ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF CHEMISTRY

DETERMINATION OF CAFFEINE IN COFFEE USING UV/VIS SPECTROPHOTOMETRY

A GRADUATE PROJECT SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDISS ABABA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

BY

TSEGAYE GUDU

ADVISORS: MESFIN REDI (Ph.D)

MERID TESSEMA (Ph.D)

JULY 2009

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Abstract

Spectrophotometric method was developed for the determination of the amount of caffeine in coffee samples. The Uv/vis spectra of pure caffeine in water and dichloromethane were obtained with peak absorption at λ_{max} 271 and 275 nm, respectively. The developed method showed linearity for the determination of caffeine in the range of $2.5x10^{-5}$ mol dm^{-3} to $1.25x10^{-4}$ mol dm^{-3}. The method also showed good precision, accuracy with limits of detection 2.14 and 1.79 mg/l in water and dichloromethane, respectively. The relative standard deviation for the determination of a $2.0x10^{-5}$ mol dm^{-3} caffeine was 4.53% and 3.72% (n=5) in water and dichloromethane, respectively. The molar decadic absorption coefficients of caffeine in water and dichloromethane at maximum wavelength are 1031.2 ± 0.036 and 1004.4 ± 0.029 m^2 mol^{-1}, respectively. Analytical parameters were investigated primarily; the method was applied to quantify the amount of caffeine in coffee samples. The method was found to be fast, simple, cost effective and environmental friendly for the determination of caffeine in aqueous solution coffee samples with satisfactory results.

Keywords: Coffee Arabica, Caffeine, Absorbance, Uv/vis spectrophotometry, Extraction

1. Introduction

1.1 Background of Coffee

It is believed that coffee tree was first originated in the Southern Nation Nationalities People Region (SNNPR) of *Kaffa* zone, in the area known today as "Mankira". Its name also believed to be derived from the name of this zone where shepherds discovered the coffee beans in the 6th century. Then, the Arabs introduced coffee from Ethiopia to Yemen during the 13th century; whereas the habit of drinking coffee was developed in the 15th century. This habit gradually spread to the rest of the world leading to the increased interest of some countries to produce coffee as a commodity on a large scale [1].

Trade in coffee, a much-prized commodity, was jealously guarded by the Arabs who would not allow foreigners to visit their coffee plantations, nor fertile coffee beans to be taken out of Arabians and cultivated in the Dutch colonies in Indian and Java. The Dutch became the main suppliers of coffee to Europe, with Amsterdam its trading center [1].

The growth of popular coffee houses, which became favorite meeting places for both social and business purpose, spread from the mid-17th century to other European countries including Austria, France, Germany, Holland and England. Coffee is now one of the valuable to primary commodities in the world, often second in value only to oil as a source of foreign exchange to developing countries. Million of people around the world earn their living from it [2].

1.2 Coffee Production

Coffee tree is indigenous to Africa, and can still found growing wild in the hills of Ethiopia. Today ,coffee is produced in several countries and it is the base of economy for many these countries such as Central America (Mexico ,Guatemala, El Savador,Nicaragua,Cost Rica, and Panama),South America (Venezuela, Colombia, Ecuador, Peru, Bolivia ,Paraguay, and Argentina), India, East Africa(Ethiopia, Kenya Tanzania, Zambia ,and Mozambique), Western and Central Africa(Ivory Coast, Cameroon, Uganda, and Angola),Brazil ,Malaysia(Vietnam, Sri

Lanka, Sumatra, Java, etc.). The two biggest producers by far are Brazil and Colombia, followed by Indonesia, Vietnam and Mexico [3, 4].

Ethiopia is the 3rd largest coffee producer in Africa after Uganda and Ivory cost. Coffee grows in most part of Ethiopia. Oromia and SNNP region comprise the largest coffee cultivated area. Wolega, EliAbaboura, Jimma, Bench Maji, Sidamo, Gedeo, East and west Harerge, South and North Omo are the largest coffee cultivated area of Ethiopia [6].

The coffee tree is a tropical evergreen shrub (genus coffee) and two beans are generally contained in each fruit, which when ripe resembles a red cheery. The two most commercial important species of Coffee are Canephora (*Robusta*) and Coffee Arabica (*Arabica*)[1,5].

Coffee Arabica is native to the southwestern highlands of Ethiopia. It is the most cultivated coffee species throughout the world. About 90% of the world's coffee production is coffee Arabica and 9% is Robusta. Coffee Arabica grown at higher altitude, requires less rain and its beans have a lower caffeine content than that of Robusta. It is mainly grown in Central and Southern America, East Africa, India. It is the most important foreign currency earner for more than 80 developing countries. It is the only species occurring in Ethiopia [7,11].

Coffee Canephora widely grown in Western and central Africa, Malaysia, Brazil, and India. It is originated in the humid lowland forest of tropical Africa, which stretches from Guinea to Uganda and Angola. It has stronger flavor than Arabica with a fully body and a woody after test, which is useful in creating blends and especially use full in instant coffee. It is grown at lower altitude [12].

1.3 The Chemistry of selected components of coffee

Coffee contains several biologically active substances, which have some capability of causing physiological effects. The chemical composition of coffee varies for different reasons such as species and variety of coffee beans and to lesser extent other factors such as agricultural activities, degree of maturation and storage conditions.

The beans of the most economically important and cultivated species of coffee,

C. Arabica, contains such as 8 % phenol polymers, 6 % polysaccharides, 4% hydroxycinnamic acids, 2 % trigonelline, 3 % minerals , 2 % water , 1 % caffeine, 0.5% Carboxylic acids 0.3 % sugar and 0.2 % lipids [12]. Some these constitute of coffee are summarized as follows.

1.3.1 Carbohydrates

Carbohydrates constitute almost half the dry weight of green coffee. Sucrose is the main oligosaccharide and *mannans* and *arabinogalactans* are the main polysaccharides. At roasting sucrose is destroyed, the polysaccharides are progressively depolymerised and eventually pyrolised at dark roasting. The percentage of carbohydrates in a cup of coffee depends on the blend, the roasting level and mode of preparation [12].

1.3.2 Carboxylic Acids

Several aliphatic acids are present in green coffee, mostly *citric, malic and quinic*. Roasting generates other acids either volatile or non-volatile and increases the proportions of *quinic* acid by breaking up Chlorogenic acids [12].

1.3.3 Lipids

Lipids are constitute of green coffee. The *diterpenes* (cholesterol-like) *kahweol* and *cafestol* are specific to coffee. The content of lipids can reach a few percent in coffee preparation or when ground coffee is percolated and not filtered but levels are in significant in filtered and instant coffees [13].

1.3.4 Trigonelline

Trigonelline comprise about 2% of the dry weight of green coffee but does not survive roasting, breaking into nicotinic acid, pyridine and other volatile compounds. It is also found in various plants and in some animal species including sea urchins and jelly fish. It also appears in mammalian urine after administration of nicotinic acid. It is one of the responsible components for bitter tasting in coffee brew. It is also thermally unstable and converted nicotinic acid and to certain flavor compounds during roasting [9].

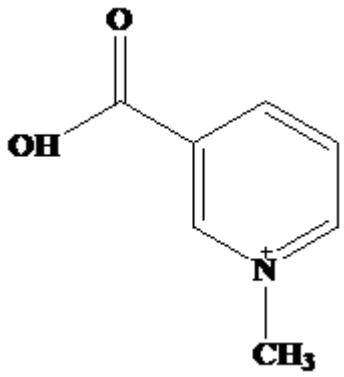


Fig. 1 The Chemical Structure Trigonelline.

1.3.5. Hydroxycinnamic acids

They are one of the major classes of phenolic compounds. They are present in a large variety of fruits and vegetables. The major representative of hydrocinnamic acid in food is caffeic acid. It is largely occurs conjugated with quinic acid as chlorogenic acid (Fig.2). Coffee, one of the most widely consumed beverages in the world, is the major dietary source of chlorogenic acid and caffeic acid is produced from chlorogenic acid by hydrolysis at elevated temperature [10,11].

Chlorogenic acid has antioxidant properties showed by its ability to scavenge various free radicals when tested. Moreover, chlorogenic acid reduces glucose uptake by favoring the dissipation of the Na^+ electrochemical gradient and inhibits the activity of hepatic glucose-6-phospates which is implicated in glucose homeostasis. When it is ingested coffee form, it increases the plasma antioxidant capacity [11].

