Yuzuncu Yil University
Journal of Agricultural Sciences
(Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi)
https://dergipark.org.tr/en/pub/yyutbd

ISSN: 1308-7576 e-ISSN: 1308-7584

Research Article

Spectrophotometric and Chromatographic Determination of Alkaloids and Nicotine Contents in Lebanese Tobacco Leaves

Sylvana ELCHAMIEH[1], Ali JABER*[2], Ghassan IBRAHIM[3], Edmond CHEBLE[4]

[1,2,3,4]Faculty of pharmacy, Laboratoire de Recherche et Développement des Médicaments et des Produits Naturels RDMPN, Lebanese University, Beirut, Lebanon.
[2]Department of Natural Sciences, School of Arts and Sciences, Lebanese American University, Beirut, Lebanon.
[3,4]Al-Kut University College, Department of Pharmacy, Wasit Governorate, Iraq.

[1]https://orcid.org/0009-0004-8143-6726, [2]https://orcid.org/0000-0002-9180-8384, [3]https://orcid.org/0000-0003-0231-3644
[4]https://orcid.org/0000-0001-9911-9025

*Corresponding author e-mail: ali.jaber.2@ul.edu.lb

Article Info

Received: 30.11.2023
Accepted: 14.05.2024
Online published: 15.06.2024
DOI: 10.29133/yyutbd.1398106

Keywords

HPLC,
Nicotiana tabacum,
Nicotine,
Tobacco,
Total alkaloids

Abstract: *Nicotiana Tabacum*; the annual herb; known as Tobacco from the Solanaceae family was known for its alkaloids and especially nicotine (NCT) content; smoking products, insecticides, anthelminthic activity and clinically proven therapeutics are examples of its uses. Herein two different methods were applied in an attempt to quantify the total alkaloids and NCT content in *Nicotiana tabacum* cultivated in Lebanon. Total alkaloids were investigated through the formation of a complex with bromocresol green under a simple spectrophotometric method. Whilst HPLC-DAD was the choice for the quantitation of NCT levels. The column was Lichrospher select B (5 µm, 250x4 mm), the temperature was set at 29 ºC and the wavelength at 260 nm. The mobile phase consisted of 2 M *O*-phosphoric acid and methanol (60:40, *v*/*v*) using isocratic elution at 1 mL/min. A linear relationship was proved under both instruments. The extraction yield of alkaloid totum ranges between 2.1 ± 0.25 and 6.8% ± 0.58 and alkaloids contents range from 12.14 ± 2.01 to 53.12 ± 4.54 mg of AE/g of extract for Ghandouriyeh and Danniye samples respectively. On the other side among the different areas which cultivated Tobacco in Lebanon, Danniye was found to have the highest NCT concentration of dry weight (2.64%) while Al-Hissa possesses the lowest content (0.75%). Even if the results were generally similar to other countries, the study showed a difference in values from one region to another.

To Cite: Elchamieh, S, Jaber, A, Ibrahim, G, Cheble, E, 2024. Spectrophotometric and Chromatographic Determination of Alkaloids and Nicotine Contents in Lebanese Tobacco Leaves. *Yuzuncu Yil University Journal of Agricultural Sciences*, 34(2): 335-345.
DOI: https://doi.org/10.29133/yyutbd.1398106

1. Introduction

For years, investigating plants ranked the highest top level among researchers around the world. Secondary metabolites were one of many topics that researchers focused on due to their various uses in agricultures, flavorings, and medicinal purposes such as usefulness for monitoring compliance in smoking cessation programs (Lerman et al., 2015; Goettel et al., 2017).

Nicotiana Tabacum; the annual herb; known as Tobacco belonging to the Solanaceae family was first distributed in tropical America and described as the “Cinderella of plant biotechnology” which

evolved into an exemplary system for both tissue culture and genetic engineering (Ganapathi et al., 2004; Bhatia et al., 2015). Therefore, its cultivation is sought after in many countries around the world.

Among the two thousand five hundred secondary metabolites (Habib et al., 2023) present in *N. tabacum*, alkaloids are the predominant and quintessential responsible for its potential biological activities. These alkaloids occur predominantly in leaves with a total alkaloid concentration of 0.2 and 8% where the potent compound; Nicotine (NCT) occupies the primary alkaloids and identifies about 90-95% of the total alkaloids (Lewis et al., 2010; Shoji et al., 2010; Sun et al., 2018). *Nicotiana*'s roots and angiosperms also contain alkaloids but with less abundant concentrations.

Note that the usability of *N. Tabacum* in tobacco products is due mainly to NCT. The latter substance interacts with nicotinic-cholinergic receptors located in the autonomic ganglia, the adrenal medulla, and within the brain. (Tiwari et al., 2020). Other secondary metabolites are the less potent pyridine alkaloids known as nornicotine and anatabine which are the most considerable minor pyridine at approximately about 4 to 5 %. Besides, myosmine (0.1%), anabasine (0.5%), cotinine, 2,3'-bipyridyl, nicotine-1'-N-oxide, N-formylnornicotine, nornicotyrine, nicotyrine, and over 20 pyridyl-type alkaloids are with minimal concentration (Yang et al., 2002; Zhang et al., 2007; Murray, 2014).

Since NCT is classified with high concentration, total alkaloids analysis is required for the quality and usability qualitatively and quantitatively. Hence, both chromatographic and non-chromatographic methods are extensively employed due to the characteristics of pyridine alkaloids, along with the abundance of literature facilitating the identification of numerous alkaloids in the plant (Li et al., 2019; Perfetti et al., 2022).

Recently, the recommendations to reduce the NCT level in cigarettes make the low NCT content trait an interest to tobacco stakeholders. Therefore, it motivated breeders to evaluate different genetic variations to investigate their effect on reducing NCT levels (Lewis, 2019; Burner et al., 2022).

