I, Ryan T. Saadawi, hereby submit this original work as part of the requirements for the degree of Master of Science in Chemistry.

It is entitled: Total Metal Analysis in Hookah Tobacco (Narghile, Shisha) – an Initial Study

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Total Metal Analysis in Hookah Tobacco (Narghile, Shisha) – an Initial Study

A thesis submitted to the

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by

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Abstract of Thesis

In this initial study, the concentrations of eighteen elements (Na, K, Mg, Ca, Al, Fe, V, Cr, Mn, Ni, Cu, Zn, Se, Mo, As, Cd, Sb and Pb) in 12 hookah tobacco formulations (moassel) were studied by ICPMS. Eight of these were formulated in the United States, and four were from the Middle East. The analyses were made from two of the hookah compartments; as homogenized material of the hookah samples as received and by separating the received samples into the actual tobacco and a hot water extract that removed as much as possible of the other formulation substances. The extractable portion that represents up to 50% in mass of the total hookah sample is made up of honey/molasses, glycerin, colorants and flavorings, but this study shows the hot water extract to be relatively free of toxic elements. The elemental ranges found for the homogenized hookah mixtures are as follows: V, 0.03 - 0.16 µg g⁻¹; Cr, 0.15 - 0.37 µg g⁻¹; Mn 8.82 - 17.2 µg g⁻¹; Ni, 0.14 - 0.64 µg g⁻¹; Cu, 0.93 - 5.67 µg g⁻¹; Zn, 3.51 - 5.62 µg g⁻¹; As, 0.01 - 0.04 µg g⁻¹; Se, 0.002 - 0.014 µg g⁻¹; Mo, 0.05 - 0.13 µg g⁻¹; Cd, 0.10 - 0.27 µg g⁻¹ and Pb, <0.001 - 0.07 µg g⁻¹. Given the very small fraction of trace metals in the extract, most of the metals are contained in the tobacco leaves. It was found that the major variation in the concentrations owe to the geographic production regions; namely the USA and the Middle East. A principal component analysis was performed as an explorative tool, and a good separation was found, when grouping according to preparation by geographic production region. Based on these results the average mass of the more toxic elements (As, Cd, Cr, Pb,) present in a hookah smoking portion of about 15 g, is smaller than that contained in a normal cigarette. However, before any true comparisons can be made on the differences between the hookah formulations and
actual dry tobaccos, the temperature at which the smoke is produced, the length of time per smoking session, any possible metal contamination from the apparatus and contamination from the charcoal used to ignite the hookah formulation must be taken into account.
Acknowledgements

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Chapter 1 - Introduction

The tobacco plant has been a valuable crop since antiquity. The leaf of the tobacco plant has been smoked in various forms throughout history. Tobacco has the ability to hyperaccumulate metals such as cadmium, lead, zinc, nickel, copper, iron and manganese to name a few, which translocates throughout aerial portions of the plant including the leaf, which is consumed. While some of the metals are nontoxic, other metals are hazardous to human health and need to be carefully considered to ascertain their biological impact.

Tobacco has become well known to contain a variety of toxic compounds, which stem from the natural plant or additives that are incorporated during the manufacturing processes turning the raw product into a ready to use consumable. Although numerous studies have been performed on tobacco products identifying toxic components, the majority of these studies have focused on cigarette, cigar, or smokeless tobacco with few studies characterizing hookah tobacco. While many have tried to compare hookah tobacco to the other tobaccos, there is little in common other than the tobacco. The tobacco matrix and way hookah tobacco is consumed greatly differs from other forms of tobacco.

There are marked differences between the Hookah tobacco and cigarette/cigar/smokeless tobacco matrices. Cigarette and cigar tobacco consists mostly of dried tobacco with various additives incorporated during the manufacturing process turning the dry tobacco into ready to use products. Hookah tobacco is a wet matrix composed of tobacco and between 30% and 50% other material such as honey, molasses, glycerin, flavorings and colorant. There is less tobacco per unit mass of
hookah matrix compared to other tobaccos and there are a variety of other components contributing the wetness of the hookah matrix that are not found in other tobaccos. This makes it essential for hookah tobacco to be analyzed in a class of its own due to the “wet” elements and lower percent of leaf tobacco per unit mass. Also, hookah tobacco is smoked through a water pipe, because of these differences between hookah tobacco and other tobacco products comparison between elements and toxic compounds are not easily made.

