

# Hazardous impact of toxic metals on tobacco leaves grown in contaminated soil by ultrasonic assisted pseudo-digestion: Multivariate study

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## Abstract

Tobacco leaves (*Nicotiana tabacum* L.), agricultural soil and pollute irrigated lake water samples were collected during 2005–2006 and analyzed for Cd and Ni by electrothermal atomic absorption spectrometry (ETAAS). A simple and efficient procedure was investigated for the complete decomposition of tobacco leaves using ultrasonic assisted acid pseudo-digestion method (UPDM). A Plackett–Burman experimental design was used as a multivariate strategy for the evaluation of seven factors/variables at once, while central composite were used to found optimum values of significant variables. The accuracy of the proposed methods was assessed by analyzing certified reference (CRM); Virginia tobacco leaves (CTA-VTL-2). The results being compared with those obtained by conventional wet acid digestion method. The result obtained by optimized method showed good agreement with the certified values and sufficiently high recovery 97.8 and 98.7% for Cd and Ni, respectively. Under optimal conditions, the detection limits ( $3\sigma$ ) were evaluated to be  $0.019 \mu\text{g g}^{-1}$  for Cd and  $0.37 \mu\text{g g}^{-1}$  for Ni. The proposed method was successfully applied to the determination of Cd and Ni in raw, processed tobacco and different branded cigarettes samples.

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## 1. Introduction

Tobacco-related diseases originate from the biological consequences of repeated inhalation exposure to numerous toxic constituents in cigarette smoke, which are produced by pyrosynthesis or liberated during combustion. Tobacco smoke has toxic, genotoxic, mutagenic and carcinogenic properties [1,2]. Metal analysis of tobacco plants is an essential feature of environmental, biological and chemical research. Robinson et al. and Romkens et al. reported that tobacco is fast growing, deep-

rooted, easily propagated, has a high biomass production and hyper-accumulators, ability to extract, accumulate and tolerate high levels of heavy metals from soils [3,4]. In Canada, the contents of such metals in cigarette tobacco have been determined as an indicator of environmental change [5].

Thus, levels of heavy metals in tobacco are higher when grown in soil contaminated with heavy metal. Other environmental factors may influence heavy metal uptake by tobacco plants including soil pH and heavy metal-containing sludge or fertilizers applied to crops. Toxic metals found in these soils cause deleterious effects due to inhalation of contaminated soil, or from the ingestion of fruits and vegetables grown in contaminated soils. It was intensively investigated that uptake of metals by plants depends on the physico-chemical makeup of the plant species and soil [6,7]. It has been known for a few decades that tobacco combustion has the potential to deliver dangerous quantities of heavy metals to the lungs if the tobacco being combusted has high initial concentrations [8]. Indeed smok-

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ing has long been considered a major source of several heavy metals in blood and various organs [9], and cadmium in particular is regarded as one of the “strong carcinogens” in tobacco smoke [10]. Cadmium is a non-essential, potentially toxic, largely pollutant ion, which is accumulated by tobacco plants and is transferred in tobacco smoke to humans [11]. Nickel is currently classified as Group 1 “carcinogenic to humans” by the International Agency for Research on Cancer (IARC) [12].

Determination of Cd and Ni in plant samples requires the use of sensitive and selective techniques such as ETAAS, using sample preparation strategies addressed to shortening and simplifying the stages previous to analysis. There are various dissolution possibilities, but in most cases these turn out to be slow, tedious and subject to contamination problems or loss of volatilization [13]. Although microwave ovens of different designs are widely used in the analytical laboratories throughout the world, some problems were arisen, the high cost, short lifetime of the digestion vessels operated at high pressures (>60 bar) and temperatures (>260 °C), long time required for cooling the digestion vessel, sample throughput is not very high and constant supervision required of the digestion procedure [14,15]. One of the techniques that have shown promise for speeding up and simplifying sample treatment, with minimal contamination, low reagent consumption, and generation of minimal residue or waste is ultrasonic assisted treatment of samples [16]. Ultrasound (US) can be considered an alternative for solid sample pre-treatment because this energy facilitates and accelerates some steps, such as dissolution, fusion and leaching, among others [17].

Concerning mechanical and chemical effects, US is known to increase the reactivity of some chemicals. The high temperature and pressure within a collapsing cavitations bubble produced by US irradiation causes the formation of free radicals and various other species. The primary chemical effects are therefore the promotion and acceleration of reactions involved in sample digestion. These localized high-energy environments formed by the application of ultrasound to solutions can be employed for the purpose of extracting elemental analytes from solid matrices, so that elemental species may then be measured by instrumental techniques such as atomic spectrometry [18,19]. US energy has been also used for extraction metals in different biological samples [20,21]. Good recoveries were obtained with diluted acids for pure organic samples. However, for samples containing inorganic part, the reported recoveries were not always quantitative.

Procedures involving optimization by multivariate techniques have been increasingly used as they are faster, more economical and effective, and allow more than one variable to be optimized simultaneously [22,23]. Among the different groups of designs, Plackett–Burman designs (PBDs), introduced in 1946 by Plackett and Burman, allow us to discover the most significant variables for a certain system with only few experiments [24]. The interest on the use of such optimization method and PBDs has been applied to optimize some sample pre-treatments [25].

