SPECTROPHOTOMETRIC DETERMINATION OF NICOTINE IN CIGARETTE TOBACCO AND BIOLOGICAL SAMPLES OF SMOKERS

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ABSTRACT

A new simple, accurate, sensitive and economical procedure for the estimation of nicotine (indirect) methods (A and B) for the determination of nicotine in bulk sample and in biological samples are described. The first method is based on the oxidation of nicotine by N-bromosuccinimide (NBS) and determination of the unreacted NBS by measurement of the decrease in absorbance of methyl orange dye (MO) at a suitable $\lambda_{\text{max}} = 507$ nm. The absorbance concentration plot is linear over the range (0.1-4.8 $\mu$g/ml). The second method is based on oxidation of nicotine by ceric sulphate in acidic medium, and determination of the unreacted oxidant by measuring the decrease in absorbance using amaranth dye; (AM) at a suitable $\lambda_{\text{max}}$ (530 nm), respectively. Regression analysis of Beer's law plots showed good correlation in the concentration ranges (0.3-5.9 $\mu$g/ml), respectively. The limits of detection as well as quantification are reported. Sex replicate analyses ($n=6$) of solutions containing three different concentrations of nicotine was carried out. The percent error and the RSD values have been reported. The proposed methods were applied to the determination of nicotine in cigarette tobacco present in Libya and in biological samples (spiked human plasma and urine) and the results demonstrate that the method is equally accurate and precise as found from the $t$- and $F$-values. The reliability of the method was established by recovery studies using standard-addition technique.

Key Words: Nicotine; Spectrophotometry; Oxidation reaction; N-bromosuccinimide; Ceric sulphate, Cigarette tobacco in Libyan markets; Spiked human plasma and Urine.
INTRODUCTION
Nicotine (pyridine, 3-(1-methyl-2-pyrrolidinyl), (S) is one of the highly toxic tobacco alkaloids present in tobacco leaves and cigarette smoke [1]. Nicotine appears to be a promising tracer for environmental tobacco smoke (ETS) because of its specificity for tobacco [2]. It is also a systemic and contact insecticide and is also used as a drug and in chemical [3]. The threshold limit value reported for nicotine is 0.05 mg/m^3 [1]. The most important symptoms of exposure to nicotine are bronchitis, emphysema, cyanosis, and exenation of the central nervous system. Excessive smoking has been implicated in lung cancer, bladder cancer, and cancer of the larynx and oesophagus [4].

The determination of total nicotine alkaloids is of particular importance to the tobacco industry and in the area of toxicology. Various analytical methods such as GC [5], TLC [6], HPLC [7-9], ASS [10], capillary electrophoresis [11], radio immuno assay [12], GC-MS [13] and circular dichroism spectropolarimetry [14] have been reported. Most spectrophotometric methods reported are based on cleavage of the pyridine ring with cyanogen bromide or chloride and condensation of the resulting compound with p-amino benzoic acid [15] and diethyl thiobarbituric acid [16] and other spectrophotometric methods [17-24], determination of outdoor Tobacco smoke exposure by distance From a smoking source [25], an electrochemically [26].

Many of these methods involve a prolonged extraction procedure prior to the determination of nicotine and the use of highly toxic cyanide [15, 16]. These methods were sophisticated to perform and/or time consuming. The oxidation reaction between NBS or Cerric in acidic medium and nicotine have not been investigated yet but for other drugs [27-37].

