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Investigation of Metals in Ethiopian Tobacco leaves and Processed Tobacco

By

Girma Regassa

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INVESTIGATION OF METALS IN ETHIOPIAN TOBACCO LEAVES AND PROCESSED TOBACCO

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INVESTIGATION OF METALS IN ETHIOPIAN TOBACCO LEAVES AND PROCESSED TOBACCO

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

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To my Wife Workua and my daughters Siyan and Heman

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ABSTRACT

INVESTIGATION OF METALS IN ETHIOPIAN TOBACCO LEAVES AND PROCESSED TOBACCO

By Girma Regassa

Advisors: Prof. B. S. Chandravanshi

Samples of tobacco leaves (Virginia type) were collected from two different regions of Ethiopia (Bilatte and Shoa Robit) which are the only places where this type of tobacco is harvested. The other three processed tobacco samples were collected from tobacco manufacturing factory in Addis Ababa, Ethiopia. After collection of the representative amounts of samples, and samples pretreatment, different digestion procedures were tested by varying reagent volumes, time of digestion, temperature of digestion, and amount of the sample to develop a procedure that consume less reagent volume, short digestion time, low temperature of digestion and smaller mass of the sample. The optimal procedure required 3:30 hours and consumes 3 mL HNO_3 (69-72 %) and 3 mL $HClO_4$ (70 %) to completely digest 0.5 g of both tobacco leaves and processed tobacco samples. The accuracy of the optimized procedure was evaluated by analyzing the digest of the spiked samples with standard solution. Recoveries of the spiked samples varied from 88.3% to 104.3% and 91.7% to 107% for leaves and processed tobacco, respectively. Concentrations of metals in the samples; (Cd, Cr, Cu, Ni, Pb and Zn) were analyzed by flame atomic absorption spectrometer employing a four point external calibration curve. The observed average metals concentrations were (mean ± $ts/N^{1/2}$, μg/g at 95% CL and N=9): Cu (4.38 ± 0.11), Zn (53.7 ± 0.96), Cd (1.2 ± 0.05) in tobacco leaves collected from Bilatte; Cu (7.3± 0.19), Ni (1.9 ± 0.08), Cd (1.3 ± 0.04), Cr (1.45 ± 0.11), Zn (33.15 ±1.9) in leaves collected from Shoa Robit; Cu (9.8 ± 0.04), Ni (2.35 ± 0.19) Cd (1.45 ± 0.023), Cr (1.65 ± 0.08), Zn (101.2 ± 0.4) in processed tobacco leaves originated from Bilatte; Cu (12.8 ± 0.11) Ni (2.2 ± 0.05) Cd (1.9 ± 0.05), Cr (1.75 ± 0 .08), Zn (83.75 ± 0 .4) in processed tobacco leaves originated from Shoa Robit; Cu (8.95 ± 0.31), Cd (1.55 ± 0.02), Cr (1.62 ± 0.11), Ni (4.7 ± 0.04), Zn (79.3 ± 0.77) in Nyala. The Concentration of

Pb was lower than detection limit of the instrument (0.1 mg/L) in all tobacco samples; Cr and Ni in tobacco leaves from Bilatte (0.05 and 0.04 mg/L respectively). This study showed that the metal contents of tobacco leaves varied with the geographical origin in which the tobacco plant grows, and the metal contents of processed tobacco were higher than the corresponding leaves. The levels of metals in Ethiopian tobacco are comparable with that of other countries.

Key words: Tobacco leaves, Processed tobacco, Trace metals, FAAS

1. INTRODUCTION

All plants have the ability to absorb and accumulate heavy metals from soil which are essential for their growth and development. Certain plants also have the ability to accumulate heavy metals that have no known biological function [1]. Tobacco plant is amenable to absorb and accumulate heavy metal species from the soil into leaves. Soil is therefore the sink for trace metals. The soi1 particles acts as scavenger for elements like Cd, Cu, Pb and Zn**.** Industrialization has contributed to the burden of trace metals in soils in urban areas. Metals that are added from the application of sewage sludge, pesticides, lime, irrigation waters and fertilizers have threatened the quality of agricultural land [2].

Zinc and copper are considered essential for plant growth [3]. They have been shown to aid in plant metabolism, RNA and ribosome formation, photosynthesis, respiration and an activator of several enzymes. However, high concentrations of these micronutrients are extremely toxic to the cell metabolism. Excess concentrations of these metals in plants may result in an alteration of root membrane permeability, tissue damage, depressed plant growth and chlorosis of new leaves. In contrast to these essential micronutrients, elements like Pb and Cd are considered non-essential for plant growth. Excess concentrations of these elements in plants have resulted in depressed nutrient uptake and the inhibition of chlorophyll formation [2].

Heavy metals are stable elements that cannot be metabolized by the body and get passed up in the food chain to human beings (bio-accumulate). High-concentration exposure is not necessary in order to produce a state of toxicity in the body. Most cases of heavy metal poisoning result from chronic low-level exposure to these hazardous environmental toxins. Heavy metals cause a significantly serious damage on human health. Cadmium is associated with bone and kidney diseases and Pb with neurological disorders. Excess Cu and Zn in the diet are associated with metabolic disorders potentially resulting in death.

While the harmful health effects of carbon monoxide, nicotine, tar, irritants and other noxious gases that are present in tobacco smoke are well known, those due to heavy

metals and other toxic mineral elements in tobacco smoke are not sufficiently emphasized. These metals are hazardous to human health. One source of toxic metals in our environment, and main reason for these metals accumulating in the body, is tobacco smoke. Cigarette smoking may be a substantial source of intake of these hazardous elements not only to the smoker but also, through passive smoking, to nonsmokers. The concern about trace metals is mainly due to their effect on human health. The pathways of metals to humans that cause the greatest concern, as they may expose many people, are the food chain and leaching into ground water [3]. Due to the healthy effect of these metals, several studies have been carried out on determinations of the level of trace metals in tobacco in the different parts of the world using different techniques. However, to the best of our knowledge, there is no report in the literature on the determination of levels of trace metals in Ethiopian Tobacco leaves. Hence this research project is intended to determine levels of trace metals, four of them are toxic (Cd, Ni, Pb Cr) and two of them are essential (Cu, Zn), in Ethiopian tobacco leaves and processed tobacco.

1.1. Literature Review

1.1.1. The Origin of Tobacco

The prehistory of tobacco begins in the Central America before birth of Christ. But the written history of tobacco begins on October 12, 1492 when Christopher Columbus reached the beaches of San Salvador in the West Indies [4]. Native Americans used tobacco before Europeans arrived in America, and early European settlers in America learned to smoke and brought the practice back to Europe, in the 15th and 16th centuries, where it became hugely popular. It was first smoked in pipes, then later used as snuff and smoked as cigars. Cigarettes became fashionable among upper class men in the late 18th century. The first introduction in to East Africa is thought have been of *Nicotiana rustica* about 1560, Portuguese was responsible [5]. In the late 1900s, machines were invented to mass-produce cigarettes [6].

1.1.2. Species of Tobacco

The genus Nicotiana, a member of the plant family Solanaceae, is represented by about 100 species and sub-species widely distributed throughout the world. The species *N. tabacum* and *N. rustica***,** commonly used for consumption, are the principal sources of tobacco [7]. Tobacco is usually a long-day plant, with a maximum height of 25 m, though the length and shape of leaf depends on the type of tobacco. Until harvesting tobacco, up to 25 leaves may be produced on alternate side of the stem. The vast majority of the world's tobaccos and virtually all, which enters world trade, is *Nicotiana Tabacum* [8]. This is classified as: Virginia, Oriental and Burley

1.1.2.1. Virginia Tobacco

It is collective name of certain sorts of tobacco, originating in what is now state of Virginia. Virginia type tobacco is about 40% world tobacco production. Virginia (flue-cured) is thus the main source of cigarette tobacco today. Major producers of this type tobacco in the world are China, USA, Brazil, India and Zimbabwe. The major exporters are the U.S., Brazil, India and Zimbabwe. It grows to a height of 1.5 to 2.5 m and have leaves about 50-70 cm long and about 30-40 cm wide [6].

Figure 1. Virginia type tobacco planted in Ethiopia.

1.1.2.2. Oriental Tobacco

Oriental tobacco is mainly grown in the countries of the eastern mediterranians (orient), hence the name. Oriental type tobacco is about 16% world tobacco production. Oriental leaf is characterized by its small size; leaf length is 7.6 to 25.4 cm and is 2-3 times the width. Average plant heights are 1 to 1.5 m. The leaves are sun-cured. The largest producers are former USSR, Turkey, Bulgaria, Greece, former Yugoslavia, Romania and Italy and largest importers are the U.S., Japan and Germany.

1.1.2.3. Burley Tobacco

Burley is light air-cured type derived from the White Burley, which arose as a mutant on a farm in Ohio in 1864. Burley is used primarily in cigarette blends. Some of the heavier leaf is used in pipe blends and for chewing. Burley leaf is characterized by high ash content. This type tobacco is about 11% of world production. The main producers and trades are the U.S., Italy, Korea, Brazil, and Mexico.

1.1.3. Distribution of Tobacco in the World

Tobacco is grown in more than 100 countries. Asia, at around 60 % of the total, is the main unmanufactured tobacco-producing region with China alone accounting for 36%. China is the world's leading producer. Other principal suppliers are the United States, India, Brazil, Turkey, Zimbabwe and Malawi. Malawi is an important producer of tobacco, but it exports 98 per cent of its crop. The Netherlands grows no tobacco of its own. Yet it is one of the world's top exporters of cigarettes and cigars. The United States is a leading importer and exporter of tobacco as well as a leading exporter of cigarettes. And there is China, the world's largest producer of raw tobacco, and the world's largest consumer and producer of cigarettes whose participation in world trade of tobacco and cigarettes is very modest [8,9].

Top ten tobacco leaf producers in year 2005 were China, Brazil, India, United States, Indonesia Turkey, Greece, Argentina, Italy, Pakistan and their productions in million metric ton are; 2.51, 0.88, 0.60, 0.29, 0.14, 0.14, 0.12, 0.12, 0.11, 0.08, respectively [10].

Table 1. Leading tobacco leaf producers in year 2001 (in thousands of metric tons) [11].

Country	**Thousands of metric tons**
China	2,661
India	701
Brazil	568
USA	450
Turkey	260

1.1.4. Distribution of Tobacco in Ethiopia

The three main types of commercial tobacco produced in Ethiopia are Virginia, oriental and burley. These types of tobacco are grown for commercial purposes by state-owned farms and by farmers around these farms. Shoa Robit, Awasa, Bilatte and Wollaita are the four tobacco farm stations. The four plantation areas cover 1,258 hectares of land. Awassa, Bilatte, Wollaita and Shoa Robit cover 250, 200, 100 and 708 hectares with annual yield of 250,000, 100,000, 150,000 and 850,000 kg/year, respectively. Virginia is grown only in Shoa Robit and Bilate. That is the farm station at Shoa Robit and Bilatte are totally covered by Virginia type tobacco. The average annual production tobacco leaves in the last three years (2003-2005) is estimated to be 1,193,870 tons. In these years Virginia production accounts for about 76.9 % of the total production followed by oriental, 19.4 %, and burley, 3.7 % [12- 13].

1.1.5. Production of Cigarette in Ethiopia

Nyala and Gissila brands have been in the market while Elleni and Delight brands were recently launched in to the market. Nyala, which is totally manufactured from Virginia type tobacco, accounts for over 86.3% of the total production by quantity and 94.5% of

the total sales value of the Ethiopian NTE. Looking at the supply side of leaf tobacco to the local cigarette manufacturing only some 45-50% of filter tobacco leaves requirements of the factory are met locally by the farm stations, the flavor tobacco is met through importation [13].

1.1.6. Use of Tobacco

Tobacco and its uses were unknown outside America before its discovery by Columbus. There are six types of tobacco use: chewing, drinking, licking, rectal insertion, nasal and oral snuffing, and smoking. Smoking is by far the most common, while rectal application is only used occasionally. Alternatively, it was swallowed and belched back, blown from one person to another, or blown into the eyes. Leaves are chewed, alone or mixed with ash, powdered shells or honey, or they are held in the mouth and sucked. The most common one is smoking. The primary intention in using tobacco is to obtain the alkaloid nicotine and, once the habit has been established, nicotine appears to fulfill both a pharmacological and psychological need [7].

