

**ADDIS ABABA UNIVERSITY  
SCHOOL OF GRADUATE STUDIES**



**LEVELS OF NICOTINE IN ETHIOPIAN  
TOBACCO LEAVES**

**By**

**ZEBASIL TASSEW**

**March, 2007**

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***To***

My wife who shared my trouble during the study

***&***

My mother

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# Levels of Nicotine in Ethiopian Tobacco Leaves

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## ***Abstract:***

*In this paper the level of nicotine in four different varieties of Ethiopian tobacco leaves was determined using High Performance Liquid Chromatography (HPLC) method of analysis. Among the variety of species Virginia tobacco leaves were found to have high nicotine level (3.26 %), followed by the native tobacco 'Gaya' (1.1 %) then burley tobacco leaves (0.65 %), finally oriental tobacco leaves were found to have lowest nicotine level, though its actual level of nicotine was not determined. Moreover it was confirmed that the nicotine level of Ethiopian tobacco leaves increases from bottom to top leaf (stalk) positions of the tobacco plant, as well it was found that nicotine level of the same species tobacco differ based on area of cultivation. The levels of nicotine in Ethiopian tobacco (obtained by this study) were found to be comparable with that in the rest of the world.*

# 1. Introduction

Now a day tobacco is money, since it is valuable cash crop, accordingly it is the most widely grown non-food crop in the world with assistance of man and with the leaf as the most valuable part of the plant. Tobacco holds an unparalleled position among crop plants in the world such as:

- 1) It is one of the very few crops entering world trade entirely on a leaf basis.
- 2) It is the most widely grown commercial non-food plant in the world.
- 3) It holds a high importance in financial and economic policies in many countries.
- 4) Consumption is by way of smoking, inhaling or chewing and is a habit forming narcotic, and although bans of its use have been attempted, its consumption marches steadily forward.
- 5) Originally having religious significance, subsequent claims of medicinal benefit have alternated with accusations of a positive danger to health [1].

Tobacco growth is restricted, by environmental factors; the majority of the tobaccos entering the world trade are produced in tropical and temperate regions. That is almost all continents are capable of growing tobacco, and hence many countries are tobacco producers. According to FAO report in 2005 the top ten leading countries to grow tobacco are listed in Table 1. Even though, the use of tobacco can be detrimental to the user, the tobacco plant remains to this day one of the most important economic crops in the world. The reason tobacco use spread quickly was that the addictive nature of its main constituent nicotine which is volatile liquid alkaloid and causes dependence. Therefore, nicotine content is an important factor, which determines the quality of tobacco from the point of view marketability of tobacco products. In small doses nicotine has a stimulating effect, increasing activity, alertness and memory. Repeat users report only relief from the symptoms of nicotine withdrawal. It also increases the heart rate and blood pressure and reduces the appetite. In large doses nicotine may cause vomiting and nausea. That is, large doses are poisonous to most animals and to

humans. Thus, nicotine level of tobacco should be quantitatively determined before their distribution in the market to minimize its risk.

Table 1. Top ten tobacco producers – 2005

| <b>Countries</b>   | <b>Million metric ton</b> |
|--------------------|---------------------------|
| China              | 2.51                      |
| Brazil             | 0.88                      |
| India              | 0.60                      |
| United State       | 0.29                      |
| Indonesia          | 0.14                      |
| Turkey             | 0.14                      |
| Greece             | 0.12                      |
| Argentina          | 0.12                      |
| Italy              | 0.11                      |
| Pakistan           | 0.08                      |
| <b>World total</b> | <b>6.38</b>               |

Source: UN Food & Agriculture Organization (FAO) [2].

According to the report of United Nations office on drugs and crime, which was done on Rapid assessment of drug abuse in Ethiopia. Peoples commonly use khat, tobacco, and alcohol, which have a share of 48.2 %, 29.9 % and 18.9 % respectively, of all type of drugs [3]. This shows that tobacco has a great place as a drug in Ethiopia following khat.

In Ethiopia, there is one huge tobacco industry known as ‘The National Tobacco Enterprise’ producing three types of tobacco products or cigarettes at this time, namely Nyala, Gissella and Delight, which are produced from three types of commercial tobacco species. These are: *Virginia*, *oriental* and *burley*. Moreover, it also uses some imported Virginia tobacco leaves. The National Tobacco Enterprise is the only industry which has mandate to organize tobacco production and processing in the country.

## **1.1. Objective of the Study**

Determination of nicotine level in tobacco is crucial to control, maintain or reduce nicotine levels below the addictive threshold as well as its toxicity level. Therefore, the knowledge of nicotine level in tobacco is especially important to tobacco industry and in the area of toxicology to control its harmful effect on health. However, there is no report in the literature on nicotine level of Ethiopian tobacco. Therefore, this paper will provide attention to quantitative determination of nicotine level of some of Ethiopian tobacco leaves based on difference of species, cultivation areas and stalk (leaf) positions. This is done using high-performance liquid chromatography (HPLC) technique of determination.

### **Specific objectives:**

- (i) To collect samples of tobacco leaf from four different tobacco-growing regions (Shewa Robit, Billate, Awassa and Wollayita) of the country based on their leaf positions and varieties of species.
- (ii) To determine the levels of nicotine in each of the collected sample by extracting the nicotine from the powdered tobacco leaves with phosphate buffer at a given pH with constant agitation in water bath for 24 hr. and using high performance liquid chromatography (HPLC) for quantitative determination.
- (iii) To compare the levels of nicotine of Ethiopian tobacco based on differences of species, farming areas and stalk (leaf) positions.
- (iv) To compare the levels of nicotine in Ethiopian tobacco with the literature values reported in the other part of the worlds.

## **2. Literature Review**

### **2.1 Tobacco**

#### **2.1.1 Tobacco History**

It is difficult to narrate the actual history of tobacco, because different literatures tried to narrate in different approach based on their interest and purpose of study. However, most of the literatures agree on the following historical facts.

##### **2.1.1.1 History of Tobacco in the World**

It is believed that tobacco began growing in the Americas about 6,000 B.C. Before the discovery of America, native Americans grew and used tobacco in areas from southern Canada to southern Brazil. The rest of the world was ignorant of all species of *Nicotiana* until 1492 when Columbus encountered the new world. When Christopher Columbus landed at San Salvador he wanted to find gold. Instead, the native Americans gave him tobacco. He thought it was worthless and threw it away. He did notice, however, that many native Americans smoked or chewed tobacco. Others sniffed it up into their nose through a tube. And also Spanish explorers and conquerors observed besides its use another unpleasant use for tobacco during battles with Native Americans. The Native Americans squirted tobacco juice into the Spaniards' eyes, which temporarily blinded them [4]. Spanish and Portuguese sailors and explorers adopted the use of tobacco. They brought their new addictions back to their homes and to there port cities they visited. Tobacco use soon spread across Europe and into other countries. The major reason for tobacco's growing popularity in Europe and other countries was its supposed healing properties. People believed tobacco had magical powers and also believed that tobacco could cure almost anything, from bad breath to cancer. They also used it to treat headaches, toothaches, asthma, stomachaches, lockjaw, heart diseases, and poisons. Even London pharmacies listed tobacco as a treatment for coughs. Moreover, a Spanish doctor named Nicolas Monardes wrote a book about the history of medicinal plants of the new world in 1571. In this he claimed that tobacco could cure 36 health problems [5].

During the 1600's, tobacco was so popular that it was frequently used as money, and literally “as good as gold!”. European traders dispersed tobacco throughout Asia and Africa in the seventeenth century [6]. Then over the years; more and more scientists begin to understand the chemicals in tobacco, as well as the dangerous health effects smoking produces. Even though that was the truth, in recent years there is growing evidence of tobacco user throughout the world.

### **2.1.1.2 History of Tobacco in Ethiopia**

In Ethiopia it was not possible to get literatures that narrate when began to use tobacco. Evidence that indicate the presence of tobacco in Ethiopia for long years ago is that there are several peoples in different part of the country that uses native tobacco in different forms, such as snuffing, chewing and pipe smoking (Gaya). However, around the beginning of 20<sup>th</sup> century tobacco factory was emerged for the first time in Dire Dawa, then in 1931 (1923 E.C.) the factory was transferred to Addis Ababa which was owned by an Armenian investor. But Italian colonists gave up production of the factory; instead they established another tobacco product factory for their soldiers' consumption. After Italian colonialists left the country, around 1943 (1935 E.C.), establishment of tobacco and matches monopole was declared by Ethiopian government [7-8]. Therefore, before 50 years, tobacco is grown for commercial purposes by state-owned farms and by farmers around these farms. The National Tobacco and Matches Corporation, which is renamed as National Tobacco Enterprise', has been given the mandate to organize tobacco production and processing in the country. It has been producing tobacco around Shewa Robit, Bilate, Wolaita and Awassa to supply its leaf processing plants and furnish the cigarette making factory in Addis Ababa [9].

### **2.1.2 Tobacco Species and Its Botany**

Tobacco belongs to a genus of short-leaved plants of the Solanaceae (nightshade) family of plants, which also includes many crop species such as tomatoes, potatoes and peppers. There

are several species of tobacco approximately more than sixty species of tobacco within the genus *Nicotiana*; among those, only *Nicotiana rustica* and *Nicotiana tabacum* are in widespread human use. *Nicotiana tabacum* is the principal species producing commercial tobacco. Some commercial tobacco is also obtained from a sister species, *Nicotiana rustica*, which is a smaller plant with fewer leaves than *N. tabacum*. However, *Nicotiana tabacum* is the most widely grown, providing virtually all the tobacco leaf used in commercial production of most countries tobacco products. It is primarily used for cigarette, cigars, chewing tobacco and snuff. Another species commonly grown in different countries, *Nicotiana rustica*, has generated interest because of its high nicotine content. It is useful in the making of insecticides and for other specialized uses [10].

Tobacco is normally grown as an annual crop and is potentially a woody, shrub like perennial. Common tobacco is 1 to 3 meters tall and has a thick, woody stem with few side branches; it has a very shallow root system. One plant typically produces 10 to 20 broad harvestable leaves that branch alternately from the central stalk. The leaf size depends on the strain. The *N. tabacum* types are one of the smaller species of tobacco varieties. The leaves supply the most important economic value of the plant and are given the most attention by botanists. Although there is variation in leaf size and shape there is general uniformity of distribution, size and shape within cultivated types. The characters which may include leaf shape, leaf angle to the stem, form of the leaf tip, attachment of leaf to the stem, structure of the attachment and leaf asymmetry. In *N. tabacum*, ovate shapes are the most common and the leaves are most usually borne directly on the main stem. The leaf surface has a matte appearance. Its narrow, trumpet-shaped flowers are dark pink to almost white. The calyx is not of notable significance but the corolla extends much beyond it and is fairly characteristic. The tabacum colour is usually pink. The five stamens are attached to the corolla tube and bear oval-shaped anthers on the ends of long filaments. The stigma is likewise borne on the end of a long style that rises to just above the mouth of the corolla. The flower structure is very simple, being biased towards self-fertilization but it is also easy to manipulate for cross breeding purposes [11].

### 2.1.3 Tobacco Types

There are different types of tobacco, the typical and the most common types even in our country, Ethiopia, are Virginia, burley and Oriental. These tobacco types are the major components of the increasingly popular blended cigarette now consumed worldwide, and represented 57 %, 11 % and 12 %, respectively, of world production in 1995. Thus, these tobaccos are widely traded internationally [12]. According to Country Information Brief, FAO report of June 1995, in Ethiopia, Virginia accounts for a little more than 74 % of the total production, followed by oriental 22 %, and barely 4 % [13].

**Virginia** also named as “Bright tobacco” because of its luster and color. It is the most extensive worldwide variety and corresponds to consumer evolution towards so-called American-blend smoking products. When bright tobacco leaf ready for harvest, it turns yellow-green, the sugar content is at its peak, and it will cure (flue-cured or dark fire-cured) to a deep golden color with mild taste.



Fig. 1. Virginia Tobacco Type

**Burley** also known as light air-cured, the white burley is seldom used today. Since red burley, a dark air-cured variety of the mid-1800s, no longer exists. Burley is cured without supplementary heat. It is the most widely grown tobacco across the United States and is the second-largest tobacco crop (behind flue-cured). Burley's flavor and aroma make it a popular tobacco for cigarette blends.



Fig. 2. Burley Tobacco Type

**Oriental** is a sun-cured, highly aromatic, small-leafed variety that is grown in Turkey, Greece, Bulgaria, and Macedonia. Oriental tobacco is frequently referred to as "Turkish tobacco", as these regions were all historically part of the Ottoman Empire. Many of the early brands of cigarettes were made mostly or entirely of Oriental tobacco; today, its main use is in blends of pipe and especially cigarette tobacco [2, 14].



Fig. 3. Oriental Tobacco Type

#### **2.1.4 Tobacco Production and Processing**

The annual tobacco cultivation cycle begins with the planting of seeds. The seeds of the tobacco plant are extremely small but with large number per plant. They are more or less ovate in shape, about 0.75 mm long, 0.53 mm broad and 0.47 mm thick. They need special care to keep them for a long time; they are long-lived, stored in dry conditions at ordinary temperature, resistant to rather high humidity, except at high temperature. The average 1000 seed weight is in the range of 0.08 - 0.09 gm [15]. Growers sow the seeds in specially prepared seedbeds of fertile, loose soil, rather than directly in the field. Seedlings are raised in carefully selected and tended seedbeds where protection is given against heavy rain and excess sun [16].

