2010).

The rate of caffeine elimination can also vary markedly within a species, including man as a function of age, endocrine status, disease, or the presence of other drugs (Ramalakshmi and Raghavan, 1999). Caffeine can, however, only reliably affect cognitive performance and mood if the dosage intervals are more than eight hours apart, but not after shorter intervals. Its use has no net restorative effects when performance and mood are decreased by sleep restriction. Therefore, caffeine users should be advised not to consume repeated doses of caffeine within a short period of time (Lee et al., 2014).

### 1.6.3 Physiological and psychological effects of caffeine on the body

Caffeine stimulates the central nervous system, an effect that may begin as early as 15 minutes after ingesting the caffeine and can last for as long as six hours. There are several types of side effects produced such as physiological effect, energy metabolism, psychoactive and neurological effect. Caffeine is rapidly and completely absorbed from gastrointestinal tract within a short period of time from consumption and then distributed throughout the body (Clarke and Macarae, 1985). However, it is not removed from the circulation until metabolized initially into paraxanthine, theophyline and theobromine then into derivative of uric acid and diaminourcil. Thus, the plasma half life of caffeine in man that is, the time required for its level to be

diminished by 50% as a result of biotransformation and excretion is 5-6 hours (Clarke and Macarae, 1985).

Most experts agree that drinking 600 mg (around 6 cups of brewed coffee) or more of caffeine per day may cause side effects. Some examples of the side effects of excessive caffeine intake include difficulty concentrating, insomnia, muscle tremors, fast heartbeat, jitteriness, heartburn, nervousness, stomach upset and irritability (David, 2012). Excessive caffeine intake during pregnancy has been linked to low birth weight, premature delivery and miscarriage. The Food and Drug Administration's recommends that women who are pregnant or trying to become pregnant consume no more than 200 mg of caffeine a day (David, 2012).

Excessive caffeine consumption should be avoided by people who are being treated for certain conditions including depression, anxiety or insomnia, heart problems, gastro esophageal reflux disease, high blood pressure and kidney disease. In these cases, decaffeinated drinks may be chosen over caffeinated ones. On the other hand, besides the physiological and psychological effects of caffeine, the chemical analysis of caffeine in coffee beans has been used as an additional tool for evaluating coffee quality. It has been reported that higher caffeine contents is associated with less quality samples compared to other Arabic samples (Belay, 2011).

Caffeine is a central nervous system stimulant which has been enjoyed by humans for many years through consumption of foods and beverages containing caffeine. However, coffee is one of the most consumed beverages throughout the world. Due to the importance of caffeine level in coffee simple analytical methods are required in order to characterize and identify the amount of caffeine in coffee beans.

Due to the above mentioned facts many physical and chemical methods have been developed for the determination of caffeine in coffee beans including spectroscopic (Gebeyehu and Bikila, 2015; Huck et al., 2005; Paradkar and Irudayaraj, 2002; Wanyika et al., 2010; Belay et al, 2008; Belay, 2011; Atomssa and Gholap, 2011), electroanalytical (Svorc, 2013) and chromatographic (Wanyika et al., 2010; Gopinandhan and Ashwiniandk, 2014) techniques. The literature survey

indicated that caffeine is determined in coffee samples mostly with the use of HPLC and UV-Vis spectrophotometer. However, relatively the above methods use solvents that are expensive and toxic which are chlorinated compounds those lead us to the chance of causing by cancer.

Therefore in this study it was aimed to develop simple, fast and inexpensive procedure by employing water as a solvent and other solvents which are less toxic and cost effective.

## 1.7. Objective

### 1.7.1. General Objective

- The main objective of this study is to investigate the possibility of determining caffeine content in aqueous solution of green coffee beans using optical methods mainly focused in the mid FT-IR spectroscopy.

### 1.7.2. Specific objectives

- To select the optimum, minimum solvent absorption interference, spectral range in mid and near IR for caffeine determination
- To develop the method for caffeine determination in aqueous solution
- To compare the amount of caffeine obtained in the new methods with that of extracted by using organic solvent (dichloromethane)

## 1.8. Statement of the problem

Like many other foods we eat and drink, the composition of coffee is complex. However coffee contains a very wide range of macro and micro nutrients and, as one of the most popular beverages consumed worldwide, it is worth considering what nutritional contribution the coffee can make to our diet. There are many compounds in coffee that are often thought to have implications upon human health. These include caffeine, micronutrients, chlorogenic acid and other bioactive components. In this study caffeine has been studied as a compound of interest due to the reason that is consumed daily in our real life through different beverages.

Caffeine has a wide spread name recognition with a controversial impact on human health. It can be determined by using different techniques. Caffeine is determined in coffee samples mostly with the use of HPLC. Caffeine was also determined using Fourier transform infrared (FT-IR) spectroscopy in combination with attenuated total reflectance (ATR) techniques and UV-Vis spectroscopy. However, combinations of ATR with FT-IR and fluorescence spectroscopy are not a very popular analytical technique for the determination of caffeine in coffee beans.

The use of attenuated total reflection (ATR) accessories in conjunction with Fourier transform infrared (FT-IR) spectrometers provides for the non-destructive measurement of samples and mid-infrared approaches have a huge potential for gaining rapid information about the chemical composition and related properties of coffee. In addition to its ability for effectively quantifying and characterizing caffeine content of coffee in aqueous solution it is also able to measure multiple chemical constituents simultaneously avoiding extensive sample preparation.

### 1.9. Significance of the study

The aim of this study is to produce a quick and cost effective method for the routine analysis of caffeine in coffee beans. Due to the large consumption of caffeine and the production capacity requirements are constantly increasing. Since there is no report in literature the amount of caffeine directly determined in aqueous solution of coffee beans by using spectroscopic techniques for example in the UV-Vis spectroscopy it is difficult to quantify directly in aqueous solution of coffee beans owing to matrix effect (Belay et al., 2008).

Developing a system based on infrared spectral information to assess the coffee quality and quantity parameters would bring economical benefits to the coffee industry by increasing consumer confidence in the quality of products. In this research work, water and other less toxic organic solvent have been used as a solvent for caffeine determination in coffee beans using optical methods. Most of the organic solvents used for coffee extraction are costly and carcinogenic than water but water is environmentally friendly, decrease cost, toxicity and time. Due to this reason, it is desirable to develop methods of caffeine determination in aqueous solution which is similar with the actual caffeine intake through coffee beverages.

## 2. LITERATURE REVIEWS ON CAFFEINE

There is much speculation but only limited evidence of coffee consumption being linked to protective effects on human health. Literatures reveal coffee beans contain efficient water soluble antioxidants, such as chlorogenic acids, caffeic acids, ferulic acids, *p*-coumaroic acids, melanoids and alkaloids (Azam et al., 2006) and their content depends mainly on the coffee species, origin and degree of roasting (Huck et al., 2005; Belay et al., 2008). The caffeine content of green coffee beans varies according to the species. During roasting there is no significant loss in terms of caffeine (Ramalakshmi and Raghavan, 1999).

Caffeine is one of the most widely used psychoactive substances in the world, its estimated global consumption being 120,000 tons per year. Caffeine is of major importance with respect to the physiological properties of coffee, and also in determining the strength, body and bitterness of brewed coffee. It also increases the effectiveness of certain drugs. Hence, it is used with some over-the-counter drugs for the treatment of conditions such as migraine and cluster headaches (Salihovic et al., 2014).

For most healthy adults, consuming moderate doses of caffeine, or about 200 to 300 mg a day, equal to about two to four cups of brewed coffee, is not harmful. Studies have shown it can help relieve pain, thwart migraine headaches, reduce asthma symptoms, and elevate mood. Although caffeine can contribute to dehydration, recent studies show that it is not dehydrating in moderate amounts, even for athletes (Nicole and Olsen, 2013).

Experimental studies that investigated the cardiovascular effects of caffeine found an increase in systolic or diastolic blood pressure (BP), increase in blood sugar, increase in gastric acid and pepsin secretion, increased plasma levels of fatty acids and decreased heart beat rate (HR) at doses as low as 1 mg/kg in children and 1.4 mg/kg in adults. Caffeine intakes of ≥1.4 mg/kg increased aortic stiffness, in-creased vascular resistance, decreased cerebral blood flow, increased plasma epinephrine, and rennin activity (Milanez, 2011).

Recently appeared methods (1996–2012) reporting the determination of caffeine in various sample matrices (environmental, biological, plants, food, etc.) cover a broad spectrum of instrumental analysis (Ally, 2013). Many methods exist for determining the methylxanthine contents of food and beverages. Some of these methods include UV-Visible spectrophotometry, electroanalytical, high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) (Igelige et al., 2014).

Most research activities have been focused on chromatographic methods. Of the above methods, HPLC has become one of the most commonly used and powerful tools available for analytical applications in many fields. Because of its versatility, low limits of detection (LOD), and accessibility, its applications can now be found in numerous methods for the analyses of industrial chemicals, agro allied chemicals, and constituents of foods and beverages (Nuhu, 2014). This method has been also the method of choice by many researchers in determining the caffeine contents of beverages (Wanyika et al., 2010), tea leaves and coffee beans (Dessalegn et al. 2008). However, HPLC is a high-priced and resource consuming technique that is not typically found in most universities especially in developing countries such as Ethiopia.

