*Original Article*

# Estimation of Nicotine in Tobacco Extract Using C8 Column in High Performance Liquid Chromatography

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## Abstract

***Background:*** Nicotine is the most used toxic substance in spite of mass media awareness. Column used in the estimation of nicotine is found to be the inhibiting factor for its estimation. ***Objective:*** To develop a method for nicotine estimation on C8 column in HPLC. ***Methods:*** The method involved a model Waters 1515 binary HPLC Pump system interfaced with a 2489 Waters UV/VIS detector, a 4.6 X 250 mm, 5µm beads Symmetry C8 column at 37°C, and an isocratic mobile phase containing 60%: 40% v/ v mixture of 10mM sodium acetate (pH 5.5) and methanol at a flow rate of 1.0ml/min at 256 nm. The method was validated for specificity, linearity, precision, accuracy, the Limit of Detection (LOD), the Limit of Quantitation (LOQ) and system suitability. ***Results:*** The HPLC Nicotine peak with average retention time of 3.415 minutes was observed on chromatogram with RSD <0.5%; linearity was greater than 0.99 with acceptable precision (within USP limits of <2.0% RSD) and USP tailing less than 2. LOD was found to be 0.200 µg/ml and Limit of Quantitation (LOQ) was found to be 0.609 µg/ml of nicotine. The average yield of the nicotine extracted by acid base extraction method from tobacco was 1.68% (range: 1.34–2.22%).***Conclusion:*** A HPLC method based on C8 column for analysis of Nicotine was developed and validated successfully.

**Keywords**: High performance liquid cheomatography, Column C8, Nicotine, Tobacco

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## Introduction

Nicotine, poisonous nitrogen-containing compound, is a tobacco alkaloid.[1] It is synthesised from numerous species of plants containing tobacco. Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine is a colorless, slightly pale yellow, hygroscopic oily liquid existing in Nicotiana tabacum L (NTL) leaves.[2] It contains a pyridine nucleus with a side chain at position-3.[3] In small quantity, in smoking, it stimulates the nervous system.

Nicotine is known to improve the health conditions of patients of psychiatric disorders, like schizophrenia abnormalities[4] and dementia patients, or those on therapy - dopaminergic neurons and axons, levodopa induced dyskinesia, skin mild cognitive dysfunction.[5] Nicotine has antimicrobial and insecticidal functions[6] and used as a natural pesticide with features of degradable, harmless and without environmental pollution challenges.[7] Tobacco addiction causes many diseases in developing countries that lead to high mortality.[8] Tobacco chewing is very common in India and causes cancer related mortality. This with tobacco smoking is a huge burden to Indian health care and over all social wellbeing. Nearly

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267 million adults (15 years and above) in India (29% of all adults) are users of tobacco, according to the Global Adult Tobacco Survey India, 2016-17. Khaini, gutkha, betel quid with tobacco and zarda are the most prevalent form of smokeless tobacco use in India. Smoking forms are bidi, cigarette and hookah.[9] To measure health related effect of nicotine it is needed to be measured in source and plasma of the addicted individuals. There are several methods of nicotine estimation including gas chromatography with flame ionization detector (GC-FID),[10] and gas chromatography-mass spectrometry (GC-MS).[11,12] Since, LC is a workhorse technique used for efficient and tedious analytical procedures,[13-15] it has been employed successfully for nicotine quantification in e-liquids such as liquid chromatography-mass spectrometry (LC-MS),[16,17] in addition to HPLC with photodiode array detection (HPLC-PDA).[18,19]

HPLC method is the rapid, simple and efficient of all. C8 column is packed with octylsilane bonded to silica, which is commonly used in reverse phase HPLC methods. The retention time for such column is less in comparison to C18 (octadecylsilane) column with 18 carbon hydrophobic chains bonded to silica particles beads because of less hydrophobicity. The density of C8 column is less in comparison to C18 column. Hence, the study aims to estimate nicotine in tobacco using C8 column in HPLC.

## Materials and Methods

*Materials and chemicals:*Nicotine standard liquid (density 1 g/ml) was from Sigma Aldrich, USA. HPLC grade Sodium acetate trihydrate and water was obtained from Finar limited, Ahmadabad, India, HPLC grade methanol was obtained from Thermo Fisher Scientific, Mumbai, India. Chloroform, glacial acetic acid, sodium hydroxide and sodium carbonate anhydrous were purchased from Empura, Merck life Science Private Limited, Mumbai, India.

