

Characterisation of Nicotine and Cancer-Enhancing Anions in the Common Smokeless Tobacco *Afzal* in Oman

Nawal M. Al-Mukhaini, Taher A. Ba-Omar, *Elsadig A. Eltayeb, Aisha H. Al-Shehi

توصيف النيكوتين والأيونات السالبة المحفزة للسرطان في التبغ عديم الدخان "أفضل" الشائع الاستعمال في عمان

نوال المخينية، طاهر باعمر، الصادق الطيب، عائشة الشحية

ABSTRACT: Objectives: *Afzal* is a common smokeless tobacco product (STP) available illegally in Oman. This study aimed to assess pH and moisture levels and determine cancer-enhancing factors in a randomly selected sample of *Afzal*. **Methods:** This study was carried out at the Sultan Qaboos University in Muscat, Oman, between April and December 2013. A package of *Afzal* was purchased from a single provider and divided into samples. The pH and moisture content of the samples were measured according to the protocols of the Centers for Disease Control and Prevention. Gas chromatography-mass spectrometry was used to analyse nicotine levels and ion-exchange chromatography (IC) was used to determine concentrations of nitrate, nitrite, chloride, fluoride, bromide, sulphate and phosphate anions. **Results:** The samples had an alkaline pH of 10.46 with high levels of total (48,770.00 µg per g of STP [µg/g]) and unionised (48,590.00 µg/g) nicotine. The concentration of nitrate (8,792.20 µg/g) was alarmingly high. The chloride concentration (33,170.80 µg/g) showed a surge on IC chromatography. The moisture content percentage was 52.00%. **Conclusion:** The moisture content percentage and chloride concentration of *Afzal* was consistent with those of other STPs. In contrast, nitrite, sulphate and phosphate concentrations were below reported levels of other STPs. All anion concentrations were below the maximum daily limit set by international health organisations. However, the high concentrations of nitrite, nitrate and nicotine and the elevated alkaline pH observed in the analysed *Afzal* samples suggest that STP users will face health risks as a result of their use.

Keywords: Smokeless Tobaccos; Gas Chromatography-Mass Spectrometry; Ion-Exchange Chromatography; Carcinogens; Nicotine; Anions; Oman.

المخلص: أفضل هو تبغ عديم الدخان ويعتبر الأكثر انتشاراً بشكل غير قانوني في سلطنة عمان. هدفت هذه الدراسة لتعيين مستويات درجة الحموضة والرطوبة وكذلك العوامل المحفزة للسرطان في عينة عشوائية من تبغ أفضل. الطريقة: نفذت هذه الدراسة في جامعة السلطان قابوس في مسقط، سلطنة عمان، في الفترة من إبريل إلى ديسمبر 2013. تم شراء كمية من تبغ أفضل من مصدر فردي وتم تقسيمها لعدة عينات. تم قياس محتوى درجة الحموضة والرطوبة للعينات طبقاً لنظام مركز السيطرة على الأمراض والوقاية منها بالولايات المتحدة الأمريكية. تم استخدام تقنية الغاز بالتخطيط اللوني وبمقياس طيفي للكتلة لتحليل مستويات النيكوتين واستخدام تقنية التخطيط اللوني للأيونات لتحديد تراكيز كل من الأيونات السالبة للنترات، والنيترات، والكلوريد، والفلوريد، والبروميد، والكبريتات والفوسفات. النتائج: أظهرت العينات درجة حموضة قلوية تعادل 10.46 ومستويات عالية من النيكوتين الكلي (48,770.00 ميكروجرام/جم) لمادة التبغ عديم الدخان والنيكوتين غير المؤين (48,590.00 ميكروجرام/جم). كان تركيز النترات (8,792.20 ميكروجرام/جم) عالياً بشكل تحذيري. كما أظهر تركيز الكلوريد (33,170.80 ميكروجرام/جم) موجة عالية بالتخطيط اللوني للأيونات. بلغت نسبة الرطوبة لتبغ أفضل نسبة 52.00%. الخلاصة: كانت نسبة الرطوبة وتركيز الكلوريد والفوسفات والنيترات والكبريتات أعلى من مستويات التبغ عديم الدخان الأخرى. وبالمثل كانت تراكيز النترات والنيترات والكبريتات والفوسفات أقل عن مثيلاتها بمنتجات التبغ عديم الدخان الأخرى. كانت كل تراكيز الأيونات السالبة أقل عن المستويات القصوى اليومية الموصى بها من قبل منظمات الصحة العالمية. ومع ذلك فإن التراكيز العالية لكل من النترات والنيكوتين ومستوى الحموضة القلوي المرتفع الملاحظ بعينات تبغ أفضل المحللة يفيد بأن مستخدمي التبغ عديم الدخان سيواجهون مخاطر صحية نتيجة لذلك الاستخدام.