Several analytical methods are used in the analysis of coffee bean components. Although these methods provide with a high level of information about the chemistry of the compound measured, but the sample requires different steps of pre-processing (e.g., extraction, purification, etc.) before and during the analysis. As different literatures indicated spectrophotometric determination of caffeine is reported as preferred method of determination such as UV-Vis spectrophotometer (Belay et al., 2008; Belay, 2011; Komes, et al. 2009; Amos, 2014; Gebeyehu and Bikila, 2015) because of its relatively low cost, rapidity, high accuracy and reproducibility.
Caffeine was also quantitatively determined with the use of Fourier transform infrared (FT-IR) spectroscopy in combination with attenuated total reflectance (ATR) techniques using a zinc selenide crystal. The method is faster and relatively simple, chloroform extract of caffeine from coffee brew having caffeine signal monitored at 1,655 $cm^{-1}$ (Singh et al., 1998). However, combination of ATR with FT-IR is not a popular analytical technique for quantitative determination of caffeine in coffee beans like UV-Vis spectrophotometer and HPLC.

Spectroscopic techniques provide a non destructive, fast and cheap determination of caffeine in green and roasted coffee beans. But UV-Vis spectrophotometer method cannot be used directly for determination of caffeine in coffee beans extracted with water owing to the matrix effect of UV-Vis absorbing substances in the sample matrix (Belay et al., 2008). In aqueous solution of coffee beans it was observed that there is spectral interference from caffeine and chlorogenic acid in the wavelength regions of 200-500 nm. Yet this method requires the extraction of caffeine from the aqueous solution of coffee beans using dichloromethane for the spectroscopic determination.

This is necessary since the caffeine spectrum is overlapped with other compounds found in coffee. Hence, the use of dichloromethane limits from wider application of UV-Vis method which is described above. Therefore, this research was to investigate the possibility of optical methods for the determination of caffeine in aqueous solution of green coffee beans by developing cost effective procedures.

According to Singh (1998) caffeine was determined from coffee beans employing chlorinated compound (chloroform) as extracting medium by using FT-IR-ATR with zinc selenide crystal. However, caffeine was not determined in the aqueous solution of coffee beans. A solution of coffee bean which is extracted with chloroform was used for FT-IR-ATR analysis. Therefore, in this method the use of chloroform may limit the wider application of this developed method.

There are also other FT-IR literature data on coffee obtained by transmission and reflectance technique indicated that only a few qualitative aspects of the spectra have been reported. The spectra obtained by transmission and reflectance are similar from qualitative point of view, in the sense that the most significant bands can be viewed in both spectrum. Also, higher intensity of peaks can be observed in the spectra that employed KBr (transmission and diffuse reflectance) relatively. The two sharp bands that can be viewed in the 3000–2800 $cm^{-1}$ have also been reported for both Arabica and Robusta roasted coffee samples (Ana et al., 2012).

Nonetheless, studies of FT-IR analysis of caffeine on soft drinks have also reported two sharp peaks at 2882 and 2829 $cm^{-1}$, with the later one being correlated with the asymmetric stretching of C-H bonds of $–CH_3$ group in the caffeine molecule and the peak region being successfully used to develop predictive models for quantitative analysis of caffeine (Paradkar and Irudayaraj, 2002).

According to Ana et al. (2012) from their previous FT-IR analysis of aqueous extract of non-defective coffee samples they confirmed that the bands at (2922, 2852 and 1658 $cm^{-1}$) are associated to caffeine. However, this conformation was performed by FT-IR-ATR analysis of aqueous extract of non-defective coffee samples spiked with standard caffeine. There was a significant increase in peak intensity with the increase in caffeine concentration. These bands are discussed and identified only in the view of qualitative analysis of caffeine in coffee bean samples.

This paper concerns on developing the methods to determine the caffeine content of green coffee beans in aqueous solution by using spectroscopic methods. Since, the amount of caffeine from coffee bean was taken by human beings through drinking of coffee beverage prepared in hot water as the extracting medium. Hence, it is always desirable to develop a method in the laboratory which is similar with the actual conditions to assess the actual intake of caffeine through coffee.

## 3. SPECTROSCOPY

Spectroscopy is the study of the interaction of electromagnetic radiation and matter, a study that encompasses a wide range of physical and chemical behavior. Molecular spectroscopy is the study of the absorption or emission of electromagnetic radiation by molecules.

### 3.1. Infrared spectroscopy

Infrared spectroscopy is study of the interaction of radiation with molecular vibrations which can be used for a wide range of sample types either in bulk or in microscopic amounts over a wide range of temperatures and physical states. The IR spectrometry technique can be used in two

variants, transmission and reflection. It is possible to test samples in any form: solid, liquid and gaseous with the use of an appropriate procedure. Aside from the conventional IR spectroscopy of measuring light transmitted from the sample, the reflection IR spectroscopy was developed using combination of IR spectroscopy with reflection theories. In the reflection spectroscopy techniques, the absorption properties of a sample can be extracted from the reflected light (Theophanides, 2012).

### 3.1.1. FT-IR-ATR spectroscopy

IR radiation within the wave number from 4000 to 400 $cm^{-1}$ is selectively absorbed by material molecules and converted into their oscillatory energy. The oscillations of molecules are of various characters, connected with their chemical structure, and depend on the type of bonds (frequency increases with increasing bond energy), relative atomic weights (frequency decreases with increasing atomic weight), spatial position of atoms in a molecule, intra- and intermolecular interaction forces (Urbaniak-Domagala, 2012). Mid-infrared spectroscopy (400–4000 $cm^{-1}$) is used to study the fundamental vibrations and associated rotational-vibrational structure of chemical bonds. Mid-infrared spectroscopy has been used as an efficient analytical tool in various fields, such as agriculture, food, oil, medical, textile and pharmaceutical.

The IR spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra. In the case of FT-IR overlapping is imminent or the absorbing peak area is narrow when compared to near IR. Because of the large number of maxima in an IR absorption spectrum, it is sometimes possible to quantitatively measure the individual components of a mixture of known qualitative composition without prior separation.

**Attenuated total reflection** (ATR) is a sampling technique used in conjunction with infrared spectroscopy which enables samples to be examined directly in the solid or liquid state without further preparation. Most of the time, this sampling technique is available for samples of aqueous solutions. Because, for non-volatile liquids a suitable sample is prepared by placing a drop of the liquid sample between two NaCl plates, forming a thin film. However, analysis of an aqueous sample in such way is complicated due to the solubility of the NaCl cell window in water.

Therefore an approach to obtain infrared spectra on aqueous solutions is to use attenuated total reflectance instead of transmission.

Light undergoes multiple internal reflections in the crystal of high refractive index. The sample is in contact with the crystal. ATR uses a property of total internal reflection resulting in an evanescent wave. A beam of infrared light is passed through the ATR crystal in such a way that it reflects at least once off the internal surface in contact with the sample. This reflection forms the evanescent wave which extends into the sample.

The ATR-IR uses the phenomenon of a complete reflection during the transition of IR radiation from an optically denser medium (prism) to thinner medium (sample). A sample is placed on the IR-transparent prism surface with a refractive index being always higher than that of the sample (Figure 4). The radiation beam is directed by one of the prism wall to the prism-sample interface at angle (θ) higher than the limiting. Under these conditions, a complete reflection occurs at the internal prism side and the beam reflected comes out through the second prism wall, where the beam intensity and absorption spectrum are recorded (Urbaniak-Domagala, 2012).

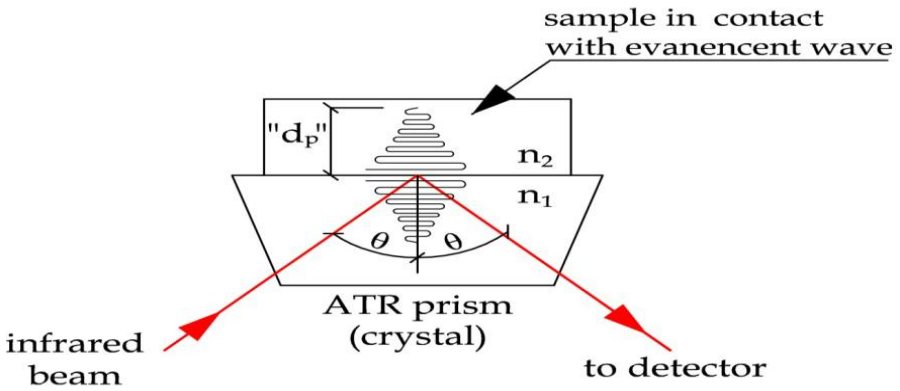



Figure 4. The schematic representation of infrared beam reflected on the crystal - sample interface in FT-IR-ATR spectrometer (Urbaniak-Domagala, 2012).

The evanescent wave penetration depth, "dp", in sample depends on the IR radiation wavelength ($\lambda$), incident angle ($\theta$), prism refractive index ($n_1$), and sample refractive index in relation to the prism ($n_{2,1}$) and is expressed by the following equation (Dechant, 1972):

$$dp = (\lambda/n1)/2\pi((\sin^2 \theta - n^2_{2,1}))^{1/2} \qquad (1)$$

The ATR module allows quick sampling of solids or liquids without preparation like the transmission methods. The trade off is that the infrared beam only penetrates the sample in microns and the method has less sensitivity than transmission measurements. The ATR crystal has a very small window which the sample can contact. Infrared light is reflected by a mirror to a conical zinc selenide crystal.

### 3.1.2. Near infrared spectroscopy

A near-infrared spectrum (12000–4000 $cm^{-1}$) is composed of combination and overtone bands that are related to absorption frequencies in the mid-infrared region. These combination and overtone bands correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same near-infrared spectrum. Therefore, near-infrared spectroscopy can result in a positive identification of each different material.