Although leaf tobacco unconsumed has many of the same compounds no matter what form it is after it’s manufactured, the filtration may affect what is consumed. Generally smoked tobaccos are lit and the smoke is filtered. Most tobacco products filter the smoke through a dry cellulose acetate fibers filter, while many compounds are still consumed¹. Hookah tobacco is consumed through the use of a water pipe type instrument. The smoke is filtered through water before being inhaled. The effects of metal retention from the smoke by the water and if the hookah contraption itself contributes metals, is unknown. Studies need to be performed to characterize and compartmentalize where metals distribute throughout the hookah and to determine what metal concentrations are actually consumed by the user.

To consume hookah tobacco, the wet tobacco matrix is put into a bowl and covered with perforated aluminum foil. Charcoal is then placed onto the foil lighting the tobacco matrix. This tobacco matrix burns at a lower temperature than cigar or cigarette tobacco so the expected metals consumed would be different for hookah tobaccos, since many metals are volatile at higher temperatures than hookah tobaccos are burned. The effects of the charcoal are also unknown and may be a contributing factor
to metals consumed during the hookah consumption process. These variables need to be accounted for to ascertain the toxic metals consumed from hookah tobacco.

Very little has been done to determine the total metals encountered by hookah consumers and the concentrations of these metals that reflect real world bioavailability. Researchers have tried to use the Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) to characterize and quantify metal concentrations in hookah tobaccos however many of the toxic metals such arsenic, mercury, and lead were below the detection limits of the ICP-OES. Other studies have tried to identify the total metals consumed, however, their simulated smoking process did not accurately reflect real life consumption so no comparisons could be made.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is an excellent approach, often used to determine metal concentrations in tobacco products. The mass spectrometer detector allows for the achievement of low detection limits and offers the capability to monitor for multiple metals at once. Many toxic metals are hazardous to the health at parts per billion (ppb) concentrations. Many instruments suitable for metal analysis do not have low enough detection limits to monitor for elements such as lead, mercury and arsenic that are toxic at low concentrations. The ICP-MS has part-per-trillion, ppt, detection limits that cannot be matched by other atomic spectroscopic methods making it far better suited for total metal analysis in tobacco products. Although ICP-MS is subjected to polyatomic and isobaric interferences, the Agilent 7500 and 7700 are equipped with a collision reaction cell to remove polyatomic interferences through kinetic energy discrimination, and multiple isotopes can be monitored and mass filtered by the quadrupole if isobaric interferences are present.
Hookah tobacco must be completely digested in order to be analyzed by the ICP-MS. With the use of the CEM MARS Xpress microwave digestion system, from 100 mg to 500 mg hookah tobacco digestion can be achieved in a short amount of time in nitric acid. The CEM MARS Xpress is a closed digestion microwave system so, high temperatures can be achieved in less time as a result of the high temperature and high-pressure capabilities of the microwave system. When working with hookah tobacco and microwave digestion, one must take caution since nitric acid mixed with glycerin may form an explosive compound - nitroglycerin. Even though the microwave system digestion chamber is considered explosion proof, it is necessary to work with the smallest sample mass possible. To reduce effects from carbon interferences, hydrogen peroxide can be added to the digested samples.

After microwave digestion, the digested hookah samples may be diluted and analyzed for total metals using the Agilent ICP-MS and either online internal standard addition to correct for instrument drift or offline addition of the internal standard to correct for variation in sample preparation. As method validation, a fortified method blank and fortified sample for each different matrix should be prepared to validate the sample prep and ensure there is no sample loss or matrix enhancement/suppression during analysis.
Chapter 2 - Total Metal Analysis in Hookah Tobacco (Narghile, Shisha) – an Initial Study

2.1 Introduction

The health hazards associated with tobacco use are well known, especially with cigarettes, cigar and smokeless tobaccos. Much attention has focused on the toxic components of these\(^2\)-\(^8\) including toxic metals such as arsenic, nickel, cadmium, mercury, lead, and chromium. Interestingly, there are few studies on tobacco that is consumed through water filtration devices such as shisha\(^9\), jurak,\(^10\) mada’a\(^11\) and goza\(^12\), which are often mistakenly lumped together and considered as “water pipes.”\(^13\). At the time of this writing, only one article has reported trace metal analysis for hookah tobacco (moassel). The study used six commercially available smoking products, four of them tobaccos and two were tobacco-free herbal products for total metal analysis in the bulk samples.\(^15\) Mainstream smoke was analyzed for selected metals, nicotine, carbon monoxide and selected polycyclic aromatic hydrocarbons (PAH). The procedure used throughout the experiment for the collection of PAH, metals, nicotine, and carbon monoxide\(^16\) has been criticized because it does not mimic that of a real smoker and may lead to unrealistic results.\(^13\) The filters used to collect the PAH’s, metal and nicotine had flow rates of 2 L/min, yet, this flow rate would be much less than that generated by an actual smoker. Most of the polycyclic aromatic hydrocarbons were reported, but were below the authors’ limit of detection given in the same article. Also most of the trace metals analyzed both in the smoke and the bulk samples, a more sensitive analytical method is required to detect and quantify trace metals, since they
were able to report on only four of the twelve metals attempted. Also they report the same limit of detection for all metals, which is highly unlikely. Furthermore, large uncertainties from run to run were reported.\(^\text{15}\)

