‘

BAND DECONVOLUTION AND FLUORESCENCE QUENCHING METHODS FOR DETERMINATION OF CAFFEINE CONTENT IN COFFEE SEED

By

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The undersigned hereby certify that they have read and recommend to the Faculty of Science for acceptance a senior project entitled "**Band deconvolution and fluorescence quenching methods for determination of caffeine content in coffee seed**" by **Menberu Mengesha W/mariam Master of Science**.

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For the memory of my mother
Aselefech W/mariam (1944-1982) E.C

Table of Contents

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List of figures

List of tables

Abstract

In this project,the concentration of caffeine in coffee in water solution is investigated. The molar decadic absorption coefficient in water is determined by preparing several concentrations and applying least square method. The obtained result is 1098.87 $\frac{m^2}{mol}$. The constant that relate the absorbance of caffeine in coffee in water solution to the extracted caffeine is developed and its value is ($\approx 2.9\, mg^{-1}$). Moreover band deconvolution method is developed to remove the interfering matrix elements in the absorption spectrum of coffee in water solution. To apply this method the interfering band is modelled by a convolution of five gaussian functions. The result obtained using this technique is consistent with other results with an error of $5-8\%$. Finally the concentration of caffeine in coffee seed is determined by correlating the fluorescence intensity of the emission spectrum of pure caffeine and coffee powder in water solution by applying quenching fluorescence. After all these methods are applicable in every part of the country to determine the concentration of caffeine in coffee seed with least cost using a universal solvent,water.

Key words: Caffeine, deconvolution, interfering band, gaussian model, absorbance, UV/Vis, and fluorescence.

Chapter 1

Introduction

Coffee is an evergreen shrub or small tree belonging to the Rubiaceae family, which comprises at least 66 species of the genus coffee [1]. The two species that are commercially exploited are coffee Arabica, which accounts for two thirds of world production, and C. Canephora, often-called Robusta coffee, with one third of global output [2]. Rubusta coffee plants and all wild coffee species have 22 chromosomes, whereas Arabica has 44 chromosomes [1,2]. Coffee C. Arabica is the only tetraploid and self-pollinated species of genus coffee [3]. Therefore, Arabica coffee and other coffee species cannot be crossed to produce a hybrid plant [2,3].

The size of the plant varies depending on the species [4]. It ranges from small woody shrubs to a large forest tree. Robusta is a high - yielding and disease -resistant tree standing up to 12 meters tall. But c. Arabica is a medium - to low - yielding tree from 5 to 6 meters tall [2].

The color of species varies from bronzed and purple green to yellow and dark green [5,6]. Moreover the flower color varies from species to species. Some species have white flowers with jasmine - like fragrance, some pink, and the others creamy to yellowish [4,5].

The term coffee derived from the southwestern massive highlands of Ethiopia, KAFFA region where the plant was first discovered blossoming [1,3,7]. Coffee has been in Kaffa since the down of history and it was from Kaffa that in early time the use of the extract of Kafa bean' spread over the entire Ethiopian highland [8,9].

Coffee is strictly tropical crop, requiring a moderately cool climate that is moist

[1]. It is reported that most Arabica coffee is grown where the average annual temperature is between 288.15 K and 298-15 K [10]. It is also reported that the optimum temperature for Arabica coffee ranges from 288.15 - 297.15 K [3]. Above 298.15 K the photosynthetic rate is reduced and leaves are damaged by continuous exposure to high temperatures. Low temperature below 288.15 K produces a white or yellow discoloration of the leaves [3]. It is cultivated successfully in equatorial region only at altitude 1200 - 1500 m, but at higher altitudes it is grown in lower laying regions [10]. It is also reported; in equatorial areas Arabica is highland crop growing from 1,000 - 2000 m [3]. The average rainfall is 1100 - 1500 mm [7].

Coffee Robusta thrives in tropical equatorial climate where the average temperature is between 297.15 K and 299.15 K [10]. It is also reported that the average temperature ranges from 295.15 K to 300.15 K [7]. Its cultivation is confined to low-lying regions sea level to 700 m [3]. The mean rainfall is 1550 - 2000 mm. Robusta requires more rainfall than Arabica but it with stands a fairly long dry period of the order of 3 month [5].

Soil for coffee should be fertile and slightly acidic. Coffee could adapt to soils derived from various parent materials with pH of 4.0 - 5.5 and organic matter content ranging from 3 - 6% [6]. The plant does not tolerate waterlogged soils [10]. Plantations are usually developed on well-drained soil prosperous in potash; enrichment by organic debris is preferable to commercial fertilizers [1,3].

Coffee is produced in 80 countries, which are spread over 4 continents: Africa, Asia, America and Australia [5]. The major coffee producing countries are Brazil, Vietnam and Colombia. Brazil is the leading coffee producing country in the world [5]. According to ICO 1986 - 1987 coffee production statistics Ethiopia is the 6^{th} coffee producing country next to Ivory cost [11].

There are four types of production system in Ethiopia: forest coffee, semi - forest coffee, garden coffee and plantation coffee. Forest coffee is found in south and south - western Ethiopia (Bale, Kaffa, West Wollega, Shekka, Metu and Jimma Zones [7,12]. It accounts 10 % of Ethiopia's total coffee production. Semi - forest coffee production system is also found in the south and south - western parts of the country. It accounts 35 % of Ethiopia's total coffee productions [12]. Garden coffee is grown in the vicinity of farmers' residences, mainly in the southern and eastern parts of the country. It accounts for about 35 % of Ethiopia's total coffee production. Plantation coffee includes that grown plantation owned by the former state and some well managed smallholder coffee farms [7.12]. The former state plantation accounts about 5 % of total production and well-managed smallholder coffee farms account 15 % of the Ethiopia's total production.

Coffee is the most important agricultural commodity ranks second only to petroleum in terms of dollars traded worldwide and is worth up to $14 billion annually [2,7]. More than 80 countries including Ethiopia, cultivate coffee, which is exported as the raw, roasted, or soluble product to more than 165 countries worldwide [5]. Coffee is exported and/or reexported by more than 121 countries. More than 50 developing countries in the world, 25 of them in Africa, depend on coffee as an export with 17 countries 25 % of their foreign exchange from coffee [7].

The agricultural based Ethiopian economy is highly dependent on c. Arabica, as it contributes more than 60 % of the countries foreign exchange earnings. No other product or service in Ethiopia has earned as much [7,9]. The labor - intensive tree - crop also provides much employment in rural areas and is the livelihood for over 15 million people in Ethiopia [7].

Coffee is a widely and extensively consumed recreational beverage. It is a worldwide favorite. Every morning every body everywhere enjoys a fresh cup of coffee to help them start the day [3]. Coffee is a big deal in Ethiopia as it is widely enjoyed [12]. It is used as stimulant beverage in almost all regions of Ethiopia. In most places of the country it is culturally prepared and served two or three times a day [9]. Coffee is also served in special circumstances such as birth, marriage, ceremonies, burials, holidays and so on [7].

Recent studies showed that daily intake of coffee were associated with a 50 percent reduction in risk of liver cancer. Additionally, there was a clear relationship in men between amount of coffee used daily and risk of liver cancer [13]. Those drinking five or more cups a day had a 76 percent reduced risk. Studies on the effect of coffee also showed that coffee contains four times the amount of cancer fighting antioxidants as green tea [5,14]

The other study from the Harvard - based Nurses' Health study and Health professionals following study examined the role of coffee in risk of bowel cancer among about 170,000 male and female participants. During a long follow up period 1,177 cases of bowel cancer were found, plus 261 cases of cancer of the lowest part of the bowel, the rectum [14]. There was no relation between caffeinated coffee (or tea) intake and cancers of the colon or rectum. However, consumption of as little as one-quarter cup of decaffeinated coffee a day reduced rectal cancer risk by 50 percent, though it had no influence on colon cancer [13].

The reduced risk of liver cancer among coffee drinkers looks quite interesting, though obviously more studies are needed before drawing any conclusions. On the other hand, the study on bowel cancer is largely negative and the findings about decaffeinated coffee are suspect [13,14].

Coffee is chemically composed of minerals, caffeine, trigonelline lipids, total chlorogenic, aliphatic acids, oligo saccharin, total polysaccharides proteins and hommic acid [15]. The chemical composition of green coffee depends on the species and variety. Other factors such as agricultural practices, degree of maturation and storage conditions determines its chemical composition to a lesser extent [15,5]. Coffee is undoubtedly one of the most complex of more commonly encountered food commodities from the point of view of chemistry [15].

The water-soluble constituents of coffee are aroma (0.1 %),‘ lipids (0.2 %), sugar (0.3 %), organic acid (0.5 %), caffeine (1 %), water (2 %), minerals (3 %), chlorogenic acid (4 %) polysaccharides (6 %) and phenol polymers (8 %) [16]. Of these components caffeine is responsible for the stimulating effect of coffee [15,12]. Caffeine, which is the main physiologically active ingredient, is classed as an alkaloid [12]. Its chemical nomenclature and chemical formula are 1,3,7 trimethyl - xanthene and $C_8H_{10}N_4O_2$ respectively [15].

Caffeine is a naturally occurring substance found in the leaves, seeds or fruits of at least in 63 plant species [17]. The most commonly known sources of caffeine are coffee and cocoa beans, kola nuts, and tea leaves [17,18].

Caffeine is odorless, white, silky, melting at 511.15 K and subliming at 451.15 K [5]. Its solubility in water increases with temperature. Caffeine is a very weak base forming unstable salts [12,15]. It is relatively stable in dilute acids and alkalis but can form a series of complexes with other coffee components.

The caffeine content of green coffees varies widely, with differences in species being the most important factor. However, even with in a species there is a very wide range of values. Robusta coffees in general have higher caffeine content with an overall mean value of 2.2 % , whilst the value for Arabicas is about 1.2 % [15].

Environmental and agricultural factors are considerably less important than genetic variation in controlling the caffeine content of green beans, and it has been reported that fertilizers, in particular potassium phosphate, magnesium, and calcium do not have a significant effects on caffeine content [3,15].

Caffeine has both positive and negative physiological effects. Caffeine stimulates the central nervous systems first at higher levels, the cortex and medulla, and finally the spinal cord at higher doses [15,18]. Mild cortex stimulation appears to be beneficial resulting in more clear thinking and less fatigue. It improves attention in study [3,18]. Its possible therapeutic use in hyperkinetic children certainly would seem advantageous when compared to the current treatment with more powerful stimulants that have concomitant adverse reactions [18]. It relaxes the smooth muscle of blood vessels and increases in heart output [3]. The occurrence of hyperesthesia, an unpleasant sensory sensation, can be stimulated by large dose of caffeine [18]. It also has an effect on the endocrine secretion of different glands and gastric secretion [3,5].

On the other hand, caffeine is a drug, which is subject to abuse [18]. Studies and reports of personal observations have shown without doubt that caffeine abuse (caffeinism) may result in a syndrome that resembles and may be confused or confounded with true psychotic states [18]. Facilitating transmitter release by increasing calcium release at neuro-muscular junctions, sever restlessness, and excitement leading to mild delirium muscular tension and twitching and cardiovascular disturbances such as tachycardia are negative effects of caffeine at large dose [15, 3]. The spinal cord is stimulated at higher doses and convulsions and death may result [18]. More than 10 g are needed for such toxicity to occur in man [15,18].

Caffeine has a physiological half-life of three and half hours to six hours [18]. Its physiological effects are observed in less than one hour. Infants do not metabolize caffeine as well as adults and thus have a half - life of about four days. Certainly, continuous ingestion of caffeine by infants can be dangerous. If a cup of coffee consumed by an adult six or seven times a day it would result in high steady concentration of caffeine in the blood [15,18]. Caffeine can accumulate in severe liver disease, when its half-life can increase to 96 hours [18].

Caffeine is not efficiently removed from the bloodstream by the kidneys and continues to circulate until metabolized, initially in to paraxanthine, theophyline and theobromine, then into derivatives of uric acid and diaminouracil, which can eventually be removed from the circulation relatively efficiently [15]. A caffeine concentration of 5 - 10 μM generally restricted to an increase in sleep latency and enhancement of alertness during fatigue or boredom [15]. At peak plasma levels of 15 - 30 μM effects may include mild anxiety, respiratory stimulation, cardiovascular effects and increased gastric secretion. Therapeutic concentrations are in the region of 50 μM and symptoms of acute toxicity may appear when levels are between 150 and 200 μM.

Caffeine sensitivity depends on many factors, such as frequency of consumption, individual metabolism and amount of intake, body weight and physical condition, moderate consumption is considered to be 300 mg [17].

Concern over to possible toxic effects and undesirable physiological effects of caffeine has lead to an increasing demand for decaffeinated green beans to the extent that some 8-10 percent of the world export market is now treated with the majority being sold in the USA and western Europe [15,19]. Decaffeination was first practiced as early as 1905 by the Hag Company in Bremen, Germany who took out a patent on their process [3]. Latter the production of decaffeinated coffee has given rise to a great number of patents through out the world [20].

There are different methods of decaffeination, though the end result is more or less the same. The three methods all widely used are: water decaffeination, solvent decaffeination and carbon dioxide decaffeination [3,20].

In water decaffeination process, the method uses water to dissolve the caffeine from green coffee beans. This is done before roasting. Typically, water in run through coffee beans, creating an extract. The extract is then passed through activated carbon to remove the caffeine. The liquor containing water soluble components but with out caffeine is then either concentrated and reincorporated on the decaffeinated beans which have been partially predried or re-circulated through the beans and the caffeine is removed. This process continually repeated [15, 20].

The other decaffeination process is solvent decaffeination. In this method solvents which can be used selectively remove caffeine from green coffee beans include methylene chloride [dichloromethane] and ethyl acetate which is a natural component of fruits, vegetables, and many other food stuffs. The process involves using a solvent the caffeine is distilled out of it, so that the used solvent can be reutilized in further round of decaffeination. After completion of decaffeination the green beans are intensely treated with steam to remove all adherent traces of the decaffeination solvent [3,15, 20].

The most recently developed technique consists of extracting the caffeine with carbon dioxide (CO_2) at super critical temperature [20]. In this process caffeine can be made to dissolve out of pre-wetted green coffee beans when contacted with super critical or liquid carbon dioxide, which is a normal constituent of air. This method does not involve the use of additional solvents, and used carbon dioxide can be passed through active carbon to remove the caffeine, so that it can be recycled [3,15]. From the three decaffeination methods mentioned above dichloromethane extraction retains the most flavor but leaves a dry taste in mouth, both water and carbon dioxide extraction blur the flavor of the beans and ethyl acetate adds a sweat fruit of flavor [5].

For some considerable time, decaffeinated coffees were known to lose much of their flavor during processing. This is less true now a days as it is now possible to produce decaffeinated coffee with qualities that are very similar to those of regular coffee [20]. The progress made in its production particularly appreciated by those who are advised to stop drinking coffee for reasons of health [20]. But decaffeinated coffees tend to be more expensive due to additional labor, equipment and material expenses required for caffeine extraction [17,20].