Figure 2. Bright leaf tobacco ready for harvest

Figiure 3. Cigarette for smoking

1.1.7. Harvesting of Tobacco

Tobacco is harvested in one of two ways. In the oldest method, the entire plant is harvested at once by cutting off the stalk at the ground with a curved knife. In the nineteenth century, bright tobacco began to be harvested by 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, and the stalks may be turned into the soil. The first crop at the very bottom of the stalks are called "sand lugs", as they are often against the ground and are coated with dirt splashed up when it rains [4, 6].

1.1.8. Topping and Suckering

At a certain stage of maturity, the plant will produce a flower cluster from its tip, as well as the tips of any suckers that remain on the plant. In order to divert more energy into the leaves, the plant is "topped" the top is cut off. Once the tobacco plants are growing well, they will begin to produce shoots from the joint of each leaf with the stalk.These secondary shoots are known as "suckers" are undesirable as they divert energy that could be directed into the leaves. They are removed in a process known as "suckering" [4, 6, 18].

1.1.9. Curing

Cut plants or pulled leaves are immediately transferred to tobacco barns, where they will be cured. Curing methods varies with the type of tobacco grown, and tobacco barn design varies accordingly. Air-cured tobacco is hung in well-ventilated barns and allowed to dry over a period of weeks. Fire-cured tobacco is hung in large barns where smoldering fires of hardwoods are kept burning. 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 to the roof, heat-curing the tobacco without exposing it to smoke.

Curing and subsequent aging allows for the slow oxidation and degradation of carotenoids in tobacco leaf. This produces certain compounds in the tobacco leaves very similar and give a sweet hay, tea, rose oil, or fruity aromatic flavor that contribute to the "smoothness" of the smoke. Starch is converted to sugar which glycates protein and is oxidized into advanced glycation end products (AGEs), a caramelization process that also adds flavor. After tobacco is cured, it is moved from the curing barn into a storage area for processing. For both cut and pulled tobacco, the leaves are then sorted into different grades [4, 6, 18].

1.1.10. Economic Importance of Tobacco in the World

Tobacco's business is the second in the World economy next to petroleum. The annual global transaction in tobacco is estimated USD 400 billion. The economic importance of tobacco growing and processing differs from country to country. At the nation's level, cigarette (sales and import) tax can be a main source of government revenue. In Russia, cigarette tax revenue contributes around 8 % to the financing of the state budget. In China, profits from state-owned amounted to the equivalent of USD 11,000 million in 1999. Japan Tobacco earned more than USD 400 million for the Japanese State in the financial year ending March 2000. Many low-income countries rely on the export of cash crops such as tobacco to pay for the service of their foreign debt. Tobacco exports made up close to 10 % of Cuba's exports in 1997-98. In Tanzania it was 15 %, in Zimbabwe over 25 % and in Malawi tobacco exports made up two-thirds of commodity exports. The cigarette companies have also been a prime source of investment in the formerly centrally-planned countries of Central and Eastern Europe, and Central Asia. Tobacco growing, processing and exports can make a significant contribution to national employment and national income. In Italy, several of the state monopoly's factories are placed in areas hit by high unemployment [9].

In Ethiopia, NTE is one of the leading companies that contributes highly to the government's annual cash out-flows (about 170 million Birr) annually in the form of VAT. Ethiopia does not export any tobacco products, but imported 600 tons of unmanufactured tobacco and around 200 million cigarettes in 1990. In 1990, Ethiopia

spent USD 7.1 million importing tobacco (0.6% of all import costs), more than double the amount spent in 1985 [12, 13]. No information is available about the recent import amount of tobacco leaf today.

1.1.11. Health Effect of Tobacco and Its Interaction with Other Chemicals

Medical research has determined that smoking is a major contributing factor towards many health problems, particularly lung cancer, emphysema, and cardiovascular disease. The International Agency for Research on Cancer (IARC) currently lists 44 individual chemical agents as "Group 1 human carcinogens" and nine of the 44 chemical agents classified as "Group 1 carcinogens" by IARC have been reported to occur in mainstream cigarette smoke. The nine agents reported are benzene, cadmium, arsenic, nickel, chromium, 2-naphthyl-amine, vinyl chloride, 4-aminobiphenyl and beryllium. The ranges that have been reported for each of the nine compounds, in micrograms/cigarette, are: benzene (0.05-104), cadmium (0-6.67), arsenic (0-1.4), nickel (0-0.51), chromium (0.0002-0.5), 2-naphthylamine (0.0002-0.022), vinylchloride (0.0013-0.0158), 4-aminobiphenyl (0.00019-0.005) and beryllium (0-0.0005). The mechanism of their carcinogenetic is well known: oxidation produces an epoxide which binds to DNA and permanently distorts it. DNA damage is the cause of cancer [6].

Tobacco smoke is an aerosol consisting of a particulate phase of liquid droplets dispersed in a gas/vapour phase. These either pass through the cigarette as mainstream smoke, or some being condensed a short distance behind the burning cone, or they are emitted into the air from the burning end as side-stream smoke. When it is inhaled, a cigarette burns at 700°C at the tip and around 60°C in the core. This heat breaks down the tobacco to produce various toxins compounds by pyrolysis of the tobacco [39]. The main point of entry of cigarette smoke into the body is via the airways, but many constituents, particularly from pipe and cigar smoke, dissolve in saliva and are absorbed in the buccal cavity or are swallowed. Cigar and pipe smokers generally do not inhale the smoke and it remains in the oral cavity, is dissolved in the saliva and absorbed through the mucous

membranes or swallowed. Alcoholic drinks have a solvent effect for the smoke constituents facilitating their absorption [14].

Tobacco use, particularly smoking, causes a range of adverse health effects, is directly implicated in a number of serious diseases, and can increase adverse effects of other chemical, physical and biological agents. Working conditions can impact adversely on health to a greater extent in smokers than non-smokers. There is evidence for synergism in the production of adverse effects (cancer) between tobacco smoking and exposure to asbestos ethanol, silica and radiation (radon, atomic bomb, X-ray), trace metals like arsenic, cadmium, nickel and lead. On the other hand, there is evidence for antagonism in the case of tobacco smoking and the carcinogenic chloromethyl ethers. Tobacco smoking affects the health risks of exposures in coal mining, pesticide handling, and in the rubber and petroleum industries. Coal miners who smoke are at greater risk of developing chronic bronchitis and obstructive airway disease but not emphysema. Lung cancer in coal miners has been attributed entirely to tobacco smoking [7].

1.1.12. Composition of Tobacco Leaves and Tobacco Smoke

Tobacco consists of carbohydrates and proteins. Other important constituents are alkaloids (0.5 to 5 %), with nicotine as the predominant compound (90 to 95 % of total alkaloids), and terpenes (0.1 to 3 %), polyphenols (0.5 to 4.5 %), phytosterols (0.1 to 2.5 %), carboxylic acids (0.1 to 0.7 %), alkanes (0.1 to 0.4 %), and alkali nitrates (0.01 to 5 %). In addition, tobacco contains traces of aromatic hydrocarbons, aldehydes, ketones, amines, nitriles, N- and O-heterocyclic compounds, pesticides, and more than 30 metallic compounds.

In general, more 4000 chemical compounds have been isolated from processed tobacco leaf. Most are leaf constituents, but some arise from growing conditions such as the soil and atmosphere in an area, while others originate from the use of agricultural chemicals, from casings, humectants and flavourings added to the leaves, from curing methods and

chemical compounds that produced as tobacco burns. When tobacco is burned in the course of smoking, many pyrolysis and other reaction products are formed [7].

1.1.13. Plants Nutrition

Plant uses mineral nutrients to build new parts of it and to carry on a wide range of chemical activities that take place in cell. The mineral nutrients are divided into two groups: macronutrients and micronutrients. Macronutrients can be broken into two more groups: primary and secondary nutrients. The primary nutrients are N, P, and K. These major nutrients usually are lacking from the soil because plants use them in large amounts for their growth and survival. The secondary nutrients are Ca, Mg, and S. There are usually enough of these nutrients in the soil so fertilization is not always needed. Micronutrients are those elements essential for plant growth, which are needed in only very small (micro) quantities. These elements are sometimes called minor elements or trace elements. The micronutrients are B, Cu, Fe, Cl, Mn, Mo and Zn [15].

On the other hand, from the biological viewpoint, metallic elements that are considered to be mineral nutrients of plants are most commonly classified into four [15-18]; these are (i) essential macronutrients, (Ca, Mg and K), are those without which plants can not complete their life cycle; (ii) essential micronutrients or trace metals, (Fe, Mn, cu, Zn, Mo, Co, V, Na, and Ga), which are needed by plants in quantities much smaller than the macronutrients and they are usually functioning in plants as components of enzyme systems [32]; (iii) essentiality not demonstrated metals, (Cr, Sr and Ni); and (iv) beneficial metals, (Al, Sr and Rb). However, their amount in plants body depends on many factors; upon their presence in air and soil, species, age, root distribution of the plant, physical and chemical nature of the soil, proportions and distributions of the elements and the general climatic conditions [16, 18].

According to Arnon and Stout (1939) for the plants nutrition to be essential: three criteria must be met. These criteria are: the plant must be unable to complete its life cycle in the absence of the mineral element; the function of the element must not be replaceable by

another mineral element; and the element must be directly involved in plant metabolism. Beneficial elements are those that can compensate for toxic effects of other elements or may replace mineral nutrients in some other less specific function such as the maintenance of osmotic pressure [19].

1.1.14. Metals in the Soil and Their Transportation in to the Plants

Metals are natural components in soil. Contamination, however, has resulted from industrial activities, such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, and generation of municipal waste. In soil, metals are associated with several fractions. These are:

1. In soil solution, as free metal ions and soluble metal complexes.
2. Adsorbed to inorganic soil constituents at ion exchange sites.
3. Bound to soil organic matter.
4. Precipitated as oxides, hydroxides, carbonates.
5. Embedded in structure of the silicate minerals.

Bioavailability of metals depends on their solubility in soil solution. Only metals associated with fractions 1 and 2 (above) are readily available for plant uptake. Some metals, such as Zn and Cd, occur primarily in exchangeable, readily bioavailable form. Cu and Pb have low transfer coefficient, and are stably bounded to the soil. Pb, occur as soil precipitate, a significantly less bio available form [1, 20, 21].

Nutrients in the soil can be transported by two different Mechanisms: by mass flow and by diffusion. Mass flow occurs when solute are transported with the convective flow of water from the soil to plant roots. The amount of nutrients reaching the root is thus dependent on the rate of water flow or the water consumption of the plant and the average nutrient concentration of water. Diffusion comes in to effect when the concentration at the root surface is either higher or lower than that of the surrounding solution. It is directed towards the root when the concentration at the root surface is decreased and a way from the root when is increased [23, 24].

1.1.15. Up Take and Accumulation of Metals by Plants

Plants have evolved highly specific mechanisms to take up, translocate, and store metal nutrients. The uptake mechanism is selective, plants preferentially acquiring some ions over others. Ion uptake selectivity depends upon the structure and properties of membrane transporters. These characteristics allow transporters to recognize, bind and mediate the trans-membrane transport of specific ions. For example, some transporters mediate the transport of divalent cations, but do not recognize mono- or trivalent ions.

In higher plants the elements enter the root of plants from the soil and move across the root, either through cell or between cells, until they reach specialized conducting cells of the vascular system. The cells of the vascular system are generally tubular, and connected end to end with similar tubular cells in a network reaching through out the plant [25].

Because of their charge, metal ions cannot move freely across the cellular membranes, which are lipophilic structures. Therefore, ion transport into cells must be mediated by membrane proteins with transport functions, generically known as transporters. Trans-membrane transporters possess an extracellular binding domain to which the ions attach just before the transport, and a trans-membrane structure which connects extracelluar and intracellular media. The trans-membrane structure facilitates the transfer of bound ions from extracellular space through the hydrophobic environment of the membrane into the cell. In many instances trace elements move through biological membranes in the form of complexes of chelates with organic ligands.

Uptake of metals into root cells, the point of entry into living tissues, is a step of major importance for the process of phytoextraction. However, for phytoextraction to occur metals must also be transported from the root to the shoot. Movement of metal-containing sap from the root to the shoot, termed translocation, is primarily controlled by two processes: root pressure and leaf transpiration. Following translocation to leaves, metals can be reabsorbed from the sap into leaf cells. A schematic representation of metal transport processes that take place in roots and shoots is shown in Figure 4.