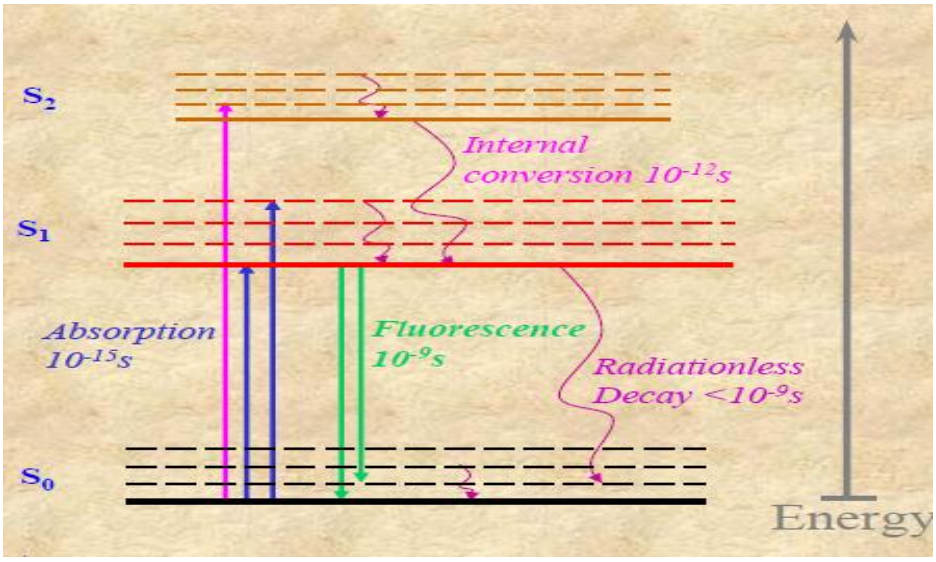
Near-infrared spectroscopy is based on molecular overtone and combination vibrations. Such transitions are forbidden by the selection rules of quantum mechanics. As a result, the molar absorptivity in the near-IR region is typically quite small. The advantage is that NIR can typically penetrate much farther into a sample than mid infrared radiation. Near-infrared spectroscopy is, therefore, not a particularly sensitive technique, but it can be very useful in probing bulk material with little or no sample preparation, may be used to observe matrix modifications and with proper calibration can be used in quantitative analysis.

In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. NIR has become a versatile technique with no sample preparation, decreased costs and analysis time, and the ability to sample through glass and packaging materials.

### 3.2. Fluorescence spectroscopy

Fluorescence occurs when a molecule absorbs light photons known as excitation, and then rapidly emits light photons as it returns to its ground state. The general principle of the phenomenon is illustrated by Jablonski diagram (Figure 5).

The first step of the fluorescence is the excitation of the molecule from the ground state ($S_0$) to the one of excited states ($S_1$, $S_2$) by the absorption of light. This is followed by a vibrational relaxation or internal conversion, where the molecule undergoes a transition from an upper excited state ($S_2$) to a lower one ($S_1$), without any radiation. Finally, the fluorescence occurs, typically $10^{-8}$ s after excitation, when the electron returns to the ground state ($S_0$). Emitting light has energy equal to the difference between energies of ground and excited states (Nawrocka and Lamorska, 2013).




Where; S = singlet state, $S_i$ = i-th electronic state, $S_0$ = electronic ground state

_____ Electronic level ---------- Vibrational level

Figure 5. Jablonski diagram.

Jablonski diagram explains stoke shifts that are emission has lower energy compared to absorption and most emission (fluorescence) occurs from the lowest vibrational level of $S_1$.

The key characteristics of fluorescence spectroscopy are its greater sensitivity and selectivity than UV-Vis spectroscopy. However, many molecules absorb strongly in the UV or visible range but do not exhibit detectable fluorescence and two wavelength selectors are available in fluorimetry. Fluorescent compounds can be identified or quantified on the basis of their excitation and emission properties. In general, the light emitted by a fluorescent solution is of maximum intensity at a wavelength longer than that of the exciting radiation. The fluorescence intensity of the solution can be recorded at the wave length of excitation/emission maxima.

Fluorescence spectrophotometry is often more sensitive than absorption spectrophotometry. In absorption measurements, the specimen transmittance is compared to that of a blank, and at low concentrations, both solutions give high signals. Conversely, in fluorescence spectrophotometry, the solvent blank has low rather than high output, so that the background radiation that may interfere with determinations at low concentrations is much less.

The high specificity of fluorescence is due to use of two spectra, i.e. excitation and emission spectra, while the high sensitivity of the technique is a result of measuring radiation against absolute darkness. Fluorescence spectrophotometry is inherently highly sensitive technique, concentrations of $10^{-5}$ M to $10^{-7}$ M can be used in order to establish linear relationship of working curve of fluorescence intensity versus concentration. In the case of fluorescence measurement its deconvolation capacity is high when it is compared to UV-Vis measurement (Nawrocka and Lamorska, 2013).

## 4. MATERIALS AND METHODS

### 4.1. Chemicals and samples

The reagents and chemicals used were standard caffeine (Fishel company, Germany), N,N-dimethylformamide (Riedel-de Haen, 99 %), acetone (Sigma-Aldrich, 99%) and dichloromethane (Sigma-Aldrich, 99%). All the reagents and chemicals used were analytical grade and were used without further purification. The coffee sample was collected from local market without considering its variety. All the glass wares were soaked overnight with detergent, washed thoroughly with water and detergent, rinsed with distilled water then dried very well before use. Distilled water was used in all experimental work.

### 4.2. Instrumentation and apparatus

The mass of standards and samples were measured using electronic balance (ARA520, OHAUS CORP., China, precision 0.01 g). Magnetic stirrer with a hot plate (Model 04803-02, Cole Parmer Instrument Company, 230 V, 50 Hz, and 2 Amp, USA) was used to dissolve the standard and the sample. Blending device (Electric motor grinder) (GEEPAS CR., Main land, China) was used for grinding green coffee bean sample.

A fluorescence emission and excitation spectrum of caffeine was recorded on Perkin Elmer Hitachi spectrofluorimeter (Flouromax-4, spectrofluorimeter, USA) with a xenon lamp which is connected to a computer supplied to data manager software. The fluorescence intensity was measured by using a 1 cm fluorescence squared quartz cuvette which is all sides transparent.

The electronic absorption of the solution was recorded on Perkin Elmer UV-Vis-NIR spectrophotometer and FT-IR-ATR spectrophotometer connected to a computer supplied to data manager software. The light source for the Perkin Elmer UV-Vis-NIR spectrophotometer is usually a deuterium discharge lamp for the UV measurement and a tungsten-halogen lamp for visible and NIR measurements. For the UV/Vis and NIR measurements a double beam UV-Vis-NIR spectrometer, Perkin Elmer Lambda 950 (Perkin Elmer, Llantrisant, CF728YW, UK) with wave length regions 170-3200 nm were used. The instrument was operated by Perkin Elmer, UV win Lab soft ware. Scanning speed 282 nm per min and with slit width 2 nm was used. For the

Mid IR measurement a sample holder of zinc selenide crystal and Fourier transform (Perkin Elmer, spectrum 65 spectrophotometer, USA) with wave number range 4000-400 $cm^{-1}$ were used.

### 4.3. Preparation of the standard caffeine solutions

Stock solution of standard caffeine was prepared first for all measurements because it is widely recognized that dilute solutions are not as stable as concentrated ones. The working standard solutions were prepared from the stock solution and all measurements were carried out in short period of time after preparation.

#### 4.3.1. Standard solutions for FT-IR spectroscopy

The stock solution of standard caffeine was prepared by dissolving 0.5 g of standard caffeine with 40 g of distilled water in 100 mL beaker and stirred using magnetic stirrer up to clear solution was formed. The solution was diluted to final weight 50.29 g in 100 mL volumetric flask and the concentration of the stock solution was calculated to 9942 mg/L. The standard solution of the required concentration for calibration was prepared by a series of dilution by using weight to weight ratio from the stock solution already prepared. Working standards were prepared by weighing 1.00, 2.01, 3.017, 4.023, 5.03 and 6.035 g, respectively, aliquots of the stock standard solution were transferred into separate volumetric flasks (25 mL). All the aliquots were diluted to 10 g of final weight of the solution with distilled water to produce concentrations of 1000, 2000, 3000, 4000, 5000 and 6000 mg/L, respectively, standard solution for the FT-IR-ATR calibration measurement.

#### 4.3.2. Standard solutions for NIR spectroscopy

The stock solution of standard caffeine was prepared by dissolving 0.47 g of standard caffeine with 40 g N,N-dimethylform amide (DMF) in 100 mL beaker and diluted to 47.73 g of final solution in 100 mL volumetric flask. A series of working standards were prepared from the stock solution having concentration of 9847 mg/L by using weight to weight ratio of dilution. Working standards were prepared by weighing 1.01, 2.03, 3.04, 4.06 and 5.07 g aliquots of the standard stock solution in to separate 25 mL volumetric flask. Each aliquot was diluting to 10 g of final

weight of the solution with DMF to produce concentrations of 1000, 2000, 3000, 4000 and 5000 mg/L, respectively.

### 4.3.3. Standard solutions for fluorescence spectroscopy

The stock solution (0.005 mol/L) of standard caffeine was prepared in 1 L volumetric flask by dissolving 0.97 g of pure caffeine in 300 mL distilled water using 500 mL beaker and made up to the mark of 1 L volumetric flask with distilled water. From the stock solution it is impossible directly to prepare the working standards. Hence, it is necessarily to come with serial of dilution which is preparing of other concentration that is less concentrated than the stock solution. To prepare the working standards 0.0001 mol/L concentration of 100 g solution was prepared from the stock solution by applying weight to weight dilution.

