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Analysis and Toxicological Evaluation of Nicotine Degradants and Impurities in Oral Tobacco Products: A Case Study of *on! PLUS* Nicotine Pouches

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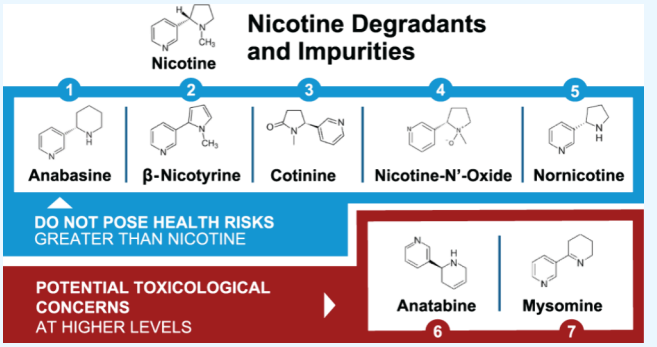
ABSTRACT: This study provides a comprehensive toxicological evaluation of seven nicotine degradants and impurities (e.g., myosmine, nornicotine, anatabine, anabasine, *β*-nicotyrine, cotinine, and nicotine-*N*′-oxide) in oral nicotine pouches. United States Pharmacopeia (USP) and European Pharmacopeia (EP) limits, International Conference on Harmonization (ICH) guidance, and available toxicity data were considered during the evaluation. The toxicological weight of evidence supports that the levels of nornicotine, anabasine, *β*-nicotyrine, cotinine, and nicotine-*N*′-oxide do not pose health risks greater than nicotine and therefore do not increase the health risks of oral tobacco products such as nicotine pouches. However, myosmine and anatabine should be closely monitored against appropriate qualification thresholds due to their potential toxicological concerns. A robust UPLC−MS/MS analytical method was developed and validated for the accurate determination of the seven nicotine degradants and impurities in *on! PLUS* nicotine pouches. The method was utilized to assess the shelf life stability of nicotine in *on! PLUS* nicotine pouches over a 12-month period under ICH long-term storage conditions. Anabasine, *β*-nicotyrine, anatabine, and nornicotine were either not detected or found below the limit of quantitation over the course of the stability study. Myosmine, cotinine, and nicotine-*N*′-oxide were found to be 0.055, 0.015, and 1.32% of the target nicotine level, respectively.



INTRODUCTION

Nicotine pouches are a type of oral tobacco product that is intended for adult use.[1,2] Unlike traditional tobacco products, such as cigarettes or chewing tobacco, nicotine pouches do not contain cut, ground, powdered, or leaf tobacco. Instead, they consist of a small pouch filled with materials such as microcrystalline cellulose, tobacco-derived or synthetic nicotine, pH adjusters such as sodium carbonate and bicarbonate, sweeteners, stabilizers, and flavorings.[3,4] Nicotine pouches are consumed by placing the pouch between the gum and lip. This placement allows for the gradual dissolution of nicotine in saliva, facilitating its absorption in the oral cavity and subsequent entry into the bloodstream.[1,5] In recent years, the popularity of nicotine pouches has increased, driven by their noncombustible nature and the associated potential reduction in tobacco and combustion-related toxicants.[6,7] By eliminating tobacco and combustion, nicotine pouches significantly reduce the exposure to toxicants typically associated with traditional tobacco products such as moist smokeless tobacco, Swedish snus, and cigarettes.[7−12] This characteristic has contributed to their increasing acceptance among individuals seeking a potentially less harmful nicotine product.