EXPERIMENTAL
Apparatus
All the spectral measurement were made using double-beam UV/Vis spectrophotometer (Biotech Engineering Ltd., UK), with wavelength range 190 –1100 nm, spectral bandwidth 2.0 nm, with scanning speed 400 nm/min, equipped with 10 mm matched quartz cells. A thermostat water bath, Buchi 461 water bath, Schwiz (France) was used to carry out the temperature studies and Magnetic Mix. 100, Thermo Scientific, UK.
Material and reagents

- All chemicals used were of analytical reagent grade of the best available quality, and all solutions were freshly prepared in doubly distilled water.
- Standard nicotine solution (Merck) was prepared by dissolving 0.01 g of pure nicotine in 50 ml of bi-distilled water and complete to 100 ml with bi-distilled water to obtain the working standard solution of 100 µg/ml.
- A stock solution of 1.0 x 10^{-3} M NBS (Aldrich Co., Ltd., Gillingham-Dorst, Germany) was freshly prepared by dissolving 0.0177 g of NBS in a least amount of warm water in a 100 ml measuring flask and then diluted with distilled water to the mark.
- A stock solution of 1.0 x 10^{-3} M ceric sulphate (Aldrich Co., Ltd., Gillingham-Dorst, Germany) was freshly prepared by dissolving appropriate weight of Ce (VI) in a least amount of warm water in a 100 ml measuring flask and then diluted with distilled water to the mark.
- A 2.0 M of HCl was prepared by adding exact volume from stock concentrated acid to bidistilled water in 500 ml measuring flask (Merck, Darmstadt, Germany, sp. gr. 1.18%, 37%) to 100 ml with distilled water.
- A solution of 2.0 M H_2SO_4, was prepared by adding exact volume from stock (98%) concentrated acid to bidistilled water in 500 ml measuring flask, and standardized as recorded [38].
- Aqueous solutions of dyes (1.0 x 10^{-3} M) methyl orange (MO) (Merck), (1.0 x 10^{-3} M) amaranth (AM) (Merck) were prepared by dissolving in an appropriate weight in 100 ml bidistilled water.

General procedure

For the first method, (method A) depends on oxidation of nicotine by addition of 0.01-4. 8 ml (100 µg/ml) to 1.8 ml of 1.0 x 10^{-3} M NBS containing 1.1 ml HCl, 2.0 M. The solution was heated in a water bath at 80±1 °C for 4.0 min. The solution was cooled and 1.7 ml (1.0 x 10^{-3} M) of MO was added, the volume was completed to 10 ml with bidistilled water. For the second method (method B) depends on oxidation of nicotine by addition of 0.01-5.9 ml nicotine (100 µg/ml) to 1.5 ml of 1.0 x 10^{-3} M Ce (VI) containing 1.2 ml of 2.0 M H_2SO_4 was added. The solution was heated in a water bath at 80±1 °C for 5.0 min, the mixture of acidic solution was cooled and 1.6 ml (1.0 x 10^{-3} M) of AM was added, the volume was completed to 10 ml with bidistilled water. The decrease in color intensity of dye was measured spectrophotometrically against a blank solution containing the same constituent except drug treated similarly, at their corresponding λ_{max} 507 (method A), 530 nm (method B),
respectively. The concentration range was determined in each case by plotting the concentration of nicotine against absorbance at the corresponding maximum wavelengths.

**Extraction and determination of nicotine in cigarette tobacco**\(^{[39]}\)

Weigh 10 g of cigarettes leaves in beaker. Add 100 ml NaOH solution and stir very well for 15 min, filter in Buchner using glass wool and press the cigarettes very well by using other beaker, transfer the cigarettes again to beaker, add 30 ml DW and stir and filter again, collect the filtrate together, (if there is any impurities re-filter), transfer the filtrate to the SF and extract by 25 ml ether, repeat the extraction 3 times, gather the 4 filtrates in conical flask, dry by using 1.0 teaspoon anhydrous potassium carbonate, filter. evaporate ether on water bath, (avoid extra heat because nicotine is hydrolyzed by extreme heating), after evaporation of ether add 4.0 ml methanol to dissolve the resulted oil, to the filtrate 14 ml of 1.0 mol/L HCl was added and the solution was made up to 100 ml with water. An aliquot of this solution was taken and analyzed as described above. The results are shown in Table 2.

