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Research Article

Simple and Effective UV Spectrophotometric Method for Nicotine Determination in Nicotine Pouches

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Abstract

As nicotine pouches have emerged as a potentially reduced-risk alternative to smoking cigarettes, accurate determination of nicotine concentration in these pouches is vital for ensuring regulatory compliance and product consistency. Although chromatographic methods such as liquid chromatography with tandem mass spectrometry (LC-MS/MS), gas chromatography with mass spectrometry (GC-MS), and gas chromatography with flame ionization detection (GC-FID) have proven effective for determining nicotine content in nicotine pouches, they involve multiple steps and time-consuming procedures. In this study, we developed and validated a simple ultraviolet (UV) spectrophotometry method for nicotine analysis in nicotine pouches. The UV spectrophotometry method simplifies the process of nicotine quantitation by reducing reagent preparation, extraction steps, and analysis time, offering ease of implementation and making it suitable for routine testing. The method was validated using 12 nicotine pouches of four flavor variants (original, citrus, mint, and wintergreen) and three nicotine levels (2, 4, and 8 mg per pouch). The method demonstrated an accuracy ranging from 95.2% to 107.7% recovery, repeatability with less than 8.02% relative standard deviation (RSD), and intermediate precision with less than 8.44% RSD. Robustness tests showed a less than 2% change for varying extraction times and devices. We demonstrated the applicability and accuracy of this UV method by comparing the nicotine quantitation results to results obtained from a standardized chromatographic Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Recommended Method (CRM). We believe this method is a valuable tool for nicotine analysis in nicotine pouches, supporting product development, quality control, and regulatory reporting.

Keywords

Nicotine, UV Spectrophotometry, Method Development, Method Validation, Nicotine Pouches

1. Introduction

Nicotine pouches are noncombustible tobacco products that serve as a potentially reduced-risk alternative to traditional tobacco and smokeless tobacco products. These pouches are tobacco-leaf-free and contain either tobacco-derived or synthetic nicotine, crystalized or granulated filler (e.g., microcrystalline cellulose [MCC]), flavors, sweeteners, and pH adjusters [1]. Accurate determination of nicotine content in these pouches is particularly important to ensure regulatory compliance and product consistency. However, the various ingredients create a challenging analytical environment, and nicotine quantification in nicotine pouches is further complicated by matrix interference.

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Various methods have been developed to analyze nicotine in nicotine pouches, mostly using separation-based techniques such as liquid chromatography (LC) and gas chromatography (GC) [2-11]. For instance, the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) recommended a method (CRM No. 62) for nicotine determination in smokeless tobacco products, including nicotine pouches [2]. This method involves homogenizing the sample in a sodium hydroxide solution, followed by liquid-liquid extraction to separate nicotine into an organic solvent, which is then analyzed by gas chromatography with flame ionization detection (GC-FID). Recently, Aldeek et al. developed and validated two methods using ultra-performance liquid chromatography (UPLC) to determine nicotine in oral tobacco products, including nicotine pouches. The first method employs UPLC with a photodiode array detector (UPLC-PDA) to measure nicotine in dissolution and mastication fractions from traditional moist smokeless tobacco, nicotine gums, and nicotine pouches [5, 11-13]. The second method combines UPLC with mass spectrometry (UPLC MS/MS) to quantify nicotine from various oral tobacco products, using a liquid-liquid extraction technique with sodium hydroxide and acetonitrile and a nicotine methyl-d_3 isotopically labeled internal standard for precise quantitation [10]. While these chromatographic methods were proven effective for accurately measuring nicotine in nicotine pouches, they require complex lab setups and labor-intensive sample preparation.