The majority of marketed nicotine pouches contain nicotine that is extracted from tobacco plants. Consequently, there is a possibility of trace amounts of related minor alkaloids, such as nornicotine, anatabine, and anabasine, being present as impurities.[13,14] Moreover, the stability of nicotine in these pouches can be influenced by various factors, including product characteristics such as pH, temperature, humidity, light exposure, and storage conditions.[15,16] These stability factors can contribute to the degradation of nicotine over time, potentially leading to the formation of nicotine degradation products, including cotinine, nicotine-*N*′-oxide, myosmine, and *β*-nicotyrine.[15,16] Therefore, it is important to evaluate product stability as part of a broader product stewardship program when evaluating the quality and shelf life of nicotine pouches. Furthermore, the Food and Drug Administration (FDA) requires shelf life stability as part of its review of new tobacco

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products in applications such as Pre-Market Tobacco Product Applications (PMTAs).[17]

The purity of nicotine used in pharmaceutical products is specified in the USP and EP.[18,19] However, these purity recommendations do not extend to tobacco products, such as nicotine pouches. In the USP and the EP, specific nicotine degradants and impurities are outlined, including nicotine-*N*′-oxide, cotinine, nornicotine, anatabine, myosmine, anabasine, and *β*-nicotyrine.[18] These pharmacopoeia guidelines serve as benchmarks for assessing the quality and purity of nicotine used in pharmaceutical products, but their application to tobacco products remains to be established.

Numerous methods have been published for the determination of nicotine degradants and impurities in e-liquids, traditional smokeless tobacco products, cigarette smoke, and plasma using a variety of techniques including LC−MS/MS, LC−Orbitrap−MS, and gas chromatography coupled to flame ionization detection (GC-FID).[15,20−24] However, few methods describe the determination of nicotine degradants and impurities in nicotine pouch products. In 2020, the CORESTA Tobacco and Tobacco Products Analysis Sub-Group (TTPA) conducted a large international proficiency study to evaluate the capabilities of laboratories in analyzing nicotine and its degradants and impurities in nicotine pouches.[25] Nineteen laboratories participated in the nicotine analysis, while 15 laboratories focused on nicotine degradants and impurities analysis. The study aimed to assess the variability of results obtained using different methodologies since there was no consensus standardized method at this time. While nicotine levels were found to be consistent across laboratories, the analysis of nicotine degradants and impurities showed variability due to the diverse methodologies employed. Based on the study findings, the TTPA recommended developing a consensus standardized method (i.e., CORESTA Recommended Method) to analyze nicotine degradants and impurities in nicotine pouches.[26] Consequently, in 2023, the TTPA completed a collaborative study for the determination of the seven nicotine degradants and impurities by adopting a modified UPLC−MS/MS method, originally published by Avagyan et al.[14,26] The resulting CORESTA Recommended Method (CRM No 105) achieved efficient separation within 6 min and demonstrated favorable extraction efficiency using a 100 mM ammonium formate buffer (pH 3) as the extraction solution. The method's accuracy was enhanced using isotopically labeled standards for each of the seven nicotine degradants and impurities. The limit of quantitation was established at 0.75 *μ*g/g. Repeatability and reproducibility values obtained in the collaborative study confirmed the method's suitability for determining nicotine degradants and impurities in nicotine pouches.

In this study, we built upon the UPLC−MS/MS method previously developed by Avagyan et al. and CORESTA (CRM No 105) for the determination of the same seven nicotine degradants and impurities. We tailored the method to suit the specific analysis of nicotine degradants and impurities in a new type of nicotine pouches known as *on! PLUS*.[1] To achieve this, we made slight modifications to the analytical method and extraction procedure, ensuring its suitability for the accurate determination of nicotine degradants and impurities in *on! PLUS* nicotine pouches. The UPLC−MS/MS method was validated and demonstrated to be fit for the analysis of nornicotine, anatabine, anabasine, cotinine, nicotine-*N*′-oxide, myosmine, and *β*-nicotyrine in nine (9) *on! PLUS* nicotine pouch products that come in three distinct flavors (Mint, Wintergreen, and Tobacco) and three nicotine levels (6 mg, 9 mg, and 12 mg). Furthermore, the method was used to assess the stability of nine *on! PLUS* nicotine pouch products under long-term stability conditions (25 ± 2 °C and 60% RH ± 5% (relative humidity)) by determining the nicotine degradants and impurities formation over a 12-month period.[27] One significant challenge we encountered was the lack of specific guidelines to determine acceptable stability limits for nicotine degradants and impurities in nicotine pouches. To address this, we employed a toxicity-based qualification approach, utilizing USP benchmarks[19] and ICH Q3B(R2) guidance[28] to determine whether nicotine degradants and impurities pose greater toxicity concerns compared to nicotine itself and provide recommendations for their stability testing requirements.