1. A metal fraction is sorbed at root surface.
2. Bioavailable metal moves across cellular membrane into root cells.
3. A fraction of the metal absorbed into roots is immobilized in the vacuole.
4. Intracellular mobile metal crosses cellular membranes into root vascular tissue (xylem).
5. Metal is translocated from the root to aerial tissues (stems and leaves).

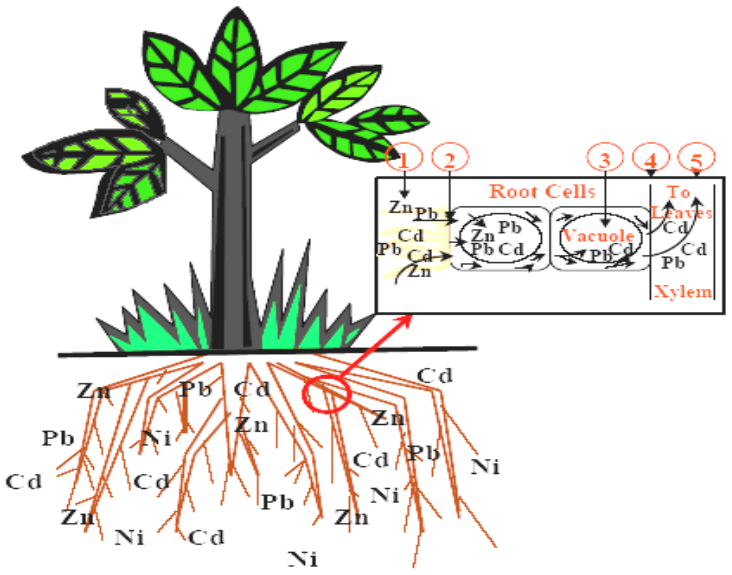


Figure 4. Metals uptake and accumulation in plants [22].

By this mechanism plants accumulate trace elements, especially heavy metals in or on their tissues. Thus plants are intermediate reservoirs through which trace elements from soil, and partly from waters and air move to man and animals. Tobacco has an exceptional capacity to accumulate metals such as Pb, Cd, and Zn in high heavy metal exposure conditions, while under lower-heavy metal exposure condition tobacco leaf is apparently similar to other leaf crops in heavy metal accumulation potential. The experimental data obtained for the presence of Pb, Cd, and Zn in the different parts of tobacco grown in an industrially polluted region showed that their amounts were mainly due to the heavy-metal containing aerosols falling from the atmosphere. Part of them, however, got into the soil, and from there penetrated via the root system into the tobacco

plants and accumulated into their different aboveground parts; particularly leaf which is used for cigarette production [25]. On burning tobacco, metals remain largely in the ash unless they become vaporized or transferred in to into the smoke stream contained in micro fragments of ash. Of the main concern with respect to the carcinogenicity of metallic smoke constituents is the amount of such component appearing in the mainstream [26]. The metals of interest whose accumulation or level in tobacco are going to be analysed are discussed briefly as the follows.

1.1.16. Cadmium

The average natural abundance of cadmium in the earth's crust has most often been reported from 0.1 to 0.5 ppm, but much higher and much lower values have also been cited depending on a large number of factors. Igneous and metamorphic rocks tend to show lower values, from 0.02 to 0.2 ppm whereas sedimentary rocks have much higher values, from 0.1 to 25 ppm. Naturally, zinc, lead and copper ores, which are mainly sulphides and oxides, contain even higher levels, 200 to 14,000 ppm for zinc ores and around 500 ppm for typical lead and copper ores. Fossil fuels contain 0.5 to 1.5 ppm cadmium, but phosphate fertilisers contain from 10 to 200 ppm cadmium. The use of cadmium-containing fertilisers and sewage sludge is most often quoted as the primary reason for the increase in the cadmium content of soils [27].

The largest source of cadmium release to the general environment is the burning of fossil fuels (such as coal or oil) or the incineration of municipal waste materials [28]. Phosphate fertilizers can contain high Cd levels due to the presence of cadmium in the phosphate rock used for their manufacture. In order to investigate the Cd concentration in phosphate fertilizers used for tobacco production, fertilizers were sampled worldwide and analyzed for Cd and phosphorus. Concentrations ranged from 0.08 ± 0.14 to 97.50 ± 8.74 g Cd/ton P_2O_5. The use of fertilizers containing high Cd concentrations should be avoided to protect the soil from gradually accumulating this element and to avoid possible additional Cd in tobacco leaves [3, 29].

Cadmium is more readily absorbed by plants than Pb. Fruit and seeds contain less Cd than leaves [30]. Tobacco plants can enrich the toxic element, Cd, from the environment-polluted soil, air and water. The amount of cadmium in tobacco depends on the variety and origin of the plant as well as on the analytical method used to determine cadmium. In the literature, cadmium concentrations in tobacco between 0.5 and 5 ppm are reported [31]. Tobacco plant leaves can accumulate Cd from the soil 10 times, than other plants' leaves [32].

Tobacco leaves naturally accumulate and concentrate relatively high levels of cadmium, and therefore smoking of tobacco is an important source of air cadmium exposure for smokers. There is slight evidence that the lower leaves of tobacco contain greater concentration of Cd than the upper part, the tips, probably due to the contamination from the soil and sand. As reported in the annual report of the research activity by Italian Tobacco Agency from September 2002-March 2003; the concentration of Cd at tips part of tobacco plant is reported to be 1.27 mg/kg where as at the lugs position is 1.97 mg/kg [33].

There are several reports of investigations, which showed that the uptake of cadmium by plant is very much influenced by zinc. Average cadmium levels in cigarettes range from 1,000 to 3,000 ppb. It has been reported that one cigarette contains about 1-2 μg of cadmium and that about 10% of the cadmium content is inhaled when the cigarette is smoked. It has been estimated that about 50% of cadmium inhaled in cigarette smoke is absorbed [27]. Cigarette construction, the use of filters and variations in the cadmium contents of tobaccos could decrease cadmium exposure by this route [7, 34]. The mean amount of Cd absorbed via the airways by smoking for smokers in Japan was calculated to be 0.89–1.78 μg/day. The values are not small in comparison with the amount of Cd absorbed from the digestive organs [35].

Cadmium can enter the blood by absorption from the stomach or intestines after ingestion of food or water, or by absorption from the lungs after inhalation. Very little cadmium enters the body through the skin. Usually only about 1 to 5% of what is taken in by

mouth is absorbed into the blood, while about 30 to 50% of that which is inhaled is taken up into the blood. However, once cadmium enters the body, it is very strongly retained; therefore, even low doses may build up significant cadmium levels in the body if exposure continues for a long time.

Cadmium may actually displace zinc in some of its important enzymatic and organ functions; thus, it interferes with these functions or prevents them from being completed. The zinc-cadmium ratio is very important, as cadmium toxicity and storage are greatly increased with zinc deficiency, and good levels of zinc protect against tissue damage by cadmium. Though cadmium has no known useful biological functions, it competes with zinc for binding sites and can therefore interfere with some of zinc's essential functions. In this way, it may inhibit enzyme reactions and utilization of nutrients. Cadmium may be a catalyst to oxidation reactions, which can generate free-radical tissue damage [7].

1.1.17. Nickel

Nickel occurs in the environment naturally, but is also discharged into the environment by man’s activities. Soil usually contains 4-80 ppm of nickel. The highest soil concentrations (up to 9,000 ppm) are found near industries that extract nickel from ore [36]. Food contains nickel and is the major source of nickel exposure for the general population. Our daily intake of nickel from drinking water is only about 2 µg. We breathe in between 0.1 and 1 µg nickel/day, excluding nickel in tobacco smoke.

Since the body’s requirement for Ni is so low, an excessive exposure from the environment, leading to toxicity, is very common, e.g. from tobacco, dental implants, stainless steel kitchen utensils and inexpensive jewellery. Toxicity by consumption, on the other hand, is rare and would require 1000 times the amount normally consumed in food [37]. An excess of Ni in tissues has several effects. It can cause damage to chromosomes and other cell structures, alter hormone and enzyme activities, and affect membrane permeability and immune function. As a result, changes in glucose tolerance, blood pressure, response to stress, growth rate, bone development, and resistance to infection are possible [39]. It is irritant to the skin, and is toxic to the cardiovascular

system, as well as being carcinogenic. However, poisoning by nickel and its salts is very rare.

The tobacco plant contains nickel most probably absorbed from the soil, fertilizing products or from pesticides. Smoking presents a significant form of exposure. The research was conducted in period 2000-2003 in Institute of Public Health by using electrothermal atomization technique. The results obtained, revealed a high content of nickel in cigarettes (2.32-4.20 mg/kg) and in tobacco leaf (2.20-4.91 mg/kg) regardless of the kind and the origin of tobacco. Nickel content in the blood of smokers (0.01-0.42 μg/L) was higher than in the blood of non-smokers (0.01-0.26 μg/L) although this difference was not statistically significant. In the urine of smokers (<0.01-8.20 μg/L) there was a significantly higher concentration of nickel than in the urine of non-smokers (<0.01-4.60 μg/L). The exposure of smokers to nickel through tobacco smoke was high regardless of the kind and the origin of tobacco and cigarettes. The content of nickel in tissue fluids established by biomonitoring shows that smokers can be far more exposed to this carcinogenic substance than non-smokers and that health risks for smokers are higher in this context [39].

It has been stated that nickel in a burning cigarette might form the volatile, gaseous compound, nickel tetracarbonyl, and thereby be introduced into the respiratory tract. Sunderman and Sunderman indicated that nickel in a burning cigarette might form the volatile, gaseous compound, nickel tetracarbonyl, which is carcinogenic at very low doses. They also stated that about 20% of nickel in the tobacco might be transferred to the mainstream smoke [38]. Kreyberg concluded that tobacco smoking and nickel exposure caused an additive effect for developing lung cancer in workers at a Norwegian nickel refinery [38]. Another report from the same refinery suggested that simultaneous smoking and nickel exposure might lead synergistically to increased risk of lung cancer.

Even though some previous authors have taken for granted that nickel in burning tobacco might be transferred to the mainstream smoke as the gaseous nickel tetracarbonyl, this chemical compound as such has never been recovered in the mainstream smoke. It is demonstrated that only negligible amounts of nickel in cigarette tobacco are likely

transferred and introduced to the respiratory tract. According to Torjussen *et al.*, chemically, nickel tetracarbonyl is formed by the reaction between finely divided metallic nickel and carbon monoxide under atmospheric pressure at low temperatures well below 100 ^{0}C [41]. Above 150 ^{0}C the nickel tetracarbonyl starts to dissociate and is fully decomposed in metallic nickel and carbon monoxide at 200 ^{0}C. Moreover, in the burning zone of the cigarette temperatures of 400–700 ^{0}C is reported. According to these authors, the average concentration of Ni in 10 unsmoked cigarettes was 1.80 μg/g. But in the ash, after smoking 15 cigarettes there is 1.75 μg g^{-1} identified. They concluded that most of the tobacco nickel was recovered in the ash. 1.1% or even less of nickel in the mainstream smoke after smoking the entire cigarettes without leaving any butt [38, 40].

1.1.18. Lead

Lead is added to soils by fallout from the atmosphere, but it is also added to soils by either accidental or deliberate dumping of lead containing wastes on soils, or from the addition of pesticides and fertilizers that contain lead [37].

The extent of occurrence of lead in the earth's crust is about 15 g/ton, or 0.002%. Significant increases in the Pb content of cultivated soils have been observed near industrial areas [39]. When released to soil, lead is normally converted from soluble lead compounds to relatively insoluble sulfate or phosphate derivatives. It also forms complexes with organic matter and clay minerals which limits its mobility in the soil. Concentrations of lead in soil solution reach a minimum between pH 5 and 6 because metal-organic complexes form in this pH range. Only a small fraction of lead in lead-contaminated soil appears to be in water-soluble form (0.2-1%). Lead uptake by plants is favored at lower pH values and in soils with low organic carbon content [38]. Pb tends to accumulate in the surface ground layer and its concentration decreases with soil depth.

