Optimizing Nicotine Extraction and Analysis Method from Tobacco Agrowaste Extract

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Abstract. Tobacco cultivation is prevalent in Indonesia and contributes significantly to the economy. However, it has negative impacts on social, health, and environmental conditions. The tobacco waste generated is classified as pre-harvest and post-harvest waste, which can be utilized to extract nicotine and recycle essential nutrients. Nicotine has various biological activities and potential health benefits. The extraction of nicotine from tobacco waste is a pressing issue to provide a valuable resource for various industries and reduce the environmental harm caused by burning tobacco waste. The study optimized the HPLC conditions for the detection of nicotine, including the mobile phase composition and flow rate, using a UV detector and a C18 column. The optimal eluent composition was Acetate Buffer: Methanol: Acetonitrile with a ratio of 30:50:20, and the optimal flow rate was 0.2 mL min^{-1}. Additionally, it was found that the nicotine content of tobacco stem samples was higher than that of tobacco dust samples. Overall, this study provides valuable information on the extraction and analysis of nicotine in tobacco samples using HPLC, which can have important implications on developing sustainable tobacco production practices to minimize the negative impacts of tobacco cultivation on social, health, and environmental conditions.

Keywords: *Nicotiana tabacum* L**,** reduce environmental harm, sustainable tobacco, ultrasonic extraction, utilization tobacco waste

1 Introduction

Tobacco (*Nicotiana tabacum* L.) is a plant that is widespread throughout Indonesia and is classified as a plantation crop. The global tobacco industry is one of the largest retail product categories in the world, encompassing various products such as cigarettes, chewing tobacco, and cigars. However, tobacco cultivation has been associated with some of negative impacts on social, health, and environmental conditions. Despite these negative impacts, the tobacco industry remains a significant contributor to the Indonesian economy. The industry provides employment opportunities for many people, particularly in rural areas where other job

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options may be limited. However, there has been increasing public concern about the negative impacts of tobacco cultivation, and efforts are being made to promote sustainable tobacco production practices that minimize these impacts [1, 2]. Tobacco waste varies so that it can be classified into two types, namely pre-harvest and post-harvest waste. Pre-harvest waste is green material waste such as leaves, stems, twigs from the harvest process. While post-harvest waste is processed waste such as tobacco dust and stems [3, 4]. Tobacco plants are known to contain a variety of chemical compounds. These include sterols, diterpenoids, sesquiterpenes, alkaloids (including nicotine), and phenolic compounds such as flavonoids, phenolic acids, and coumarins. These compounds have been found to have various biological activities and potential health benefits [5, 6, 3]. Until now, tobacco waste has only been handled by burning. Burning tobacco stems has a negative impact on the environment because there is still nicotine in tobacco stems, and has negative environmental impacts, including the release of greenhouse gases and air pollution [7]. Other utilization, namely as composting, is also not optimal due to the characteristics of harder tobacco stems and the need for further processing which requires time and further research [8, 9]. Recent study also tried utilizing tobacco stems into bio briquettes, pellets, and liquid smoke [10].

Tobacco leaf waste is known to still contain high nicotine. Nicotine is also can be secreted from the tobacco plant roots. Some amount of nicotine is released to the soil environment through root exudation. Nicotine released in rhizosphere improves nitrogen, calcium, iron, and zinc uptake; and thus, promotes seedlings emergence and vigour, chlorophyll contents and growth of maize as a subsequent crop. Previous research also stated that the nitrogen content of nicotine is detected in the roots and will be absorbed by the soil and then distributed to all parts of the plant, except seeds [11]. Nicotine was also found in the stem although not as high as that found in the leaves [7]. Other waste such as tobacco dust, Tobacco dust an agro-industrial waste can be applied to the soil to recycle essential nutrients such as nitrogen (N), phosphorous (P) and potassium (K) back into the soil that plant has taken up from the soil [12].