Tobacco plays a crucial role in Lebanon's economy, with tobacco leaf production totaling approximately eight thousand tons in 2015. Around 25000 families derive economic benefits from the cultivation and production of tobacco and its associated products, highlighting its crucial role in both the Lebanese economy and society (Jaber et al., 2020).

However, to date, the NCT content of *N. tabacum* cultivated in Lebanon has not yet been studied. Hence, the objective of this study was to measure the overall alkaloid and NCT content using spectrophotometric methods and HPLC-DAD, respectively. Extracts of *N. tabacum* from four different Lebanese regions were subjected to this study. The type of tobacco under examination was the authentic Saada Six. This designation originates from the Saadiyat Laboratory of the Regie. Developed through a combination of Bulgarian and Azmirly tobacco, cultivation of this variety commenced in 1973 (Jaber et al., 2020 and 2022).

2. Material and Methods

2.1. Chemicals and reagents

All chemicals were of analytical or HPLC grade. The water utilized in all processes was sourced from a system of water purification (TKA MICROMED, Germany), ensuring its ultrapure quality. Methanol was procured from Sigma Aldrich (USA); 85% orthophosphoric acid was obtained from Fisher Scientific Company (USA); Chloroform, aluminum chloride hexahydrate, toluene, sodium hydroxide, Folin-ciocalteu reagent, ascorbic acid, and ammonia solution were purchased from BDH (England). Ethyl acetate, gallic acid, rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and sodium carbonate anhydrous were purchased from Sigma Aldrich (USA). The samples were measured using both an analytical and a digital balance (Melter Toledo). The dried leaves were ground using a POLYMIX (PX-MFC 90 D) grind mill. The absorbance of the solutions was assessed using a VWR UV-6300PC double-beam spectrophotometer, and a HEIDOLPH rotavapor apparatus was used to concentrate the different extracts.

2.2 HPLC system

All measurements were conducted using an HP 1100 Series LC system (Hewlett Packard, Palo Alto, CA, USA) fitted, in the order, with a quaternary pump, degasser compartment, thermostatted column, and diode-array detector (DAD). The whole HPLC system was managed by the HP

Chemstation chromatography software. The stationary phase utilized was an Agilent Lichrospher select B (250 x 4 mm, 5 µm) column produced by Agilent (Germany). The pH adjustment required the use of the CG 820 (SCHOTT GERATE, made in West Germany) pH meter.

2.3. Plant material

The *N. tabacum* L. samples were collected from four different Lebanese regions on June 2018, more specifically from Hisah (34°35'47"N, 36°3'17"E, 33 m MSL), Yohmor El-Chkif (33°18′45″ N 35°31′3″ E, 530 m MSL), Ghandouriyeh (33°16′20″N 35°25′52″E, 530 m MSL), and Sir Al-Danniye (34°23'09" N 36°01'47" E, 898 m MSL). A voucher specimen (No. 1805, 1806, 1807, and 1808) has been deposited at the Pharmacognosy Department, Faculty of Pharmacy of the Lebanese University.

The specimens were conveyed to the laboratory and maintained at ambient temperature until processing. The harvested plant materials underwent an initial natural drying phase (shaded and at room temperature) for four weeks. Subsequently, the leaves were separated from the dried plant material. The leaves were then finely ground using a manual grinder. Lastly, the ground materials were stored in a tightly sealed container for future utilization.

2.4. Plant extraction

2.4.1. Ultrasound-assisted extraction (UAE)

Briefly, 150 mL of sulphuric acid (0.05 M) was added to 10 g of the powdered leaf samples. An ultrasonic cleaner bath USC100T (VWR, Malaysia) was used for the UAE. Ultrasonic-assisted extraction was conducted at room temperature for a duration of 30 minutes, employing a sonication power of 30 W and a frequency of 45 kHz. The samples were then filtrated and the aqueous solution was basified to pH 9-10 with a few drops of NH_4OH (25%, m/m). The obtained sample was extracted with chloroform (3 x 25 mL). After vigorous shaking the organic phases were collected and dried with Na_2SO_4 and concentrated to dryness under reduced pressure to obtain the alkaloid totum (Jaber, 2017).

2.4.2. Extraction by maceration (ME)

Extraction by maceration was carried out with 10 g of powdered leaf samples in 150 mL of sulfuric acid (0.05 M) at room temperature for 24 hours. After maceration, the aqueous extracts were treated as described in Section 2.4.1.

2.4.3. Extraction yield

The extraction efficiency was determined using the Equal 1.

$$\text{Yield of leaves extract \%} = \frac{W_2}{W_1} \text{ x } 100 \quad (1)$$

Where W_1 represents the dry weight of the utilized material and W_2 is the weight of the alkaloid totum.

2.5. Total alkaloid content (TAC)

TAC estimation in the *N. tabacum* extract was carried out through the Bromocresol Green (BCG) spectrophotometry method, utilizing atropine as a standard (John et al., 2014). The procedure followed is as follows, in different separatory funnels, accurately measured aliquots (0.2, 0.4, 0.6, 0.8, 1 and 1.2 mL) of aqueous atropine standard solution (0.1 mg/mL) were added to 5 mL of phosphate buffer (Na_2HPO_4) (pH = 4.7, adjusted with citric acid 0.2 M) along with 5 mL of BCG solution (prepared by dissolving 69.8 mg of BCG in 3 mL of 2 M NaOH, and 5 mL of distilled water. The solution was gently heated, and then the volume was adjusted to 1000 mL with distilled water) shaken vigorously with 5 mL of chloroform. Following thorough mixing, the mixture was allowed to stand for 3 minutes. The extracts were then gathered in 10 mL volumetric flasks and subsequently diluted to reach the mark with chloroform. Likewise, solutions of *N. tabacum* extracts were determined using the same procedure. The absorbance of the complex in chloroform was measured at 417 nm against a reagent blank prepared as above without atropine. The calibration curve was plotted for the calculation of the content of total

alkaloids. The whole experiment was conducted in three replicates. The total alkaloid content was expressed as mg of AE/g of extract.