*Preparation of nicotine (NCT) extract:*Acid-base extraction method was used to extract nicotine from commercially available chewable tobacco. This method is based on the alkaloid property of nicotine, which involves different solubility levels in water and an organic solvent. The 5g of tobacco leaves was boiled with 350ml of water at 80±5◦C for 20 min, then 5g of sodium carbonate was added and the mixture was continuously heated for 10 min. After filtration, the obtained filtrate was checked by pH strip paper and adjusted to pH 12 using 1N sodium hydroxide. The filtrate was then mixed with 50ml chloroform in a separatory funnel with stop cock closed. The separatory funnel was allowed to rest vertically on a stand to allow the organic and water phase to separate into layers. The bottom layer is drained carefully into a beaker. The process was repeated once more with 50ml chloroform. Both of the drained filtrate was combined and poured on a wide petri-dish 150mm × 25 mm. The dish was placed on a dry block incubator at 50°C for one hour to obtain crude extracts. The process of evaporation was carried out in a fume hood to avoid unwanted hazards. A yellow dried residue was obtained. The dried extract was then dissolved in 10ml HPLC grade methanol. A brownish gummy insoluble substance formed which was mostly nucleic acid, whereas the soluble suspension was filtered into a clean test tube through a 0.2µm PTFE syringe filter. Each extraction was performed in duplicate, the obtained extracts were protected from light and stored at 4◦C for analysis.

*Determination of nicotine content in the extracts:*The concentration of extracted nicotine was measured by injecting 10µl of the dissolved extract in HPLC system (Table 1). Waters HPLC system equipped with 1515 binary HPLC Pump, 2707 Waters auto-sampler, Waters column oven, and 2489 Waters UV/VIS detector was utilized. Waters Breeze 2 software was used for data acquisition and system suitability calculations.The samples were filtered through 0.2µm nylon filter into a 2ml autosampler vial and10µl injection of sample was injected on column in triplicate by the autosampler. Chromatogram, retention time, response and other parameters were estimated by Waters breeze 2 software (Figure 1).

**Table 1:** Chromatographic parameters for determination of nicotine

| Parameter | Condition |
|---|---|
| Method | Reverse phase high performance liquid chromatography |
| Column | X Bridge C8 (Waters, USA) 250mm × 4.6mm, and 5µm particle size |
| Flow rate | 1 ml/minute |
| Detection | UV detector, 259nm PDA detector, 37°C |
| Column temperature | 37°C |
| Injection volume | 10µl |
| Mobile Phase | Sodium acetate buffer (pH 5.5; 10mM): Methanol (60% : 40% v/ v) |

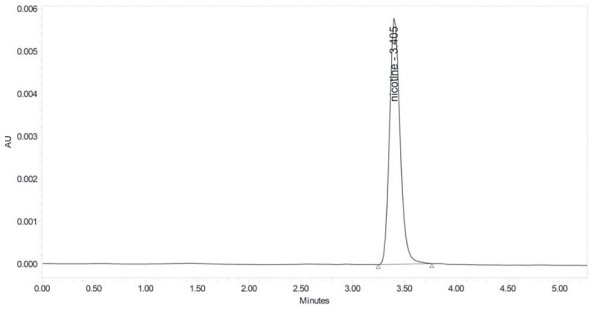


**Figure 1:** Chromatogram of nicotine sample 5µg/ml; $t_R$=3.405 min. with 10µl injection; Mobile phase: Sodium acetate buffer (pH 5.5;10mM) : Methanol (60% : 40% v/v); flow rate 1ml/min; Column C8 Symmetry (4.6 × 250mm,5µm beads); detector:UV 256 nm.