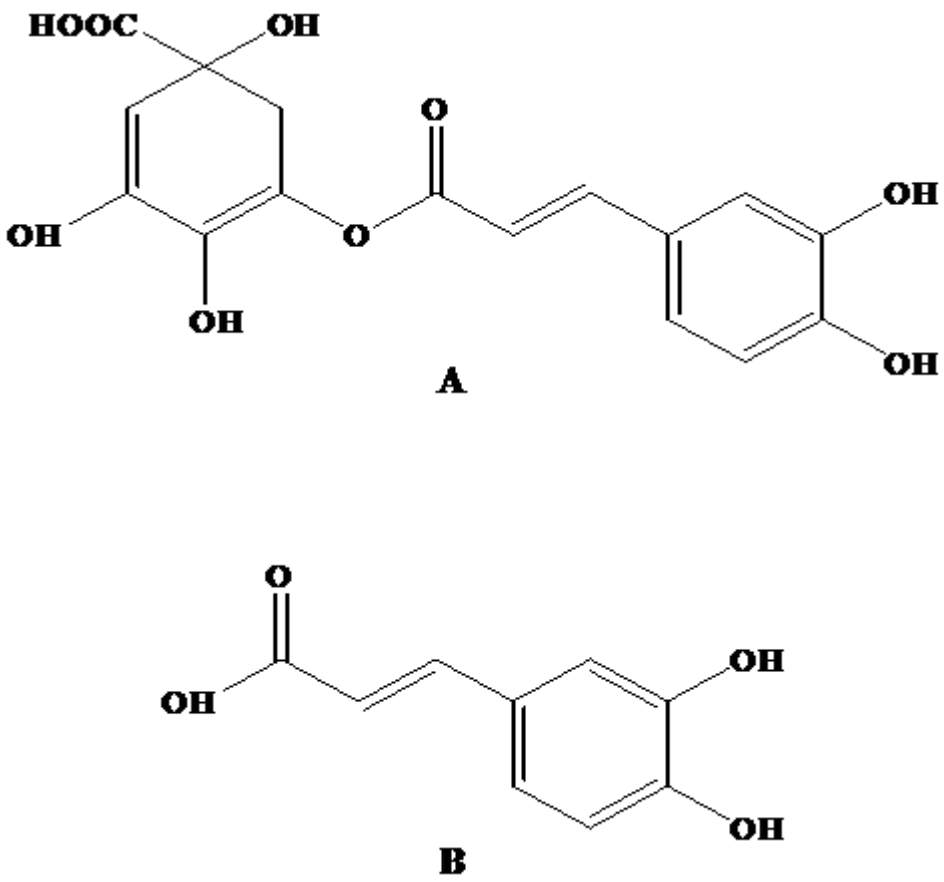


Fig. 2 The Chemical Structure of Chlorogenic (A) and Caffeic acid (B).

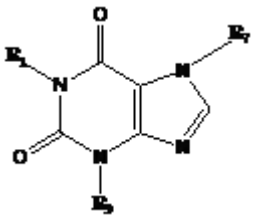
However, among these components of coffee, trigonelline and hydroxycinnamic acids are the major variables that affect for the determination of caffeine in coffee sample. The presence of these acids in green coffee extracts, they affect the quantitative determination of caffeine in coffee using Uv/vis spectrophotometer. From the literature report these coffee components absorb with λ_{max} 271, 275 and 330nm for trigonelline, caffeic acid and chlorogenic acid, respectively [12]. Developing suitable method for removing these acids from caffeine is the first work in direct absorption method for the determination of caffeine.

2. Caffeine

2.1 Caffeine

Caffeine is a naturally occurring alkaloid which belongs to a class of organic compounds called methylxanthines. Chemically speaking caffeine belongs to the alkaloid family and the methylxanthine group. Caffeine or *1,3,7-trimethylxanthine* is a xanthine molecule with methyl groups replacing all of the three hydrogen's bound to nitrogen in xanthine. The closely related substances theophylline or 1, 3-dimethylxanthine and theobromine or 3, 7-dimethylxanthine are members of *N*-methyl derivates of xanthine [12,13, 14].

Table 1: Structure of Xanthine and its naturally occurring *N*-Methyl Derivatives [13]

Structure	R_1	R_3	R_7	Compound
	H	H	H	Xanthine
	CH_3	CH_3	H	Theophyline
	H	CH_3	CH_3	Theobromine
	CH_3	CH_3	CH_3	Caffeine

In pure form, caffeine is a crystalline white powder moderately soluble in water and a wide range of organic solvent such as ethanol, ethyl acetate methanol, benzene and etc. When it is removed from the sources plant and reduced to its most natural state, it forms a white powder. This powdered form of caffeine is actually the scientific definition of bitter, that is why many beverages containing caffeine also contains copious amounts of sugar or other sweeteners. Its molecular weight is 194.19 g/mol. with chemical formula $C_8H_{10}N_4O_2$, melting point is 236 °C, which sublimes at 178 °C at atmospheric pressure and its specific gravity is 1.2 [13].

2.2 Occurrence

Caffeine occurs naturally in over 60 plant species of which the most well-known are coffee, tea and cocoa-beans, all of which have been consumed for many years. It is produced commercially by extraction from natural sources. Coffee is the major sources of caffeine. Coffee is one of the

most popular consumed beverages in the world. Consequently, the ingestion of caffeine, an important ingredient of coffee as well as tea, has become a daily habit for many. A large amount of caffeine is found in coffee seeds (mainly in endosperms). Caffeine is found in coffee associated to trigonelline and chlorogenic acids. Coffee has both the highest and the most variable caffeine content among dietary products containing this alkaloid [14, 15].

The Caffeine content of coffee beans is mainly expressed as percentage of dry weight. The bean of most cultivars of Arabica coffee (Coffea Arabica) contains up to 1.0% caffeine, but Robusta coffee (Coffea canephora) contains about 2.0% caffeine. Although these species are not commonly used for beverages, Coffea dewevrei (1.2%) and Coffea libberica (1.4%) also contains high concentration of caffeine [16].

Nowadays, coffee is one of the most popular drinks across the world and the total annual worldwide production of green coffee is estimated to be about six million tons and the coffee industry is second next to oil with an associated revenues of $60 billion world wide. The roasting of green coffee beans yields the apparently bitter and dark coffee beverage, in which caffeine is responsible for the mild stimulating properties and to a particular proportion of its apparent to bitterness. Coffees grown in different parts of the world and differ genetically in their composition; as a result the amount of caffeine per gram of the coffee bean varies depending on the species of beans, geographic areas, and the preparation process[11,17].

Carbonated beverages are also, one source of caffeine for all ages of human being. Caffeine is added to soft drinks as a part of flavor profile as its bitterness taste enhance other flavors. The variability of the caffeine content in carbonated beverage occurs among the brands, since most of the caffeine content of these products are added from other natural sources [13].

2.3 Absorption and Metabolism

Caffeine enters into human body through different sources such as food stuffs, beverage and medications. Caffeine absorption from the gastrointestinal tract is rapid and virtually completed about 45 minutes after ingestion. The absorbed caffeine is readily disturbed through out the entire body. It passes across the blood brain barriers, through the placenta into amniotic fluid and the foetus, and into the breast milk [16,18].

Caffeine is metabolized by *demethylation* and *oxidation.* It is metabolized in the liver through a series of *N- demethylation* and *purine ring oxidation* reaction to yield a mixture of mono and dimethylxanthines and methyluric acids. The main dimethyl xanthine metabolites are *theophyline* (4 %), *theobromine* (12 %) and *paraxanthine* (*1, 7-dimethylxanthine*) (80 %). *Paraxanthine* is not found in foods but it is the main metabolite of caffeine. Formation of the other metabolites; *1,3 7- trimethyluric acid* and *6-amino-5 (N-formylmethylamino)-1,3 dimethyluracil* together account for 15% of caffeine elimination. The metabolism is slowed during pregnancy and in women taking oral contraceptives. On the other hand, the metabolism rate of caffeine is grater in smokers than non-smokers [17, 18, 19].

The half – life of caffeine, the time required for the body to eliminate one-half of the total amount of caffeine consumed at a given time varies widely among individuals according to such factors as response, age, sex, liver function , pregnancy, some concurrent medications, smoking and the level of enzymes in the liver needed for caffeine metabolism. Caffeine has a half - life which is faster than theobromine. It is completely metabolized by human body; only 1-5 % of ingested caffeine is recovered unchanged in the urine. Infant up to the age of 8-9 months have a greatly reduced ability to metabolize caffeine, excreting about 85 % of the administered caffeine in the urine unchanged [20].

The half- life in the newborn child range from 5-100 hrs. But it gradually approaches that of an adult. By 6 months of age caffeine's half- life is about 20-30 % shorter in females than in males. The half life in females using oral contraceptive steroids is approximately twice that observed for ovulatory females. Pregnancy slows down the metabolism of caffeine. The metabolic half –life increases steadily from 4 hrs during the first trimester to 18 hrs during the third trimester. Obesity also slows down caffeine metabolism. Smoking is another factor which affects the metabolism of caffeine. It accelerates the rate at which caffeine is eliminated [19,20].

2.4 Beneficial Effects of Caffeine

Caffeine is a plant based alkaloid which stimulates the central nervous system. The stimulatory effects of caffeine usually results in an increased ability for mental activity for sweets by simulating the production of those adrenal hormones that cause blood sugars to be increased. Stimulatory effects of caffeine results in increased capacity for both mental activity

and muscular work. The weakness, depression and discomfort from excess of alcohols can be nullified with black coffee or hypodermic injections of caffeine. Insensibility from hashish is believed to be ended by the use of caffeine medication. Even the dullness and sense of depression from a little too much tobacco is helped by coffee [17,19.21].