There are several methods for extracting nicotine from tobacco or tobacco waste. One method is using an aqueous two-phase system and solvent reverse extraction [13]. Another study been conducted to extract nicotine from tobacco leaves using maceration and acid-base extraction methods [14]. The recent efficient method to extract nicotine from tobacco waste is ultrasonic assisted extraction. The basic principle of ultrasonic extraction is to increase mass transfer due to ultrasonic acoustic waves that will cause cavitation effects. The cavitation effect is a process of micro-bubble formation that breaks the cell wall, and the solvent diffuses into the cell so that the alkaloid compounds inside the cell are extracted. The advantages of ultrasonic extraction are that it is fast, energy-efficient and uses a smaller amount of solvent [15, 3]. Finding ways to utilize tobacco waste such as stem or dust to extract nicotine is a pressing issue due to the negative impacts of tobacco cultivation on social, health, and environmental conditions. The extraction of nicotine from tobacco waste has the potential to provide a valuable resource for various industries and reduce the environmental harm caused by burning tobacco waste.

2 Materials and method

2.1 Sample preparation

Tobacco dust was dried using an oven at 105 °C for 15 min. This drying aims to remove the water content present in the sample and to remove impurities present in the sample. Then sieving is carried out using 100 mesh to obtain the same size simplisia. The tobacco stem waste was washed to remove impurities, then cut and dried with solar heat for approximately

7 d. The dried tobacco stem waste was ground using a grinder until it became tobacco powder. After that, this tobacco powder is pulverized using a dish mill and sieved using 80 mesh so that the size becomes uniform.

2.2 Calculation of moisture content

Amount of 10 g sample was put into an empty crucible cup and then dried using an oven at 100 °C for ± 5 h. After that, it was placed in a desiccator for 24 h to remove the water content. After being vacuumed in a desiccator, the sample was then weighed and compared the results before and after entering the desiccator. The calculation of water content uses the following formula in Equation (1):

$$\% \text{ Moisture Content } = \frac{W - W1}{W} \times 100\ \% \quad (1)$$

Where,
W = Weight of sample + initial crucible
W1 = Weight of sample + crucible after drying

2.3 Ultrasonic extraction

The solvents used in this study were methanol and water. Amout of 10 g sample was dissolved with 100 mL of solvent and then extracted using ultrasonic for 30 min with a frequency of 20 Hz. The filtered filtrate was added with 10 mL of HCl and continued with evaporation using a rotary evaporator at 55 °C (methanol solvent) and 70 °C (water solvent) until the crude extract was obtained.

2.4 Fractionation by liquid-liquid extraction

Fractionation is done to remove unwanted compounds. The concentrated extract of 1 g was dissolved with 10 mL of HCl and then sonicated for 30 min. The addition of HCl aims to make nicotine base molecules into salts so that they dissolve in the water phase. Next, 10 mL of chloroform was added to separate non-polar compounds. The solution was flipped for about 5 min until two layers were formed, namely the water phase and the organic phase. The water phase solution was collected and then added with NaOH little by little until a solution pH of 13 was obtained. Next, 10 mL of chloroform was added and extracted using a rotary evaporator at 45 °C until the chloroform evaporated completely. The concentrated extract obtained was dissolved with solvent and will be used for analysis.

2.5 Preparation of nicotine standard curve

In this study, a standard solution of nicotine liquid vape with 99.9 % purity was used. Stock solution was made with a concentration of 2 mg L^{-1}, then diluted using mobile phase. Making a series of standard solutions is done by making three different concentration levels, namely low concentration (0.01 mg L^{-1} and 0.03 mg L^{-1}), medium concentration (0.05 mg L^{-1} and 0.09 mg L^{-1}), and high concentration (0.09 mg L^{-1}). The purpose of making standard solutions with different concentrations is to determine the detector response that appears at the three levels of solution concentration.