2.6. Quantitative analysis of nicotine by HPLC-DAD

The procedure was carried out at 29 °C using a Lichrospher select B (250 x 4 mm, 5 µm). The mobile phase consisted of 0.2 M orthophosphoric acid and methanol (60:40, v/v) in isocratic mode. The mobile phase was filtered before injection through a Whatman filter paper 0.45 µm (Whatman, Maidstone, UK), and delivered at a flow rate of 1 mL/min, and the injection volume was 20 µL. Quantification was done using signals detected at 260 nm.

The characteristics and procedures of the HPLC method were validated following the guidelines set forth by the International Conference of Harmonization (ICH). The validation encompassed various parameters, including specificity, linearity, recovery, precision, limit of detection (LOD), and limit of quantification (LOQ).

A stock solution of nicotine was prepared in methanol at 2 mg mL^{-1}. First of all, 20 µL of standard, sample, spiked sample, and mobile phase (blank) were injected separately in order to evaluate the specificity. The linearity response was established by injection (n = 3) of a series (six levels) of NCT work solutions ranging from 0.64 to 1200 mg mL^{-1}. The standard calibration curves were generated by plotting concentrations against the peak area of the analyte. The repeatability of the method was checked by analyzing six replicate samples of NCT and calculating the percent relative standard deviation (% RSD). The intermediate precision (ruggedness) was checked by repeating the linearity test for 3 consecutive days and calculating the RSD between 3 days for area, slope, and intercept. Additionally, the LOD and the LOQ for this method were determined based on the standard deviation (σ) of y-intercepts from regression analysis and the slope (m) of the calibration curve, as Equations 2 and 3, respectively.

$$\mathrm{LOD} = 3.3\frac{\sigma}{\mathrm{m}} \tag{2}$$

$$\mathrm{LOQ} = 10\frac{\sigma}{\mathrm{m}} \tag{3}$$

2.7. Statistical analyses

The experiments were performed in triplicates. The analysis of variance (ANOVA) followed by the Tukey test (p-value < 0.05) using the SPSS 21.0 software package (Chicago, IL, USA) was used to determine statistically significant differences between means. The coefficients of determination (r^2) were determined using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

3. Results and Discussions

Sample preparation is a vital part of any investigation of a biological analysis, regardless it is qualitative or quantitative. The importance of this step lies in the necessity to bring out the whole chemicals from the sample or only target substances in the selective extraction cases. In addition, the later application of chromatographic techniques needs a preliminary preparation giving a sample free of unwanted constituents that may hinder the analysis and ruin the column (Petruczynik, 2012).

Indeed, in this work, the first step was to establish the optimal selective extraction method of total alkaloid content from *N. tabacum*. The extraction procedure from an aqueous acidic medium was based on the basic properties of alkaloids. Then, the alkalinization (pH>7) by the addition of ammonia solution will stimulate the release of free alkaloids.

Leaf of *N. Tabacum* was collected from two fields from the north governorate (Danniye, Al-Hissa) and two from the south governorate (Ghandouriyeh, Yohmor El-Chkif). In order to choose the more appropriate extraction method sample undergoes two different extraction techniques, namely ultrasound-assisted extraction and maceration. The obtained extraction yields were 1.95 ± 0.15 and 1.19 ± 0.35 % for UAE and maceration respectively. Therefore, the % yield obtained by the UAE method nearly doubled that obtained by maceration, and UAE therefore was adopted for the subsequent works.

The extraction yield of alkaloid totum (Table 1) was found to range between 2.1±0.25 and 6.8 % ± 0.58 for Ghandouriyeh and Danniye samples respectively. The variance in extraction yield among the four examined samples is statistically significant ($p<0.05$).

To quantify total alkaloid content, a spectrophotometric method was applied. Atropine was used as standard, thus a range of different concentrations (2 and 14 mg mL^{-1}) was used for constructing the calibration curve. Upon mixing the different standard solutions with BCG, a yellow color complex was formed with maximum absorption at 417 nm. The results, as illustrated in Figure 1, indicate that the absorbance of the complex follows Beer's law within the concentration range of atropine (R^2=0.989).