It is important to differentiate between the various water filtration practices used to consume tobacco for the tobacco formulation and smoking styles are vastly different from one technique to another. The toxic metals associated with hookah tobacco are the subjects of interest for this initial study. Although hookah consumption as known today has been a regular part of Middle Eastern and Eastern cultures for decades, hookah use, in particular, has been increasing rapidly in Western Cultures.\(^\text{17-19}\) Yet, only few analytical studies have been reported\(^\text{9}\); hence, the health hazards are essentially unknown. This is further complicated by the fact that unlike cigarette or cigar tobaccos, the hookah tobaccos are complicated as they are mixtures with glycerin, honey/molasses, colorants and flavorings, all contributing to the smoking experience and all complicating the ultimate analytical matrix. Furthermore, the charcoal used to ignite the tobacco formulation is also a potential contaminant source for the smoker as are parts of the smoking apparatus; e.g. the ceramic bowl.

Tobacco is a metal accumulating plant, taking up many of both inorganic as well as organic substances from the soil, and may incorporate or metabolize these components. Tobacco plants have been shown to accumulate high levels of cadmium, lead, zinc, nickel, copper, iron, manganese, etc.\(^\text{20-24}\); hence, there are many reports for these elements in cigarette tobacco and cigarette smoke.\(^\text{2, 25, 26}\) While it is noteworthy to discuss the health effects associated with tobacco use and to acknowledge that tobacco use on any level poses a health hazard\(^\text{27-30}\), differentiation between the different
smoking styles and the various tobaccos is necessary to begin to authoritatively address actual health implications.\textsuperscript{31} To do so in the most rigorous fashion requires many more samples than could be reasonably done in this initial study, which is intended to provide an initial report to stimulate additional research in this important area. These studies will need to take place in the multiple hookah smoking compartments – the special hookah charcoal, ceramic bowl, other parts of the apparatus, tobacco, filtration water, both side-stream and mainstream smoke, to name some.

There is much debate over the health hazards associated with hookah consumption.\textsuperscript{32-36} Often hookah consumption is compared to cigarette smoking,\textsuperscript{16,37} yet it is difficult to directly compare hookah consumption to cigarette, cigar or smokeless tobaccos, since these different tobaccos are not consumed in the same fashion and widely differ in sample composition. While a larger amount of hookah tobacco is consumed for a greater length of time, anywhere from twenty minutes to two hours per session, relative to the cigarette at three to ten minutes per session, the hookah smoke is filtered through water and also aged before reaching the smoker.\textsuperscript{31} Hookah tobacco is generally comprised of wet tobacco, glycerin/molasses or honey, and a flavoring such as strawberry, grape, apple, etc. Cigarette tobacco is generally composed of a blend of different dried tobaccos that is processed, and aged. Since modern cigarettes are a dry tobacco, this results in a greater tobacco density per unit weight than hookah tobacco and the burn temperature of cigarette tobacco is considerably higher than burning hookah tobacco. Cigarette values are usually in the range of 800–900 °C during a puff
and 700–800°C during the natural smolder between puffs.\textsuperscript{38} For the hookah tobacco the tobacco mixture in the bowl rarely exceeds 200 °C.\textsuperscript{39, 40}

A typical hookah smoking apparatus consists of the following components: water jar, the body, a hose insert, a check valve, and the bowl as shown in Figure 1.