### **2.1.4.1 Transplanting**

After the plants have reached a certain height, they are transplanted into fields. This was originally done by making a relatively large hole in the tilled earth with a tobacco peg, then placing the small plant in the hole. Various mechanical tobacco planters were invented throughout the late 19th and early 20th century to automate this process, making a hole, fertilizing it, and guiding a plant into the hole with one motion.

### **2.1.4.2 Topping and Suckering**

When the tobacco crop is about half-grown, flower buds begin to appear. These flower heads are removed or “topped” to prevent seed formation, forcing the plant to focus on leaf production. The result is larger, thicker, darker leaves that mature more uniformly and contain more nicotine. Topping may be done by hand or with special machines that cut the flower heads and sacrifice a few leaves. Topping requires two or three trips over the field to catch all the plants.

Topping of plants also stimulates the growth of secondary stems from the base and/or leaf axils. These “suckers” must also be removed to assure uniformity and quality. While chemicals are available to suppress suckering, these may not be allowable under organic certification standards. The alternative is removal by hand every seven to ten days. Suckering is one of the most labor-intensive activities in tobacco production, as many plants sucker two or three times before harvest [17].

### **2.1.4.3 Harvest**

Tobacco is harvested in one of two ways, stalk-cutting and priming. In the oldest method, by stalk-cutting, the entire plant is harvested at once by cutting off the stalk at the ground with

knife. In the nineteenth century, tobacco began to be harvested by priming that is pulling individual leaves off the stalk as they ripened. The leaves ripen from the ground upwards, so a field of tobacco may go through several "pullings" before the tobacco is entirely harvested. "Cropping" or "pulling" are terms for pulling leaves off tobacco. Leaves are cropped as they ripen, from the bottom of the stalk up [17, 33].

#### **2.1.4.4 Curing**

In tobacco technology "Curing" usually refers to the changes undergone by harvested fresh leaves under regulated conditions of temperature and humidity [35]. Cut plants or pulled leaves are immediately transferred to tobacco barns (kiln houses), where they will be cured. The curing environment can be manipulated by controlling heat, humidity and air movement. It is the process that brings about the rapid destruction of chlorophyll, giving leaves their yellow appearance, converting starch into sugar and removing moisture. Curing and subsequent aging allows for the slow oxidation and degradation of different components of variety of tobacco leaf, as a result it can produce certain compounds in the tobacco leaves that could give a sweet tea, rose oil, or fruity aromatic flavor that contribute to the "smoothness" of the smoke. Before tobacco is cured, the leaves contain 80-85 % water. After the curing cycle, there is essentially no water left. Curing involves three essential steps: yellowing, leaf drying and stem drying.

The Yellowing Stage is a continuation of the ripening process and is thought to be the most important part of the curing process. The leaf is still alive during the yellowing phase, which allows it to carry on certain chemical, catalytic, enzymatic, bacteriological activities that are needed to convert starch to simple sugar, break down chlorophyll and producing aromatic products. The Science behind Yellowing is that the stoma allows a continuous exchange of carbon dioxide, oxygen and water through the leaf. As this exchange occurs, yellow pigments become visible as chlorophyll breaks down. Then, the leaf drying stage is where the leaf tissue is dried to a particular moisture level. Finally, the stem drying Stage is referred to as the 'killing out stage' because all moisture is removed from the stem and leaf killing them.

Curing methods varies with the type of tobacco grown, and tobacco barn design varies accordingly. The following curing methods of tobacco are commonly used.

### **Flue Curing**

Flue-cured tobacco was originally strung onto tobacco sticks, which were hung from tier-poles in curing barns. These barns have flues which run from externally-fed fire boxes or furnaces, heat-curing the tobacco without exposing it to smoke. Used mainly in the manufacture of cigarettes, flue-cured tobacco is lemon, orange, or mahogany in color, with high sugar content and a medium-to-high nicotine content. Flue curing requires a closed building equipped with a system of ventilation and a source of heat. When heat and humidity are controlled, a leaf color change, moisture is quickly removed, and the leaves with stems dry.

### **Air Curing**

Air-cured tobacco is hung in well-ventilated barns and allowed to dry over a period of weeks. This group includes the original air-cured tobaccos of South and Central America, the cigar tobaccos (subdivided into wrappers, binders, and fillers, depending on their use), and the burley tobaccos. These have low sugar content but vary in nicotine content. Air curing requires an open framework in which sticks of leaves (or whole plants) are hung, protected from wind and sun. Leaf color changes from green to yellow, as leaves and stems dry slowly.

### **Fire Curing**

Fire-cured tobacco is hung in large barns where smoldering fires of hardwoods are kept burning. Fire-cured tobacco, generally dark brown, is used mostly for pipe tobacco mixtures, snuff, and chewing tobacco and has a low sugar but high nicotine content. Fire curing employs an enclosed barn similar to that used for flue curing. Small fires are built on the floor, and the leaves cure in a smoke-laden atmosphere. Whereas flue curing takes 6 to 8 days, fire curing, using far lower temperatures, may take up to 4 weeks.

## **Sun Curing**

Sun curing is the drying of uncovered sticks or strings of leaf in the Sun. Of all Sun-cured tobaccos, the best known are the so-called oriental tobaccos of Turkey, Greece, Yugoslavia, and nearby countries. These are used in cigarettes and have characteristic aromas. They are low in both sugar and nicotine [18].

The purpose of curing is to produce dried leaf of suitable physical properties and chemical composition. Among the compounds in the nitrogen containing group there was a major shift distribution, a transfer of insoluble to soluble materials. However, only small amounts were lost as volatile nitrogen compounds. There is almost a loss of more than 1 % of total nitrogen and a reduction of about 0.1 % in protein nitrogen and nicotine. For instance, for a particular study the composition of nicotine 1.10 % of green leaf reduced to 0.97 % after flue-curing process [33].

After tobacco is cured, it is moved from the curing barn into a storage area for processing. If whole plants were cut, the leaves are removed from the tobacco stalks in a process called stripping. For both cut and pulled tobacco, the leaves are then sorted into different grades. Finally, the leaf bundled based on their variety and grade and then transported to tobacco product manufacturing industries or ready for market.

### **2.1.5 Tobacco Leaves Position (Stalk Position)**

There are a number of different styles or leaf positions on a tobacco plant as shown on figure 4. These leaf positions include priming, lugs, cutters, leaf and tips. These leaf positions are recognized by the shape, size and the leaf position on the stem as well as chemical attributes.

**Tips** are the short, narrow leaves from the top of the plant. These leaves often have higher nicotine content than other leaf positions.

**Leaf** occurs near the top of the plant which is long and wide with a large stem. Leaf is the thickest of all leaf positions and has moderate nicotine content.

**Cutters** are the leaves above lugs and are normally wider long than others. But they are thin with a narrow stem.

**Lugs** are the lowest leaves on the plant and are short wide leaves, which are typically thin and papery with low nicotine content.

**Primings (flyings)** consist of the oldest, most mature leaves grown at the bottom of the stalk. These leaves are very thin and a brownish yellow or tan in color. Flyings generally show a certain degree of injury from age and/or disease. Thus a few leaves on the stalk that go into this group [19].

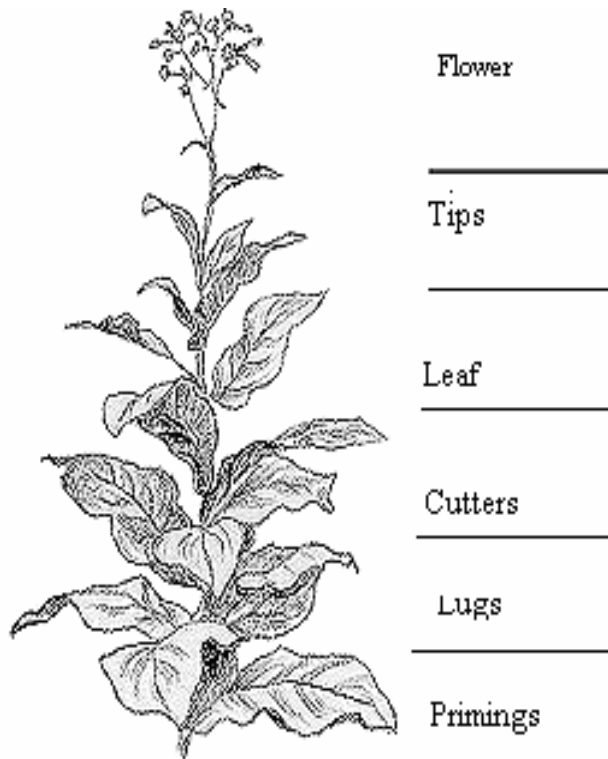


Fig. 4. Tobacco plant: names for leaf positions

The difference in chemical composition among leaves from various stalk positions is well known. For instance, the nicotine content increased from bottom to top of the plant that is low in lower leaves and high in the tips, but total sugar content is the reverse. Even in the study of single leaves total alkaloid was lowest at the base portion of the leaf and gradually increased (in the arrow shape) towards the tip of the leaf [20-21]. In particular study the nicotine content of each stalk (leaf) positions Primings, Lugs, Cutters, Leaf and Tips for flue cured tobacco was reported as 2.12 %, 2.49 %, 2.59 %, 3.17 % and 3.27 % respectively [22]. Therefore, separation of tobacco into distinct stalk positions during harvest is extremely important to producing a quality, high-demand product regardless of the buying company. Cigarette manufacturers and leaf dealers have different preferences for the styles of tobacco they desire to meet customer needs. For example, some cigarette manufacturers would like to see greater production of tip grades for use in blending while others would like to buy less lower stalk tobacco [23].

## **2.2 Nicotine**

The tobacco leaf is composed of 85-90 % water, mineral matter and organic compounds. The organic compounds may be considered as organic acids, carbohydrates and alkaloids. From which alkaloids make the tobacco to be used by human beings. Alkaloids are a group of nitrogen-containing bases. Most of them are drugs. Only a few (like caffeine) are derived from purines or pyrimidines, while the large majority is produced from amino acids [24]. Among the alkaloids, nicotine is recognized as an essential component in tobacco cultivation. It is found in the nightshade family of plants (Solanaceae), predominantly in tobacco, and in lower quantities in tomato, potato, eggplant (aberigine), and green pepper. It is also found in the leaves of the coca plant. It is the principal tobacco alkaloid comprising about 95% of the total alkaloid content [25-26]. Nicotine content is an important factor, which determines the quality of tobacco from the point of view of the marketability of tobacco products, since nicotine is addictive in nature.

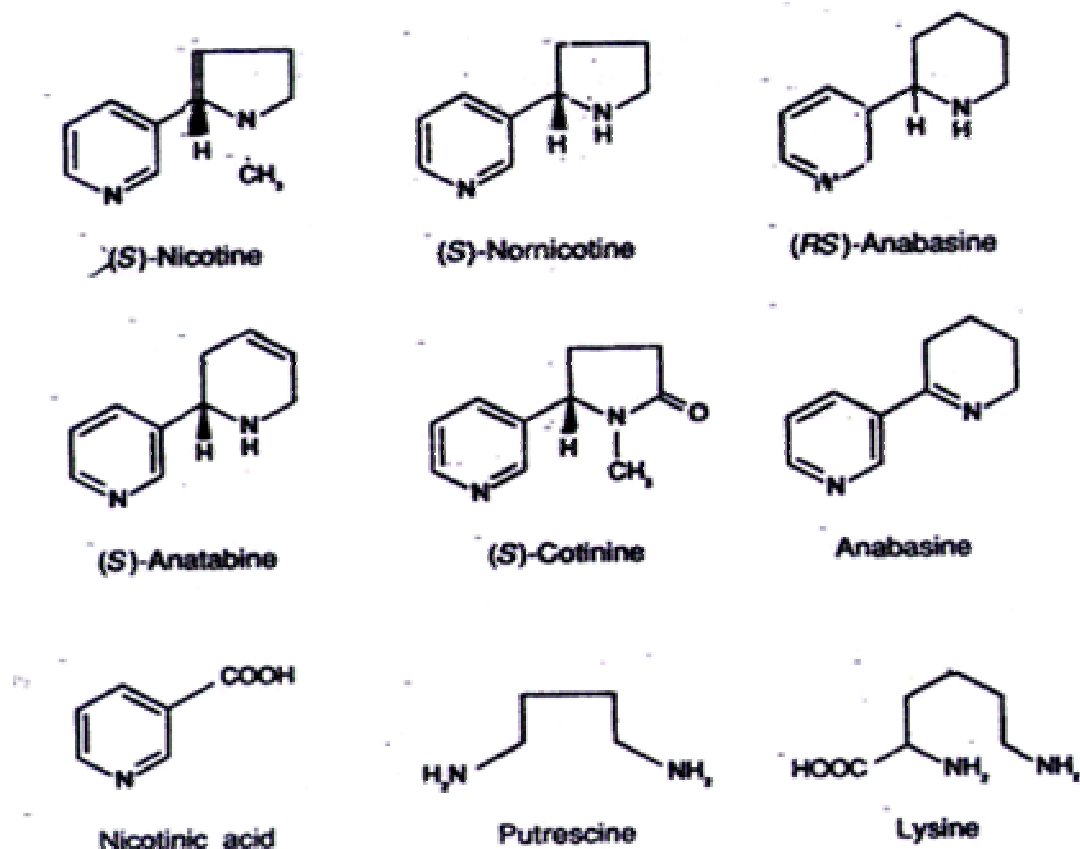


Fig. 5. Structures of nicotine and some other alkaloids found in tobacco [33].

