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Determination of Heavy Metals in Tobacco Leaves and Their Growing Soils in Assosa District, Benshangul Gumuz Regional State, Ethiopia

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Abstract: Tobacco is a commercial plant that can accumulate heavy metals in its leaves. In this study, we investigated the levels of selected heavy metals (Mn, Cu, Zn, Cd, and Pb) in tobacco leaves and their growing soils from Assosa District, Benshangul Gumuz Regional State, Ethiopia. Both samples were collected from five tobacco farmlands. The samples were digested before quantitative determinations of the metals via inductively coupled plasma optical emission spectroscopy (ICP-OES). Repeatability and recovery studies were used to evaluate the efficiency of the method. Repeatability was evaluated by analyzing replicate samples and expressed as relative standard deviations (RSD) were below 10.0. Recovery or percent recovery (%R) studies, which were performed by spiking known concentrations of metals into the samples, ranged from 85.67 - 111.33% and 84.25 - 114.00% for tobacco leaves and soil samples, respectively, indicating that the employed method is valid for the analysis of the metals. The concentrations of metals in tobacco leaf samples were: 0.75 -1.18 mg/kg, Mn; 0.11-0.21 mg/kg, Cu; 0.58-1.16 mg/kg, Zn; and not quantified (NQ) - 0.01 mg/kg, Cd. In soil samples, their concentrations were: 1.59-8.04 mg/kg, Mn; 0.32 - 0.38 mg/kg, Cu; and 0.29 - 1.26 mg/kg, Zn; and 0.17 - 0.31 mg/kg, Pb. Except in one tobacco leaf sample, Pb was below the limit of quantification (LOQ). Similarly, in all soil samples, the concentration of Cd was below LOQ. The findings indicate that the concentrations of the studied heavy metals in both tobacco leaves and soil samples are below their WHO permissible limits in soil and plants. However, the levels of Zn in Amba and Basha tobacco leaves were higher than the WHO permissible limit in plants.

Keywords: Tobacco leaves, Soils, Heavy metals, Digestion

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INTRODUCTION

Metals are involved in many biological processes of living systems. Plants can absorb and accumulate a wide range of essential, micro-essential, and toxic metals from their growing soils (1). In particular, toxic heavy metals accumulated in plants may affect their metabolic activities by interacting with DNA and proteins and causing oxidative deterioration of biological macro-molecules (2). Metals such as Zn, Cu, Mn, Mo, Co, and Ni are essential because they are necessary in different biological processes and developmental pathways (3). However, essential metals can be harmful to plants when their uptakes are higher than the required levels (4,5). For instance, an excess of Zn causes metabolic disorders and possibly results in the plant's death (6).

On the other hand, metals like Pb, Cd, As, Hg, Cr, and Se are highly toxic to plants and humans, even in small amounts (7–9). Pb and As are classified as carcinogenic to humans (10), while Cd is associated with bone and kidney diseases (11). Previous studies indicated that cigarette smokers had higher concentrations of Cd (6), Pb (12), and

As (13) in their biological samples than nonsmokers. Nonsmokers can also take in some amounts of toxic metals when cigarette smokers smoke in their vicinity (12). Therefore, monitoring the levels of heavy metals in tobacco is essential for protecting the environment and the health of human beings.

Tobacco (*Nicotiana tabacum L.*) is a commercial plant since its leaves are commonly used for making cigarettes (14). Traditionally, people use it also for snuffing, chewing, and pipe smoking (15). The plant may absorb and accumulate heavy metals from the soil through its roots (16–18). For example, the tobacco plant absorbs Cd from the soil and may accumulate the metal in its different parts: leaves, roots, and stems (9). The accumulated heavy metals in the leaves may transfer from the cured and processed tobacco to the cigarette smokers (9,19). Plants take up varied amounts of metals based on metal types and the content in which they occur in the soil and the plant species (1). The transfer of these metals from the soil to the plants is influenced by a number of factors, including the type of the soil, pH, chemical speciation of the metal, and the quality of water used for watering (20,21). The use of fertilizers and pesticides may result in greater metal concentrations in soils and plants (22).