The other benefits of caffeine include reduced risks of Parkinson's disease, colon cancer, diabetes, decrease in exercise induced myocardial flow reserve and increase and in both sexual motivation and locomotors activity on female mating behavior. Caffeine expands blood vessels and consequently the brain receives more oxygen. It helps in preventing a positive energy balance and obesity. Caffeine is also an accepted drug for intramuscular application to treat arterial hypotension [22,23].

2.5 Side effects of Caffeine

There is no requirement for caffeine in the diet. A moderate caffeine intake, 50-200 mg of caffeine, is generally sufficient for a mild stimulation. Excessive Caffeine intake can lead to a fast heart rate, dieresis (excessive urination), nausea and vomiting, restless, anxiety, depression tremors. Over doses of caffeine may result in hypertension, hypotension, tachycardia, fever, delusions, hallucination, and cardiac arrest [24, 25, 26].

Caffeine is thought to increase alertness, reduced sleep tendency and produce adverse effects in the central nervous system such as insomnia (difficulty in getting to sleep or staying a sleep). One of the negative effects of caffeine is its ability to disrupt sleep cycles, causing less deep sleeps , particularly when intake caffeinated beverage with in a few hours of bed time. Caffeine can create panic attacks and interfere with medications taken to calm the system. Caffeine does reduce dopamine, a chemical produced by the brain that affects the brain pleasure centers. This can create more depressions and anxiety [27, 28, 29].

For people with high blood pressure or high blood sugar levels (diabetes) the negative effects of caffeine should be understood. Caffeine can raise these levels. Any type of heart problems can be affected by caffeine. The negative effects of caffeine is increasing heart rate can create problems for people with heart conditions and in high doses, caffeine can induce irregular heart beats in healthy people. Caffeine increase acidic responses in the stomach

which may lead to excusive bowel movements and is associated with stomach discomfort [29].

2.6 Physiological Effects of Caffeine

Caffeine is a powerful central nervous system stimulant; a mild diuretic and has been used as a mild anti-depressant. It has many physiological effects such as, gastric and secretion, diuresis and stimulation of the central nervous and the cardio vascular system. In addition, caffeine, interference with the uptake and storage of calcium by the sarcoplasmic reticulum. It also increases the respiratory rate and causes bronchodilation and stimulates lypolgysis [25,29].

The stimulatory action of caffeine involves antagonism of adenosine receptors which are present in brain, blood vessels, kidneys, heart, the gastrointestinal tract and the respiratory hierarchy [28,29] . Adenosine is an adenine molecules attached to a ribose or deoxyribose sugar molecules. The similarity in chemical structure between the adenine portion of adenosine and the caffeine molecules is the key to how caffeine works [13,30]. Adenosine when bound to receptors of nerve cells, slow down nerve cell activity during sleep. Caffeine, being structurally similar to adenosine, has the potential to occupy adenosine receptors sites. When the caffeine molecules binds to the receptors doesn't cause the cells to slowdown; instead , the caffeine blocks the receptors and thereby blocks the regulatory functions adenosine and produces the stimulatory effect [13,36,39] .

In addition, caffeine blocks the receptors and thereby disorders the regulatory function of adenosine and produces the stimulatory effect. The resulting effect in turn increases nerve activity that causes the release of hormone epinephrine, which leads to several effects such as higher heart rate, increased blood pressure and release of glucose by the liver [33,40,41]. Adenosine inhibits the release of many neurotransmitters including noradrenalin, dopamine, acetylcholine and glutamate. By blocking the receptors, caffeine facilitates the release of neurotransmitters and through this mechanism it also promotes wakefulness. Caffeine enhances muscular performance by lowering threshold for sarcoplamic Ca^{2+} release through the ryandoine receptor [13]. The physiological effects of caffeine associated with cardio vascular system are believed to be the result of multiple actions. These include antagonistic effects on

adenosine receptors, activation of the sympatic nervous system (release of catecholamine from adrenal medulla), stimulation of adrenal cortex (release of corticosteroids), renal effects (Diuersis, natriursis and activation of reninangiotensin aldosterone system)

2.7 Methods of Analysis

The development of Chemical and instrumental methods for the separation, identification and quantitative analysis of individual components in food and beverages are extremely important. Recently, the food industry as well as academic and governmental institutions needs to assess the nutritional value of food and beverage for human health. These days caffeine is being utilized as flavor- enhancer by most of the beverage (soft drinks), coffee and tea companies. So, the development of simple, fast, cost-effective environmentally friendly analytical methods for monitoring caffeine for the quality of products containing caffeine is highly needed.

Many analytical methods have been developed for the determination of caffeine and the quality control of products containing caffeine. The most commonly used methods for the determination of caffeine in beverages include various analytical methods such as High Performance Liquid Chromatographs (HPLC), Thin Layer Chromatography (TLC), Solid Phase-Extraction And High Performance Liquid Chromatography (SPE-HPLC) ,Capillary Zone Electrophoresis (CZE), Fourier Transform Infra-red (FT-IR),and Electrochemical methods have been reported. Although these methods have been usually used widely for caffeine determination, the methods require very costly instrumentation, higher skilled technician and complicated and time consuming procedures [16,18,19,26,27,37,38,40].

UV/Vis spectrophotometric method became important means in the analyses of caffeine as various methods have been reported for the determination of caffeine in soft drinks, coffee and tea. The method is cost effective, uses fewer reagents, easily performed, is less time consuming and highly accurate. On the other hand, the use of Spectrophotometric provides a simple and rapid method for the determination of caffeine in tea and coffee and their products this is because of the characteristics observation peak of caffeine at 271–276 nm [32, 33, 34, 35].

Rapid and simple Uv/Vis Spectrophotometric procedures were applied for the determination of caffeine in coffee mixtures. Interfering impurities are removed from the sample

by preferential extraction of caffeine with chloroform from an alkali solution and subsequent passage of the extract through a column of activated alumina [8].

Spectrophotometric determination of caffeine and theophylline in pure alkaloids and its application in pharmaceutical formulations. The method is based on the oxidation of caffeine and theophylline with sodium metaperiodate in the presence of acetic acid followed by coupling with 3-methyl 2-benzo thiazolinone Hydrazone hydrochloride (MBTH), to produce colored product with λ_{max} of 630 nm [20]. Simultaneous spectrophotometric determination of paracetamol and caffeine in pharmaceutical preparation in solid phase has been reported [41].

Simultaneous determination of caffeine and acetylsalicylic acid in analgesics by Uv/Vis spectrophotometetry [32]. Kinetic spectrophotometric method for rapid determination of caffeine in food samples have been reported [17].

Spectrophotometric determination of caffeine in coffee and tea products with special reference to coffee and chicory mixtures. Most methods used for determining caffeine involve the preparation of a suitably clarified water extract from which the caffeine is extract is weighed and its caffeine content is obtained from a Kjeldahl determination of nitrogen. This determination can, however, be avoided by spectrophotometer measurement of the chloroform extract but, as chloroform is an unstable solvent for spectrophotometric method, it is found that capable of confining all spectrophotometric measurements to aqueous solution. First the examination was made suitably method for preparing clarified aqueous extracts of the samples. For removing interfering substances, by using heavy magnesium oxide and zinc Ferro cyanide, was found to remove chlorogenic acid, but did not remove trigonelline, which has an absorption peak close to the 273 nm peak of caffeine [36].This Method was used satisfactorily it is excessively time consuming. Interfering impurities as trigonelline and chlorogenic acid have been removed by treatment with heavy magnesium oxide and Ferro cyanide before the aqueous extracts were analyzed spectrophotometrically.

This procedure gave inconsistent results particularly when applied to coffee mixtures. Because of this limitation; rapid, simple, cost effective and environmentally friendly method for determining caffeine in coffee is highly needed. In economic terms, coffee is one of the most valuable agricultural products exported by developing countries in Central and South America, South-east Asia and Africa. Beans of Arabica and Robusta coffee respectively contain about 1 % and 2 % caffeine. The demand for decaffeinated coffee has been increased. This is because of a

growing belief that ingestion of a large amount of caffeine has adverse effects on health. Decaffeination is usually performed by physico-chemical treatments, such as water diffusion, solvent extraction, and supercritical carbon dioxide oxidation. However, the last two methods have high operational costs and usually produce waste products that require further treatments. In order to overcome these problems, a specific process is necessary. The water decaffeination method is considered to be an efficient, cost-effective, simple and environmentally friendly for the determination of caffeine in coffee sample.

In addition, in the production of decaffeinated coffee the manufacturer needs how much caffeine is still in the coffee to ensure product quality. Consequently, the manufacturers want to replace the time-consuming and complicated method with a method that is faster easier and cheaper to use.

2.8 Objective of the study

In the manufacturing of decaffeinated coffee the manufacturer needs to know how much caffeine is in the coffee to ensure products quality. One of the requirements of a good coffee is whether it contains a reasonable moderate quantity of caffeine or not. Therefore quantitative determination of the amount of caffeine in green coffee is necessary.

- The general objective of this study is to develop a simple, rapid, cost-effective, and environmentally friendly method for the quantitative determination of caffeine in aqueous solution of raw coffee samples grown in Ethiopia.