## 2.2.1 History and Name

Nicotine is named after the tobacco plant *Nicotiana tabacum*, which in turn is named after Jean Nicot, a French ambassador, who sent tobacco and seeds from Portugal to Paris in 1550 and promoted their medicinal use. Nicotine was first isolated from the tobacco plant in 1828 by German chemists, Posselt and Reimann. Its chemical empirical formula was described by Melsens in 1843, and it was first synthesized by Pictet and Crepieux in 1893 [27].

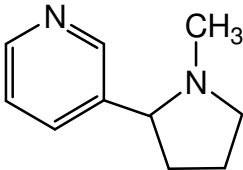
## 2.2.2 Physical and Chemical Properties of Nicotine

Nicotine is also called 3-(1-methyl-2-pyrrolidinyl) pyridine according to the IUPAC nomenclature. It is a bicyclic compound with a pyridine cycle and a pyrrolidine cycle. The

molecule possesses an asymmetric carbon and so exists in two enantiomeric compounds. In nature, nicotine only exists in the S shape, which is laevogyrate. Some physical properties of Nicotine are listed on Table 2.

Nicotine is a liquid alkaloid, pale yellow to dark brown oily liquid with a slight fishy when warm or unpleasant pungent odor with a sharp burning persistent bitter taste. As a nitrogenous base, it is strongly alkaline in reaction and forms salts with acids and double salts with many metals and acids that are usually solid and water-soluble. Nicotine is hygroscopic, miscible with water below 60 °C; on mixing nicotine with water the volume contracts. It is very soluble in alcohol, chloroform, ether, kerosene and the like. Nicotine has  $pK_1 = 6.16$ ;  $pK_2 = 10.96$  at 15 °C, and its 0.05 M solution has a pH of 10.2. It can be stored at room temperature that is below 30 °C by protecting from light and air [28-29].

Table 2. Physical properties of nicotine

|                    |   |
|--------------------|---|
| Chemical structure |  |
| Formula            | $C_{10}H_{14}N_2$   |
| Molecular Weight   | $162.26 \text{ g.mol}^{-1}$   |
| Melting point      | $-79 \text{ }^\circ\text{C}$  |
| Boiling point      | $247 \text{ }^\circ\text{C}$  |
| Rotatory index (S) | $\alpha_D = -169.3 \text{ at } 20^\circ\text{C}$                                    |
| Density            | $d = 1.00925$   |
| Refractive index   | $n = 1.530$   |
| Vapor pressure     | $0.006 \text{ kPa at } 25 \text{ }^\circ\text{C}$                                   |
| Viscosity          | $2.7 \text{ mPa}\cdot\text{s at } 25 \text{ }^\circ\text{C}$                        |
|                    | $1.6 \text{ mPa}\cdot\text{s at } 50 \text{ }^\circ\text{C}$                        |
| Surface Tension    | $37.5 \text{ dynes/cm at } 25.5 \text{ }^\circ\text{C}$                             |
|                    | $37.0 \text{ dynes/cm at } 36.0 \text{ }^\circ\text{C}$                             |

### 2.2.3 Nicotine Content of Tobacco Leaves

Nicotine is present in the unburnt tobacco and is volatilized in the combustion process. Nicotine may be measured in the tobacco itself, although this is much less common than measurement of yields. When measured in the tobacco, nicotine is reported as *concentration*, or *content*. The concentration is usually expressed as percentage by weight, and it is important to know whether it is by dry weight or wet weight [30].

The nicotine content did not differ significantly among the different localities. The nicotine content is higher in the lamina than the midrib and increase from bottom to top of the plant. The mean nicotine content of the leaf averaged over growing regions was  $(2.1 \pm 0.22)$  % [33]. The nicotine content of *Nicotiana tabacum* varieties grown commercially generally ranges from 0.3 to 3 %, though 5 % and even 7 % have been recorded in some heavy bodied tobaccos. Yields above this level are found in some *Nicotina rustica* varieties grown especially for commercial nicotine extraction. From the dried leaves of and *Nicotina rustica* nicotine occurs to the extent of 2 to 8 % composed to citric acid and malic acid [30]. Commercial nicotine is entirely produced from tobacco varieties which have high nicotine content and which have been specifically grown for this purpose (nearly always *Nicotina rustica*). It is also extracted from the waste product or byproduct of tobacco industry [16].

### 2.2.4 Factors Affecting Nicotine Content in Tobacco

Nicotine level is a key index for evaluating the quality of tobacco, and is closely correlated with many factors [32]. Some of the factors affecting nicotine level of tobacco are the following.

**Tobacco species or type** is the most determinant factor that affects nicotine level of tobacco. There are different tobacco species in the world which have different nicotine content. For instance, among the type of tobacco Maryland and Turkish (oriental) are low in nicotine; the

flue-cured (Virginia), Burley, and Connecticut Cigar wrapper are medium; and the Pennsylvania and especially *N. rustica* are high in nicotine level [33].

**Soil type** is another factor that affects nicotine content. The quality of tobacco is highly correlated with the nicotine content and good quality tobacco requires fertile, well-drained, moist soil. Soil can affect leaf size, texture, and color. Sandy soils tend to produce a relatively large leaf that is light in color and body, fine in texture, and burns with a weak aroma. Heavier soils, which contain silt and clay, tend to produce a small, dark leaf with a heavy body and a strong aroma when burned [18]. Moreover, the amount of root development as conditioned by depth of soil influences the total nicotine content of tobacco plant. Deep soil favor development of extensive root system, since nicotine elaboration primarily localized near the root tip then stored in the leaf and plants having the largest root system accordingly elaborate the largest total content of nicotine [34]. On contrary damage of the root by any means reduce the nicotine content of the tobacco plant [22].

**Nitrogen nutrition** is also related with soil type since almost all nitrogen consumed by tobacco plant from the soil as ammonium and nitrate compounds. Since N is 17.3 % of the molecular weight of nicotine, the amount of nitrogen (N) supplied has direct relation with nicotine content of the tobacco plant. The nitrogen content of the soil also depends on the type of soil, and also by the introduction of nitrogenous fertilizer [35].

**Topping height and time** is also affect nicotine content of the tobacco plant, which also related with nitrogen supply of tobacco plant. The reason is nitrogen absorbed after topping effectively incorporated in to nicotine compared with nitrogen absorbed before topping. The more N supply after removing the apex, results the higher the nicotine concentration in the leaves [33 & 36]. Similarly, as the height of the position of topping increase, meaning large apex removed the thicker, darker and larger leaf produced as a result nicotine content of the tobacco plant become high [37].

**Degree of maturity or ripening of tobacco** has also great effect on nicotine content of tobacco plant. As the tobacco plant maturity increase, it turns to yellow quicker, had a higher yield and quality index, with higher alkaloid (nicotine) and sugar content and less total nitrogen [38].

**Curing and yellowing period of tobacco** have also slight effect on the level of nicotine of the tobacco leaf, that is extended yellowing period will increase level of nicotine of the tobacco leaf [22]. But during aging and curing process of tobacco there is a marked decrease of nicotine with a concomitant increase of nicotine transformation products such as nicotinic acid, nicotine amide, oxynicotine, cotinine and the like [33].

### **2.2.5 Pharmacology**

**Pharmacokinetics:** As nicotine enters the body, it quickly gets distributed through the bloodstream and can cross the blood-brain barrier. On average it takes about seven seconds for the substance to reach the brain. The amount of nicotine inhaled with tobacco smoke is a fraction of the amount contained in the tobacco leaves (most of the substance is destroyed by the heat). The amount of nicotine absorbed by the body from smoking depends on many factors, including the type of tobacco, whether the smoke is inhaled, and whether a filter is used. For chewing tobacco, often called dip, snuff, or snus, which is held in the mouth between the lip and gum, the amount released into the body tends to be much greater than smoked tobacco.

**Pharmacodynamics:** Nicotine acts on the nicotinic acetylcholine receptors. In small concentrations it increases the activity of these receptors, among other things leading to an increased flow of adrenaline, a stimulating hormone. The release of adrenaline causes an increase in heart rate, blood pressure and respiration, as well as higher glucose levels in the blood. Cotinine is a break-down product of nicotine which remains in the blood for up to 48 hours and can be used as an indicator of a person's exposure to smoke. In high doses, nicotine will cause a depolarizing block of the nicotinic acetylcholine receptor, which is the reason for its toxicity and its effectiveness as an insecticide [39].

### **2.2.6 Toxicology**

Nicotine is a very toxic substance, and serious or fatal poisoning may occur as the result of the ingestion, inhalation or skin absorption of only small amounts. The lethal dosage of nicotine is 50 mg/kg for rats and 3 mg/kg for mice. 40–60 mg can be a lethal dosage for adult human beings. This makes it an extremely deadly poison. It is more toxic than many other alkaloids such as cocaine, which has a lethal dose of 1000 mg [40-41].

### **2.2.7 Use of Nicotine**

Nicotine is most frequently encountered in tobacco products for smoking, chewing, sniffing and tobacco. And also as an adjunct to smoking cessation programmes (gums, patches) [29]. Moreover, nicotine has found wide use as pesticide and insecticide (now rare). Dilute solutions are used on fruit and vegetables to control insects such as aphides plant lice, whiteflies, leaf hoppers, etc. It may also used to control certain animal skin parasites. Because the residual effect of nicotine last, at most, for only a few hours after application. For spray application, nicotine must be incorporated in a solution of soap or with emulsifiable oil or must be mixed with hydrated lime or another powdered carrier to use as a dust [41].

### **2.3 Healthy effect of Tobacco/ Nicotine**

Tobacco is the leading cause of drug use related mortality world over. Besides nicotine, tobacco consists of nearly 4000 substances including particles and gases, 1000 of them are produced during smoking. These include the known carcinogens and carbon monoxide which impair the oxygen carrying capacity of blood [42]. Cigarette smoking is associated with coronary heart disease, stroke, ulcers, and an increased incidence of respiratory infections. Smoking is the major cause of lung cancer and is also associated with cancers

of the larynx, esophagus, bladder, kidney, pancreas, stomach, and uterine cervix [43]. Smoking is also the major cause of chronic bronchitis and emphysema. Today, with universal awareness of the health risks, a billion adults worldwide make the choice to smoke.

Nicotine is the major addictive substance in cigarette tobacco. Nicotine is given off by burning tobacco and carried into the respiratory tract on tar droplets and in the vapor phase. As a weak base, nicotine may exist in an ionized or a non-ionized form. The relative proportions of these two forms, determined by the pH of the smoke, affect where nicotine is most readily absorbed into the body. At the acidic pH of most cigarette smoke, absorption occurs predominantly in the lungs, but with the alkaline smoke produced by cigars and pipe tobacco, nicotine being predominantly non-ionized, is absorbed mainly in the mouth. Absorption into the blood stream is rapid, and concentrations of nicotine in the blood rise rapidly during smoking. Nicotine is metabolized, mainly in the liver, and the two major metabolites are cotinine and oxynicotine.

Nicotine is a powerful psychoactive agent that has a variety of central and peripheral nervous system effects, as well as effects on the cardiovascular, endocrine, gastrointestinal, and skeletal motor systems. Self administration of tobacco leads to physical nicotine dependence and psychological dependence on smoking behavior, with withdrawal symptoms associated with smoking cessation. The action of nicotine on the brain occurs rapidly after smoking, and this is believed to provide optimal reinforcement for nicotine dependence.

The direct contribution of nicotine to tobacco-associated diseases is unclear, as it is inhaled along with many other substances in tobacco smoke. The role of nicotine is to maintain the addiction and other substances in tobacco smoke, particularly tar and some of the gaseous components such as carbon monoxide, are the main direct causes of disease [29].

### **3. Experimental**

This paper mainly concerns to determine only nicotine content of tobacco leaves using HPLC. Therefore, most of the apparatus, equipments, chemicals and procedures were similar with that of Sandurs and Blume [44] work to determine major tobacco alkaloids. Nevertheless, different modifications were made based on the objective, conditions and availability of equipments for this study.

#### **3.1 Apparatus and Equipments**

The high performance liquid chromatography system (HPLC 600 PerkinElmer) with Totalchrom software consists of Series-200 Pump, Autosampler (automatic injector), UV/Vis detector, column oven and vacuum degasser. The column used was Beckman Coulter C-18 reversed phase column (4.6 mm x 25 cm) made in USA. The spectrophotometer (CE4400/UV/Vis double beam scanning), pH-meter (inoLab pH level 2, D-82362 Weilhelm, Germany), Drying Oven (Heraeus Instruments Vacutherm VT 6025), Weighing Balance (ACCULAB sartorius group ALC-2104), Water bath with agitator (GFL, D-30938 Bergwedel, Germany), and Sonicator (BANDELIN SONOREX RK514) were used.