*Method validation:*The developed method was validated for the parameters like linearity, precision, accuracy, recovery, and system suitability as described here.A stock solution of nicotine 100ppm was prepared by dissolving 1µl liquid nicotine standard in 10 ml of mobile phase. Different concentrations of standard were prepared from the stock of 1, 2, 5, 10 and 25µg/ml in mobile phase. The standards were filtered through 0.2µm nylon filter into a 2ml auto-sampler vial and injected on column in triplicate by the auto-sampler. Slope and intercept were estimated by Waters Breeze 2 software.The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple sampling of homologous sample. It can be expressed as repeatability and intermediate precision.Repeatability is to use of analytical procedure within a laboratory over a short period of time using the same analyte with the same equipment and is expressed as the percent Relative Standard Deviation (RSD). The repeatability of the method was analyzed by measuring six replicates of nicotine at 100% assay concentration of 5µg/ml.Intermediate precision of the method was checked by repeating the entire procedure for three consecutive days and calculating the RSD inthree consecutive days.Accuracy was established by analyzing standard (5µg/ml) three times at 50%, 100% and 150% of the assay level. The average results were calculated against the true standard concentration and percent recovery was calculated.

*Limit of detection (LOD) and Limit of quantification (LOQ):*Limit of detection (LOD) and Limit of quantification (LOQ). The calculation of both LOD and LOQ were based on the standard deviation (SD) of the response (σ) and the slope of the calibration curve (S) for nicotine standards (n=5). The LOD and LOQ were expressed according to the following equationsThe LOQ is lowest reliable concentration in the calibration curve that could be quantified by the analytical method.

*System suitability:*Data from six injections of 5µg/ml standard (at 100% assay concentration) was utilized for calculating system suitability parameters like retention time, USP tailing, number of theoretical plates and area calculated by waters Breeze 2 software.

## Results and Discussion

The standard curve of nicotine standards showed linearity in the range of 1-25µg/ml (Figure *2*) with a linear equation of y = 6675.7x – 1590.8 as obtained in linear regression analysis. The coefficient of correlation was r=0.9999855 and goodness of fit $R^2$=0.9999711.This was in well agreement with USP recommendation of $R^2$> 99%. The method passed the test for repeatability as determined by per cent RSD 1.01%of the area of the peaks of six replicates injection at 100% assay concentration. The method passed the test for intermediate precision as percent RSD obtained with intraday as well as for 3 different days were within the limits of 2% (Table 2).The accuracy of the analyte in mentioned in Table 3. The % recovery at 50%, 100% and 150% were found to be 98.3 to 101.5% with percent RSD well below 2%.Limit of Detection and Limit of Quantification (LOD and LOQ)- Limit of Detection (LOD) was calculated based on the equation as described in Section 2.3.3. LOD was found to be 0.200µg/ml and Limit of Quantitation (LOQ) was found to be 0.609µg/ml of nicotine.System suitability test was performed by running six injections of standard 5µg/ml at 100% assay level. % RSD of the response of six injections was found to be less than 2% and the theoretical plate count was above 2000 with USP tailing 1.12.The variation in retention time was less than 1%. Suitability test of the current method passed all USP criteria (Table 4).The concentration of the extract found by HPLC analysis and total amount of nicotine in 10ml of extract was calculated, starting amount of tobacco taken 5g. The amount of nicotine per tobacco leaf extract ranged from 1.34 to 2.22% (Table 5).

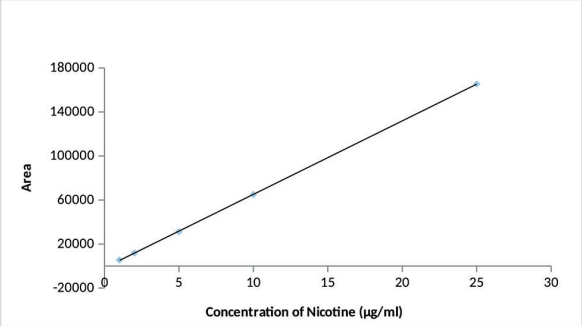


**Figure 2:** Standard curve of nicotine (r=0.9999855; $R^2$=0.9999711; y = 6675.7x – 1590.8)

**Table 2:** Precision results for the HPLC method at different assay concentrations

| Concentration (µg/ml) | Intra-day Variation | | Inter-day Variation | |
|---|---|---|---|---|
| | Mean (n=6) | (%RSD) | Mean (n=12) | (%RSD) |
| 1 | 1.02 | 1.98 | 1.02 | 1.89 |
| 2 | 2.03 | 1.07 | 2.02 | 1.08 |
| 5 | 5.06 | 1.04 | 5.06 | 0.98 |
| 10 | 10.08 | 0.92 | 10.11 | 1.03 |
| 25 | 25.27 | 0.93 | 25.20 | 0.93 |