مفتاح الكلمات: منتجات التبغ عديم الدخان؛ جهاز كروماتوغرام الغازات بالتخطيط اللوني وبمقياس طيفي للكتلة؛ التخطيط اللوني للأيونات؛ مواد مسرطنة؛ نيكوتين؛ الأيونات السالبة؛ عمان.

ADVANCES IN KNOWLEDGE

- This study shows that *Afzal*, an illegal smokeless tobacco product (STP) commonly available in Oman, contains high levels of nitrites, nitrates and nicotine and an elevated alkaline pH.

APPLICATION TO PATIENT CARE

- This study may encourage health officials and relevant government departments to continue increasing awareness among young *Afzal* users about the potential health risks associated with this STP.
- Health practitioners should be aware of *Afzal* use among patients and the medical complications that could arise as a result.

ACCORDING TO THE INTERNATIONAL Agency for Research on Cancer, smokeless tobacco products (STPs) are carcinogenic.¹ Understanding the chemical composition of these products is therefore the first step in assessing their potential toxicity.² The majority of STPs contain compounds that are potentially detrimental to health, including tobacco-specific nitrosamines (TSNAs); nitrosodimethylamine; polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene); and heavy metals.³ Additionally, they generally have elevated alkaline pH and moisture levels and contain nicotinic secondary metabolites and anions such as nitrite (NO_2^-) and chloride (Cl^-), which may enhance cancer formation.⁴ The most harmful carcinogens in STPs are TSNAs, which normally form during the preservation, fermentation, maturation and storage stages of the tobacco process.^{3,5} The presence of certain agents in tobacco—including nicotine, elevated moisture levels, a high pH level and NO_2^- and nitrate (NO_3^-) concentrations—usually facilitates the accumulation of high concentrations of TSNAs.⁶ As such, STPs must be analysed and their compounds quantified in order to estimate their potential health risk.

The nicotine content of a specific STP is the primary determinant of both its appeal and cause of addiction among users.⁷ The absorption of nicotine is strongly associated with the pH and moisture content of the product.⁸ The naturally occurring form of nicotine is the unprotonated form, which is easily absorbed by the oral mucus membranes into the blood stream and delivered directly to the brain.² Within STPs, an alkaline pH value helps make more of the total nicotine absorbable.⁹ Furthermore, the moisture content of an STP enhances microbial activity, resulting in an elevated formation of TSNAs.^{10,11} Microflora in the buccal cavity cause the reduction of NO_3^- to NO_2^- , which is more toxic to humans.¹² In turn, NO_2^- forms the nitrosating agent of the alkaloids in the tobacco (mainly nicotine and its secondary metabolites), which leads to the formation of carcinogenic nitrosamines.⁴

Afzal is a commonly used STP available illegally in Oman. In order to educate consumers, public health officials and regulatory policy-makers regarding the levels of toxicants in *Afzal*, analyses of the contents of this STP are needed. Previous research has determined the composition of heavy metals in *Afzal*.¹³ The aim of this study was therefore to measure pH and moisture

levels and assess cancer-causing agents in a random sample of *Afzal*.

Methods

This study was carried out at the Sultan Qaboos University (SQU) in Muscat, Oman, between April and December 2013. A single package of 4.00 kg of *Afzal* was purchased from a single source in order to maintain uniformity. The pH levels and moisture content of the sample were tested on the day of purchase according to the methods described below. The product was subsequently labelled with the date of purchase as well as the original pH level and moisture content so that any changes that occurred after storage could be noted. The sample was then kept refrigerated at 4 °C in plastic bags until analysis.