Conversely as it was reported by [5], transgenic plants with a 70 percent reduced caffeine contents were created by scientists from Nara Institute of Science and Technology in Japan. The researchers constructed transgenic coffee Canephora plants, which had been designed to repress a gene that activated one of the three N-methyl transferase enzymes such as CaXMT1, CaMXMT1 (theobromine syntheses), and CaDXMT1 (caffeine synthase); it will be possible to grow coffee plants, which do not produce caffeine. But using genetically - engineered plants is, controversial as critics fear the altered genes could be handed on to other species and may also affect human health [21].

Moreover, Brazilin scientists found a rare variety of coffee plant that should provide the world's first cup of naturally decaffeinated but full - tasting coffee [21]. They bred 3000 Ethiopian coffee plants and found three bushes, all derived from the same plant were virtually caffeine free, containing 15 times less stimulant than commercial strains [5,21]. This discovery would eliminate the need of caffeine extraction processes, which are not only expensive, but cause the loss of flavors and aromas.

After all Ethiopia is the origin of naturally decaffeinated coffee. This makes the country economically beneficial, as a demand of decaffeinated coffee increases in the world. Therefore, it is very important to develop a method that is used to determine the caffeine content of coffee seed in different parts of the country with least cost.

For the determination of caffeine in liquors different researches are conducted, using analytical methods such as titrimetery, polarography, GC, HPLC and spectrophotometery have been reported [23]. Titrimetric method suffers the disadvantage of undergoing interference from many redox reagents and using a large amount of sample. The chromatographic methods are limited to small industrial laboratories due to the use of expensive equipment and the demand for more operator attention. The polarographic method suffers from electrochemical impurities in the food. Spectrophotometery is a fast and simple method for caffeine determination due to the recent development of new technologies [5]. Moreover standard procedure was setup for determination of caffeine contents in coffee seeds by using optical method particularly by UV/Vis spectrophotometer [5].

Even though the method developed in [5] is fast, and simple to other mentioned methods, it uses different stages of extraction using a solvent dichloromethane, which is expensive and not available everywhere. Further more as to my knowledge, it is not observed whether there exist a relation between the absorbance of the spectra of caffeine in coffee dissolved in water solution and the absorbance of spectra of caffeine extracted by dichloromethane and deconvoluted by Gaussian fit from the same solution. In addition there is no a method developed to determine the caffeine content in water solution due to high interference of UV absorbing matrix elements in coffee in water solution.

Therefore the general objectives of this thesis are: (i) To determine the relationship between the spectra of caffeine in coffee dissolved in water solution and the spectra of caffeine extracted by dichloromethane and deconvoluted by Gaussian fit from the same solution. (ii) To develop mathematical deconvolution method that enables to determine the caffeine content in coffee seed without using chemicals. (iii) To determine the concentration of caffeine in coffee seed using flourimeter. The specific objectives are: constructing a calibration curve for different concentrations using pure caffeine. Determining molar decadic absorption coefficient and transition dipole moment from the calibration curve applying least square method. Removing interfering matrix element using band deconvolution method. Setting a relationship between the spectra of caffeine in coffee dissolved in water solution and the spectra of caffeine extracted by dichloromethane. Depending on the relationship between the spectra of caffeine in coffee dissolved in water solution and the spectra of caffeine extracted by dichloromethane determining the concentration of caffeine content in coffee seed in water solution. Determining the concentration of caffeine in coffee seed using band deconvolution method. Determining the concentration of caffeine by fluorescence emission.

Chapter 2

Theory

2.1 Spectroscopy

Spectroscopy is an experimental charting of the energy level structure of physical systems. For that purpose the transition processes, spontaneous or induced between different energy states are studied, and spectroscopy, therefore, normally means analysis of various types of radiation - electromagnetic or particle emission [31]. Spectroscopy is about light and matter and how they interact with one another. Studies of light and matter have often been interwoven, with spectroscopy playing an important role in the emergence and validation of quantum theory in the early twentieth centaury [25]. Spectroscopy is a tool for studying the structure and dynamics of molecules. The structure of atoms and molecules is based on spectroscopy investigations [26].

Spectroscopy provides tools to explore the micro world of atoms and molecules. Wavelength measurements of spectral lines allow the determination of energy levels of the atomic or molecular system [32]. The line intensity that is proportional to the transition probability measures how strongly the two levels of a molecular transition are coupled. The natural line width of a spectral line helps to determine the mean lifetime of excited molecular states. Measurement of Doppler width yields the velocity distribution of the emitting or absorbing molecules. Pressure broadening and pressure shifts of spectral lines are used to extract information about collision processes and inter atomic potentials.

2.1.1 Electromagnetic Radiation in Vacuum

The electric and magnetic fields are in phase and mutually perpendicular as a consequence of Maxwell's equations [24]. Physically, Electric field results from electric charges or from time dependent magnetic fields, while magnetic fields result from electric currents or time - dependent electric field. Maxwell's equations can be manipulated to give classical wave equations from the time and space dependence of the electric and magnetic field. A significant achievement of this picture was the relation derived between the speed of light c and the product of the electric permittivity ϵ_0 and magnetic permeability μ_0 of free space:

$$c^2\mu_0\epsilon_0 = 1 \tag{2.1}$$

Maxwell's equation are postulates that predict about the physical property of light. In vacuum, they are:

$$\nabla \cdot \overrightarrow{E} = 0 \tag{2.2}$$

$$\nabla \cdot \overrightarrow{B} = 0 \tag{2.3}$$

$$\nabla \times \overrightarrow{E} = \frac{-\partial \overrightarrow{B}}{\partial t} \tag{2.4}$$

$$\nabla \times \overrightarrow{B} = \mu_0\epsilon_0 \frac{\partial \overrightarrow{E}}{\partial t} \tag{2.5}$$

where, $\epsilon_0 = 8.854 \times 10^{-12} C^2 m^{-1} J^{-1}$, and $\mu_0 = 4\pi \times 10^{-7} \frac{N}{A^2}$

After some manipulation Maxwell's equation can be recast in the following form to give classical wave equation:

$$\nabla^2 \overrightarrow{E} = \epsilon_0\mu_0 \frac{\partial^2 \overrightarrow{E}}{\partial t^2} \tag{2.6}$$

$$\nabla^2 \overrightarrow{B} = \epsilon_0\mu_0 \frac{\partial^2 \overrightarrow{B}}{\partial t^2} \tag{2.7}$$

From the symmetrical relationship between the magnetic and electric field, there are two quantities, the scalar potential ϕ and the vector potential $\overrightarrow{A}$, from which $\overrightarrow{E}$ and $\overrightarrow{B}$ can be derived:

$$\overrightarrow{E} = -\nabla\phi - \frac{\partial \overrightarrow{A}}{\partial t} \tag{2.8}$$

$$\overrightarrow{B} = \nabla \times \overrightarrow{A} \tag{2.9}$$

Hence the vector potential also obeys the classical wave equation,

$$\nabla^2 \overrightarrow{A} = \mu_0 \epsilon_0 \frac{\partial^2 \overrightarrow{A}}{\partial t^2} \tag{2.10}$$

2.1.2 Electromagnetic Radiation in Material Media

All macroscopic aspects of the static and dynamics of the electromagnetic field of the material media described by Maxwell's equation:

$$\nabla \cdot \overrightarrow{D} = \rho_f \tag{2.11}$$

$$\nabla \cdot \overrightarrow{B} = 0 \tag{2.12}$$

$$\nabla \times \overrightarrow{E} = -\frac{\partial \overrightarrow{B}}{\partial t} \tag{2.13}$$

$$\nabla \times \overrightarrow{H} = \overrightarrow{J} + \frac{\partial \overrightarrow{D}}{\partial t} \tag{2.14}$$

where the electromagnetic properties of material media may be taken in to account through relations, $\overrightarrow{D} = \epsilon \overrightarrow{E}$, $\overrightarrow{B} = \mu \overrightarrow{H}$ and $\overrightarrow{J} = \sigma \overrightarrow{E}$ known as constitutive relations.

When an external electric field is applied to an insulator, the matter responds with an induced polarization that partially cancels the field due to out side charges. Although the charges in an insulator are not mobile as they are in conductor, an external field has the ability to displace the charges in length of polar bonds [24]. The resulting net dipole moment per unit volume is called the polarization, and in linear electric materials it is proportional to the electric field. If there are N such molecules per unit volume the macroscopic polarization$\overrightarrow{P}$ is given by:

$$\overrightarrow{P} = N \overrightarrow{p} \tag{2.15}$$

$$\overrightarrow{P} = \epsilon_0 \chi_e \overrightarrow{E} \tag{2.16}$$

$$\overrightarrow{D} = \epsilon_0 (1 + \chi_e) \overrightarrow{E} \tag{2.17}$$

Similarly, when ordinary matter is placed in an external magnetic field, induced magnetization, $\overrightarrow{M}$, the magnetic dipole moment per unit volume, results. Unlike the polarization $\overrightarrow{P}$, the magnetization can either reinforce or oppose the applied field. The bound and free charges in electric polarization are analogous to the bound and free currents in magnetic polarization. The magnetic field $\overrightarrow{H}$ is given by:

$$\overrightarrow{H} = \frac{\overrightarrow{B}}{\mu_0} - \overrightarrow{M} \tag{2.18}$$

The bound currents are those due to the induced magnetic moments and the alignment of permanent moments, possessed by the atoms molecules that comprise the sample.

$$\overrightarrow{M} = \chi_m \overrightarrow{H} \tag{2.19}$$

$$\overrightarrow{B} = \mu_0(1 + \chi_m)\overrightarrow{H} \tag{2.20}$$

Applying the curl operation to both sides of Equation (13), we obtain

$$\nabla \times (\nabla \times \overrightarrow{E}) = -\nabla \times \frac{\partial \overrightarrow{B}}{\partial t} = -\frac{\partial}{\partial t}\left(\nabla \times \overrightarrow{B}\right) \tag{2.21}$$

$$\nabla \times (\nabla \times \overrightarrow{E}) = \nabla(\nabla \cdot \overrightarrow{E}) - \nabla^2 \overrightarrow{E} \tag{2.22}$$

$$\nabla(\nabla \cdot \overrightarrow{E}) - \nabla^2 \overrightarrow{E} = -\mu_0 \frac{\partial^2}{\partial t^2}\left(\epsilon_0 \overrightarrow{E} + \overrightarrow{P}\right) \tag{2.23}$$

$$\nabla^2 \overrightarrow{E} - \nabla(\nabla \cdot \overrightarrow{E}) - \frac{1}{c^2}\frac{\partial^2 \overrightarrow{E}}{\partial t^2} = \frac{1}{\epsilon_0 c^2}\frac{\partial^2 \overrightarrow{P}}{\partial t^2} \tag{2.24}$$

for transverse fields (some times called solenoidal or radiation fields) satisfy,

$$\nabla \cdot \overrightarrow{E} = 0 \tag{2.25}$$

Transverse equations therefore satisfy the inhomogeneous wave equation,

$$\nabla^2 \overrightarrow{E} - \frac{1}{c^2}\frac{\partial^2 \overrightarrow{E}}{\partial t^2} = \frac{1}{\epsilon_0 c^2}\frac{\partial^2 \overrightarrow{P}}{\partial t^2} \tag{2.26}$$

In classical physics Newton's second law describes the motion of particle. For charged particles in electromagnetic field the force referred to in Newton's second law is the

Lorentz force [33],

$$\overrightarrow{F} = e(\overrightarrow{E} + \overrightarrow{V} \times \overrightarrow{B}) \tag{2.27}$$

In equation (2.27), the magnetic contribution to the Lorentz force is dropped. Because optical phenomena do not normally involve relativistic particle velocities, we can safely disregard the magnetic force. Under the influence of electromagnetic field the electron experiences a Lorentz force, and the equation of the force without considering the damping force is, and the equation of motion is,

$$m\frac{d^2\overrightarrow{x}}{dt^2} = e\overrightarrow{E}(\overrightarrow{r}, t) - K_s\overrightarrow{x} \tag{2.28}$$

When a field is applied, each atoms electron is displaced by some $\overrightarrow{x}$ from its original position. Thus each atom has a dipole moment,

$$\overrightarrow{p} = e\overrightarrow{x} \tag{2.29}$$

If the density of atoms is denoted by N, then the density of dipole moment is,

$$\overrightarrow{P} = N\overrightarrow{p} = Ne\overrightarrow{x} \tag{2.30}$$

The Maxwell equation (2.26) tells us how the electric field $\overrightarrow{E}$ depends up on the dipole moment density $\overrightarrow{P}$ of the medium. Newton's equation (2.27) tells us how the electron displacement $\overrightarrow{x}$ depends upon the $\overrightarrow{E}$. Equation (2.30) connects these basic equations by relating $\overrightarrow{P}$ to $\overrightarrow{x}$. The electron oscillator model thus ties together the Maxwell equation with Newton's law of motion. Solutions of these coupled equations will provide the model's predictions about the mutual interaction of light and matter. For linearly polarized wave the electric field at the position of the atom has the form,

$$\overrightarrow{E}(z, t) = \hat{\epsilon}E_0\cos(\omega t - kz) \tag{2.31}$$

where E_0 is the amplitude and $\hat{\epsilon}$ a unit vector in x- direction. If the electric field in equation (2.31) is to be a solution of coupled Maxwell-Newton equations, it must be the driving field in the Newton equation (2.28),

$$\frac{d^2\overrightarrow{x}}{dt^2}+\omega_0^2\overrightarrow{x}=\hat{\epsilon}\frac{e}{m}E_0\cos(\omega t-kz) \tag{2.32}$$

This equation has the solution,

$$\overrightarrow{x}=\hat{\epsilon}\left(\frac{\frac{e}{m}E_0}{\omega_0^2-\omega^2}\right)\cos(\omega t-kz) \tag{2.33}$$

The polarization can also be described by polarizablity ,

$$\overrightarrow{p}=e\overrightarrow{x}=\alpha(\omega)\overrightarrow{E} \tag{2.34}$$

Relating equations (2.32), (2.33) and (2.34) the polarizablity is given by,

$$\alpha(\omega)=\frac{e^2}{m}\frac{1}{\omega_0^2-\omega^2} \tag{2.35}$$

Thus the dipole moment density is,

$$\overrightarrow{P}=\hat{\epsilon}\left(\frac{\frac{Ne^2}{m}}{\omega_0^2-\omega^2}\right)E_0\cos(\omega t-kz) \tag{2.36}$$

This solution for the polarization provides the source term on the right hand side of the Maxwell equation (2.26),

$$\left(-k^2+\frac{\omega^2}{c^2}\right)\hat{\epsilon}E_0\cos(\omega t-kz)=-\frac{N\alpha(\omega)\omega^2}{\epsilon_0 c^2}\hat{\epsilon}E_0\cos(\omega t-kz) \tag{2.37}$$

To satisfy this equation k must satisfy a more general dispersion relation

$$k^2=\frac{\omega^2}{c^2}\left(1+\frac{N\alpha(\omega)}{\epsilon_0}\right)=\frac{\omega^2}{c^2}n^2(\omega) \tag{2.38}$$