The working standards were prepared by weighing 0.76, 1.53, 3.10, 5.89, and 11.34 g, respectively, added aliquots of the standard solution in to separate 50 mL volumetric flask and diluting to 25 g final weight of the solution with distilled water to produce concentrations of 3.06 x $10^{-6}$, 6.14 x $10^{-6}$, 1.24 x $10^{-5}$, 2.358 x $10^{-5}$, and 4.534 x $10^{-5}$ mol/L, respectively. For the fluorescence measurement highly concentrated solutions are not suitable because fluorescence spectroscopy is very sensitive technique and needs low concentration. However, fluorescence intensity is directly proportional with concentration of diluted solution. But at higher concentration inner filter effect have to be considered because at higher concentration the solution as a whole is not uniformly exciting.

### 4.3.4. Standard solutions for UV/Vis spectroscopy

The stock solution (0.005 mol/L) of standard caffeine was prepared in 50 mL volumetric flask by dissolving 0.05 g of pure caffeine in 20 mL dichloromethane using 100 mL beaker and made up to the mark of 50 mL volumetric flask with the solvent. From the stock solution it is impossible directly to prepare the working standards. Hence, it is necessarily to come with serial of dilution which is preparing of other concentration that is less concentrated than the stock solution. To prepare the working standards 0.0005 mol/L concentration of 25 g solution was prepared from the stock solution by applying weight to weight dilution.

The working standards for UV/Vis calibration were prepared by employing weight to weight dilution. Five working standards solution of pure caffeine in the range of ($1.57x10^{-5}$ – $2.5x10^{-4}$ mol/L) were prepared in separate 25 mL volumetric flask.

### 4.4. Coffee sample preparation

Green coffee beans were ground and screened through 250 μm sieve to get a uniform texture. Then accurately weighed amount of sieved coffee was dissolved in distilled water for fluorescence and FT-IR analysis and in DMF for NIR analysis. The solution was stirred using magnetic stirrer and heated gently to remove caffeine easily from the solution. In addition the solution was filtered out with Whatman filter paper by a glass funnel to get clear solutions as far as possible. Finally the filtrate of green coffee beans was used for qualitative and quantitative analysis by using spectroscopic techniques (UV-Vis-NIR, fluorescence and FT-IR-ATR).

#### 4.4.1. Coffee sample preparation for UV/Vis determination

Using UV/Vis spectrophotometer direct determination of caffeine in aqueous solution of coffee bean is not possible in which spectral overlapping is very high due to matrix effect. Hence, to overcome this difficulty it is to extract with dichloromethane by using the procedure developed by Belay et al. (2008). Accurately weighed amount of sieved coffee bean was dissolved in 25 mL distilled water, stirred using magnetic stirrer and heated gently to remove caffeine easily from the solution. Then the aqueous solution of coffee bean was filtered and the filtrate was stored for liquid - liquid extraction.

#### 4.4.2. Liquid – liquid extraction

The filtrate of aqueous coffee solution prepared above was mixed with equal volume of dichloromethane for the extraction of caffeine from coffee beans. The solution was stirred for about 10 minute and using separatory funnel caffeine was extracted from the solution using dichloromethane. The extraction of caffeine was performed four times with a total volume of 100 mL dichloromethane. The extracted caffeine from each round was stored in a flask for quantitative determination of caffeine using UV/Vis spectrophotometer.

### 4.5. FT-IR-ATR measurement

In order to collect sample spectrum first an open beam background spectrum were collected for the chamber through which infrared radiation is directed to a detachable ATR zinc selenide crystal mounted in a shallow trough for sample containment. The background spectrum with no sample this gives a characteristic background that shows the instrument operating normally. Background spectrum of the pure solvent (distilled water) was also collected in order to select the best free spectral region, minimum solvent interference for analysis. The ATR crystal was carefully cleaned with the solvent and dried between measurements to ensure the best possible sample spectra.

The maximum peak of absorption for the aqueous solution of standard caffeine was obtained by scanning the standard solution from 4000–400 $cm^{-1}$ and the spectrum over the wave number range (2825–2982) $cm^{-1}$ with a good absorption spectrum of standard caffeine have been selected for quantitative analysis. Then after, the spectrum of the standard solution was collected by taking a required amount of the standard solutions in to the sample holder for each measurement. A small amount of liquid to cover the cell is enough when taking a sample it is not good to allow liquid to run down the plate. Finally the filtrate of green coffee bean solution in required amount was measured with the same working principle as the standard solutions and spectrum were obtained in absorbance unit.

#### 4.5.1. The standard calibration curve for FT-IR-ATR

To determine the percentage of caffeine in green coffee beans it is necessary to set up calibration using standard caffeine. The solution of standard caffeine having concentration range (1000 – 6000 mg/L) was run to obtain the linear range of sample analysis. The linear calibration curve of standard caffeine was obtained by integrating the peak area of the FT-IR- ATR spectrum separately and then, the integrated peak area was sum up to get the total peak area of the standards. Then from the calibration graph of peak area versus concentration the concentration of green coffee bean was determined by integrating the peak area of the coffee bean over the same range as the standards integrated.

### 4.6. NIR measurement

To determine the $\lambda_{max}$ of absorption for standard caffeine in DMF first the free spectral range of the solvent was determined by scanning the solvent DMF over the wave length range (800–2500 nm) and the range over 1200–2110 nm was selected in which the region have low absorption by the solvent relatively. The $\lambda_{max}$ for standard caffeine was obtained by scanning the standard solution over the range 1200–2110 nm and the result gave an absorption spectrum, which was characterized by a single intensive absorption band located in the NIR region at around $\lambda_{max}$ = 1922 nm. But there are also other bands in the NIR range of interest which are very broad and less intense bands having absorption close to the solvent. Due to this reason the absorption band at $\lambda_{max}$ = 1922 nm was selected for quantitative analysis.

### 4.7. Fluorescence measurement

The $\lambda_{max}$ of excitation for standard caffeine was determined by scanning the standard solution over the wavelength range (240–360) nm and the result gave an absorption spectrum, which was characterized by a single intensive absorption band located in the UV range at $\lambda_{max}$ = 272 nm. To collect excitation spectrum, emission wave length was set then find wavelength on excitation, which gives maximum emission intensity. To collect emission spectrum, excitation wavelength was set in order to find wavelength on emission, which gives maximum excitation intensity.

Set the excitation wavelength at 272 nm and the $\lambda_{max}$ of emission was determined by scanning the standard solution 250–500 nm. The spectrum was best at 385 nm, which is far from the Rayleigh and Raman scattering. For quantitative analysis the excitation property was used. Set the emission wavelength at $\lambda_{max}$ = 385 nm and scanning the solutions over the range 240 – 360 nm to obtain the maximum excitation intensity.

#### 4.7.1. The standard calibration for fluorescence

To determine the percentage of caffeine in green coffee beans it is necessary to set up calibration using different concentrations of standard caffeine. The solution of standard caffeine having concentration range ($4.5 \times 10^{-5} – 3.06 \times 10^{-6}$ mol/L) was run to obtain the linear range of sample analysis. From the fluorescence spectrum of the standard solutions the maximum excitation

intensity was collected and calibration graph of concentration versus maximum excitation intensity was constructed. Then from the graph of intensity versus concentration, the amount of caffeine in green coffee beans was determined.

### 4.8. UV/Vis measurement

To determine the $\lambda_{max}$ of absorption for standard caffeine in dichloromethane first the free spectral range of the solvent was determined by scanning the solvent DCM over the wave length range (200–500 nm). The $\lambda_{max}$ for standard caffeine was obtained by scanning the standard solution over the wave length range (200–500 nm) and the result gave an absorption spectrum, which was characterized by a single intensive absorption band at $\lambda_{max}$ = 275 nm. To determine the percentage of caffeine in coffee beans calibration graph of absorbance versus concentration was constructed for the standard solutions. Then, the amount of caffeine in green coffee bean was calculated using the given equation of line.

### 4.9. Statistical analysis

Statistical analysis in chemistry language is related to using of chemometric methods to improve chemical measurement processes, and extract more useful information from chemical and physical measurement data. Some statistical software and methods are used to analyze raw data obtained from experiments.

The procedures developed for caffeine determination showed that the spectrum of caffeine was not complex, it has been resolved or deconvolated. Therefore, no need of using multivariate analysis calibration techniques simply quantitative analysis of caffeine was employed by using univariate calibration technique for all methods developed. In this research work the data obtained were analyzed by using origin statistical software (version 6.0). For all methods triplicate measurements of sample were performed. The result was expressed as mean ± standard deviation for all replicate measurements. The data were also subjected to one way analysis of variance (ANOVA) using origin soft ware (version 6.0) to test the significance differences in the mean values of caffeine obtained by the three methods (NIR, FT-IT-ATR, and fluorimeter).

## 5. RESULT AND DISCUSSION

### 5.1. FT-IR-ATR

#### 5.1.1. Integrated peak area versus concentration relation

To determine the percentage of caffeine in aqueous solution of green coffee beans different concentration of standard caffeine were prepared and the absorption spectrum of the standard solutions was observed over a wave number range (2825-2982) $cm^{-1}$. The absorption curves for different concentrations (1000, 2000, 3000, 4000, 5000 and 6000 mg/L) of standard caffeine are shown in Figure 6.