Lead uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of Pb whilst simultaneously greatly restricting its translocation to above ground parts. It is agreed that the bulk of the Pb taken up by plants remains in the

roots. Absorption of Pb (as Pb) in soil increases with increasing pH between 3.0 and 8.5. However, Blaylock and coworkers [1] reported that in soil with a pH between 5.5 and 7.5 Pb solubility is controlled by phosphate or carbonate precipitates and very little Pb is available to plants even if they have the genetic capacity to accumulate it. Pb in soil is classified as a weak Lewis acid, which implies a strong covalent character to many of the ionic bonds it forms in soils and plants. Pb present in the soil is nearly always tightly bound to organic or colloidal material or in a precipitated form, all of which serve to reduce the uptake of Pb by plant roots. Pb retention in the roots is based on binding of Pb to ion exchangeable sites on the cell wall and extracellular precipitation, mainly in the form of Pb carbonate deposited in the cell wall. After being taken up by roots, the localization of Pb is greater in roots than in other parts of the plants.

Uptake and accumulation rates of lead vary among species and within species. Tobacco *(Nicotiana tabacum)* leaves accumulate between 24 and 49% of total soil lead concentrations [2]**.** The tobacco plant absorbs lead from the soil and around 5-6% of that in cigarettes is inhaled in the smoke. Lead concentrations in the smoke from one cigarette were found to range from 0.017 to 0.98 µg [40]. Higher blood lead and erythrocyte protoporphyrin levels have been demonstrated in heavy smokers exposed to lead; these could have been partly due to contaminated cigarettes acting as vectors. Other studies on occupationally exposed workers showed a progressive increase in blood lead with an increase in the number of cigarettes smoked [7].

Lead has no known biological function, and is highly toxic and accumulates in man. Lead is absorbed by foodstuffs (particularly green leafy vegetables) growing on soil where lead is present. There is also evidence, which supports that the lower leaves of tobacco contain greater concentration of metals than the upper part, the tips. As reported in the Annual report of the research activity by Italian Tobacco Agency from September 2002-March 2003; the concentration of Pb in tips leaf part of tobacco plant is reported to be 22.31/kg where as at the lugs position is 32.20 mg/kg [3].

1.1.19. Zinc

Zinc is one of the essential micronutrients required for optimum crop growth. The movement of zinc is highly dependent upon the soil moisture, and this may be the reason why, particularly in arid and semi-arid areas, zinc deficiency is more frequently seen. The solubility of zinc is highly dependent upon soil pH. Presence of calcium carbonate decreases the availability of zinc due to higher soil pH. The poor zinc availability in alkaline calcareous soils is precisely due to the formation of zinc carbonate. High levels of soil phosphorus are also commonly responsible for zinc deficiency. Presence of excess amount of copper can also reduce zinc availability because the absorption of both cations is through the same mechanism, which causes interference in the uptake. On the contrary, application of magnesium can enhance zinc availability and uptake by the roots. According Toward in 1941, the average content of Zn in commercial tobacco is 51-84 ppm [4]. Cogbill and Hobbs in 1957 reported that 24-33 ppm of Zn in tobacco and 0.7-2.5 ppm in smoke [26].

Zinc is an essential component of various enzyme systems for energy production, protein synthesis, and growth regulation. Zinc deficient plants also exhibit delayed maturity. Zinc is not mobile in plants so zinc-deficiency symptoms occur mainly in new growth. Poor mobility in plants suggests the need for a constant supply of available zinc for optimum growth. The most visible zinc deficiency symptoms are short internodes and a decrease in leaf size. Delayed maturity also is a symptom of zinc-deficient plants.

Zinc deficiencies are mainly found on sandy soils low in organic matter and on organic soils. Zinc deficiencies occur more often during cold, wet spring weather and are related to reduced root growth and activity as well as lower microbial activity decreases zinc release from soil organic matter. Zinc uptake by plants decreases with increased soil pH. Uptake of zinc also is adversely affected by high levels of available phosphorus and iron in soils.

Zinc deficiency most often occurs when zinc intake is inadequate or poorly absorbed, when there are increased losses of zinc from the body, or when the body's requirement

for zinc increases. Signs of zinc deficiency include growth retardation, hair loss, diarrhea, delayed sexual maturation and impotence, eye and skin lesions, and loss of appetite. Zinc toxicity has been seen in both acute and chronic forms. Intakes of 150 to 450 mg of zinc per day have been associated with low copper status, altered iron function, reduced immune function, and reduced levels of high-density lipoproteins (the good cholesterol). Since Cd binds to sulfhydryl groups more tightly than Zn, it can easily inhibit Zn-metalloenzymes by displacing Zn from enzyme active sites. This competition between Cd and Zn is evidenced by the fact that excess Zn can prevent Cd toxicity, and Zn deficient animals are more susceptible to Cd's toxic effects.

Zinc is an essential element, necessary for sustaining all life. It is estimated that 3000 of the hundreds of thousands of proteins in the human body contain zinc prosthetic groups. It stimulates the activity of approximately 100 enzymes, which are substances that promote biochemical reactions in our body. Zinc supports a healthy immune system, is needed for wound healing, helps maintain our sense of taste and smell, and is needed for DNA synthesis. Zinc also supports normal growth and development during pregnancy, childhood, and adolescence. It is also required in plants for leaf formation, the synthesis of indole acetic acid (auxin) and anaerobic respiration (alcoholic fermentation)

1.1.20. Copper

Copper is a metal that occurs naturally throughout the environment, in rocks, soil, water, and air. It can enter the environment through releases from the mining of copper and other metals, and from factories that make or use copper metal or copper compounds. Copper can also enter the environment through waste dumps, domestic waste water, combustion of fossil fuels and wastes, wood production, phosphate fertilizer production, and natural sources (for example, windblown dust, from native soils, volcanoes, decaying vegetation, forest fires, and sea spray). Therefore, copper is widespread in the environment. Soil generally contains between 2 and 250 ppm copper, although concentrations close to 17,000 ppm has been found near copper and brass production facilities [1].

Copper is more available at low pH levels than at high pH levels because metals are bound very tightly to the soil or exist in solid minerals at high pH, that is copper uptake decrease as soil pH increases. Some copper in the environment is less tightly bound to soil or particles in water and may be soluble enough in water to be taken up by plants and animals [42]. Copper deficiencies are mainly reported on sandy soils, which are low in organic matter. Increased phosphorus, and iron availability in soils decreases copper uptake by plants. Even though the content of Cu in tobacco depends on different factors, according to Collins in 1961, a flue-cured tobacco contains Cu in the range of 51-84 ppm [4]. Cogbill and Hobbs in 1957 reported that 17 –37 ppm of Cu in tobacco and only 1-4.6 ppm in smoke. However, Voss and Nicol found that 76.6 ppm Cu in tobacco [26].

Copper is necessary for carbohydrate and nitrogen metabolism and, inadequate copper results in stunting of plants. Copper is also required for lignin synthesis which is needed for cell wall strength and prevention of wilting. Deficiency symptoms of copper are dieback of stems and twigs, yellowing of leaves, stunted growth and pale green leaves that wither easily.

Copper can be taken into our body upon ingestion of water or soil that contains copper or by inhalation of copper-containing dust. Copper is essential for good health. It is involved in numerous biochemical reactions in human cells. Copper is a component of multiple enzymes, and it is involved with the regulation of gene expression, mitochondrial function cellular metabolism, connective tissue formation, as well as the absorption, storage, and metabolism of iron. However, exposure to higher doses can be harmful. Long term exposure to copper dust can irritate your nose, mouth, and eyes, and cause headaches, dizziness, nausea, and diarrhea. EPA does not classify copper as a human carcinogen because there are no adequate human or animal cancer studies. The Food and Nutrition Board of the Institute of Medicine has developed recommended dietary allowances (RDAs) of 340 micrograms (μg) of copper per day for children aged 1–3 years, 440 μg/day for children aged 4–8 years, 700 μg/day for children aged 9–13 years, 890 μg/day for children aged 14–18 years, and 900 μg/day for adults [43].

1.1.21. Chromium

Chromium occurs naturally in the Earth's crust. Continental dust is the main source of exposure to natural chromium present in the environment. As a result of human activities, however, chromium is released into the environment in larger amounts. The general population is exposed to chromium by eating food or food supplements, drinking water, and inhaling air that contain chromium [44].

Chromium is a highly toxic non-essential metal for microorganisms and plants. Due to its widespread industrial use, chromium (Cr) has become a serious pollutant in diverse environmental settings. The hexavalent form of the metal, Cr (VI), is considered a more toxic species than the relatively less mobile Cr (III) form [47, 48]. The presence of Cr in the environment has selected microbial and plant variants able to tolerate high levels of Cr compounds. The diverse Cr-resistance mechanisms displayed by microorganisms, and probably by plants, include biosorption, diminished accumulation, precipitation, reduction of Cr (VI) to Cr (III), and chromate efflux [49]. Some of these systems have been proposed as potential biotechnological tools for the bioremediation of Cr pollution. Chromium causes growth retardation, reduces the number of palisade and spongy parenchyma cells in leaves, results in clotted depositions in the vascular bundles of stems and roots, and increases the number of vacuoles and electron dense materials along the walls of xylem and phloem vessels.

Under normal conditions, the concentration of Cr in the plant is < 1 μg /g. Chromium uptake by plants is mainly non-specific, probably as a result of plant uptake of essential nutrients and water. Plants can absorb both Cr (VI) and Cr (III). Chromium (VI) is more toxic than Cr (III) and is more easily transported inside the plant. Chromium (VI) uptake has been reported to occur by an active mechanism, whereas Cr (III) uptake is passive, Many studies have reported that plants absorb Cr (VI) better than Cr (III), based solely on plant concentration data and the observation that Cr (VI) is more toxic to plants than Cr(III) [48].

Chromium toxicity can occur when soil is contaminated with Cr (VI) and the pH is high or when soil is contaminated with Cr (III) or Cr (VI) and the pH is low. In the latter case, Cr (VI) will be reduced to Cr (III), which will then equilibrate with the soil solution. Chromium is precipitated at higher pH values as the Cr (III) hydroxide and is unavailable to plants [49].

1.2. Description of Sample Collection Sites

Bilatte is located in Southern Ethiopia, which is 300 km from Addis Ababa. It is in Wolaita zone and 80 km from the capital city Southern People National Administrative Region, Awasa. Shoa Robit is located in the Northern Ethiopia 215 km from Addis Ababa. It is in Amehera region. The distance between the two sample sites is about 500 km. Both places are located in East African Rift Valley. Therefore, the two areas have more or less similar weather conditions. The National Tobacco and Matches Corporation (NTMC), which is renamed as National Tobacco Enterprise, has been given the mandate to organize tobacco production and processing in the country. The company operates a cigarette factory located at the head office of the company in Addis Ababa.

1.3. Objectives of the Study

1.3.1. General Objective

The main objective of this project research is to determine the extent of the accumulation of essential metals (Zn and Cu) and toxic (Cd, Pb, Ni and Cr) in Ethiopian tobacco leaves and processed tobacco (Virginia)

1.3.2. Specific Objectives

1. To collect samples of tobacco leaves from two tobacco growing region (Shoa Robit and Bilatte) and three samples from the factory manufacturing cigarette in Addis Ababa.
2. To determine selected trace metals composition: both essential (Zn, Cu,) and toxic metals (Cr, Pb, Ni and Cd) by flame atomic absorption spectrometer (FAAS).
3. To compare the levels of the metals in Virginia type tobacco cultivated in the two regions (Shoa Robit and Bilatte).
4. To determine the effect of processing from harvesting to cigarette production on the metallic composition by comparing the levels of the metals in leaves and factory processed tobacco.
5. To compare the processed tobacco from Bilatte and Shoa Robit with Nyala
6. To compare the level of metals in Ethiopian tobacco with that in the other countries (literature values).

2. EXPERIMENTAL

2.1. Instrumentation and Apparatus

Ceramic pestle and mortar were used for grinding and homogenizing of both tobacco leaves and processed tobacco; digital analytical balance (**METTLER Toledo, Model AT250**, Switzerland) and oven **(J.P.SELECT, Spain)** were used for weighing and drying the samples respectively. Quick-fit round bottom flasks (150 mL) fitted with reflux condenser were used in Kjeldahl apparatus hot plate to digest the samples. **BUCK SCIENTIFIC MODEL 210VGP** (East Norwalk, USA) Atomic Absorption Spectrophotometers equipped with deuterium ark background correctors was used for analysis of the metals (Cu, Cd, Cr, Ni, Pb, Zn).