There is a growing demand for faster and simpler methods that provide timely feedback to ensure product quality during product development and manufacturing. UV absorption and electronic circular dichroism in the UV region have been studied to characterize nicotine and its analogs in diverse solvent systems [14, 15]. Although UV spectrophotometry is extensively utilized as a detection technique of nicotine in separation-based methods [5, 8, 12, 13, 16, 17], there is a paucity of studies investigating the use of UV spectrophotometry as an independent analytical method for this purpose [18, 19]. Matrix interference hinders the accurate determination of the target analyte, and this limitation undermines the reliability of standalone UV spectrophotometric methods, especially for products with complex formulations. In this study, we developed and validated a UV spectrophotometric method for the quantitative determination of nicotine in nicotine pouch products. This method incorporates a flavor-blank correction to mitigate matrix interference. This simple yet innovative approach enabled accurate nicotine quantitation in flavored nicotine pouches while offering more efficient analysis compared to traditional chromatographic methods. By establishing a reliable and efficient UV spectrophotometric method, this study aims to provide a simple and cost-effective alternative analytical approach that can be readily adopted for routine testing and rapid assessment of nicotine pouch products.

2. Materials and Methods

2.1. Chemicals

Optima grade acetonitrile was purchased from Thermo Fisher Scientific (Wyman, MA, USA). Free base neat nicotine (-)-nicotine was acquired from Sigma-Aldrich (Burlington, MA, USA). ISO 17034 certified solution of nicotine (10 mg/mL) was provided by Restek Corporation (Bellafonte, PA, USA).

2.2. Reagent Preparation

2.2.1. 10:90 (v/v) Acetonitrile/Type 1 Water Solution

Acetonitrile/water (10:90 volume ratio) solution was prepared using Type 1 water (resistivity ≥ 18.2 MΩ·cm) produced by a Milli-Q Plus water purification system (Millipore Sigma, Burlington, MA, USA). This solution was used for the calibration solution preparation and sample dilution.

2.2.2. Intermediate Calibration Standard Solution (0.5 mg/mL)

An intermediate calibration standard solution was prepared using the ISO 17034 certified nicotine stock solution (10 mg/mL nicotine). One milliliter (mL) of the ISO 17034 certified nicotine stock solution was diluted with the 10:90 (v/v) acetonitrile/water solution in a 20 mL volumetric flask to produce final concentration of 0.5 mg/mL.

2.2.3. Working Calibration Standard Solutions

The nicotine calibration standards were prepared using 25 mL volumetric flasks. For each calibration level, specific volumes (0.250, 0.500, 0.750, 1.000, 1.500, and 2.000 mL) of the intermediate calibration standard solution (0.5 mg/mL) were added to six separate 25 mL volumetric flasks. Each flask was then diluted to a final volume of 25 mL with the 10:90 (v/v) acetonitrile/water solution, resulting in final concentrations of 5.0, 10.0, 15.0, 20.0, 30.0, and 40.0 µg/mL for levels one through six, respectively.

2.2.4. Nicotine Fortification Solution (100 mg/mL) for Accuracy Experiments

To prepare the 100 mg/mL nicotine fortification solution, 0.500 ± 0.025 g of free base neat nicotine (-)-nicotine was weighted into a 5 mL volumetric flask and diluted to a final volume of 5 mL with the 10:90 (v/v) acetonitrile/water solution.

2.3. Nicotine Extraction from Nicotine Pouch Products

Nicotine pouch fillers from the nicotine pouch product samples and the flavored zero (0) mg nicotine reference

samples were extracted in Type 1 water for nicotine quantitation. For the nicotine pouch product samples, multiple pouches of a nicotine pouch product were cut, and the pouch filler was used to form a composite. Flavored zero (0) mg nicotine reference samples were prepared as the reference blank for the UV measurement of nicotine pouch products. These reference samples, which matched the flavors of the test samples (original, citrus, mint, and wintergreen), were used to create flavor-specific reference standards. The use of flavor-specific references for UV measurement enabled the minimization of matrix interference due to the flavor compounds. For each replicate analysis, one gram (g) of filler was weighed into a 50 mL polypropylene centrifuge tube. Then, 30 mL of Type 1 water was added to the extraction tube, and samples were shaken manually by hand for 1 minute. The extraction tubes were centrifuged at 4000 RPM for 5 min using a Thermo Scientific Sorvall ST 40R unit (Waltham, MA, USA). After centrifugation, 1 mL of the clear supernatant phase from each extracted sample was transferred to separate 50 mL polypropylene centrifuge tubes and diluted with 39 mL of the 10:90 (v/v) acetonitrile/water solution. The diluted samples were filtered using a 2-µm polyvinylidene difluoride (PVDF) syringe filter and added to a quartz cuvette for analysis. Figure 1 shows a schematic representation of the developed extraction method.