The standard solution was made using the multilevel dilution method and then put in a 5 mL volumetric flask and diluted using the mobile phase until it reached the mark. After that, filtering was done using Millipore and allowed to stand for 15 min. Replication was carried out three times. The nicotine standard solution series was injected into the inverted

HPLC system as much as 20 µL. The chromatogram results obtained will show the peak area and nicotine concentration. Nicotine standard curve can be determined using the equation (2) below.

$$y = bx + a \quad (2)$$

2.6 Observation of optimum wavelengths

Determination of the maximum absorption wavelength is done to determine the wavelength of nicotine which will then be used in the analysis process using HPLC. Observations were made using a Uv-Vis Spectrophotometer in the range of 225 nm to 300 nm. Some references make wavelength observations in the range of 200 nm to 300 nm. After obtaining the maximum wavelength, a calibration curve was determined using the concentration of the nicotine standard solution series.

2.7 Thin layer chromatography

The mobile phase used in this thin layer chromatography (TLC) is acetate buffer:methanol:acetonitrile. Methanol and acetonitrile are organic solvents that are most used as mobile phases in reversed phase chromatography. Acetonitrile has a low UV-cut off making it suitable for use in applications that require low UV detection wavelengths. Acetonitrile also has a low viscosity and high boiling point. Acetonitrile is classified as a polar-aprotic solution and methanol is classified as a polar-protic solution. These two solutions are used as mobile phases because they have a strong effect on the selectivity of chromatography. Acetate buffer can maintain pH and is used as a mobile phase mixture for the analysis of easily ionized compounds.

Mobile phase optimization was performed by comparing mobile phase compositions. The mobile phase (eluent) used was buffer acetate:methanol:acetonitrile. A sample of 20 µL was bottled on an F254 chromatograph plate. The plate was then inserted into the chamber that already contained the eluent. After that, the plate was dried and then checked under UV light to see the resulting spot.

2.8 HPLC analysis

High performance liquid chromatography (HPLC) is a liquid chromatography analysis technique used both in qualitative analysis in the form of separation of compounds and in quantitative analysis, namely the determination of the number of compounds in a solution. The principle of HPLC is that a sample in the form of a solution is injected into a column containing a stationary phase and a mobile phase, then given high pressure so that the mobile phase can elute the sample out of the column and detected by a detector which then produces a chromatogram14.

A mixture of mobile phases with three different compositions was used as the mobile phase in the analysis using HPLC. The mobile phase was analysed using three types of solutions, namely samples, samples mixed with standards, and 0.045 mg L^{-1} standards. The mobile phase used was acetate buffer:methanol:acetonitrile with three different compositions, namely 40:54:6, 30:50:20, and 37:51:12. After obtaining the optimal eluent composition, testing was carried out to obtain the optimal flow rate. The flow rates observed were 0.2 mL min^{-1}, 0.3 mL min^{-1}, and 0.4 mL min^{-1}. Retention time (Rt) was measured when HPLC conditions were constant and stable. Retention time observation is done by observing the retention time of the sample peak. This retention time will be compared with nicotine

standard solution. Quantitative analysis is the identification of the amount of analyte levels in a sample or extract. Quantification can be determined by measuring peak height and area.

2.9 Determination of sample resolution on HPLC

The specificity of a method is determined by looking at the resolution of the peak produced by the chromatogram. A 20 µL of the extracted solution was filtered using a Millipore and left for 15 min. Next, the extract was injected into a reversed-phase HPLC instrument using a UV detector. UV detector is used because it is adjusted to the wavelength of nicotine. The column used was C18 column as stationary phase and acetate buffer solution:methanol:acetonitrile as the mobile phase with volume optimization at a flow rate of 0.2 mL min^{-1}. Repetition was done three times. Determination of resolution is done by entering the difference in retention time and width and half height of nicotine peak into the resolution calculation formula. Resolution will be calculated if there are two peaks that have retention times that are close to each other.

2.10 Determination of percent recovery and variation of nicotine standard solution coefficient

Nicotine standard solution as much as 20 µL with low concentration levels (0.01 mg L^{-1}), medium concentration levels (0.05 mg L^{-1}), and high concentration levels (0.09 mg L^{-1}) that have been filtered using Millipore and left for 15 min, injected into a reversed phase HPLC instrument. The stationary phase was a C18 column, and the mobile phase was acetate buffer:methanol:acetonitrile with the most optimal volume with a flow rate of 0.2 mL min^{-1}. Replication was done three times. Nicotine concentration was obtained by entering the AUC (Area Under Curve) obtained into the standard curve equation. After that, recovery, standard deviation, and variation coefficient were calculated.