**Procedure for spiked human urine**

A 50 ml volume of nicotine – free human urine taken in a 125 ml separating funnel was spiked with 2.5 mg nicotine. Ten milliliters of 1.0 M NaOH was added, mixed and kept aside for 3.0 min. Then, 25 ml of ethyl acetate was added, shaken well for about 15 min and the upper organic layer was collected in a beaker containing anhydrous sodium sulphate. The water-free organic layer was transferred into a dry beaker and solvent removed by evaporation on a hot water bath. The dry residue was dissolved in glacial acetic acid and transferred into a 25 ml calibrated flask, and diluted to the mark with the same acid. The resulting solution equivalent to 100 µg/ml nicotine was diluted with the same acid and analyzed by following the procedure described above.

**Procedure for spiked human plasma samples**

Aliquots of 1.0 ml of plasma were spiked with different concentration levels of nicotine. The spiked plasma samples were treated with 0.1 ml of 70% perchloric acid and vortexes for 1.0 min. The samples were centrifuged for 20 min at 13000 rpm. The supernatants were transferred into test tubes and neutralized with 1.0 M NaOH solution.

**Stoichiometric relationship**

The stoichiometry of the reaction between nicotine and the oxidants at the selected conditions were established by the molar ratio method. In this method 1.0 ml of 1.0 \(x\ 10^{-3}\) M NBS
(method A) $1.0 \times 10^{-3}$ M Ce (VI) (method B) is kept constant and variable concentrations (0.1-6.0 ml) of nicotine ($1.0 \times 10^{-3}$ M) were added. The absorbance was measured at $\lambda_{\text{max}}$ against blank solution prepared in the same manner. The absorbance values were then plotted against the molar ratio $[\text{D}]/[\text{O}]$ as shown in Figure 6.

RESULTS AND DISCUSSION

First Method (A)
The proposed spectrophotometric methods are based on the reaction between nicotine and measured excess of NBS and subsequent determination of the latter by reacting it with a fixed amount of MO dye, and measuring the absorbance at 507 nm. The reaction takes place completely after 4.0 min., was heated in a thermostat water bath at 80±1 °C. This method make use of the bleaching action of NBS on the dye, the decolorization being caused by the oxidative destruction of the dye. nicotine when added in increasing concentrations to a fixed concentration of NBS consumes the latter and there will be a concomitant decrease in the concentration of NBS. When a fixed concentration of dye is added to decreasing concentrations of NBS, a concomitant increase in the concentration of dye is obtained. Consequently, a proportional increase in the absorbance at the respective $\lambda_{\text{max}}$ is observed with increasing concentrations of nicotine, the color remains constant for at least 48 h.

Second Method (B)
The method involves two steps namely:

- Oxidation of nicotine with Ce(SO$_4$)$_2$ in acidic medium by heating in water bath 80±1 °C.
- Determination of unreacted oxidant by measuring the decrease in absorbance of dye at a suitable $\lambda_{\text{max}}$.

\[
\text{Nicotine} + \text{Ce(SO}_4\text{)}_2 \xrightarrow{\text{Dil. H}_2\text{SO}_4} \text{Oxidized products} + \text{Ce (III)} + \text{Ce(SO}_4\text{)}_2 \quad \text{(Unreacted)}
\]

\[
\text{Unreacted Ce(SO}_4\text{)}_2 + \text{Amaranth dye} \rightarrow \text{Ce (III)} + \text{Unreacted Amaranth dye}
\]

Pink colour, $\lambda_{\text{max}}$ 530 nm

The influence of each of the following variables on the reaction was tested.

Effect of NBS concentration

The influence of NBS concentration was studied in the range from $10^{-4}$ - $10^{-2}$ M, as final concentration. The optimum results were obtained with 1.8 ml of $1.0 \times 10^{-3}$ M.
Effect of ceric sulphate concentration
The influence of Ce (VI) concentration was studied in the range from $10^{-4}$ to $10^{-2}$ M, as final concentration. The optimum results were obtained with 1.5 ml of $1.0 \times 10^{-3}$ M; higher concentration of Ce(SO$_4$)$_2$ caused the color to disturbed.