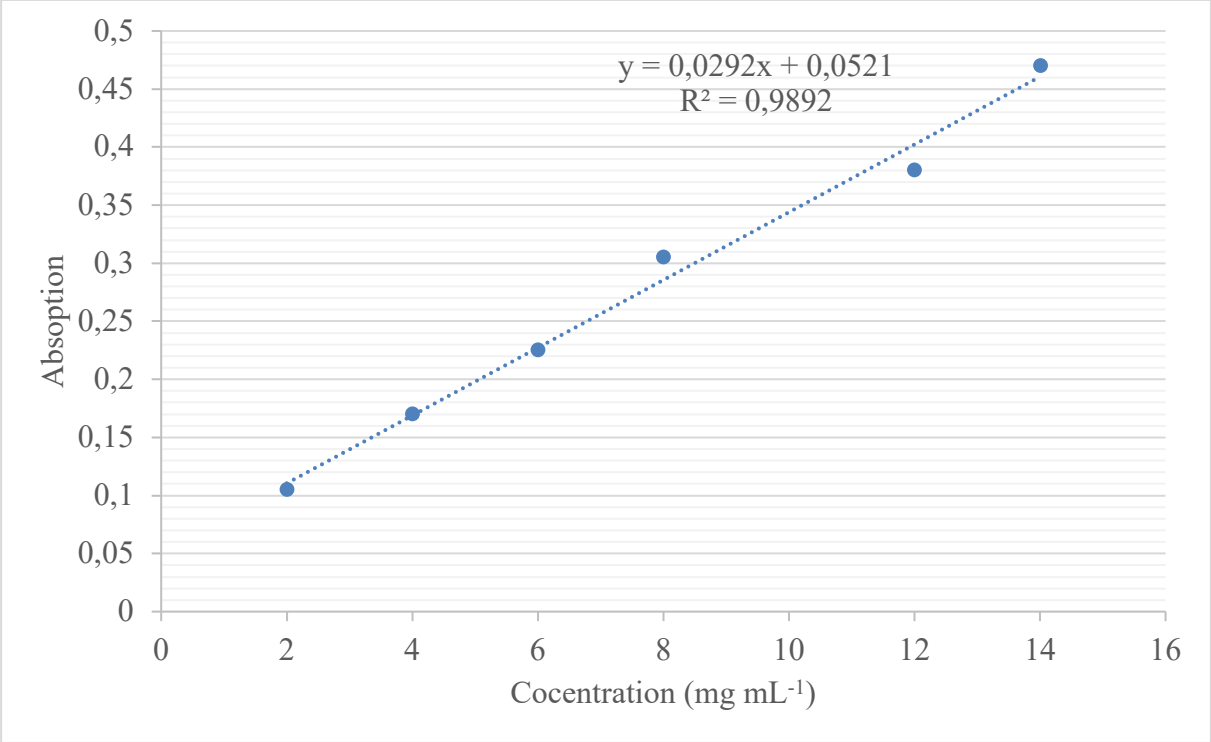

Figure 1. Calibration curve of standard atropine solutions.

At pH<7 most compounds containing tertiary amine groups or quaternary ammonium salts, form with BCG yellow complexes extractable by chloroform (Khoi, 1983). This spectrophotometric procedure is simple and sensitive, and BCG can react with the alkaloids having nitrogen inside their structure (Fazel et al., 2010; Ajanal et al., 2012; Liu and Liu, 2015; Salamah and Ningsih, 2017). After the protonation in an acidic medium, the protonated nitrogen (quaternary ammonium cations) is the target for the reaction with the BCG compound.

As above mentioned, tobacco is one of the richest plants in pyridine alkaloids (Figure 2). Among the tobacco-containing alkaloids, NCT is the major compound, besides many other minor alkaloids (Clemens et al., 2009; Jacob et al., 2022). Thus in their reaction to BCG, the NCT will be the most influential.

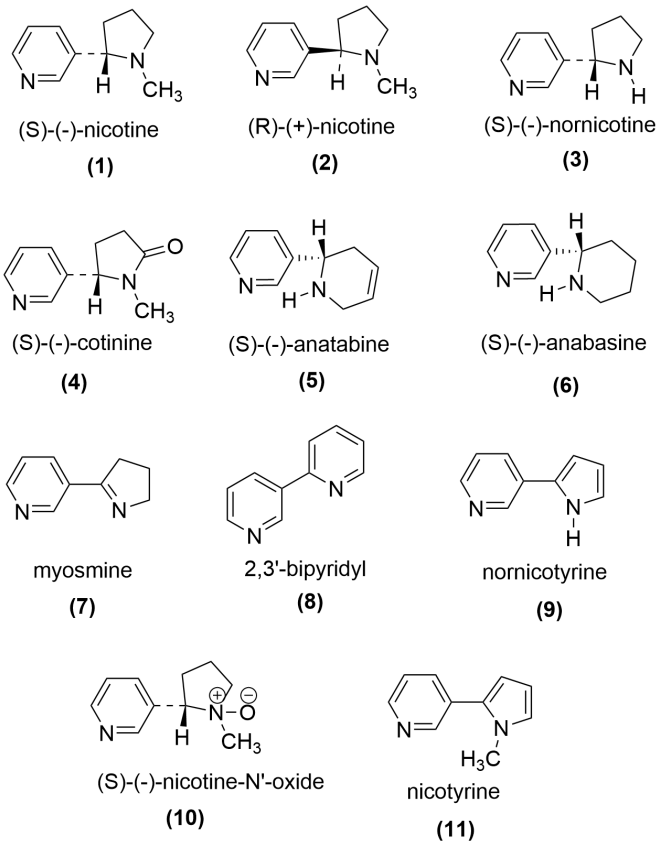

Figure 2. Nicotine and some related tobacco alkaloids chemical structures.

The nitrogen in the pyrrolidine cycle, holding a pka of 8, is the stronger basic site in the nicotine structure, and the pH of the used buffer solution is equal to 4.7 (pka>pH). Thereby, the nitrogen involved in the pyrrolidine will be protonated forming the ion-pair complex with BCG (Figure 3).

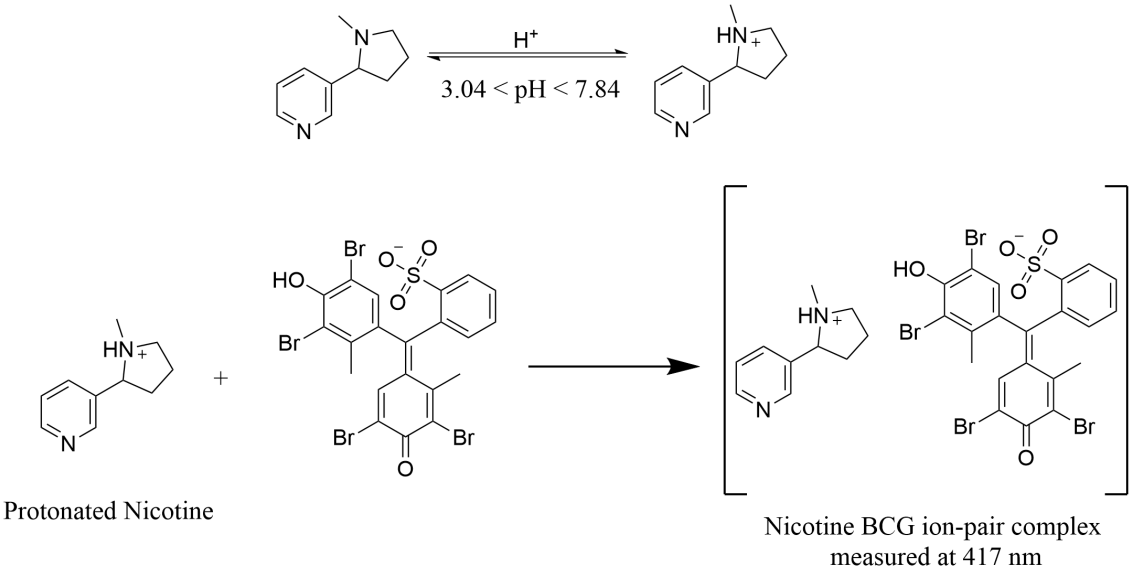

Figure 3. Structure of Nicotine and formed ion pairs.