**Table 3:** Accuracy results for the method at different assay concentrations

| % spiked or assay sample | Replicate | % recovery | % mean recovery | %RSD |
|---|---|---|---|---|
| 50% ( 2.5 µg/ml) | 1 | 99.1 | 98.7 (2.47) | 0.41 |
| | 2 | 98.7 | | |
| | 3 | 98.3 | | |
| 100% (5 µg/ml) | 1 | 101.2 | 99.7 (4.99) | 1.41 |
| | 2 | 99.5 | | |
| | 3 | 98.4 | | |
| 150% (7.5 µg/ml) | 1 | 99.8 | 101.2 (7.59) | 1.22 |
| | 2 | 101.5 | | |
| | 3 | 102.2 | | |

**Table 4:** System suitability test (no adjacent peaks were observed at the retention time of nicotine)

| Replicate injection | Retention time $t_R$ | Area | USP tailing | USP plate count |
|---|---|---|---|---|
| 1 | 3.405 | 31958 | 1.138 | 5607.119 |
| 2 | 3.412 | 32037 | 1.1416 | 5611.324 |
| 3 | 3.397 | 31694 | 1.1508 | 5656.237 |
| 4 | 3.426 | 31581 | 1.137 | 5597.425 |
| 5 | 3.414 | 31423 | 1.15 | 5486.142 |
| 6 | 3.436 | 31092 | 1.12 | 5404.258 |
| Mean | 3.415 | 31630.83 | 1.140 | 5560.418 |
| SD | 0.014 | 349.862 | 0.011 | 63.173 |
| % RSD | 0.413 | 1.106 | 0.986 | 1.136 |

**Table 5:** Percentage content of nicotine in dried and crushed tobacco leaves

| Tobacco leaves | Tobacco amount (g) | Concentra-tionof nic-otine in the extract (µg/ml) | Total amount of nicotine present in the extract (g) | Nicotine (%) |
|---|---|---|---|---|
| Saraisa, Samastipur | 5.00 | 7.5 | 0.075 | 1.5 |
| Saraisa, Muzaffarpur | 5.00 | 8.3 | 0.083 | 1.66 |
| Saraisa, Vaishali | 5.00 | 11.1 | 0.111 | 2.22 |
| Saraisa, Dar-bhanga | 5.00 | 6.7 | 0.067 | 1.34 |

Althoughwe could quantify the nicotine present in the tobacco leaves extract directly, the procedure can be used for other sources of tobacco, such as e-cigarette and chewing gum etc., after proper extraction. C18 column is more hydrophobic in comparison to C8 column; hence the retention time is different.[20] The elution time of nicotine under the proposed method is much less than other methods indicating suitability for rapid determination of nicotine. The theoretical plates for proposed method were adequate. Also, the peak shape of nicotine was reasonably good and principal peak was well separated from the mobile phase interference. Furthermore, the method uses a mobile phase without ion-pair reagent, so longer column equilibration time was avoided as mentioned in other methods. Under the proposed method, chromatographic conditions stabilized in less than 45 minutes.There are many methods developed on C18 column almost none is done on C8 column. The present study tried to contribute in the direction of developing one of such methods.

C8 has a lower degree of hydrophobicity, which may cause faster retention time for non-polar compounds. As the retention is short in C8 for the analyte, it can be run on a short length column. There is also less tailing in C8 column compared

to C18 column. Moreover, this procedure can bring down the cost of the analysis,as C8 is relatively cheaper to C18 column.

## Conclusion

A simple HPLC method using a C8 type column is developed for the analysis of nicotine in tobacco extracts and other sources. The method is specific for nicotine and validated with respect to various analytical parameters. The method is faster and simple for the analysis of nicotine present in different substance of abuse containing tobacco formulations as well as plant nicotine extracts.

**Conflict of interest:** None declared.

**Ethical approval:** Not applicable.

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**Authors' contribution:** All authors were equally involved in conception, research design, data collection, analysis, manuscript preparation, revision and finalization.

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