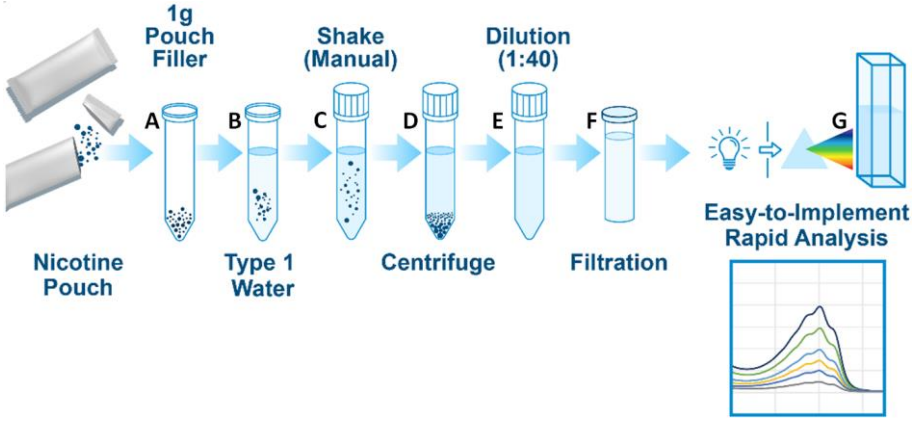

Figure 1. *Schematic representation of the sample extraction procedure consisting of (A) weighing sample, (B) addition of 30 mL Type 1 water, (C) shaking for 1 min, (D) centrifugation for 5 min, (E) dilution with the 10:90 (v/v) acetonitrile/water solution, (F) filtration, and (G) transferring to 10 mm pathlength cuvette for measurement.*

2.4. Nicotine Quantitation Using UV Spectrophotometry

The UV spectra of the samples were collected using a Perkin Elmer Lambda 35 UV-Vis spectrophotometer (Shelton, CT, USA), which features a double-beam configuration. The UV absorbance of the samples was measured in the wavelength region of 200 nm to 400 nm with a slit width of 1 nm. The spectra were collected with a data interval of 1 nm and a scan speed of 480 nm/min. The sample solution and a blank reference solution were measured simultaneously using a pair of 10 mm pathlength quartz cuvettes for the measurements. One quartz cuvette was filled with sample solution and placed in the sample holder, while the other was filled with the corresponding reference blank and placed in the reference holder. The 10:90 (v/v) acetonitrile/water solution was utilized as the reference blank for measuring the calibration standard solutions. When a nicotine pouch sample was measured, a corresponding 0 mg nicotine reference sample with the sample flavor as the test sample was used as the reference blank. Collected spectra were saved as the instrument default file format and exported to CSV file format for further analysis. A linear curve obtained from the calibration solutions was utilized to determine nicotine contents in the samples quantitatively.

2.5. Method Validation

A total of 12 nicotine pouch products were purchased from retail stores. The products were the same brand, including four flavor variants (original, citrus, mint, and wintergreen) and three nicotine strengths (2, 4, and 8 mg). The target nicotine concentrations of the pouch products were expected to be approximately 7.5, 15, and 30 mg/g for the 2, 4, and 8 mg nicotine pouches, respectively. To minimize product variability, such as can-to-can variability, nicotine pouch fillers from multiple cans were gathered and combined into a single container to create a composite sample for each of the twelve products. The flavored 0 mg nicotine reference products (original, citrus, mint, and wintergreen) were acquired from Altria Client Services (Richmond, VA, USA) and used as the reference blank for the UV measurement. The matrix interference with the nicotine absorbance peak at 260 nm was subtracted by using the specific 0 mg nicotine-flavored reference solution that was selectively prepared for each sample