#### **3.2 Chemicals**

The standard nicotine 98 % (Riedel-de Haen AG Seelze-Hannover-62867, Germany) was used as received. Distilled water (distilled in EPHARM), Methanol HPLC grade (Fisher Scientific UK Limited), phosphoric acid (Riedel-de Haen, Germany) and triethylamine (Riedel-de Haen, Germany) were used for preparation of isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine. A 25 mM phosphate buffer (pH 7.8) was the extraction solvent which was prepared from potassium dihydrogen orthophosphate,  $\text{KH}_2\text{PO}_4$ , (Fisher Scientific UK Limited) and disodium hydrogen orthophosphate,  $\text{Na}_2\text{HPO}_4$ , (BDH Chemicals Ltd. England) according to the procedure stated in British Pharmacopoeia (BP) [45].

All the apparatus, equipment and chemicals (except nicotine standard) were used from the chemical laboratory of Ethiopian Pharmaceuticals Manufacturing Sh. Co. (EPHARM).

### **3.3 Samples and Sample Collection**

Samples were collected based on their leaves (stalk) positions, species and place of cultivation.

#### **3.3.1 Sample Site Robi**

Robi or commonly known as Shewa Robit is in northern part of Ethiopia specifically in northern Shewa zone of Amahara region, which is about 230 km far from Addis Ababa. Most of the tobacco farms around this town are owned by the farmers whom produce Virginia type tobacco leaf by the supervision and technical assistance of the tobacco enterprise professionals, and finally farmers sold their entire product to the branch office of the main factory of the National Tobacco Enterprise found there.

Virginia type tobacco was collected from Robi at different leaf positions. The leaf positions were identified and collected separately by the help of agricultural professionals of the state farm; these were *lugs*, *cutters*, *leaf* and *tips* (from bottom to top). As different literatures suggest, these positions have different nicotine content that is why the samples were collected separately based on the leaf position. Thus 4- samples, obtained from different plants of four separate leaf positions of tobacco, were collected in these sample sites by omitting the bottom leaf position (priminges) which was not available at the time of collection of samples. Although the time of harvesting and particular farm site of the sample was different, cured samples of the tobacco leaf at the respective leaf position of the Robi site were also collected from the National Tobacco Enterprise for comparison of cured and sun-dry tobacco leaves. That is another four samples of cured leaf were obtained. Totally 8- samples were analyzed from this site.

#### **3.3.2 Sample Site Billate**

Billate is situated in southern part of the country in the region of southern nation nationalities, Wollayita zone and about 330 km, 80 km, and 50 km away from Addis

Ababa, Awassa and Wollayita Sodo, respectively. The area receive rain fall between 750-800 mm annually, its highest temperature arrive about 32 °C in hot season. In this site the samples were collected from the state farms of the factory. Like that of Robi, similar species Virginia type of tobacco obtained, methods and number of collected samples were the same except cured leaves. Thus only four samples were collected in these sample sites.

### **3.3.3 Sample Site Awassa**

Awassa is also situated in southern part of Ethiopia about 250 km away from Addis Ababa, specifically Kikea and Galley (7 km to 10 km from Awassa) were sample sites of Burley and Oriental tobacco leaf, respectively. Burley and Oriental type of tobacco species were collected from this site, so two samples were collected here.

For these two species though each samples were collected based on their different leaf positions, all leaves positions of each species were dried and analyzed together, without separation of their leaf positions for simplicity of sample management. Since unlike that of Virginia type drying or curing style has no exaggerated effect on Burley and no effect on Oriental type of tobacco, no need of comparing with cured tobacco. Therefore, two samples were analyzed from this site.

### **3.3.4 Sample Site Wollayita**

Wollayita situated in southern part of Ethiopia about 360 km away from Addis Ababa. Here there are many peoples that use native tobacco in different style; the most commonly used is pipe smoking (Gaya). The leaves used for this purpose were collected as a sample for analysis of its nicotine content.

Totally fifteen (15) samples were collected from all selected sample sites.

### **3.4 Preparation of solutions**

Different solutions were prepared in this study; these were for extraction of nicotine phosphate buffer, buffered mobile phase and nicotine standard solutions.

#### **3.4.1 Phosphate Buffer**

A 25 mM phosphate buffer (pH 7.8) was used as the extraction solvent of nicotine from the leaves of tobacco. The extracting solvent was prepared by dissolving 3.55 g of disodium hydrogen orthophosphate,  $\text{Na}_2\text{HPO}_4$ , and 3.4 g of potassium dihydrogen orthophosphate,  $\text{KH}_2\text{PO}_4$ , in a liter of water according to the procedure stated in British Pharmacopoeia (BP) [45]. Finally, its pH was adjusted to 7.8 using a little amount of a normal of sodium hydroxide solution.

#### **3.4.2 Buffered Mobile phase**

An isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine was used. This mobile phase was prepared once almost a volume of 3 liter for entire analysis. To prepare 3 liter of mobile phase 1.8 liter of distill water, 6 mL of phosphoric acid and 1.2 liter of HPLC grade methanol was mixed together in large beaker and stirred thoroughly to good homogeneity of the solution, then using the pH meter with constant stirring of the solution the pH was adjusted to pH 7.25 by pipetting trimethylamine.

#### **3.4.3 Preparation of Nicotine Solution**

The standard nicotine (98 %) was taken and then different concentrations of nicotine standard solutions were prepared for constructing calibration curve. To prepare each nicotine standard solution (0.1 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM), first 10 mM stalk solution was prepared by diluting 1.639 mL of 6.1 M (98 %) standard nicotine solution to a liter of volume using water as a solvent in volumetric flask. And then by diluting 1 mL, 5 mL, 10 mL, 15 mL, 20 mL, 25 mL and 30 mL of 10 mM stalk solution in to a separate 100

mL volumetric flask up to volume with water, the above listed concentrations of standard nicotine, respectively, prepared.

### **3.5 Procedure for Nicotine Determination**

Dried leaves of tobacco samples were grounded in a mortar with a pestle then sieved with about 2 mm mesh, and the meal was oven dried at 60 °C for more than 24 h to a constant dry weight. The milled samples were weighed in to 0.5 g lots and extracted with 10 mL of 25 mM phosphate buffer (pH 7.8) in water bath adjusted at 30 °C for 24 h with constant agitation. The aqueous extract was filtered through filter paper (Ø150 mm, S & Sc. GmbH, Germany) and the filtrate stored in refrigerator (ALGOR) until injected to HPLC. When the HPLC system ready for analysis, the extracts were diluted to ten-fold with water and sonicated for good homogeneity and degassing of the sample (extract) solution. Finally, each extract was filtered through a 0.45 µm cellulose filter (Sartorius AG. 37070, Goettingen, Germany) and sealed in a screw-capped septum vial to permit automatic injection of a 10 µL aliquot.

All quantitative determinations were made with triplicate injections of the standard samples of seven different concentrations to prepare calibration curve. And also triplicate extractions were performed for each leaf sample, thus each three extracts of a given leaf sample were injected only once to have a triplicate result of each leaf sample. The content of nicotine of each sample then quantitatively determined based on the calibration curve plotted using standard samples.

### **3.6 Conditions used by HPLC**

The high performance liquid chromatography system (HPLC 600 PerkinElmer) with Totalchrom software consists of Series-200 Pump, Autosampler (automatic injector), UV/Vis detector, Column oven and vacuum degasser. The pump generates almost 2090 psi at a flow rate of 1 mL/minute throughout the analysis, when an isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine was used. The buffered mobile phase was prepared once for the whole analysis with great care by

adjusting the intended pH value. Since as the pH of the mobile phase not constant, it is difficult to get stable result especially on retention time. And also it has great effect on the life time of the C-18 reversed phase column because the column can not tolerate pH more than 7.5 according to the manufacturer company caution. The column used was Beckman Coulter C-18 reversed phase column (4.6 mm x 25 cm) made in USA. Furthermore the mobile phase was used after filtration through a 0.45  $\mu\text{m}$  cellulose filter and then five minute sonication. There was no critical consideration on adjusting the column temperature, simply room temperature was used as a column temperature for this work.

UV/Vis detector was adjusted to detect nicotine at its maximum wavelength absorbance of 259 nm (this is shown in section 4.3). Then the mobile phase allowed to flow through the column or the line of flow of the instrument for some minutes until stable absorbance of the mobile phase observed. After observation of stable absorbance of the mobile phase using the instrument software it was set to out-zero, and the automatic injector was obeyed by the software programme to inject automatically 10  $\mu\text{L}$  of sample from the septum vials. As a result the nicotine peak was appeared at about 7.3 sec. at a flow rate of 1 mL/minute.

## 4. Results and Discussion

### 4.1 Method of Analysis

There are numerous reports in the literature on the detection and quantitation of tobacco related alkaloids from various tissues by a variety of methods. Nicotine, for example, has been analyzed in both animal and plant tissues by paper, thin-layer (TLC) and gas chromatography [46-48]. In addition, atomic absorption, potentiometric determination, circular dichroism spectropolarimetry, and non-aqueous capillary electrophoresis with electrochemical detection (NACE-ED) have been applied to the determination of nicotine in tobacco [49-52]. To provide reliable quantitative values these methods often involve lengthy sample preparation that is expensive fractionation and extraction procedures including lengthy refluxing, dialysis, and multiple solvent extraction and irreversible derivatization of the alkaloids. Moreover; most of them are not applied to specifically for the determination of nicotine, since they determine nicotine content as total nicotine with other related alkaloids [44].

Recently, interest in nicotine determinations in the urine of smokers has prompted the analysis of nicotine by high performance liquid chromatography (HPLC) [53]. In addition, Jane [54] has described that the separation of nicotine from common drugs of abuse by isocratic HPLC on silica gel columns. A high performance liquid chromatographic technique was developed to quantitate the major alkaloids from both fresh green and air-dried tobacco leaf tissue [44]. HPLC method with diode-array detection for the determination of nicotine and its metabolites, cotinine and caffeine, in meconium is described [55]. According to literatures implication the current standard methods for the detection and quantification of nicotine in tobacco leaves involve either an HPLC method using UV detection or a GC method using a nitrogen specific detector [56-57]. Besides that HPLC is highly applicable and reliable method for both separation and quantitation of nicotine from the sample containing a mixture of many alkaloids extracted from plant tissue. Further it was possible to find and use HPLC easily as compared with other instruments and methods of analysis for the determination of nicotine in tobacco leaves. Therefore, for these reasons HPLC method of determination of nicotine is preferred for this research work.

## 4.2 Retention Time of Nicotine

Then the nicotine peak appeared in the chromatogram at about 7.3 seconds at a flow rate of 1 mL/minute as shown on Figure 6 and 7, and also at about 22 seconds when a flow rate adjusted to 0.5 mL/minute. Though separate sharp peaks of nicotine observed and the peaks of other metabolites of nicotine alkaloids (such as nornicotine, anabesine, anatabine etc.) resolved at 0.5 mL/minute the former flow rate of 1 mL/minute was used for subsequent work for saving time to analyze many samples, in addition the resolution of other metabolites of nicotine was not the concern of this study. Representative chromatographs of the standard samples and the extract of nicotine are shown on Figure 6 and 7, which are obtained under the stated conditions on section 3.6.