\textbf{Figure 1.} Typical hookah smoking apparatus, green arrows represent flow of smoke

Clearly there are multiple compartments to be considered as possible sources of metal contamination. In this report, we consider only the “tobacco.” The jar is usually made of glass and holds anywhere from 0.5 to 1.5 liters of water. The amount of water placed in the jar is of personal preference and its purpose is to cool and filter the smoke as well as making it less harsh by mixing with water vapor. The body of the hookah is usually
made of stainless steel, copper or aluminum and passes into the water-containing jar. There is no precise length of the body and it varies from 10 cm to 40 cm. The hose insert is where the hose of choice is plugged into the body. The bowl is usually made of clay or ceramic, another possible source of trace metal contamination. The tobacco, ten to forty grams on average depending on the size of the bowl, is placed into the bowl and covered with perforated aluminum foil to allow air-flow. The bowl is placed at the top of the body as shown in Figure 1. Charcoal is placed on top of the aluminum foil. Upon inhalation suction forces air through the head, burning the tobacco and producing the smoke, some of which is then forced down the body, into and out of the water and through the hose into the smoker’s respiratory system. It is clear that hookah smoking is more complex than other types of smoking. All of these complications, from the nature of the hookah tobaccos to the multiple entry points for trace contaminants, to the smoker’s respiratory system, make for a complex and multi-compartmented analytical challenge.

A number of studies indicated tar,$^{35}$ nicotine,$^{16, 35, 41}$ and carbon monoxide levels$^{35, 41, 42}$ utilizing a hookah. However no rigorous studies have been done to determine metal profiles in hookah tobacco or the actual inhaled metal species. Due to the paucity of studies and an accompanying literature regarding basic chemical analysis, we begin with an initial metal analysis study of various hookah tobaccos. This study identifies the metals and their concentrations in twelve different hookah tobaccos, both USA and Middle Eastern formulations. Also a water-soluble extract experiment was done on the tobacco mixture.
2.2 Materials and Methods

2.2.1 Instrumentation

An Agilent 7700x inductively coupled plasma mass spectrometer (ICPMS, Agilent Technologies Santa Clara, CA, USA), equipped with a CETAC Micromist nebulizer (CETAC, Omaha, NE, USA), was utilized for the determination of total metals in hookah tobacco formulations. The instrument was set to monitor the following metals: $^{23}$Na, $^{24}$Mg, $^{27}$Al, $^{43}$Ca, $^{51}$V, $^{52}$Cr, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{60}$Ni, $^{63}$Cu, $^{66}$Zn, $^{75}$As, $^{78}$Se, $^{95}$Mo, $^{111}$Cd, $^{121}$Sb, $^{207}$Pb. The following $^{6}$Li, $^{45}$Sc, $^{72}$Ge, $^{89}$Y, $^{115}$In, $^{159}$Tb, $^{209}$Bi were used as internal standards to cover the full mass range.

2.2.2 Reagents and Standards

Doubly deionized water (DDW) 18 MΩ generated from a Milli-Q system (Bedford, MA, USA) was utilized. Ultrace II ultra-high purity nitric acid (HNO$_3$) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Trace metal grade HNO$_3$ was obtained from Fisher Scientific (Pittsburg, PA, USA). Multi-elemental standards, 1000 $\mu$g ml$^{-1}$ and 10 $\mu$g ml$^{-1}$ stock solutions used for both spiking and calibration curves were obtained from Spex Certiprep (Metuchen, NJ, USA). Internal standard mix ICP-MS-IS-3 and trace metals in drinking water (TMDW) certified reference materials (CRM) were obtained from High-Purity Standards (Charleston, SC, USA). The standard reference material 1573a (tomato leaves) was obtained from the National Institute of Science and Technology (NIST, Gaithersburg, MD, USA).

2.2.3 Sample collection and preparation

Twelve hookah tobacco samples were obtained as an initial representation of domestic and imported tobacco matrices from five different manufacturers. These
hookah tobaccos were chosen based on flavor indicated, manufacturer, and country of origin. Eight of the samples are from domestic suppliers (Starbuzz and Social Smoke), while the other four are imported from the Middle East, (Al Fakher, Romman and Nakhla). All hookah tobaccos were homogenized under liquid nitrogen using a Freezer/Mill 6870 (Spex Sample Prep, Metuchen, NJ, USA). During homogenization samples were pre-cooled for 20 minutes in a liquid nitrogen bath then homogenized in 3 cycles for the duration of 4 minutes. Approximately 30 grams of hookah matrix was ground then stored in acid cleaned 60 mL polytetrafluoroethylene (PTFE) bottles and covered with parafilm until analysis. Water extraction was performed on all twelve of the homogenized samples. ~1.5g of tobacco was weighed out and placed into a 15 mL acid washed vial. 3mL of 60°C DDW was added to vial and vortexed on high for 3 min. Sample was then centrifuged at 17000 g force for 25 min and decanted into a new acid washed vial. Procedure was done three times per sample for a total of 9mL of DDW. Samples were then acidified with trace metal grade HNO₃ and ran on the ICP-MS.