The specific objective of this study is to:

- Develop simple, rapid, cost-effective, and environmentally friendly Uv/vis spectrophotometric method for the quantitative determination of caffeine in aqueous solution of raw coffee samples grown in Ethiopia particularly Bench Maji, Jimma Limmu and Sidama Arbegona.
- Compare the results of this work with literature methods.

3. Brief Theory of Spectrophotometry

3.1 Ultraviolet Spectrophotometry

The absorption of ultraviolet/visible radiation by molecules leads to transitions among the electronic energy levels of the molecule and for this reason the alternative title *electronic spectroscopy* is often preferred. Most organic compounds having chromophoric groups absorb ultraviolet light and show ultraviolet absorption in this range of wavelength. Consequently, Spectrophotometry is of limited utility in this range of wavelengths.

3.2 Nature of Molecular Absorption

When continuous radiation passes through a transparent material, a portion of the radiation may be absorbed. If that occurs the residual radiation when it is passed through a prism, yields a spectrum with gaps in it, called an *absorption spectrum***.** As a result of energy of absorption, atoms or molecules pass from a state of low energy (the initial, or grounds state) to a state of higher energy (the excited state).The electromagnetic radiation that is absorbed has energy excited and ground states.

In the case of ultraviolet and visible Spectrophotometry, the transitions that result in the absorption of electromagnetic radiation in this region of the spectrum are transitions between

electronic energy levels. As a molecule absorbs energy, an electron is promoted from an occupied orbital to an unoccupied orbital of higher potential energy

Molecular absorption is more complex than atomic absorption because many more potential transitions exist. A molecule may absorb light energy in three ways.

1) By raising an electron to a higher energy level (electronic)
2) By increasing the vibration of constituent nuclei (vibration)
3) By increasing the rotation of molecules about its axis (rotational)

$$E_{total} = E_{electronic} + E_{vibrational} + E_{rotational} + \cdots$$

When a molecule interacts with photons in Uv/Vis region, the absorption of energy results in displacing an outer electron (valence electron) in the molecule is given by the equation:

$$\Delta E = E(excited) - E(ground) = h\nu = hc / \lambda$$

3.3 Band Spectrum

For molecules the Uv/Vis absorption usually occurs over a wide range of wave lengths, because molecules normally have many excited modes of vibration and rotation at room temperature. In fact the vibration of molecules cannot completely frozen out even at absolute zero. Consequently, a collection of molecules generally has its members in many states of vibration and rotational exaction.

The energy levels for these states are quite closely spaced, corresponding to energy differences considerably smaller than those of electronic levels. The rotational and vibration levels are thus super imposed on the electronic levels. A molecule may there fore undergo electronic and vibration rotational excitation simultaneously.

Because of there are so many possible transitions each, differing from the others by only a slight amount, each electronic transition consists of a vast number of lines spaced so closely that the spectrophotometer cannot resolve them. Rather, the instrument traces an envelope over the entire pattern. What observed from these types of combined transitions is that the Uv/vis spectrum of a molecule usually consists of a broad band of absorption centered clear the wavelength of the major transition [44,45].

3.4. Principles of Absorption Spectrophotometry

The higher the number of molecules capable of absorbing light of a given wavelength the higher the extent of light absorption. Furthermore, the more effectively a molecule absorbs light of a given wavelength, the greater the extent of light absorption. There are two laws that govern the absorption of light by molecules.

Beer's law relates the absorption to the concentration of absorbing solute, and Lambert's law relates the total absorption to optical path length. They are most conveniently used as the combined Beer's Law:

$$A = \varepsilon c l$$

Where A is the absorbance of the solution, c is molar concentration of solute (mole/m^3), ε is molar decadic absorption coefficient (m^2 mole $^{-1}$), and l is optical path length (m). The molar decadic absorptivity coefficient ε, is constant for a particular compound at a given wave length, and is most commonly expressed as ε_{max} the molar absorptivity at an absorption band maximum. The size of the absorbing system and the probability that the electronic transition will take place control the absorptivity, which ranges from 0 to 10^6. values above 10^6 are termed as high intensity absorption, while values below 10^3 are low intensity absorptions. Forbidden transitions have absorptivities in the range from 0 to 1000.

The Beer-Lambert Law is rigorously obeyed when a single species gives rise to the observed absorption. This may not be obeyed, however when different forms of the absorbing molecule are in equilibrium, when solute and solvent form complexes through some sorts of association, when thermal equilibrium exists between the ground electronic state and a law lying exited states or when fluorescent compounds or compounds changed by radiation are present[46].

3.5 Instrumentation

The typical ultraviolet–visible spectrophotometer consists of light source, a monochromatic, and a detector. The light source is usually a deuterium lamp which emits electromagnetic radiation in the ultraviolet region of the spectrum. These core light source a tungsten lamp, is used for wavelength in the visible region of the spectrum. The monochromatic is a diffraction grating; its role is to spread the beam of light into its component wave length.

The light that passes through the sample cell reaches the detector which records the intensity of the transmitted light (I) emanating from the light source which is split into two beams the detected light is taken to be equal to the intensity of light entering the sample (I_0).

The sample cell must be constructed of a material that is transparent to the electro-magnetic radiation being used in the experiment. For spectra in the visible range of the spectrum, cells composed of glass or plastic are generally suitable. For measurements in the ultraviolet regime of the spectrum however; glass and plastic cannot be used because they absorb wavelengths radiation. Instead cells made of quartz must be used since quartz does not absorb radiation in this region.

The instrument design just described is quite suitable for measurements at only one wavelength. If a complete spectrum is desired this type of instruments has some deficiencies. A mechanical system is required to rotate the monochromatic and provides a scan of all desired wavelengths. This type of system operates slowly, and therefore requires more time to record a spectrum [46].

4. Experimental

4.1 Instrumentation

Measurements were performed with UV/Vis spectrophotometer (SPECRONIC® GENESYS™ 2PC.) with 1cm quartz cuvette. The spectrophotometer was interfaced with computer and other laboratory apparatus were used.

4.2 Reagents and Samples

Dichloromethane (Aldrich, Germany), Caffeine (Evan, England), Sodium Hydroxide (pool, England) and de-ionized water (AAU, Ethiopia) were used through out the study. Coffee samples *(Coffea Arabica)* were collected from the southern and south east of Ethiopia in particular from Bench Maji, Sidama Arbegona and Jimma limmu with out considering their varieties. Each coffee sample was measured three times and the mean value and standard deviation and relative standard deviation for each were calculated. The coffee samples used for this study were raw coffee beans.

4.3 Spectrophotometric Procedure

An aqueous caffeine stock solution was prepared by dissolving 2.91 mg caffeine in 100 ml. Working solutions of caffeine were subsequently prepared by appropriate dilution of the stock solution with de-ionized water. Similarly, a stock solution of caffeine was prepared by dissolving 1.45 mg of caffeine in 50 ml of dichloromethane and diluting to a series of 25 ml standard volumetric flasks.

For the spectrophotometric determination of caffeine the wavelengths were studied within the interval 220-450 nm using 7.5×10^{-5} mol dm^{-3} of aqueous standard caffeine solution. The wavelength of a single intense absorption located in the Uv range at λ_{max} = 271nm was determined by scanning 7.5×10^{-5} mol dm^{-3} of aqueous standard caffeine solution. It was observed

that as the concentration of caffeine was varied over the range from $2.5x10^{-5}$ to $1.25x10^{-4}$ mol dm^{-3}. It was accompanied by appropriation enhancement in the monitored absorption intensity over such wide concentration range. Similarly, the Uv/Vis spectra was measured over the range 220-450 nm using $1.0x10^{-4}$ mol dm^{-3} standard caffeine solution dissolved in 25 ml dichloromethane. The resulted band showed that an absorption spectrum, which had an intensive peak found at λ_{max}=275 nm. A linear relationship between absorbance and the analytes concentration was investigated over the rang $2.5x10^{-2}$ to $1.25x10^{-1}$ mol/m^3 at λ_{max}=275 nm. The absorbance of the solution was measured at wavelength of maximum absorbance for the two solvents and the calibration curves were constructed. The absorptivity coefficient of caffeine in each solvent is calculated from the slope.

4.4 Background Absorbance Correction

The absorption spectra results indicated the existence of overlapping absorption bands, mainly from acidic components of coffee. This was confirmed by determining the caffeine contents of the dichloromethane extracts. The values are shown in Table 5. Attempts were made to eliminate these interfering bands by drop wise addition of 0.1 M NaOH solution (particularly to aqueous extracts) as described previously, but complete elimination of the interferents.

In order to eliminate the background absorption bands from the direct absorption measurements, corrections were made by employing the “base line” procedure, considering the mean of the readings obtained in the interval between 260-290 nm (at the wavelength on the ascending and descending portion of the curve at equidistance on either side of the maximum caffeine peak) from the reading at maximum for caffeine. The results of the determination of caffeine in coffee samples were obtained after subtracting the interferents’ peak from the total caffeine spectra.