The index of refraction with the absence of damping force is real and becomes,

$$n(\omega)=\left(1+\frac{\frac{Ne^2}{m\epsilon_0}}{\omega_0^2-\omega^2}\right)^{\frac{1}{2}} \tag{2.39}$$

In the presence of damping force equation (2.32) can be modified as,

$$\frac{d^2\overrightarrow{x}}{dt^2} - \gamma\frac{dx}{dt} + \omega_0^2\overrightarrow{x} = \hat{\epsilon}\frac{e}{m}E_0\Big[\cos(\omega t - kz) - j\sin(\omega t - kz)\Big] \tag{2.40}$$

where γ in equation (2.40) is the damping constant.

$$\overrightarrow{x}(t) = \Re\hat{\epsilon}\frac{\frac{e}{m}E_0 e^{-j(\omega t - kz)}}{\omega_0^2 - \omega^2 - j\gamma\omega} \tag{2.41}$$

The polarizablity in damping case is complex and given by,

$$\alpha(\omega) = \frac{\frac{e^2}{m}}{\omega_0^2 - \omega^2 - j\gamma\omega} \tag{2.42}$$

The complex nature of polarizablity leads to the complexity of the index of refraction.

$$n^2(\omega) = 1 + \frac{\frac{Ne^2}{m\epsilon_0}}{\omega_0^2 - \omega^2 - j\gamma\omega} = [n_{\Re}(\omega) + jn_{\Im}(\omega)]^2 \tag{2.43}$$

The electric field is given by,

$$\overrightarrow{E} = \hat{\epsilon}E_0 e^{-j(\omega t - kz)} \tag{2.44}$$

But the wave vector is,

$$k = \frac{\omega}{c}[n_{\Re}(\omega) - jn_{\Im}(\omega)] \tag{2.45}$$

Then,

$$\overrightarrow{E} = \hat{\epsilon}E_0 e^{\frac{-\omega}{c}n_{\Im}(\omega)z} e^{-j[\omega t - \frac{\omega z}{c}n_{\Re}(\omega)]} \tag{2.46}$$

The intensity is proportional to the square of the amplitude of the electric field and given by

$$I(\omega) = I_0 e^{-\sigma(\omega)z} \tag{2.47}$$

$\sigma(\omega)$ is the absorption coefficient or extinction coefficient, and given by,

$$\sigma(\omega) = 2[n_{\Im(\omega)}]\frac{\omega}{c} = \frac{Ne^2}{\epsilon_0 mc}\frac{\gamma\omega^2}{(\omega_0^2 - \omega^2)^2 + \gamma^2\omega^2} \tag{2.48}$$

2.1.3 Semiclassical Treatment of the Interaction of Radiation with Matter

Much of spectroscopy is concerned with the absorption of radiant energy as a system goes from one stationary state to another under the influence of electromagnetic radiation [26]. When an atomic or molecular system with energy level E_n subject to the oscillating electric field of incoming monochromatic light, the Hamiltonian is expressed as the sum of a zero order part $\hat{H}_0$ that does not depend on time, and the perturbation $\hat{H'}$ ' that does [24]. The Schrodinger equation then takes the form

$$[\hat{H}_0 + H^{\hat{'}}(t)]|\psi\rangle = i\hbar\frac{\partial}{\partial t}|\psi\rangle \tag{2.49}$$

Assuming that the zero orders Eigen functions and eigen values are known: $\hat{H}_0|m\rangle = E_m|m\rangle$, and the zero - order wave function $\psi_m \equiv |m\rangle$ as a basis for expanding the perturbed wave function:

$$|\psi\rangle = \sum C_m(t)e^{-i\frac{E_m t}{\hbar}}|m\rangle \tag{2.50}$$

Equation (2.50) present the perturbed state as superposition of the stationary states expressed in the Schrdinger representation. Substituting the superposition function (wave function) into equation (2.49)

$$\sum[\hat{H}_0 + \hat{H}^{'}(t)]C_m(t)e^{-i\frac{E_m t}{\hbar}}|m\rangle = i\hbar\sum\frac{\partial}{\partial t}\left\{C_m(t)e^{-i\frac{E_m t}{\hbar}}|m\rangle\right\} \tag{2.51}$$

The sum runs over an infinite number of eigenstates indexed by the letter m. Taking one of these stats and calling it state n; and multiplying both sides of equation (2.51) by the complex conjugate of the wave function for state n, and then integrating over all space. The result is:

$$\sum C_m(t)e^{-i\frac{E_m t}{\hbar}}\left\{\langle n|\hat{H}_0|m\rangle + \langle n|\hat{H}^{'}|m\rangle\right\} = i\hbar\sum\frac{\partial}{\partial t}\left\{C_m(t)e^{-i\frac{E_m t}{\hbar}}\langle n|m\rangle\right\} \tag{2.52}$$

The Eigen functions are orthonormal: $\langle n|m\rangle = \delta_{mn}$; so in the infinite sum on the right hand side, only the term m=n survives. And since $\langle m|\hat{H}_0|n\rangle = E_n\delta_{mn}$, the first sum on the left hand side is similarly reduced to one term. Thus

$$\dot{C_n}(t) = \frac{-i}{\hbar}\sum C_m(t)e^{-i\omega_{mn}t}\langle n|\hat{H}^{'}(t)|m\rangle \tag{2.53}$$

where $\omega_{mn} \equiv \frac{(E_m - E_n)}{\hbar} = -\omega_{nm}$

$$\dot{C_n}(t) = \frac{-i}{\hbar} \sum C_m(t) e^{-i\omega_{mn}t} \hat{H}'_{nm}(t) \tag{2.54}$$

Equation (2.55) says that the time dependence of any one coefficient is a function all the other time dependent coefficient. To solve this problem we use weak perturbation limit as it assumes that $\hat{H}'$ is small enough that the coefficients never depart greatly from their unperturbed values. Assuming that before the start of perturbation the system was in a definite initial state indexed by i. So we can replace the coefficients on the right hand of equation (2.54) side by $C_m(t) \approx C_m(0) = \delta_{mi}$ eliminating all except one term in the summation. Using the index f, for final state where $f \neq i$ for the state whose coefficient to calculate. That is,$C_i(0) = 1$ and $C_f(0) = 0$. The resulting differential equation is then integrated to give :

$$C_f(t) = \frac{-i}{\hbar} \int_0^t exp(i\omega_{fi}t') \langle f|\hat{H}'|i\rangle dt' \tag{2.55}$$

The probability that the system is in state f at time t , given that it was in state i at time zero, is given by the square of the amplitude of the coefficient:

$$P_f(t) = \frac{1}{\hbar^2} \left| \int_0^t exp(i\omega_{fi}t') \hat{H}_{fi}(t) \right|^2 \tag{2.56}$$

Considering the case where $\hat{H}_{fi}(t)$ is due to a time varying electromagnetic field. In a source free region this field can be expressed by magnetic vector potential,$\overrightarrow{A}$, fulfilling the condition $\nabla \cdot \overrightarrow{A} = 0$, according to the Lorentz condition, $\nabla \cdot \overrightarrow{A} + \frac{1}{c}\frac{\partial \phi}{\partial t} = 0$

$$\hat{H}' = \frac{ie\hbar}{m} \overrightarrow{A} \cdot \nabla = \frac{-e}{m} \overrightarrow{A} \cdot \overrightarrow{P} \tag{2.57}$$

The vector potential can be written as follows:

$$\overrightarrow{A} = \frac{\overrightarrow{A_0}}{2} \left[exp(i(\overrightarrow{k} \cdot \overrightarrow{r} - \omega t)) + exp(-i(\overrightarrow{k} \cdot \overrightarrow{r} - \omega t)) \right] \tag{2.58}$$

$$\overrightarrow{A} = \Re \overrightarrow{A_0} exp(i(\overrightarrow{k} \cdot \overrightarrow{r} - \omega t)) \tag{2.59}$$

The size of molecule is typically much smaller than the wavelength of light used in spectroscopy, So we can often neglect the gradiant of the field and in fact its spatial dependence altogether. So $\hat{H}^{'}(t) = -\overrightarrow{\mu} \cdot \overrightarrow{E}(t)$ corresponding to energy of a dipole in time varying but spatially constant, electric field [24]. Since $\overrightarrow{k} \cdot \overrightarrow{r} \approx \frac{2\pi r}{\lambda} << 1$ over the typical dimension of a molecule, the exponential function can be expanded about $\overrightarrow{k} \cdot \overrightarrow{r} = 0$,

$$e^{i\overrightarrow{k} \cdot \overrightarrow{r}} = 1 + (i\overrightarrow{k} \cdot \overrightarrow{r}) + \frac{1}{2}\left(i\overrightarrow{k} \cdot \overrightarrow{r}\right)^2 + ... \tag{2.60}$$

The first term in this series leads to electric dipole allowed transition, and the second to magnetic dipole and electric quadruple allowed transition Taking the first term of equation (2.60) gives, the following from matrix element:

$$\hat{H}^{'}_{fi}(t) = \frac{ie\hbar}{m}\Re(e^{-i\omega t})\langle f|\overrightarrow{A_0} \cdot \nabla|i\rangle, \quad k \longrightarrow 0 \tag{2.61}$$

Considering the vector potential points in z - direction, the resultant matrix elements is:

$$\hat{H}^{'}_{fi}(t) = \frac{ie\hbar A_0}{m}\Re(e^{-i\omega t})\langle f|\frac{\partial}{\partial z}|i\rangle \tag{2.62}$$

Thus, $i\hbar\langle f|\frac{\partial}{\partial z}|i\rangle = -\langle f|p_z|i\rangle$ in equation(2.62) Using the comutator relation $[z, p_z] = i\hbar$

$$p_z = \frac{im}{\hbar}(\hat{H}_0 z - z\hat{H}_0) = \frac{im}{\hbar}[\hat{H}_0, z] \tag{2.63}$$

The matrix element is thus

$$\langle f|p_z|i\rangle = \frac{im}{\hbar}(\langle f|\hat{H}_0 z|i\rangle - \langle f|z\hat{H}_0|i\rangle) = \frac{im(E_f - E_i)}{\hbar}\langle f|z|i\rangle = im\omega_{fi}\langle f|z|i\rangle \tag{2.64}$$

The z component of the dipole moment operator is $\mu_z = -ez$, the matrix element can be written:

$$\hat{H}^{'}_{fi}(t) = \frac{-e}{m}\Re(e^{-i\omega t})\langle f|\overrightarrow{A_0} \cdot \overrightarrow{p}|i\rangle = i\omega_{fi}\Re(e^{-i\omega t})\langle f|\overrightarrow{A_0} \cdot \overrightarrow{\mu}|i\rangle \tag{2.65}$$

But,$E_0 = -\omega A_0$ and $\Re(e^{-i\omega t}) = \frac{1}{2}\left[e^{-i\omega t} + e^{i\omega t}\right]$,we get

$$\hat{H}^{'}_{fi}(t) = \frac{-i\omega_{fi}}{2\omega}\left(\overrightarrow{\mu}_{fi} \cdot \overrightarrow{E_0}\right)\left(e^{-i\omega t} + e^{i\omega t}\right) \tag{2.66}$$

Transitions that are made possible by a non-zero value of the transition dipole,$\mu_{fi} = \langle f|\mu|i\rangle$ are referred to as electric dipole allowed transition [24,26]. Substituting the matrix element given in equation (2.66) in to equation (2.55)

$$C_f(t) = \frac{-i}{\hbar}\int_0^t exp(i\omega_{fi}t^{'})dt^{'}\left[\frac{-i\omega_{fi}}{2\omega}\left(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0}\right)\left(e^{-i\omega t}+e^{i\omega t}\right)\right] \tag{2.67}$$

$$C_f(t) = \frac{-i\omega_{fi}}{2\hbar\omega}\left(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0}\right)\left[\frac{e^{i(\omega+\omega_{fi})t}-1}{\omega+\omega_{fi}} - \frac{e^{-i(\omega-\omega_{fi})t}-1}{\omega-\omega_{fi}}\right] \tag{2.68}$$

Equation (2.68) reveals that the amplitude of the final state is expected to be large when ever $\omega = -\omega_{fi}$ or $\omega = \omega_{fi}$. For the condition $\omega = \omega_{fi}$, the second term can take on large values and be of major importance in determining $C_f(t)$. The system goes from lower energy level i to higher one f, and a transition is absorption. On the other hand, for the condition $\omega = -\omega_{fi}$, the first term in square bracket is of major importance in determining $C_f(t)$. For such cause the transition is stimulated emission. For absorption process:

$$C_f(t) = \frac{-i\omega_{fi}}{2\hbar\omega}\left(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0}\right)\left(\frac{1-e^{-i(\omega-\omega_{fi})t}}{\omega-\omega_{fi}}\right) \tag{2.69}$$

$$P_f(t) = \frac{\omega_{fi}^2|(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0})|^2}{4\hbar^2\omega^2}\frac{\sin^2(\frac{\Delta\omega t}{2})}{(\frac{\Delta\omega t}{2})^2} \tag{2.70}$$

Let $f(t+\Delta\omega) \equiv \frac{\sin^2(\frac{\Delta\omega t}{2})}{(\frac{\Delta\omega}{2})^2}$

$$\frac{\sin^2(\frac{\Delta\omega t}{2})}{(\frac{\Delta\omega}{2})^2} = 2\pi t\delta(\omega-\omega_{fi}) = t\delta(\nu-\nu_{fi}) \tag{2.71}$$

$$P_f(t) = \frac{|(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0})|^2}{4\hbar^2}t\delta(\nu-\nu_{fi}) \tag{2.72}$$

$$\omega_{fi}(t) = \frac{P_f(t)}{t} = \frac{|(\overrightarrow{\mu}_{fi}\cdot\overrightarrow{E_0})|^2}{4\hbar^2}\delta(\nu-\nu_{fi}) \tag{2.73}$$

Where equation (2.73) is the Fermi's golden rule. But the total energy density is ,

$$u(\nu) = \int\rho(\nu)d\nu = \frac{1}{2}\epsilon_0{E_0}^2 \tag{2.74}$$

$$\omega_{fi}(t) = \frac{P_f(t)}{t} = \frac{|\overrightarrow{\mu}_{fi} \cdot \hat{e}|^2 \rho(\nu_{fi})}{2\epsilon_0 \hbar^2} \tag{2.75}$$

Where $\hat{e}$ in equation(2.75) is a unit vector in the direction of the electric field. Since there is no preference for any one direction, in randomly oriented sample such as gas or liquid, the x, y, and z component the transition moment squared must be equal [26,27].

$$|\mu_{fi}|^2 = 3|\mu_{fix}|^2 \tag{2.76}$$

$$\omega_{fi}(t) = \frac{|\overrightarrow{\mu}_{fi}|^2 \rho(\nu_{fi})}{6\epsilon_0 \hbar^2} \tag{2.77}$$

2.1.4 Comparisons of Theory with Experimental Quantities

According to Beer's law for an absorbing solute in a non-absorbing solvent, the decrease in intensity of radiation as it penetrates a distance dx is proportional to $I(\frac{w}{m^2})$ the radiation intensity; to $C(\frac{mol}{l})$ molar concentration and dx (m), the path length [33]. Beer's law in differential form is given by,

$$-dI = \alpha(\nu) I C dx \tag{2.78}$$

$$-dI = 2.303\varepsilon(\nu) C I dx \tag{2.79}$$

The molar absorptivity is generally expressed in units of$(\frac{m^2}{mol})$. If N is the number of molecules per m^3 (cubic meter) in the initial state i (essentially equal to the total number of molecules for electronic spectroscopy), equation (2.79) also written as,

$$-dI = N h \nu \omega_{fi} dx \tag{2.80}$$

$$\omega_{fi} = \beta_{fi} \rho(\nu_{fi}) \tag{2.81}$$

where β_{fi} in equation (2.81) is the Einstein's absorption coefficient.