2.2.4 Sample digestion for total metal analysis

All samples were prepared and analyzed in quadruplicate, fortifying the fourth sample with a final concentration of 100 ng g⁻¹ of multi elemental standard. Prepared samples (0.40 -0.50g) were weighed directly into Teflon microwave digestion vessels and 7g trace metal grade HNO₃ was added to each vessel. Covered sample vessels were allowed to pre-digest overnight. Prior to microwave digestion, 3g DDW was added to each vessel. Samples were subjected to microwave digestion using a CEM MARS Xpress microwave system (CEM, Matthews, NC, USA). Digestion occurred in two steps. The sample vessels were first ramped to 120°C over 10 min and held for 5 min. Then
samples were ramped up to 200°C over 15 min then held for 15 min before they were allowed to cool and subsequently vented. The digested solution was then diluted to 30g with DDW. Prior to analysis samples were diluted a second time taking 5g of the first dilution and diluting to a final weight of 10g. NIST SRM 1573a was digested with each sample set to assure a valid response to leafy material.

2.2.5 Total Metal analysis
A final concentration of 5 ng g$^{-1}$ was used as the internal standard. Standards were re-analyzed and check standards were run every 8 samples. Blanks, sample fortification and High-Purity Standards CRM-TMDW and NIST SRM 1573a were analyzed with each batch of hookah tobacco sample.

2.3 Results and Discussion
The aim of this initial study was to begin a series of comprehensive studies on hookah tobacco, in view of the lack of sufficient analytical information related to possible toxic substances. This study focuses on trace elements, specifically metals present in the twelve different tobaccos, both Middle Eastern and USA formulations. Metal analysis includes toxic metals derived from the 12 tobacco formulations; 8 of USA preparation and 4 from the Middle East. Prior to running these hookah samples, the methods were tested against the tomato leaves SRM 1573a the results are included in the Supplement. Eighteen minor and trace metals were investigated; Na, K, Mg, Ca, Al, Fe, V, Cr, Mn, Ni, Cu, Zn, Se, Mo, As, Cd, Sb and Pb. Our sample set is relatively small,
but yields results sufficient to stimulate a major study, for which considerable external funding is required.

Total elemental analysis was carried out on the wet crude tobacco samples and hot water extracted samples. The hot water was used to remove as much as possible of the water-soluble glycerin, molasses, etc. from the tobacco. The results obtained for the total elemental analysis in these 12 tobacco formulations are summarized in Tables 1 and 2 where the average of three determinations per hookah sample are indicated plus the standard deviation (SD).

| Sample | As | Sb | Se | Br | I | K | Na | Mg | Al | Si | P | S | Cl | Ar | Ca | Fe | Mn | Zn | Cu | Cr | Ni | Sn | Pb | Bi |
|--------|----|----|----|----|---|---|----|----|----|----|----|---|---|----|----|----|---|----|----|----|----|----|----|----|----|
| NE 1   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 2   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 3   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 4   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 5   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 6   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 7   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 8   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 9   | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 10  | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 11  | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| NE 12  | 13 | 0.0 | 0.7 | 3.0 | 1.5 | 0.5 | 1.0 | 0.7 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.05 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
Table 1. Results of the total elemental analysis performed on the twelve samples. The results are the average of 3 replicates in ng g\(^{-1}\) with ± std. dev. SD. USA denotes the American formulations and ME represents Middle Eastern formulations. Cells in green highlight show ME toxic element formulations and are contrasted with the USA counterparts in blue. Clearly from this sample set the ME formulations are about 2 times more toxic than the USA formulations, especially for the toxic elements with lead showing the greatest difference between the two.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cu</th>
<th>Zn</th>
<th>Mn</th>
<th>Fe</th>
<th>Mg</th>
<th>Ca</th>
<th>K</th>
<th>Al</th>
<th>Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>7.0</td>
<td>0.05</td>
<td>1.38</td>
<td>1.24</td>
<td>2.91</td>
<td>0.91</td>
<td>0.14</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>USA</td>
<td>3.9</td>
<td>0.03</td>
<td>2.27</td>
<td>1.29</td>
<td>0.98</td>
<td>0.14</td>
<td>0.02</td>
<td>0.03</td>
<td>0.32</td>
</tr>
<tr>
<td>USA</td>
<td>2.7</td>
<td>0.02</td>
<td>1.24</td>
<td>0.88</td>
<td>0.86</td>
<td>0.12</td>
<td>0.01</td>
<td>0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>USA</td>
<td>1.4</td>
<td>0.01</td>
<td>0.87</td>
<td>0.86</td>
<td>0.86</td>
<td>0.12</td>
<td>0.01</td>
<td>0.01</td>
<td>0.22</td>
</tr>
</tbody>
</table>