4.5 Real Sample Preparation and Extraction

Coffee bean was ground with a commercial blendor. The ground was sieved through test sieve with a mesh size of 212 μm (Retsch®, Germany). Approximately 100 mg of ground coffee was dissolved in approximately 20 ml of de-ionized water in a beaker and diluted to 50 ml with de-ionized water. Then, the solution was heated in boiling water bath at about 60^0C for one hour in hot plate while stirring. After allowing the residue to settle, the solution was filtered using a glass filter to remove particles from the solution.

For measuring the volume of the final purified aqueous solution of coffee sample, first the mass of a standard flask (m_f), which was used to contain the purified aqueous filtrate, was measured using an electronic balance. After filtration, the total mass the weighed standard flask with the aqueous purified filtrate (m_t) was measured. The mass of the aqueous purified filtrate was calculated using in the total mass of the standard flask with the filtrate (m_t) and mass of initially measured standard flask (m_f). Subsequently the volume of the filtrate was measured using the net mass (m_t-m_f) and the density of water. This aqueous filtrate solution was taken as a test sample for quantitative determination purpose.

In the case of dichloromethane extraction, the procedure was similar up to the clean up step. Afterwards, that caffeine was extracted using equal volume of aqueous extract. After measuring the volume of the coffee solution filtrate (aqueous extract), half of the measured volume was used for extraction with dichloromethane (volume ratio 1:1). For removal of interferents, liquid-liquid extraction with dichloromethane was performed using 250 ml separately funneled. The extraction was carried out with similar volume of aqueous extract, by gentle swirling of the mixture to mix the layers by inverting the funnel several times. Then, using separatory funnel caffeine was extracted with dichloromethane from the solution. The caffeine extracted with dichloromethane was stored in a measured mass of volumetric flask. Using the mass of the extract and density of dichloromethane the volume of the extract was calculated. The coffee solution in de-ionized water and the extract were measured in the range between 220-450 mm.

5. Results and Discussion

5.1 The Study of Absorption Spectra

For spectrophotometric determination of caffeine, the spectrum was recorded in the region 220-450 nm using a $7.5x10^{-5}$ mol dm^{-3} standard caffeine solution which was prepared by diluting a stock solution with 50 ml de-ionized water. The spectrum gave an absorption band, which was characterized by a single intense peak at λ_{max} =271 nm, as shown in Fig.3.

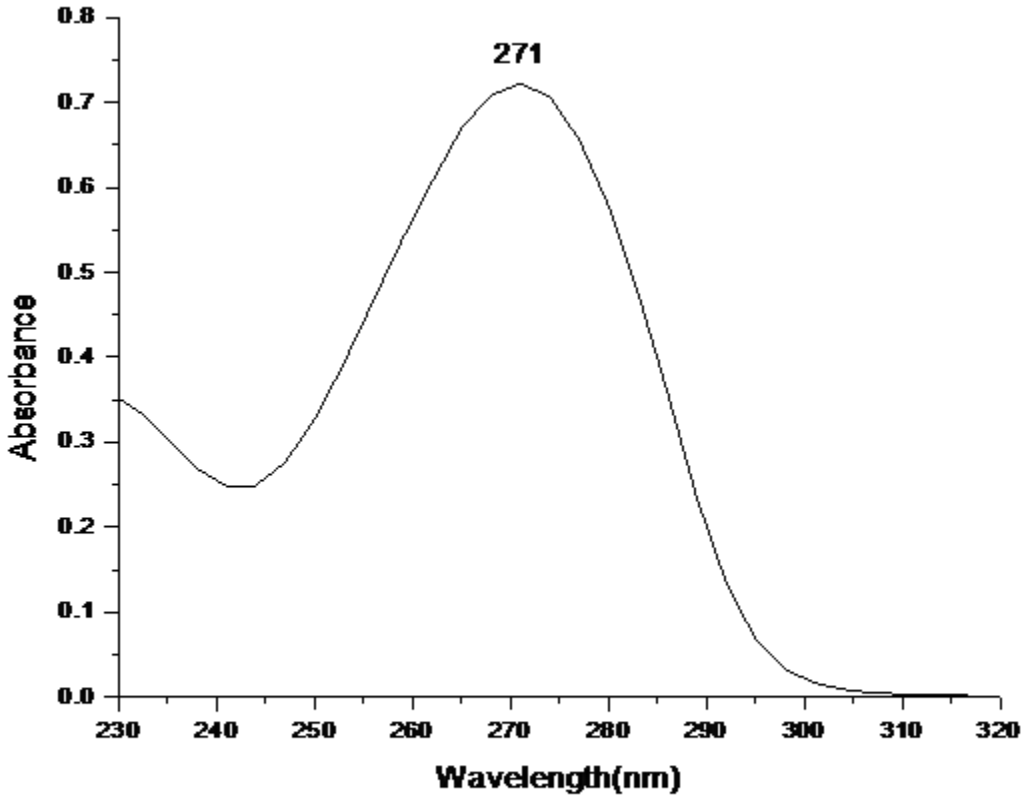


Fig.3 Uv/vis Spectrum of Caffeine in water

A linear function increase in the absorbance was observed as the concentration of caffeine was varied between $2.5x10^{-5}$ to $1.25x10^{-4}$ mol dm^{-3}. The obtained results are depicted in Table 2.

Table 2: Performance Data of the Uv/Vis Spectrophotometric Method for the Determination of Caffeine.

Concentration of caffeine (mol dm^{-3})		Absorbance in	
		Water	Dichloromethane
Water	Dichloromethane		
$2.5x10^{-5}$		0.301	0.309
$5.0x10^{-5}$		0.514	0.531
$7.5x10^{-5}$		0.828	0.812
$1.0x10^{-4}$		1.018	0.996
$1.25x10^{-4}$		1.338	1.324

Similarly, absorbance measurement was carried out using $1.0x10^{-4}$ mol dm^{-3} standard caffeine solution prepared by diluting a stock solution with 25 ml dichloromethane. The spectrum result showed a maximal absorption at λ_{max} =275 nm as shown in Fig.4.

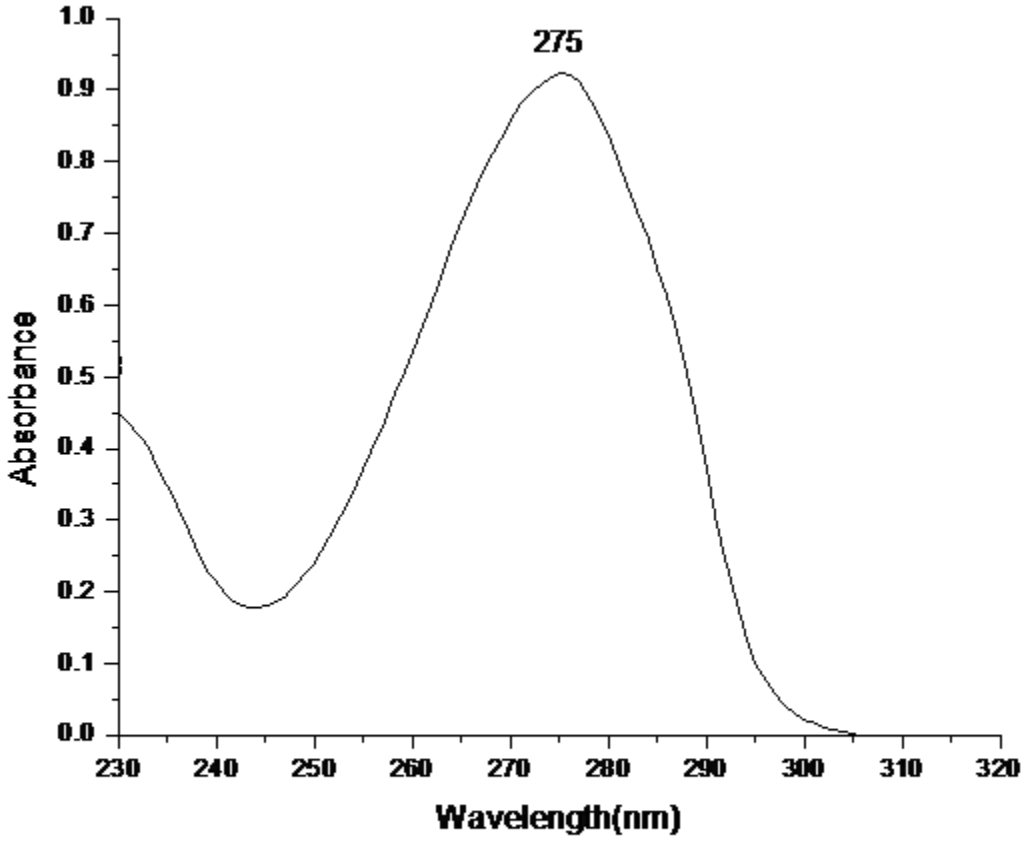


Fig. 4 Uv/vis spectrum of caffeine in Dichloromethane

In order to establish the range in which linearity between absorption intensity and concentration exists for caffeine, different concentration of standard solutions of caffeine, ranging from 2.5×10^{-5} to 1.25×10^{-4} mol dm^{-3} were prepared. The linear relationship of absorbance and concentration in the concentration range measured (Table.2).