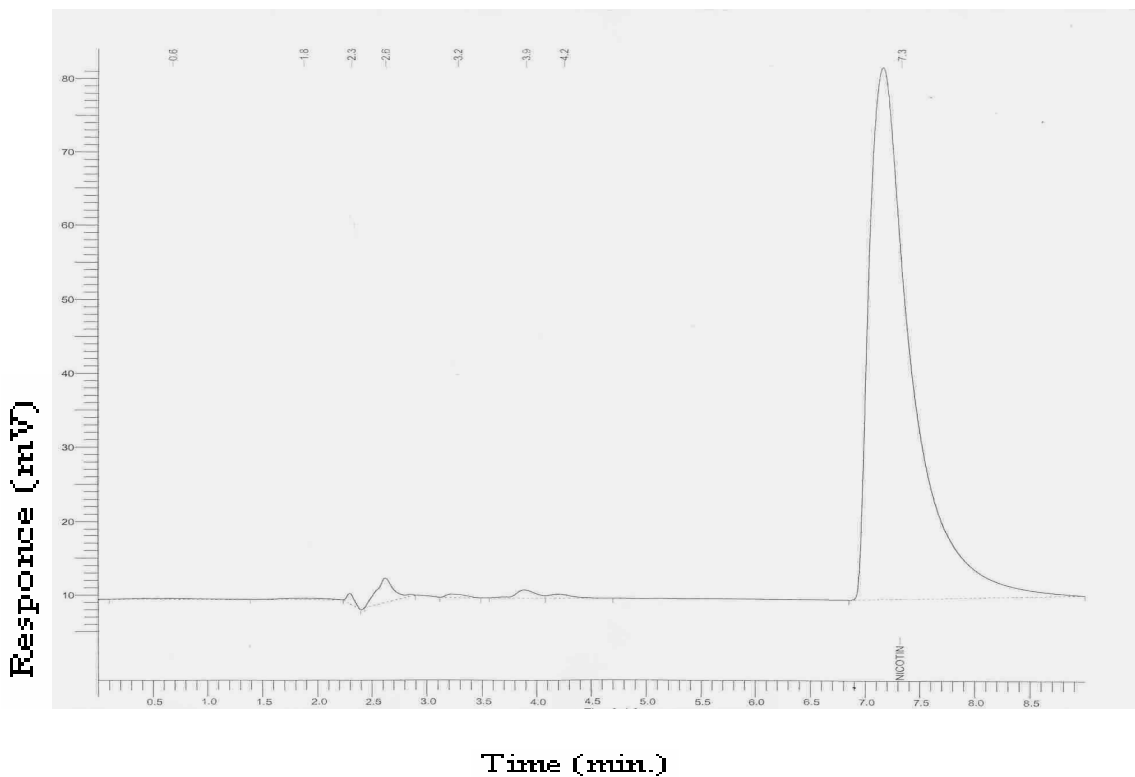


Fig. 6. Chromatogram of 2.0 mM 98 % of standard nicotine (at 7.3 s)

(Flow rate of 1 mL/minute, injection volume of 10  $\mu$ L, C-18 reversed phase column, isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25)

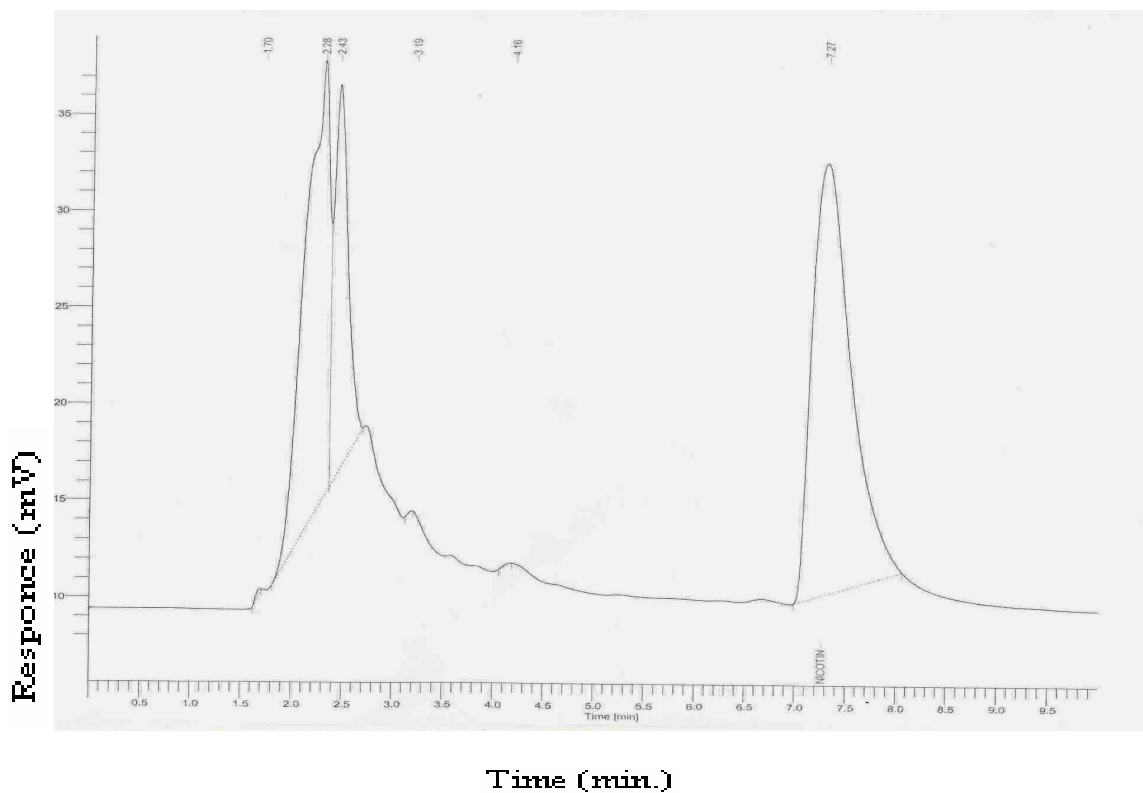


Fig. 7. Chromatogram of extracted nicotine ( at 7.27 s) from Billate Virginia tobacco leaves (Flow rate of 1 mL/minute, injection volume of 10  $\mu$ L, C-18 reversed phase column, isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25)

### 4.3. Selection of Maximum Wavelength for the Detection of Nicotine

The HPLC used for this analysis was equipped with UV-detector; therefore there was a need to know the maximum wavelength absorbance of nicotine. The  $\lambda_{\text{max}}$  nicotine are found to be 238 nm, 254 nm, 259 nm and 282 nm [58]. To select the preferred  $\lambda_{\text{max}}$  of nicotine for the analysis 10  $\mu$ L of 1 mM standard nicotine was injected to HPLC and the peak area at each of the above four wavelengths was recorded separately. The response peak area for 259 nm was the largest. Moreover, by preparing 0.1 mM standard solution of nicotine the UV-Vis spectrophotometer was also run to know the maximum absorbance of nicotine, thus its maximum peak was at 259 nm as shown on Figure 8, thus this maximum wavelength of nicotine was selected for subsequent analysis.

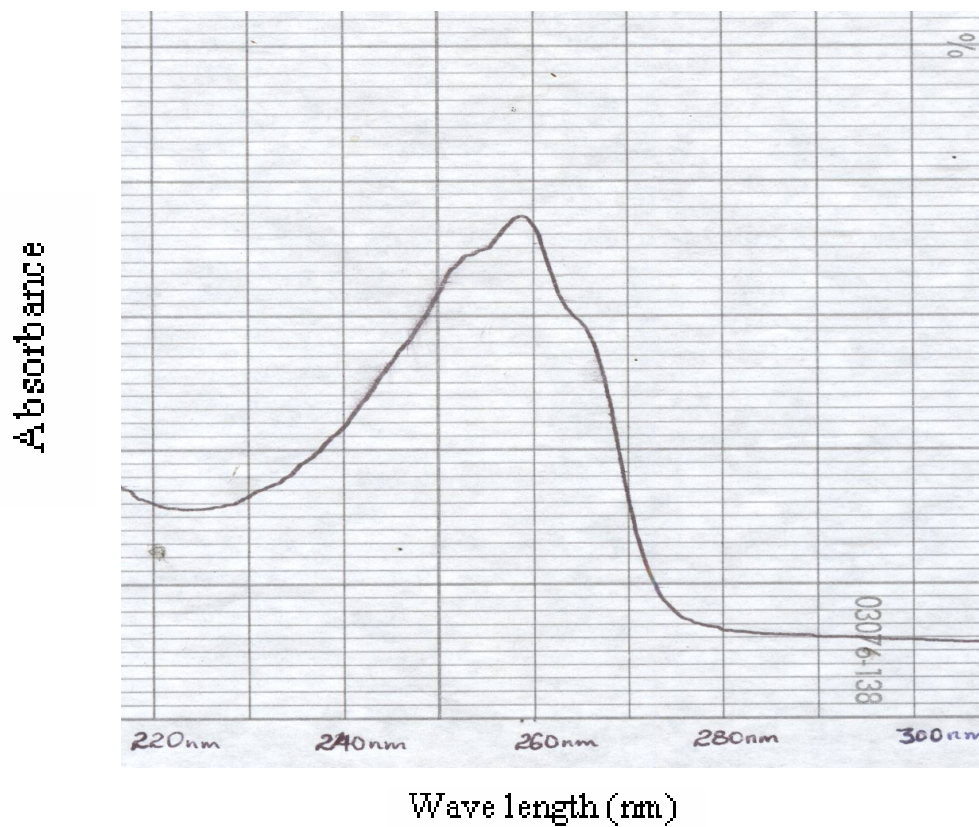


Fig. 8. UV spectrum of 0.1 mM nicotine standard solution

#### 4.4 Calibration Curve

In chemical analysis especially by HPLC method constructing the calibration curve is crucial for the determination of unknown concentrations of analyte. The calibration curve was obtained by plotting the peak area as a function of concentrations of nicotine in the standard solutions with its goodness of fit, regression line, ( $r^2$ ) 0.9971. The data are given in Table 3. The calibration curve is shown in Figure 9.

Table 3. Data obtained for calibration curve for the determination of nicotine

| Concentration (mM) of Nicotine | 1 <sup>st</sup> peak area (μV.s) | 2 <sup>nd</sup> peak area (μV.s) | 3rd peak area (μV.s) | Mean of peak area (μV.s) | S.D. (μV.s) | RSD (%) |
|--------------------------------|----------------------------------|----------------------------------|----------------------|--------------------------|-------------|---------|
| 0.1                            | 116613                           | 109306                           | 108946               | 111621.27                | 4326.27     | 3.86    |
| 0.5                            | 449604                           | 464479                           | 432635               | 448905.93                | 15933.31    | 3.55    |
| 1                              | 987920                           | 939112                           | 1003338              | 976789.83                | 33528.60    | 3.43    |
| 1.5                            | 1776018                          | 1653110                          | 1776061              | 1735063.00               | 70973.38    | 4.09    |
| 2                              | 2170625                          | 2364663                          | 2256979              | 2264089.00               | 97214.20    | 4.29    |
| 2.5                            | 2619273                          | 2603977                          | 2692866              | 2638705.33               | 47523.94    | 1.80    |
| 3                              | 3158852                          | 3252556                          | 3017050              | 3142819.33               | 118568.77   | 3.77    |

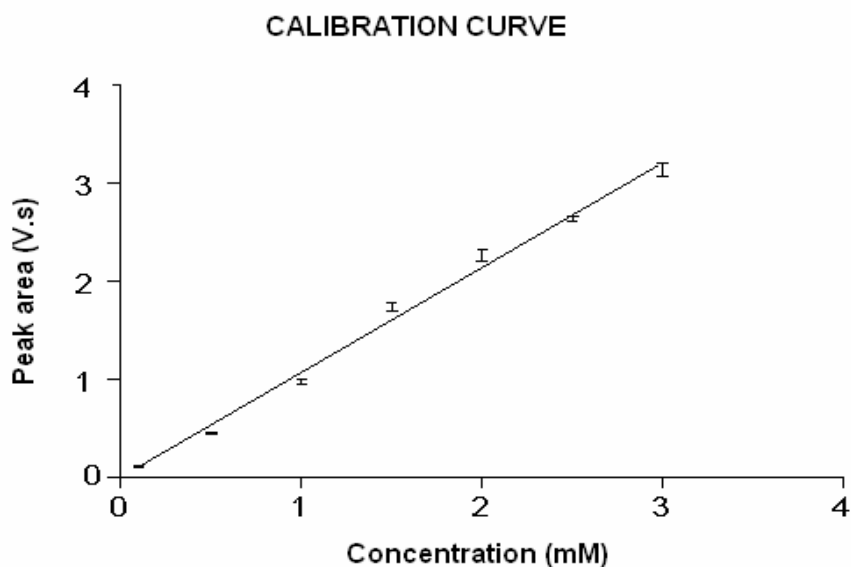


Fig. 9. Calibration Curve Plot forced to pass through the Origin

Characteristics of the curve are:

Slope  $1071000 \pm 12900$

Y-intercept 0.0000

X-intercept 0.0000

Regression line,  $r^2 = 0.9971$ .

## 4.5 Limit of Detection

In general terms, detection limit of analytical method refers to the minimum concentration of a substance that can be reported with 99% confidence to be greater than zero [59]. Determining the detection limits of analysis differ based on the analysis type. For HPLC case, detection limit is the minimum amount of species that can be reliably seen on the chromatogram. Experimentally, the detection limit is the amount of substance that will give a peak whose height is some multiple (commonly multiples of 2 or 3) of the baseline noise which is the signal to noise ratio (S/N) [60]. However, the S/N ratio is misleading for most techniques. Therefore, for our case it is simple to determine the detection limit from the data obtained for plotting calibration curve. When plotting calibration curve, without forcing the curve through the origin, there are two possibilities of the raw data of the standard samples. These are negative concentration (X-intercept) or positive response (Y-intercept) as a result of instrument interferences (such as stray light in a UV-detector). The other most probable possibility is positive concentration (X-intercept) or negative response (Y-intercept). In a latter case, at a point below a certain positive amount (concentration) of analyte, there is no response from the chromatograph detector. This amount (concentration) is the detection limit for the analysis [61].

In this study, the detection limit for the analysis was determined according to TotalChrom User's guide by solving the calibration curve without forcing to pass through the origin using the software known as 'IN STAT' or 'PRISM' to determine the X- intercept or the detection limit as shown on Figure 10. Thus the detection limit of the analysis is found to be 13.98  $\mu\text{M}$  of nicotine.

The 13.98  $\mu\text{M}$  of nicotine can also be expressed as percentage value by going through simple analytical calculations. Since it is known that M Wt. of nicotine = 162.26, Detection limit = 13.98  $\mu\text{M}$ , 0.5 g tobacco leave sample used for extraction and the extract diluted to 100 mL (50 mL for some cases). For the case of 100 mL dilution it will be 0.045% of nicotine.