By relating equation (2.79) and equation (2.80)

$$\omega_{fi} = \frac{2.303\varepsilon(\nu) C I}{N h \nu} \tag{2.82}$$

$$I = \frac{cu}{n} \tag{2.83}$$

$$C = \frac{N}{N_A} \tag{2.84}$$

$$\omega_{fi} = \frac{2.303c\varepsilon(\nu)u(\nu)}{N_A h\nu n} \tag{2.85}$$

The total transition rate ought to be integrated over the band replacing $u(\nu)$ by $\int \rho(\nu)d\nu$ and $\frac{\varepsilon(\nu)}{\nu}$ in the integrand.

$$\omega_{fi} = \frac{2.303c}{N_A hn} \int_{band} \frac{\rho(\nu)\varepsilon(\nu)}{\nu} d\nu \tag{2.86}$$

Relating equation (2.77) and equation (2.86)

$$\frac{|\overrightarrow{\mu}_{fi}|^2 \rho(\nu_{fi})}{6\epsilon_0 \hbar^2} = \frac{2.303c}{N_A hn} \rho(\nu_{fi}) \int_{band} \frac{\varepsilon(\nu)}{\nu} d\nu \tag{2.87}$$

$$|\mu_{fi}|^2 = \frac{(6\epsilon_0 \hbar^2) 2.303c}{N_A hn} \int_{band} \frac{\varepsilon(\nu)}{\nu} d\nu \tag{2.88}$$

2.2 Convolution and Deconvolution

The experimental spectrum observed using a spectrometer could be significantly different from true line profile. This discrepancy being caused by resolving power of the instrument in the absence of absorbing medium [27]. The resultant effect can be expressed mathematically as a convolution of the actual intensity with the instrument function as,

$$I_{ob}(\nu_0 - \nu_e, 0) = \int S(\nu - \nu_0) I(\nu - \nu_e) d\nu \tag{2.89}$$

where $I_{ob}(\nu_0 - \nu_e, 0)$ is the observed emission in the absence of absorbing medium, $I(\nu - \nu_e)$ is the actual emission, and $S(\nu - \nu_0)$ is the instrument response function. The observed transmission $I_{ob}(\nu_0 - \nu_e, 0)$ is a smearing of a true profile by a given instrument response function. Therefore deconvolution of the observed spectra with slit function gives the true transmission [30]. Deconvolution is the procedure of undoing that smearing in an effort to see what the true profile would look like had the instrument or measuring system perfectly rendered it [29]. The term convolution is explained by using three gaussian functions in Fig.2.1.

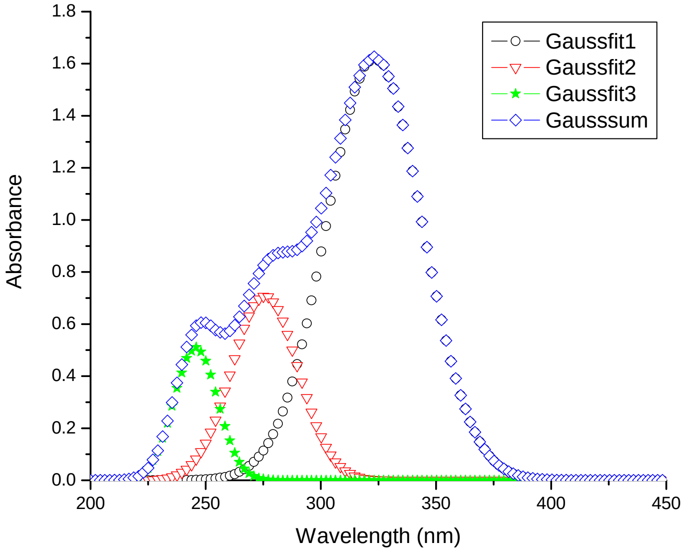

Figure 2.1: *The observed spectrum as a convolution of three gaussian functions .*

2.2.1 Absorbance Measurement

The absorption curves of many organic and inorganic substances are composed of superimposed broad bands [27]. Gaussian distributions are used to model the absorbance property of an object, since they closely approximate the physical process involved in electronic transition absorptions. Each absorption process is represented by three parameters: amplitude, bandwidth, and the location of the peak wavelength. The absorption profile of a given object is represented by several overlapping Gaussian distributions. The total absorption distribution A is given by:

$$A = \sum_i A_i = A_1 + A_2 + A_3 + ... + A_n \tag{2.90}$$

where A is the total absorption distribution, and A_i is the i^{th} individual absorption.

$$A_{i\lambda} = a_i exp\bigg(- \Big(\frac{\lambda - p_i}{\omega_i} \Big)^2 \bigg), i = 1, 2, 3..., n \tag{2.91}$$

The individual absorption processes are modelled by equation (2.91), where $A_{i\lambda}$ is the value of the i^{th} absorption at wavelength λ,and a_i,p_i and ω_i representing the amplitude, peak wavelength and half width (at half-height) of the Gaussian for the i^{th} Gaussian absorption distribution, respectively.

In the presence of absorbing medium, the observed intensity is depleted by the absorbing medium and the observed intensity takes in the form [27],

$$I_{ob}(\nu_0 - \nu_e, N) = \int S(\nu - \nu_0) I(\nu) e^{-N\sigma(\nu)l} d\nu \tag{2.92}$$

Equation (2.92) cannot be solved analytically, since the expression for the absorption cross section, $\sigma(\nu)$ is complicated function. If it is intended to find out the intensity at the resonance, that is when $\sigma(\nu) = \sigma(\nu_0)$, the exponential term can be taken out of the integral, and the expression for the observed intensity is given by:

$$I_{ob}(\nu_0 - \nu_e, N) = e^{-N\sigma(\nu)l} \int S(\nu - \nu_0) I(\nu - \nu_e) d\nu \tag{2.93}$$

If the instrument response function is ignored, for instrument with high resolving power,

$$I_{ob}(0, N) = e^{-N\sigma(\nu)l} I_{ob}(0, 0) \tag{2.94}$$

$$\frac{I_{ob}(0, N)}{I_{ob}(0, 0)} = e^{-N\sigma(\nu)l} \tag{2.95}$$

$$\ln\left(\frac{I_{ob}(0, 0)}{I_{ob}(0, N)}\right) = N\sigma(\nu)l \tag{2.96}$$

$$A = N\sigma(\nu)l \tag{2.97}$$

where $\sigma(\nu) = \frac{\varepsilon(\nu)}{N_A}$

$$A = \varepsilon(\nu)Cl \tag{2.98}$$

Equation(2.98) represents Beer's law. An important extension of beer's law is the "law of additivity" which states that the absorption of the radiation by one species will be unaffected by the presence of other materials, whether they absorb or not.

$$A = \sum_i \varepsilon_i C_i l \qquad , i = 1, 2, 3, ..., n \tag{2.99}$$

2.3 Ultraviolet Absorption Spectroscopy

The ultraviolet and visible regions of the electromagnetic spectrum are associated with large enough kinetic energy. Hence the energy that is absorbed will affect the energy states of electrons occupying the molecular orbital within the molecule. When the energy of the radiation is equal to the energy of transition for an electron to be promoted to the exited state then energy will be absorbed by that electron and be promoted to the higher energy molecular orbital. For this electronic transition within molecules energy should be [35] in the UV region of the electromagnetic spectrum.

Three distinct types of electrons are involved in organic molecules. First are the electrons involved in saturated bonds, such as those between carbon and hydrogen in paraffin's. These bonds are called σ bonds. The very high amount of energy required to excite electrons in σ bonds is usually more than that produced by UV light. For this reason paraffin compounds do not absorb UV radiation and are therefore frequently very useful as solvents [30,35].

The second types are electrons involved in unsaturated bonds in hydrocarbons. These bonds usually involve one σ bond and one π bond. Electrons that are not involved in bonding between atoms are the third types involved in organic molecules. These are called n electrons. Organic compounds containing nitrogen, sulfur, or halogens, however, all contain electrons that are non-bonding. Because n electrons can become excited by UV radiation, any compound that contains atoms with n electrons absorb UV radiation. The types of compounds that absorb UV radiation are those with unbounded electron (n electrons) and those with a conjugated double bond system (π electrons) such as aromatics.

Molecular UV absorption spectroscopy is useful for the determination of molecules containing π bonds or n electrons. Included among these compounds are conjugated olefins, aromatic compounds, and many natural products from both animal and plant life.

Ultraviolet absorption spectroscopy is not a widely used tool for qualitative analysis. It is however a powerful tool for quantitative determination of compounds that absorbs UV and is used in chemical research or industrial processing [30].

Spectroscopic techniques are based on the measurement of absorption or emission of electromagnetic radiation [31]. In visible or UV spectroscopy the absorption or emission process is a result of an electronic rearrangement in atoms or molecules. In the absorption process the atom or molecule first in ground electronic state; on absorbing an incident radiation, the system rises to a higher energy electronic excited states [33]. The emission is the reverse of absorption process; that is a molecule already in an excited state makes a transition by emission of energy in the form of electromagnetic radiation. In each case the energy absorbed or emitted is proportional to the frequency of electromagnetic radiation.

2.3.1 Application of UV/Vis Spectroscopy

UV absorption spectroscopy has many applications. It is used to determine: polynuclear aromatic compounds that may cause cancer, natural products such as steroids or chlorophyll, the distinction between forms such as aromatic and quinanoid structure that absorb at different wavelengths [35]. UV/Vis analysis has been used to identify the compounds found in the various parts of plants such as leaves, pollen, and roots. Interaction with UV/Vis light is an essential feature of photosynthesis and hence living tissue [30].

In addition the method has been used for the determination of impurities in organic samples, such as industrial plant streams [31]. In the field of agriculture, UV spectroscopy can be used for the determination of pesticides on plants, the content of some compounds in plant seeds, and leaves. In the medical field it can be used for the analysis of enzymes, vitamins, hormones and alkaloids [35]. These measurements are used in the diagnosis of diabetes, kidney damage and myocardial infection.

2.3.2 Sensitivity of UV Spectroscopy

Ultraviolet absorption analysis is frequently quite sensitive. It is not unusual to detect compounds in concentrations as low as 1 ppm [35]. The sensitivity determines the lowest concentration levels that can be determined quantitatively by this method. Concentrations lower than the sensitivity limits cannot be measured. Other reliable results are only obtained at concentrations 10 times as high as the sensitivity limits. The highest concentration level that can be measured is limited by deviations from Beer's law [34]. The absorption by all solutions of high concentration deviate from Beer's law. When the deviation is high, the absorbance changes only slightly, where as the sample concentration varies greatly. Small errors in the measurement of absorption lead to large errors in the determined concentration. This problem can frequently be overcome by simply diluting the sample to more desirable concentration range [30,34].

2.4 Fluorescence Spectroscopy

If a chemical system absorbs energy and then loses energy by emitting electromagnetic radiation, the phenomenon of luminescence has occurred. When the source of energy is chemical in nature, the result is called chemiluminescence. For biological source of energy, the luminescence is bioluminescence [36]. When both the absorbed and emitted energy is in the form of electromagnetic radiation (usually in the ultraviolet and visible region), either fluorescence or phosphorescence has occurred [37].

In fluorescence,the spin multiplicities of the ground and emissive excited states are the same. In most organic molecules, the ground state is a singlet state (all spins paired). Fluorescence occurs when a molecule has been promoted to an excited singlet state by absorption and then decays back to the ground singlet state by emission. The energy level diagram for a hypothetical molecule, illustrating the principal process concerned with fluorescence is shown in Fig.2.2.

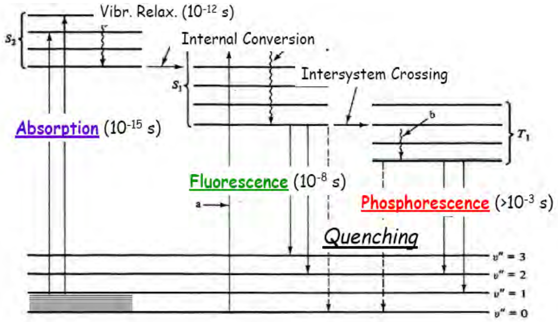

Figure 2.2: *Energy level diagram for hypothetical molecule illustrating the principal processes concerned with fluorescence.*

Once a molecule is in the lowest vibrational level of its first excited singlet state three processes are in competition. One is the emission of photon in which the molecule under goes a radiational transition from an excited singlet state to that of higher vibrational state of the ground electronic state. This emission is called fluorescence and has a life time of 10^{-9} to 10^{-7} s. The fluorescence spectrum will not change as a function of the region of excitation, since fluorescence originates from the lowest vibrational level of the first excited state [36].

The second deactivation process is internal conversion to the ground state. A radiationless processes, it is of sufficient probability for some systems to compete effectively with fluorescence, and has a life time of 10^{-13} to 10^{-11} s. The third process competing with fluorescence is inter-system crossing: a change in state of molecule from a singlet state to a triplet state or viceversa [37]. The process is dependent up on vibrational coupling between the two states and has a life time of approximately 10^{-8} to 10^{-7} s. These two processes , internal conversion and inter-system crossing in effect determine the amount of fluorescence that will be observed and the decay time of fluorescence. These processes are extremely dependent up on the environment, particularly temperature, solvent, and impurities.

2.4.1 Fluorescence Efficiency

The fluorescence efficiency ϕ_f is the fraction of the incident radiation which is re-emitted as fluorescence. It is the ratio of number of emitted photons to the number of absorbed photons or the ratio of the intensity of fluorescence I_f to the intensity of absorption I_a.

$$\phi_f = \frac{I_f}{I_a} = \frac{\Gamma_f}{\Gamma_f + \Gamma_{nr}} \tag{2.100}$$

Γ_f= the rate of constant for fluorescence emission.

Γ_{nr}=the rate of constant for non-radiative deactivation.