MATERIALS AND METHODS

Reagents. Optima grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Wyman, MA). 6 N ammonium hydroxide was purchased from RICCA, (Pocomoke, MD), and 1 M acetic acid was purchased from Fluka Analytical (Pittsburgh, PA). Anabasine-d_4, Cat.# A637180, *β*-nicotyrine-d_3, Cat#: N445002, cotinine-d_3, Cat.# C725005, nicotine-*N*′-oxide-d_3, Cat# N427492, nornicotine-d_4, Cat.# N757010, myosmine-d_4, Cat.# M835010, were purchased from Toronto Research Chemicals (North York, Canada). ISO 17034 nicotine degradants and impurities standard solution containing anabasine, anatabine, *β*-nicotyrine, cotinine, myosmine, nicotine-*N*′-oxide, and nornicotine at 1 mg/mL in acetonitrile, Cat.# G34-113070-01, was purchased from O2si smart solutions (Charleston, South Carolina).

Instrumentation. The determination, identification, and quantitation of nicotine degradants and impurities in all samples were performed using a Waters (Milford, MA) Acquity I-Class Ultra Performance Liquid Chromatography system coupled to a Xevo-TQD triple-quadrupole tandem Mass Spectrometer (UPLC−MS/MS). The UPLC was equipped with a binary solvent manager, temperature-controlled autosampler, temperature-controlled column compartment, Waters BEH C18 analytical column (2.1 × 50 mm, 2.5 *μ*m), and Waters BEH C18 VanGuard precolumn (2.1 × 5 mm, 1.7 *μ*m).

Preparation of 70:30 (v:v) Methanol/Water Extraction Solution. 70:30 methanol/Type 1 water was prepared by adding 700 mL of methanol and 300 mL Type 1 water into a 1 L solvent bottle. The solution was mixed well and stored for sample extraction and dilution and for calibration solution preparation.

Preparation of UPLC Solutions. The aqueous mobile phase (10 mM ammonium acetate) solution was prepared by mixing 10 mL of 1 M acetic acid with 900 mL of Type 1 water. This solution was then combined with 13 mL of 6N ammonium hydroxide, and the pH was adjusted to 10 ± 0.1 and measured using a Mettler-Toledo (Columbus, OH) Seven Compact pH meter. The UPLC needle wash solution was made by combining 950 mL of acetonitrile with 50 mL of Type 1 water in a 1 L solvent bottle. The seal wash solution was prepared by combining 300 mL of Type 1 water with 700 mL Optima grade methanol in a 1 L solvent bottle.

Preparation of Individual Internal Standards (IS) (2.5 mg/mL) Solutions. Individual solutions for each IS were prepared by dissolving 25 mg of neat, isotopically labeled

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internal standard into separate 10 mL volumetric flasks and diluting to volume with acetonitrile, resulting in a 2.5 mg/mL concentration for each internal standard.

Preparation of Mixed Internal Standards (IS) Solution (100 μg/mL) − Mixed Internal Std. Stock Soln. The mixed IS solution was prepared by mixing 4 mL of each of the IS solutions (2.5 mg/mL) in a 100 mL volumetric flask and diluting to volume with a methanol/Type 1 water (70:30, v/v) solution, resulting in a 100 μg/mL concentration mixed stock of internal standards. This solution was used for the preparation of calibration standards and for spiking samples for sample extraction.