The resulting reaction forms a yellow color complex, which indicates the mono-anionic form of BCG. The latter complex is the result of the electrostatic attraction between the mono-protonated alkaloids and the mono-anionic dye. The data obtained are presented in Table 1.

Table 1. Extractions yields (%) of alkaloids totum expressed; Alkaloid contents in the plant extracts expressed in terms of atropine equivalent (mg of AE/g of extract)

Samples	Extractions yields of alkaloids totum	mg of AE/g of extract
Danniye	6.80 ± 0.59^{a}	12.14 ± 2.01^{d}
Al-Hissa	4.13 ± 0.38^{b}	51.46 ± 5.44^{e}
Ghandouriyeh	2.10 ± 0.24^{c}	53.12 ± 4.54^{e}
Yohmor El-Chkif	4.93 ± 0.45^{b}	34.14 ± 3.98^{f}

Each value is the average of three analyses ± standard deviation, in each column values marked by the same letters (a-f) are not significantly different ($P < 0.05$), where AE is atropine equivalent.

The alkaloid contents were examined, a sample from Ghandouriyeh showed a higher content of alkaloids atropine equivalents, although it was the lowest extractive yield value. This observation applied to the three other samples, and the order of extractive values was reversed in the total alkaloid contents.

NCT is produced in the root of the tobacco plant before it moves to the leaves and the lateral parts (Baldwin et al., 1993). The NCT content depends on many factors such as the type of tobacco, nitrogen availability, temperature, light, and moisture (Yasinok et al., 2009). There's a relationship between the amount of nitrogen and the concentration in tobacco. In early development, N uptake is believed to be active so the concentration of NCT reaches its maximum at the late growth stage, especially after removing terminal buds in the middle and upper leaves (Wang et al., 2008). Therefore, the different bioclimatic levels in Lebanon, even in short distances, will probably lead to differences in the alkaloids and NCT contents.

Later, a simple and rapid HPLC–DAD method was developed for the quantification of NCT. The method validation was achieved according to the International Conference on Harmonization (ICH) (Singh, 2015). For specificity evaluation, as mentioned before, separately 20 µL from the standard, sample, spiked sample, and a mobile phase (blank) were injected into the chromatographic system. The chromatogram results showed that there are no interfering peaks at the retention time of NCT, which confirmed the method's specificity (Figure 4). Then a serial of different standard solutions (0.64 to 1200 mg mL^{-1}) was used for the linearity evaluation. A graph was constructed by plotting the peak area of NCT from each standard solution against the corresponding concentration. The regression analysis produced the linear equation $y= 30.177x+38.105$ with a correlation coefficient (R^2) of 0.9967, demonstrating a high linear relationship between the analyte's concentration and the peak's area. As a result, it has been found that the current analytical procedure is linear in the given range.

The LOD and LOQ values were calculated from the standard deviation of the y-intercepts of regression lines, and the calibration curve slope (Zahreddine et al., 2021; Beldar et al., 2022). The obtained LOD and LOQ were 132.207 mg mL^{-1} and 400.629 mg mL^{-1} respectively.

The precisions were tested by the evaluation of the repeatability of the adopted method. The intra-assay and inter-assay (expressed as % RSD) variations of retention times and concentrations were checked. Repeated measurements of standard solutions, 13 replicates (intraday) over three days (interday), were done. The intraday % RSD were 0.166 and 0.33% for retention times and concentrations respectively, while the interday % RSD were 0.27 and 0.71%.

The accuracy of the assay method was evaluated through recovery studies conducted at three concentration levels over three days. The percent recovery was determined by comparing the measured concentrations of NCT with the added concentrations. The percent recovery fell within the range of 91% to 112%, and the average percent relative standard deviation (% RSD) was 1.7%. These values are within the accepted limits, typically ranging from 80% to 120%, indicating the method's accuracy (Shikanga et al., 2012), and not more than 5% (Koetz et al., 2017), respectively. These values imply the applicability of the method for NCT analysis.

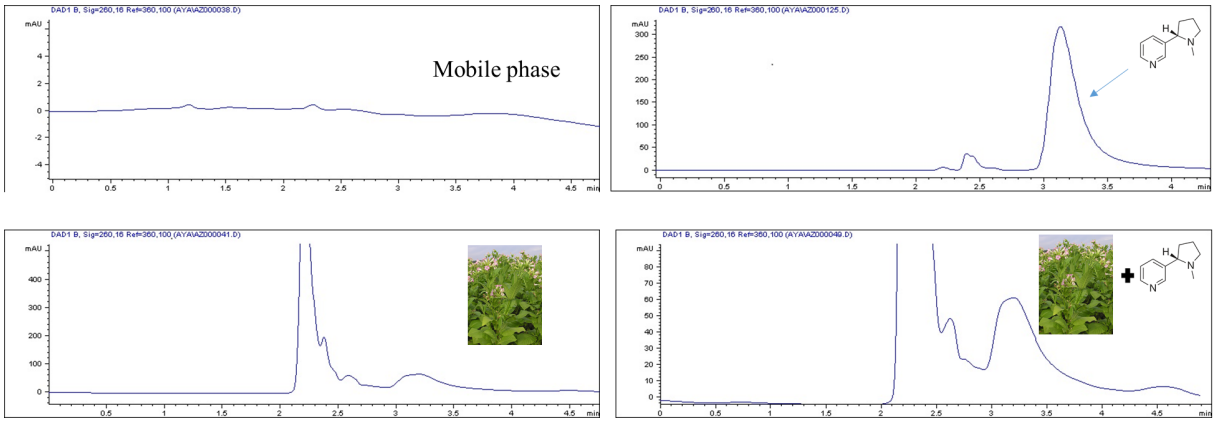

Figure 4. Chromatograms of the blank, the standard NCT, the N. tabacum extract, and spiked extract.