The aim of present study was to develop UPDM method for the extraction of toxic metals from tobacco leaves. For this pur-

pose, PBD was used as a multivariate strategy for the evaluation of the effects of varying several variables at once. Parameters influencing UPDM such as pre-sonication time [PSt], sonication time [St], temperature of ultrasonic bath [Bt], particle size [Ø], sample mass [SM], volume of HNO<sub>3</sub> [A1], and volume of a mixture of HNO<sub>3</sub>:H<sub>2</sub>O<sub>2</sub> [A2] were regarded as factors. Further optimization was done by a 2<sup>3</sup> + star central composite design (CCD), which involved 16 experiments. Optimum values of the variables were selected for the extraction of Cd and Ni from raw and processed tobacco leaves. An acid digestion induced by electric hot plate was used also for comparative purposes. The uptake of Cd and Ni in tobacco leaves grown in agricultural land irrigated with polluted lake water was evaluated. The obtained values were compared with those obtained from different branded cigarette. The water and soil samples were also analyzed.

## 2. Experimental

### 2.1. Apparatus

Agate ball mixer mill (MM-2000 Haan, Germany), was used for grinding the dried raw processed and branded tobacco leave samples, to reduce the particle size. Sieve made of nylon with mesh size of <30 and <65 µm. The UPDM was carried out with an ultrasonic bath (Sonicor, Model No. SC-121TH, programmable for temperature ranging from 0 to 90 °C with intensification frequency of 35 kHz). Centrifugation was carried out using a WIROWKA Laboratoryjna type WE-1, nr-6933 centrifuge (speed range 0–6000 rpm, timer 0–60 min, 220/50 HZ, Mechanika Pheczyjzna, Poland). A PerkinElmer Model 700 (Norwalk, CT, USA) atomic absorption spectrometer, equipped with a graphite furnace HGA-400, pyrocoated graphite tube with integrated platform, an autosampler AS-800, were used for the analysis. The instrumental parameters are shown in Table 1.

### 2.2. Reagents and glassware

Ultrapure water obtained form ELGA labwater system (Bucks, UK) was used throughout the work. 1000 ppm standard solutions of Cd and Ni were prepared by dilution of certified standard solutions, Fluka kamika (GmbH CH-9471, Buchs, Switzerland. Mg(NO<sub>3</sub>)<sub>2</sub> stock standard solution, 5.00 g L<sup>-1</sup>, used as a chemical modifier, was prepared from Mg(NO<sub>3</sub>)<sub>2</sub> Merck (Darmstadt, Germany). Pd stock standard solution, 3.00 g L<sup>-1</sup>, used as a chemical modifier, was prepared from Pd 99.999% Sigma Aldrich (Milwaukee, WI, USA). Concentrated 65% HNO<sub>3</sub> and 30% H<sub>2</sub>O<sub>2</sub> were spectroscopic grades (Merck). Certified standard reference material Virginia tobacco leaves (ICHTJ-cta-VTL-2) was purchased from International Atomic Energy Agency, Vienna (Austria). All glassware was used previously treated for a 24 h in 5 M HNO<sub>3</sub> and rinsed with double distilled water and then with ultra pure water.

Table 1  
Measurement conditions for electrothermal atomization AAS 700

Parameters	Cd	Ni
Lamp current (mA)	4	30
Wave length (nm)	228.8	232.0
Dry temperature (°C)/dry time (ramp/hold) (s)	140/15/5	140/15/5
Ashing temperature (°C)/ashing time ramp/hold (s)	850/10/20	1400/10/20
Atomization temperature (°C)/atomization time/ramp/hold (s)	1650/0/5.0	2500/0/5.0
Cleaning temperature (°C)/cleaning (ramp/hold) (s)	2000/1/3	2600/1/3
Chemical modifier 10 (μL)	Mg(NO <sub>3</sub> ) <sub>2</sub> + Pd(NO <sub>3</sub> ) <sub>2</sub> (0.01 + 0.015 mg)	Mg(NO <sub>3</sub> ) <sub>2</sub> (0.05 mg)
Common parameters		
Slit width = 0.7 L		
Sample volume = 10 (μL)		
Cuvette = Pyrocoated graphite tube		
Back ground correction = D <sub>2</sub> lamp		
Carrier gas (argon) = 200 mL min <sup>-1</sup>		

### 2.3. Sample collection and pre-treatment

#### 2.3.1. Water

Two-year survey (2005–2006) was conducted to collect five to ten water samples each month from Manchar lake (Sehwen Sindh, Pakistan) (26°3′N:67°6′E), and analyzed for different physico-chemical parameters. The analytical data quality was ensured through careful standardization, procedural blank measurements, and duplicate samples. For Cd and Ni analysis the sample solutions were concentrated five times by evaporating the water at 60 °C on an electric hot plate, filtered and kept at 4 °C till further analysis.

#### 2.3.2. Soil

Soil samples were collected from an agricultural land irrigated with contaminated lake water in two growing seasons, where tobacco is cultivated. Composite soil samples consisting of ten subsamples were collected from the upper layer (0–30 cm) of each year with two replicates. The treatment and physico-chemical analysis were reported in our previous work [26]. Total Cd and Ni were determined by digestion of 0.2 g samples using HNO<sub>3</sub>–HCl (aqua regia) by conventional wet acid digestion method, while leaching Din test based on the German Standard Method was used for water extractable metals (DIN 38414-S4) [27].

#### 2.3.3. Tobacco leaves

Tobacco leaf samples were collected from the agricultural lands irrigated with polluted lake during 2005–2006, fifty samples per year ( $n=100$ ). The processed tobacco leaves were collected from local industry ( $n=50$ ), where the same raw tobacco leaves are processed for preparing local unbranded cigarette. The samples once in the laboratory were washed with ultra pure water and dried for 48 h in an oven at 65 °C. Twelve different branded cigarettes ( $n=10$ ) were purchased from local market for comparison purpose. The raw, processed and different branded cigarettes tobacco were ground in vibrational agate ball mill for 5 min using a power of 60%. The powdered samples were sieved through nylon sieve to obtain particle size [φ] 30–65 μm and were stored in closed polyethylene bottles and kept in a refrigerator at 4 °C until analysis.