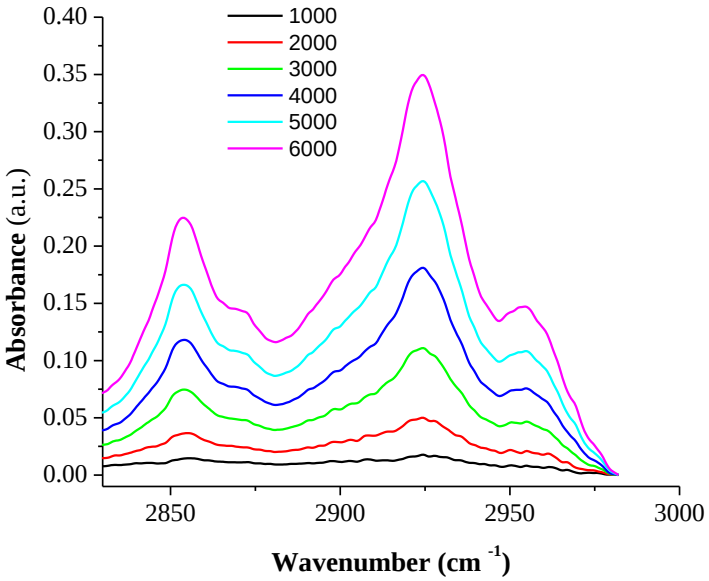



Figure 6. FT-IR-ATR absorption spectrum of different concentrations of standard caffeine in water. Where, the numbers at the top of the figure are the respective concentrations of standard caffeine in mg/L.

To construct the calibration graph of concentration versus integrated peak area the given spectrum was treated with baseline correction and separately integrated over the range (2982–2882 $cm^{-1}$) and (2880–2825 $cm^{-1}$). The peak area was obtained from the integrated FT-IR-ATR spectrum by adding the peak areas integrated separately. The baseline corrected and integrated spectrum is shown in Figure 7.

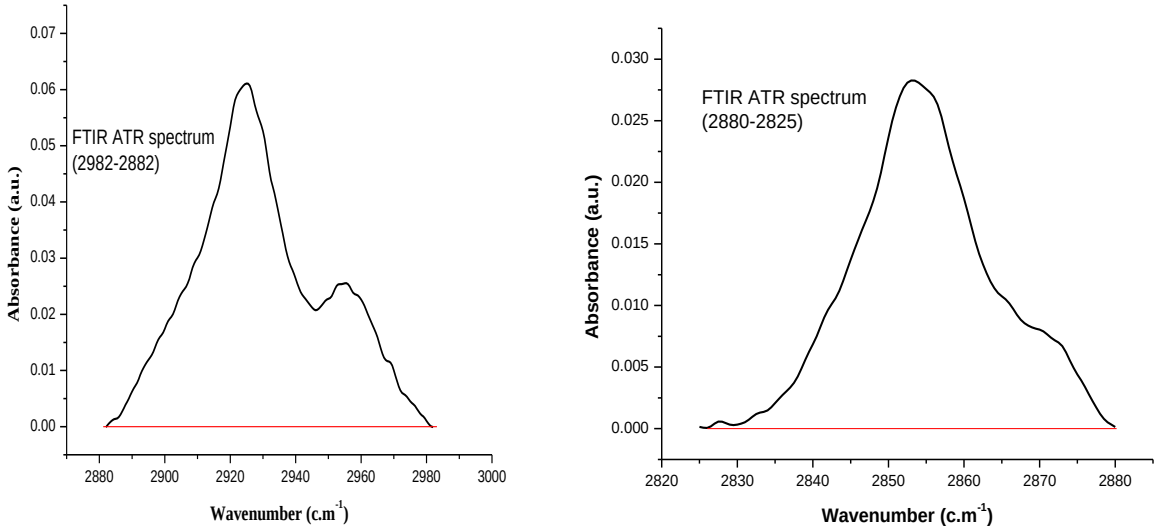



Figure 7. The treated (base line corrected) FT-IR-ATR spectrum of standard caffeine in water.

The integrated peak area versus concentration graph of the calibration curve is shown in Figure 8 which showed that linear relationship between the integrated peak area and concentrations of standard solutions.

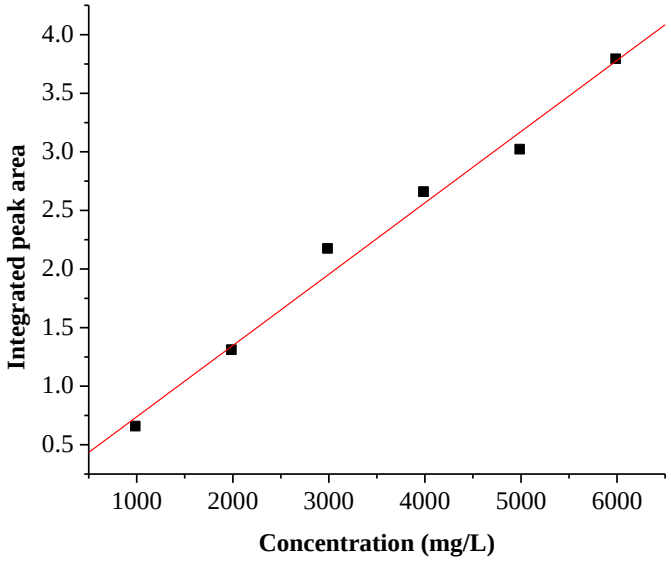



Figure 8. Graph of concentration versus integrated peak area for standard caffeine in water.

The calibration curve obtained for FT-IR-ATR analysis of caffeine have correlation factor of (R = 0.993) and the standard calibration curve was linear over the range (1000-6000) mg/L of standard caffeine with equation (Y = 0.13045 + 0.000608X, where, Y indicates the sum of integrated peak area and X indicates concentration in mg/L). The quantitative amount of caffeine in aqueous solution of green coffee bean (mg/L) was then determined using the standard curve.

The structure of caffeine comprises of 10 C-H bonds (from the three $CH_3$ groups consisting of nine C-H bonds), one C=C bond (the only unsaturated in the cyclic structure), one C=N bond, two C=O bonds (carbonyl group in the cyclic structure), 10 C-N bonds and one C-C bond. Caffeine molecule has the characteristics of three methyl group cyclic structure hence this stretching vibration may play an important role in the qualitative and quantitative analysis of caffeine in aqueous solution of coffee beans.

FT-IR-ATR analysis of caffeine on aqueous solution of green coffee beans was characterized with two sharp peaks at around 2855 and 2924 $cm^{-1}$ these bands are correlated with the symmetrical and asymmetrical stretching of C–H bonds of methyl ($–CH_3$) group in the caffeine molecule and the peak region over the wave number range of 2982-2825 $cm^{-1}$ was successfully used for quantitative analysis of caffeine in green coffee beans.

### 5.1.2. FT-IR-ATR measurement of caffeine in green coffee beans

The standard caffeine dissolved in water and the filtrate of aqueous coffee solution have similar FT-IR absorption spectrum over the wave number range (2825-2982) $cm^{-1}$ which have a maximum absorption at around 2855 and 2924 $cm^{-1}$. The two spectra are exactly similar to each other both in peaks and shapes. The similarity in peak and shape of the two spectra show there is no overlap band from other components of coffee in these regions, and this shows the specificity of the method.

There are also other FT-IR literature data on coffee obtained by transmission and reflectance techniques with similar spectrum in which the two sharp bands that can be viewed in the 3000–

2800 $cm^{-1}$ have been reported qualitatively for both Arabica and Robusta coffee samples (Ana et al., 2012). Studies of FT-IR analysis of caffeine on soft drinks have also reported two sharp peaks at 2882 and 2829 $cm^{-1}$, the peak region being successfully used to for quantitative analysis of caffeine (Paradkar and Irudayaraj, 2002). The FT-IR-ATR spectrum of green coffee beans dissolved in water is given in Figure 9.

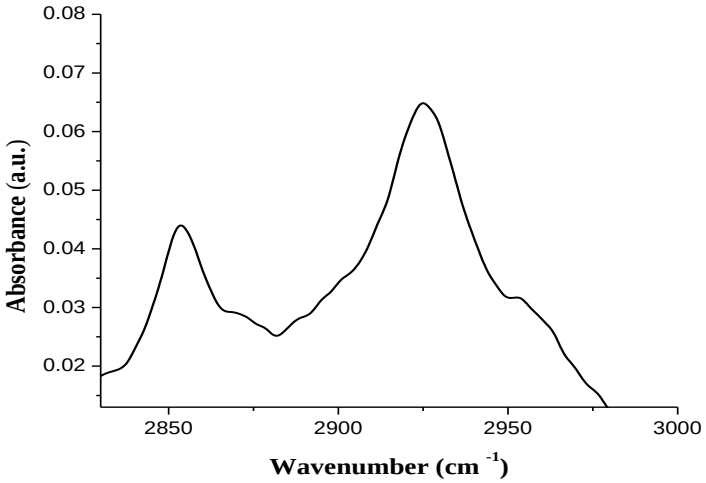



Figure 9. FT-IR-ATR spectrum of green coffee beans dissolved in water

The given spectrum was baseline corrected and integrated separately over the range (2982–2882 $cm^{-1}$ and 2880–2825 $cm^{-1}$). Then the integrated peak area was added and the amount of caffeine was determined from the linear standard calibration. The percentage of caffeine for triplicate measurement of coffee obtained from local market is given in the Table 3.