type. Therefore, only nicotine showed a distinct absorbance peak at 260 nm among the other ingredients in the samples. The nicotine standards were analyzed on three separate days to evaluate the calibration model for the coefficient of determination (R^2) and percent relative concentration residual (% RCR). The 0 mg nicotine flavored reference products (original, citrus, mint, and wintergreen) were fortified with known amounts of nicotine to determine if the analytical method could accurately measure the analyte concentration in the presence of sample matrix components. The reference products were fortified with the nicotine fortification solution (100 mg/mL) at two concentration levels and analyzed in triplicate according to the procedure described in the Materials and Methods section. The accuracy of the analytical method was evaluated by calculating the recovery for each sample at each fortification level. The lowest standard, highest standard, and 2 and 8 mg original pouch samples were analyzed six times to determine instrument precision. Repeatability was estimated by analyzing six replicates of each nicotine pouch sample within a single day (n=6). Two analysts estimated intermediate precision by analyzing six replicates of each nicotine pouch sample over three separate days (n=18). The robustness was evaluated by intentionally varying the extraction time and device. Samples were analyzed in triplicate for each robustness experiment using 4 mg original flavor pouch samples. The impact of variations in the extraction time on extraction efficiency was evaluated by varying the extraction time for 30 seconds, 1 minute, and 2 minutes. The impact of a vortex mixer on the sample extraction was also evaluated by comparing the handshaking and vortex mixing using a Fisher Vortex Genie 2 vortex mixer (Fisher Scientific, Pittsburgh, PA, USA) for 1 minute. The percent change was calculated to compare the samples prepared per the robustness modification results versus those prepared per the standard method (e.g., 1 minute manual extraction time). The stability of the intermediate calibration standard solution and the nicotine fortification solution was evaluated using replicates of the solutions diluted to near the mid-point of the calibration range. The results from the day of preparing the intermediate calibration standard solution and the nicotine fortification solution (T_0) were compared to newly prepared sets of diluted solutions at different time points. The stability time points for the intermediate calibration standard solution were Day 1 (T_0), Day 2, Day 3, and Day 10, while those for the nicotine fortification solution were Day 1 (T_0), Day 3, Day 7, Day 14, and Day 30. These comparisons were made using the same intermediate calibration standard solution and nicotine fortification solution prepared on T_0 and stored under refrigerated conditions (4 ± 3 °C). Six replicates of diluted solutions were prepared and analyzed at each time point. The calibration standard stability under the refrigerated conditions was also evaluated using the lowest and highest standard solutions at Day 1 (T_0), Day 3, Day 7, Day 14, and Day 30. The sample extract stability was determined using the 4 mg nicotine pouch samples (original, citrus, mint, and wintergreen). The short-term stability of the sample extracts was evaluated under ambient conditions at 25 °C. The centrifuged samples were stored in the extraction vessel at ambient temperature before proceeding to the dilution step. For the long-term stability test, the sample extracts were stored under refrigerated conditions (4 ± 3 °C) with corresponding reference samples. The long-term stability was determined by comparing the results from T_0 and those obtained from different time points at Day 1 (T_0), Day 3, Day 7, Day 14, and Day 30. The system suitability was used to verify the instrument performance while obtaining accurate and precise data. System suitability evaluation was performed before each analytical batch by measuring the standard deviation (SD) noise of the zero absorbance at 260 nm (instrument noise), and by averaging the peak position and absorbance at the peak maximum from three measurements of the lowest calibration standard. The instrument noise was collected over 180 seconds while both reference cell holder and sample cell holder left empty. The acceptable SD noise was less than or equal to the limit, 0.00005. The acceptable absorbance peak position of the lowest calibration standard was 260 nm ± 1 nm. The absorbance at the peak maximum (i.e., 260 nm ± 1 nm) was compared with the previous analysis. The acceptable absorbance peak height was ± 10% of the previous result.