### 2.4. Digestion procedures

#### 2.4.1. Ultrasound-assisted pseudo-digestion method (UPDM)

For UPDM optimization, HNO<sub>3</sub> [A1], mixture of HNO<sub>3</sub>:H<sub>2</sub>O<sub>2</sub> [A2] (1:1, v/v), pre-intensification time (2–10 min) intensification or sonication times (1–5 min), particle size (30–65 μm), sample mass (25–100 mg) and the temperature of ultrasonic bath was ranged between 40 and 80 °C were tested. To evaluate the efficiency of the process, the results obtained with the UPDM were compared with those obtained from CDM, using electric hot plate on same certified and real samples. To optimized the different analytical variables, six replicate of certified tobacco leaves, at (–) and (+) level for SM were taken in polytetrafluoroethylene (PTFE) flasks (25 mL capacity), concentrated acids A1 and A2 were added at two level, minimum (–) and maximum (+) separately, and kept at room temperature, marked as PSt (without ultrasonic stirring), after different time intervals (2–10 min), the flasks were placed inside the ultrasonic water bath and were subjected to ultrasonic energy at 35 kHz for different time intervals (1–5 min). The temperature range of ultrasonic water bath was 40–80 °C. After sonication for different time intervals, the content of flasks were diluted with 5 mL deionized water and subjected to ultrasound energy for 2 min and centrifuge at 3000 rpm. Final solution was made up to 10 mL with ultrapure water, and collected in polyethylene flask and kept at –4 °C. Blanks were also treated in the same way. After studying the effect of the different variables involving in UPDM method, the remaining collected raw, processed and different branded tobacco samples were prepared according to optimized conditions prior to determine the analytes understudy.

#### 2.4.2. Conventional digestion method (CDM)

An acid digestion method induced by electric hot plate was used in order to discover the total content of toxic metals and also for comparative purposes. Triplicate samples of Standard reference Material Virginia Tobacco Leaves (ICHTJcta-VTL-2) and duplicate 100 mg subsamples of dried tobacco leaves ( $n=10$ ) were weighed in 50 mL Pyrex flasks. Added 5 mL volume of a freshly prepared mixture of concentrated

Table 2  
Variable and levels used for the Plackett–Burman and central composite designs in the factorial design

Variables	Symbol	Low level (–)	High level (+)
Pre-sonication time (min)	PSt	2	10
Sonication time (min)	St	1	5
Temperature of ultrasonic bath (°C)	Bt	40	80
HNO <sub>3</sub> (mL)	A1	1	2
HNO <sub>3</sub> :H <sub>2</sub> O <sub>2</sub> (mL)	A2	1	2
Sample mass (mg)	SM	25	100
Particle size (µm)	Ø	30	65

HNO<sub>3</sub>–H<sub>2</sub>O<sub>2</sub> (1:1, v/v), to each flask and solutions were heated on electric hot plate at 80 °C, for 2–3 h, till the clear transparent digests were obtained. The final solutions were collected in polyethylene flask, for the determinations of Cd and Ni, by ETAAS. The resulting digests obtained from both methods were analyzed by ETAAS by delivering 10 µL aliquots and 10 µL appropriate modifiers to the atomizer. The concentrations were obtained directly from calibration graphs after correction of the absorbance for the signal from an appropriate reagent blank.

## 2.5. Experimental design

### 2.5.1. Plackett–Burman design

The PBD was used as a screening approach with the aim of establishing the significant factors that influence the proposed method and selecting suitable digestion conditions. The application of this experimental design reduced the development time of the methods and provided less ambiguous digestion conditions, hence facilitating data interpretation. For the evaluation of seven factors at two levels PBDs with only twelve experiments is described instead of the  $2^7 = 128$  required for a full factorial design. This work was carried out using the Minitab (Release 13 of MINITAB) Version 5.1 [28,29]. This optimization method permits estimation of the principal effects of the variables studied as well as the values for each (+) representing the maximum and (–) the minimum are shown in Table 2. Twelve experiments were carried out for completing the design matrix, the resulting values (1–12) are being the % recovery of Cd and Ni average value of six replicates. Sometimes, the effect of some factors (less significant) can lead to wrong results on the importance of the (most significant) variables. In addition, the evaluations of interactions between factors are also important. Such interactions are not allowed by a design of this type, and to evaluate them, the effects of some variables were omitted, and the effects of the significant factors were again studied CCD.

### 2.5.2. Central 2<sup>3</sup> + star orthogonal composite design

Having screened out the variables that did not have a significant effect on the response, the remaining three factors were optimized to provide the maximum metal recovery. A CCD with 6 degrees of freedom and involving 16 experiments was performed to optimize the variables, A2, Bt and St for the Cd and Ni determination in tobacco. The factors that were shown to

be insignificant by the PBDs were fixed at convenient values as PSt = 6 min, SM = 100 mg, and particle size [Ø] were <35 µm for all runs.