In Ethiopia, tobacco is grown by individual farmers and state-owned farms for commercial purposes. For example, Virginia (74%), Oriental (22%), and Burley (4%) are the three most commonly cultivated tobacco varieties on state farms, which are used for cigarette production (15). Most of these farms are located in Shewa Robit (North Showa), Billate, Hawasa, Wolaita (Sidamo), and Nura-Era (Hararghe), where 500 to 900 MT-cured tobacco leaves are produced every year on 2000 hectares of land (14). People in Ethiopia have become accustomed to Khat (48.2%), tobacco (29.9%), and alcohol (18.9%), indicating the high prevalence of tobacco addiction next to Khat (23).

In Assosa District, Benshangul Gumuz Regional State, western Ethiopia, tobacco is widely grown, by individual farmers. People of this region use tobacco leaves for chewing and making traditional cigarettes. During tobacco production, farmers use agrochemicals, which may lead to the enrichment of the soils with heavy metals such as Cu, Zn, Cd, and others (24). Therefore, in this study, the levels of selected heavy metals: Zn, Cd, Pb, Mn, and Cu in tobacco leaves and their growing soils from Assosa District were determined using ICP-OES. To the best of our knowledge, there is no previous report on the levels of heavy metals in tobacco leaves and their growing soil from the study area.

MATERIAL AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade. HNO_3 (69%) and HCl (37%) supplied by Loba chemie pvt. Ltd (Jehangir villa, Mumbai, India), and H_2O_2 (30%) from RDH Labor chemi kalien GmbH & Co KG (Seelze, Germany) were used for digestion of tobacco leaf and soil samples. Stock standard solutions of the metals (Mn, Cu, Zn, Pb, and Cd), 1000 mg/L prepared as nitrate for each metals in 0.5 mol/L HNO_3 were purchased from Luba Chemical Pvt. Ltd (Mumbai, India).

Instrument and Apparatus

A ceramic mortar and pestle were used for the grinding of tobacco leaves and soil samples. An analytical balance KERN ABS-N · ABJ-NM, Model ABS 120-4N (Balingen, Germany) was used for weighing samples. A heating oven, M 3040 P, with a working temperature ranging from 50 to 250 °C (Elektro-mag, Turkey) was used for the drying of samples. Glass beakers (100-400 mL) and an aluminium hot plate magnetic stirrer (CB/SB series, SB 302) form The Carl roth GmbH + Co.KG (Karlsruhe, Germany) were employed for wet digestion of tobacco and soil samples. Inductively coupled plasma optical emission spectroscopy (ICP-OES), Optima 8000, supplied by PerkinElmer, Inc. (Waltham, USA) was used for the quantitative determination of targeted heavy metals.

Study Area and Period

The study area was conducted in Assosa District, Benshangul Gumuz Regional State, Ethiopia, which is at 10°04'N longitude, 34°31'E latitude, and 1572 m elevation above sea level. Assosa City, the capital city of the regional state, is located 659 km away from Addis Ababa, Ethiopia, which has an estimated area of 49,289.46 km^2. It has a tropical climate and gets about 1236 mm of annual rainfall and an average temperature of 28.2 °C. Tobacco is widely grown in the regional state, including Assosa District, for local consumption as cigarettes and chewing its leaves. The study was conducted from May to October 2017.

Sample Collection

Representative tobacco leaf samples were systematically collected from five different tobacco farmlands in Assosa District, namely, Tsentseha, Belmili, Ateto, Amba, and Basha. From each farmland, five samples were collected from different sites. At each sampling site, four leaves per plant, from the bottom to the tip of the stalk position, were collected from five tobacco plants. The collected samples from the same farmland were combined to form a single composite sample (14). Similarly, soil samples were collected at 0.0 - 30 cm depth from the same sites where tobacco leaf samples were collected. The collected soil samples from the same farmland were combined and homogenized to form a single composite sample. Both tobacco leaves and soil samples were collected in polyethylene bags and transported to Jimma University Analytical Chemistry laboratory for further pretreatments.