2.6. Comparative Study with the CORESTA Recommended Method for the Quantitation of Nicotine

The CORESTA recommended method (CRM No. 62) was utilized as a standard method for nicotine determination in the nicotine pouch products. The results were compared with those obtained using the UV method. A fresh set of 4 mg nicotine pouch products for all four flavor variants (original, citrus, mint, and wintergreen) was employed in this comparative study. For the CRM No. 62 testing, 1 gram (g) of the sample was extracted using a mixture of water, sodium hydroxide (NaOH), and methyl t-butyl ether (MTBE) solvent, with quinoline serving as the internal standard. Following extraction, an aliquot of the organic phase was analyzed in triplicate using a GC-FID system (Agilent Technologies 7890, Agilent, Wilmington, DE). The GC-FID system was operated with an Agilent Technologies 7693 automatic liquid sampler system, which included an auto-injector, a 150-sample tray, and an HP-5 column (cross-linked 5% phenyl methylpolysiloxane, 30 m x 0.32 mm x 0.25 µm) (Agilent, Wilmington, DE).

3. Results and Discussion

3.1. Spectral Collection

The calibration solutions exhibited a nicotine absorption peak at 260 nm. However, extracts from nicotine pouch samples showed an elevated baseline around 260 nm when using a

10:90 (v/v) acetonitrile/water solution as the reference blank. This background interference hindered the accurate quantification of nicotine in flavored samples. To address this issue, flavored 0 mg nicotine reference products with the same flavor as the test samples were used as the reference blank for the measurements, effectively minimizing background interference. Figure 2 displays the UV spectra of the standard solutions, the reference 0 mg nicotine samples (original, citrus, mint, and wintergreen), and test samples, each collected with the corresponding flavored 0 mg nicotine reference product.