Preparation of Intermediate Standard Solution 1 (100 μg/mL) − Interm Std. Soln #1. The intermediate standard solution 1 was prepared by transferring 1 mL of the ISO 17034 certified nicotine degradants and impurities standard solution (1 mg/mL) to a 10 mL volumetric flask and diluting to volume with a methanol/Type 1 water (70:30, v/v) solution.

Preparation of Intermediate Standard Solution 2 (10 μg/mL) − Interm Std. Soln #2. The intermediate standard solution 2 was prepared by transferring 1 mL of the intermediate standard solution 1 (100 μg/mL) to a 10 mL volumetric flask and diluting to volume with a methanol/Type 1 water (70:30, v/v) solution.

Preparation of Calibration Standards. The nicotine degradants and impurities calibration standards were prepared in 10 mL volumetric flasks according to Table S1 (See Supporting Information). To prepare the calibration standards, 0.020 mL of the mixed IS solution (100 μg/mL) was first added to seven volumetric flasks. Calibration standard levels 1−3 were prepared by adding 0.025, 0.1, and 0.25 mL of intermediate nicotine degradants and impurities standard solution 2 (10 μg/mL) to separate 10 mL volumetric flasks and diluting to volume with a methanol/Type 1 water (70:30, v/v) solution. Calibration standards 4−7 were prepared by adding 0.05, 0.1, 0.25, and 0.5 mL of intermediate nicotine degradants and impurities standard solution 1 (100 μg/mL) to separate 10 mL volumetric flasks and diluting to volume with a methanol/Type 1 water (70:30, v/v) solution. The final calibration concentrations were 0.025, 0.10, 0.25, 0.50, 1.0, 2.5, and 5.0 μg/mL for standard levels 1 to 7, respectively.

Nicotine Degradants and Impurities Extraction from *on! PLUS* Nicotine Pouches. One pouch (∼0.7 g) per replicate was cut in half and both filler and pouch material were transferred into a 40 mL amber glass vial and weighed. Next, samples were fortified with an IS nicotine degradants and impurities mixed solution (60 μL of 100 mg/mL). After IS fortification, 30 mL of methanol/Type 1 water (70:30, v/v) extraction solution was added to each of the extraction vessels and samples were shaken using a vortex mixer for 30 min at 70% motor speed. Then, the extraction vessels were centrifuged at 1500 rpm for 5 min. The supernatant was filtered using 0.2 μm PVDF syringe filter directly into an autosampler vial for UPLC−MS/MS analysis.

UPLC−MS/MS Method for the Quantitation of Nicotine Degradants and Impurities. The UPLC−MS/MS instrument included a Waters Acquity I-Class UPLC system coupled to a Xevo-TQD triple-quadrupole mass spectrometer. Chromatographic separation was achieved using a Waters Acquity C18 column (50 mm × 2.1 mm i.d., 2.5 μm). Mobile phase A was 10 mM ammonium acetate buffer and mobile phase B was methanol. The flow rate was 0.4 mL/min for 6 min runtime. The initial composition was 90% ammonium acetate buffer and 10% methanol, and the gradient is ramped to 40% ammonium acetate buffer and 60% methanol until 3.5 min. The column is then flushed with primarily organic eluent (10% ammonium acetate buffer and 90% methanol) from 3.6 to 4.6 min. The eluents were returned to the original condition at 5 min and held constant for 1 min to allow re-equilibration of the system. The column and autosampler temperatures were maintained at 45 (±1) °C and 10 (±1) °C, respectively, and the standards and sample extracts injection volumes were 1 μL. Analyses were performed by electrospray ionization (ESI) using multiple reaction monitoring (MRM) in positive-ion mode. Mass spectrometry (MS) parameters were: 0.5 kV capillary voltage, 150 °C ion source temperature, 500 °C desolvation temperature, 20 L/h cone gas, and 1000 L/h desolvation gas.