**Table 3**. The mean percentage of caffeine obtained by FT-IR-ATR spectrophotometer.

| Coffee sample name | Mass of coffee (g) | Mass of solution (g) | Mass of caffeine (g) | Percentage of caffeine in coffee (% w/w) | Mean value of three independent measurement ± SD |
|---|---|---|---|---|---|
| Sample A | 2.05 | 10.00 | 0.0334 | 1.629 | 1.52 ± 0.093% |
| | 2.00 | 10.05 | 0.0293 | 1.465 | |
| | 2.00 | 10.00 | 0.0294 | 1.470 | |

Using the proposed method the percentage of caffeine in green coffee beans was determined. This is the simplest and cost effective method in which water was used for the whole experimental parts. The sample was collected from a local market in which the origin of the coffee sample is not known. The target of the present work is not to determine and compare the percentage of caffeine in coffee beans cultivated in different areas rather it is to validate the developed fast, accurate, sensitive and cost effective methods for caffeine determination. The percentage of caffeine in aqueous solution of green coffee beans was directly investigated using FT-IR-ATR spectrophotometer. The mean percentage of caffeine in coffee beans determined for three independent measurement of one coffee sample was 1.52 ± 0.093 %.

Table 3 shows that the result obtained is comparable with the highest caffeine content of Arabica coffee samples as reported by (Gebeyehu and Bikila, 2015) for different Ethiopian Arabica coffee samples grown in Wembera, Goncha, Zegie and Burie determined by UV-Vis spectroscopy using dichloromethane for extraction to be 1.53 ± 0.003, 1.41 ± 0.04, 1.29 ± 0.033 and 0.97 ± 0.049, respectively. Another study using HPLC method also showed caffeine content variability as reported by (Dessalegn et al., 2008) ranging from 0.6 to 1.21%, 0.7 to 1.82% and 0.9 to 1.62% among 9, 21 and 38 coffee Arabica genotypes, respectively. Therefore, these values are in reasonable degree of agreement with the value of the present work.

Studies have indicated that the chemical composition of green coffee beans mainly depends on the variety of the coffee, although slight variations are possible due to agro-climatic conditions, agricultural practices (processing and storage), its species, origin and weather of the plantation (Wang, 2012, Gebeyehu and Bikila, 2015). Hence the variation of caffeine content of coffee samples may be due to the difference come from geographical origins.

### 5.2. NIR spectroscopy

#### 5.2.1. Absorption versus concentration relation

To determine the percentage of caffeine in DMF solution of green coffee beans different concentrations of standard caffeine in DMF were prepared and the maximum absorbance of the standard solutions was recorded at $\lambda_{max}$ = 1922 nm. The absorbance versus wave length graph of different concentrations of standard caffeine is shown in Figure 10.

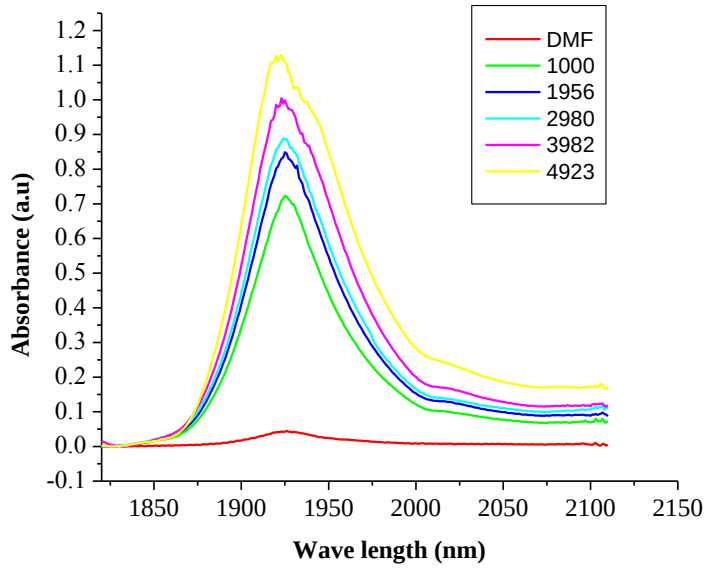



Figure 10. NIR absorption spectrum of different concentrations of standard caffeine in DMF. Where, the numbers at the top indicates concentrations of standard caffeine in mg/L.

The graph of maximum absorbance versus concentration of standard caffeine in DMF is shown in Figure 11.

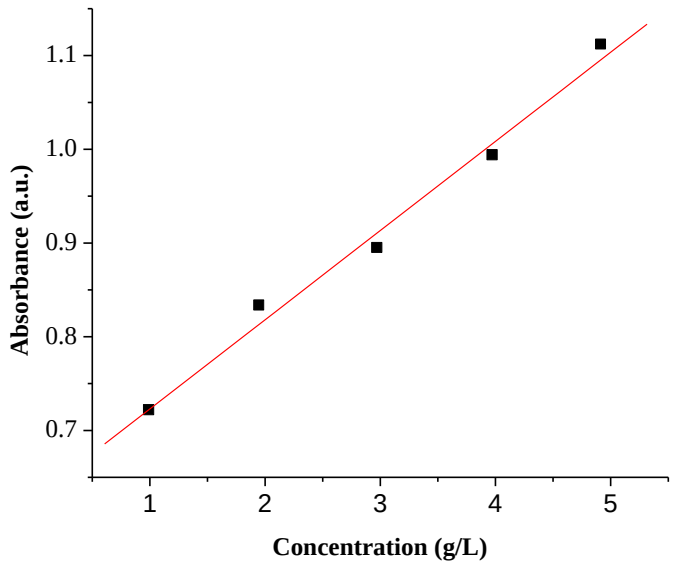



Figure 11. Absorbance versus concentration graph of standard caffeine in DMF.

The calibration curve obtained for NIR determination of caffeine in DMF have correlation factor of (R = 0.994) and the standard calibration curve was linear over the range (1000-5000) mg/L of standard caffeine in DMF with equation ($Y = 0.62786 + 9.51x10^{-5} X$, where Y indicates maximum absorbance and X indicates concentration in g/L). The quantitative amount of caffeine in DMF solution of green coffee bean (g/L) was determined using the standard curve.

### 5.2.2 NIR measurement of caffeine in green coffee beans

NIR spectrophotometric method cannot be used directly for the determination of caffeine in aqueous solution of coffee beans. In the NIR region water absorbs strongly, the free spectral range is not wide and on the free spectral range available the absorption of aqueous solution of caffeine is not significant. However, it is necessarily to use other solvents which are available for the NIR determination of caffeine in coffee beans. For this method, DMF was selected as a solvent which is less carcinogenic than chlorinated solvents, its ability to dissolve caffeine very well and having free spectral range on the studied region.

The NIR spectrum of coffee beans dissolved in DMF is given in Figure 12.

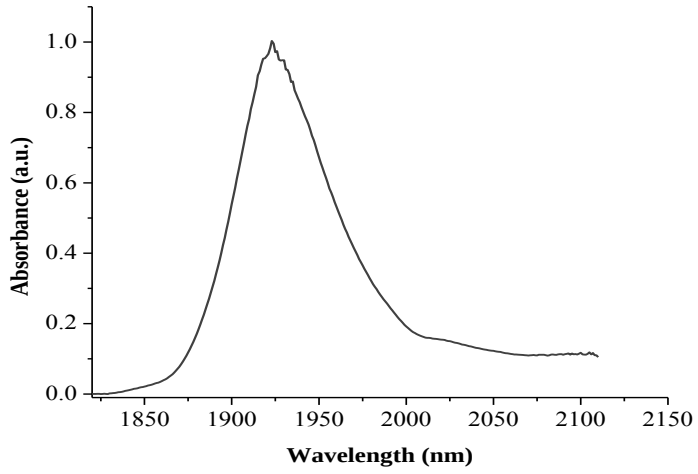



Figure 12. NIR spectra of coffee dissolved in DMF.

From the spectrum given in Figure 12 the NIR spectra of standard caffeine and the filtrate of coffee bean solution in DMF have similarity. The two spectra's are similar in shape and peak.

Due to this reason, the region over the range (2110–1820 nm) was used for quantitative analysis of caffeine in coffee beans. The percentages of caffeine for triplicate measurement of coffee sample collected from local market dissolved in DMF are given in Table 4.

**Table 4**. The mean percentage of caffeine obtained by NIR spectroscopy.

| Coffee sample name | Mass of coffee (g) | Mass of solution (g) | Mass of caffeine (g) | Percentage of caffeine in coffee (% w/w) | Mean value for three independent measurement ± SD |
|---|---|---|---|---|---|
| Sample A | 2.05 | 10.00 | 0.0343 | 1.68 | 1.50 ± 0.14% |
| | 2.00 | 9.980 | 0.0289 | 1.44 | |
| | 2.00 | 10.00 | 0.0281 | 1.41 | |

Using the present method the percentage of caffeine in DMF solution of green coffee beans was directly investigated using NIR spectrophotometry. The mean percentage of caffeine in coffee beans determined for three independent measurement of one coffee sample was 1.50 ± 0.14 %. There is no literature data on the percentage of caffeine in coffee beans which is directly determined in DMF solution using NIR spectrophotometer. However, this result was interesting it is very comparable with percentage obtained using FT-IR spectrophotometry for the same coffee sample.

The molecular overtone and combination bands seen in the NIR range are typically very broad, leading to complex spectra; thus being a difficult task to assign particular features to specific chemical components. It brings a great challenge to build a high-quality prediction model for unknown set of samples. Due to this reason, literatures indicated that, in the NIR region determinations are performed by employing multivariate calibration technique. However, the present work was developed a method of caffeine determination in coffee beans using univariate calibration technique which can overcome the difficulty of NIR region for direct determination of caffeine in coffee beans.