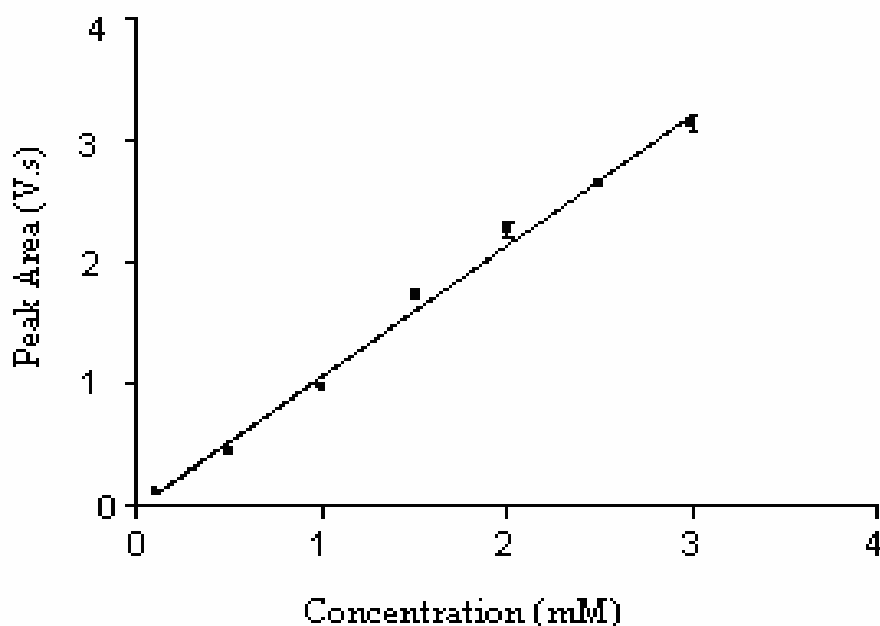


Fig. 10. Calibration Curve Plot without forcing to pass through the Origin

Characteristics of the curve are:

Slope  $1078000 \pm 24300$

Y-intercept  $-15070 \pm 43810$  ( $\mu\text{V.s}$ )

X-intercept  $0.01398$  (mM)

Regression line,  $r^2 = 0.9904$ .

#### 4.6. Recovery for Method of Extraction

The terms recovery or recovery factor (symbol  $R$ ) are at present used to indicate the yield of an analyte in a preconcentration or extraction stage in an analytical method, which can be determined as follows, If  $R_A$  is measured using a standard addition or spike procedure,

$$R_A = \frac{Q_A(O + S) - Q_A(O)}{Q_A(S)}$$

Where  $Q_A(S)$  is the quantity of analyte A added (spike value) and  $Q_A(O + S)$  the quantity of A recovered from the spiked sample and  $Q_A(O)$  from the original sample [62].

Though recovery of this method was reported as being greater than 95 %, it was tried to determine the actual recovery for this study. According to the IUPAC recommendation stated above the recovery of the method of analysis used in this study was determined by simultaneous extraction of nicotine from the powdered tobacco leaves in which 1 mM concentration of standard nicotine solution was added (O + S), and the powdered tobacco leaves with out addition of standard (O). The recovery was  $97.2 \pm 0.07 \%$ . This is shown in Table 4.

Table 4. Data for recovery experiment

| Tobacco leaf | Triplicate concentrations (mM) |                 |                 | Mean (mM) | S.D. (mM) | RSD (%) |
|--------------|--------------------------------|-----------------|-----------------|-----------|-----------|---------|
|              | 1 <sup>st</sup>                | 2 <sup>nd</sup> | 3 <sup>rd</sup> |           |           |         |
| O            | 1.564                          | 1.504           | 1.435           | 1.501     | 0.065     | 4.330   |
| O + S        | 2.498                          | 2.434           | 2.487           | 2.473     | 0.034     | 1.375   |

$$\text{Recovery} = \frac{[(2.473 \pm 0.034) - (1.501 \pm 0.065)] \times 100}{1\text{mM}} = 97.2 \pm 0.07 \%$$

## 4.7 Nicotine Level of Ethiopian Tobacco Leaves

The nicotine level of Ethiopian tobacco mainly Virginia, others burley, oriental and native tobacco ‘Gaya’ were determined by HPLC technique. The results are presented and discussed below based on their leaf (stalk) positions, places of cultivation and variety of species.

### 4.7.1 Nicotine Level of Robi Tobacco Leaves

As stated in subtopic of sample collection from the sample site Robi Virginia tobacco leaves were collected and analyzed. The nicotine level of Robi tobacco leaves is presented based on leaf position and drying style with their rough comparison.

Table 5. Level of nicotine of Robi Virginia Tobacco Leaves by percentage (%)

| Stalk (leaf) position | Samples of sun-dry leaf |             | Samples of cured leaf |             |
|-----------------------|-------------------------|-------------|-----------------------|-------------|
|                       | Nicotine content (%)    | RSD (%)     | Nicotine content (%)  | RSD (%)     |
| Lug                   | 1.75 ± 0.05             | 2.86        | 1.07 ± 0.04           | 3.74        |
| Cutter                | 2.04 ± 0.06             | 2.94        | 1.22 ± 0.06           | 4.92        |
| Leaf                  | 2.18 ± 0.04             | 1.83        | 2.87 ± 0.10           | 3.48        |
| Tip                   | 2.81 ± 0.12             | 4.27        | 3.60 ± 0.08           | 2.22        |
| <b>Average</b>        | <b>2.20 ± 0.07</b>      | <b>3.18</b> | <b>2.19 ± 0.07</b>    | <b>3.20</b> |

#### 4.7.1.1 Nicotine Level of Sun-dry Robi Tobacco Leaves

Sun-dry tobacco leaves for this study mean that as soon as the leaves collected were not exposed to direct sun light and dried. However, first they were allowed to pass through the yellowing process (gradual conversion of green to yellow leaf) by preventing the green leaf from direct sunlight. This yellowing process took almost 12 to 14 days, and then yellow leaves of tobacco were exposed to direct sun light to under go slow drying process. Finally dried leaves were obtained with in a week.

The nicotine level of sun-dry tobacco leaves is shown on Table 5 and Figure 11 based on the leaf positions *lugs*, *cutters*, *leaf* and *tip* with respective RSD for their triplicates. The result shows that the nicotine level of tobacco leaves increase from bottom leaf position (Lugs) to upper leaf position (Tip) this is supported by literatures. And their average nicotine level was determined to be 2.2 %.

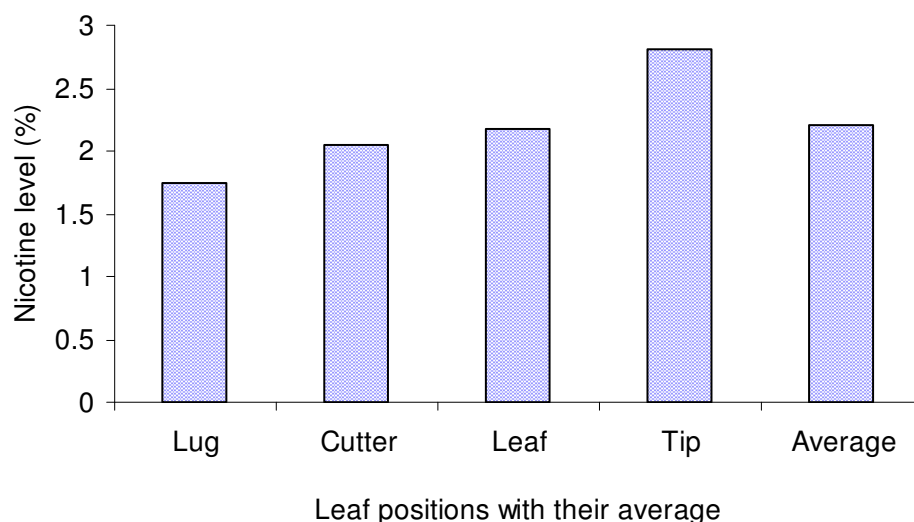


Fig. 11. Nicotine level of sun-dry Robi Virginia based on their leaf position

#### 4.7.1.2 Nicotine Level of Cured Robi Tobacco Leaves

Curing tobacco leaves is a process of drying tobacco leaves in the controlled heat and humidity. Sample of cured tobacco leaves were collected from Ethiopian tobacco enterprise chemical laboratory leaves store and analyzed for simple evaluation and comparison purpose. The nicotine levels in cured leaves collected from the factory were also determined based on their leaf positions and the results are presented in Table 5 and Figure 12. The average nicotine level of cured Robi tobacco leaves was found to be 2.19 %. This result is almost comparable with results presented on literatures that tell us nicotine level of Virginia tobacco [34].

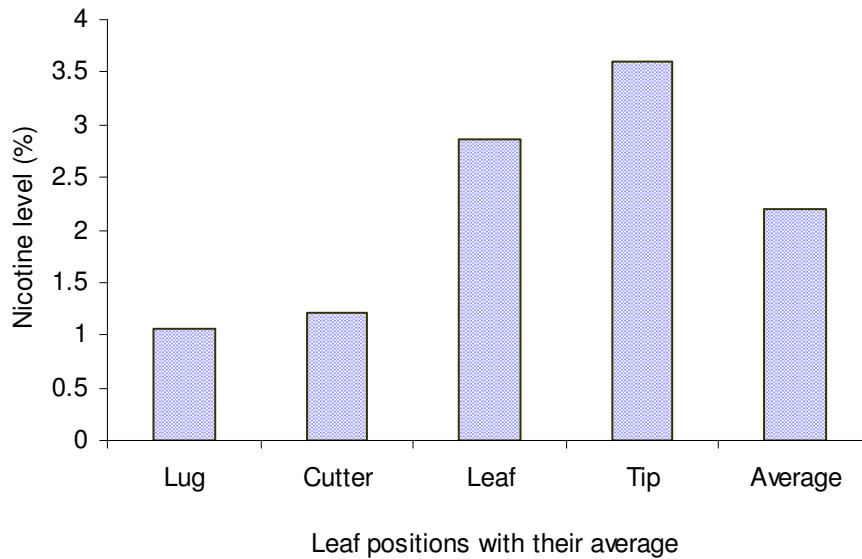


Fig. 12. Nicotine level of cured Robi Virginia based on their leaf position

#### 4.7.1.3 Comparison of Nicotine Level of Sun-dry and Cured Robi Tobacco

Drying or curing style of tobacco leaves makes slight effect on their nicotine level. Thus though the comparison is done roughly significant difference of nicotine level was noticed between the sun dry and cured leaves of Robi tobacco.

This comparison is rough comparison, the reason why saying 'rough' comparison is that the cured and sun dry sample leaves have been the same only by their place of cultivation (Robi) and species (Virginia). They were different in different aspects. For instance cured sample leaves were collected, cured and stored without the control or supervision of the researcher. Different leaf positions samples were simply taken from the laboratory, and then analyzed and compared with sun dry sample leaves which were collected and dried with great care.

The results of the nicotine levels of the two samples (sun-dry and cured) are presented in Table 5 and compared in Figure 13. These results indicate that the nicotine level of cured leaves are slightly less than that of sun dry leaves as observed on their average nicotine level of leaf positions. Although the nicotine level of each leaf position result is showing increment on leaf positions from bottom to top (lug to tip) as expected, the comparison of the two drying style for each leaf position does not show consistency.

The other observation in relation to their nicotine level was that the leaf positions of sun dry leaves show slight increment (from 1.75 % to 2.81 %) as compared with that of cured leaves that show relatively inflated increment (from 1.07 % to 3.60 %). This may be accounted by maturity levels of collected sample leaves. That is sun dry leaves were collected once with great care based on their leaf positions and selectively matured leaves were collected on different tobacco stems or plants. Whereas the cured sample leaves were collected by priming tobacco leaf harvesting style as stated by the collector which means that all the leaf positions were not collected at the same time rather they were collected based on their maturity stages. That is lugs first, cutter second, then leaf, finally tip position, which were collected at least with in a week difference of each positions. Therefore if the maturity level of the leaf positions appraised inaccurately, they may be collected without keeping the exact maturity period. These have great effect on nicotine level of each leaf positions. In other words, when these leaves were collected one leaf position may be premature, the other mature and the remaining may be post mature which leads to less, moderate and higher nicotine level respectively.