2.2. Chemicals, Reagents and Standard Solutions

Chemicals and reagents that were used in the analysis were all analytical grade except HNO_3. 69%-72% HNO_3 (SUPREME ENTERPRISES, AMALA CANTT, India) and 70 % $HClO_4$ (Analar®, BDH, England) were used for digestion of tobacco samples. Stock standard solutions of the metals (Zn, Cr, Cu, Ni, Pb, and Cd), 1000 mg/L calibration standards BUCK SCIENTIFIC, prepared as nitrates for each element in 2% HNO_3) were used for the preparation of calibration curves for the determination of metals in the samples. Deionized water was used for preparation of standard solutions, dilution and for cleaning (rinsing) purpose.

2.3 Procedures

2.3.1. Sample Collection, Preservation, and Handling

Laboratory analysis requires less than one gram of sample. However, a good sample contains enough samples to represent the area sampled. Therefore, the larger the area is, the larger the sample size needs to be. Sample size also varies with crop types. For crops

with large leaves, like tobacco, a sample of l0-15, leaves are adequate for nutrient analysis.

Depending on the availability of tobacco plant, representative amount of leaves from Bilatte and Shoa Robit were collected. The leaves were collected from a minimum of 12 mature tobacco plants per samples and four leaves per plant staring from bottom to the tips by stalk position. The collected samples were washed with tape water and rinsed three times with deionized water to make them free of extraneous substances, including soil and dust particles, and foliar spray residues that may influence analytical results [50]. Another three samples were, processed Virginia whose origin was Bilatte, Shoa Robit, and Nyala sample. These processed samples, which were ready for use after warping, were prepared in the laboratory of the cigarette factory in exactly the same procedure of Nyala manufacturing. The preparation was carried out by an expert of laboratory in the factory. All the three processed samples were about 50 g each.

Both types of samples, the leaves and the processed, were sealed in washed and rinsed with deionized water and dried polyethylene bag and transported to the laboratory where further sample pre-treatments were made. Drying at temperatures under 80^{o}C may not remove all combined water and may result in poor homogenization and incorrect analytical results. Drying temperatures above 80^{o}C may result in thermal decomposition and reduction in dry weight [51]. Accordingly, after chopping the samples with plastic knife; all the samples were dried in the oven for 24 hour at 80^{o}C at which the mass of the samples became constant. The dried samples were well grinded by using pestle and mortar and kept in the desiccator until digestion [52].

2.3.2. Digestion of Tobacco Samples

Applying the optimized procedure, 0.5 g of well-powdered tobacco sample was added into a round bottom flask (150 mL). To this flask 3.0 mL HNO_3 (69-72 %) and 3 mL $HClO_4$ (70%) were added and the mixtures were digested on a micro Kjeldahl digestion apparatus by setting the temperature first to 150 ^{0}C for 10 minutes and then increased to

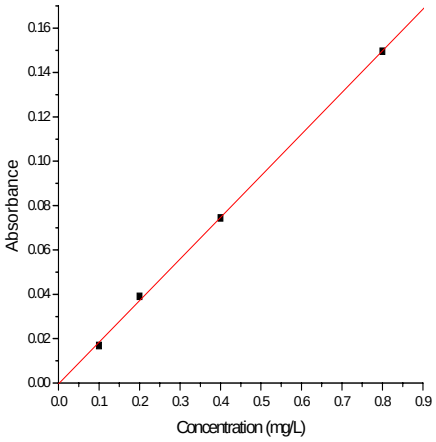
350 °C for 3:20. After total of 3:30 h digestion, the residue of the digest was allowed to cool by leaving it for about 15 minutes at room temperature. After cooling, about 8 mL of deionized water was added to dissolve the precipitates formed on cooling and to minimize the dissolution of a filter paper by the digest residue while filtrating with Whatman®, (110 mm, dia), filter paper. The round bottom flask was rinsed by using another 8 mL deionized water and filtered and the filtrate was diluted to 25 mL with deionized water. Triplicate digestions were carried out for each sample. The digested samples were kept in the refrigerator, until the level of all the metals in the sample solutions were determined by FAAS. The blank solutions were prepared by digesting the mixture of reagents following the same digestion procedure and diluted to 25 mL with deionized water.

2.3.3. Determination of Metals in the Tobacco Samples (Analytical Procedure)

Atomic absorption spectroscopic standard solutions containing 1000 mg/L were used for preparing intermediate standards (10 mg/L) and working standards by using deionized water. Working standards of metals solutions were prepared by diluting the intermediate standards solutions of the metal with deionized water (Table 2). Four points of calibration curve were established by running the prepared working standard solutions in flame atomic absorption spectrometer (BUCK SCIENTIFIC MODEL 210GP). Immediately after calibration, the sample solutions were aspirated into the AAS instrument and direct readings of the metal concentrations was recorded. Three replicate determinations were carried out on each sample. The same analytical procedure was employed in the determination of elements in each six digested blank. The operating conditions of AAS employed for each analyte are given in Table 3.

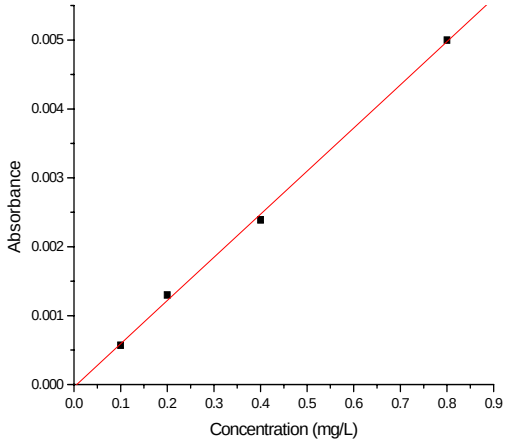
Table 2. List of working standards for determination of metals in all tobacco samples using flame atomic absorption spectrometer

No	Element	Concentration of standards, in mg/L	Correlation Coefficient
1	Cd	0.01, 0.02, 0.04, 0.08	0.99974
2	Cu	0.1, 0.2, 0.4, 0.8	0.99982
3	Cr	0.05, 0.1, 0.2, 0.4	0.99876
4	Ni	0.05, 0.1, 0.2, 0.4	0.99927
5	Pb	0.1, 0.2, 0.4, 0.8	0.99937
6	Zn	0.1, 0.2, 0.4, 0.8	0.9997



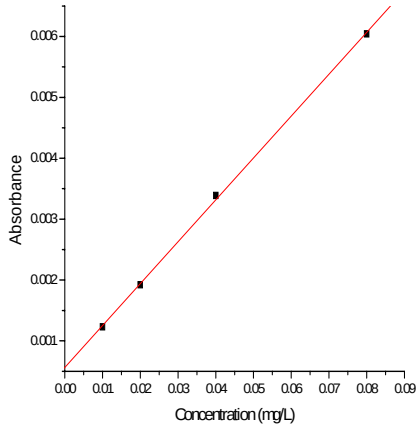
A = 0.18757C - 3.48261E-4
R^2 = 0.9997

Figure 5a. Calibration graph of Zn standard solution



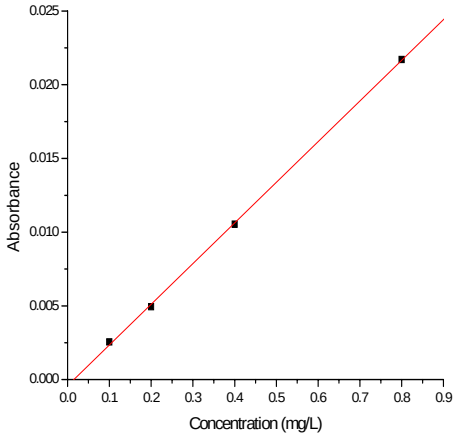
A = 0.00626C - 3.34783E-5
R^2 = 0.99937

Figure 5b. Calibration graph of Pb standard solution



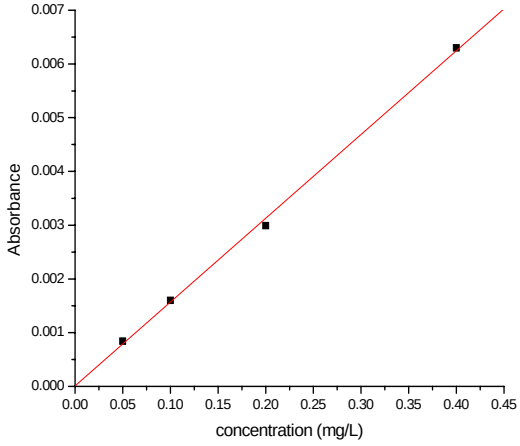
A = 0.06878 + 5.65652E-4
R^2 = 0.99974

Figure 5c. Calibration graph of Cd standard solution.



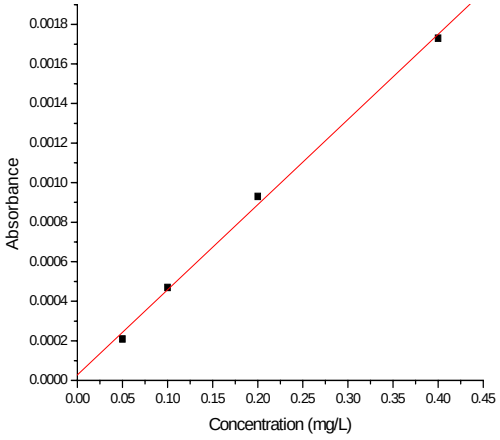
A = 0.02757C - 4.02826E-4
R^2 = 0.99982

Figure 5d. Calibration graph of Cu Standard solution



A = 0.01559C + 9.13043E-6
R^2 = 0.99927

Figure 5e. Calibration graph of Ni standard solution



A = 0.0043C +2.82609E-5
R^2 = 0.99876

Figure 5f. Calibration graph of Cr standard solution.

Table 3. Instrumental operating conditions for determination of metals in tobacco samples using flame atomic absorption spectrometer.

Element	Wave length (nm)	Detection limit (mg/L)	Slit width (nm)	Lamp current (mA)
Cd	228.9	0.005	0.7	2.0
Cu	324.8	0.02	0.7	1.5
Cr	357.9	0.05	0.7	2
Ni	234	0.04	0.2	7
Pb	283.2	0.1	0.7	2.0
Zn	213.9	0.005	0.7	2

2.3.4. Study of Recovery (Procedure of Spiking)

To confirm the efficiency of developed optimized procedures, spiking experiments in which known volume and concentration of standard solutions, were employed. From the stock solution (1000 mg/L) an intermediate standard solutions (10 mg/L) were prepared for all the metals. 13 µL of Cd, 30 µL of Cr, Ni, Pb, 50 µL of Cu, and 70 µL of Zn from 10 mg/L solutions were added to 0.50 g tobacco leaves collected from Shoa Robit. The same amounts of solutions were added to processed tobacco sample collected from Bilatte and Nyala except the volume of Cd added was increased to 30 µL (since the amount Cd was increased in samples). Then samples were digested with the optimized procedures for leaves and processed tobacco samples. After diluting the digested samples to 25 mL with deionized water, they were analyzed with the same procedure followed for the analysis of tobacco samples. As used for original samples triplicate spiked samples were prepared and triplicate readings were recorded. The results are given in Table 5**.**

2.3.5. Determination of Method Detection Limits

Detection limit is the lowest concentration level that can be determined to be statistically different from an analyte blank or the minimum concentration that can be detected by the analytical method with a given confidence limit [53, 54]. There are numerous ways of determining detection limits of a given measurement. According to EPA (Environmental Protection Agency) of America it is the minimum concentration of a substance that can be measured and reported with 99% confidence that the analytical concentration is greater than zero. The generally accepted and common definition of method detection limit is the concentration that gives a signal three times the standard deviation of the blank or background signal [55-57]. In this work, after digestion of six blank solutions, triplicate reading was obtained for each sample. Then the method detection limit of each element was calculated as three times the standard deviation of the blank ($3\sigma_{blank,}$ n = 18), which is summarized in Table 6.