Effect of acid medium
The different types of acid were examined (HCl, HClO$_4$, H$_2$SO$_4$, H$_3$PO$_4$, CH$_3$COOH and HNO$_3$). The most suitable acid to achieve maximum yield of redox reaction was found to be 1.1 ml of 2.0 M HCl for (method A) and 1.2 of 2.0 M H$_2$SO$_4$ was added on using AM, for (method B) Figure 3.

Effect of temperature and time
The reaction takes place completely after 20 min at room temperature 25±1 °C. The oxidation process of nicotine with NBS is catalyzed by heating in a thermostat water bath at 80±1 °C for 4.0 min. using MO dye. The oxidation process of nicotine for the (method B) Ce(SO$_4$)$_2$ in acidic medium was catalyzed by heating in a thermostat water bath of 80±1 °C for 5.0 min, using AM. After oxidation process, the solution must be cooled at least for 2.0 min before addition of dye.

Effect of sequence of additions
The effect of sequence of additions on the oxidation process of nicotine was studied by measuring the absorbance of solution prepared by different sequence of additions against a
blank solution prepared in the same manner. Experiments showed that (Oxidant-Acid-Drug) for both methods, gave the best results.

**Effect of dye concentration**

The optimum volume of dye used for production of maximum color intensity was 1.7 ml of $1.0 \times 10^{-3}$ M (MO) for (method A), 1.6 ml of $1.0 \times 10^{-3}$ M (AM) for (method B) Figure 4. The effect of time after the addition of dye indicated that shaking for 1.0 min was sufficient to give reliable results for all dyes. The color remains constant for at least 24 h.

**Stoichiometric ratio**

The stoichiometry of the reaction between nicotine and the oxidant at the selected conditions was established by the molar ratio method. In this method 1.0 ml of $1.0 \times 10^{-3}$ M NBS for (method A), 1.0 ml of $1.0 \times 10^{-3}$ M Ce(VI) for (method B) is kept constant and variable concentrations (0.1 - 6.0 ml) of nicotine ($1.0 \times 10^{-3}$ M) were added using micropipette. The absorbance was measured at $\lambda_{\text{max}}$ against blank solution prepared in the same manner. The absorbance values were then plotted against the molar ratio [D]/[O]. The stoichiometry of [D]/[O] at the selected conditions showed that the inflection of the two straight lines at 0.63 for (method A) 0.71 AM for (method B), Figure 6.

**Reproducibility of the method**

The reproducibility of the method was assessed by carrying out seven replicate analyses of a solution containing 3.0 µg/ml of nicotine in a final solution volume of 25 ml. The standard deviation and relative standard deviation of absorbance values were found to be ±0.011 and 0.56 %, respectively.

**Analytical data**

Beer's law was verified up to (0.1-4.8 µg/ml) for method A, and (0.3-5.9 µg/ml) for method B as shown in (Figure 5) and Ringbom limits, molar absorptivities, Sandell sensitivities, regression equations and correlation coefficients were calculated and recorded in (Table 1). The limits of detection (K=3) and quantitation (K=10) were established according to IUPAC definitions [40] are recorded in (Table 1).

In order to determine the accuracy and precision of the methods, solution containing three different concentrations of nicotine were prepared and analyzed in six replicates (Table 2).
Effect of ml added of acid (2.0 M HCl using NBS, 2.0 M H₂SO₄ using Ce(VI)); nicotine (4.0 µg/ml), 1.1 ml of (1 x 10⁻³ M) NBS, reaction temperature 80±1 °C; reaction time: 4.0 min

Effect of ml added of dyes on 4.0 µg/ml nicotine, (MO in case of NBS, AM in case of Ce(VI)), reaction temperature 80±1 °C; reaction time: 4.0 min

Stability of colour

The pink or red colour formed was stable for at least 24 h under optimum conditions.