Sample Preparation and Analysis

Before drying at 105 °C in an oven, tobacco leaf samples were washed with distilled water. The samples were digested following the method reported by Imran et al. (2013) (25) with some modifications. The dried samples were then ground using mortar and pestle. Subsequently, 0.5 g was taken into a 125 mL Erlenmeyer flask. Then, a mixture of 5 mL HNO_3 and 4 mL H_2O_2 was added. After that, the flask was covered with a watch glass and gently heated on a hot plate for 30 minutes. After cooling, 5 mL HNO_3 were added and gently heated for a few minutes. Then, 5 mL of $HCl:HNO_3$ (1:1) were added and heated for 15 minutes. Finally, the resulting digest was cooled and filtered using Whatman No 42 filter paper into a 50 mL volumetric flask. The remaining volume was filled to the mark with double-distilled water and made ready for the subsequent analysis of the target metals.

Similarly, soil samples were dried in the open air, ground, and sieved through a 2 mm sieve. Then, 0.5 g was taken into a 125 mL Erlenmeyer flask. Subsequently, after adding 12 mL of aqua regia $HCl:HNO_3$ (3:1, v/v) and 2 mL of H_2O_2 the flask was covered with a watch glass and heated on a hot plate at 110 °C for 45 min. Eventually, the resulting digest was cooled and filtered using Whatman No 42 filter paper into a 50 mL volumetric flask. Then, the remaining volume was adjusted with double-distilled water and made ready for the subsequent analysis of the target metals.

During the construction of calibration curves, a series of standard solutions were prepared from the stock standard solution of each target metal by dilution. Laboratory blank samples were prepared by a mixture of reagents, following the procedures used for digestion of tobacco leaves and soil samples. Replicate samples are prepared and measured.

Data Analysis

One-way ANOVA at a confidence level of 95% was employed to compare the levels of the studied heavy metals among tobacco leaves and soil samples. Pearson correlation coefficient was used to evaluate the correlation between the target metals content in tobacco leaves and their farmland soils. The obtained concentrations of the heavy metals were reported as mean ± RSD.

Quality Control

All laboratory glassware was socked in 10% HNO_3 overnight and rinsed with double distilled water before use. Double distilled water was used to dilute, clean, and prepare all solutions throughout the laboratory work.

Precision and Accuracy

The precision of the results was evaluated by the relative standard deviation of the results obtained from triplicate samples (n=3). The accuracy of the results was evaluated by recovery studies. For a recovery study, a standard solution containing a mixture of 2 mg/L Mn, Zn, and Cu and 0.2 mg/L of Pb and Cd was spiked onto tobacco and soil samples collected from Tsentseha farmland. The spiked samples were then digested in the same earlier procedure used for tobacco leaves and soil samples. The resulting digests were diluted with double-distilled water to the required volume for subsequent analysis. All samples were prepared and measured in replicates.

Limits of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) of each element were determined based on the blank reagent method (26). Accordingly, five reagent blanks were digested and diluted following the procedure used for tobacco leaves and soil samples. Each digest was then analyzed in triplicates. Finally, the LOD and LOQ of each element were determined by using the standard deviation of the blank (SD) and slope of the calibration curve (S) as indicated by Eq.1 and Eq.2, respectively (26). Table 1 shows the instrumental detection limit (IDL), LOD, and LOQ of the studied metals in tobacco leaf and soil samples.

$$LOD = 3SD/S \quad \text{(Eq. 1)}$$

$$LOQ = 10SD/S \quad \text{(Eq. 2)}$$

Where: SD and S are the standard deviation of the blank and slope of the calibration curve, respectively.

RESULTS AND DISCUSSION

Validation of the Procedure

The accuracy of the digestion procedure was evaluated by determining the percent of recovery (%R) of the spiked tobacco leaves and soil samples due to the lack of certified reference materials in our laboratory (27). Table 1 shows the %R for tobacco leaf and soil samples. The obtained recoveries for the spiked tobacco samples ranged from 85.67 ± 5.52 to 111.33 ± 5.19%. Similarly, the obtained recoveries for soil samples ranged from 84.25 ± 3.27 - 114.00 ± 6.14%. These values are within the accepted range for the analysis of heavy metals in plant and soil samples, indicating that the employed digestion procedure has the required accuracy for analysis of the target heavy metals. The precision studies, expressed as RSD for both tobacco leaves and soil samples, were < 10%, which is also acceptable for the analysis of the target metals.