3. RESULTS AND DISCUSSION

3.1. Sample Site Selection for Collection of Samples

Samples were collected from two-tobacco plantation areas (Shoa Robit and Bilatte) which account for > 76% of total tobacco plant production in Ethiopia and are the only places where Virginia type tobacco is planted. Almost 65% of the world's tobacco is classified as flue-cured (Virginia). The processed samples were collected from cigarette factory, located at Addis Ababa, which is the only factory for manufacturing cigarette in Ethiopia. For this study, Virginia type tobacco was chosen because of the above-mentioned reasons and Nyala type cigarette which is totally manufactured from Virginia type tobacco and accounts more than 89% of total cigarette production in Ethiopia [14]. Therefore, the availability of samples of interest was taken in to consideration in site selection of sample site.

3.2. Optimization of Digestion Procedure

Concentrated perchloric acid is a powerful oxidizing agent when hot. However, due to the risk of explosion, perchloric acid was used in mixture with nitric acid which serves not only to dilute the perchloric acid but also to ensure the easily oxidizable compounds are broken by reaction with nitric acid first at low temperature before the perchloric acid starts to exert its oxidizing power at 160^{o}C [63]. It is recommended that the sample size should be less than 1 g for the reason of safety when perchloric acid used for digestion [58]. Refluxing is compulsory, when a sample is decomposed by open ashing to determine volatile trace elements like Cd [59].

Using these reagents and 0.5 g sample different digestion methods were tested and the procedure that produce clear solution, consumed minimal reagent volumes and shorter digestion time, with acceptable sample masses of tobacco samples was selected from the tested alternatives [6, 61]. Optimizing of the digestion procedure involved some changes of parameters such as reagent volume, digestion temperature and digestion time.

Accordingly, thirteen procedures were tested for digestion of tobacco samples (Table 4). Based upon above listed criteria, the optimal digestion procedure chosen was the one that fulfilled the stated criteria for complete digestion of 0.5 g of the dry sample powders, with 3 mL HNO_3 (69-72 %) and 3 mL $HClO_4$ (70 %) for total of 3:30 hour. The mixture was heated smoothly for 10 minutes by adjusting the temperature to 150 ^oC. After 10 minutes when the evolution of oxides of nitrogen ceased the mixture was heated strongly by adjusting the temperature 350 ^oC. The procedures that required higher reagent volume, longer digestion time, and which resulted in the formation of turbid digests and colored digest solutions were rejected.

Table 4. Procedures tested during optimization of methods for digestion of tobacco samples.

No	Sample size	Reagent added	[a]Intial Temp. Kjeldahl appar. (fixed at)	Final Temp. Kjeldahl appar. (fixed at)	[b]Digestion time	Nature of the digest after filtration
1	0.5 g	5 mL HNO_3 (69-72 %) 2 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Yellowish and turbid
2	0.5 g	5 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Clear but pale yellowish color
3	0.5 g	4 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Clear but slightly pale yellowish color
4	0.5 g	4 mL HNO_3 (69-72 %) 2 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Clear but yellowish color
5	0.5 g	3 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Clear and almost colorless color
6	0.5 g	3 mL HNO_3 (69-72 %) 2 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:0	Clear and pale yellow
7	0.5 g	2 mL HNO_3 (69-72 %)	150 ^oC	350 ^oC	3:0	Clear but pale yellow

		2 mL $HClO_4$ (70%)				color
8	0.5 g	3 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	3:3 0	Clear and colorless Optimum
9	0.5 g	3 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	4:0	Clear and colorless
10	0.5 g	4 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	4:0	Clear and colorless
11	0.5 g	3 mL HNO_3 (69-72 %) 2 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	4:0	Clear but pale yellowish color
12	0.5 g	2 mL HNO_3 (69-72 %) 2 mL $HClO_4$ (70%)	150 ^oC	350 ^oC	4.0	Clear but pale yellowish
13	0.5 g	3 mL HNO_3 (69-72 %) 3 mL $HClO_4$ (70%)	150 ^oC	310 ^oC	4:0	Clear but slightly yellowish color

[a]Intial temperature for 10 minutes

[b] The total time in hour for digestion including smoothly heating for 10 minutes

3.3. Evaluation of Analytical Figures of Merit

3.3.1. Precision of Results

Analyst will be concerned with the question of precision (repeatability of results), that is, the agreement between a set of results. It can be determined by standard deviation, variance, coefficient of variance, relative standard deviation, and range of series measurements. In this study the precision of the results were evaluated by the pooled standard deviation and relative standard deviation of the results of triplicate samples (n = 9) and three reading obtained for sample. These parameters are useful in estimating and reporting the probable size of indeterminate errors. The result of analysis was reported with corresponding standard deviation at 95% confidence limit and relative standard

deviation. It can be seen that the values of percentage relative standard deviations (%RSD) are less than 10% for all the mean concentrations except for the concentration of Ni in processed tobacco leaves from Bilatte, which has percentage relative standard deviation equal to 10.3% (Table 7). This shows the precision of the results obtained by this method is good.

3.3.2. Validation of Optimized Procedure

The efficiency of the optimized procedure was evaluated by analyzing the digests of spiked samples for both leaves and processed tobacco samples. The recoveries of metals in the spiked tobacco samples were 88.3 % to 104.3 %, 93.8% to 106.4%, and 91.7% to 107% for tobacco leaves from Shoa Robit, processed tobacco leaves from Bilatte and Nyala sample, respectively. These values are within the acceptable range for analyses of biological samples such as plants. The results are given in Table 5 and Figure 6. Generally, good recoveries were obtained for all the metals. As can be seen from the graph, percentage recovery of all metals in all samples are within the range of 100 ± 10% except for Ni whose recovery is slightly below this range in sample collected from Shoa Robit. Thus, on the average good recoveries were obtained for all elements in all the samples validating that the optimized procedure has good accuracy.

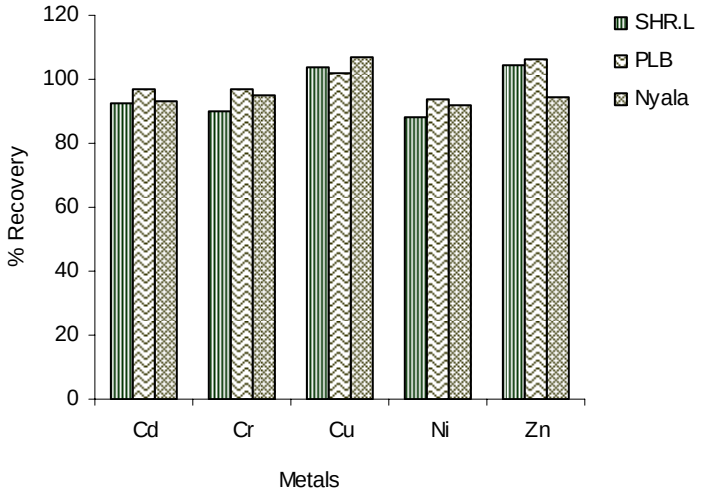
Table 5. Analytical results obtained for validation of the optimized procedure after spiking with standard solutions.

Element	Sample type of tobacco	Amount added (µg/g)	Amount before addition (µg/g)	Amount found (µg/g)	(%)Recovery ± %RSD
Cd	SHRL	0.26	1.3 ± 0.04	1.54 ± 0.07	92.3 ± 6.01
	PBL	0.6	1.45 ± 0.023	2.03 ± 0.03	96.7 ± 1.9
	Nyala	0.6	1.55 ± 0.23	2.11 ± 0.06	93.3 ± 3.6
Cr	SHRL	0.6	1.45 ± 0.11	1.99 ± 0.12	90 ± 7.8
	PBL	0.6	1.65 ± 0.08	2.23 ± 0.03	96.7 ± 1.7

	Nyala	0.6	1.62 ± 0.11	2.19 ± 0.08	95 ± 4.5
Cu	SHRL	1.0	7.3 ± 0.19	8.34 ± 0.02	104 ± 0.3
	PBL	1.0	9.8 ± 0.04	10.82 ± 0.08	102.1 ± 1
	Nyala	1.0	8.95 ± 0.31	10.02 ± 0.24	107 ± 3.1
Ni	SHRL	0.6	1.96 ± 0.08	2.49 ± 0.02	88.3 ± 0.9
	PBL	0.6	2.35 ± 0.19	2.91 ± 0.08	93.8 ± 3.6
	Nyala	0.6	4.7 ± 0.04	5.25 ± 0.25	91.7 ± 6.1
Zn	SHRL	1.4	33.15 ± 1.9	34.61 ± 0.25	104.3 ± 0.9
	PBL	1.4	101.2 ± 0.4	102.69 ± 0.4	106.4 ± 0.5
	Nyala	1.4	79.3 ± 0.77	80.62 ± 0.37	94.3 ± 0.6

SHRL -Tobacco leaves collected from Shoa Robit
Pro.BF -Processed tobacco leaves collected from Bilatte

Note: Measured results are average of three samples recorded three times each (N = 9) at 95% confidence limit, mean ± st/$N^{1/2}$.



SHRL -Tobacco leaves collected from Shoa Robit.
PLB -Processed tobacco leaves from Bilatte.

Figure 6. Percentage recoveries of metals determined in the three types of samples spiked with standard solution.

3.4. Determination of Metals

As described in section 3.2 the digestion mixture consisted of 3 mL HNO_3 (69-72%) and 3 mL $HClO_4$ (70%) for the decompositions of 0.5 g of both type, leaves and processed, samples. However, this amount of acid mixture is large enough to introduce high values of the analyses in the blank solution. Therefore it was necessary to determine limits of method detection for the developed procedure. For this reason, a blank solution consisting of the mixture of digestion reagents was used for the calculation of method detection limits. Detection was calculated as three times the standard deviation of the blank. The values of limits of detection for each element are summarized in Table 5. It is likely that the large volume (6 mL) of the mixture of reagents (HNO_3 and $HClO_4$) used in the digestion of tobacco samples could have contributed to the high observed method detection. Concentrations of the metals in the digested and diluted blank solutions of both leaves and processed samples were determined with FAAS employing external calibration graphs. In a similar way, the average concentrations of the six trace metals (Cu, Cr, Cd, Ni, Cu and Zn) in digested and diluted solutions of tobacco samples were determined with FAAS. The detail information is discussed in the following section.

Table 6. Method Detection Limits, (n = 18, DLM =$3\sigma_{blank}$ and in μg/g), for tobacco samples.

Element	**Cd**	**Cr**	**Cu**	**Ni**	**Pb**	**Zn**
Method detection limit (μg/mL)	0.005	0.05	0.02	0.04	0.1	0.005
Method detection limit (μg/g)	0.18	1.04	0.9	1.7	1.52	0.67

Table 7. Average metal concentration and percentage relative standard deviation of tobacco samples.

Type of sample	Concentration of metals (µg/g)					
	Cd	**Cu**	**Cr**	**Ni**	**Pb**	**Zn**
BL	1.2 ± 0.05	4.38 ± 0.11	ND	ND	ND	53.7 ± 0.96
% RSD	5.4	3.2	-	ND	-	2.3
SHRL	1.3 ± 0.04	7.3 ± 0.19	1.45 ± 0.11	1.96 ± 0.08	ND	33.15 ± 1.9
% RSD	3.9	3.4	9.8	5.3	-	7.4
PBL	1.45 ± 0.023	9.8 ± 0.04	1.65 ± 0.08	2.35 ± 0.19	ND	101.2 ± 0.4
%RSD	2	0.5	6.3	10.3	-	0.5
PSHRL	1.9 ± 0.05	12.8 ± 0.11	1.75 ± 0.08	2.2 ± 0.05	ND	83.75 ± 0 .4
%RSD	3.4	1.1	5.9	2.9	-	0.6
Nyala	1.55 ±0.07	8.95 ± 0.31	1.62 ± 0.11	4.7 ± 0.04	ND	79.3 ± 0.77
%RSD	5.8	4.5	8.8	1.1	-	1.2

ND-not detected
BL-Bilatte leaves
SHRL- Shoa Robit Leaves
PBL.-Processed leaves from Bilatte
PSHRL. - Processed leaves from Shoa Robit

Note: Each measured results are average of three samples analyzed three times each (n = 9) at 95% confidence limit

3.4.1. Distribution Pattern of Metals in Different Tobacco Samples

Trace metals play an important role in the life processes. Some of these elements are toxic for the human bio-system even at a very low level of intake. These toxic elements are present in foods and plant leaves. Increasing industrialization and associated pollution of the biosphere, uptake from the soil fertilizer, pesticide treatment, storage, processing, packing and others are the main contributions for large amounts of these trace metals in plants. The use of sewage sludge, pesticides, irrigation waters and fertilizers on agricultural land has made some of that land of questionable quality for production of food for humans and animals. The distribution and accumulation of metals in tobacco leaves are the reflection of the mineral composition of the soil and environment in which the tobacco plant grows. Therefore, the actual metal content of tobacco vary considerably according geographic origin, the use of fertilizers with different chemical compositions and other characterizing features such as water for irrigation.