Validation method

The proposed method was successfully applied to determine nicotine in cigarettes tobacco and in biological samples (spiked human plasma and urine). The accuracy of the proposed methods is evaluated by applying standard addition technique, in which variable amounts of the nicotine were added to the previously analyzed portion of extracted nicotine from cigarettes tobacco and in biological samples (spiked human plasma and urine). The results recorded in (Table 3), The results are in good agreement with those obtained by the reported method [17-19], by Student’s t-test (for accuracy), and variance ratio F-test (for precision) [41], at 95% confidence level as recorded in (Table 4). The results showed that the t- and F- values were lower than the critical values indicating that there was no significant difference between the proposed and reported methods. The proposed method was more accurate with high recoveries.
Fig. 5. Calibration curve of nicotine µg/ml, using NBS and Ce(VI) (1.0 x 10^{-3} M) and dyes, MO and AM (1.0 x 10^{-3} M), reaction temperature 80±1 °C; reaction time: 4.0 min

Fig. 6. Molar ratio method for nicotine (1.0 x 10^{-3} M) using NBS and Ce(VI) (1.0 x 10^{-3} M) and dyes, MO and AM (1.0 x 10^{-3} M), reaction temperature 80±1 °C; reaction time: 4.0 min

Table 1. Optical and regression characteristics of nicotine for the proposed method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NBS</td>
</tr>
<tr>
<td>λ_{max} (nm)</td>
<td>507</td>
</tr>
<tr>
<td>Stability / h</td>
<td>24</td>
</tr>
<tr>
<td>Beer’s law limits (µg/ml)</td>
<td>0.1 – 4.8</td>
</tr>
<tr>
<td>Ringbom limits (µg/ml)</td>
<td>0.3 – 4.5</td>
</tr>
<tr>
<td>Molar absorptivity (L/mol cm)</td>
<td>2.76 x 10^4</td>
</tr>
<tr>
<td>Sandell sensitivity (ng/cm^2)</td>
<td>5.88</td>
</tr>
<tr>
<td>Detection limits (µg/ml)</td>
<td>0.058</td>
</tr>
<tr>
<td>Quantitation limits (µg/ml)</td>
<td>0.193</td>
</tr>
<tr>
<td>Regression equation^a^:</td>
<td></td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.170</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>8.4 x 10^{-8}</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9996</td>
</tr>
<tr>
<td>RSD^b %</td>
<td>0.59</td>
</tr>
<tr>
<td>Calculated t-values (2.57)^c</td>
<td>0.49</td>
</tr>
<tr>
<td>Calculated F-test (5.05)^c</td>
<td>2.27</td>
</tr>
<tr>
<td>Stochiometric ratio [D]/[O]</td>
<td>1.0 : 0.63</td>
</tr>
</tbody>
</table>

^a A = a + bC where C is concentration of nicotine in µg/ml and A is absorbance.

^b Relative standard deviation for six determinations.

^c Values in parentheses are the theoretical values for t- and F values at 95% confidence limits and five degrees of freedom.
Table 2. Evaluation of the accuracy and precision of the proposed procedures for nicotine.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Taken µg/ml</th>
<th>Found µg/ml</th>
<th>Recovery %</th>
<th>RSD a %</th>
<th>RE b %</th>
<th>Confidence Limits c</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS</td>
<td>1.0</td>
<td>1.02</td>
<td>102.0</td>
<td>0.55</td>
<td>1.23</td>
<td>1.02 ± 0.0125</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.98</td>
<td>99.00</td>
<td>0.69</td>
<td>1.60</td>
<td>1.98 ± 0.0316</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.01</td>
<td>100.25</td>
<td>0.76</td>
<td>0.82</td>
<td>4.01 ± 0.0329</td>
</tr>
<tr>
<td>Ce(SO₄)₂</td>
<td>1.0</td>
<td>0.97</td>
<td>97.0</td>
<td>0.73</td>
<td>1.39</td>
<td>0.97 ± 0.0135</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>2.98</td>
<td>99.33</td>
<td>0.84</td>
<td>1.07</td>
<td>2.98 ± 0.0318</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.01</td>
<td>100.2</td>
<td>0.91</td>
<td>1.02</td>
<td>5.01 ± 0.0510</td>
</tr>
</tbody>
</table>

a Relative standard deviation for six determinations.