## 3. Results and discussion

We adopted ultrasound energy to enhance the action of acid on decomposition of plant material, for the determination of Cd and Ni by ETAAS. By this proposed method to avoid interference problems which most frequently occur in slurry and direct introduction of biological samples to graphite furnace due to high organic matter, as for from the reliable approaches for routine analysis. The all blanks, standards and sample solution were made in 0.1 M HNO<sub>3</sub>, to minimize the matrix interferences. Seven factors were selected to be examined by proposed UPDM. Two factors are related to the acids, which are volume of HNO<sub>3</sub> [A1] and HNO<sub>3</sub>–H<sub>2</sub>O<sub>2</sub> [A2], pre-sonication time [PSt] prior to subjecting US energy, sonication time [St], temperature of ultrasonic bath [Bt], sample mass [SM] and particle size of samples [Ø]. The ultrasound energy was fixed at a frequency of 35 kHz for all experiments. Extraction efficiency, expressed as percentage, was calculated as the ratio between element content obtained by US treatment and those obtained with CDM, based on the equation:

$$\% \text{ Recovery} = \frac{[\text{Metals obtained by USD}]}{[\text{Metals obtained by CDM}]} \times 100$$

A recovery close to 100% would show quantitative extraction of both metals. The results of the design (% recovery) are shown in Table 3 and visualized by using a Pareto chart of the standardized effect, Figs. 1 and 2 for Cd and Ni, respectively. From inference tests, the results produced a minimum *t*-value at the 95.0% confidence interval of 2.8, and we consider that a factor is significant when the *t*-value for a certain factor is higher than 2.8. Significance effects were checked by analysis of the variance (ANOVA) and using *P*-value shown in Table 4. These values express how much the probability of a factor is due to random errors.

Table 3  
Plackett–Burman design for the significant variable determination (*n* = 6)

Run	PSt	St	Bt	SM	Ø	A1	A2	% Recovery (mean ± S.D.)	
								Cd	Ni
1	+	–	+	–	–	–	+	67.5 ± 4.6	58.8 ± 4.1
2	+	+	–	+	–	–	–	55.8 ± 3.9	50.6 ± 2.6
3	–	+	+	–	+	–	–	71.1 ± 5.2	66.2 ± 5.3
4	+	–	+	+	–	+	–	54.4 ± 4.0	51.7 ± 3.5
5	+	+	–	+	+	–	+	70.3 ± 4.7	60.4 ± 5.4
6	–	+	+	–	+	+	–	86.5 ± 5.8	74.6 ± 8.2
7	–	+	+	+	–	+	+	98.6 ± 7.6	97.3 ± 6.0
8	–	–	+	+	+	–	+	65.3 ± 4.8	61.2 ± 3.8
9	–	–	–	+	+	+	–	43.8 ± 2.2	28.7 ± 3.3
10	+	–	–	–	+	+	+	46.2 ± 3.0	38.5 ± 3.5
11	–	+	–	–	–	+	+	61.8 ± 4.3	54.5 ± 4.8
12	–	–	–	–	–	–	–	38.2 ± 2.7	34.2 ± 3.0

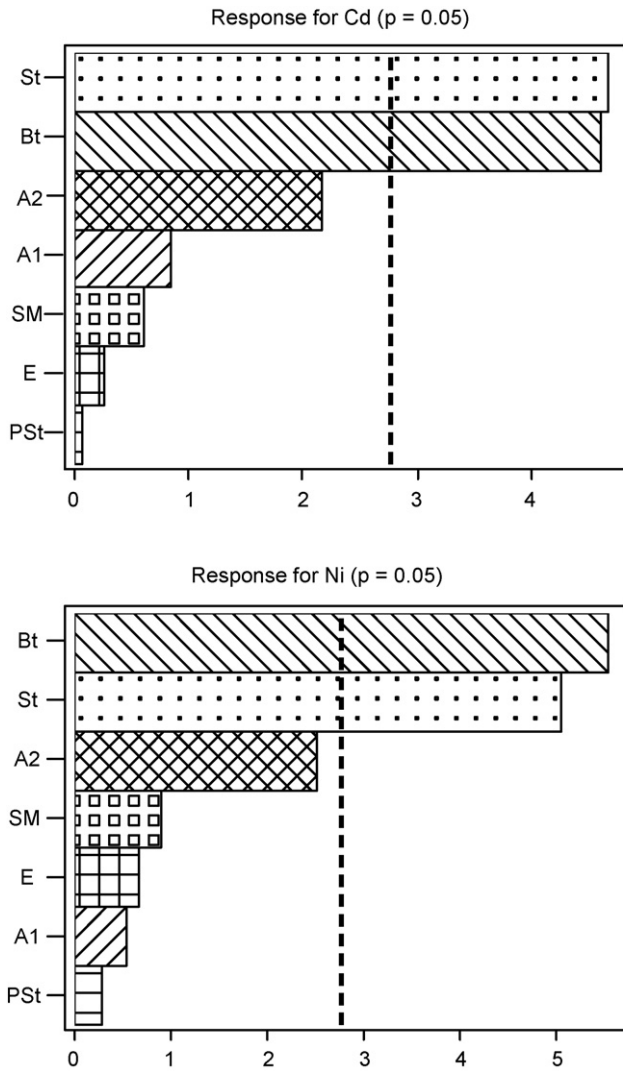


Fig. 1. Pareto chart of the standardized effects.

### 3.1. Estimated effects of variables

For both elements, the most significant effect was found for variable Bt, in the order of Ni > Cd, while reverse the case for St. The maximum recoveries of both elements were observed at 80 °C. It can be seen in experiment 5 of Table 3 that at (–) level of Bt with optimum values of other variables, the % recovery of Cd and Ni were 70.3 and 60.4, respectively. The influence of pre-sonication time (kept the concentrated acid treated samples at room temperature for different time interval) was not significant, it was observed that at low level of PSt, both elements were quantitatively extracted (experiment 7) when other parameters were at optimum level. Sonication time is in the range of 1–5 min; however, it can be seen in Table 3, that the maximum recoveries of both elements were achieved after 5 min at temperature 80 °C of US bath, as compared to those obtained by CDM. In previous studies developed by El-Azouzi et al. the St was about 180 min [30]. From the results of the PBDs (Table 3), it is clearly observed that high amounts (+) of HNO<sub>3</sub>:H<sub>2</sub>O<sub>2</sub> mixture (A2) provided a significantly high recovery for Cd and Ni