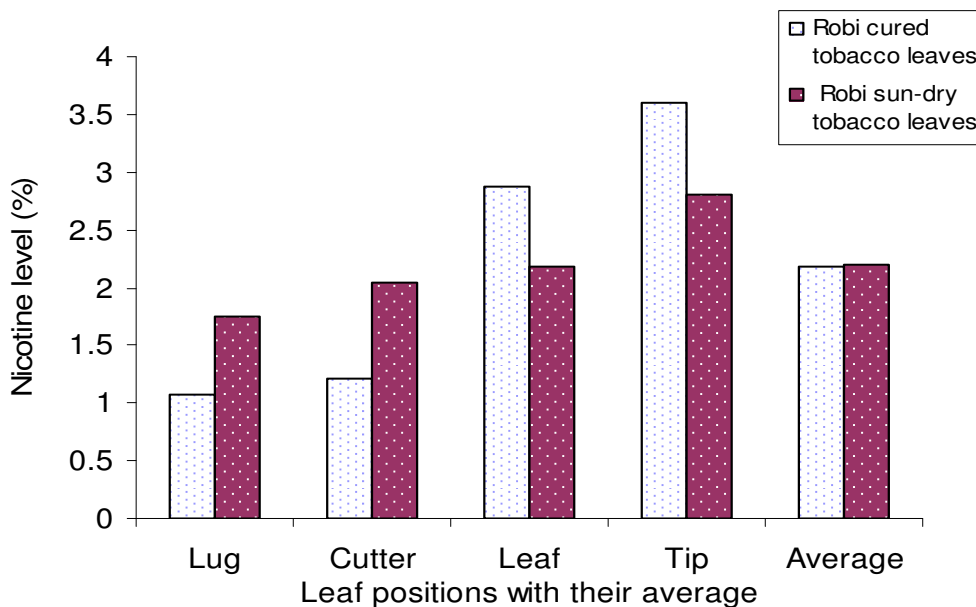


Fig. 13. Comparison of nicotine level of cured and sun-dry Robi Virginia tobacco

#### 4.7.2 Nicotine Level of Billate Tobacco Leaves

Like that of Robi, from the sample site of Billate Virginia tobacco species were collected based on the leaf (stalk) positions. Collected tobacco leaves samples were allowed to go through sun drying process as stated above for the Robi samples sun drying style. Finally after the dried leaves of Billate Virginia tobacco obtained, they were analyzed for their level of nicotine.

The nicotine levels of tobacco leaf samples are given in Table 6 with respective RSD of triplicate samples of each leaf positions. The average nicotine level of the Billate tobacco leaves was determined as 3.26 % which is slightly higher nicotine level of all the samples analysis result of this study, but not out of the expected result of nicotine level of Virginia tobacco. The leaf positions nicotine level also increase from bottom (lugs) to upper (Tip) leaves as stated on the literature [22-23]. The bar graph of leaf positions nicotine level is shown in Figure 14.

Table 6. Nicotine level of Billate Virginia (sun-dry) by percentage (%)

| <b>Leaf samples based on stalk<br/>(Leaf) position</b> | <b>Nicotine level (%)</b> | <b>RSD<br/>(%)</b> |
|--|---------------------------|--------------------|
| Lug  | 2.24 ± 0.07               | 3.13               |
| Cutter   | 2.48 ± 0.11               | 4.44               |
| Leaf   | 3.29 ± 0.18               | 5.47               |
| Tip  | 5.03 ± 0.27               | 5.37               |
| <b>Average</b>   | <b>3.26 ± 0.16</b>        | <b>4.91</b>        |

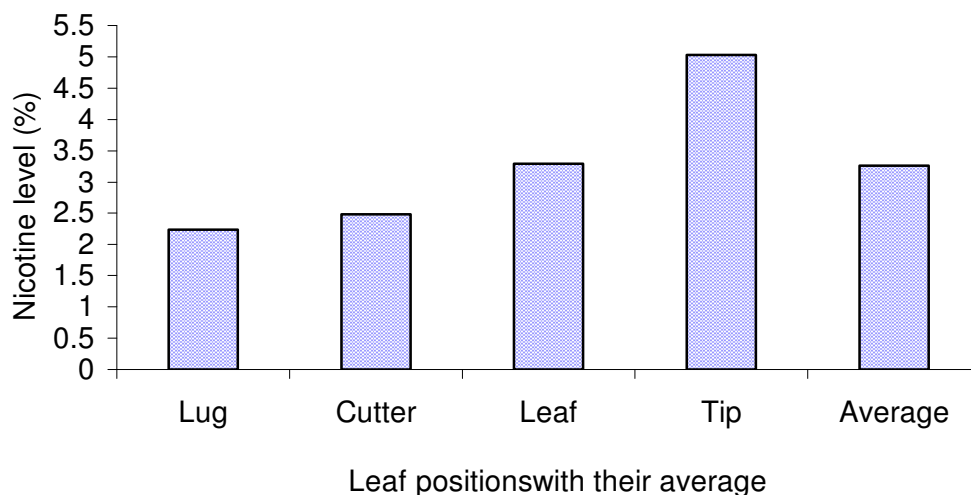


Fig. 14. Nicotine level of Billate Virginia (sun-dry) by percentage (%)

#### 4.7.3 Comparison of Nicotine Level of Virginia Tobacco Leaves based on Area of Cultivation

Virginia tobacco leaves were collected from two samples sites Robi and Billate. The two sites Virginia tobacco leaves samples were enforced to pass through almost by the same process. That is they were collected in the same season, conditions, and also pass through the same drying style sun drying with the same process of nicotine analysis. Therefore, it was possible to compare nicotine level of them appropriately. This is presented in Table 7 with their respective leaf positions. The bar graph of their comparison is shown in Fig. 15.

Table 7. Comparison of nicotine level of Virginia tobacco based on area of cultivation

| Leaf samples based on stalk (Leaf) position | Nicotine level of Billate (%) | Nicotine level of Robi (%) |
|---|-------------------------------|----------------------------|
| Lugs  | 2.24 ± 0.07                   | 1.75 ± 0.05                |
| Cutter                                      | 2.48 ± 0.11                   | 2.04 ± 0.06                |
| Leaf  | 3.29 ± 0.18                   | 2.18 ± 0.04                |
| Tip   | 5.03 ± 0.27                   | 2.81 ± 0.12                |
| <b>Average</b>                              | <b>3.26 ± 0.16</b>            | <b>2.20 ± 0.07</b>         |

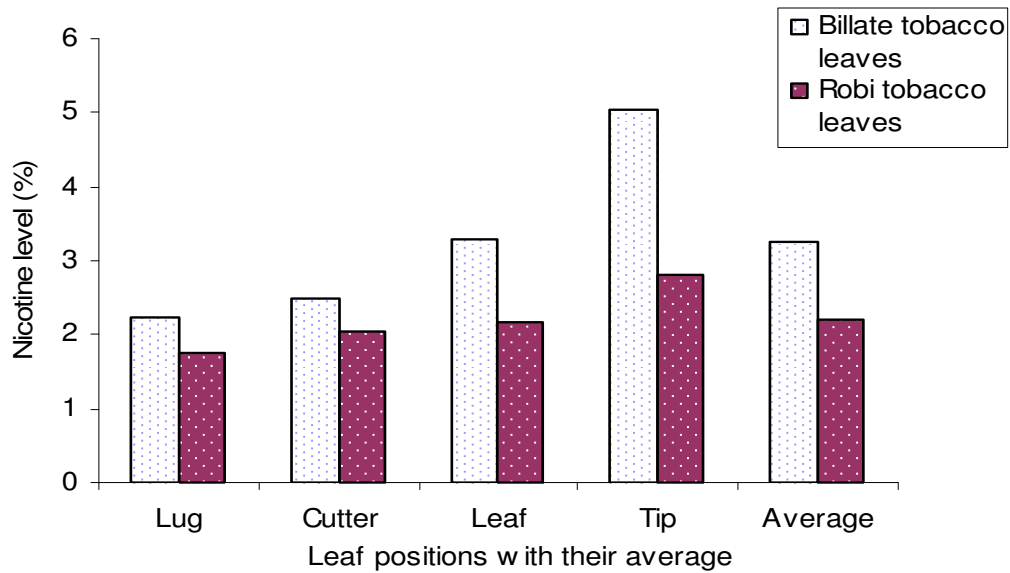


Fig. 15. Comparison of nicotine level of Virginia tobacco based on area of cultivation

As shown on Table 7 and Figure 13, the nicotine level of Billate tobacco leaves is higher than that of Robi tobacco leaves. This is shown by the average nicotine level of the leaf positions as well as nicotine level of each leaf positions. This may be accounted by many factors. The most suspected factor is that the fertility of the cultivation area of tobacco in Billate is greater than that of Robi. In view of the fact that the cultivation area of tobacco in Billate is state owned so controlled and cared by agricultural professionals; where as Robi cultivation area of tobacco are owned by farmers who have responsibility to control, provide care by the supervision of agricultural professionals. This makes great difference on nicotine content of tobacco leaves of the two cultivation areas. Moreover atmospheric conditions such as temperature, rainfall, humidity of the cultivation area, cultural practice of cultivation, maturity level and soon contribute significant difference on nicotine level of the two places tobacco leaves.

#### **4.7.4 Nicotine Level of Awassa Burely and Oriental Tobacco Leaves**

From Awassa sample site two species of tobacco were collected. These were burley and oriental tobacco species. As literatures suggest that these two species have low nicotine level. Consequently the analysis of nicotine level of burley and oriental tobacco leaves using this selected method of analysis was difficult for oriental and slightly difficult for burley species. Accordingly to increase the concentration of nicotine in the extracts of these species for analysis, it was tried to dilute the extract to five fold and also even without dilution by deviating from the procedure of method of analysis, which state that ten fold dilution. The five fold dilution modification provides somewhat fair result for burley but not for oriental tobacco species leaves nicotine level.

Like other method of analysis HPLC was not analyzing nicotine as total alkaloid. It is powerful method of analysis to analyze each alkaloid separately with well validated and set of conditions. Therefore, when these two species of tobacco leaves, especially oriental tobacco leaves, analyzed by previously stated HPLC set of conditions their nicotine peak (appeared at about retention time of 7.2 sec) is very small as compared with other related alkaloid peaks (appeared at retention time of about 2.0 to 3.5 sec) on the chromatogram. Thus these other related alkaloid peaks suppress all the smallest peaks and the instrument couldn't integrate well the small nicotine peak. The chromatograms of burley and oriental tobacco species have shown on Figure 16 and 17.

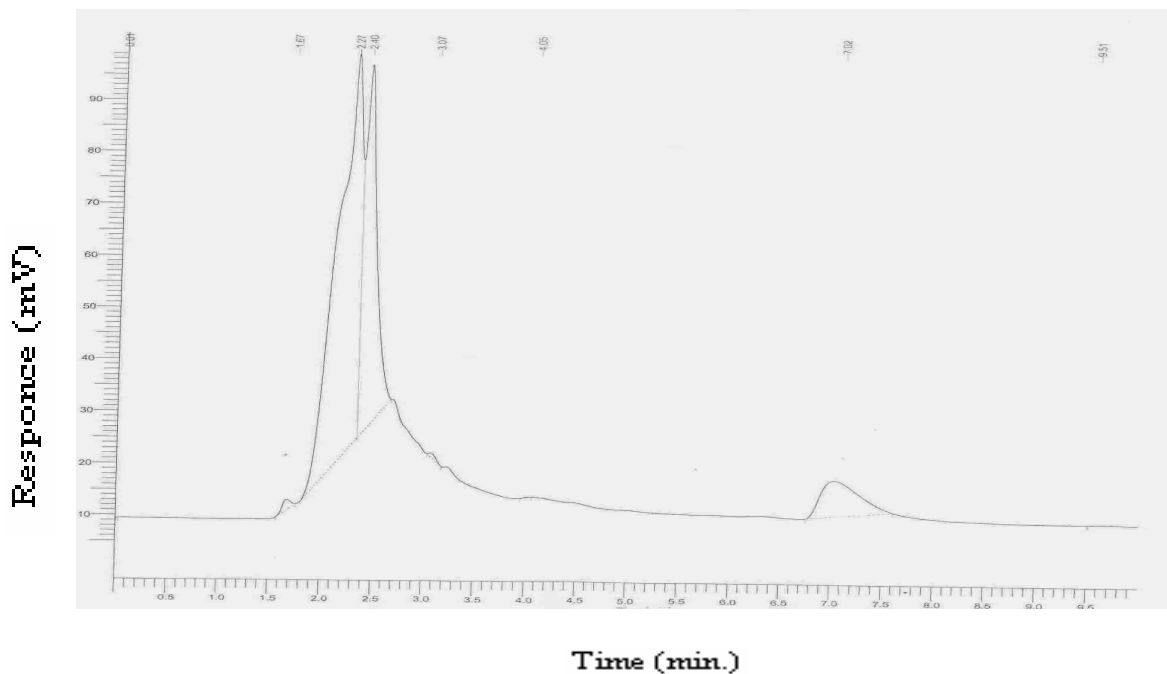


Fig. 16. Chromatogram of extracted nicotine (at 7.12 sec.) and other related alkaloid from Burley type of Awassa tobacco leaves.