| ME     | 7.0 | 0.05 | 1.38 | 1.24 | 2.91 | 0.91 | 0.14 | 0.03 | 0.25 |
| ME     | 3.9 | 0.03 | 2.27 | 1.29 | 0.98 | 0.14 | 0.02 | 0.03 | 0.32 |
| ME     | 2.7 | 0.02 | 1.24 | 0.88 | 0.86 | 0.12 | 0.01 | 0.01 | 0.22 |
| ME     | 1.4 | 0.01 | 0.87 | 0.86 | 0.86 | 0.12 | 0.01 | 0.01 | 0.22 |
Table 2. Results of the total elemental analysis performed on the twelve samples, the results are the average of 3 replicates in μg g⁻¹. Generally, these metals appear to be somewhat higher in the ME formulations relative to their USA counterpart for this particular sample set, but not as exaggerated as those lower concentration metals shown in Table 1. Al, Mn and Cu standout with more prominent differences between ME and USA.

In Table 2 the metals are shown in μg g⁻¹ (μg of metal per g of tobacco formulation). For these particular samples Table 1 shows clearly that the USA formulations, for the most part are 2x or less trace metals in the μg g⁻¹ (ppm) range. This observation is particularly notable for the toxic metals (highlighted in the table) and especially for lead, which is markedly higher in the ME tobaccos. Without far more information on the tobacco growth and processing, it is impossible know with certainty the causes of these differences. However, likely causes are environmental factors such as the growth soil contamination or with the tobacco processing equipment. As indicated below, contamination from the other formulation ingredients is unlikely. In Table 2, aluminum is the predominant element of those in the higher ppm range; aluminum > potassium >manganese >calcium > magnesium. In this range, the predominance of these trace metals from ME tobacco is not as apparent as with the sub-ppm level metals shown in Table 1.

Next, the hot water extracted portion was studied, which is composed primarily of honey or molasses, glycerin and flavorings. It was found that the trace metals in these extracts contribute only minimally to the total amount of the elements analyzed from the complete sample formulation (see Supplement Figure S1a-c), indicating the tobacco leaves are the most relevant material in the hookah tobacco formulation with respect to the metals analyzed. Minimal trace metals in the extracts is worthy of note, since the
contribution of the tobacco leaves represents ~50% or higher total mass, but contains
the major contribution of the metals studied. This factor is important if any comparison
with cigarette tobacco is attempted. While cigarettes contain preponderantly tobacco
leaves, an equal portion of hookah tobacco formulation, relative to cigarette tobacco,
will have appreciably less concentration for the metals analyzed, because of the dilution
factor. The concentration levels of toxic metals are lower than those reported for
cigarette tobacco as seen in Table 3.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>0.03 - 0.16</td>
<td>N/A</td>
<td>N/A</td>
<td>0.81 - 1.10</td>
</tr>
<tr>
<td>Cr</td>
<td>0.15 - 0.37</td>
<td>N/A</td>
<td>N/A</td>
<td>0.30 - 1.58</td>
</tr>
<tr>
<td>Mn</td>
<td>8.82 - 17.2</td>
<td>N/A</td>
<td>34.1 - 50.9</td>
<td>95.1 - 161</td>
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<tr>
<td>Ni</td>
<td>0.14 - 0.64</td>
<td>N/A</td>
<td>N/A</td>
<td>0.37 - 1.18</td>
</tr>
<tr>
<td>Cu</td>
<td>0.93 - 5.67</td>
<td>N/A</td>
<td>5.62 - 11.2</td>
<td>2.94 - 5.79</td>
</tr>
<tr>
<td>Zn</td>
<td>3.51 - 5.62</td>
<td>N/A</td>
<td>4.65 - 23.5</td>
<td>9.04 - 25.9</td>
</tr>
<tr>
<td>As</td>
<td>0.01 - 0.04</td>
<td>N/A</td>
<td>N/A</td>
<td>0.25 - 1.07</td>
</tr>
<tr>
<td>Se</td>
<td>0.002 - 0.014</td>
<td>N/A</td>
<td>N/A</td>
<td>0.18 - 0.34</td>
</tr>
<tr>
<td>Mo</td>
<td>0.05 - 0.13</td>
<td>N/A</td>
<td>N/A</td>
<td>0.38 - 0.88</td>
</tr>
<tr>
<td>Cd</td>
<td>0.10 - 0.27</td>
<td>0.78 - 2.78</td>
<td>0.26 - 0.62</td>
<td>0.02 - 0.05</td>
</tr>
<tr>
<td>Pd</td>
<td>&lt;0.001 - 0.07</td>
<td>1.33 - 3.61</td>
<td>10.2 - 27.3</td>
<td>0.60 - 7.93</td>
</tr>
</tbody>
</table>