Nicotine Degradants and Impurities Quantitation. The quantitation method was designed to utilize a quadratic calibration model, represented by the equation ($y = ax^2 + bx + c$), where (y) is the response factor (RF) relative to the internal standard, and (x) is the concentration (μg/mL) of the standards. The coefficients (a), (b), and (c) are determined through the calibration process. The origin was excluded from the model to ensure accuracy. Given the wide range of concentrations used, a weighting factor of ($1/x$) was applied. This weighting factor assigns different levels of importance to the data points, with higher concentrations (larger (x) values) having less influence on the calibration curve compared to lower concentrations. This approach was chosen because the variance of the response increases with concentration, thereby improving the precision and accuracy of the calibration at lower concentrations. Examples of the seven nicotine degradants and impurities calibration curves can be found in the Supporting Information, Figure S1. Waters MassLynx V4.2 software (Milford, MA) was used to integrate the standards chromatograms and generate the corresponding calibration curve for each nicotine degradant and impurity. The concentration of nicotine degradants and impurities in samples (μg/mL) was determined using the calculated RF of the sample, the slope, and the intercept obtained from the calibration equation. The concentration of the nicotine degradants and impurities based on sample weight (mg/g), was determined using the calculated μg/mL concentration of nicotine degradants and impurities, weight of the sample analyzed, and extraction volume. The mg/g concentration was then used to determine the weight percentage (%) relative to the target nicotine value in products.

Method Validation. The nicotine degradants and impurities standards were analyzed on three separate days to evaluate the calibration model for slope, intercept, coefficient of determination (R^2), and percent relative concentration residual (%RCR). Due to the low and not detected levels of nicotine degradants and impurities in *on! PLUS* pouches, reference 0 mg nicotine *on! PLUS* pouches were produced in Mint, Wintergreen, and Tobacco flavors for the purpose of method validation. For the accuracy experiment, the reference 0 mg nicotine *on! PLUS* pouches were fortified at low (2.87 μg/g) and high (107 μg/g) levels for the seven nicotine degradants and impurities. Accuracy of the analytical method was then evaluated by calculating the recovery. The lowest standard, highest standard, and a fortified matrix sample were analyzed six times to determine instrument precision.

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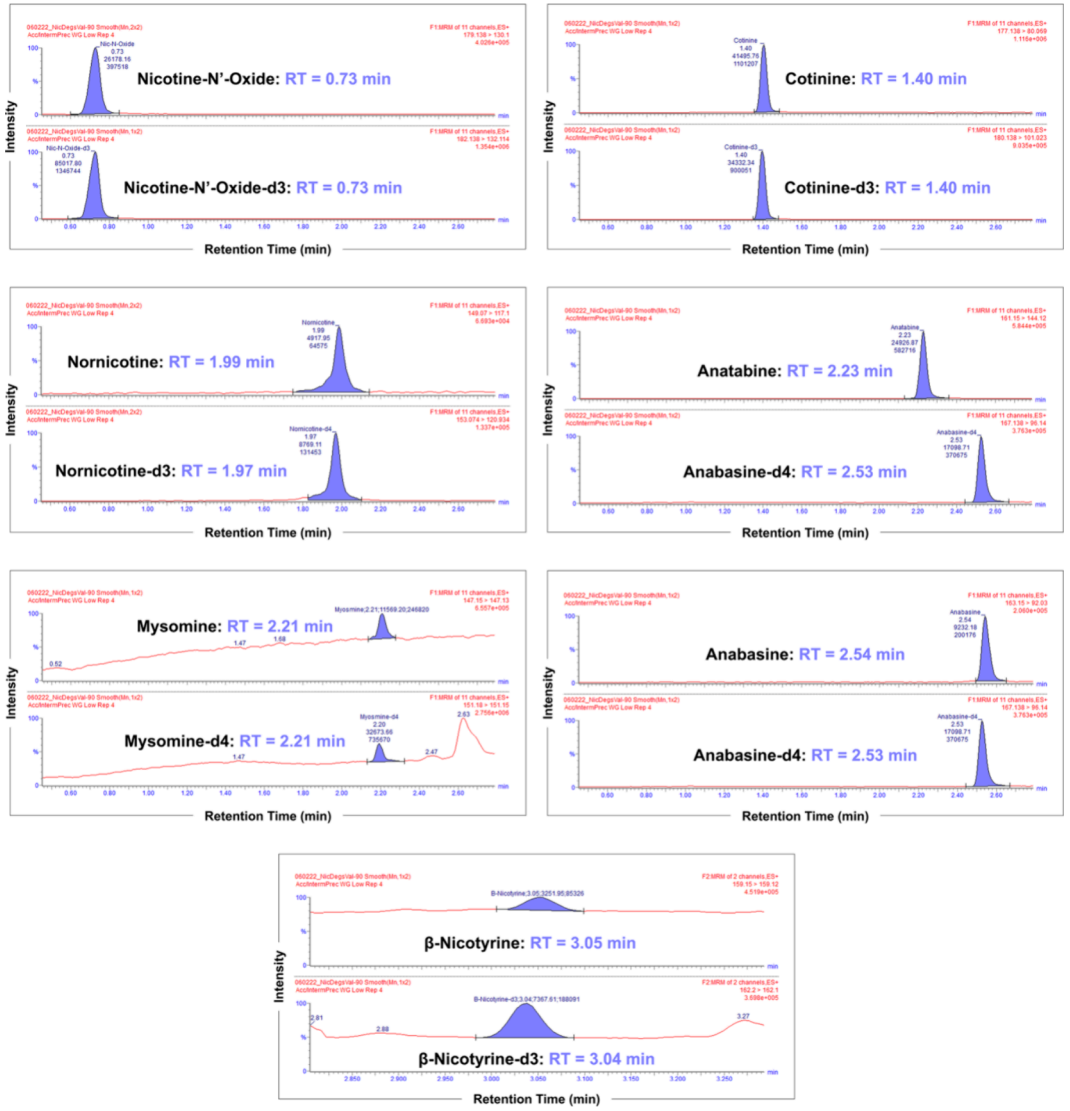