5.2 Calibration Curves

Calibration curves for caffeine in water and dichloromethane were constructed by plotting absorbance values as a function of the analyte concentration. Each point of the calibration graphs was the average of three replicates. The molar decadic absorptivity coefficient, ε, was calculated from the slope of the calibration curves. The molar decadic absorptivity coefficient, determined from the slope was used for the quantitative determination of caffeine in real sample.

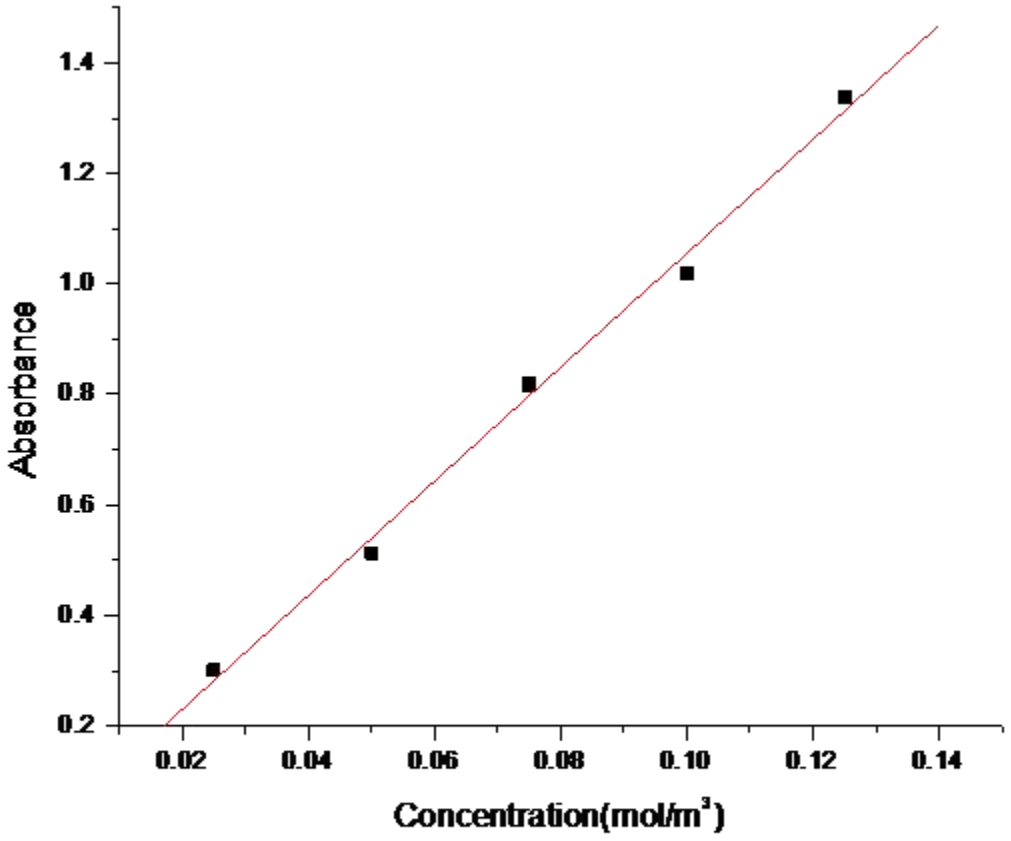


Fig.5 Linear plot of Concentration versus Absorbance in Water

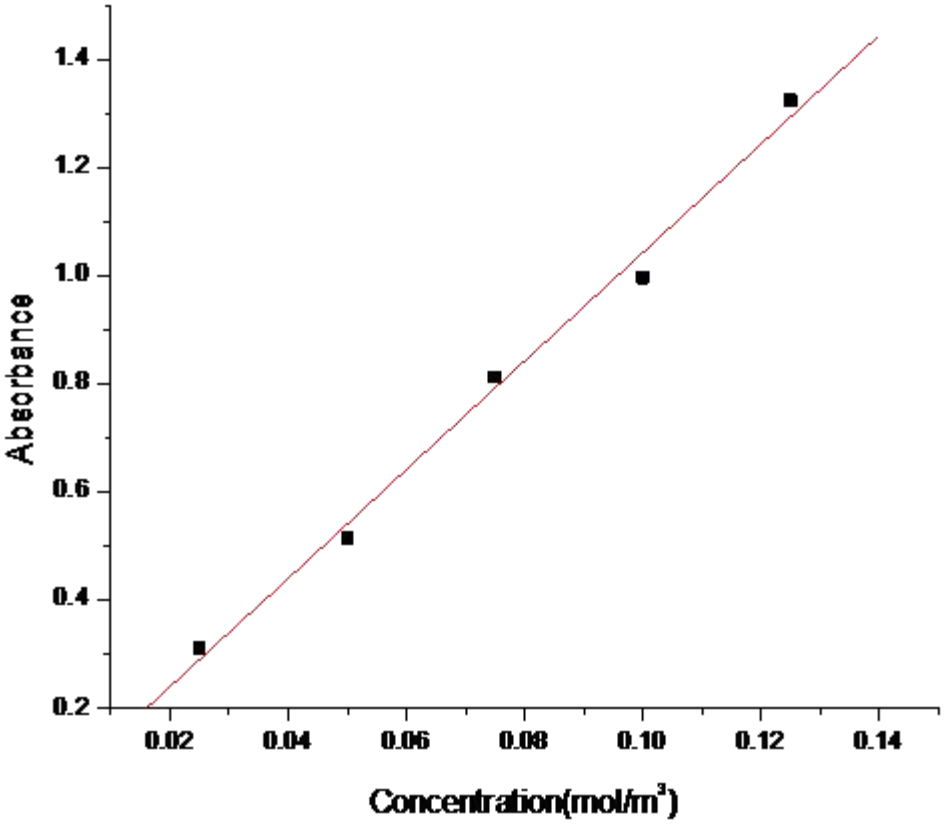


Fig.6 Linear plot of Concentration versus Absorbance in Dichloromethane

5.3 Analytical Parameters

Beer's law was valid in the concentration range between 2.5×10^{-5} to 1.25×10^{-4} mol/dm^{-3} standard caffeine solutions prepared in de-ionized water and dichloromethane. The correlation coefficients indicated a good linear correlation between absorbance and caffeine concentration. The overall results are summarized in Table 3.

Table 3: Characteristics of the calibration graphs for the determination of Caffeine

Parameter/Characteristics	Solvent	
	De-ionized water	**Dichloromethane**
λ_{max} (nm)	271	275
ε (m^2 mol^{-1})	1031.2 ± 0.036	1004.4 ± 0.029
Correlation Coefficient (*r*)	0.997	0.998
Regression equation	$A = 0.0244 + 10.312\ c$	A =0.0379+10.044 C

where *c* is Concentration in mol m^{-3}, $n = 5$

To evaluate the precision and the detection limit, a $2x10^{-5}$ mol dm^{-3} standard caffeine solution in each solvent was used. The relative standard deviation for five replicate measurement of caffeine was 4.53 % and 3.72 % in water and dichloromethane respectively. The limit of detection (three times the blank standard deviation divided by the slope of the equation) was 2.14 mg/l and 1.79 mg/l in water and dichloromethane respectively. On the basis of these results the work was extended to the spectrophotometric determination of caffeine in real sample.

5.4 Determination of Caffeine in Coffee Samples

The developed spectrophotometric method for determining caffeine in coffee has been tested for real sample. However, coffee contains a complex a mixture of biologically active substances.

Many studies imply that trigonelline, chlorogenic and caffeic acids are the primary interferents in the quantitative determination of caffeine in coffee sample using UV/Vis spectrophotometry. These acids show bands closer to the caffeine band. For removing these bands closer to caffeine peak a suitable method has been developed.

Portions of aqueous extracts were used for spectrophotometric measurements made on suitable diluted solutions before extraction of caffeine with dichloromethane. As Search for simple method was made for removing the interferences, which show absorption peak closer to the peak of caffeine. Then, the clarified aqueous extracts were treated with drop wise aqueous solution of 0.1 M NaOH. Spectrophotometric measurements for aqueous extracts showed that

well resolved with drop wise alkali solution. But complete elimination was not readily obtained. The spectrophotometric method used for determining caffeine was therefore, modified for complete removal of interferents from aqueous extracts. It was expected that complete extraction of caffeine from coffee mixture could be effected with dichloromethane extraction.

The samples were treated as described in the sample preparation section. The samples were collected from Ethiopia Coffee, tea quality and liquoring center to get a coffee treated under the same conditions. All coffee are green and washed at the standard of the center. Three samples were analyzed for caffeine content using aqueous extracts and dichloromethane extracts. The results from the developed method are described below.

5.4.1 Determination of Caffeine in Aqueous Extracts

Initially, an investigation was made regarding the suitability of method for preparing clarified aqueous extract of the sample. The first spectrophotometric measurement for the aqueous extract, without any drop of alkali solution, showed that, all spectra had maxima at which the absorption peak of caffeine was expected. These overlapped bands are clearly seen in the spectral bands of coffee sample (Fig.7) dissolved in de-ionized water.