In order to assess pH levels, the package of *Afzal* was divided into three samples of 2.00 g each. Each sample was then mixed with 20 mL of deionised water. The pH levels were measured using a pH meter (Benchtop pH Meter, Mettler-Toledo International Inc., Columbus, Ohio, USA) with two-point calibration to an accuracy of two decimal places using standard pH buffers (4.00 and 7.00). The pH was measured with continuous stirring at the first five, 15 and 30 minutes to ensure no variation in the results. The mean pH value was obtained in accordance with standard protocols from the Centers for Disease Control and Prevention (CDC).¹⁴ Three samples of 5.00 g each of *Afzal* were dried to determine their moisture content. This was measured by obtaining the difference in weight of the samples before and after they had been dried in an oven for three hours at 99 ± 1 °C. The mean moisture value was reported as a percentage of the original weight and compared to CDC protocols.¹⁴

Ion-exchange chromatography (IC) analysis was performed for NO_2^- , NO_3^- , Cl^- , fluoride (F^-), bromide (Br^-), phosphate (PO_4^{2-}) and sulphate (SO_4^{2-}) ions. Dried *Afzal* samples were ground into a homogenous powder and three samples of 1.00 g each were transferred into three 50 mL centrifuge tubes. Subsequently, 20 mL of Milli-Q® water (Merck KGaA, Darmstadt, Germany) was added to each tube. Samples were sonicated for 30 minutes followed by centrifugation at 3,000 revolutions per minute (rpm) for 30 minutes. Finally, the samples were filtered through a 0.45 µm cellulose acetate

polytetrafluoroethylene membrane nylon syringe filter (Whatman[®], Sigma-Aldrich Corp., St. Louis, Missouri, USA) to remove solids before the solution was injected into the 881 Compact IC Pro Anion System with an 858 Professional Sample Processor (Metrohm AG, Ionenstrasse, Switzerland). In order to ensure the accuracy of the readings, each sample was injected into the IC instrument twice. Each sample was diluted five-fold before IC analysis. The IC method used was validated and applied by the Environmental Engineering Laboratory of the College of Engineering at SQU.

The conditions of the analysis were as follows: column size of 250.00 x 4.00 mm; flow rate of 0.7 mL/minute; suppressed conductivity detector; ambient temperature of 21–23 °C; pressure of 13.7 megapascals; and a sample size of 20 µL. The eluent was prepared by dissolving 678,400.00 µg (3.2 mM/L) of 98% pure sodium carbonate (VWR International Ltd., Lutterworth, Leicestershire, UK) and 168,000.00 µg (1.0 mM/L) of sodium bicarbonate with 99% purity (VWR International Ltd.) in 2 L of deionised ultrapure water. The suppressor regenerating solution used was 50 mM of analytical-grade sulphuric acid (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany). In order to form a five-point calibration curve, 1,000 parts per million of each anion was concentrated using six anion standard concentrations (Fluka[®] Analytical, Sigma-Aldrich Corp.). The NO₂⁻ standard was laboratory-prepared from 98% pure sodium NO₂⁻ (Merck KGaA). Deionised water at a purity of 18 megohms (Ultrapure Private Limited, Singapore) was used throughout the analysis.

The mean total nicotine value of *Afzal* samples was obtained from gas chromatography-mass spectrometry (GC-MS) analysis. The nicotine analysis procedure was performed as previously described.^{15,16} A total of 5 mL of a 2 M solution of sodium hydroxide and 50 mL of extraction solution (methyl tert-butyl ether) with quinoline as an internal standard was added to a 1.00 g sample of *Afzal*. Sample vials were shaken on an orbital shaker at 160 rpm for two hours. The resulting extract was transferred to a 2 mL autosampler vial. The analysis was performed by injecting 1 mL from each vial into a Clarus[®] 600 Gas Chromatograph (PerkinElmer Inc., Waltham, Massachusetts, USA) fitted with an Rtx[®]-5MS capillary column (Restek Corp., Bellefonte, Pennsylvania, USA) of 30.00 m x 0.25 mm x 0.25 µm film thickness at a maximum temperature of 350 °C and coupled to a Clarus[®] 600C Mass Spectrometer (PerkinElmer Inc.). Ultra-high-purity helium (>99.9%) was used as a carrier gas at a constant flow of 1.0 mL/minute. The injection, transfer line and ion source temperatures were 290, 280 and 280 °C, respectively.

The ionising energy was 70 electron volts. The injected volume for each sample was 1 µL with a split ratio of 80:1. The oven temperature was initially 80 °C for five minutes and then accelerated at a rate of 10 °C per minute to 280 °C for 30 minutes. Unknown compounds were identified by comparing the spectra obtained with National Institute of Standards and Technology (NIST) Software, Version 11 (United States Department of Commerce, Gaithersburg, Maryland, USA) and NIST Mass Spectral Library, Version 14 (United States Department of Commerce). All chemicals used for GC-MS analysis were of analytical grade (Sigma-Aldrich Corp.).