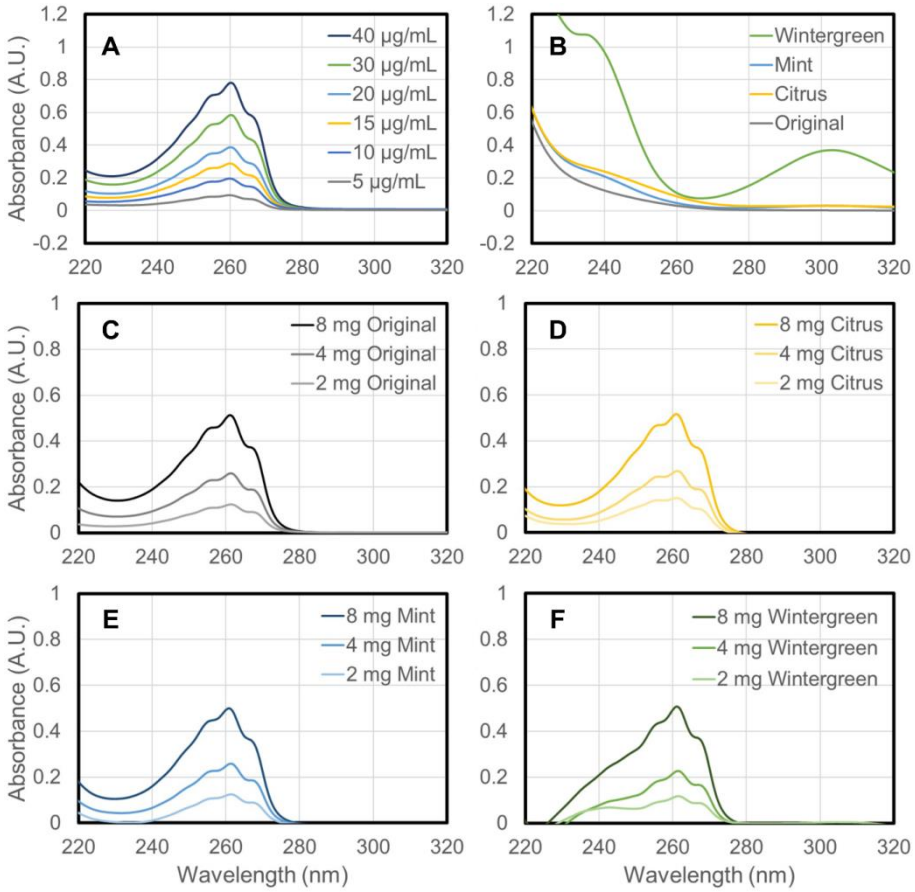

Figure 2. *UV spectra of (A) the standard solutions, (B) four flavor variant reference samples containing 0 mg nicotine, and (C) original, (D) citrus, (E) mint, (F) wintergreen flavor nicotine pouch samples collected with the corresponding flavored 0 mg nicotine reference product.*

3.2. Method Validation

The working range of the calibration model was assessed by a linear fit using six working calibration standard solutions. The nicotine concentrations of the calibration solutions were 5, 10, 15, 20, 30, and 40 µg/mL, respectively. The calibration curve for this method was set appropriately to provide an optimal absorbance range of 0.1 – 0.8, below one according to the Beer-Lambert law. The calibration standard curves demonstrated the coefficient of determination (R^2) greater than 0.999, and the percent relative concentration residuals (% RCR) were less than 4% for all calibration curves over three days. The method's accuracy was validated by evaluating the fortification recovery as described in the Materials and Methods section. Three replicates of each fortified sample were analyzed, and the percent recovery was determined by dividing the measured nicotine concentration by the nominal nicotine concentration of the fortified nicotine. The average recovery ranged from 95.2 – 107.7% for all flavored nicotine pouch products' low and high fortification levels (Table 1). Instrument precision was determined by calculating the percent relative standard deviation (% RSD) obtained from six measurements of standards and sample extracts, which was found to be less than 0.11% RSD. The repeatability, estimated from the six replicate analyses performed on the same day for each sample, was less than 8.02% RSD. Intermediate precision was determined to be less than 8.44% RSD by analyzing six replicate samples over three days (n=18 replicates) (Table 2). The robustness of the method was evaluated to study the influence of varying the extraction time and device on the nicotine extraction efficiency. Compared to the standard extraction procedure (manual hand extraction for 1 minute), the percent change values obtained from different extraction times and different extraction devices were less than 2%. The intermediate standard solution was stable for 10 days in an

amber glass bottle with a screw cap at 4 (±3) °C with a percent change ranging from 1.34 to 2.65%. Calibration curve standard solutions were stable for one month in an amber glass bottle with a screw cap at 4 (±3) °C with a percent change of less than 5%. The nicotine fortification solution was stable for 14 days with a percent change of less than 7% under the refrigerated conditions. Short-term stability of sample extracts stored in extraction vessels at ambient temperature exhibited less than 4% change over 24 hours. Sample extracts were stable for one month when stored in an extraction vessel at 4 (±3) °C with a percent change less than 5%.

Table 1. *Accuracy data (% Recovery) at low (15 mg/g) and high (30 mg/g) fortification levels in 0 mg nicotine fillers with different flavors including % RSD (n=3).*

Sample Name	Fortification Level (mg/g)	Mean % Recovery	% RSD (n=3)
Original 0 mg Nicotine – Low Level	15.90	101.12	2.63
Original 0 mg Nicotine – High Level	31.64	95.22	0.56
Citrus 0 mg Nicotine – Low Level	15.86	100.86	0.50
Citrus 0 mg Nicotine – High Level	31.71	96.21	0.11
Mint 0 mg Nicotine – Low Level	15.87	99.93	0.75
Mint 0 mg Nicotine – High Level	31.71	96.27	2.17
Wintergreen 0 mg Nicotine – Low Level	14.52	107.66	1.88
Wintergreen 0 mg Nicotine – High Level	29.07	103.37	0.27