After some steps the intensity of fluorescence is given by,

$$I_f = 2.303\phi_f I_0 \varepsilon C l \tag{2.101}$$

2.4.2 Stokes Shift

Two basic types of spectra can be produced by fluorescence spectrometer. In a fluorescence spectrum or emission spectrum the wavelength of exciting radiation is held constant (at a wavelength at which the analyte absorbs) and the spectral distribution of the emitted radiation is measured. In excitation spectrum the fluorescence signal at a fixed emission wavelength, is measured, as a wavelength of the exciting radiation is varied [36]. The maximum in the fluorescence spectrum of a compound occurs at a longer wavelength than the maximum in the absorption spectrum. The wavelength difference between the absorption and fluorescence maxima is called the stokes shift. Often the stokes shift is large (20-50 nm) specially in polar solvents [37]. The stokes shift between the absorption and fluorescence maxima is described in fig.2.3.

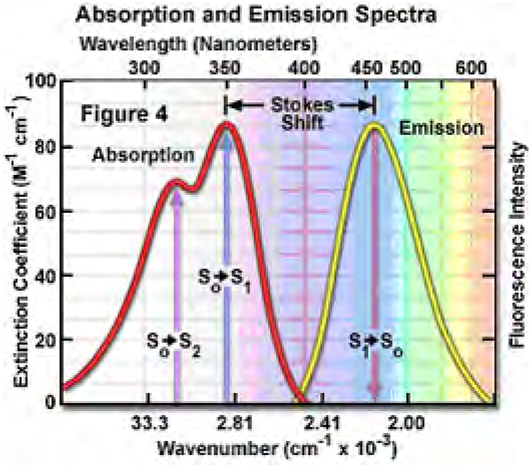

Figure 2.3: *Stokes shift between absorption and emission spectra of a molecule.*

2.4.3 Fluorescence Quenching

Fluorescence quenching is a process, which decreases the intensity of fluorescence emission [36]. In quenching a sample constituent decrease the fluorescence quantum yield for the analyte. The more obvious method for fluorescence quenching is intermolecular electronic energy transfer due to collision:

$$M^{*} + Q \rightarrow M + Q^{*} \tag{2.102}$$

Here an excited analyte molecule (M^{*}) transfers excitation energy to a quencher molecule Q causing de-excitation of M and forming an excited quencher molecule, Q^{*}. If Q^{*} is a fluorescent species, its fluorescence (called sensitized fluorescence) may then be observed. This phenomenon can allow one to observe fluorescence from a molecule (Q) that may be difficult to excite directly [36]. Quenching often follows the the stern-volmer equation:

$$\frac{{\phi^{0}}_{f}}{\phi_{f}} = 1 + K_{sv}(Q) \tag{2.103}$$

where ${\phi^{0}}_{f}$ and ϕ_{f} are the fluorescence quantum yields for the analyte in the absence of quencher and presence of quencher at concentration (Q) respectively and K is known as the stern-volmer quenching constant which turns out to be proportional to the life time of fluorescent species in the absence of quencher, τ_{0}.

2.4.4 Sensitivity and Selectivity of Fluorimetry

The key characteristics of fluorescence spectroscopy is its high sensitivity. Fluorimetry may achieve limits of detection several orders of magnitude lower than those of most other techniques [36]. Sensitivity is highly dependent on fluorescence quantum yield of analyte and the extent to which blank signals (such as impurity fluorescence, Rayleigh and Raman scatter) are minimized. For intensely fluorescent compound (such as polycyclic aromatic hydrocarbons) detection limit is 10^{-11} to 10^{-12} M. Fluorimetry is more selective than UV/Vis absorption spectrometry, since many molecules absorb strongly in the UV or visible range but do not exhibit detectable fluorescence and two wavelength selectors are available in fluorimetry.

Chapter 3

Materials and Methods of the Experiment

In this unit, laboratory apparatus, instruments, chemicals, and experimental procedures will be presented.

3.1 Apparatus and Instrument

The laboratory apparatus used for the experiment are measuring cylinders, beakers volumetric flasks, pipettes, spatula, quartz cuvette, and magnetic stirrer with hot plate, funnel, separatory funnel, glass filter, and 250 micro meter sieve. The instruments used for the experiment are: Electrical beam balance for measuring mass of coffee powder (SA.120, number1450, graduated with a division of 10^{-2} g level, Boulder company), Electrical microbalance for measuring mass of caffeine (M5.SA, number 6500355, graduated with a division of 10^{-4} g, sauter company) Electric motor grinder for grinding the coffee beans, the double beam UV/vis spectrophotometer with a model UV/Vis/NIR spectrometer lambda 19, wavelength range 170 nm-3200 nm, wavelength accuracy 0.1 nm and slit width 1 nm or 2 nm as required. The spectrophotometer was interfaced with personal computer that is used to be operated by UVCSS application software. Spectrofluorophotometer to measure fluorescence intensity.

3.2 UV/Vis Spectrophotometer

A modern recording photoelectric UV/Vis spectrophotometer is used to measure the absorption or emission of electromagnetic radiation by using a photo detector, in the wavelength range.

It consists of five sections or areas: (1) radiation source (2) monochromator (3) sample area, (4) photometer, and (5) detection area. The main components of the spectrophotometer in this section are shown in Fig.3.1.

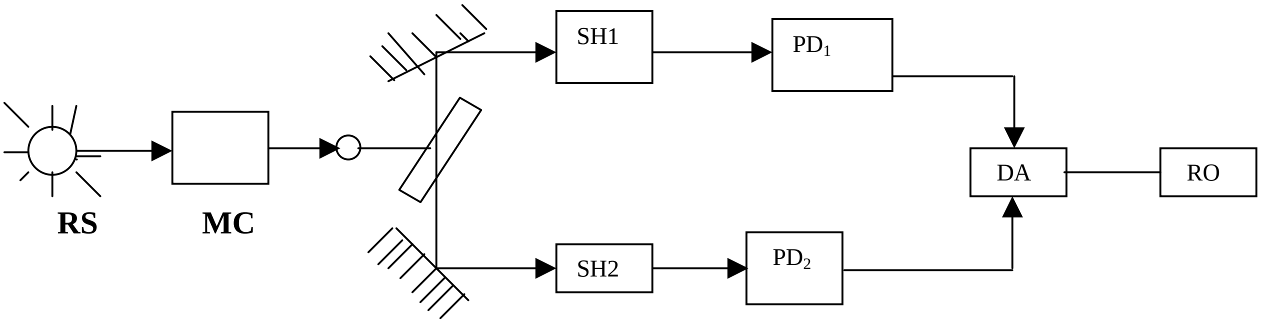

Figure 3.1: *schematic diagram of double beam UV/Vis spectrophotometer.*

Light (Radiation) source (RS), monochromator (MC), chopper (C), Beam splitter (BS), Sample holder (cuvette) for reference beam (SH1), Sample holder (cuvette) in the direction of sample beam (SH2), photo detectors (PD1) and (PD2), difference amplifier (DA) and read out (RO).

There are two types of radiation sources, in the UV/Vis spectrophotometer, from the entire ultraviolet to near IR region [31]. For the UV region the radiation source is Deuterium discharge lamp that emits polychromatic UV- radiation, which can then be filtered into monochromatic UV - radiation. For the visible region the radiation source should be changed to a tungsten filament similar to the ones found in a common incandescent light bulb. The monochromatic or wavelength selector disperses the light from radiation source into its separate wavelength. The wavelength selector consists of an iterance slit, a collimating lenses, dispersing device a focusing lens and an exit slit. A radiation of only a particular wavelength leaves the monochromator through an exit slit. The monochromatic light that emerges from exit slit is pulsed by a chopper and split into sample and reference beams by the beam splitter. A reference beam passes through a sample holder or a quartz cuvette that contains only a solvent. The sample beam passes through a sample holder or a quartz cuvette that contains a sample, solution. The radiation beams that pass through the detectors is amplified by difference amplifier, and finally reaches the recorder (read out), where the results are recorded digitally, in a personal computer attached to a spectrophotometer.

3.3 Instruments to Measure Fluorescence

The instrumentation for observing photoluminescence has many similarities to that used for measuring absorption in the UV region of the spectrum. The major difference between fluorescence and absorption instrument are; in florescence spectroscopy a more powerful source is used, the emitted radiation is viewed at 90^o to the incident radiation, and two wavelength selectors are required to control the wavelength of incident and emitted radiation independently.

In this instrumentation, the sample is excited by radiation from high power xenon arc or mercury lamp at a wavelength determined by a wavelength selector. The emitted radiation is usually observed at the right angle to the incident beam through a second wavelength selector. A detector and read out device completes the system. The sample is contained in a cell similar to that used for absorbance measurement except that in most cases all four sides polished. The main component of fluorescence spectroscopy are described in Fig. 3.2.

Light source (S), Excitation monochromator (EX-MC), sample area (S A), Emission monochromator (EM-MC), Detector (DE), and read out(RO).

The most commonly used source of fluorescence measurement is high power xenon arc which is usually operated at 150 watts. It provides a light out put from 190-1200 nm [37]. The excitation monochromator is used to screen out the wavelength of light not absorbed by a target compound. It allows a selected band of light energy to pass through and excite the sample. The emission monochromator screen out stray light,

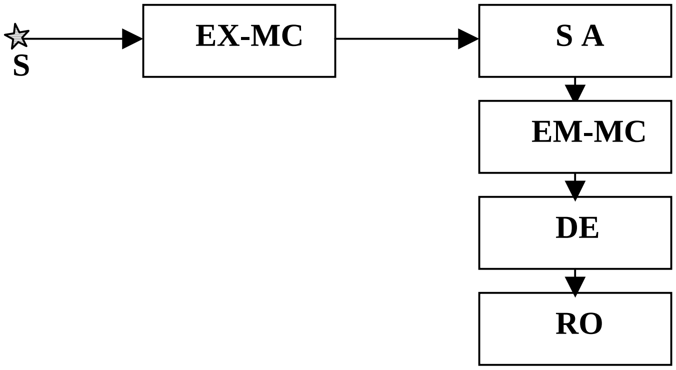

Figure 3.2: *Lay out of a typical fluorescence spectrometer .*

Rayleigh and Raman scattering, allowing primary wavelengths of light specific to the compound to pass through. The light passing through the emission monochromator is detected by photomultipliers, or photodiodes which convert incident light into electric current.

3.4 Chemicals and Samples

The chemicals used for the experiment are: dichloromethane (99.6 % A.C.S, reagent, ALDRICH) and de-ionized water. Moreover, two different types of samples were used in this experiment: caffeine and coffee beans. Caffeine powder from (Evan pharmaceutical company, England). Coffee bean samples from (Bench-maji, yirgacheffe, Teppi and Wollega) which are treated under the same condition were collected from Ethiopia coffee and tea quality and liquoring center. In addition Rhodamine die, high intense fluorescence emitting substance, is used to determine caffeine content by fluorescence quenching.

3.5 Personal Computer

In this experiment, the computer interfaced to spectrophotometer is used to measure physical quantities such as absorbance, amplitude, peak wavelength, wave number and bandwidth. The application software that is designed to operate with spectrophotometer contains different application programs to generate: absorbance difference, transmittance ratio, wavelength, wave number and multiple wavelengths as required. Moreover, it is used to draw graph of wavelength versus absorbance, wavelength versus transmittance, and wave number versus molar decadic absorption coefficient per wave number. The scanning speed of the computer is adjusted as required.

Similarly, the computer interfaced to spectrofluorometer is used to measure physical quantities such as fluorescence intensity and peak wavelength.

3.6 Method of the Experiment

3.6.1 Calibration of the Experiment

To determine the percentage of caffeine in different coffee seeds, it is necessary to set up calibration using pure caffeine. For calibration, pure caffeine and de-ionized water as a solvent were used. The procedures for calibration are:

I. Caffeine is weighed using electrical microbalance graduated at a microgram level.

II. Water is weighed using electrical beam balance graduated at milligram level.

III. The caffeine powder dissolved in water and stirred for 15 minutes using magnetic stirrer. Using the data collected from step (I) and step (II), the number of moles is determined by dividing the actual weight of caffeine to its molecular weight. Finally by taking the ratio of number of moles to volume of the water, the concentration C of the caffeine is determined. The absorption spectrum of this solution was under taken using UV/VIS spectrophotometer. Similarly the experiment is repeated for different concentrations and the absorption spectrum of these concentrations were collected. From the graph of absorbance versus concentration, the molar decadic absorption coefficient determined using Beer's law.

3.6.2 Least Square Method

One does not accept the molar decadic absorption coefficient value determined from one known concentration; rather, several concentrations are prepared and the corresponding absorbance value plotted against concentrations [35].

In any real experiment there will be a random errors arising from the limitation of the experiment. In the equation $A = \varepsilon Cl$, it is customarily assumed that concentrations are known (i.e. more accurately than the absorbance value). The deviation of experimental values from the equation may then be written $A - \varepsilon C_i l = e_i$. The deviations e_i , are squared and the sum of them for all the experimental points. It is required that this sum of the squares of the deviation be minimum. This is achieved by setting the derivative with respect to the adjustable parameter, ε or εl equal to zero.

$$\frac{\partial \sum_i {e_i}^2}{\partial \varepsilon l} = \frac{\partial \sum_i}{\partial \varepsilon l}\left[A_i^2 - 2\varepsilon l C_i A_i + (\varepsilon l C_i)^2\right] = 2\sum_i (\varepsilon l {C_i}^2 - C_i A_i) = 0 \tag{3.1}$$

The least square condition is therefore,

$$\varepsilon l = \frac{\sum_i A_i C_i}{\sum_i {C_i}^2} \tag{3.2}$$

To find the best value of molar decadic absorption coefficient (by this criterion) one should therefore multiply each absorbance value by its corresponding concentration and find the sum of these products. The sum is divided by the sum of squares of the concentrations.

3.7 Setting Up Experimental Procedure

To determine the caffeine concentration in coffee seeds in water solution two main experimental procedures are necessary. These procedures are to determine the absorbance of the spectrum coffee in water solution and to determine the absorbance of the spectrum of caffeine extracted from coffee dissolved in de-ionized water.

3.7.1 Procedures to Determine the Absorbance of Coffee in De-ionized Water Solution

For this set up, sieved coffee powder and de-ionized water are used; The procedures are:

I. To get a uniform texture of coffee powder, it is sieved by microsieve.

II. 200 mg (sample type from Yirgacheffe), 160 mg (sample type from Teppi), 120 mg (sample type from west Wollega) and 80 mg (sample type from Bench- Majji) of sieved coffee powder are measured and added to 50 mL of de-ionized water.

III. Each solutions of coffee powder is stirred by magnetic stirrer for an hour by putting the flask containing the solution in a water bath and adjusting the temperature at 313 K.

IV. Each solutions is filtered using glass filter.

V. The filtered solution is weighed by an electronic balance and divided in to two equal volumes.

VI. 225 g of water is measured and added to half of the solutions (i.e. in 25 g of coffee water solution) to make 1 to 10 dilution. Finally using UV/Vis spectrophotometer the absorption spectra of each solution is collected

3.7.2 Procedures for the Determination of Caffeine Content from Coffee Solution using Extraction Method

In this experimental procedure, caffeine is extracted from each solutions (i.e. from 100 mg of coffee dissolved in 25 g of water, 80 mg of coffee dissolved in 25 g of water, 60 mg of coffee dissolved in 25 g of water and 40 mg of coffee dissolved in 25 g of water) that are organized in (3.7.1) with out dilution.