For method validation purposes, blank samples of deionised water were run 10 times to calculate the limits of detection (LOD) and limits of quantitation (LOQ) of the tested analytes. Blank reagent samples of the tested anions were also used to subtract the results of all tested anion standards and samples. The LOD was calculated using the following equation:

$$\text{LOD} = 3 \times \text{SD}_{\text{blank}} \times \text{Concn}_{\text{sample}} / I_{\text{net}}$$

Where SD_{blank} is the standard deviation for the signal recorded on the blank for the corresponding element studied and Concn_{sample} is the concentration in µg/L of the respective sample aspired. The I_{net} value was calculated as below:

$$I_{\text{net}} = I_{\text{sample}} - I_{\text{blank}}$$

Where I_{sample} and I_{blank} are the signal intensities recorded for the sample and blank, respectively. The LOQ was approximately 10 times the LOD for all studied anions. Each analysed standard and sample was injected three times. This reflected high precision as it was automatically calculated using the relative standard deviation for each analyte. Six-point calibration standards curves were plotted at 50, 100, 150, 200, 250 and 300 parts per billion for each analyte.

The percentage of unprotonated nicotine was calculated using the pH and the pKa value of the pyrolic nitrogen of nicotine (pKa = 8.02), which was substituted into the Henderson-Hasselbalch equation.¹⁴ The total amount of unprotonated nicotine was then calculated by multiplying the percentage of unprotonated nicotine by the total nicotine as follows:¹⁴

$$\text{Total nicotine} = (W_{\text{ISTD}} / W_{\text{sample}}) \times (A_{\text{nicotine}} / A_{\text{ISTD}})$$

Where W_{ISTD} is the weight of the internal standard (6,250.00 µg), W_{sample} is the weight of the *Afzal* sample (1.00 g), A_{nicotine} is the area of nicotine from the chromatogram and A_{ISTD} is the area of the internal standard. Unprotonated nicotine was calculated