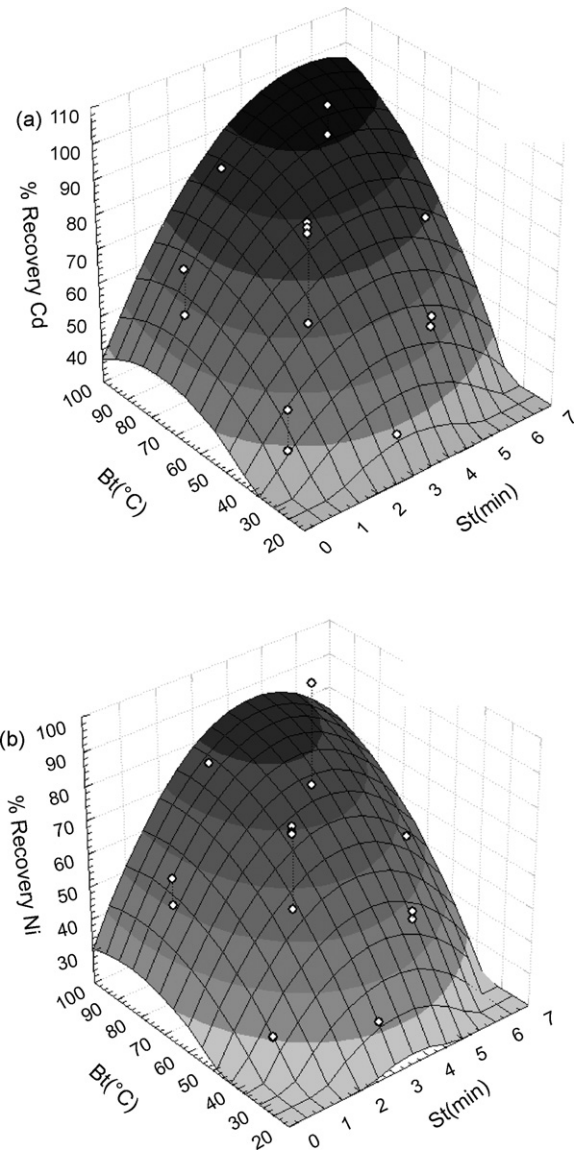


Fig. 2. Three-dimensional (3D) surface plot of % recovery (a) for Cd and (b) for Ni.

as compared to alone acid (A1). The concentrated HNO<sub>3</sub> alone at (+) level, gave 86.5 and 74.6% recovery of Cd and Ni, respectively, although the other variables were at optimum levels. The particle size attempted for metal pseudo-digestion in this work with the use of ultrasonic bath ranged from upto 30 to 65 μm. It was reported in literature that smaller particles size of samples required smaller acid concentration and volume and the sonication time [31]. It was also reported in literature that particle size can be considered as insignificant due to high energy supplied by US energy (frequency 35 kHz) [32]. It was observed in experiment 7 the optimum recoveries of Cd and Ni were obtained when ϕ have (–) level, i.e. <35 μm. There was significant difference between 25 and 100 mg sample mass for both metals at 0.05 probabilities. It can be seen in experiment 6 and 7 (Table 3), SM at (–) level produced 86.5 and 74.6% recovery of Cd and Ni, respectively, i.e. 12.1 and 22.7% less than those values obtained at SM (+) level. The results indicated that 100 mg sample mass was sufficient for optimum recovery of both metals.

Table 4  
Estimated effects of main variables for % Cd, and Ni

	Cd					Ni			
	<sup>a</sup> DF	<sup>b</sup> SS	<sup>c</sup> MS	F-ratio	<sup>d</sup> P-value	<sup>b</sup> SS	<sup>c</sup> MS	F-ratio	<sup>d</sup> P-value
PSt	1	0.300	0.300	0.005	0.948	4.70	4.70	0.084	0.787
St	1	1380	1380	21.7	0.010	1420	1420	25.3	0.007
Bt	1	1350	1350	21.3	0.010	1701	1701	30.3	0.005
SM	1	23.8	23.8	0.375	0.573	44.5	44.5	0.793	0.423
Ø	1	4.00	4.00	0.063	0.815	25.5	25.5	0.455	0.537
A1	1	44.5	44.5	0.701	0.450	16.1	16.1	0.287	0.620
A2	1	299	299	4.71	0.096	348	348	6.22	0.067
St × A2	1	53.3	53.3	0.263	0.322	44.5	44.5	0.180	0.682
ST × Bt	1	113	113	0.093	0.168	124	124	0.065	0.145
Error	2	88.1	44.05			55.7	27.85		
Total	11	3356				3784.			

<sup>a</sup> DF = degrees of freedom.

<sup>b</sup> SS = sum of squares.

<sup>c</sup> MS = mean squares.

<sup>d</sup> P-value = probability level.

### 3.2. Optimization by central composite design

Taking into account that temperature of ultrasonic bath [Bt] is efficient for the recovery of metals under study and has a strong interaction with sonication time [St] and acid mixture [A2], as demonstrated by results of the PBDs. So these three factors were optimized to provide the maximum recovery of Cd and Ni. A central  $2^3$  + star orthogonal composite design with 6 degrees of freedom, involving 16 experiments were performed to optimize these variables. The factors that were shown to be insignificant by the PBDs were fixed at convenient values; therefore, the mass of tobacco leaves were 100 mg,  $\phi < 35 \mu\text{m}$  and PSt at 6 min, while A1 was omitted in this experiment, for all runs. The experimental field definition for this design is given Table 2, while Table 5 shows the central composite design together with the % response obtained for Cd and Ni. The study of estimated response surfaces for variables, [Bt]/[St] for Cd and Ni, showed the optimum values of these variables for both elements Fig. 2(a and b). The comments for each element are the following.

