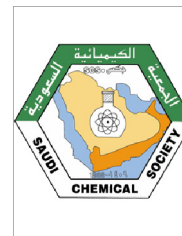




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ORIGINAL ARTICLE

Analytical determination of nicotine in tobacco leaves by gas chromatography–mass spectrometry

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Abstract A preliminary investigation using gas chromatography–mass spectrometry (GC–MS) to analyze the nicotine contained in tobacco leaves was carried out. Nicotine is an alkaloid and tobacco leaves was extracted with methanol and determined by GC–MS. The detection limit for nicotine was at the ppm level for non selective monitoring and the nanogram level for selective detection. This is a simple chromatography–mass spectrometry method for the analysis of nicotine in tobacco leave. Compared to other currently utilized methods for the detection of nicotine in tobacco leaves, the GC–MS provided advantages of high sensitivity, nicotine specific detection and lower instrumentation cost.

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1. Introduction

Nicotine is an alkaloid found in the nightshade *Solanaceae* family of plants, predominantly in the leaves of tobacco, and in lower concentration in tomato, eggplant, and in green pepper. They are also found in the leaves of the coca plant. Most of the medicinal higher plants extractable organic compounds in sufficient quantities to be economically useful as chemical feed stocks or

raw materials for various scientific, technological and commercial applications. Industrial oil, resins, tannins, saponins, nicotine, natural rubber gums, waxes, dyes, pharmaceuticals and many other products from economically important plants serve as sources (The Merck Index, 1989). Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine is a colourless, less to pale yellow, hygroscopic oily liquid present in the leaves of *Nicotiana tabacum*.

Nicotine is one of the most highly toxic compounds belonging to the tobacco alkaloids (The Merck Index, 1989). Several chromatographic techniques have been applied to describe for the determination of nicotine in various plants extract (Burrows et al., 1971; Beckett and Triggs, 1996; Isaac and Rand, 1972; Feyerabend et al., 1995; Dow and Hall, 1978). Various solvent extraction techniques followed by gas chromatographic-mass spectrometric analysis (Thompson and Ho, 1982; Watson, 1977; Grubner et al., 1980) and liquid chromatography (LC) with ultra-violet absorbance detection (Davis, 1986; Moore et al., 1993) are the most useful techniques employed for the determination of nicotine in the leaves of tobacco. A most useful technique described for the determination of

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nicotine by Moore, et al. and cotinine in plasma after a single extraction procedure using gas chromatography-nitrogen selective detection. The electron capture detector used for the determination of nicotine after chemical derivatization using heptafluorobutyric anhydride. For the determination of nicotine and N-methylnicotinium ion at the picogram level using electrochemical detection by very useful and selective LC method has been developed (Mousa et al., 1985).

Bangladesh is an agricultural country. Vegetables, crops, tobacco and fruits are grown here in plenty, mainly in the winter season. Different types of tobacco herbs are the most commonly used because they are cheap and available all over the Bangladesh throughout the season. The concurrent use of tobacco with alcohol is one of the most common drug combinations in the United States. There is a general consensus that nicotine modifies the acute effects of alcohol.

The effects of nicotine are very important as sex-related differences have been noted. Generally, nicotine appears to be less reinforcer in women than in men for maintaining cigarette smoking and this may be due to sex differences in the sensitivity to nicotine's interoceptive cues (Perkins, 1999). Hormones are the most important factors for understanding many drug effects in women. As for example, cocaine and amphetamine are responsible to influenced by varying the level of estrogen and progesterone associated with the follicular and luteal phases (Evans et al., 2002; Justice and Wit de, 1999; Mitchell et al., 1995). Mello *et al.*, reported that cigarette smoking and alcohol self-administration in women and found that almost three-quarters of women increased smoking during the luteal phase of their menstrual cycle, as measured by inter-cigarette interval. On the other hand, additional important evidence for the influence of menstrual cycle phase on nicotine's effects is seen during withdrawal. Perkins *et al.* report that nicotine withdrawal symptoms for severity is greater during the luteal phase of the cycle than during the follicular, which may be explain why others have reported more smoking during this phase (Mello, 2007; Benowitz et al., 1988). Cigarette smoking has a relatively short duration of action of nicotine administration. The levels of nicotine peak for blood are achieved typically within the time it takes to consume the cigarette (5 to 10 minutes) and decline quickly (Pomerleau et al., 1994). However, alcohol via the oral route has a slower onset and longer duration of action. In this paper, a simple, chromatographic method is described for the determination of nicotine in the leave and stems of tobacco.

2. Material and methods

2.1. Chemicals

Nicotine standard was purchased from Sigma-Aldrich Company with purity 99.9%. Methanol (BDH, UK), dichloromethane and water (Merck, Germany) were of HPLC grade. Anhydrous sodium sulfate (Merck, Germany) was cleaned by heating at 200 °C before use. Silica gel (60–120 mesh, Loba, India) was activated at 400 °C for 12 h prior to use.