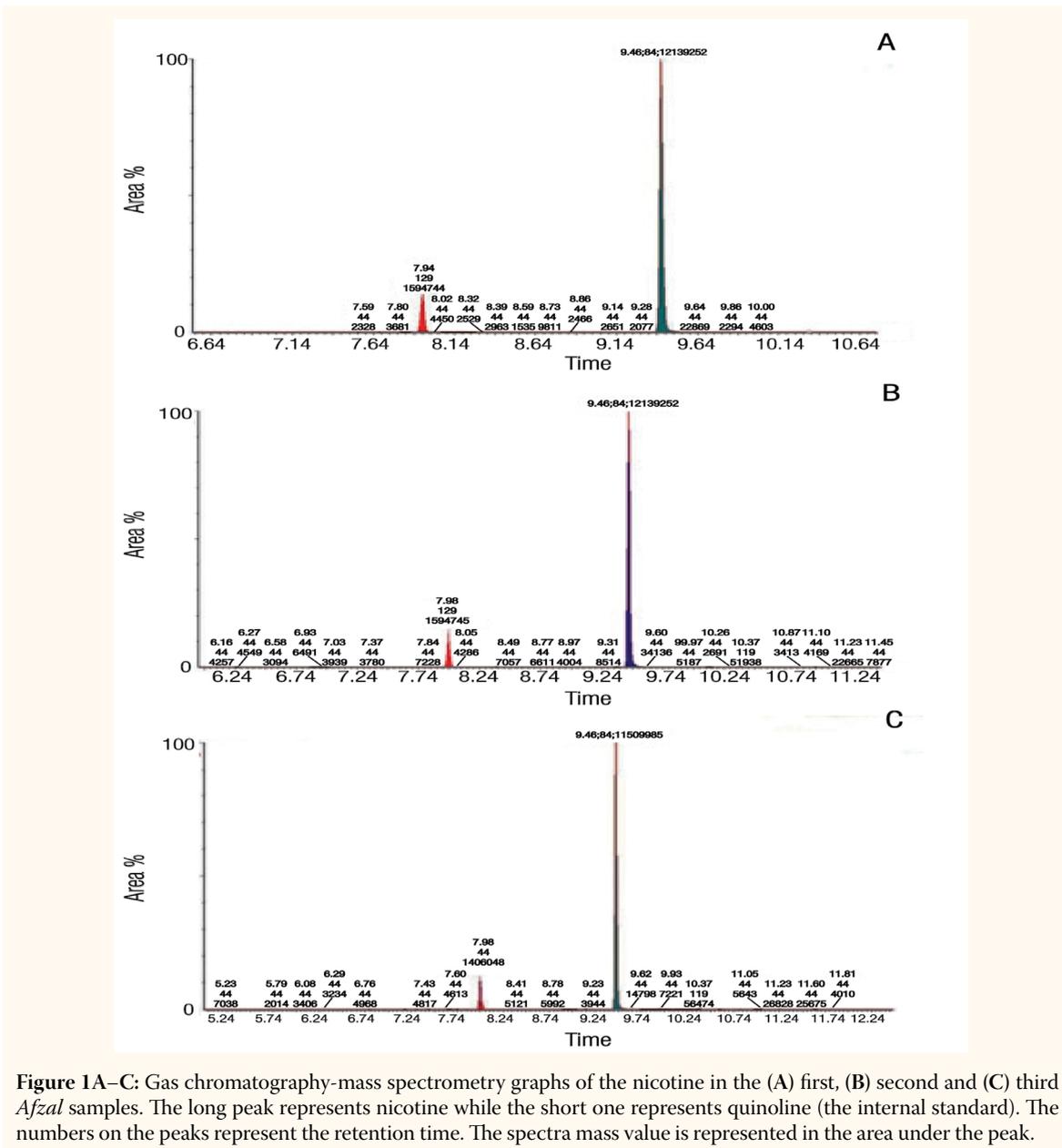


Figure 1A–C: Gas chromatography-mass spectrometry graphs of the nicotine in the (A) first, (B) second and (C) third *Afzal* samples. The long peak represents nicotine while the short one represents quinoline (the internal standard). The numbers on the peaks represent the retention time. The spectra mass value is represented in the area under the peak.

according to the following Henderson-Hasselbalch equation:¹⁷

$$pH = pK_a + \log (B/BH^+)$$

Where B is the amount of unprotonated nicotine, BH⁺ is the amount of ionised nicotine, pK_a is 8.02 and pH is 10.46. The percentage of unprotonated nicotine was calculated according to the following formula:

$$\% \text{ unprotonated nicotine} = \frac{(B/BH^+)}{[(B/BH^+) + 1]} \times 100$$

This was then used to calculate the total free nicotine as per the below formula:

$$\text{Total free nicotine} = \text{total nicotine} \times \% \text{ unprotonated nicotine} / 100$$

Excel spreadsheet software, Version 2010 (Microsoft Corp., Redmond, Washington, USA) was used for the statistical analysis. For each *Afzal* sample, the mean pH value, moisture content and nicotine concentrations were calculated. This study did not require ethical approval as it involved a chemical analysis of *Afzal*.

Results

The minimum detection limit values of the samples revealed satisfactory sensitivity of the IC method. The calibration of the linearity (r value) of the tested anion standards ranged between 0.996–0.999. The chromatograms from both the GC-MS and IC analyses validated the methods, as evidenced by the consistency