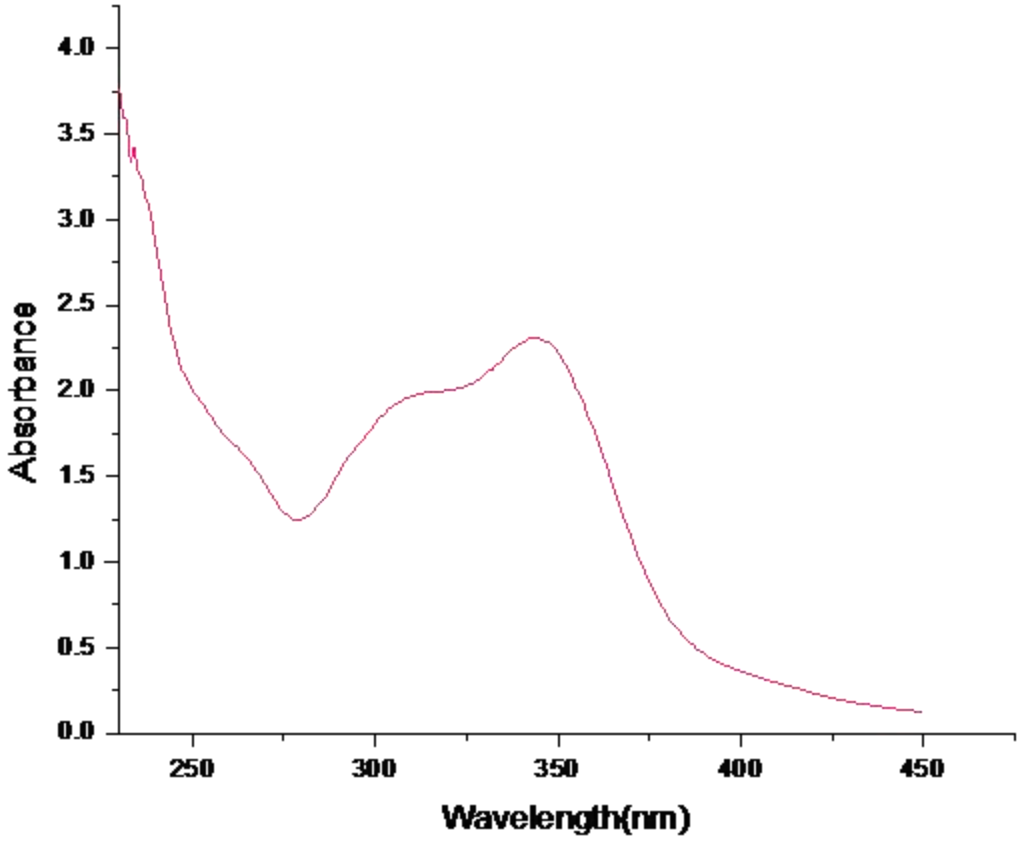


Fig. 7 Absorption spectra of Aquous extract of Coffee sample

In order to remove interfering bands, aqueous solution of 0.1 M NaOH was used as the modifier of the method. With drop wise addition of 0.1 M NaOH, the overlapped bands start to resolve. After successive drops, into aqueous extract of the coffee solution, the spectra bands (Fig. 8) completely shifted to longer wavelength reduced, but the peak of caffeine intensified.

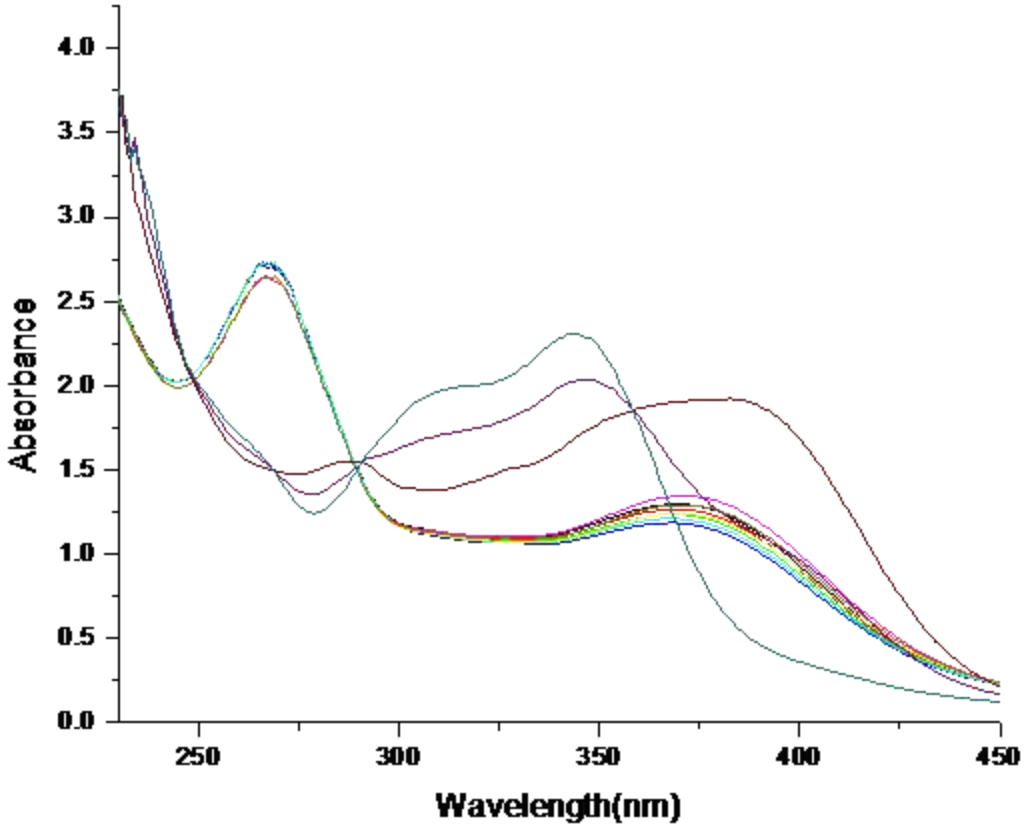


Fig.8 Absorption Spectra of Aqueous Extract of Coffee Sample With Successive Addition of 0.1 M NaOH

After all the bands from the other components of coffee were separated from band of caffeine one spectrum was taken as a test sample for quantitative determination of caffeine for subsequent base line correction (Fig.9).

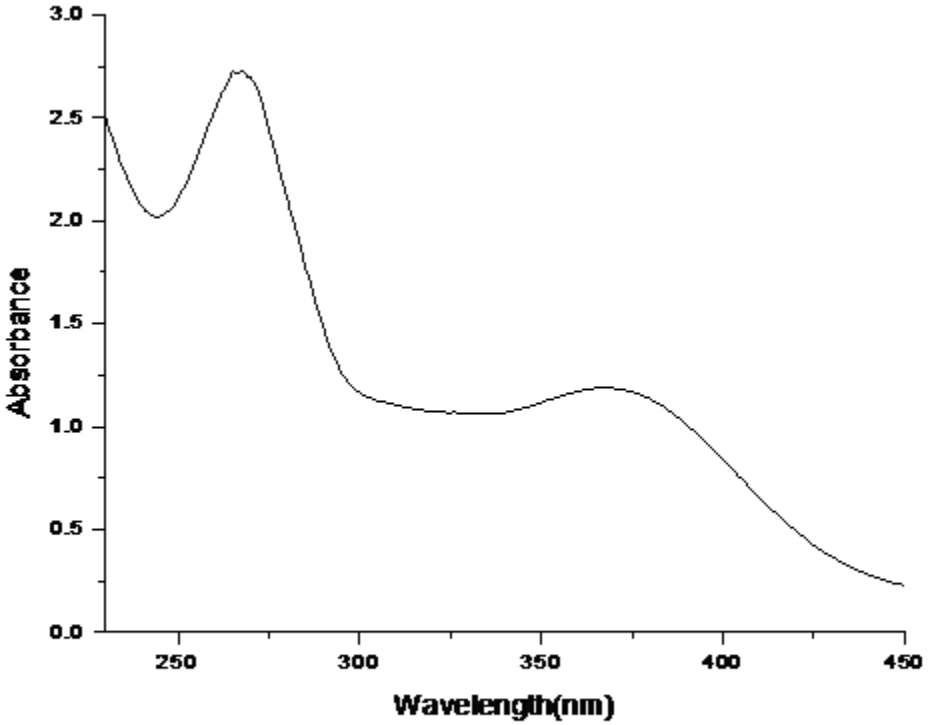


Fig.9 Absorption spectra of Caffeine in aqueous extract before base line correction

However, complete elimination was not obtained by drop wise addition of alkali solution, except resolving the bands. It was suspected that the presence of these bands might have positive interferences in aqueous extracts for the determination of caffeine in coffee samples. The determination of caffeine in the presence of these interferents by this method, the interferents made it impossible to obtain the maximum absorption of caffeine.