Table 3. Concentration ranges obtained for some of the studied elements, compared
with reported ranges for cigarettes. All results are in μg g⁻¹

Grouping by USA and Middle Eastern formulations for this sample set is
illustrated in Figure 2 for concentration variations of all the metals including toxic Cd,
Pb, As and Cr, although these results cannot be generalized until a much larger sample
set can be investigated.
Figure 2. Box chart representation of the concentrations measured for the twelve samples separated by the geographic formulation region. The boxes represent, from bottom to top, the 10th to the 90th percentiles or 80% of the data points lie within the box; the center square is the median with the horizontal line as the mean. The *s mark the ranges. ME, Middle East; USA , United States of America. The coincidence of the mean and the median and narrower ranges are found more extensively for the USA formulations with the standard deviations following this trend.
The boxes in Figure 2 show the 10th to 90th percentiles as well as other useful figures of merit. The box charts from Figure 2 are value added since the medians and ranges also are included. For this figure the overlaps or lack thereof are readily noted.

**Figure 3.** Bar graph representation of the results from total metal analysis of the twelve hookah samples, separated by the geographic production region. Error bars represent the SD. ME - Middle East with dark bars and USA- with light bars.

This approach, applied to a larger sample set meeting appropriate power calculations, will have high value in evaluating the more extensive amount of data from a future study. The bar graph data representations are shown in Figure 3 and provide the usual mean/std. dev. Information. It is readily seen that the error bars for some metals are relatively large. These errors are much smaller if two individual charts are made for the 8 USA samples and the 4 Middle Eastern samples, respectively. For example, with lead...
the difference in the hookah tobacco between the two formulation regions is very large, as seen from Table 1 and this is reflected in the error bars when grouping the regional samples together as done in Figure 3.

An exploratory principal components analysis was performed. The PCA generates principal components as new variables that are linear combinations of the original variables; the new variables are orthogonal in the original variable space. Analyzing the data with respect to principal components can thus sometimes effectively reduce the number of variables, especially if a large percentage of the total variation is described by a few principal components. Based on the total concentrations of the 18 metals analyzed, and using the first three principal components resulting from the analysis, the three dimensional Figure 4 was constructed. Clearly the geographic region of hookah formulation can be used as a classifier variable to group the samples in the space as two sets of samples (generating the blue and red colors). From the loadings plot it is possible to see that Al, Ca, Mg and Cu have the largest contribution to PC-1, while Ca, Mg and Cu to PC2. The first three principal components can explain more than 99% of variation in the samples. In Figure 3c the cumulative X-Variance for the first seven principal components calculated in the PCA model illustrating the high influence of PC-1.
Figure 4. Principal component analysis performed on the data obtained for the eighteen elements. (a) Scores in three-dimensional representation, where the geographic region of formulation was used as a classifier. In red are given the USA sample formulations, and in blue the ME formulations. The importance of PC-1 is readily shown (b) Loadings represented in a three dimensional plot show Al, Ca, Mg and Cu as the most relevant metals for the model in (a). (c) Graph of the cumulative X-Variance for the first seven principal components calculated in the PCA model illustrating the high influence of PC-1.

The sample grouping by geographic formulation region shows that with for these 12 samples, it is possible to distinguish USA formulated samples from the Middle Eastern ones, and the PCA used as an exploratory tool (illustrative in this case) shows its potential for a much larger sample set. As an example, power sample size calculations based on the averages and standard deviation results obtained in this analysis, at a power goal of 0.9000 (90% confidence that the sample set will produce statistically viable results), and an error type I of $p < 0.05$, it is necessary to have 30 samples per manufacturing region, to appropriately make a comparison between trace metals in the hookah samples across the two geographic regions. PCA analysis have been proven useful in evaluating other commercial products such as tequila, orange juice, wine and potatoes.
While the differences observed for toxic elements between samples are high, the overall range is lower than that reported for cigarette tobacco.\textsuperscript{3, 25, 26, 47} We found in the crude hookah tobacco a range of concentrations for As from 0.01 - 0.04 µg g\textsuperscript{-1} while previous reports show 0.25 - 1.07 µg g\textsuperscript{-1} for the cigarette tobacco.\textsuperscript{25} Interestingly Cu from the hookah tobacco is the only metal to be near the range reported for cigarettes: 0.93 - 5.67 µg g\textsuperscript{-1} compared with cigarette 5.62 - 11.2 µg g\textsuperscript{-1}.