2.2. Sample collection

There are different types of tobacco available in Bangladeshi markets all year round. The dried tobacco leaves samples were

collected from one of the biggest tobacco industry at Dhaka Metropolitan City, Bangladesh in April 2009. Five fresh tobacco leaves samples were collected from southern district of Kushtia after harvest. The leaves samples were washed by tap and de-ionized water to remove dusts and any other foreign particles. After collection, the sample was kept in a polyethylene bag with aluminum foil protected cover and stored in refrigerator to avoid any deterioration. After harvesting, the apples are stored at 2 °C.

2.3. Isolation and preparation of crude extracts

After having washed, the leaves were cut into small pieces and dried by sunlight or oven below temperature 40 °C. The dried leaves samples were pulverized into powder form. The dried powder (0.1 g) was extracted three times with methanol (5 ml 3×) by sonication at 30 min. It was then filtered and the filtrate was evaporated near to dryness by Kuderna-Danish evaporator. The extract was passed through the cleanup column (i.d. = 1 cm), which was filled with cotton in the bottom. An activated silica gel (10 g) soaked with solvent was loaded into the cleanup column (5 cm), which was then topped with 1.5 cm of anhydrous sodium sulfate. Five milliliters of solvent were added to wash the sodium sulfate and the silica gel. The pre-concentrated dried crude extracts, 1 ml of each extract sample, were then separately transferred into the column, and the vessel was rinsed twice with 2 ml loaded solvent, which was also added to the column. Sixty milliliters of loaded solvent were added to the column and allowed to flow through the column at a rate of 3–5 ml/min, and the eluent was collected. The collected eluent from the cleanup procedure was reconcentrated to 2 ml by using K-D concentrator. Finally the extract (2 ml) from leaves was filtered through a 0.45 μ m Millex HA filter (Millipore, Molsheim, France) prior to GC-MS analysis.

2.4. GC-MS analyses

2.4.1. Preparation of samples from markets for GC-MS analyses

The methanol extract (1 ml) was diluted with 5 ml of methanol and the samples were filtered through 0.45 μ m membrane filters (Molsheim, France) prior to GC-MS analysis.

2.4.2. Identification and quantification of marker in the leaves samples

The GC-MS analysis of the methanolic crude extract of tobacco leaves samples was performed using a Varian GC-MS (Model Varian CP 3800, Varian, Inc. Scientific Instruments, Lake Forest, CA 92630-8810, USA) equipped with a VF-5 fused silica capillary column (30 m $\times$ 0.25 i.d. mm film thickness 0.25 μ m, Varian, USA). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. Injector and mass transfer line temperature were set at 250 and 300 °C, respectively. The oven temperature was programmed from 50 to 200 at 8 °C/min, and then held isothermal for 20 min and finally raised to 300 °C at 10 °C/min. Diluted samples (1/100 v/v, in methanol) of 0.2 μ l were manually injected in the split less mode. Identification of compounds of the methanolic crude extract was based on GC retention time on VF-5 capillary column, computer matching

of mass spectra with standards (Mainlab, Replib and Tutorial data of GC–MS systems). The reference compound, nicotine was used as marker. The marker was accurately weighed and dissolved in methanol to produce a series of concentrations. Standard calibration curves were established by plotting the peak areas against different concentrations of the reference compounds (varying from 5.0 to 1000 ng on column for nicotine). The external standard method was used for quantification of the marker in the samples of leaves extract from different places.

The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three different standard concentrations.

2.5. Statistical analyses

Data are expressed as means of triplicate measurements. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Means were compared by Tukey-HSD and LSD (least significant differences). Differences at $P < 0.05$, considered to be significant.

3. Results and discussion

3.1. Collection of the samples

Table 1 shows the locations in Bangladesh where the samples were collected. The tobacco leaves were collected in the late

afternoon, when leaves are less turgid and, therefore, less likely to be damaged and energy substrate levels are high to facilitate long storage life.

3.2. Concentrations of the marker in methanolic leaves extracts of tobacco

The GC–MS method applied is a modification of that reported by Stuart et al. (1988) for the analysis of nicotine present in tobacco. In the present study, a programmed method was used for simultaneous assay of the authentic marker nicotine. The standard was determined in a single GC–MS run. The standard was resolved and eluted at 10.16 min, with respect to nicotine (Fig. 1). The marker (5, 50, 500, 750 and 1000 ng on column for nicotine) showed a good linearity in the range from 5.0 to 1000 ng in the calibration curves that were obtained by GC–MS analysis. The reference marker was present in the chromatographic profiles of the samples from various locations when the sample solution was analyzed by GC–MS (Fig. 2). The peak of nicotine was confirmed by comparison of the retention times with reference standard.

To assess the precision of these methods, standard solutions of nicotine were determined six times on the same day and over a six-day period. The results showed a very good precision, ranging from 5 to 100 µg/ml. The accuracy of the method was evaluated through recovery studies. The recovery experiments were performed at three concentrations (5, 50 and 100 ng) of the standard added to sample solutions, in which the marker content had been determined, using a sample from Dhaka.