Regarding caffeine content determination a fast, simple and cost effective procedure was developed using NIR spectrophotometer in green coffee samples with reduced amount of organic solvent used. The sensitivity of spectroscopic measurements relies on band intensities, even the spectra obtained for the NIR measurement of caffeine in DMF was very intense band relative with other less intense bands in which spectral information is repeated throughout the successive overtones and combination regions.

Table 4 shows that the caffeine content of the same coffee sample investigated using FT-IR and NIR was comparable amount with different solvents used. The coffee sample has the caffeine level in the range of the caffeine content of different coffee Arabica investigated using different methods reported previously (Dessalegn et al. 2008; Gebeyehu and Bikila, 2015). The NIR spectrophotometric method employed in this study for the quantification of caffeine in coffee sample is found to be relatively easy, fast and cheap which can be used as alternative method. The major instrument required is a computerized UV/Vis/NIR spectrophotometer. This method may therefore be applicable for the rapid, simple and accurate quantification of caffeine in coffee beans.

### 5.3. Fluorescence

#### 5.3.1. Concentration and fluorescence intensity relation

To determine the percentage of caffeine in aqueous solution of green coffee beans different concentration of standard caffeine were prepared and fluorescence intensity was collected. The standard caffeine dissolved in water and the coffee solutions have an emission and excitation spectrum but there is difficulty for quantification of caffeine in aqueous solution of coffee beans using the emission property due to strong overlapping. Therefore, to overcome this difficulty it is necessary to quantify the amount of caffeine using the excitation intensity. Hence, Fluorescent compounds can be identified or quantified on the basis of their excitation or emission properties.

The fluorescence intensity versus wavelength spectrum of standard caffeine is shown in Figure 13.

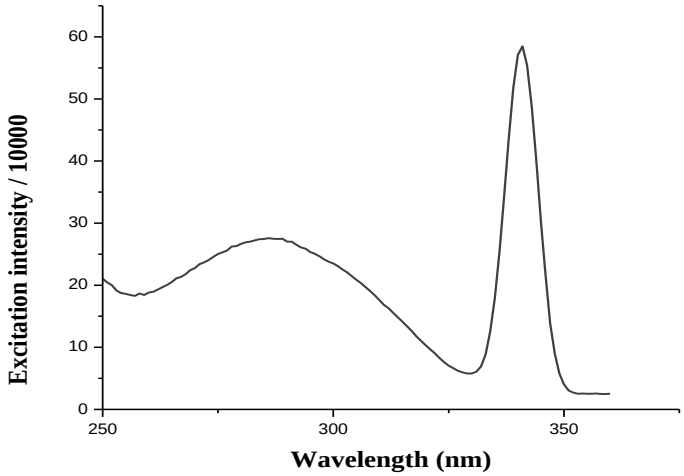



Figure 13. Fluorescence excitation spectrum of standard caffeine in water.

The graph of maximum excitation intensity versus concentration of standard caffeine is shown in Figure 14.

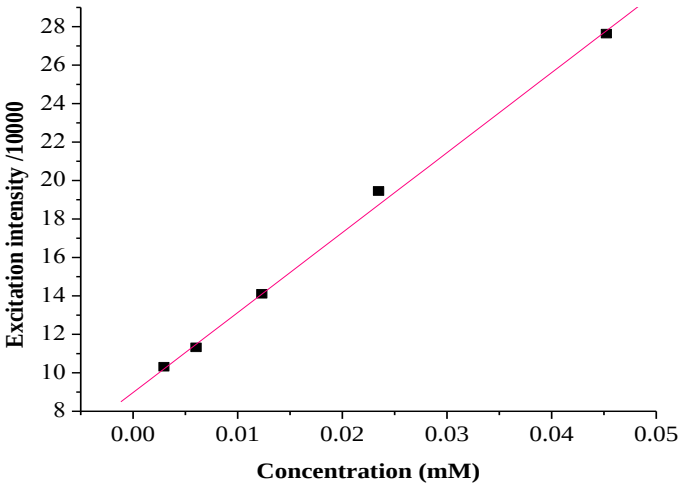



Figure 14. Graph of maximum excitation intensity *vs* concentration of standard caffeine.

From the given linear calibration curve correlation factor was (R = 0.998) and the calibration curve was linear over the range with equation ($Y = 4.15867x10^{9} X + 8.974x10^{4}$, where Y

indicates maximum excitation intensity and X indicates concentration). The quantitative amount of caffeine in green coffee bean (mol/L) was then determined using the calibration curve.

### 5.3.2. Fluorescence measurement of caffeine in green coffee beans

Using the proposed method the percentage of caffeine in aqueous solution of green coffee beans was determined employing fluorescence spectroscopy. It was determined from the excitation intensity of caffeine setting the emission wave length on 385 nm and scanning over the range (240–360 nm) to collect the maximum excitation intensity. The fluorescence spectrum of coffee beans dissolved in distilled water is given in Figure 15.

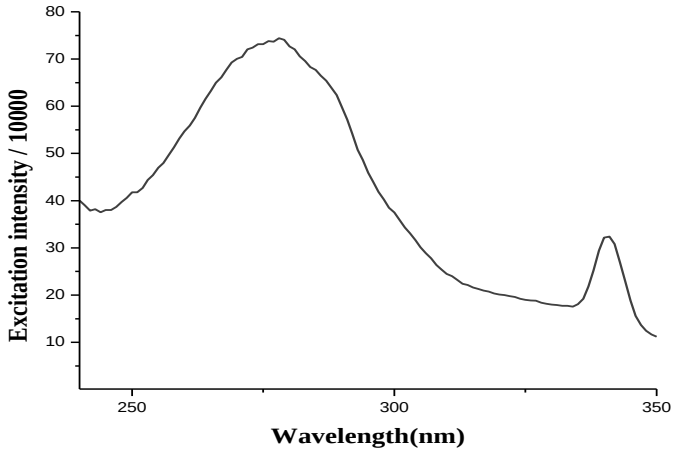



Figure 15. Fluorescence excitation spectrum of coffee dissolved in water

The mean percentage of caffeine in aqueous solution of green coffee beans for triplicate measurement of coffee sample collected from local market is given in Table 5.

**Table 5**. The mean percentage of caffeine obtained by fluorescence spectroscopy.

| Coffee sample name | Mass of coffee (g) | Mass of solution (g) | excitation intensity at $\lambda_{max}$ = 267 nm | Mass of caffeine (g) | Percentage of caffeine in coffee (% w/w) | Mean value of triplicate measurement ± SD |
|---|---|---|---|---|---|---|
| Sample A | 0.5 | 65 | 2452490 | 0.00056816 | 1.434 | 1.497 ± 0.05 % |
| | 0.5 | 75 | 2275033 | 0.00052549 | 1.530 | |
| | 0.5 | 70 | 2428590 | 0.00056241 | 1.529 | |

Table 5 shows that the mean percentage of caffeine in coffee beans determined for independent measurements of the sample was 1.497 ± 0.05 %. There is no literature data on the percentage of caffeine in coffee beans which is directly determined in aqueous solution of coffee beans using fluorescence spectroscopy. However, the result is comparable with the result obtained in this paper using FT-IR-ATR and NIR spectroscopy. Therefore, the mean percentage result was in a good agreement with the percentage obtained using FT-IR-ATR with the same solvent available. Furthermore, this method may be used as a fast, sensitive and cost effective method of caffeine determination.

### 5.4. Comparison of results obtained by three newly developed methods for caffeine determination

In the present study, three different methods were developed for the quantitative determination of caffeine in green coffee beans by using water and DMF as a solvent employing the same procedure for all methods. Hence, all the results were comparable with the percentage of caffeine in Arabica green coffee beans determined by using other methods such as UV/Vis spectrophotometer and HPLC method. It is also necessary to compare the present developed methods for caffeine determination and to describe the advantage and disadvantage of each method. The advantage and disadvantage of the present developed methods are given in Table 6.

**Table 6**. The advantage and disadvantage of the three methods

| | Methods developed for caffeine determination | | |
|---|---|---|---|
| | FT-IR-ATR | NIR | Fluorescence |
| Advantage | Uses water as a solvent<br>Simple procedure<br>Short time of analysis<br>Simple preparation<br>Can perform simultaneous analysis of mixtures | Simple procedure<br>Simple preparation<br>Cost effective instrument<br>Short time of analysis | Uses water as a solvent<br>Very sensitive and selective<br>Simple preparation |
| Disadvantage | Needs high concentration | Needs high concentration<br>Not possible to determine using water | Highly depends on concentration<br>Relatively complicated<br>Not available every where |

The analytical parameters such as correlation factor (R), linear range, limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD) of each method are given in Table 7.

**Table 7**. The analytical parameters for the three developed methods

| Methods | Liner range | R | LOD | LOQ | RSD |
|---|---|---|---|---|---|
| FT-IR-ATR | (1 – 6) g/L | 0.993 | 0.15 g/L | 0.5 g/L | 5.9 |
| NIR | (1 – 5) g/L | 0.994 | 0.3 g/L | 1 g/L | 9.3 |
| Fluorescence | ($5.95 \times 10^{-4}$ – $87.3 \times 10^{-4}$) g/L | 0.998 | $1.75 \times 10^{-4}$ g/L | $5.82 \times 10^{-4}$ g/L | 3.7 |

The data were also subjected to one way analysis of variance (ANOVA) using origin soft ware (version 6.0). However, the ANOVA result indicated that at 5% significance level, the means are not significantly different.