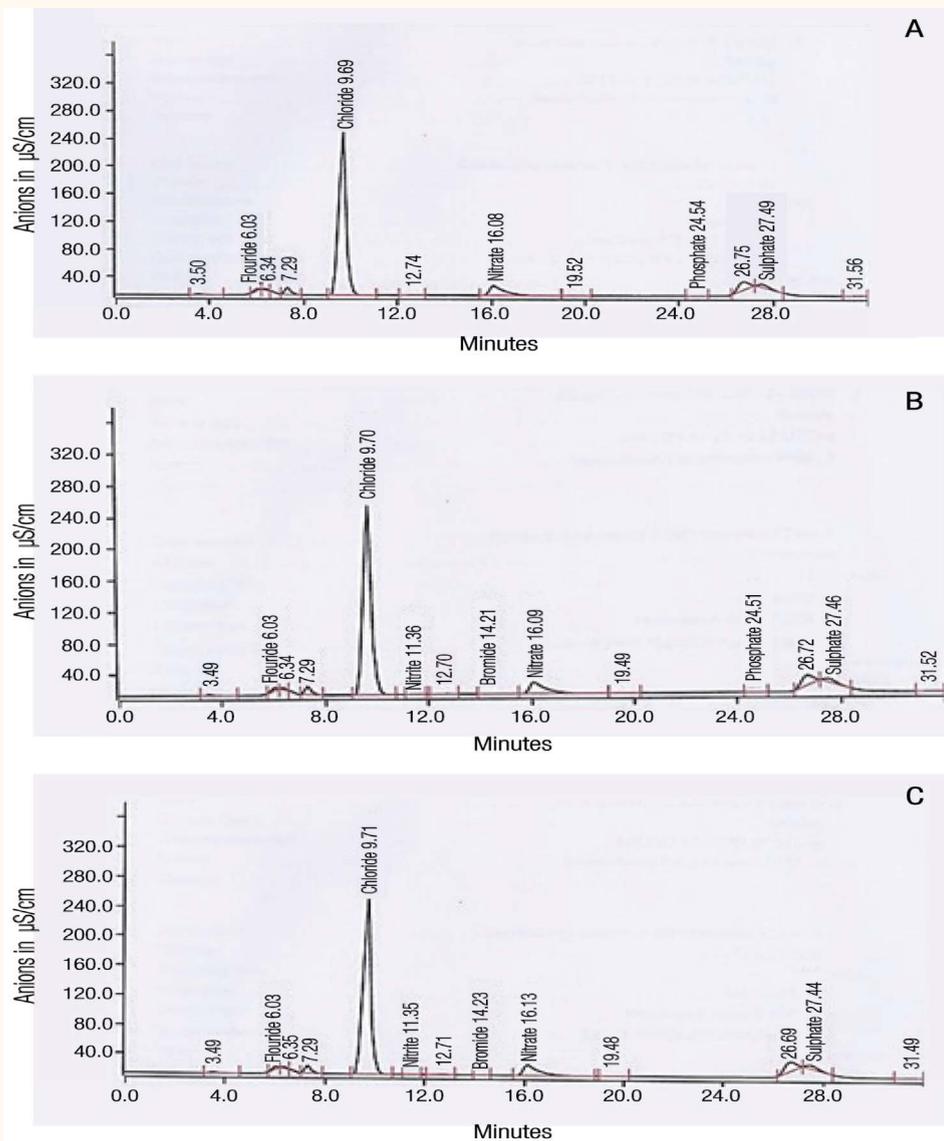


Figure 2A–C: Ion-exchange chromatograms of the anions in the (A) first, (B) second and (C) third *Afzal* samples. Each sample was injected twice into the instrument. There was a high chloride peak in all samples. Numbers on top of each anion peak represent the retention time.

$\mu\text{S}/\text{cm}$ = microSiemens/cm.

and repeatability of the results [Figures 1 and 2].

The *Afzal* samples had a mean pH of 10.46 and moisture content of 2.60 g. The calculated moisture content percentage was 52.00%. With regards to nicotine, the mean total nicotine and unprotonated nicotine levels were 48,770.00 μg per g of STP ($\mu\text{g}/\text{g}$) and 48,590.00 $\mu\text{g}/\text{g}$, respectively. The percentage of unprotonated nicotine was 99.64%. The chromatograms of the *Afzal* nicotine analysis showed two peaks—a long peak representing nicotine and a shorter one representing the internal standard [Figure 1]. The ion-exchange chromatograms also showed a long peak for Cl⁻ [Figure 2]. Table 1 shows the anion concentrations within the *Afzal* samples. Cl⁻ concentrations were highest at 33,170.00 $\mu\text{g}/\text{g}$

while NO₂⁻ was the lowest at 6.22 $\mu\text{g}/\text{g}$. The nitrate concentration (8,792.20 $\mu\text{g}/\text{g}$) was alarmingly high. The order of the ion concentrations from highest to lowest was as follows: Cl⁻, NO₃⁻, SO₄²⁻, F⁻, Br⁻, PO₄²⁻ and NO₂⁻.