The result for the recoveries of nicotine was in the range of 83–96%. The limit of detection (LOD) of the GC–MS method, established at signals three times that of the noise for nicotine, was 2.5 ng.

The GC–MS procedure was applied to the determination of the marker in the leaves samples from different regions. As shown in Table 1, all the analyzed samples showed a significant range in the concentrations of the marker, in samples from the same region and from different regions. The variation may be ascribed to environmental conditions and variation in sample sourcing.

The leaves selected for this experiment were of similar size, fresh weight and dry weight; however, the chemical

Table 1 Concentration of nicotine in the Bangladeshi tobacco leaves samples.

S. No.	Tobacco leave	Nicotine (%)
1	Tobacco Industry, Dhaka	1.8
2	Kursha, Kushtia	0.9
3	Mirpur, Kushtia	2.5
4	Amla, Kushtia	3.1
5	Poradah, Kushtia	2.8
6	Halsha, Kushtia	3.6
7	Blank	ND

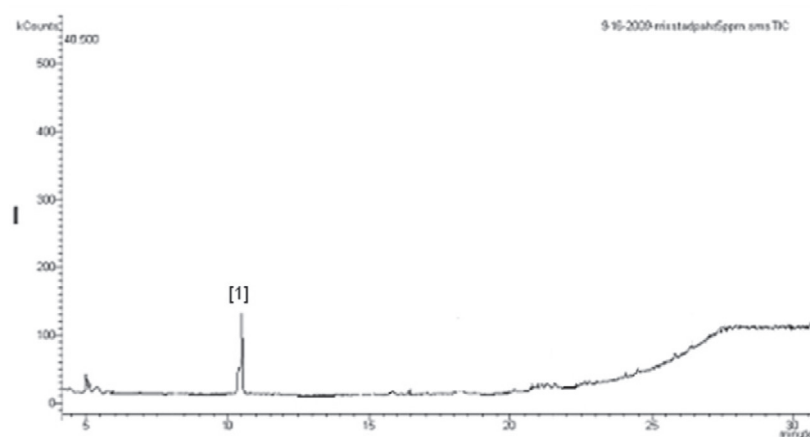


Figure 1 Chromatogram of the marker, nicotine (The Merck Index, 1989).

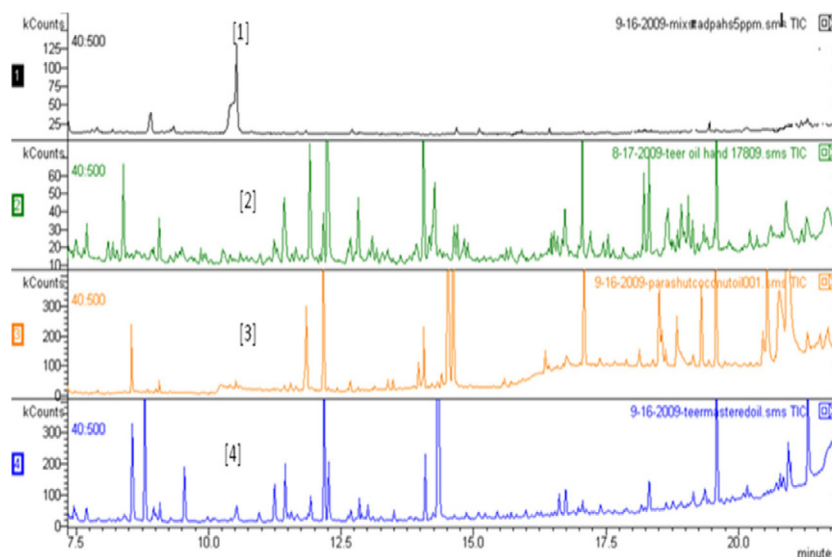


Figure 2 An overlay chromatogram of the methanol extract of tobacco leaves; (1) Standard; (2) Tobacco Industry Dhaka; (3) Kursha; (4) Mirpur.

composition of the leaves could be affected by the soil fertility levels and age (Schmidt, 1971; Mayer et al., 1975). The values obtained for the markers appear to fall within the range reported for the marker in leaves (Guyot et al., 1997; Mohamed and A., 2000). However, the overall levels of the marker concentration were considerably higher in samples from Halsha, Kushtia. Nicotine was the main component found in leaves at concentrations ranging from 0.9% to 3.6% of total dry leaves weight (Table 1). Table 1 summarizes the concentration of nicotine and shows that this differs at different sampling stations. The concentration at Halsha is relatively higher than the other sampling stations. The highest concentration for toxic nicotine was found 3.6% dry weight at Halsha, Kushtia and the lowest value was recorded as 0.9% dry weight at Kursha, Kushtia. The GC–MS results showed that the relative concentrations of the marker varied considerably.

4. Conclusions

The GC–MS chromatographic profiles of the Bangladeshi tobacco samples collected from different locations were qualitatively similar but the results showed variations in the concentrations of the marker. The GC–MS finger-printing could be used in authentication of tobacco samples and formulations.

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