Two kinds of samples were made, namely sample solution and sample solution added to nicotine standard (addition). The sample solution was made by taking 500 µL of sample extract into a 5 mL volumetric flask, then diluted with mobile phase until it reached the mark. Addition solution was made by adding 150 µL of standard solution into the sample solution into a 5 mL volumetric flask and then diluted with mobile phase until it reached the mark. Both solutions were filtered using a Millipore and left in air for ± 2 min, then injected into the HPLC instrument on the reverse phase. The stationary phase was a C18 column, and the mobile phase was a solution of acetate buffer:methanol:acetonitrile with optimal volume and flow rate of 0.2 mL min^{-1}. Replication was done three times. The standard nicotine content added to the sample is the difference between the value of the addition sample content and the sample content.

3 Results and discussion

The urgency to find and utilize tobacco waste such as stem or dust to extract nicotine is driven by several factors. Firstly, tobacco waste is generated in large quantities during the processing and manufacturing of tobacco products. If not properly managed, this waste can have negative environmental impacts, including greenhouse gas emissions and air pollution. Secondly, there is a growing concern about the negative impacts of tobacco cultivation on social, health, and environmental conditions. By finding new ways to utilize tobacco waste, the negative impacts of tobacco cultivation can be minimized. Additionally, the extraction of nicotine from tobacco waste has the potential to provide a valuable resource for various industries, including pharmaceuticals, agriculture, and bioenergy.

Determination of water content aims to determine the maximum limit of water content in simplisia, in which used tobacco stem and dust. The high amount of water in simplisia can be a medium for the growth of bacteria and fungi which can damage the compounds contained in simplisia. In general, the requirement for water content in a simplisia is no more than 10 %. Based on calculations, the moisture content in tobacco dust waste is 1.96 % and 3.66 % for tobacco stems. Qualitative testing to determine the presence of nicotine compounds in the sample is done using the TLC method. Qualitative analysis on TLC is determined by comparing the Rf (Retardation Factor) value of the sample with the Rf value of the standard compound. The presence of alkaloid compounds is evidenced by the appearance of an orange colour after the plate on which the sample is photographed is sprayed with Dragendorff reagent. The tobacco dust extract sample showed 2 Rf values, namely 0.44 and 0.76 (Figure 1A). While the tobacco stems extract sample obtained 3 Rf values, namely 0.91, 0.77, and 0.37 (Figure 1B). The Rf value obtained from the Nicotine standard is 0.77 (Figure 1C), so that the spot suspected of nicotine alkaloid compounds is the second spot for tobacco dust and tobacco stem samples because the sample Rf value is similar to the standard's Rf. In this TLC optimization, the composition of the mobile phase composition of buffer acetate:methanol:acetonitrile which is the most optimal. The resulting stains were calculated by Rf and the composition that produced Rf between 0.2 to 0.8 was chosen for optimal results. Based on the results of the comparison of mobile phases with three compositions (Table 1), namely the comparison of mobile phases of acetate buffer:methanol:acetonitrile with a composition of 40:54:6, 30:50:20, and 37:51:12, it was found that the comparison of 40:54:6 which has an average Rf value closest to the Rf value of the nicotine standard for both tobacco dust and tobacco stem samples.