Discussion

In the current study, samples of *Afzal* were found to have an alkaline pH; this is known to encourage the formation of TSNAs, which are potent carcinogens.⁶ An alkaline pH environment also plays an important role in causing nicotine to become more absorbable, thus increasing its addictiveness.⁸ The elevated alkaline pH of *Afzal* noted in the current study may therefore

Table 1: Anion concentrations in *Afzal* samples determined by ion-exchange chromatography

| <i>Afzal</i> sample* | Anion levels in µg/g | | | | | | |
|-----------------------------------|----------------------|------------------|-------------|-------------|---------------|-------------|--------------|
| | Fluoride | Chloride | Nitrite | Bromide | Nitrate | Phosphate | Sulphate |
| 1A | 16.73 | 1,639.21 | 0.29 | 4.49 | 432.49 | 1.98 | 73.23 |
| 1B | 16.67 | 1,639.71 | ND | ND | 432.91 | 1.92 | 74.59 |
| 2A | 17.11 | 1,677.85 | 0.36 | 4.30 | 447.34 | 1.77 | 75.06 |
| 2B | 17.05 | 1,678.62 | 0.32 | 4.30 | 446.46 | ND | 76.59 |
| 3A | 17.05 | 1,656.96 | 0.30 | 1.91 | 438.56 | ND | 76.05 |
| 3B | 17.06 | 1,658.87 | 0.30 | 1.90 | 439.90 | ND | 75.12 |
| Mean concentration ± SD | 16.95 ± 0.19 | 1,658.54 ± 17.36 | 0.31 ± 0.03 | 3.38 ± 1.35 | 439.61 ± 6.38 | 1.89 ± 0.11 | 75.11 ± 1.17 |
| Concentration | 339.00 | 33,170.80 | 6.22 | 67.60 | 8,792.20 | 37.80 | 1,502.20 |
| Instrument detection limit in ppm | 0.02 | 0.02 | 0.03 | 0.02 | 0.02 | 0.03 | 0.03 |

ND = not detected; SD = standard deviation; ppm = parts per million.

*Three *Afzal* samples were tested, of which each was injected twice into the ion-exchange chromatography instrument (A and B).

increase health risks for users, including the possibility of heightened addiction rates and carcinogenicity. Substances such as slaked lime are often added to the tobacco blend during the curing process, thereby increasing the alkaline pH.⁷ The *Afzal* samples in this study had a higher pH value (pH 10.46) than the value described in the quality standards used by GOTHIA TEK® (Swedish Match, Stockholm, Sweden) for *snus* (pH 8.50), a moist powder tobacco which is highly regulated and monitored by the Swedish National Food Act.¹⁰ It has been shown that the pH value of *snus* is affected by its storage temperature.¹⁰ Similarly, a pH range of 9.2–10.0 has been reported for other STPs including *khaini* and *gul* from India, *toombak* from Sudan and snuff products from South Africa.¹⁵ However, traditional Alaskan *Iq'mik* appears to have the highest alkalinity (pH 11.00).¹⁸ Bearing in mind the pKa of nicotine, any increase in pH levels would favour transformation of the nicotine to its unprotonated form.

Moisture content is another major factor enhancing the permeability of both nicotine and TSNAs into the mucosal membrane of the mouth, since both are water-soluble.^{7,19} Higher nicotine levels are found in moist snuff in comparison to the dry form.¹ Additionally, it has been found that American moist snuff contains higher levels of NO₂⁻ and Cl⁻ than the dry type.² Thus, the observed moisture content of *Afzal* in the current study (52.00%), which was similar to that of the American moist snuff (51.00%), can be expected to contain similarly elevated nicotine, NO₂⁻ and Cl⁻ levels. The moisture content of the *Afzal* samples in the current study was also higher than that reported for the Indian tobacco *Bidi* (10.26%), but within the range of *snus* (45.00–60.00%).^{2,10,20}

Nicotine content in STPs is considered low at ≤7,500.00 µg/g, moderate between 10,300.00–11,400.00 µg/g and high at >11,400.00 µg/g.²¹ This is worth noting since, besides addiction, nicotine may cause other physiological effects such as an increase in pulse rate, blood pressure and plasma free fatty acids and the mobilisation of blood sugar and catecholamines in the blood.^{22,23} A high nicotine content has been found in *toombak* (40,600.00 µg/g) and *Iq'mik* (42,700.00 µg/g).^{18,24} However, the nicotine content of *Afzal* in the current study exceeded these values (48,770.00 µg/g); as evidenced by Henningfield *et al.*, nearly all of its nicotine content would have been in the unprotonated form because of its high pH level.²¹ As is the case with *snus*, STPs with higher unprotonated nicotine content hold the highest market share.²⁵