Table 2. Quantitation of nicotine in the different regions in Lebanon

Samples	% Nicotine in dry weight
Al-Hissa	0.75 ± 0.29^{a}
Yohmor El-Chkif	$1.76 \pm 0.42^{a,b}$
Danniye	2.64 ± 0.61^{b}
Ghandouriyeh	1.03 ± 0.22^{a}

Each value is the average of three analyses ± standard deviation, values marked by the same letters are not significantly different ($P < 0.05$).

Due to the variability in NCT content among tobacco plants, manufacturers have the option to substitute commonly used, nicotine-rich varieties such as *Nicotiana rustica* with lower-nicotine alternatives (Tengs et al., 2005). To offer consumers tobacco products with decreased health risks, companies employ genetic engineering and plant breeding techniques to cultivate tobacco with significantly lower NCT levels, which is then utilized in the manufacturing of cigarettes (Food and Drug Administration, 2018).

The findings herein show that NCT levels in the tobacco from north Lebanon, vary between 0.75% and 2.64%, while in the south 1.03 - 1.76%. In general, the level of NCT in Lebanese tobacco is comparable with that in the rest of the world.

Tobacco plants contain 2– 4% alkaloids of their total dry weight (Saitoh et al., 1985), while the NCT content varies from 0.3 to 3% in dry weight, in some cases, 5 to 7% are also reported (Tassew and Chandravanshi, 2015; Tayoub et al., 2016). Gonzalez-Coloma (Gonzalez-Coloma et al., 2010) report about 2-6% of NCT content of the leaves of *N. rustica* and *N. tabacum*.

In contrast, 6.7% of NCT content was found in Virginia variety, 4.9% in Burlip, 4.84% in Katrina, 4.67% in Shk al-bent, 4% in Zegrin, 3.3% in Basma of NCT in dry-weight leaves (Tayoub et al., 2016).

On the other hand, in Ethiopian tobacco leaves, levels of nicotine vary between the four varieties of Ethiopian tobacco including Burley tobacco (0.650%), Oriental tobacco leaves (≤0.05%), Virginia tobacco (3.26%), and native tobacco 'Gaya' (1.10%). Also, Tepecik and Ongun (2020) reported that NCT contents were greater in the second harvest (between 0.28-0.86%) than in the first harvest (0.19-0.74%) (Tepecik and Ongun, 2020). Moreover, the levels of NCT of the same species vary in different areas of cultivation (Tassew and Chandravanshi, 2015). A study carried out in China (Wang et al., 2008) reported that the NCT level depended on leaf position and different treatments, with levels ranging from 0.78 to 3.26%. The Bulgarian oriental tobacco was found to be 2.3% in the leaves (Popova et al., 2018).

Conclusion

To quantify the levels of total alkaloids and NCT content in dry weight Lebanese tobacco, extraction of total alkaloids was conducted. Then, spectrophotometric and chromatographic methods were used for quantification. The extraction yield of alkaloid totum ranges between 2.1 ± 0.25 and 6.8% ± 0.58 and alkaloid contents range from 12.14 ± 2.01 to 53.12 ± 4.54 mg of AE/g of extract for Ghandouriyeh and Danniye samples respectively. On the other side among the different areas which

cultivated Tobacco in Lebanon, Danniye was found to have the highest NCT concentration of dry weight (2.64%) while Al-Hissa possesses the lowest content (0.75%). This study of tobacco breeding in Lebanon shows that NCT levels are comparable with other countries.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The Author(s) declare(s) that there are no conflicts of interest.

Funding Statement

The authors are grateful to the Lebanese University; Faculty of Pharmacy department- Lebanon- for providing all the chemicals necessary to carry out this project.

References

Ajanal, M., Gundkalle, M., & Nayak, S. (2012). Estimation of total alkaloid in Chitrakadivati by UV-Spectrophotometer. *Ancient Science of Life*, *31*, 198-201. https://doi.org/10.4103/0257-7941.107361

Baldwin, I. T., Oesch, R. C., Merhige, P. M., & Hayes, K. (1993). Damage-induced root nitrogen metabolism in *Nicotiana sylvestris*: Testing C/N predictions for alkaloid production. *Journal of Chemical Ecology*, *19*, 3029–3043. https://doi.org/10.1007/BF00980600

Beldar, V., Laddha, K. S., Dudure, R. H., Fayed, M. A. A., & Jadhao, M. (2022). An approach for developing a simple and quick method for separation of asiatic acid and asiaticoside rich fraction From *Centella Asiatica* and simultaneous determination by reversed-phase high-performance liquid chromatography. *Yuzuncu Yil University Journal of Agricultural Sciences*, 33, 18-28. https://doi.org/10.29133/yyutbd.1175779

Bhatia, S., Sharma, K., Dahiya, R., & Bera, T. (2015). *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*, Oxford, UK: Elsevier Inc.