Table 1. Recovery results of tobacco leaves and soil samples.

Analyte	IDL	LOD	LOQ	%R ± RSD	
				Tobacco	Soil
Mn	0.0014	0.07	0.08	105 .67± 5.21	84.25 ± 3.27
Cu	0.0097	0.068	0.0701	111.33 ± 5.19	96.00 ± 8.53
Zn	0.0059	0.125	0.132	85.67 ± 5.52	107.25 ± 5.87
Cd	0.0027	0.005	0.008	108.50 ± 9.78	114.00 ± 6.14
Pb	0.042	0.05	0.06	95.00 ± 9.12	106.67 ± 7.16

The concentration of Metals in the Tobacco Leaves

In the present study, the levels of three micronutrients: Mn, Cu, and Zn as well as two non-essential (toxic) metals: Cd and Pb were determined in tobacco leaves grown in Assosa District, Ethiopia. Table 2 presents the obtained concentrations of the studied metals in tobacco leaf samples.

Table 2. Average of metal concentrations in tobacco leaf samples (mean ± RSD, n = 3).

Sample site	Concentration of metals (mg/kg)				
	Mn	Cu	Zn	Cd	Pb
Tsentsehalo	1.09 ± 1.19	0.11 ± 0.74	0.58 ± 1.14	0.01 ± 1.23	ND
Belmili	0.96 ± 0.52	0.15 ± 0.03	0.58 ± 0.28	NQ	NQ
Ateto	1.09 ± 0.72	0.20 ± 0.10	0.74 ± 0.74	NQ	NQ
Amba	0.75 ± 1.26	0.21 ± 1.26	1.16 ± 1.56	0.01 ± 0.61	0.07 ± 0.95
Basha	1.18 ± 0.39	0.17 ± 0.03	1.05 ± 1.32	0.01 ± 9.52	NQ
WHO limits in plants (28)	-	10	0.6	0.02	2

NQ: not quantified; ND: not detected

As can be seen from Table 2, the concentrations of Mn in tobacco leaf samples ranged from 0.75 mg/kg - 1.18 mg/kg. The highest concentration of Mn (1.18 mg/kg) was found in the tobacco leaves of Basha farmland, and the lowest (0.75 mg/kg) was detected in the tobacco leaves of Amba farmland. The concentrations of Cu in the tobacco leaves ranged from 0.11 to 0.21 mg/kg. The highest concentration of Cu (0.21 mg/kg) was determined in tobacco leaf samples collected from Amba farmland. The lowest concentration of Cu was detected in tobacco leaves of Tsentsehalo farmland. The obtained concentrations of Zn ranged from 0.58 to 1.16 mg/kg. The lowest concentration of Zn was detected in tobacco leaf samples collected from Tsentsehalo and Belmili, whereas the highest concentration was obtained in tobacco leaves from the Amba farmland.

The findings revealed that the studied tobacco leaf samples have lower concentrations of Cu and Zn than tobacco leaf samples from state farms of Billate and Shewa Robit, Ethiopia (14). This variation could be due to the soil type, pH, use of agrochemicals (fertilizers and pesticides), climatic conditions, and tobacco variety (24). The findings show that the levels of studied heavy metals in the tobacco leaves were below their WHO permissible limits, except for the levels of Zn in tobacco leaves from Amba and Basha farmlands (Table 2).

In the current study, except for tobacco leaf samples collected from Amba farmland, which contained 0.07 mg/kg of Pb, other tobacco leaf samples had Pb concentrations either below LOD or LOQ. Likewise, the concentration of Cd in tobacco leaf samples collected from Belmili and Ateto farmlands was also below LOQ value. However, tobacco leaf samples collected from Tsentsehalo, Amba, and Basha farmlands exhibited similar Cd content, i.e., 0.01 mg/kg, which is much lower than the earlier report from Billate and Shewa Robit, Ethiopia (14).

In general, the trend of concentrations of the studied metals in tobacco leaf samples collected from Tsentsehalo, Belmili, Ateto, and Basha farmlands showed the following order: Mn > Zn > Cu > Cd. However, tobacco leaf samples collected from Amba farmland exhibited a different order: Zn > Mn > Cu > Cd > Pb.