A correlation analysis among the different metal concentrations in the full sample set was performed with the matrix results are shown in Figure 5. The correlations for p\textless0.05 are shown in red. The statistical significance (p\textless0.05, n=12) for the positive correlation between toxic elements (Cd, As, Pb, Cr) with common soil elements such as Fe and Al indicates that the plant uptake of these metals occurs in the same manner regardless of the growth region. The higher concentrations observed in the Middle Eastern brands may be the result of higher concentrations of these metals in the soil or other environmental effects, such as metal release from machinery to the tobacco during the formulation process. Lead is noted as remarkably different.

Because the exposure to toxic elements for the hookah smoker is limited to the maximum amount of starting material, using a typical portion of 15 g, we can estimate an average maximum possible exposure to the toxic elements per smoking session. Figure 6 shows the maximum mass of toxic elements; however, ultimately inhaling that entire elemental mass via the smoke is highly unlikely, in as much as a residual amount of the hookah tobacco remains in the bowl due to the lower burn temperatures.
Figure 5. Correlation matrix obtained from the concentrations of eighteen elements analyzed. *r* and *p* are shown. **Bold red** are values for which *p* < .050. The matrix suggests that Fe is reasonably correlated with Mg, Al, and V. That is, the tobacco plant is likely to metabolize all three elements if available in the soil. For toxic lead - Ni and Cu correlate well.
Figure 6. Average amount of toxic elements present in a typical smoking portion of 15 g used in a smoking session of hookah. The average of each element and the standard deviation is expressed for the twelve samples analyzed. A similar plot, for example using only the 8 USA hookah formulations, shows smaller error.

The particular way of burning the hookah formulation generates a maximum temperature of $<450$ °C in the region nearest the charcoal and a maximum temperature nearest to the bowl exit of $<200$ °C$^{16}$. These relatively low and varying temperatures can be important factors on the release of toxic elements from the tobacco leaves. While most of the molasses and glycerin are decomposed during a smoking session, the results obtained in this study show only a small contribution of these materials to the total amount of toxic elements, therefore, the toxic metal amounts in the smoke produced are expected to be low and is one of the subjects in our continuing studies on the analytical aspects of hookah smoking.
2.4 Conclusion

The concentrations of 18 elements in 12 hookah tobacco samples were measured. This study represents a first step to our overall goal to better understand the release of metals and organic materials to the hookah smoker plus determining if smoking this popular device is similar to cigarette smoking in the release of noxious substances. The results based on the total elemental analysis of 12 hookah formulations show that the tobacco leaf portions are the main source of toxic elements and that the content of these is in the lower ranges of previous reports for cigarette tobacco\textsuperscript{3, 25, 47}. While for this initial study, the geographic origins of formulation lead to variability for the amount of toxic elements, the geographic origin for the other metals does not generate as notable a difference.

The concentration of toxic elements per portion smoked is diluted by the molasses and glycerin primarily, which can represent up to a 50% of the total mass of the hookah formulation. When normalized to the metal totals in cigarettes the amount of toxic metals per smoking session are expected to be lower in the hookah smoke if the smoke tracks the amounts found in the hookah formulations.

It is noted that the most important measure regarding the toxic metals is the amount reaching the smoker’s respiratory system from the smoke. This project is now ongoing in these labs. However, the starting point by investigating the concentrations of trace metals in the hookah tobacco neat, is important to assure a good reference point for future studies, which will carry forward with the more formulations provided by the most popular suppliers. In view of the multiple analytical compartments in hookah
smoking that need investigation, further studies are necessary to clarify the state of
hookah smoking and its relationship to human health risks.

2.5 References

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Chapter 3 - Conclusions

The results presented by this study demonstrate leaf tobacco is the major component contributing toxic elements in the hookah sample, rather than the molasses/glycerin/honey/colorant/flavoring components. Since the hookah matrix is comprised of up to 50% these other components, lower ranges of metal toxins were found than cigarettes are reported to contain. Principle component analysis (PCA) indicated metal content and concentrations were similar in tobaccos from the same region but there was variability in hookah tobacco depending on its country of origin. While American tobaccos had similar metal concentration profiles when compared to other American brands and Middle Eastern metal profiles were similar from Middle Eastern brand to brand, there were stark differences between comparing American to Middle Eastern tobacco for certain elements.

Metal profiling is just one of many studies that need to be performed on hookah tobacco. There are many more toxic components found in tobacco and it is not known how the matrix (other than tobacco) and manner in which the tobacco is smoked may affect the consumer requiring many future studies.