Burner, N., McCauley, A., Pramod, S., Frederick, J., Steede, T., Kernodle, S. P., & Lewis, R. S. (2022). Analyses of diverse low alkaloid tobacco germplasm identify naturally occurring nucleotide variability contributing to reduced leaf nicotine accumulation. *Molecular Breeding : New Strategies in Plant Improvement*, *42*, 1-15. https://doi.org/10.1007/s11032-021-01274-5

Clemens, K. J., Caillé, S., Stinus, L., & Cador, M. (2009). The addition of five minor tobacco alkaloids increases nicotine-induced hyperactivity, sensitization, and intravenous self-administration in rats. *International Journal of Neuropsychopharmacology*, *12*, 1355–1366. https://doi.org/10.1017/S1461145709000273

Fazel, S., Hamidreza, M., Rouhollah, G., & Verdian-Rizi, M. (2010). Spectrophotometric determination of total alkaloids in some Iranian medicinal plants. *Journal of Applied Horticulture*, *32,* 17-20. https://doi.org/10.37855/jah.2010.v12i01.15

Food and Drug Administration. (2018). *Tobacco Product Standard for Nicotine Level of Combusted Cigarettes* Access date: 03.08.2022. https://www.govinfo.gov/content/pkg/FR-2018-03-16/pdf/2018-05345.pdf

Ganapathi, T. R., Suprasanna, P., Rao, P. S., & Bapat, V. A. (2004). Tobacco *(Nicotiana tabacum* L.)-A model system for tissue culture interventions and genetic engineering. *Indian Journal of Biotechnology, 3,* 171-184.

Goettel, M., Niessner, R., Mueller, D., Scherer, M., Scherer, G., & Pluym, N. (2017). Metabolomic Fingerprinting in Various Body Fluids of a Diet-Controlled Clinical Smoking Cessation Study Using a Validated GC-TOF-MS Metabolomics Platform. *Journal of Proteome Research*, *16*, 3491–3503. https://doi.org/10.1021/acs.jproteome.7b00128

Gonzalez-Coloma, A., Reina, M., Diaz, C. E., & Fraga, B. M. (2010). Natural product-based biopesticides for insect control. In *Comprehensive Natural Products II: Chemistry and Biology*. https://doi.org/10.1016/b978-008045382-8.00074-5

Habib, M. A., Islam, M. M., Islam, M. M., Hasan, M. M., & Baek, K.-H. (2023). Current Status and De Novo Synthesis of Anti-Tumor Alkaloids in Nicotiana. *Metabolites*, *13*, 623. https://doi.org/10.3390/metabo13050623

Jaber, A. (2017). *Matrices MALDI bithiophéniques spécifiques aux alcaloïdes: Étude des mécanismes fondamentaux et applications*. Université d'Angers, France.

Jaber, A., Cheble, E., Abdel Sater, F., Soukariyeh, R., & Khalil, A. (2020). Biological activities of total oligomeric flavonoids enriched extracts of *Nicotiana tabacum* from eight lebanese regions. *Int. J. Pharm. Sci. Rev. Res., 61*, 70–77.

Jaber, A., Hajjar, S., El Riachi, M., Abdel Sater, F., & Cheble, E. (2022). GC-MS Analysis of essential oil and anticancer activities of extracts from discarded leaves of *Nicotiana Tabacum* Linn. *46*, 291–307. https://doi.org/10.33483/jfpau.991289

Jacob, P., Chan, L., Cheung, P., Bello, K., Yu, L., St Helen, G., & Benowitz, N. L. (2022). Minor Tobacco Alkaloids as Biomarkers to Distinguish Combusted Tobacco Use From Electronic Nicotine Delivery Systems Use. Two New Analytical Methods. *Front. Chem.*, *10*. https://doi.org/10.3389/fchem.2022.749089

John, B., C T, S., George, S., & Reddy, V. R. K. (2014). Spectrophotometric estimation of total alkaloids in selected justicia species. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*, 647–648.

Khoi, A. A. M. E. (1983). Spectrophotometric promethazine hydrochloride determination using bromcresol green. *Journal of Pharmaceutical Sciences*, *72*, 704–705. https://doi.org/10.1002/jps.2600720629

Koetz, M., Santos, T. G., Rayane, M., & Henriques, A. T. (2017). Quantification of atropine in leaves of atropa belladonna: Development and validation of method by high-perfomance liquid chromatography (HPLC). *Drug Analytical Research*, *1*, 44-49. https://doi.org/10.22456/2527-2616.74150

Lerman, C., Schnoll, R. A., Hawk, L. W., Cinciripini, P., George, T. P., Wileyto, E. P., Swan, G. E., Benowitz, N. l, Heitjan, D. F., & Tyndale, R. F. (2015). Use of the nicotine metabolite ratio as a genetically informed biomarker of response to nicotine patch or varenicline for smoking cessation: A randomised, double-blind placebo-controlled trial. *The Lancet Respiratory Medicine*, *3*, 131–138. https://doi.org/10.1016/S2213-2600(14)70294-2

Lewis, R. S. (2019). Potential Mandated Lowering of Nicotine Levels in Cigarettes: A Plant Perspective. *Nicotine & Tobacco Research*, *21*, 991–995. https://doi.org/10.1093/ntr/nty022

Lewis, R. S., Bowen, S. W., Keogh, M. R., & Dewey, R. E. (2010). Three nicotine demethylase genes mediate nornicotine biosynthesis in Nicotiana tabacum L.: Functional characterization of the CYP82E10 gene. *Phytochemistry*, *71*, 1988–1998. https://doi.org/10.1016/j.phytochem.2010.09.011

Li, X., Liu, F., Wang, H., He, F., Yang, R., & Zhao, M. (2019). Gas Chromatography-Mass Spectrometry Method for Simultaneous Detection of Nine Alkaloids in Tobacco and Tobacco Products by QuEChERS Sample Preparation. *Analytical Sciences: The International Journal of the Japan Society for Analytical Chemistry*, *35*, 849–854. https://doi.org/10.2116/analsci.19P063

Liu, Y., & Liu, C. (2015). Determination of Total Alkaloids in Different Parts of Actinidia arguta by Spectrophotography. International Conference on Materials, Environmental and Biological Engineering (MEBE 2015) https://doi.org/10.2991/mebe-15.2015.31

Murray, J. (2014). *Nicotine and what Else?: HPLC Elution Optimization for the Analysis of Alkaloids Found in Electronic Cigarettes*. University of Tennessee at Chattanooga, United States.