All of the anions analysed in this study were below the recommended daily intake limits determined by international health organisations (F⁻ = 33.30 µg/kg/day; Cl⁻ = 9,000.00 µg/kg/day; NO₂⁻ = 60.00 µg/kg/day; Br⁻ = 400.00 µg/kg/day; NO₃⁻ = 370.00 µg/kg/day; PO₄²⁻ = 13,330.00 µg/kg/day; and SO₄²⁻ = 8,330.00 µg/kg/day).^{26–32} Although anions are vital to the human body, they can be toxic at excessive levels. Sodium chloride is often added to tobacco blends to improve the flavour of the product and act as a preservative.^{2,10} However, high quantities of this compound may contribute to several disease mechanisms, including chronic inflammation, tumour promotion and co-carcinogenesis (mutations in the gastric epithelium).³³ Although the Cl⁻ content of *Afzal* in the current study showed a high peak compared to other anions, it was below the recommended daily uptake.²⁷ In the USA, STPs were found to contain higher Cl⁻ levels

in the moist form (95,000.00 µg/g) than the dry form (23,100.00 µg/g); this finding was similar to the levels in moist *snus* sold in the USA at the same time.² The reported Cl⁻ content in unused *snus* (35,300.00 µg/g) was close to that of *Afzal* as observed in the current study (33,170.80 µg/g).¹⁹ Quantities of Swedish *snus* are well monitored as they are typically sold in 1.00 g tea bag-sized sachets,¹⁹ in contrast, *Afzal* users usually self-determine doses (typically a pinch-sized amount per single dose ranging from 1.00–2.00 g) which are then repeated as desired.¹³ As a result, Cl⁻ consumption will increase due to repeated *Afzal* use during the day.¹⁶ Few studies have investigated the other anions tested in this study (including F⁻, Br⁻, SO₄²⁻ and PO₄²⁻). Stepanov *et al.* analysed SO₄²⁻ (4,560.00–12,300.00 µg/g) and PO₄²⁻ (309.00–1,300.00 µg/g) levels in American STPs; their findings were higher than anion levels indicated in the current study (1,502.20 µg/g and 37.80 µg/g, respectively).⁴

NO₃⁻ is an endogenous component of tobacco which originates from the presence of inorganic fertilisers in the soil.³⁴ In saliva, it is converted to NO₂⁻ (the more toxic form) by the oral microflora and NO₃⁻ reductase, leading to various toxic products such as methaemoglobin, which reduces oxygen transport and can lead to cyanosis.^{35,36} In addition, the formed NO₂⁻ promotes the conversion into nitrosating agents which in turn facilitate the formation of endogenous TSNA from tobacco alkaloids and dietary amines.⁴ Besides the oral reduction of NO₃⁻ to NO₂⁻, there is a natural endogenous formation of NO₃⁻ processes in other areas of the gastrointestinal tract.¹² The NO₃⁻ content reported in unused *snus* (1,220.00 µg/g) was much lower than that of the *Afzal* samples in the current study (8,792.20 µg/g).¹⁹ Borgerding *et al.* reported that the NO₂⁻ value of American STPs was >20.00 µg/g, although Hoffmann *et al.* have reported that values range much higher in moist snuff.^{2,37} Stepanov *et al.* reported that newer STPs generally contain <10 µg/g of NO₂⁻.⁴ However, the NO₂⁻ content of *Afzal* found in the current study (6.22 µg/g) was below all of these levels, including the GOTHIA TEK[®] limits for *snus* (7.00 µg/g).³⁸ Nevertheless, *Afzal* use is potentially harmful due its elevated NO₃⁻ content which can be reduced in the body to NO₂⁻ and subsequently to carcinogens (in the form of TSNA). Critically, elevated NO₃⁻ levels have been found to result in an increased risk of gastric cancer.³⁹ Many tobacco manufacturing companies are now attempting to produce STPs with low NO₃⁻ and NO₂⁻ levels in order to reduce their toxicity and limit TSNA formation during tobacco processing.⁴

Conclusion

Afzal was analysed chemically for toxicants that enhance cancer formation. There was a surge in both nicotine and Cl⁻ content, with alarmingly elevated NO₃⁻ levels. The levels of certain factors, including pH value, nicotine content and NO₃⁻, were above those reported for other international STPs, while the levels of Cl⁻ and moisture content were consistent and the levels of NO₂⁻, SO₄²⁻ and PO₄²⁻ were below those found in other STPs. All tested anions were below the maximum recommended daily intake advised by international organisations. However, the frequent use of *Afzal* poses a health risk to users which may potentially manifest as cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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