### 3.3. Cadmium

Ultrasound energy has significant effect on % recovery of Cd from plant matrixes. It was observed that at low level (–) of St, the recovery of Cd is 69.2%, which was 29.5% lower as compared to the value obtain at high level of St (run 7 and 8). It can be seen, from Fig. 2(a), that the UPDM efficiency is increased when the temperature was higher, at low level (–) the percentage recovery of Cd was 56.3% (Table 5 run 6). The recovery of Cd was 90.0% at (–) level of A2, which is higher as compared to Ni at same condition (run 4). The percentage recovery of Cd was peaked when St and Bt at the maximum levels.

### 3.4. Nickel

From center composite design (Table 5), it was observed that the % recovery of Ni was increased when the temperature was

at high level, while at low level (–) the recovery of Ni is 48.6% (run 6 in Table 5), although the other two variables A2 and St were at (+) levels. In this case, the extraction efficiency is directly proportional to the temperature (Bt). The other effective variable is St, at (+) level as shown in Table 5 (run 8), the recovery of Ni was enhanced 40.2% as compared to the run 7 at low level (2 min). It is also indicated in the estimated response surface (Fig. 2(b)). The other effective variable is  $\text{HNO}_3:\text{H}_2\text{O}_2$  mixture volume [A2]. It was noted that at (–) level of A2, the % recovery of Ni obtained 67.6 (run 4), to increase the volume of A2, enhance 30.2% recovery of Ni (run 8) at optimum values of other two variable (St and Bt).

Table 5

Central  $2^3$  orthogonal composite design for the sonication time/ultrasonic water-bath temperature/ $\text{HNO}_3:\text{H}_2\text{O}_2$  ( $n = 6$ )

Run	St	Bt	A2	% Recovery (mean ± S.D.)	
				Cd	NI
1	–	–	–	48.8 ± 1.4	30.5 ± 2.2
2	+	–	–	53.4 ± 1.5	46.3 ± 2.1
3	–	+	–	55.6 ± 2.9	49.6 ± 3.8
4	+	+	–	90.0 ± 6.4	67.6 ± 2.5
5	–	–	+	36.8 ± 1.5	30.6 ± 1.8
6	+	–	+	56.3 ± 3.4	48.6 ± 3.9
7	–	+	+	69.2 ± 3.3	57.6 ± 3.0
8	+	+	+	98.7 ± 7.4	97.8 ± 4.3
9	– $a^1$	$a_0^f$	$b_0^f$	51.6 ± 3.3	45.3 ± 2.7
10	+ $a^2$	$a_0^f$	$b_0^f$	69.4 ± 5.4	55.3 ± 2.3
11	$c_0^f$	– $b^1$	$b_0^f$	38.6 ± 3.7	32.4 ± 2.7
12	$c_0^f$	+ $b^2$	$b_0^f$	82.8 ± 5.1	76.6 ± 6.4
13	$c_0^f$	$a_0^f$	– $c^1$	53.7 ± 3.1	48.9 ± 3.5
14	$c_0^f$	$a_0^f$	+ $c^2$	83.0 ± 4.6	73.6 ± 5.3
15	$c_0^f$	$a_0^f$	$b_0^f$	81.5 ± 4.0	71.8 ± 4.5
16	$c_0^f$	$a_0^f$	$b_0^f$	79.8 ± 2.9	71.3 ± 4.8

$a^1 = -0.7272 \text{ min}$ ,  $a^2 = 12.727 \text{ min}$ ;  $b^1 = 26.3641 \text{ }^\circ\text{C}$ ,  $b^2 = 93.6359 \text{ }^\circ\text{C}$ ;  
 $c^1 = 0.6591 \text{ mL}$ ,  $c^2 = 2.409 \text{ mL}$ .

<sup>a</sup>  $f_0 = 60 \text{ }^\circ\text{C}$ .

<sup>b</sup>  $f_0 = 1.5 \text{ mL}$ .

<sup>c</sup>  $f_0 = 6 \text{ min}$ .

Table 6  
Determination of Cd and Ni in standard reference Materials Virginia Tobacco Leaves (ICHTJ-cta-VTL-2) ( $\mu\text{g g}^{-1}$  dried basis)

CRM/metals	Certified values	CDM $\bar{x} \pm t/\sqrt{n}^a$	% Recovery	% relative error	USD $\bar{x} \pm t/\sqrt{n}^a$	% Recovery	% Relative error	$t$ critical=2.57
ICHTJ-cta-VTL-2								
Cd	1.520 $\pm$ 0.17	1.530 $\pm$ 0.10	100.6	0.65	1.501 $\pm$ 0.078	98.7	−1.3	0.12 <sup>a</sup>
Ni	1.980 $\pm$ 0.21	1.984 $\pm$ 0.21	100.2	0.20	1.937 $\pm$ 0.16	97.8	−2.2	0.57

<sup>a</sup> Average value  $\pm$  confidence interval ( $P=0.05$ ), ( $n=6$ ).

### 3.5. Analytical figure of merit

The equations for the linear range of the Cd and Ni calibration curves were the following:

$$y = (2.88 \times 10^{-2} \pm 2.4 \times 10^{-4})(\text{Cd}) \\ + (1.8 \times 10^{-3} \pm 1.0 \times 10^{-4}) \quad (R^2 = 0.9999)$$

$$y = (1.1 \times 10^{-3} \pm 1.9 \times 10^{-5})(\text{Ni}) \\ + (4.0 \times 10^{-4} \pm 2.9 \times 10^{-5}) \quad (R^2 = 0.9993)$$

where  $y$  is integrated absorbance and the concentration in each case expressed as  $\mu\text{g L}^{-1}$ . The linear range of the calibration curve reached from the detection limit up to 10, and  $100 \mu\text{g L}^{-1}$ , for Cd and Ni, respectively. Characteristic masses were 1.0 and 32  $\mu\text{g}$  for Cd and Ni.