Perfetti, T. A., Dube, M. F., & Coleman, W. M. (2022). Qualitative and Quantitative Analysis of Nicotine, Nicotine Derivatives, and Nicotine-Related Alkaloid Optical Isomers: A Review. *Contributions to Tobacco & Nicotine Research*, *31*, 175–185. https://doi.org/10.2478/cttr-2022-0018

Petruczynik, A. (2012). Analysis of alkaloids from different chemical groups by different liquid chromatography methods. *Open Chemistry*, *10*, 802–835. https://doi.org/10.2478/s11532-012-0037-y

Popova, V., Ivanova, T., Stoyanova, A., Georgiev, V., Hristeva, T., Nikolova, V., Docheva, M., Nikolov, N., & Damyanova, S. (2018). Phytochemicals in leaves and extracts of the variety "Plovdiv 7" of Bulgarian oriental tobacco (*Nicotiana tabacum* L.). *Trends in Phytochemical Research, 2*, 27–36.

Saitoh, F., Noma, M., & Kawashima, N. (1985). The alkaloid contents of sixty *Nicotiana* species. *Phytochemistry*, *24*, 477–480. https://doi.org/10.1016/S0031-9422(00)80751-7

Salamah, N., & Ningsih, D. S. (2017). Total alkaloid content in various fractions of *Tabernaemonata sphaerocarpa* Bl. (Jembirit) leaves. *IOP Conference Series: Materials Science and Engineering*. https://doi.org/10.1088/1757-899X/259/1/012017

Shikanga, E. A., Kamatou, G. P. P., Chen, W., Combrinck, S., & Viljoen, A. M. (2012). Validated RP-UHPLC PDA and GC–MS methods for the analysis of psychoactive alkaloids in Sceletium tortuosum. *South African Journal of Botany*, *82*, 99–107. https://doi.org/10.1016/j.sajb.2012.05.004

Shoji, T., Kajikawa, M., & Hashimoto, T. (2010). Clustered Transcription Factor Genes Regulate Nicotine Biosynthesis in Tobacco. *The Plant Cell*, *22*, 3390–3409. https://doi.org/10.1105/tpc.110.078543

Singh, J. (2015). International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. *Journal of Pharmacology & Pharmacotherapeutics*, *6*, 185–187. https://doi.org/10.4103/0976-500X.162004

Sun, B., Tian, Y.-X., Zhang, F., Chen, Q., Zhang, Y., Luo, Y., Wang, X.-R., Lin, F.-C., Yang, J., & Tang, H.-R. (2018). Variations of Alkaloid Accumulation and Gene Transcription in *Nicotiana tabacum*. *Biomolecules*, *8*, 114. https://doi.org/10.3390/biom8040114

Tassew, Z., & Chandravanshi, B. S. (2015). Levels of nicotine in Ethiopian tobacco leaves. *SpringerPlus*, *4*, 649. https://doi.org/10.1186/s40064-015-1448-y

Tayoub, G., Sulaiman, H., & Alorfi, M. (2016). Determination of nicotine levels in the leaves of some *Nicotiana tabacum* varieties cultivated in Syria. *Herba Polonica*, *61,* 23–30. https://doi.org/10.1515/hepo-2015-0028

Tengs, T. O., Ahmad, S., Savage, J. M., Moore, R., & Gage, E. (2005). The AMA proposal to mandate nicotine reduction in cigarettes: A simulation of the population health impacts. *Preventive Medicine*, *40*, 170–180. https://doi.org/10.1016/j.ypmed.2004.05.017

Tepecik, M., & Ongun, A.R. (2020). Determination of some quality parameters of oriental type tobacco based on harvest times. *Turkish Journal of Agricultural Research*, 7(2), 156-162. https://doi.org/10.19159/tutad.691727

Tiwari, R. K., Sharma, V., Pandey, R. K., & Shukla, S. S. (2020). *Nicotine Addiction: Neurobiology and Mechanism*. *23*, 1–7. https://doi.org/10.3831/KPI.2020.23.001

Wang, S. S., Shi, Q. M., Li, W. Q., Niu, J. F., Li, C. J., & Zhang, F. S. (2008). Nicotine concentration in leaves of flue-cured tobacco plants as affected by removal of the shoot apex and lateral buds. *Journal of Integrative Plant Biology, 50,* 958-964. https://doi.org/10.1111/j.1744-7909.2008.00684.x

Yang, S. S., Smetena, I., & Huang, C. B. (2002). Determination of tobacco alkaloids by gas chromatography with nitrogen-phosphorus detection. *Analytical and Bioanalytical Chemistry*, *373*, 839–843. https://doi.org/10.1007/s00216-002-1380-1

Yasinok, A. E., Sahin, F. I., Eyidogan, F., Kuru, M., & Haberal, M. (2009). Grafting tomato plant on tobacco plant and its effect on tomato plant yield and nicotine content. *Journal of the Science of Food and Agriculture*, *89*, 1122–1128. https://doi.org/10.1002/jsfa.3555

Zahreddine, Z., Jaber, A., Haidar, S. A., Hosri, C., Ibrahim, G., & Cheble, E. (2021). HPLC-DAD multi-residue method for determination of florfenicol, penicillin and tetracycline residues in raw cow milk. *Journal of Clinical and Laboratory Research*, *2*, 1–8.

Zhang, J., Ji, H., Sun, S., Mao, D., Liu, H., & Guo, Y. (2007). Selective Determination of Pyridine Alkaloids in Tobacco by PFTBA Ions/Analyte Molecule Reaction Ionization Ion Trap Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*, *18*, 1774–1782. https://doi.org/10.1016/j.jasms.2007.07.017