Table 2. *% RSD from repeatability (n=6) and intermediate precision data within 3 days (n=18) for the 12 nicotine pouches including 4 flavor variants (original, citrus, mint, and wintergreen) at 3 nicotine levels (2, 4, and 8 mg).*

Nicotine Pouch	% RSD Day 1, (n=6)	% RSD Day 2, (n=6)	% RSD Day 3, (n=6)	% RSD 3 Days, (n=18)
2 mg Original	2.51	1.52	2.09	5.10
4 mg Original	1.71	2.82	6.31	5.19
8 mg Original	2.89	5.20	1.37	4.07
2 mg Citrus	2.15	5.88	2.38	3.91
4 mg Citrus	0.93	0.94	2.28	6.07
8 mg Citrus	1.56	6.24	1.61	7.28
2 mg Mint	3.10	4.33	6.04	5.10
4 mg Mint	1.38	1.65	1.88	5.09
8 mg Mint	1.93	2.44	2.98	6.02
2 mg Wintergreen	3.20	2.57	3.17	8.44
4 mg Wintergreen	3.30	2.47	8.02	7.71
8 mg Wintergreen	0.92	1.02	1.36	1.41

3.3. UV Method Compared to GC-FID for Nicotine Quantification

Flavored 4 mg nicotine pouch products were analyzed using the GC-FID method (CRM No. 62) and UV method. The GC-FID method was used as a standardized method to provide the target nicotine concentration of the nicotine pouch samples. The nicotine concentration estimated by the UV method was compared to the target concentrations, and the

mean percent difference values were assessed. The mean percent differences between the two methods were less than 4.44%, and the results verified the capability of the UV method for reliable determination of nicotine concentration in the nicotine pouch samples. Table 3 summarizes the nicotine concentrations obtained using the GC-FID and UV methods.

Table 3. *Determination of nicotine content in 4 mg nicotine pouches, including 4 flavor variants (original, citrus, mint, and wintergreen) using GC-FID and UV methods.*

Nicotine Pouch	Nicotine Concentration (mg/g)		% difference
	GC-FID	UV	
4 mg Original	14.50	14.85	2.36
4 mg Citrus	14.68	14.42	1.80
4 mg Mint	15.49	15.86	2.33
4 mg Wintergreen	14.20	14.86	4.44

4. Conclusion

A simple and effective UV spectrophotometric method was developed and validated for the nicotine determination in various flavored nicotine pouches. It was observed that matrix interference due to flavors can affect nicotine quantification and hinder an accurate determination of nicotine in flavored nicotine pouch products. A key consideration of this method is the use of flavor-matched blanks. Products without nicotine but with the same flavor as the test samples were utilized as references for each measurement to address the matrix interference. This approach ensures accurate nicotine quantification by minimizing the impact of matrix interferences. The method was successfully evaluated for various analytical method validation parameters, including linearity, accuracy, instrument precision, repeatability, intermediate precision, robustness, and stability. Compared to a standardized method for determining nicotine in nicotine pouches (CRM No. 62), this UV method provided comparable data, demonstrating its accuracy and reliability. This UV method offers several practical advantages, such as reduced extraction and analysis time, ease of implementation and maintenance, and suitability for routine testing and quick assessment during product development.

Abbreviations

LC	Liquid Chromatography
GC	Gas Chromatography
UPLC	Ultra-performance Liquid Chromatography
FID	Flame Ionization Detection
PDA	Photodiode Array
MS	Mass Spectrometry
UV	Ultraviolet
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
CRM	CORESTA Recommended Method
MCC	Microcrystalline Cellulose
ISO	International Organization for Standardization
PVDF	Polyvinylidene Difluoride
% RCR	Percent Relative Concentration Residual
RSD	Relative Standard Deviation
SD	Standard Deviation

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Statements and Declarations

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Author Contributions

Seok Chan Park: Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing

Fadi Aldeek: Conceptualization, Data curation, Resources, Supervision, Writing – review & editing

Conflicts of Interest

The authors declare no conflicts of interest.

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