The detection and quantification limits, given by

$$\text{LOD} = 3 \times \frac{s}{m}$$

and

$$\text{LOQ} = 10 \times \frac{s}{m}$$

respectively, where  $s$  is the standard deviation of ten measurements of a reagent blank and  $m$  is the slope of the calibration graph. The LOD of 0.05 and  $0.15 \text{ ng mL}^{-1}$  and LOQ of 0.17 and  $0.5 \text{ ng mL}^{-1}$  calculated for Cd and Ni, respectively.

In order to confirm the applicability of the UPDM process to the determination of Cd and Ni in CRM Virginia tobacco leaves samples result shown in Table 6, the analytical characteristics, precision of the methods, expressed as the relative standard deviation (R.S.D.) of a minimum of 6 independent analyses of the same sample, provided values ranging from 5.2 to 8.26% as a function of the element considered and its concentration level.

### 3.6. Application for real samples

The proposed methodology was applied to the duplicate of each raw, processed and branded tobacco samples, to extract the Cd and Ni. The mean metals concentration expressed as  $\bar{x} \pm t/\sqrt{n}$ , for  $n=100$  (raw tobacco leaves samples),  $n=50$  (processed tobacco leaves samples) and  $n=10$  (branded cigarette samples), are shown in Table 7.

### 3.7. Evaluation of Cd and Ni in water, soil and tobacco samples

The results of physico-chemical analysis of lake water and agricultural soil are shown in Table 8. The pH of agricultural soil and lake water was found in the range of 6.4–6.8 and 7.4–8.0, respectively. The level of Cd and Ni in lake water used for irrigation an agricultural land where tobacco is cultivated, were found in the range of 4.22–7.4 and  $10.5\text{--}40.9 \mu\text{g L}^{-1}$ , respectively during 2-year study 2005–2006. The agricultural soil contains total Cd (3.9–9.7) and Ni (13.1–28.6)  $\text{mg kg}^{-1}$ , on dried weight basis (DW). The availability of metals was calculated as the ratio of water extractable concentration to the total concentration for each metal ( $C_{\text{water extractable}}/C_{\text{aqua regia}} \times 100$ ). The water extractable contents of Cd and Ni in soil were observed in the

Table 7  
Analytical results obtained for Cd, and Ni in Tobacco ( $\mu\text{g g}^{-1}$  dried basis)

Samples	Cd $\bar{x} \pm t/\sqrt{n}$	Ni $\bar{x} \pm t/\sqrt{n}$	$\mu\text{g } 10 \text{ g}^{-1}$	
			Cd	Ni
Raw tobacco leaves <sup>a</sup>	6.85 $\pm$ 1.1*	2.82 $\pm$ 0.61	68.5	28.2
Unbranded cigarette <sup>b</sup>	7.12 $\pm$ 1.2	2.94 $\pm$ 0.54	71.2	29.4
Diplomat <sup>c</sup>	3.71 $\pm$ 0.15	1.21 $\pm$ 0.11	37.1	12.1
Morven gold <sup>c</sup>	5.18 $\pm$ 0.14	1.38 $\pm$ 0.12	5.18	13.8
Red & white <sup>c</sup>	3.84 $\pm$ 0.18	1.57 $\pm$ 0.15	38.4	15.7
Gold flake <sup>c</sup>	3.89 $\pm$ 0.13	1.36 $\pm$ 0.092	38.9	13.6
Gold leaf <sup>c</sup>	4.55 $\pm$ 0.21	1.77 $\pm$ 0.081	45.5	17.7
Boss <sup>c</sup>	4.52 $\pm$ 0.18	1.75 $\pm$ 0.076	45.2	17.5
Channel <sup>c</sup>	2.78 $\pm$ 0.16	1.28 $\pm$ 0.098	27.8	12.8
Capstan <sup>c</sup>	3.32 $\pm$ 0.21	1.36 $\pm$ 0.087	33.2	13.6
Camel <sup>c</sup>	4.09 $\pm$ 0.25	2.11 $\pm$ 0.14	40.9	21.1
Benson and heggen <sup>c</sup>	3.36 $\pm$ 0.41	1.27 $\pm$ 0.12	33.6	12.7
Dunhill <sup>c</sup>	2.11 $\pm$ 0.14	1.28 $\pm$ 0.095	21.1	12.8
Marlboro <sup>c</sup>	2.12 $\pm$ 0.3	1.01 $\pm$ 0.11	21.2	10.1

<sup>a</sup> ( $n=100$ ).

<sup>b</sup> ( $n=50$ ).

<sup>c</sup> ( $n=10$ ).

\* Average value  $\pm$  confidence interval ( $P=0.05$ ).

Table 8  
Physico-chemical parameters of agricultural soil and lake water

Parameters content	Soil	Water
pH	6.40–6.80	7.40–8.01
Cadmium	18.5 $\pm$ 2.8 <sup>a</sup> (0.880 $\pm$ 0.12) <sup>b</sup>	5.01 $\pm$ 1.5 <sup>c</sup>
Nickel	20.1 $\pm$ 3.4 <sup>a</sup> (0.48 $\pm$ 0.07) <sup>b</sup>	31.5 $\pm$ 9.4 <sup>c</sup>

<sup>a</sup> ( $\text{mg kg}^{-1}$ ).

<sup>b</sup> Water extractable metals.