The levels of the studied heavy metals in the present tobacco leaf samples were lower than those reported in the literature (14,29). For instance, the level of Mn obtained in the current raw tobacco leaves are much lower than the Mn level reported from tobacco cigarettes of different countries, such as Spain (112.03 mg/kg) (30), Nigeria (44.67-297.69 mg/kg) (31), Pakistan (45.03 mg/kg) (32), and Mexico (108-244.26) (33). These variations are may be due to the difference in the level of heavy metals in the soil where the plant has been cultivated. Similar to other plants, tobacco absorbs heavy metals from the soil and accumulates them in its leaves, stems, and roots (9). The processes of accumulation of metals in soils and their absorption by plants with subsequent translocation and bioaccumulation in leaves are well known (24,34). The other possible reason for the high concentration of Mn in the cigarettes could be the contamination of some heavy metals in the manufacturing process (31).

The concentration of Heavy Metals in the Soil Samples

The concentrations of the target heavy metals were also determined in the soil samples collected from tobacco farmlands. Table 3 shows the concentrations of the studied heavy metals in the soil samples.

In the studied soil samples, the observed concentrations of Mn ranged from 1.59 to 8.04 mg/kg. Its lowest and highest concentrations were recorded in soil samples collected from Belmili and Tsentsehalo farmlands, respectively. The content of Cu in the studied soil samples varied from 0.32 to 0.38 mg/kg. The levels of Zn ranged from 0.29 to 1.26 mg/kg. The lowest and highest concentrations of Zn were recorded in soil samples collected from Ateto and Basha farmlands, respectively. The findings demonstrated that the content of Mn, Cu, and Zn, in the studied soils, were lower than the WHO permissible limits in soil (28).

Table 3. Average of metal concentrations in soil samples (mean ± RSD, n=3).

Sample site	Concentration of metals (mg/kg)				
	Mn	**Cu**	**Zn**	**Cd**	**Pb**
Tsentseha	8.04 ± 0.28	0.35 ± 0.45	0.69 ± 1.14	ND	0.17 ± 0.87
Belmili	1.59 ± 0.20	0.38 ± 1.76	0.45 ± 0.29	ND	0.18 ± 0.38
Ateto	7.28 ± 0.55	0.37 ± 0.41	0.29 ± 2.44	NQ	0.24 ± 1.03
Amba	6.38 ± 0.72	0.35 ± 0.30	0.43 ± 2.33	NQ	0.22 ± 1.72
Basha	5.98 ± 0.54	0.32 ± 1.05	1.26 ± 1.19	ND	0.31 ± 0.59
WHO limits in soil(28)	-	36	50	0.8	85

The Pb content of the soil samples ranged from 0.17 to 0.31 mg/kg. The highest concentration of Pb was obtained from the Basha farmland soil sample, whereas the lowest was observed from the Tsentsehalo farmland soil sample. The concentration of Cd in all the studied soil samples was either below LOQ or LOD and thus reported as NQ or ND, respectively. In general, soil samples from the Tsentsehalo, Belmili, Amba, and Basha farmlands showed the following order of metal content: Mn > Zn > Cu > Pb. However, for Ateto farmland, the observed order was different: Mn > Cu > Zn > Pb. The slight variations in the heavy metal content may be due to the difference between the growing soils or anthropogenic sources such as fertilizers and pesticides used during the cultivation of the plant or other proximate crops (21).

Statistical Analysis

One-way ANOVA was used to compare the variations of the concentrations in the heavy metals in the studied samples. Pearson correlation coefficient was also employed to evaluate where the heavy metal content of tobacco leaves is either related to their growing soil metal content or not (35). The ANOVA results showed the presence of a statistically significant difference at 95% ($P < 0.05$) confidence level in the concentrations of Mn, Cu, and Zn in tobacco leaf samples and Mn, Zn, and Pb in soil samples. The observed variations may be due to the difference of mineral content in the farmland soils.

Table 4 indicates the Pearson correlation coefficient. The content of Mn in tobacco leaves has shown a weak positive correlation with Mn in the soil, which indicates the low dependence of Mn in the tobacco leaves on its amount in the farmland soil. Similarly, Cu in soil and Cu in tobacco leaves have a negligible correlation. However, the content of Zn in tobacco leaves exhibited a moderately positive correlation with the level of Zn in the soil. This shows that the concentration of Zn in tobacco leaves is moderately dependent on the level of Zn in the farmland soil.