### 5.5. Comparison of results obtained by the present developed methods with UV/Vis spectrophotometry

To validate the newly developed methods it is necessarily comparison of the result using standard method or with other accepted methods. The present methods developed for caffeine determination were also compared with the results obtained by using currently developed method UV/Vis spectrophotometry. The method was reported by many researchers as preferred method of caffeine determination (Belay et al., 2008; Belay, 2011; Komes, et al. 2009; Amos, 2014; Gebeyehu and Bikila, 2015) because of its relatively low cost, rapidity, high accuracy and reproducibility.

#### 5.5.1. Determination of caffeine content by UV/Vis spectrophotometer method

The absorbance of five working standards solution of pure caffeine in the range ($1.57x10^{-5}$ – $25x10^{-5}$ mol/L) was recorded and the absorbance *vs* concentration graph was constructed. The UV/Vis absorption spectrum of different concentration of standard caffeine for calibration is given in Figure 16.

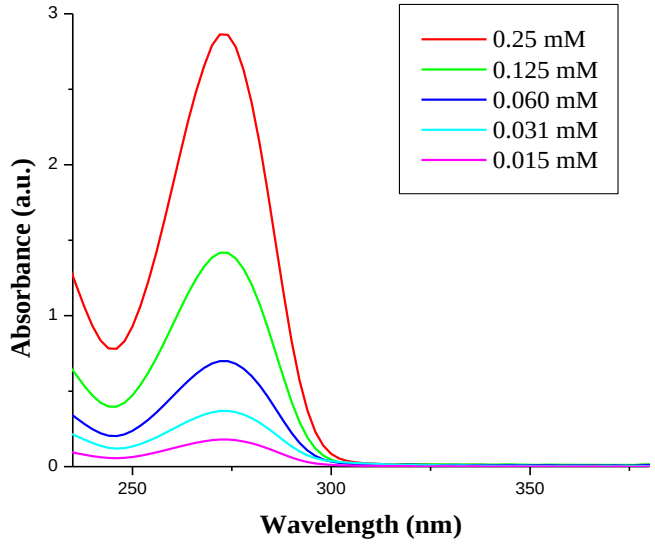


Figure 16. UV/Vis absorption spectrum of standard caffeine

From the spectrum Absorbance *vs* concentration graph of different concentration of standard caffeine was constructed and is shown in figure 17.

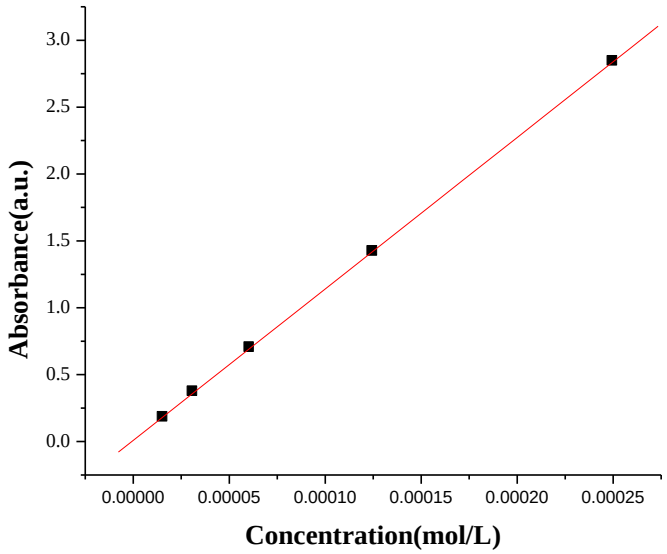



**Figure 17.** Graph of absorbance versus concentration of standard caffeine

From the linear calibration curve correlation factor was (R = 0.99999) and the calibration curve was linear over the range with equation ($Y = 1.1322x10^4 X + 0.00861$, where Y indicates maximum absorbance and X indicates concentration). The quantitative amount of caffeine in coffee sample (mol/L) was then determined using the calibration curve.

Belay et al. (2008) reported that UV/Vis spectrophotometer cannot be used directly for determination of caffeine in aqueous solution of coffee due to sample matrix effect. To overcome this difficulty the coffee samples was first dissolved in water and extracted with dichloromethane based on the procedure developed by Belay et al. (2008). After extraction, the absorbance of the solution was measured using UV/Vis spectrophotometer and the maximum absorbance was obtained at 275 nm. The UV/Vis absorption spectra of caffeine extracted from coffee beans using dichloromethane is shown in Figure 18.

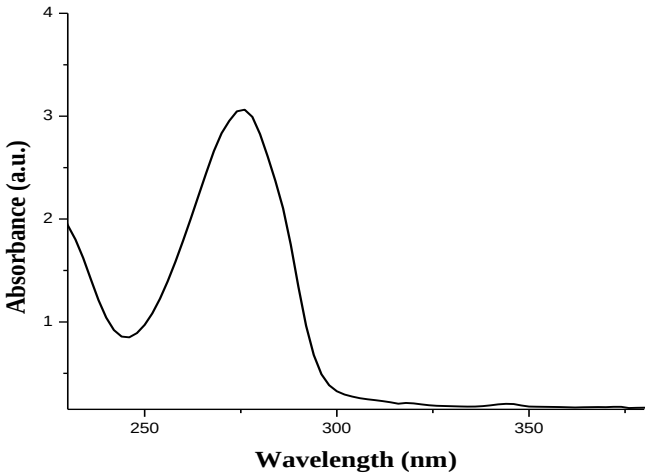



Figure 18. UV/Vis spectra of caffeine extracted by dichloromethane

The mean percentage of caffeine determined from UV/Vis analysis of green coffee beans extracted using dichloromethane is given in Table 8.

**Table 8**.The mean percentage of caffeine for three independent measurement of coffee sample collected from local market obtained by UV/Vis spectrophotometer

| Coffee sample name | Mass of coffee (g) | Extracting volume(mL) | $A_{max}$ (a.u.) | Mass of caffeine(g) | Percentage of caffeine in coffee(%w/w) | Mean for triplicate measurement ± SD |
|---|---|---|---|---|---|---|
| Sample A | 0.33 | 100 | 2.65 | 0.00453 | 1.373 | 1.397 ± 0.02 % |
| | 0.33 | 100 | 2.72 | 0.00465 | 1.409 | |
| | 0.33 | 100 | 2.73 | 0.00466 | 1.410 | |

Table 8 shows the percentage of caffeine in green coffee beans extracted with dichloromethane was found 1.397 ± 0.02 % for the same coffee sample with the newly developed optical methods.

**Table 9.** Comparison of the means of each of the three newly developed methods with the mean obtained by UV/Vis spectrophotometer using t-test at 95% confidence level.

| Methods | Mean ± SD | Degree of freedom | $t_{calculated}$ | $t_{critical}$ | Remark |
|---|---|---|---|---|---|
| FT-IR-ATR | 1.52 ± 0.093 % | 4 | 2.05 | 2.132 | No significantly different |
| NIR | 1.50 ± 0.14 % | 4 | 1.26 | 2.132 | No significantly different |
| Fluorescence | 1.497 ± 0.05 % | 4 | 1.97 | 2.132 | No significantly different |

The results obtained using the three newly developed methods are comparable with the results obtained using UV/Vis spectrophotometer (Gebeyehu and Bikila, 2015) and HPLC (Dessalegn et al., 2008). This was further confirmed by applying t-test to compare the means of the three newly developed methods with the mean of caffeine obtained by using UV/Vis Spectrophotometry for the same coffee sample. The results indicated that at 95% confidence level the means are not significantly different. Therefore, the present developed methods are relatively easy and cost effective.

## 6. CONCLUSION

A simple, rapid and inexpensive FT-IR-ATR procedure was developed for direct determination of caffeine content in aqueous solution of green coffee beans. Water was used for the whole process which is the cheapest solvent found everywhere, environmentally friendly and can help to perform experiments without suffering from the toxic nature of different organic solvents. The procedure was very short and simple, aqueous solution of coffee bean was filtered and the filtrate was used for rapid FT-IR-ATR analysis without further purification or pre-treatment. The use ATR accessories in conjunction with FT-IR spectrometers provides for the non-destructive measurement of sample and the ATR accessory also allows for easy and reproducible as well as fast analysis of liquid samples with just a few drops required.

Caffeine was also directly determined in aqueous solution of green coffee beans using fluorescence spectroscopy. Green coffee beans were dissolved in water, filtered out and the filtrate was used directly for fluorescence analysis. The developed method for analysis of caffeine is simple, cheap, fast and highly sensitive. The percentage of caffeine determined by this method is comparable with the previously reported results using UV-Vis and HPLC methods. NIR spectroscopy can also be used as alternative choice of caffeine determination using reduced amount of organic solvent (DMF) and univariate calibration technique. The method is simple, cheap and fast relatively, the result obtained by this method is in a good agreement with the present methods determined in aqueous solution of coffee beans.

The percentage of caffeine in green coffee bean was also determined by using previously developed UV/Vis spectrophotometric method for comparison. The caffeine content of the green coffee bean in % w/w was found to be 1.397 ± 0.02 using UV/Vis spectrophotometer for the same coffee sample. The results obtained using the three newly developed methods are comparable with the results obtained using UV/Vis spectrophotometer. It was further confirmed by applying t-test and the results indicated that at 95% confidence level the means are not significantly different.

From the experimental point of view the present developed methods have their own advantage and disadvantages. These advantages and disadvantages can help us to select the appropriate methods according to the need. The means of the three methods are also compared by applying one way analysis of variance (ANOVA) and based on the ANOVA result at 5% significance level the means are not significantly different.

Therefore, a quantitative analysis of caffeine in coffee beans become feasible by employing the present proposed optical methods with a simple, short time of analysis and inexpensive procedure. These analytical methods may therefore, be recommended for the rapid, simple, safe and cost effective determination of caffeine in coffee beans.

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