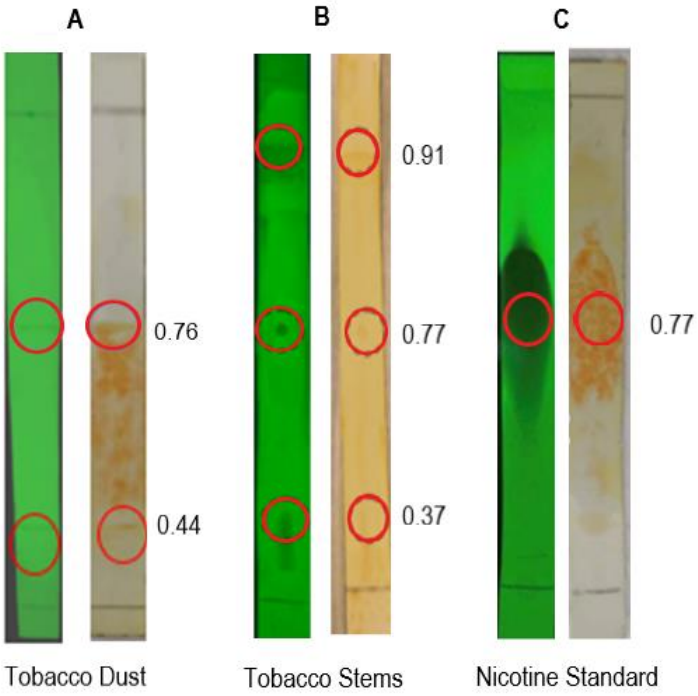

Fig. 1. Comparison of thin layer chromatogram results between extract sample of Tobacco dust (A), extract sample of Tobacco stems (B), and nicotine standard (C).

Table 1. Screening of optimum mobile phase composition based on nicotine Rf.

Mobile phase composition (acetate buffer:methanol:acetonitrile)	Nicotine Rf of Tobacco dust	Nicotine Rf of Tobacco stems
40:54:6	0.773 ± 0.014	0.773 ± 0.026
30:50:20	0.729 ± 0.027	0.746 ± 0.026
37:51:12	0.747 ± 0.033	0.728 ± 0.337

Wavelength determination was carried out using Uv-Vis Spectrophotometry. Based on research, the wavelength of nicotine reads at 254 nm to 260 nm showed maximum absorbance. This length determination uses three series of standard solutions, including concentrations of 0.09, 0.07, and 0.05 mg L^{-1} which are representatives of the five different concentrations of standard series solutions. The three standard series solutions were then detected on a UV Spectrophotometer at a wavelength of 225 nm to 300 nm. This is because the maximum absorption wavelength of nicotine is in that range. In the standard series with concentrations of 0.09 mg L^{-1} and 0.07 mg L^{-1} showed the same wavelength of 259 nm. While at a concentration of 0.05 mg L^{-1} showed a wavelength of 257 nm. When averaged, the maximum absorption wavelength obtained is 258.3. The difference in wavelength obtained can be caused by the presence of other compounds contained in the standard solution, because the standard solution used is a liquid vape or e-cigarette solution containing 99.9 % natural pure nicotine (CAS No: 54-11-5) 10 % and Propanediol 90 %. According to previous research, the wavelength of nicotine detected on a spectrophotometer depends on the pH contained in nicotine [16]. Changes in pH can cause nicotine to be protonated so that chemical shifting is formed which results in a peak shift in wavelength, in this case it can be caused by Propanediol contained in liquid vape. The wavelength obtained from UV Spectrophotometry is then used for observation on the UV detector HPLC (Table 2).

Table 2. Screening of optimum UV-Vis Spectrophotometer Wavelength for nicotine detection

Sample concentration	Optimum wavelength for Tobacco dust extract	Optimum wavelength for Tobacco stems extract
0.05 mg L^{-1}	257 nm	257 nm
0.07 mg L^{-1}	259 nm	259 nm
0.09 mg L^{-1}	259 nm	260 nm
Average	258.33 nm	258.67 nm

Optimization of HPLC conditions was carried out twice, namely optimization of eluent composition (mobile phase) and optimization of flow rate. The detector used is a UV detector. This is because nicotine has a chromophore that can absorb electromagnetic radiation. The stationary phase (column) used is octadecylsilane column (C18). The column is commonly used to detect nicotine. The eluent system used was isocratic with a wavelength of 259 nm. Optimization of the eluent composition was carried out with three different types of eluent composition, namely the ratio of acetate buffer:methanol:acetonitrile ratio of 40:50:10; 40:45:15; and 30:50:20. The injected samples consisted of samples diluted two times, a mixture of samples and 0.09 mg L^{-1} standards with the same amount, and 0.045 mg L^{-1} standards.