<sup>c</sup> ( $\mu\text{g L}^{-1}$ ).

range of 3.92–4.64 and 1.55–2.39%, respectively. Therefore, the element of primary concern regarding soil pollution, Cd, had the highest availability. This result agrees with other investigations [33].

Smoking of tobacco leaves is one of the principal routes of exposure to heavy metals. Metals contained in tobacco leaves originate from root uptake and transfer to the shoots and also from deposition of aerosol particles on the leaves. Our results are consistent with other studies that tobacco grown in soils with higher available Cd and Pb levels has correspondingly higher levels in the tobacco leaves [34,35].

It is likely that cigarettes made from tobacco grown in various geographical regions or under different agricultural conditions will have different levels of the heavy metals in the tobacco filler and thus, generate different levels in the smoke. Cd oxide generated during the burning of cigarettes is highly bioavailable. Approximately, 10% of the inhaled Cd oxide is deposited in lung tissues, and another 30–40% is absorbed into systemic blood circulation of smokers. Smokers have 4–5 times higher Cd levels in blood and 2–3 times greater amounts of Cd in their kidneys than in non-smokers [36]. The average concentration of Cd in raw tobacco and cigarette made from it, was observed as  $6.85 \pm 0.8$  and  $7.12 \pm 1.2 \mu\text{g g}^{-1}$  DW, respectively. The concentration of Cd was 3.94% higher in processed tobacco used as filler for local unbranded cigarette as compared to raw tobacco, may be due to different processing steps and using additives for flavoring. In present work, for comparative purpose we analyzed 12 branded cigarettes. It was observed that the level of Cd in our study samples, are 3–4 times higher than those obtained from different branded cigarette tobacco. These results are 5–6 times higher than cigarette produced in United Kingdom ( $0.90 \mu\text{g g}^{-1}$ ) and Korea ( $1.02 \mu\text{g g}^{-1}$ ), respectively [37].

As in the case with Cd, tobacco plants absorb Ni from the soil and concentrate it in the leaves [9]. The concentrations of Ni in raw tobacco ranged from 2.42 to  $3.22 \mu\text{g g}^{-1}$  (DW), with an average of  $2.82 \mu\text{g g}^{-1}$ , are 1.5–2 times higher than other branded cigarette understudy. Nickel reacts with carbon monoxide in tobacco smoke to form a highly toxic carbonyl compound, which is believed to be a potential carcinogen. Nickel has been long known to produce nasal and lung cancers. Ni dermatitis is one of the common forms of allergic contact dermatitis. Thus, Ni in tobacco smoke may be responsible for some of the irritant properties of smoke in the lungs [38]. Elevated exposure to heavy metals (Cd and Ni) from smoking contributes to increased risk for lung disease, and other systemic maladies such as peripheral artery disease and complications of pregnancy [39–41]. In epidemiological studies of the association between Ni and cancer risk, Ni has been examined either alone or in combination with Cd. Jarup et al. reported a statistically significant increased risk of prostate cancer mortality with relatively high occupational Ni exposure [42]. Ni as a trace element that is ‘probably’ essential, given its role in Ni-containing enzymes found in plants and microorganisms. However, evidence that Ni has similar functions in humans is not currently available. In contrast, Ni compounds can display tumor promoting capability via a number of mechanisms including inhibition of intercellular communication, the induction of DNA deletions

Table 9

Correlation coefficient ( $r$ ) of Cd and Ni in water, soil (total and water extractable) with tobacco leaves ( $n = 100$ )

	Cd	Ni
Metals in water	0.971	0.902
Water extractable metals in soil	0.816	0.878
Total metals in soil	0.809	0.683

and aberrations, production of DNA-protein cross-links, oxidative damage, inhibition of nucleotide excision repair and an increase in DNA methylation leading to inactivation of gene expression [43].

Correlation coefficient ( $r$ ) of Cd and Ni in water, soil (total and water extractable) with tobacco leaves (Table 9) shows that there is a strong relationship among the concentrations of both metals in raw tobacco samples with lake water concentration. Strong correlation was also recorded among the available concentrations of metals from agricultural soil and their respective concentrations in raw tobacco leaves. This suggests that the concentrations of Cd and Ni in irrigated water and water extractable metals in the agricultural soil can be used as a predictor of metal concentration in tobacco leaves.

The local unbranded cigarette formed from tobacco grown on agricultural land irrigated continuously with contaminated lake water, have low price as compared to the different branded cigarette. So the high consumption of local unbranded cigarette due to poverty, create health effects of toxic metal, transfer from tobacco via smoke to the lungs indicates that habitual smokers may be risking additional harm from high levels of Cd and Ni possibly other toxic contents. In this area a survey study by us confirmed that the mortality rate is high due to lung diseases.

#### 4. Conclusions

The proposed ultrasound-assisted pseudo-digestion method can be used for the determination of toxic metals in plant samples with minimum time and reagents, using inexpensive device ultrasonic bath. The advantage of multivariate experimental design and optimization techniques becomes important in establishing a valid methodology with relatively small number of experiments. In the present study, the application of factorial design as factor screening showed that  $\text{HNO}_3:\text{H}_2\text{O}_2$  mixture (A2), temperature of ultrasonic bath (Bt) and the exposure to ultrasonic energy (St) was the most significant variable for maximum recovery of Cd and Ni from CRM and real samples of tobacco leaves. For comparative purpose 12 branded cigarettes were analyzed. It was observed that the level of Cd in our study samples is 3–4 times higher than those obtained from different branded cigarettes. A significant flux of heavy metals, among other toxins, reaches the lungs through smoking tobacco contained high level of Cd and Ni. Consequently, contaminated soil and irrigation with contaminated water usually avoided for tobacco cultivation.



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