Figure 1. UPLC−MS/MS chromatograms of all seven nicotine degradants and impurities and their corresponding isotopically labeled internal standards, fortified at low level in 0 mg nicotine *on! PLUS* Wintergreen samples.

Preliminary test results showed all analytes, except nicotine-*N*′-oxide, are either below the limit of quantitation (LOQ) or nondetectable (ND) for nine *on! PLUS* nicotine pouch products including Mint, Wintergreen, and Tobacco flavors at three nicotine levels (6 mg, 9 mg, and 12 mg). Therefore, repeatability and intermediate precision experiments were evaluated by fortifying the reference 0 mg nicotine *on! PLUS* pouches (e.g., Mint, Wintergreen, and Tobacco flavors). The final fortification level used for the repeatability and intermediate precision experiments was 2.5 μg/mL. Repeatability was evaluated by analyzing six replicates of the fortified 0 mg samples in 1 day (n = 6). Intermediate precision was estimated by analyzing 6 replicates on 3 separate days (n = 18). The specificity of the method was addressed by evaluating a variety of product matrices for coelution in the region of the analyte and internal standard peaks. We evaluated the lowest standard to ensure it was above the LOQ based on having a signal-to-noise of greater than 10.

Stability Study Protocol. The stability study was conducted on the nine (9) *on! PLUS* nicotine pouch products listed above. The purpose of the study was to evaluate the seven nicotine degradants and impurities over the shelf life of these products. The stability study involved testing three production lots in their final packaging to ensure capturing any inherent variability in the manufacturing process. As a part of the stability study, the analytical test method detailed herein was utilized to measure all specified nicotine degradants and impurities at 0, 2, 3, 4, 6, 9, and 12 months. Zero (0) month represents the baseline for the initial measurement at the start of the stability study, rather than the values at the time the samples were produced. During the study, all products were stored according to the ICH (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) guideline for "Stability Testing of New Drug Substances and Products" Long-term storage conditions (25 ± 2 °C and 60% RH ± 5% (relative humidity)).[27] Three individually prepared replicates were analyzed for each sample, at each time point.