I. 33 g dichloromethane is added to a coffee solution prepared under procedure (3.7.1) and stirred again for 10 minutes.

II. The above mixture is added to a separatory funnel. Due to the density difference between the two solvents, dichloromethane with the extracted caffeine is easily separated from coffee in water solution.

III. The dichloromethane with extracted caffeine is stored in a volumetric flask.

IV. The above steps (I-III) are repeated three times since more than 95 % of caffeine is extracted up to third round which is the same with the result reported in [3].

V. The weight of dichloromethane with extracted caffeine in volumetric flask is measured and the result will be recorded.

Using UV/Vis spectrophotometer the absorption spectra of each solution of extracted caffeine is collected.

The method of the above separation technique totally could not remove the possible interferences with caffeine spectra [5]. To remove the interfering matrix elements band deconvolution method is applied using one Gaussian function. Finally the absorbance of the spectra of extracted caffeine for each solution after Gaussian fit are recorded.

The physical quantities that relate the absorption spectra of coffee in water solution and the absorption spectra of caffeine extracted from these solutions are described in table 3.1.

Tab. 3.1 The absorbance of absorption spectra of caffeine in coffee
in water and extracted caffeine.

mass coffee	λ_{max} (nm) coffee	$A_{max}$of coffee	λ_{max} (nm) caffeine	A'_{max}(caffeine)	$\frac{A_{max}}{A'_{max}}$
100 mg	272.8	0.8298	276	0.7276	11.36
80 mg	272.8	0.7215	276	0.717	10.05
60 mg	272.8	0.5048	276	0.57037	8.85
40 mg	272.8	0.3368	276	0.3528	9.54

3.8 Band Deconvolution Method

A UV/Vis spectrophotometer cannot be used directly for determination of caffeine in coffee in water solution because the observed spectrum is a convolution of caffeine spectra and the spectra of other water-soluble elements. In this advanced mathematical method, gaussian distributions are used to model the absorption properties of objects since they closely approximate in electronic transitions absorptions.

The absorption profiles of the interfering band (matrix element) of coffee in water solution are represented by five overlapping gaussian distributions. The process of modelling can be expressed as:

$$A = A_1 + A_2 + A_3 + A_4 + A_5 \tag{3.3}$$

where A is the total absorption distribution and A_i is the i^{th} individual absorption.

$$A_{i\lambda} = a_i exp\left(- \left(\frac{\lambda - p_i}{\omega_i} \right)^2 \right), i = 1, 2, 3 \qquad and \lambda = 200 - 450 nm \tag{3.4}$$

Steps for band deconvolution method

1. Isolating the actual caffeine spectrum from caffeine in coffee in water solution

by fitting five Gaussian distributions.

2. Comparing the deconvoluted caffeine spectrum with pure caffeine spectrum.
3. Determining the amount of caffeine in coffee seed using the absorbance of deconvoluted caffeine spectrum.

3.9 Calibration of the Experiment by using Spectrofluorophotometer

To observe whether pure caffeine fluoresce or not; 2 mg of caffeine is measured and dissolved in 200 mL of water. As it is observed by spectrofluorophotometer, caffeine does not fluoresce in water solution. So it is imposable to determine the concentration of caffeine in coffee powder dissolved in water directly by selective excitation. If a molecule which absorbs UV radiation does not fluoresce it must have disposed of excess energy by some other means in order to return to the ground state. One way is fluorescence quenching in which an excited molecule transfers its excitation energy to a quencher molecule, causing de-excitation of an excited molecule, and forming an excited quencher molecule. This phenomenon can allow one to observe fluorescence from a quencher molecule that may be difficult to excite directly. For calibration of the experiment Rhodamine molecule which has intense fluorescence and caffeine is used as analyte. 2 mg of Rhodamine is dissolved in 200 mL of water, and 10 ml of Rhodamine solution is diluted by adding 90 mL of water (1 to 10 dilution). 2 mg of caffeine is dissolved in 200 mL of water. After that the mixture of both solutions are prepared according to the ratio shown in table 3.2.

Tab.3.2 Concentration of pure caffeine for calibration in terms of weight to weight ratio using Rhodamine dye.

Concentration	Caffeine solution in (mL)	Rhodamine solution in (mL)
1-1	10	10
1-2	5	10
1-3	5	15
1-4	5	20
1-5	5	25

The emission spectra of these solutions are collected by fixing the excitation wavelength of rhodamine at 540 nm. From the fluorescence spectra , the peak wavelength and the fluorescence intensities are recorded.

3.10 Procedure for the Determination of Caffeine using Fluorometry

To determine the concentration of caffeine in coffee powder dissolved in water solution, 200 mg of coffee powder is dissolved in 200 mL of de-ionized water and stirred by magnetic stirrer for an hour by controlling the temperature as usual. Then it is filtered using a glass filter. Similar to the calibration procedure, 2 mg of rhodamine is dissolved in 200 mL of de-ionized water, and 10 mL of this solution is diluted by adding 90 mL of de-ionized water to it. Then the mixture of both solutions are prepared as shown in the following table.

Tab. 3.3 Concentration of caffeine in coffee powder in water solution in terms of weight to weight ratio using rhodamine dye.

Concentration	Coffee solution in (mL)	Rhodamine solution in (mL)
1-1	10	10
1-2	5	10
1-3	5	15
1-4	5	20
1-5	5	25

Chapter 4

Results and Discussions

In this unit the results of the experiments are presented based on the experimental procedure in the previous unit.

4.1 Absorbance versus Concentration Relation

The absorption spectra of pure caffeine for different concentrations are observed using UV/Vis spectrophotometer. The peak absorbance of each concentration of the absorption spectra of pure caffeine in water solution is at peak wave length of 272.8 nm. The absorbance versus wavelength graph of the absorption curve for different molar concentrations of pure caffeine are shown in Fig. 4.1.

To determine the molar decadic absorption coefficient and the transition dipole moment of caffeine in water solution, a series of solutions each with a known concentration was prepared; and the absorbance of each solution is measured using UV/Vis spectrophotometer as shown in table 4.1.

Tab. 4.1 Physical quantities from the absorption spectra of different concentrations of caffeine.

C $(\frac{mol}{m^3})$	A	$\frac{A}{C}(\frac{m^3}{mol})$	$\varepsilon(\frac{m^2}{mol})$	$\mu_{nm}(cm)$	$I_A(\frac{m^2}{mol})$
0.04873	0.5465	11.21	1121	11.50×10^{-30}	129.45
0.07097	0.7416	10.45	1045	11.10×10^{-30}	120.57
0.08770	1.0	11.40	1140	11.27×10^{-30}	124.41
0.11	1.2	10.90	1090	11.3×10^{-30}	124.01

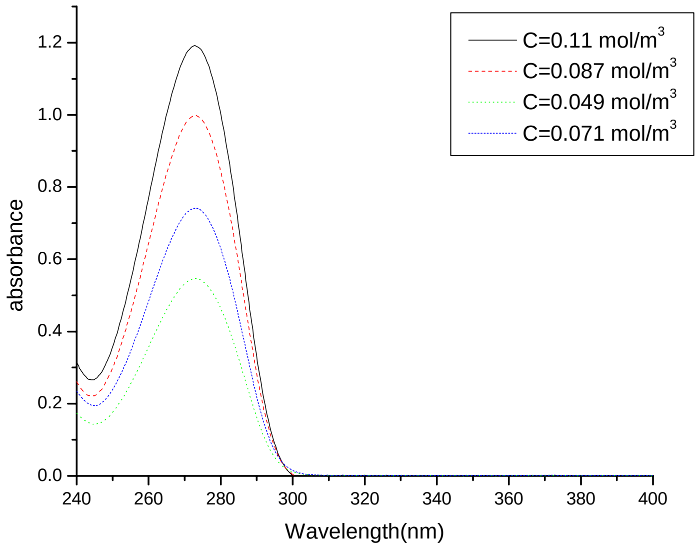

Figure 4.1: *Absorbance versus wavelength graph for different concentrations of caffeine.*

The molar decadic absorption coefficient of caffeine in water is calculated using least square method and its value is $1098.87\frac{m^2}{mol}$. This is a constant for a given type of transition occurring with in a particular sample. Moreover the value of transition moment was calculated using the integrated molar absorptivity and its value is $(11.28 \times 10^{-30} \pm 0.14 \times 10^{-30})cm$. The values of these constants are the same as the values that are reported in [5]. All the above peak absorbance of a solution for a given concentration is found at a maximum wavelength of 273 nm. The average integrated area under the curve is $(124.62 \pm 3.16)\frac{m^2}{mol}$. The electronic type transition for pure caffeine is $\pi^* \leftarrow \pi$ transition and this transition is the cause for absorption.

The concentration versus absorbance graph for the quantities described in table 4.1 is shown in Fig. 4.2.

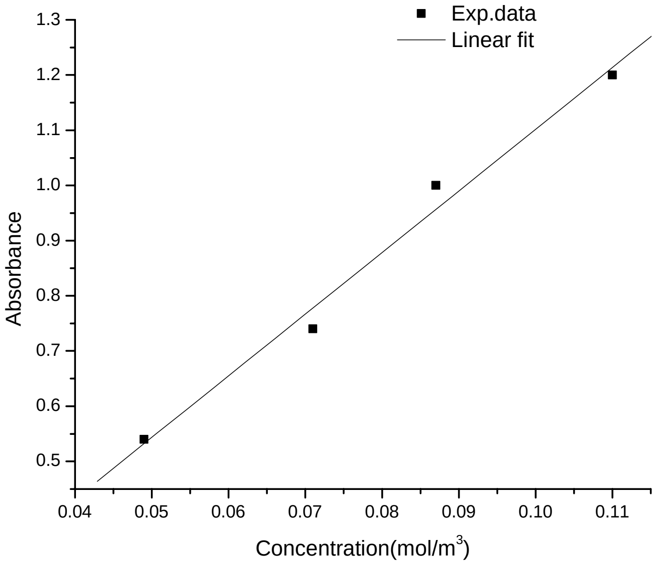

Figure 4.2: *Concentration versus absorbance graph of pure caffeine.*

4.2 Technical Procedures Developed to Determine the Caffeine Content in Coffee Seeds Using wWater as a Solvent

Even though there are more than three interfering matrix elements, when coffee powder is dissolved in water, it is possible to determine the caffeine content in coffee seeds using the cheapest universal solvent, water. The methods are discussed in section 4.2.1.

4.2.1 Determining the Constant that Relate the Absorption Spectrum of Caffeine in Coffee Dissolved in Water and Caffeine Extracted by Dichloromethane

To determine this constant, the extensive quantity, the peak absorbance of caffeine in coffee in water solution is compared with the intensive quantity the concentration or mass of caffeine extracted by using dichloromethane. The extraction is made three times, since about 95% of caffeine has been extracted up to third round of extraction.

It is also observed that the extracted caffeine is a convolution of a pure caffeine spectrum and one interfering matrix element. This interfering matrix element is removed by fitting it with one Gaussian model as shown in the following table:

Table 4.2 parameters of a gaussian model for deconvolution of the interfering matrix element of the absorption spectra of extracted caffeine.

Sample type	Amplitude	Peak wavelength (nm)	Band width (nm)
Teppi	0.043	300	18.31
Yirigacheffe	0.060	300	20.5
West Wollega	0.017	300	19.28
Bench majji	0.042	300	17.42

For 100 mg of coffee powder dissolved in 67.5 mL of water, the absorbance of the absorption spectrum observed at wavelength of 272.8 nm is 3.07. Caffeine is extracted from the same mass of coffee powder using 67.5 mL of dichloromethane and the absorbance of its absorption spectrum is 0.7276 at the peak wavelength of 276 nm. The comparison between the absorption spectra of caffeine in 100 mg of coffee in water solution to the absorption spectra of extracted caffeine from the same mass after deconvoluting by one gaussian function are described in Fig. 4.3.

Similarly, 80 mg of coffee powder is dissolved in 67.5 mL of water, and the absorbance of its absorption spectrum is 2.67 at a wavelength of 272.8 nm. The peak absorbance of the absorption spectrum of caffeine extracted from the same mass using 67.5 mL of dichloromethane is 0.717 at a peak wavelength of 276 nm. The absorption spectra of 80 mg of coffee powder dissolved in water and and caffeine extracted from the same mass is described in Fig. 4.4.

For the third sample, 60 mg of coffee powder is dissolved in 67.5 mL of water and the absorbance of its absorption spectrum is 1.87 at the wavelength of 272.8 nm. The peak absorbance of the absorption spectrum of caffeine extracted from the same mass using the same volume of dichloromethane is 0.57037 at a peak wavelength of 276 nm.The absorption spectra of 60 mg of coffee powder dissolved in 67.5 mL of water and caffeine extracted from the same mass using dichloromethane is shown in Fig 4.5.

Finally 40 mg of coffee powder is dissolved in 67.5 mL of water,and the absorbance

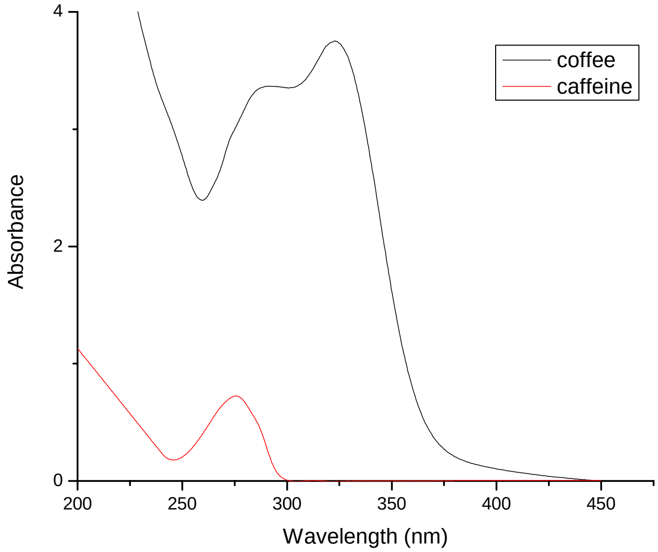

Figure 4.3: *Absorption spectra of coffee and caffeine in 100 mg of coffee.*

of its absorption spectrum at the wavelength of 272.8 nm is 1.25. The peak absorbance observed for the absorption spectrum of caffeine extracted from the same mass using 67.5 mL of dichloromethane is 0.3528 at a peak wavelength of 276 nm.The absorption spectra of 40 mg of coffee powder dissolved in 67.5 mL of water and caffeine extracted from the same mass using the same volume of dichloromethane is described in Fig. 4.6.

The peak absorbance of caffeine in coffee in water solution is recorded at the wavelength of 272.8 nm. This wavelength is the maximum wavelength at which the peak absorbance of pure caffeine in water solution is observed. The peak absorbance of extracted caffeine in dichloromethane is at a wavelength of 276 nm. This is the maximum wavelength, at which the peak absorbance of pure caffeine is observed in dichloromethane solution. In this comparison, the volume of water in which coffee powder is dissolved should be the same as the volume of dichloromethane from which caffeine is extracted. According to Beer's law, the peak absorbance of absorbing medium is inversely proportional to the volume. So the absorbance of caffeine in coffee in water solution is a multiple of constant, $B = 250/67.5 = 3.7$

The result of comparison is summarized in table 4.3.