The amount of pure caffeine was determined at the wavelength of maximum absorption and points of the ascending and descending portions of the curve equidistant from the maximum. The results of the determination of caffeine in coffee samples were obtained after subtracting the interferents' peak from the total caffeine spectra (Fig.10)

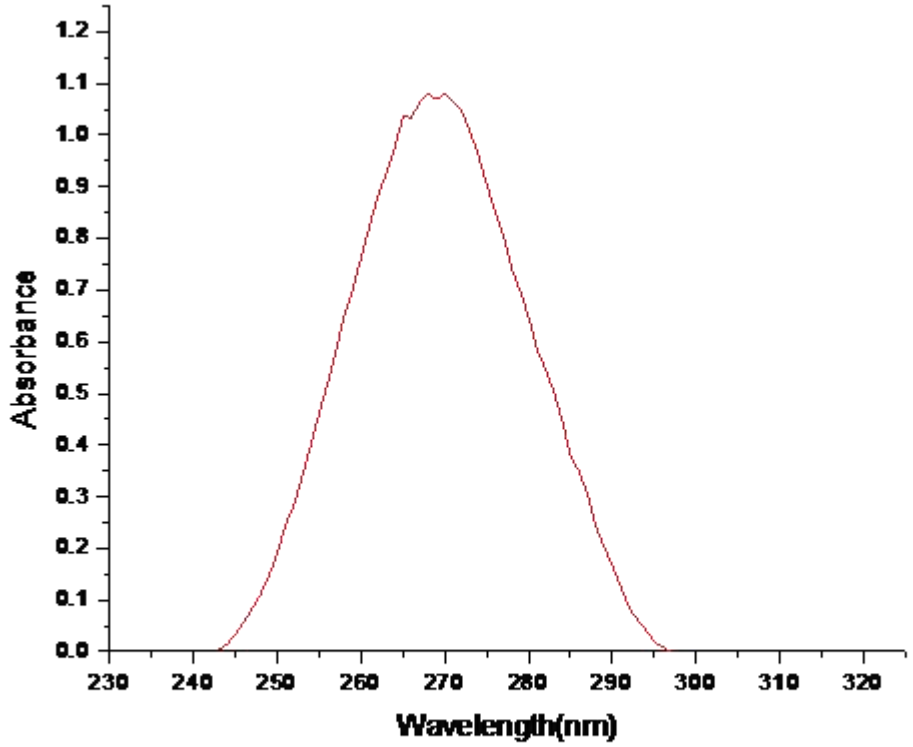


Fig. 10 Absorption Spectrum of caffeine in aqueous extract after base line correction

5.4.2 Determination of Caffeine in Dichloromethane Extracts

This extraction procedure has an advantage that interfering substances such as chlorogenic acid, and trigonelline acid, which are usually found in coffee associated with caffeine, are completely removed, as the former is fixed by aqueous solution of 0.1 M NaOH. Trigonelline, Caffeic acid and chlorogenic acid being insoluble in dichloromethane remained in the aqueous solution and by this procedure did not cause interference.

Studies of the ultra-violet absorption spectra of dichloromethane extracts showed that all the spectra has maximum at 276 nm which are similar to that of caffeine itself. Uv/Vis spectra of caffeine in dichloromethane extracts were found to be specific as no interfering peaks were observed during spectrophotometric measurement of caffeine in dichloromethane extracts, as it was also evidenced by the peak purity in Fig. 11.

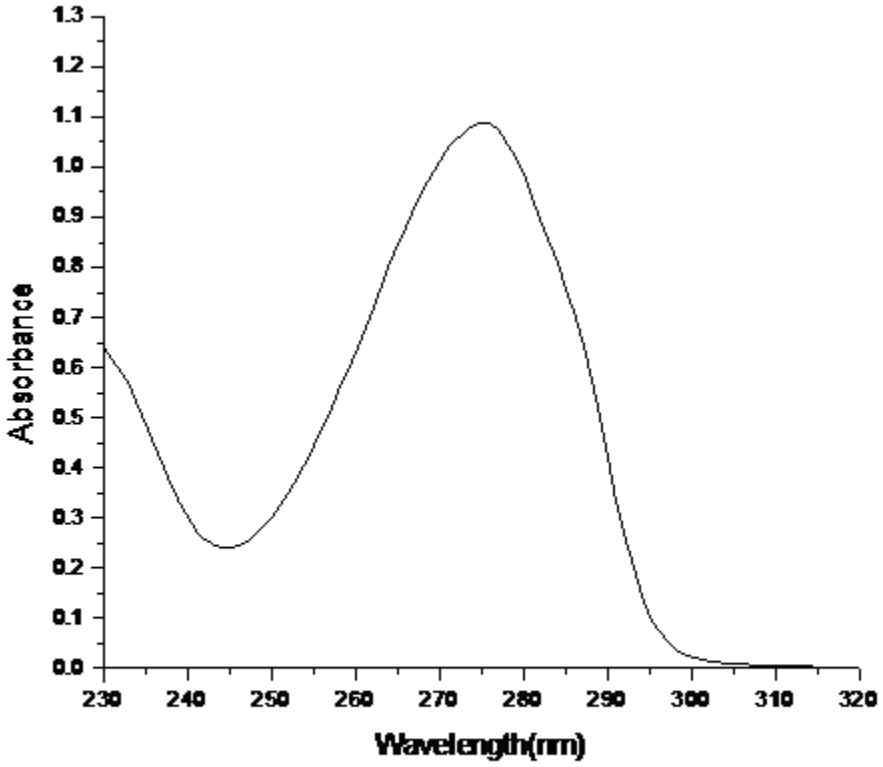


Fig.11 Absorption spectrum of Caffeine in Dichloromethane extract

5.5 Application of the Method

The proposed method was applied to determine the caffeine content in coffee samples using aqueous extracts. For this purpose coffee samples were dissolved in water. The samples of each type were analyzed by the proposed method, by measuring the final volume of each extracts. The quantitative results of this analysis are shown in Table 4.

Table 4: Results of aqueous extracts in three replicate of each sample

	Sample source		
	Bench Maji	**Jimma limmu**	**Sidama Arbegona**
Weight of coffee Sample(mg)	100.1	99.9	100.2
Volume of aqueous solution (ml)	47.23	46.56	48.38
Absorbance Measured	1.075	1.029	1.036
	1.055	1.037	1.077
	1.046	1.018	1.006
	1.080	1.020	1.070
Calculated caffeine Weight (mg)	1.06	1.03	1.11
	1.05	1.01	1.03
	1.08	1.02	1.07
Percentage of caffeine (w/w %)	1.06	1.03	1.11
	1.05	1.01	1.03
	1.08	1.02	1.07
mean ± Standard deviation	1.06±0.02	1.02±0.01	1.07±0.04
Relative standard deviation (RSD) (%)	1.88	0.98	3.74

The results obtained from the proposed method were compared to the literature extraction method using dichloromethane extracts for analysis of caffeine in real sample. Table 5 summarizes the analytical results of the determination of caffeine in coffee samples collected from different parts of Ethiopia using the dichloromethane method. The good agreement between the results of the aqueous and dichloromethane extracts indicates the successful applicability of this method for aqueous extract.

Table 5: Results of dichloromethane extracts in three replicate of each sample

	Sample source		
	Bench Maji	**Jimma limmu**	**Sidama Arbegona**
Weight of coffee Sample(mg)	99.8	100.1	100.3
Volume of extracts (ml)	49.29	47.55	46.96
Absorbance Measured	1.088	1.097	1.099
	1.087	1.095	1.097
	1.079	1.085	1.105
Calculated caffeine Weight (mg)	1.04	1.01	0.998
	1.03	1.01	0.996
	1.03	0.997	1.00
Percentage of caffeine (w/w %)	1.04	1.01	0.996
	1.03	1.01	0.994
	1.03	0.997	1.00
	1.04	1.01	0.996
Mean ± Standard deviation	1.03 ± 0.01	1.01 ± 0.01	0.99 ± 0.01
Relative standard deviation (RSD) (%)	0.97	1.00	1.01

The results of this method were compared with the results of the same published analytical methods that have been applied for the determination of caffeine in coffee sample. Each method has its own advantage and limitation in terms of simplicity and reagent use. Compared with the literature methods, the present method shows out standing advantage of simplicity of instrument, faster cost effective and environmentally friendly. Results from the present method are in good agreement with those obtained by previously reported (Table 6).

Table 6: Comparisons of results for the determination of Caffeine by current method and published methods

Percentage of caffeine found in (w/w %)	Reference
1.36 ± 0.03	[8]
1.30	[33]
0.97 - 1.20	[35]
0.98 - 1.10	**This work**

6. Conclusions and Recommendation

The UV/Vis Spectrophotometric method has been developed for the determination of caffeine in coffee samples. The proposed method is more simple, rapid, cost-effective and environmentally friendly than most of the previously reported methods. The aqueous extraction and dilution procedures are simple and require no special technique. The method does not involve use of any special reagent, nor does it include tedious filtration or extraction procedure. Furthermore, literature methods were mainly applied to determine caffeine using liquid-liquid extraction; most of them were not applied to aqueous extracts for the determination of caffeine. The current method provides a new technique for the determination of caffeine in coffee sample using aqueous extracts with good precision and accuracy. Moreover the developed method, it should also be applied to Higher Performance Liquid-chromatography (HPLC) for comparison. Because

HPLC is powerful official method in quantitative determination of samples, which may more assure the feasibility of the developed method.

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