3.4.2. Concentration of Metals in Tobacco Leaves

Leaves of plants are one of the major parts of plant tissue in which both essential and toxic metals can be accumulated. They are the main part of plant tissue through which metals can transfer from soil to animals in food chain. Tobacco plant is known to easily absorb heavy metals from soil and accumulate them in the leaves.

This study confirmed that the concentration of metals in leaves varies with the type of sample analysed (the leaves and processed) and the geographical origin of sample. Highest concentration of Zn was determined in both tobacco leaves samples from Bilatte and Shoa Robit. However, higher average content of Zn (53.7 μg/g dry mass) was determined in tobacco leaves collected from Bilatte than tobacco leaves, which were collected from Shoa Robit (33.15 μg/g dry mass). In contrast to Zn, average Cu concentration was found to be higher in Shoa Robit's tobacco leaves than that of Bilatte. Followed by Zn the concentration of Cu was relatively higher in both tobacco leaves samples. The tobacco leaves collected from Shoa Robit (7.3 μg/g dry mass) were higher

in concentration of Cu than that collected from Bilatte (4.38 µg/g dry mass). Except the amount Zn, all the metals were higher in Shoa Robit than tobacco leaves collected from Bilatte.

From six metals evaluated in this study, Pb was found to be lower than the detection limit of the instrument in both leaves samples. Ni was determined lower than instrument detection limit in leaves collected from Bilatte. However, its average concentration in tobacco leaves collected from Shoa Robit was found to be 1.96 µg/g dry mass. The amount of Cr obtained in the tobacco leaves from Shoa Robit was found to be 1.45 µg/g dry mass. However, its concentration in Bilatte leaves was determined below the detection limit of the instrument.

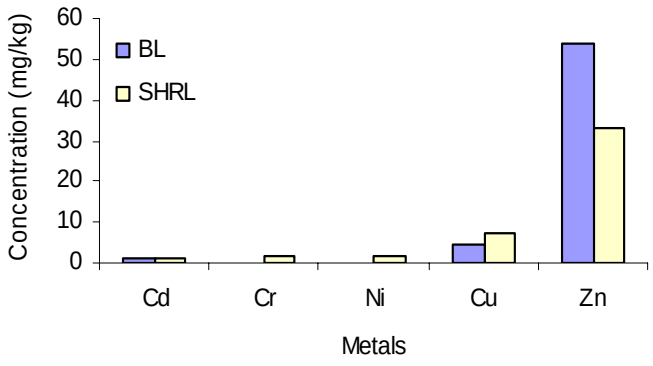


Figure 7. Distribution of metals in tobacco leaves collected from Bilatte and Shoa Robit.

The concentration patterns of metals in both areas follow almost the same trend. The concentration in tobacco leaves collected from Shoa Robit is Zn > Cu >Ni > Cr > Cd. That of Bilatte is also in the trend of: Zn > Cu > Cd. These comparative results were given in Table 6. From the two samples studied both highest concentration of metal (Zn) and the minimum concentration of metal (Cd) were detected in the leaves collected from Bilatte.

The results of this study showed that the metal contents of tobacco leaves varied with the geographical origin in which the tobacco plant grows. The concentration of metals in

plant leaves depends on different factors: the physical and chemical property of the soil the availability of metals to the soil and the interactions metals to inhabit each other from the availability to the plant. Even though there are no industrial activities in both areas of sampling, the natural weathering of rocks, agricultural activities like using fertilizer, herbicide, and water for irrigation could contribute to these concentrations of metals determined in tobacco leaves. The relatively excess amount of Zn in the leaves from both areas could be due to its higher natural abundance of it in the soil.

Both areas of sampling were using phosphate fertilizer, which has significant contribution to metals concentration in tobacco leaves. Ordinary super phosphate fertilizer is produced by reacting H_2SO_4 with phosphate rock. The resulting product will contain all of the heavy metal constituents found in the phosphate rock. Concentrations of other heavy metal in phosphate fertilizers vary considerably, depending on the phosphate rock source. Some metals of possible significance such as Cr, Pb, Ni, are of less concern than Cd, either because they are not as readily absorbed by plants from phosphate fertilized soils or their apparent relative effects on human health are less than that of Cd [62].

In comparison to Billatte, Shoa Robit is more exposed to human activity and the farms were used for long time, which could have significant contribution to gradual accumulation of metals in tobacco farms through agricultural activities. This could be suggested as one of the reasons for the higher concentration of most metals determined in Shoa Robit than Bilatte. As indicated previously metals like Ni, Pb, and Cr in Bilatte leaves sample were below the detection limit of the instruments. The concentration Cu determined in tobacco leaves collected from Bilatte was smaller than that from Shoa Robit.

3.4.3. Concentration of Metals in Processed Tobacco Leaves

The average metal concentrations in processed tobacco leaves whose origin from Bilatte were Cd (1.45), Cu (9.8), Ni (2.35), Cr (1.65), and Zn (101.2) μg/g dry mass. The amounts of metals determined in the processed tobacco leaves whose source from Shoa Robit were Cd (1.9), Cu (12.8),Ni (2.2) Cr (1.75), Zn (83.75) μg/g dry mass. In the same way Cd (1.55), Cu (8.95), Cr (1.62), Ni (4.7), Zn (83.75) μg/g dry mass concentrations were found in Nyala.

Here also large amount Zn was observed in processed tobacco sample collected from Bilatte in comparison to the others. The pattern of concentration was in the order of Zn > Cu > Ni > Cr > Cd in processed tobacco sample from Bilatte and Zn > Cu >Ni > Cr > Cd that originated from Shoa Robit. The concentration of Ni was found to be higher in processed tobacco sample from Bilatte than the processed tobacco sample from Shoa Robit. The concentration order of metals in Nyala tobacco is Zn > Cu > Ni > Cr > Cd. In all the processed tobacco samples, the amount of Pb was found to be lower than the detection limit of the instruments.

Nyala type processed tobacco sample is different from other two processed tobacco in that it is the mixture of tobacco leaves from Shoa Robit, Bilatte, which account about 45-50%, and imported leaves from different country (Brazil, Zimbabwe, and India). In comparison to the metals concentration of processed tobacco from Bilatte and Shoa Robit almost all the metals content of Nyala was found to be in the range of the two samples. The concentration Cr in Nyala was slightly less than both the processed tobacco leaves from Bilatte samples and collected from Shoa Robit. However in comparison to both processed samples from Bilatte and Shoa Robit higher Ni (4.7 μg/g dry mass) concentration was determined in Nyala sample. This concentration of Ni in Nyala could be suggested to come from tobacco leaves, which were imported. The comparative results were presented in Figure 9 &10 for non-essential and essential trace metals respectively.

3.4.4. Comparison of Metals in Leaves and Processed Tobacco Samples

The variation in composition of metals in the leaves and in processed tobacco was observed for all the detected metals. Large increments in concentration can be seen from the graph particularly for some metals such as Zn and Cu the change is almost a double.

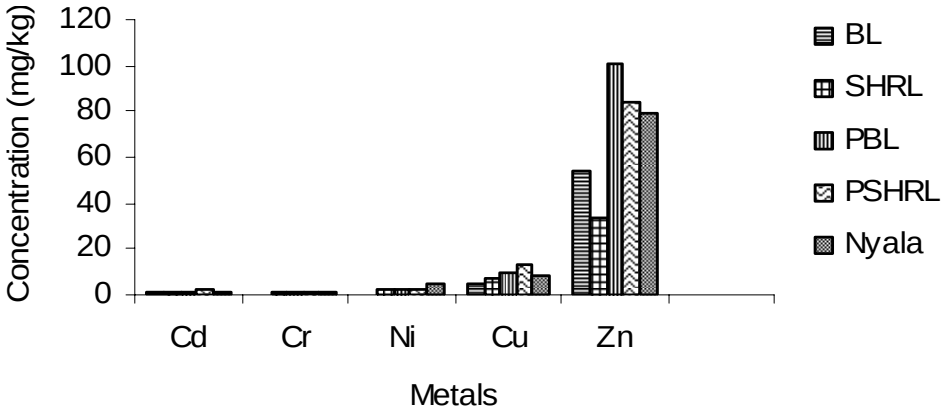


Figure 8. Average concentration of trace metals in tobacco samples.

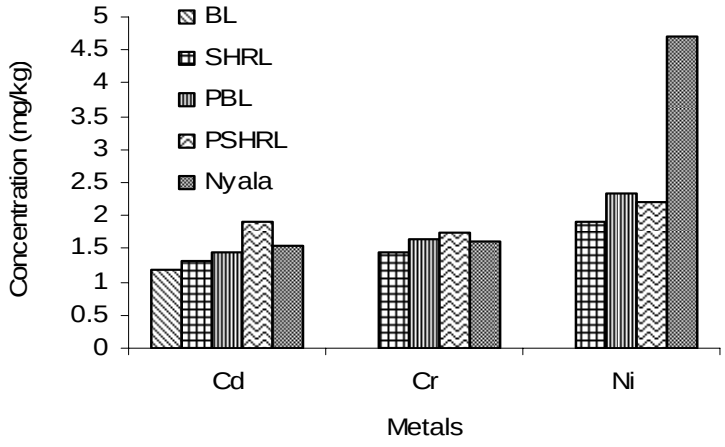


Figure 9. Average concentration of Cd, Cr and Ni in tobacco samples.

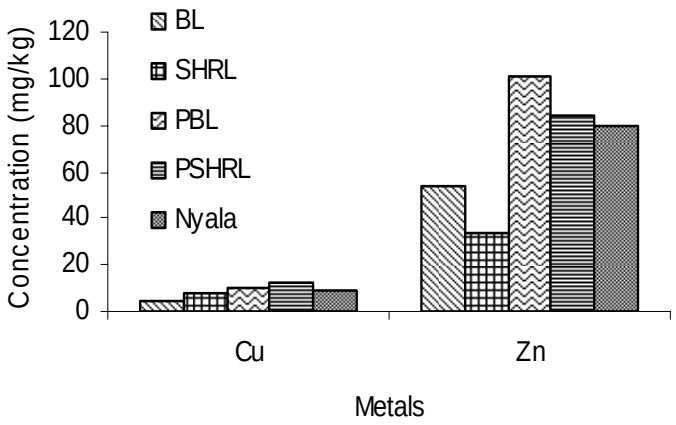


Figure 10. Average concentration of Cu and Zn in tobacco samples.

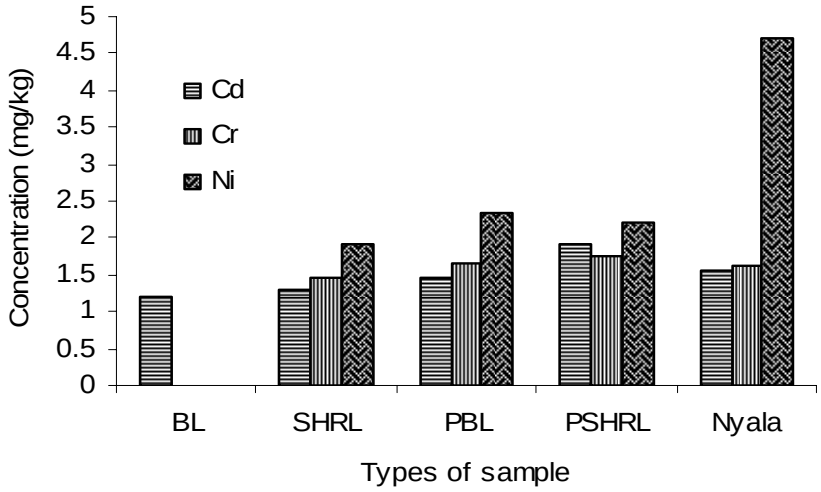


Figure 11. Comparison of metals Cd, Cr and Ni within type of samples.