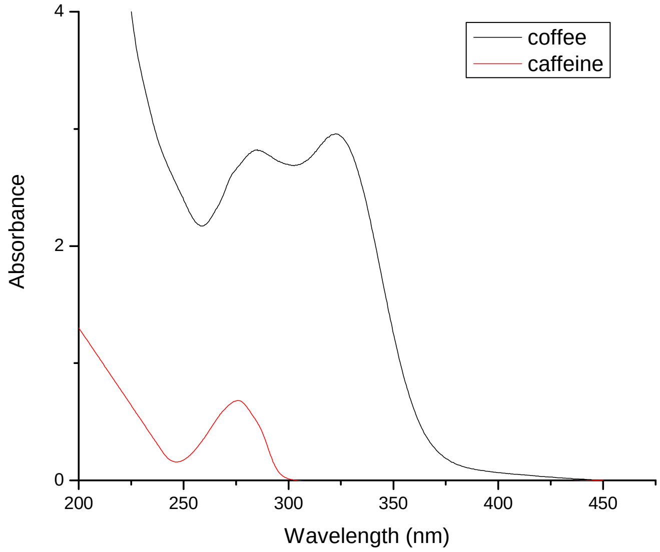

Figure 4.4: *Absorption spectra of coffee and caffeine in 80 mg of coffee.*

Tab.4.3: the relation between the extensive quantity (absorbance of coffee) in water solution and intensive quantity(mass of extracted caffeine) for different samples.

Sample type	m_i(coffee) mg	A_i(coffee)	m_i(caffeine mg)	$k_i = \frac{A_i}{m_i(caffeine)}$ (mg)$^{-1}$
Y. Cheffee	100	3.07	0.953	3.22
Teppi	80	2.67	0.939	2.85
W. wollega	60	1.87	0.739	2.53
B. Majji	40	1.25	0.457	2.73

As it is observed from the table above there is no much variation between the ratios of peak absorbance of caffeine in coffee in water solution to the mass of caffeine extracted using a dichloromethane from the different samples. So the constant is the slope of the, extensive quantity, peak absorbance of caffeine in coffee in water solution to the, intensive quantity, mass of caffeine. The value of this constant is (2.90) mg^{-1} by applying least square method.

Employing this constant the mass of caffeine as well as the percentage of caffeine in 100 mg, 80 mg, 60 mg and 40 mg of coffee powder dissolved in 67.5 mL of water is presented in the following table.

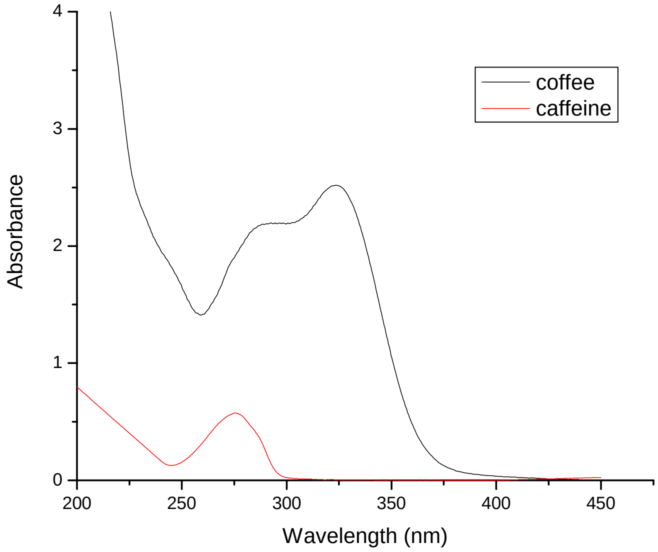

Figure 4.5: *Absorption spectra of coffee and caffeine in 60 mg of coffee.*

Tab. 4.4: The mass of caffeine and the percentage of caffeine for different samples collected from different regions at the same condition.

Sample type	Mass (coffee) mg	Mass (caffeine) mg	% of caffeine
Y.cheffee	100	1.06	1.06
Teppi	80	0.921	1.15
W.Wellega	60	0.645	1.07
B. Majji	40	0.431	1.07

As it was reported by [24], the percentage of caffeine determined by HPLC is 1.1 %. It was also reported that using UV/Vis spectrophotometer and dichloromethane as a solvent for the extraction of caffeine the percentage of caffeine is almost 1.1 % [5]. Hence the result obtained using water, as a solvent is consistent with the other results.

After all this method is the simplest method with least cost which is applicable in every part of the country to determine the caffeine concentration of ordinary coffee with simple mathematical knowledge.

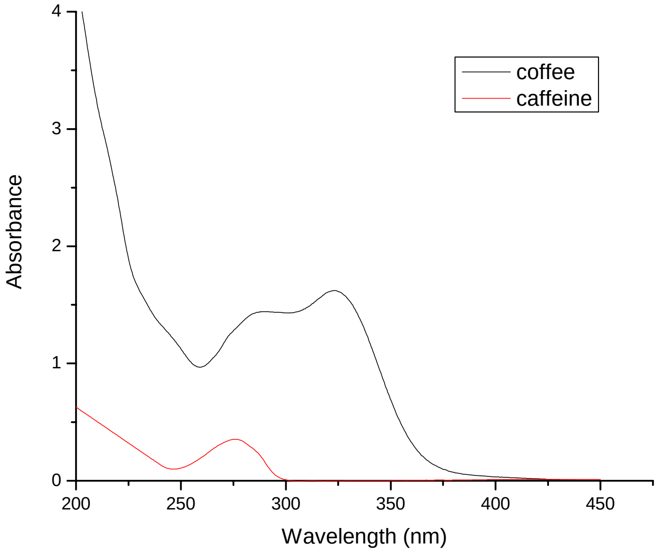

Figure 4.6: *Absorption spectra of coffee and caffeine in 40 mg of coffee.*

4.2.2 Band Deconvolution Method for the Determination of the Percentage of Caffeine from Coffee Seeds

As it is mentioned in the previous section, the absorption spectrum of coffee in water solution is a convolution of pure caffeine spectrum and the other interfering matrix elements. It is possible to obtain the pure caffeine spectrum by removing all the interfering matrix elements using band deconvolution method. As it was mentioned in the previous chapter, the interfering matrix element is modelled by five Gaussian distributions. The parameters describing the model for interfering matrix element in 40 mg of coffee powder dissolved in 67.5 mL of water are explained in the following table.

Tab. 4.5: Obtained absorption parameters for modelling interfering band.

Band No	Peak wavelength (nm)	Half width (nm)	Absorbance
1	323.30	41.80	1.62
2	294.80	18.99	0.45
3	275.72	28.68	0.71
4	245.72	18.99	0.51
5	169.85	71.29	5.24

In the following figure five absorption bands are generated by equation (3.2) using the above absorption parameters. The convolution of Gaussian distributions is formed by using origin software. The five Gaussian distributions for modelling the interfering bands are shown in Fig.4.7.

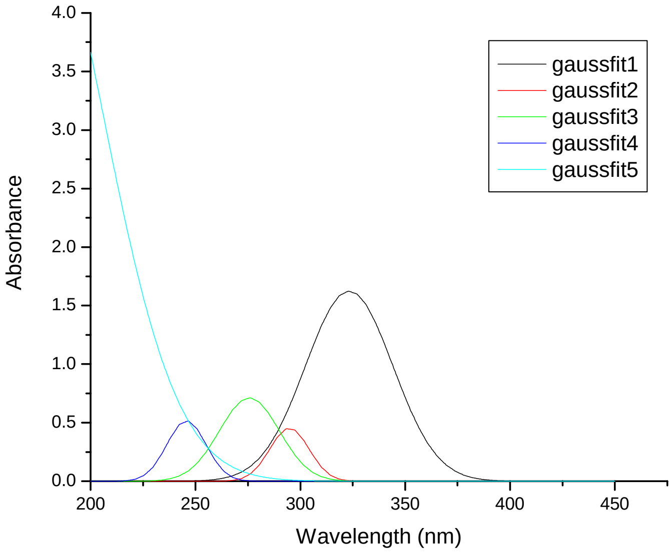

Figure 4.7: *Absorption band model for interfering matrix element.*

Generally it is mentioned that the absorption spectrum of coffee in water solution is the convolution of the pure caffeine spectrum and other interfering matrix elements. In this approach the interfering matrix elements are modelled as a convolution of five Gaussian models. The comparison between the absorption spectra of the original interfering band and the model is shown in Fig. 4.8. The actual caffeine spectrum is deconvoluted from the absorption spectrum of 40 mg of coffee dissolved in 67.5 mL of

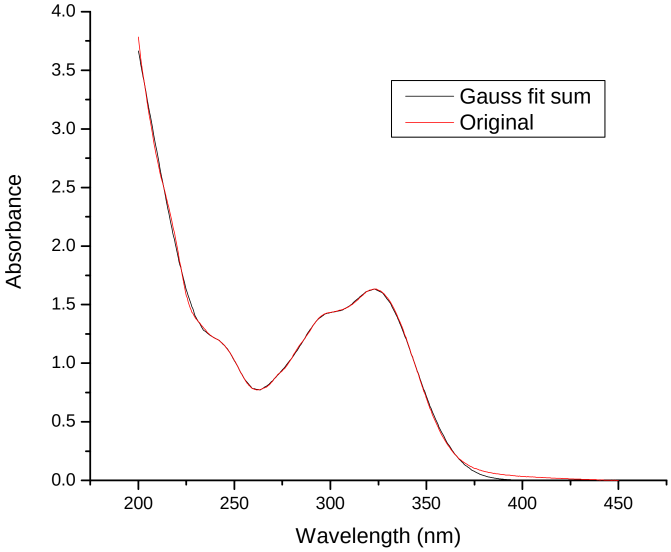

Figure 4.8: *Comparison of the original interfering band with a convolution of five Gaussian model.*

water solution and is compared with the spectrum of caffeine extracted from 40 mg of coffee powder using 67.5 mL of dichloromethane solution. As it is observed the peak absorbance of the absorption spectrum of deconvoluted caffeine is 0.3385 at a peak wavelength of 272.8 nm. But the peak absorbance of the absorption spectrum of extracted caffeine is 0.3528 at a peak wavelength of 276 nm. The result shows that the error is only 4%.The comparison between the absorption spectra of deconvoluted and extracted caffeine is shown in Fig. 4.9. Generally it is observed that the absorption spectrum of coffee powder dissolved in water solution is the convolution of the actual caffeine spectrum and five Gaussian distributions.So the absorption spectrum of coffee as a convolution of the actual caffeine spectrum and the five absorption bands are shown in Fig. 4.10.

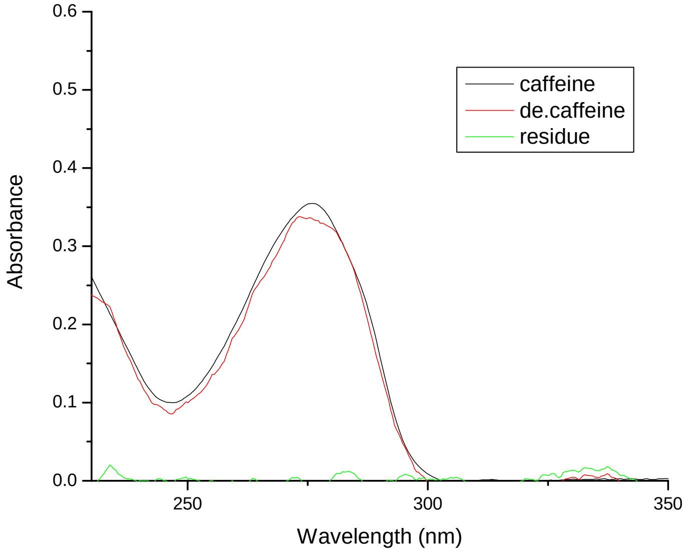

Figure 4.9: *Comparison of the deconvoluted caffeine spectrum with the spectrum of extracted caffeine.*

For the absorption spectra of 60 mg, 80 mg, and 100 mg of coffee powder dissolved in water solution the above model can be modified by multiplying the absorbance of the above Gaussian distributions by a constant, $k = \frac{A_{peak}(x \quad mg)}{A_{peak}(40 \quad mg)}$. Employing the above relation the constants are shown in table 4.6.

Tab. 4.6 A constant relating the peak absorbance of the absorption spectrum for different masses of coffee powder in water solution.

No	Sample type	Mass (coffee) in mg	Constant (k)
1	Y. Cheffe	100	2.31
2	Teppi	80	1.82
3	W.Wellega	60	1.55

Employing the above constants the absorption parameters of interfering bands for other samples are described in the following table.

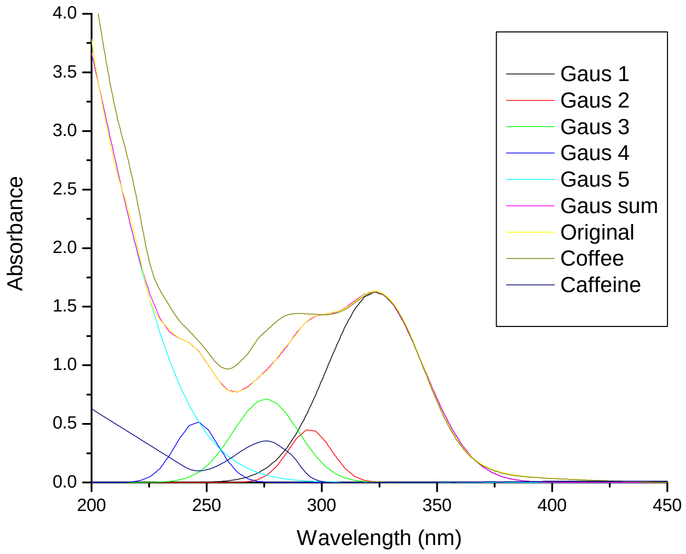

Figure 4.10: *Absorption spectrum of coffee as a convolution of caffeine and five Gaussian model.*

Mass of coffee in (mg)	Peak wavelength in (nm)	Band width (nm)	Absorbance
60	323.30	41.80	2.51
	294.80	18.99	0.69
	275.72	28.68	1.10
	245.72	18.99	0.79
	169.85	71.29	8.13
80	323.30	41.80	2.95
	294.80	18.99	0.81
	275.72	28.68	1.29
	245.72	18.99	0.92
	169.85	71.29	9.54
100	323.30	41.80	3.74
	294.80	18.99	1.03
	275.72	28.68	1.64
	245.72	18.99	1.17
	169.85	71.29	12.10

Tab.4.7 Absorption parameters for interfering band in 60, 80, and 100 mg of coffee in water solution.

The absorption spectrum of caffeine can be determined by deconvoluting the interfering band model from coffee absorption spectrum. The absorbance of the absorption spectrum of deconvoluted caffeine for different samples are shown in table 4.8.