Table 4. The Pearson correlation coefficient of metal content in tobacco leaves and soil samples.

		Soil sample			
		Mn	**Cu**	**Zn**	**Pb**
Tobacco leaves	Mn	0.23	-0.33	0.57	0.40
	Cu	0.04	0.01	-0.30	0.49
	Zn	0.22	-0.64	0.32	0.67

Comparison of the Level of Metals with Literature Values

Many researchers have reported the concentration of metals in the raw tobacco leaves and the processed product, the tobacco cigarettes. Table 5 shows the reported concentrations of the studied heavy metals in tobacco cigarettes and raw tobacco leaves. As it can be seen in Table 5, very high concentrations of Cd (5.90-7.94 mg/kg) and Pb (17.21-74.78 mg/kg) were reported in tobacco cigarettes from Nigeria (31). Cd and Pb are non-essential and highly toxic metals, even at low concentrations, for plants and humans (16). Compared to the reported values, the raw tobacco leaves considered in this study showed lower concentrations of the studied metals. The observed concentrations of the target heavy metals in Assosa district tobacco leaves were also lower than those reported in tobacco leaves collected from other regions of Ethiopia: Shewa Robite and Billate farmlands (14). The observed variations are may be due to the soil nature, repeated use of fertilizers and other agrochemicals on these farmlands rather than the farmlands considered in the present study. Generally, the observed concentrations of heavy metals in the tobacco leaves of Assossa District could be due to the availability of lower amounts of heavy metals in the farmland soils, which were lower than the WHO permissible limits in soil and plants (28). As a result, heavy metals' absorption and accumulation by tobacco plants in the study area were lower than those reported in raw tobacco leaves and tobacco cigarettes.

Table 5. Comparison of heavy metal levels (mg/kg dry mass) of the studies tobacco leaves with literature values.

Type	Metal concentrations (mg/kg)					Country	Reference
	Cd	**Cu**	**Mn**	**Zn**	**Pb**		
Tobacco Cigarettes	5.90-7.94	18.26-34.94	44.67-297.69	47.02-167.31	17.21-74.78	Nigeria	(31)
	0.18	NR	112.03	NR	0.60	Spain	(30)
	0.50	7.89	45.03	8.57	14.39	Pakistan	(32)
	0.18-3.07	NR	108-244.26	32.30-72.0	NR	Mexico	(33)
	1.73-2.02	Nd	Nd	Nd	0.38-1.16	Ireland	(36)
	0.45	14	NR	27	1.94	India	(9)
	0.65	Nd	Nd	Nd	0.27	Brazil	(37)
	0.18	4.13	NR	NR	0.64	China	(38)
	1.55	8.95	NR	79.3	ND	Ethiopa (Nyala)	(14)
Raw Tobacco Leaves	0.21	11.5	NR	NR	0.57	China	(38)
	1.20-1.30	4.38-7.30	NR	33.2-53.7	ND	Ethiopia (Billate & Shewa Robit)	(14)
	0.01	0.11-0.21	0.75-1.18	0.58-1.16	ND	Ethiopia (Assosa)	This study

NR Not reported; ND Not detected

CONCLUSION

In this study, the concentrations of toxic heavy metals (Cd and Pb), as well as micro-essential heavy metals (Cu, Mn, and Zn), were determined in tobacco leaves and their growing soil samples collected from five different tobacco farmlands in Assosa District, Benishangul-Gumuz Regional State, Ethiopia. Relatively, the highest concentrations of Mn were observed in both tobacco leaves and soil samples. However, the obtained concentrations of Cd and Pb were low in the studied samples. The study results demonstrated that the concentrations of the micronutrients (Mn, Cu, and Zn) in tobacco leaves and soil samples were below the WHO permissible limits in soils and plants. However, tobacco leaves collected from Amba and Basha farmlands contained slightly higher concentrations of Zn than the WHO permissible limit in plants. The heavy metal content of the studied tobacco leaves was much lower than the values reported in the literature.

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