Test results using three different mobile phase compositions showed that the composition of acetate buffer:methanol:acetonitrile with a ratio of 30:50:20 is the optimal composition, because in this composition, the resulting peak shows the compound can be separated properly. The flow rate variations tested were 0.4 mL min^{-1}, 0.3 mL min^{-1}, and 0.2 mL min^{-1}. The injected samples were samples diluted two times, samples and standards of 0.09 mg L^{-1} with the same amount, and standards of 0.045 mg L^{-1}. From the results of the HPLC chromatogram, the optimal flow rate is at a flow rate of 0.2 mL min^{-1} because it has a

small HETP value and the resolution value meets the requirements, which is a value of more than 1.5. The total HPLC running time was 20 min for each injection.

After obtaining the optimal eluent composition and flow rate, HPLC can be used to identify nicotine in tobacco dust waste and tobacco stems as shown in Table 3. HPLC is the choice in identifying nicotine because it is able to separate substances based on polarity well and has high selectivity and sensitivity so that it can detect very small levels. The diluted sample is then filtered using a Millipore to remove foreign particles in the mobile phase that can clog the column and interfere with the analysis process. The eluent used must be sonicated using an ultrasonic device first for 1 h. The purpose of this sonication is to remove the presence of bubble particles that can inhibit the column which can interfere with HPLC analysis. Before injecting the sample, purging is done first. The purpose of purging is to remove bubbles that are still contained in the eluent and removed through the hose. These bubbles can interfere with the detection process in HPLC. After ensuring that the eluent has no bubbles, the HPLC application is set and then the sample injection is carried out. Consistently, it can be seen that the extraction results using methanol solvent show higher nicotine levels than the extraction with distilled water solvent. The higher nicotine extraction levels using methanol solvent can be attributed to the solvent's ability to dissolve the lipids and waxes in the tobacco samples, which in turn increases the extraction efficiency. Methanol is a polar solvent and has a higher solubility for polar compounds such as nicotine, compared to non-polar solvents such as distilled water. Average nicotine based on peak area calculations on HPLC chromatograms shows extracts from tobacco stem samples have higher nicotine content than tobacco dust.

Table 3. Nicotine concentration based on HPLC results of various extraction sample from Tobacco dust and Tobacco stems.

Extract sample	Solvent	Retention time	Peak area	Nicotine concentration (mg L^{-1})	Average (mg L^{-1})
Tobacco dust	Ultrasonic (methanol)	13.604	441.92	0.110	**0.094**
		13.527	380.12	0.101	
		13.561	135.55	0.071	
	Ultrasonic (aquadest)	13.721	315.86	0.032	**0.029**
		13.740	226.14	0.023	
		13.697	328.21	0.033	
Tobacco stems	Ultrasonic (methanol)	13.658	1442.46	0.232	**0.269**
		13.667	1436.41	0.231	
		13.680	2140.98	0.344	
	Ultrasonic (aquadest)	13.718	102.47	0.016	**0.069**
		13.735	168.53	0.027	
		13.713	1019.06	0.164	

4 Conclusion

The conclusion drawn from the study is that the use of methanol solvent results in higher nicotine extraction levels compared to distilled aquadest solvent. Additionally, the study found that tobacco stem samples have a higher nicotine content compared to tobacco dust samples. These findings are based on the analysis of HPLC chromatograms that were used

to calculate the average nicotine content based on peak area. Overall, the study's findings suggest that methanol solvent is a more effective solvent for extracting nicotine from tobacco waste, and that tobacco stems are a more valuable source of nicotine compared to tobacco dust. These findings have implications for the development of new strategies for utilizing tobacco waste and extracting valuable resources from it.

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