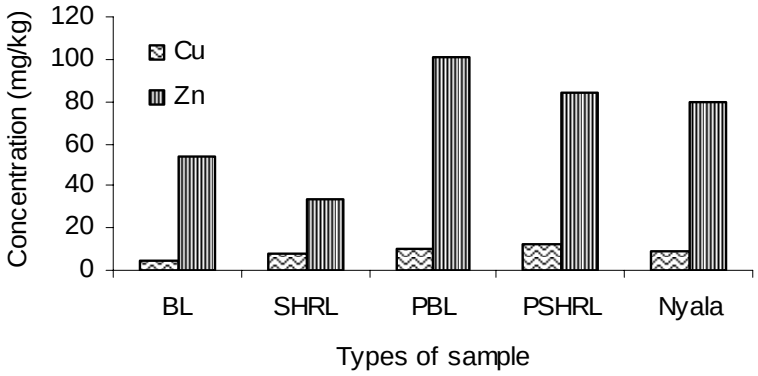


Figure 12. Comparison of metals Cu and Zn within type of samples.

This change in concentration of metals could be due to treatments and handling of tobacco leaves starting from harvesting to the cigarette manufacturing in the factory. During harvesting, transportation from the farm to the site of curing, transportation from site of curing to the factory and system of the storage could make leaves of tobacco be contaminated with dusts and soil, which contain these metals. The processes of packing and packing materials, curing system, treatments in the factory and chemical additives (casing activities) in the manufacturing, could have contribution for the contamination the tobacco leaves with the metals.

The other factor that made the large difference in concentration of metals could be: during the collection of tobacco leaves for analysis, in this study, the collected samples were washed with tape water and rinsed with deionized water. Since there is no such treatment in processed tobacco or in manufacturing of cigarette, the extraneous substances including soil and dust particles, and foliar spray residues could introduce extra metal contamination. Particularly tobacco leaves from the lower part were highly contaminated with soil. Therefore, metals from the soil that were deposited on the leaves could contribute to the high level of metals in the processed tobacco.

3.4.5. Comparison of Observed Metals Concentration with Literature Values

Determination of trace metals in tobacco leaves and cigarettes has received considerable attention due to their toxicity even at lower concentration. Many researchers have reported the concentration of metals in cigarette tobacco as well as tobacco leaves. Murty *et al.* [68] in compared Cd and Pb content of Indian flue cured tobacco (Virginia) with the flue cured tobacco leaves of different country such as America, Germany. New Zealand and Canada. The results reported by these researchers are given in the Table 8.

Table 8. Comparison of cadmium and lead concentration of present study with literature report (μg/g dry mass) [63].

Metals	*Present study	India	America	Germany	New Zealand	Canada
Cd	1.2 -1.3	0.218 - 0.494	1.7 - 2.9	1.07 - 2.3	0.23 - 0.56	1.25 - 7.02
Lead	ND	0.311 - 0.416	0 - 200	2.4 - 4.3	0.48 - 0.55	0.8 - 9.15

*Present study -Ethiopia

ND-not detected

Another literature report [4] indicated that the contents of some metals such as Cu (14.9-21.1), Zn (51-84), Ni (less than 1) μg/g dry mass, in flue cured tobacco leaves. Cogbill and Hobbs reported that 24-33 μg/g dry mass of Zn in tobacco [4]. Stojanoric *et al.* revealed a high content of nickel in cigarettes (2.32-4.20 μg/g dry mass) and in tobacco leaves (2.20-4.91 μg/g dry mass) regardless of the kind and the origin of tobacco [39]. Saldivar, *et al.* [64] reported the average concentration of Cd in both tobacco leaves and cigarettes in Mexican produced-tobacco of four different type of tobacco. The average concentration of cadmium in cigarettes was 4.41 ± 0.67 and 2.65 ± 0.99 μg/g dry mass for tobacco leaves.

Moulin *et al.* [65] analyzed 755 tobacco leaves samples from chief tobacco-producing regions of three major types (Flue-cured, Burley, and Oriental) obtained from 13 countries during 2001-2003. They found that cadmium concentrations in the samples ranged from 0 to 6.78 μg/g dry mass and variations in Cd concentrations were also found in all countries. The report also indicated that Cd contents of flue cured tobacco leaves as India (0.33 ± 0.13), France (1.46 ± 1.35) and processed one from USA (0.51 ± 0.05) μg/g dry mass.

As presented above, different researcher at different time and country reported their findings of metal contents in tobacco leaves. The concentration of lead in present study was below the detection limit of the instrument. However, contents of tobacco leaves in present study are expected to be lower than the leaves from other countries like America.

Cadmium is a potentially toxic metal that has been classified as a known human carcinogen by the International Agency for Research on Cancer. Due to this Cd has got high attention from researchers. As compared the report of Murty *et al*. [63], Table 8 the concentration of Cd in presently studied flue cured tobacco was higher than flue cured tobacco of India, New Zealand and within the range of other countries (America, Germany, and Canada) flue cured tobacco concentration. The level of Cd in present study was with in the range of the literature values, which can range from 0 to 6.78 [66]. However, in comparison with the flue-cured leaves from India and France, Cd content in present study was found to be higher than that of Indians' and lower than Frances' flue cured leaves [65].

The concentration of Ni was reported to range from less than 1 to 4.91 μg/g dry mass [4, 39] Therefore, the concentration of Ni in the present study was found to be with in the range of literature value.

As it was mentioned above the concentration of Cu is lower than the literature value which ranges from 14.9-21.14 μg/g. The level of Zn in the tobacco leaves in this study is also within the range of literature value, which ranges from 24 to 81 μg/g dry mass.

Precise reported information was not obtained on the content of Cr in tobacco leaves from literatures.

In this study, the processed tobacco samples were taken from the factory at the end, which were ready to be rolled and used for smoking. Even though there could be contamination on transportation of cigarette, rough comparison of metal content of cigarette from different country can be made with present study. There are a number of different studies of metal contents in cigarettes. Some these are as follows:

Vastarella *et al.* [33] determined the content of Cd, Ni, Pb and Cr content in tobacco from various European countries such as UK, France, Belgium, Italy and Germany. Another researcher Ei-Amri, *et al.* [66] also reported the concentration of some metals in cigarette from different countries including Libya, India, USA, Iran, and Yugoslavia. Zhang, *et al.* [66] analyzed the Cu, Zn, Ni, contents of three kinds of Japan cigarette. Nnorom *et al.* [52] reported Cd content of in cigarette available in Nigeria. The report also compared the metal contents of cigarette from different country.

Table 9. Comparison of Cr, Cu, and Zn concentration of present study with literature report (μg/g dry mass) [66].

Metals	[a] Present study	Libya	India	USA	Iran	Yugoslavia
Cr	1.65-1.75	4.55-4.85	3-8.2	0.24-6.3	4.3-6.2	NR
Cu	8.95-12.8	32.8-41.14	NR	NR	NR	18.9
Zn	79.3-101.2	63.5-95.82	15-31	4.1-54	51-56	51

[a] Present study- The contents of metals includes all the processed tobacco leaves collected from Bilatte, Shoa Robit and Nyala, Ethiopia.

Table 10. Comparison of Cd concentration in present study with literature report [57] (μg/g dry mass).

Metal	[a]Present study	Cameroun	France	Germany	Switzerland	UK	USA
Cd	1.63	1.3	2.3	1.8	1.27	1.34	1.6

[a] Average concentration all the three processed samples.

Table 11. Comparison of Cd, Ni, Pb, Cr, Zn, concentration in present study with literature report in μg/g dry mass [33, 67].

Metals	Ave. of BL & SRHL (Ethiopia)	Nyala (Ethiopia)	UK	France	Belgium	Italy	Germany	Japan
Cd	1.67	1.55	1.62	1.59	1.22	1.96	1.96	1.04
Ni	2.27	4.7	5.62	8.91	9.44	8.60	9.11	3.24
Pb	ND	ND	14.27	15.55	14.70	22.03	17.20	NR
Cr	1.7	1.62	0.11	0.05	0.06	0.07	0.07	NR
Zn	92.7	79.3	NR	NR	NR	NR	NR	75.77

ND-not detected

NR-not reported

BL Leaves from Bilatte

SRHL- Leaves from Shoa Robit

Data presented in Table 9 clearly indicated that concentration of Cr in present study was lower than the concentration Cr in Libya, India, Iran cigarette but with in the range of cigarette from USA. Another report [33, 67] (Table 11) has indicated that Cr concentration in the present study is higher than the report values. Therefore, from the

results given in Tables 9 and 11 one could conclude that the concentration in the present study is within the range minimum concentration obtained in cigarette collected from France (Table 11) and maximum concentration determined in Indian cigarette (Table 9).

As presented in Table 8 and 10 the Cd concentration of present study is within the range of minimum concentration determined in Japan's cigarette [67] and maximum concentration obtained in the France cigarette [52] (Table 9). The concentration of Pb obtained by this method is less than all the literature report. Similarly Ni in processed Shoa Robit and Bilatte is less than all other literature report. However, the Ni concentration determined in Nyala is within the range minimum report (2.32 μg/g) [42] and maximum content of Germany's (9.11 μg/g dry mass) cigarette [33].

Comparative results given Tables 9 and 11 revealed that the Zn concentration in present study is found to be higher than the literature value. In contrast to Zn, the amount of Cu is found to be lower than the literature values. The concentration of lead in present study is lower than its concentration in processed tobacco from countries in the table 11. It has been demonstrated that most of the lead in green plant parts originate from deposition of air borne lead from automotive sources and thus the lead content of tobacco leaves in this study can be expected to be low as such occurrences are minimum in Ethiopia [63]. Even the lead in the soil is not in soluble form to be available to plant as compared to other metals.

In general, the concentrations of metals observed were more or less comparable with the reported literature values. However, relatively lower concentrations of Cu were observed in this study in comparison to the reported values

4. CONCLUSIONS AND RECOMMENDATIONS

An efficient digestion procedure was developed and validated through recovery studies. The optimal digestion procedure allowed the use of acids with minimum volumes leading to reduced blank concentration, and lowers the method detection limit. Further more, this condition allowed most elements analyzed with greater precision and accuracy.

This investigation revealed the dependence of metal accumulation in tobacco on the geographical origin in which tobacco plant grows and type sample (leaves or processed leaves). Although the data set is relatively small to draw authoritative conclusions about the metals content of tobacco leaves and processed tobacco leaves, the investigation has indicated the presence of trace metals (Cu, Zn, Cd, Ni, Cr), provided baseline data for comparison, give good awareness for general people (smokers and non-smokers), Ethiopian Tobacco Enterprise and health organizations. In addition, this study revealed comparability of the metal component of Ethiopian tobacco with other countries' tobacco. The concentrations of most of the metals detected are found to be within the range of literature value, except copper, which was found to be slightly lower than literature value.

The concentration of metals in tobacco leaves depends on different physical and chemical property of the soil, the availability of metals to the soil and the interactions of metals to inhabit each other from the availability to the plant. Therefore, to draw strong conclusion, parallel to the study of metal content in the leaves further investigations are needed on the physical and chemical property of soil, the metal content of soil, water for irrigation and fertilizers. In general, basic and valid research should be conducted on tobacco leaves starting from the soil and soil inputs to the cigarette.

Cadmium occurs naturally in soil at concentrations that can differ considerably, depending on the soil type, but which are usually less than 1 μg/g [66]. Further enrichment of Cd in agricultural soils in many parts of the world can occur due to the long-term use of Cd-rich phosphate fertilizers and Cd contaminated sewage sludge

application, as well as from atmospheric deposition. Even though there is no industrial activity and no contaminated sewage sludge application in both the sample sites (Bilatte and Shoa Robit), the Cd concentration was found to be comparable with industrialized countries and greater than that naturally available in the soil. Fertilizer could be the main contributor for such un-expected concentration of Cd in these un-industrialized and free of human impact sample sites. Therefore, there should be further investigation on the Cd content of fertilizer and fertilizers, which are free of Cd should be used.

This study revealed that there was large difference between the metal content of tobacco leaves and processed tobacco. The large increment in composition of metals in processed tobacco was observed for all the detected metals. This indicated that the metal contents that determined in cigarette are not only the content of leaves itself but also the metal originated from contamination of leaves in the process, starting from harvesting to cigarette manufacturing. Therefore, to control further contamination of tobacco leaves with toxic metals, well treatments in handling, transportation and storage of tobacco leaves are recommended.

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