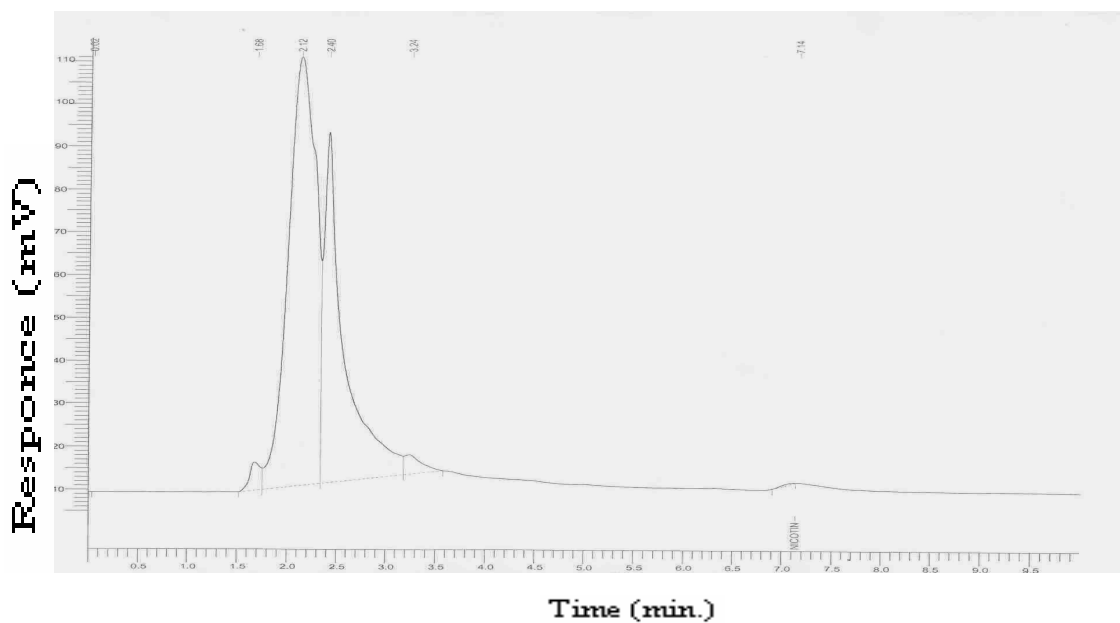


Fig. 17. Chromatogram of extracted nicotine (at 7.14 sec.) and other related alkaloid from Oriental type of Awassa tobacco leaves.

(Flow rate of 1 mL/minute, injection volume of 10  $\mu$ L, C-18 reversed phase column, isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % phosphoric acid buffered to pH 7.25)

After many trials the nicotine peak for burley tobacco was integrated and the result of nicotine level of burley tobacco is reported as shown in Table 7. However, it was not possible to get well integrated nicotine peak area for oriental tobacco leaves by this method of analysis and set of conditions, and hence the level of nicotine could not be quantified reliably. This implies the method of analysis or set of conditions need further modification or change to determine nicotine level of oriental tobacco leaves. Consequently the analysis to quantitative determination of nicotine level of oriental tobacco leaves was abandoned in this study.

Table 8. Nicotine level of Awassa Burley by percentage (%)

| Burley leaf sample                              | Nicotine level (%) | RSD (%) |
|---|--------------------|---------|
| Mixture of 1:1:1:1 proportion of leaf positions | 0.65 ± 0.02        | 3.07    |

#### 4.7.5 Nicotine level of Wollayita Native Tobacco Leaves ‘Gaya’

For simple comparison and evaluation of nicotine level of native tobacco leaves with commercial tobacco leaves, it was tried to analyze the nicotine level of ‘Gaya’ of Wollayita which is one of well known native tobacco species in Ethiopia.

As the result is shown in Table 8, the nicotine level of Wollayita ‘Gaya’ is not very high as expected by many peoples. The nicotine level of ‘Gaya’ as determined by this method of analysis is 1.11 % which is fair level of nicotine as compared with other tobacco species, especially with Virginia note that this study analyze only nicotine level of ‘Gaya’ taken from a particular sample site and which also collected by the help of information provided by a user of this native tobacco leaves. Therefore to provide broad report on native tobacco nicotine level, it needs further analysis by taking many sample sites and broad analysis on related species of native tobacco with sufficient elaboration. Moreover, it is better to realize it does not mean that ‘Gaya’ is free from or has less level of other alkaloids and other harmful components of health.

Table 9. Nicotine Level of Wollayita ‘Gaya’ by Percentage (%)

| <b>‘Gaya’ leaf sample</b>                       | <b>Nicotine level (%)</b> | <b>RSD (%)</b> |
|---|---------------------------|----------------|
| Mixture of 1:1:1:1 proportion of leaf positions | 1.11 ± 0.05               | 4.50           |

#### 4.7.6 Comparison of Nicotine Level of Ethiopian Tobacco Leaves based on their Species

The nicotine levels of Ethiopia tobacco leaves of individual species with their respective sample site have been present above. The results show that the nicotine levels of Ethiopian tobacco leaves are not much different from the nicotine levels of other countries as reported in different literatures [34]. As cigarette manufacturers and tobacco leaf dealers want, the lowest and moderate levels of tobacco leaves are required for good tobacco market. Thus based on marketability point of view, nicotine level of Ethiopian tobacco is not in difficult situation. However, it require great care to limit or keep the nicotine level of tobacco leaves in lowest and moderate levels, especially for Virginia tobacco species and particularly the tip leaf position leaves.

Table 10. Comparison of nicotine level (%) based on their species

| <b>Type of species of tobacco</b> | <b>Average nicotine level (%)</b> |
|-----------------------------------|-----------------------------------|
| Virginia (Billate)                | 3.26 ± 0.16                       |
| Virginia (Robi)                   | 2.20 ± 0.07                       |
| Burley (Awassa)                   | 0.65 ± 0.02                       |
| Oriental (Awassa)                 | Around detection limit (0.045 %)  |
| Gaya (Wolayita)                   | 1.11 ± 0.05                       |

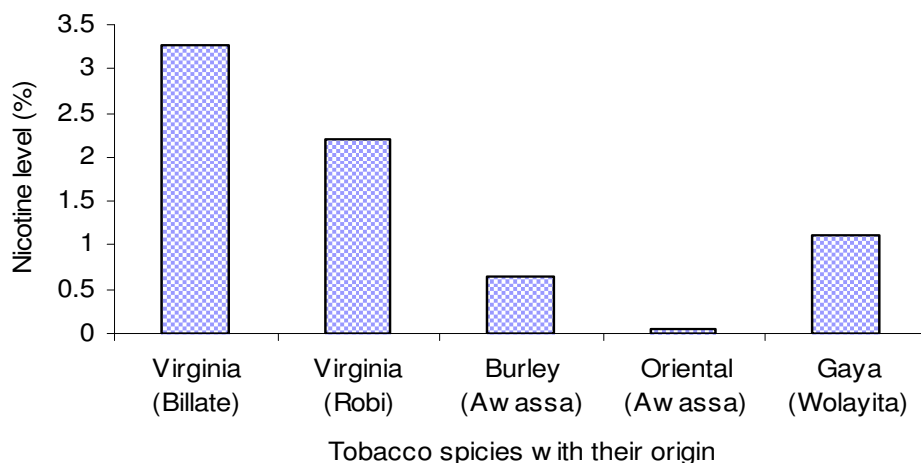


Fig. 18. Comparison of nicotine level Ethiopian tobacco species

As the results shown in Table 10 and Figure 18, the nicotine level of Billate Virginia tobacco leaves is large as compared with others, followed by Robi Virginia tobacco leaves. Native tobacco leaves ‘Gaya’ possess middle place of nicotine level, and then burley tobacco leaves contain very low nicotine. Finally the lowest of all is oriental tobacco leaves nicotine level, though its accurate level of nicotine was not determined by this study the chromatogram of oriental tobacco leaves shows that the presence of nicotine in trace level. That is very small un-integrated peak of nicotine is observed in the chromatogram of oriental tobacco leaves.

#### 4.7.7 Nicotine Level of Ethiopian Tobacco Leaves as compared with other part of the World Tobacco Leaves

The nicotine content of *Nicotiana tabacum* varieties grown commercially generally ranges from 0.3 to 3 %, though 5 % and even 7 % have been recorded in some heavy bodied tobaccos [30]. Thus, the nicotine content (level) of Ethiopian tobacco leaves found by this study is almost in the range of other countries commercial tobacco leaves. So it is possible to realize that the nicotine level of Ethiopian tobacco leaves is almost similar or in the range as compared with other countries tobacco leaves nicotine level.

Moreover, the nicotine content of Virginia tobacco of Billate obtained from this study is almost comparable with the results reported by different exporter countries to Ethiopia in different years, but nicotine content of Virginia tobacco of Robi seems a little less than that

of the reported nicotine content of tobacco by those exporter countries. This comparison is shown on Table 11 and Figure 19.

Table 11. Nicotine level of Virginia tobacco as reported by exporters and importer

| Exporter country     | Year imported to Ethiopia | Nicotine (%)                     |  |
|----------------------|---------------------------|----------------------------------|--|
|                      |                           | Reported by the exporter country | Analysis result of National Tobacco Enterprise ( Ethiopia) |
| Italia               | 1995                      | 3.092                            | 3.001  |
| China                | 1998                      | 3.020                            | 2.998  |
| India                | 1999                      | 2.986                            | 2.979  |
| Brazil               | 2001                      | 3.124                            | 3.122  |
| Zimbabwe             | 2006                      | 3.180                            | 3.172  |
| * Ethiopia (Billate) | 2007                      | -                                | 3.26   |
| * Ethiopia (Robi)    | 2007                      | -                                | 2.20   |

\* Results obtained by the study.

Source: National Tobacco Enterprise of Ethiopia.

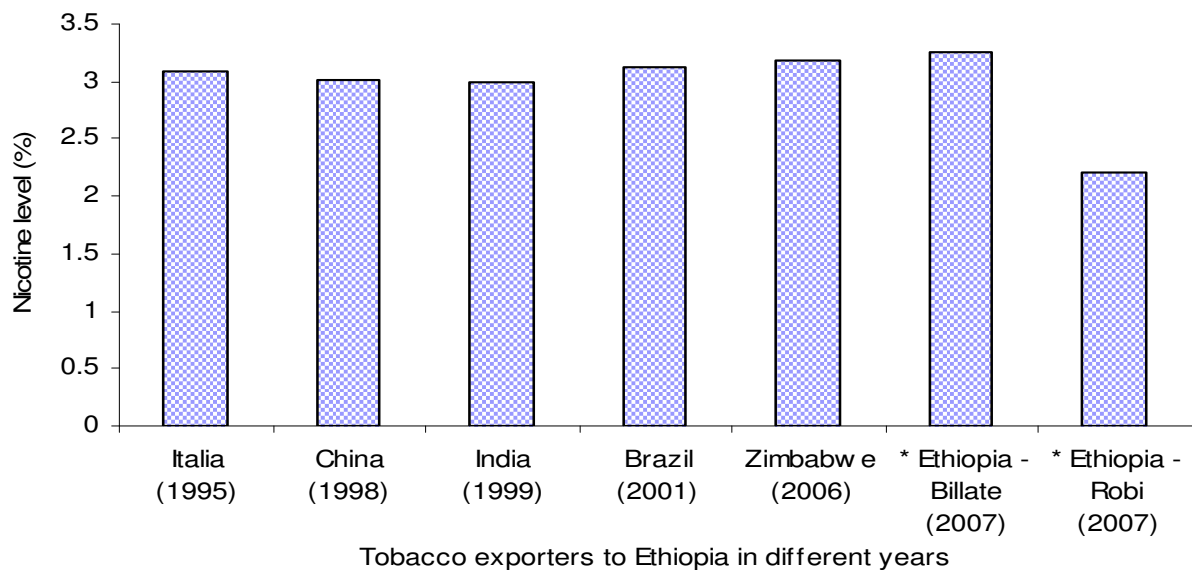


Fig. 17. Comparison of nicotine level of Virginia tobacco of Ethiopia with that of other countries.

## 5. Conclusion

Ethiopian tobacco leaves based on their leaf (stack) positions, places of cultivation and variety of species were analyzed for their nicotine level by using High Performance Liquid Chromatography (HPLC) technique. Among the variety of species Virginia tobacco leaves have high nicotine level (3.26 %), followed by the native tobacco 'Gaya' (1.1 %) then burley tobacco leaves (0.65 %), finally oriental tobacco leaves have found with lowest nicotine level, though its actual level of nicotine was not quantified reliably by this method of analysis of nicotine. Based on their leaf (stack) positions the nicotine level of Ethiopian tobacco leaves like that of other countries increase from bottom to top (tip) leaves. The nicotine level of especially the tip positions of Virginia tobacco leaves supposed to be controlled and care should be taken by the tobacco product manufacturer. Furthermore this study has found that nicotine level of a given tobacco species cultivated in different places is different this is may be due to the fertility of the land, nitrogen content, cultural practice, environmental and atmospheric conditions difference, however these factors need further justification by further studies. For the reason that the method of analysis used by this study is more applicable to determine the nicotine level with other related alkaloids simultaneously, the nicotine level of oriental tobacco species could not be quantified reliably, due to the appearance of very small peak of nicotine (which cannot be integrated well). Therefore to determine nicotine level of oriental tobacco the method should be modified or changed to suppress the large peaks of other nicotine related alkaloids appeared before the appearance of the smallest nicotine peak on the chromatogram. Finally it was tried to compare the level of nicotine of Ethiopian Virginia type tobacco (obtained by this study) with other countries Virginia type tobacco nicotine level, thus the results were found to be comparable with that in the rest of the world.

Lastly it can be recommended that this study will give preliminary information about tobacco in Ethiopia for other researchers who intend to study further on this area.

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