Tab.4.8 The mass and percentage of caffeine obtained for different samples using band deconvolution method.

mass(coffee)in mg	$A_{deconvoluted\ caffeine}$	λ_{peak}	mass(caffeine) in mg	%
40	0.3385	272.8	0.407	1.02
60	0.5431	272.8	0.652	1.08
80	0.6690	272.8	0.804	1.01
100	0.842	272.8	0.976	1.01

To obtain the above result the residue is considered as baseline and the absorbance is recorded taking the residue as a reference point.From the above table it is possible to conclude that the result obtained by applying band deconvolution method is consistent with HPLC result with an error of 5-10 %.

4.3 Fluorometric Method for the Determination of the Concentration of Caffeine in Coffee Seeds

Even though pure caffeine dissolved in de-ionized water has no emission spectrum, it is possible to determine the concentration of caffeine in coffee powder dissolved in water, by a process of fluorescence quenching. This process allows one to observe wether the quenching effect of pure caffeine is linear or not for different concentrations. The emission spectra of Rhodamine due to the quenching effect of different concentrations of caffeine is shown in Fig.4.11.

Moreover coffee dissolved in water solution has no fluorescence. Hence to observe the emission spectra, different concentrations of coffee solutions are used as quencher and Rhodamine solution is used as energy donor as it was discussed in the experimental procedure. The emission spectra of Rhodamine due to the quenching effect of different concentrations of coffee in water solution is described in Fig.4.12.

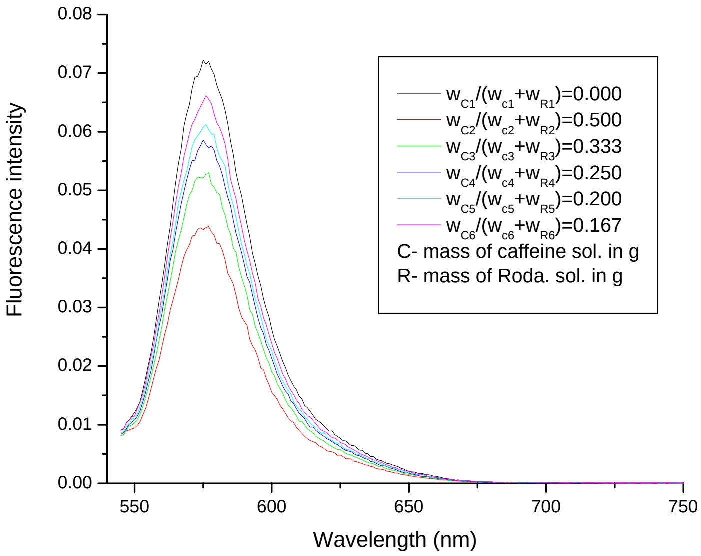

Figure 4.11: *Fluorescence spectra of Rhodamine due to the quenching effect of different concentrations of caffeine in water solution.*

According to the above calibration and experimental procedure the fluorescence intensity of Rhodamine due to the quenching effect of pure caffeine and coffee powder in water solution are measured using spectrofluorophotometer and the result is described in the following table.

Tab.4.9 The fluorescence intensity of the emission spectra of rhodamine due to quenching effect of coffee and caffeine of different concentrations.

Conc. of coffee/caffeine	$\lambda_{max}(nm)$	I_F rhod. due to caf. conc.	I_F rhod. due to cof. con.
0.000	575	722	722
0.167	575	645	635
0.200	575	612	624
0.250	575	586	585
0.333	575	527	526
0.500	575	433	421

The correlation between the fluorescence intensity of the emission spectra of 2 mg of pure caffeine and 200 mg of coffee powder in water solution from the above

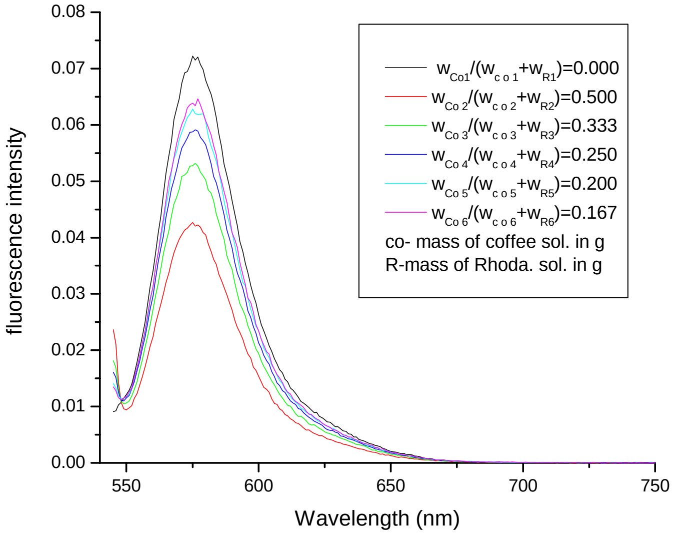

Figure 4.12: *Fluorescence spectra of Rhodamine due to the quenching effect of different concentrations of coffee in water solution.*

table reveals that the intensities for a given concentration are almost equivalent. This experimental result is elaborated in Fig.4.13.

The result clearly confirms that 200 mg of coffee powder contains 1.906 mg of caffeine, which is 0.953 % in concentration. The correlation between the calculated and experimental value of of caffeine concentration is described in Fig.4.14.

The percentage of caffeine obtained using scale up factor, band deconvolution and fluorescence quenching methods are compared with Standard procedures to determine the percentage of caffeine in coffee seeds by UV/Vis spectrophotometer (extraction method using dichloromethane) are shown in table 4.14.

Tab. 4.10 Comparison of percentage of caffeine obtained by different methods.

Sample type	band dec. meth.	scale up meth.	fluore. quench.	extraction meth.
Bench maji	1.02	1.07	-	1.12
West Wollega	1.08	1.07	-	1.18
Teppi	1.01	1.15	-	-
Yiriga cheffee	1.01	1.06	1.0	1.06

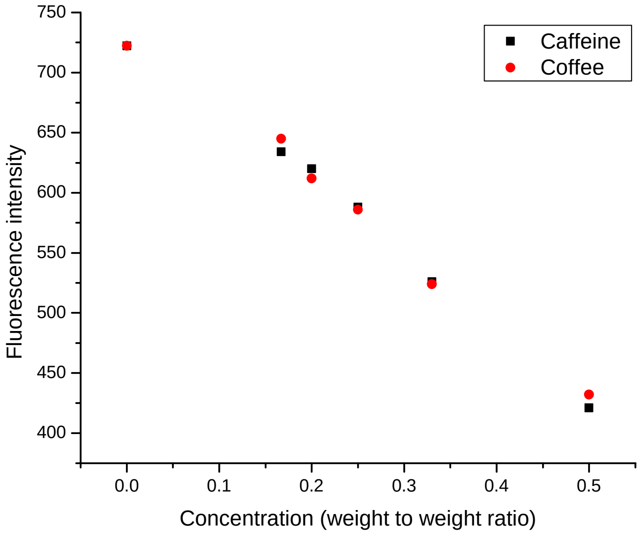

Figure 4.13: *Correlation between the quenching effect caffeine and coffee in water solution using rhodamine as energy donor molecule.*

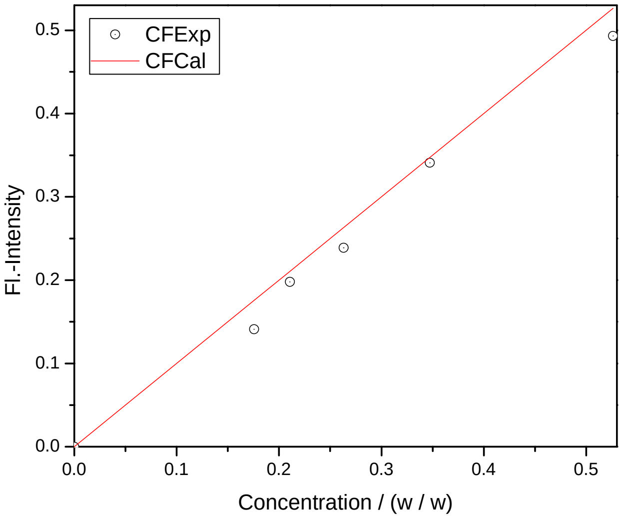

Figure 4.14: *Correlation between calculated and experimental value of caffeine concentration in coffee.*

Chapter 5

Conclusion

The demand of decaffeinated coffee is highly increased in the world due to physiological and psychological effects of caffeine on health. Even though many researches were conducted to determine the concentration of caffeine in coffee seeds, some suffer the disadvantage of using large amount of samples and undergoing interference from many redox reagents with the determination. With the rest of the methods the use of expensive equipment and toxic chemicals prevent their application in small laboratories which are available in developing country like Ethiopia.

The objective of the study was to determine the concentration of caffeine in coffee seed using water as the solvent. Water is the cheapest solvent found everywhere and helps the experimentalist to perform his experiment without suffering from the toxic nature of other chemical solvents. To achieve this objective three methods are investigated. In the first method the constant that relate the absorbance of caffeine in coffee in water solution to the mass of extracted caffeine was found and its value is 2.9 mg^{-1}. Employing this constant one can determine the concentration of caffeine in ordinary coffee seed with the least cost and simple mathematical knowledge.

In the second method band deconvolution method was used to remove the interfering matrix elements. In this method the interfering matrix elements in the absorption spectrum of coffee dissolved in water solution are modelled by five Gaussian functions, and the model is applied for other samples by changing the absorbance, while fixing the other parameters. The results obtained by these methods are consistent with the result reported in [5] with an error of only 5-8 %. As the result specially band deconvolution method has useful application for analysis of naturally decaffeinated coffee seeds with least cost.

Finally, caffeine does not exhibit emission spectrum, in water. Thus, indirect approach was employed for a comparative study of the caffeine content of coffee by correlating fluorescence quenching produced by pure caffeine and coffee powder dissolved in water using rhodamine dye as energy donor molecule. The experimental result reveals that, both coffee and caffeine stock solutions show approximately the same fluorescence quenching. This implies that the major constituent responsible for the quenching is caffeine, and thus the quenching effect attributed to the concentration of caffeine.

In the future detailed research should be conducted to determine the concentration of caffeine in coffee seed to overcome the mentioned errors in band deconvolution method as well as fluorescence quenching.

Bibliography

[1] P.P. Sybil, etal, ”Encyclopedia of Science and Technology”, McGraw Hill, pp 105-107, 1992.

[2] E. Illy, ”The complexity of coffee”,Scientific American Inc., June 2002

[3] M.N. Clifford and K.C Wilson, ”Coffee botany, biochemistry, and production of beans and beverage”,London, 1987.

[4] P.B. Norton, J.Joseph, ”The new Encyclopedia Britannica” Vol. 3, pp 200-205, 1768.

[5] A. Bealay, ”Standard procedures to determine the percentage of caffeine in coffee seeds by UV/Vis spectrophotometer”, A.A.U, M.Sc. thesis, 2005.

[6] F. L. Wellman, ”Coffee, Botany, cultivation and utilization”, Leonard Hill, 1961.

[7] Coffee and tea authority, ”Cradle of the wonder bean”, pp 5-6, 1999.

[8] B. W/mariam, ”History of Kaffa people and its states”, pp 41, 2004.

[9] M. Wondimu, ”Kaffa coffee”, Vol.1 No1 ECEA, 1998.

[10] T.Teklu, ”Coffee farming and marketing system in Ethiopia”, B.A. thesis, pp 5-10 ,2003.

[11] Rene coste, ”Coffee: the plant and the product”, Macmillan press, 1992.

[12] EEPA, ”Ethiopian coffee production potential and export marketing”, pp 2, 2002.

[13] A.Tavani,etal ”coffee and tea intake and risk of cancers of the colon and rectum”, Wiley-Liss,INC., 1997.

[14] K.Davids, ”Health benefits of coffee”, the coffee review, 2001.

[15] R.J Clarke and R.Macrae, ”Coffee chemistry”, Vol.1, Elsevier, 1985.

[16] K. Ture, ”All optical qualitative and quantitative determination of caffeine in various coffee beans grown in Ethiopia”, A.A.U, M.Sc., pp 1-5,2005.

[17] Smu Dining service, ”Caffeine, Are there grounds to concern?”,pp 1-3,2001.

[18] S.Bolton, and G.Null, ”caffeine,psychological effects,use and abuse, Orthomolecular psychiatry” ,Vol.10, No.3, pp 202-211, 1981.

[19] A.E. Haarer, ”Arabian coffee planting andcultivatn modern way”, Mon. Bull Indian coffee, Vol. 14,pp 7,1950.

[20] R.coste, ”coffee,the plant an the product”,Macmilan press, pp 240-249, 1992.

[21] Ogita, H.Uefuji,Y,Yamaguchi, N.Koizumi, and H.Sano, ”Nature”,Vol.423, 2003.

[22] A.Guzin, ”Derivative spectrophotometer determination of caffeine in some beverages”, Turk.J.Chem, 26(2002), 295-3002.

[23] Analytical chemistry umea university, ”Determination of caffeine in Vietnam coffee using HPLC”,Sweden, 2002.

[24] Jeanne,L.mchale, ”Molecular spectroscopy”,prentice-HALL,Inc., 1999.

[25] P.W. Millonni, ”Lasers”,John wiely and sons,1988.

[26] Gordon M.Barrow, ”Introduction to molecular spectroscopy, McGRAW-Hill, 1962.

[27] Asfaw A., ”Optical measurement of number density, Vapour pressure and broadening parameters of Na in sub micro torr region”,JQSRT 74(2002) 655-662, Elsevier.

[28] Asfaw A., ”A fast method of modeling spectral lines”,JQSRT 70(2001) 129-137 Elsevier.

[29] Q. Chen,T.L.V.Cheung, S.west land, ”physical modeling of spectral re flectance”, center of color design techinology, universiyy of Leeds, UK, 1988.

[30] T.H. Couw, ”guide to modern methods of instrumental analysis”, Wiely & sons,inc.,pp 161-230, 1972.

[31] Svanberg, ”Atomic and molecular spectroscopy”, Springer-Verlag, Berlin Heideleberg, 1992.

[32] Demtröder, ”Laser spectroscopy”, Berlin, pp 5-100, 1995.

[33] J.michl, E.W.Thulstrup, ”Spectroscopy with polarized light”,VCH, PP 25-28, 1986.

[34] R.P.Bauman,”Absorption spectroscopy”,John wiley & sons,Inc., New york, pp 370-389, 1963.

[35] J.W. Robinson,"Undergraduate Instrumental Analysis", Mrcel Dekker,Inc. ,New york,pp 119-150, 1970.

[36] D.Rendell, "Fluorescence and phospheroscence", John Willy and sons, New york, 1987.

[37] J.R. Lakowicz, "Principles of fluorescence spectroscopy", Klumer academic, New york, 1996.

DECLARATION

I here by declare that this thesis is my original work and has not been presented for a degree in any other University. All sources of material used for the thesis have been duly acknowledged.

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