b Relative error.

c 95% confidence limits and five degrees of freedom

Table 3. Determination of nicotine in cigarette tobacco in Libyan markets and biological samples using standard addition technique.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Taken µg/ml</th>
<th>NBS</th>
<th>Ce(VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added µg/ml</td>
<td>Found* µg/ml</td>
<td>Recovery %</td>
</tr>
<tr>
<td>American Legend a **</td>
<td>0.0</td>
<td>1.03</td>
<td>103.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.05</td>
<td>102.50</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.97</td>
<td>99.00</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>4.01</td>
<td>100.25</td>
</tr>
<tr>
<td>Marlloborro b **</td>
<td>0.0</td>
<td>0.97</td>
<td>97.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.05</td>
<td>101.00</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.99</td>
<td>99.00</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.96</td>
<td>99.50</td>
</tr>
<tr>
<td>Urine</td>
<td>0.0</td>
<td>0.98</td>
<td>98.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.98</td>
<td>99.00</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.97</td>
<td>99.00</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.03</td>
<td>100.75</td>
</tr>
<tr>
<td>Spiked human plasma</td>
<td>0.0</td>
<td>0.98</td>
<td>98.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.07</td>
<td>103.50</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.96</td>
<td>98.66</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.03</td>
<td>100.75</td>
</tr>
</tbody>
</table>

* Average of six determinations.

** Amount of sample: 0.1 gm. After treatment diluted to 50 ml, nicotine found in µg/ml of the final solution.

a Cigarette smoke, made in EU, under the authority of the Trade mark Owners, by KTG, INC.
b. Cigarette smoke, Karelia light, Karelia tobacco company INC, made in Greece, nicotine content 0.7 mg.

Table 4. Determination of nicotine in cigarette tobacco in Libyan markets and in biological samples using the proposed and reported methods.

<table>
<thead>
<tr>
<th>Samples</th>
<th>NBS</th>
<th>Ce(VI)</th>
<th>Reported method[17]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery</td>
<td>t-test</td>
<td>F-value</td>
</tr>
<tr>
<td>American Legend(^a)*</td>
<td>98.80</td>
<td>1.22</td>
<td>2.49</td>
</tr>
<tr>
<td>Marlloborro(^b)</td>
<td>99.17</td>
<td>0.89</td>
<td>1.99</td>
</tr>
<tr>
<td>Urine</td>
<td>97.89</td>
<td>1.08</td>
<td>2.07</td>
</tr>
<tr>
<td>Spiked human plasma</td>
<td>97.19</td>
<td>0.96</td>
<td>2.55</td>
</tr>
</tbody>
</table>

Theoretical value for t- and F-values for five degrees of freedom and 95% confidence limits are 2.57 and 5.05, respectively.

* Amount of sample: 0.1 gm. After treatment diluted to 50 ml, nicotine found in µg/ml of the final solution.

\(^a\) Cigarette smoke, made in EU, under the authority of the Trade mark Owners, by KTG, INC.

\(^b\) Cigarette smoke, Karelia light, Karelia tobacco company INC, made in Greece, nicotine content 0.7 mg.

CONCLUSION

The proposed method was advantageous over other reported visible spectrophotometric and colorimetric methods, related to their high reproducibility, high sensitivity, less time consuming and using simple and inexpensive reagents. Moreover, these methods allowed the determination of nicotine up to 0.1 µg/ml, in addition to simplicity, rapidity, precision and stability of colored species for more than 24 h.

REFERENCES