Toxicity-Based Qualification. We conducted a toxicity-based qualification of seven nicotine degradants and impurities, including nornicotine, anatabine, anabasine, cotinine, nicotine-*N*′-oxide, myosmine, and β-nicotyrine. This qualification was

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Table 1. Accuracy Data (%Recovery) at Low (2.87 μg/g) and High (107 μg/g) Fortification Levels in 0 mg *on! PLUS* Nicotine Pouches with Different Flavors Including %RSD (n = 3)

0 mg nicotine *on! PLUS* sample and fortification level	nicotine-*N*′-oxide % recovery (%RSD)	cotinine % recovery (% RSD)	nornicotine % recovery (% RSD)	anatabine % recovery (% RSD)	myosmine % recovery (% RSD)	anabasine % recovery (% RSD)	β-nicotyrine % recovery (% RSD)
mint low level	97.4 (0.54)	99.6 (1.27)	99.8 (3.97)	99.3 (2.73)	97.8 (4.53)	98.5 (4.17)	103.5 (3.59)
wintergreen low level	108.6 (4.11)	114.4 (5.41)	107.3 (0.56)	112.5 (5.48)	99.3 (5.16)	110.6 (8.71)	106.2 (3.41)
tobacco low level	95.9 (1.21)	93.5 (0.72)	99.5 (1.31)	97.7 (0.49)	94.1 (3.07)	95.7 (0.59)	94.4 (2.31)
mint high level	101.4 (1.34)	104.3 (1.62)	95.7 (7.28)	107.3 (1.82)	92.2 (3.10)	104.0 (2.47)	103.0 (3.34)
wintergreen high level	100.0 (1.38)	101.5 (0.89)	97.6 (5.08)	104.8 (4.66)	100.0 (4.57)	98.0 (5.31)	102.8 (2.43)
tobacco high level	96.5 (0.33)	94.9 (0.73)	97.3 (1.46)	97.7 (0.35)	99.7 (1.95)	94.3 (0.40)	95.3 (3.29)

based on existing scientific data regarding their occurrence, absorption, distribution, metabolism, and excretion (ADME). Additionally, we considered their potentials for local effects, sensitization, general systemic effects resulting from acute and repeated exposures, reproductive and developmental toxicity, carcinogenicity, and genotoxicity. Toxicity data were collected from PubMed, PubChem, eChemPortal, the European Chemicals Agency, the Lhasa Carcinogenicity Database, and the Registry of Toxic Effects of Chemical Substances (RTECs). Additionally, official publications and evaluations issued by authoritative expert groups were considered to ensure the accuracy and reliability of the collected toxicity information. The toxicity implications of any structural differences relative to nicotine were also assessed and limits from the USP nicotine monograph[19] were applied as benchmarks for mitigating potential toxicity concerns. Any nicotine degradants and impurities with the potential to exert a level of toxicity greater than that associated with nicotine itself were assigned ICH Q3B(R2)-based qualification limits to mitigate potential toxicological risks over product shelf life.[28] Under ICH, qualification is the process of acquiring and evaluating data to establish the biological safety of an individual degradant or impurity at the level(s) specified. Because nicotine is a pharmacologically active substance and our acceptance criteria are based on relevant safety considerations, the ICH qualification thresholds for new drug products were considered appropriate for establishing acceptable limits for nicotine degradants and impurities. Per ICH Q3B(R2), the reporting, identification, and qualification thresholds for impurities in drug products are based on the maximum daily dose of active pharmaceutical ingredient (API). For a nicotine dose less than 10 mg/day, the maximum total daily intake would correspond to 1% nicotine or up to 50 μg/day (whichever is lower). For a nicotine dose within the range of 10−100 mg/day, the threshold would be either 0.5% nicotine or 200 μg/day, and for doses >100−2000 mg/day either 0.2% or 3000 μg/day (whichever is lower). Therefore, by assuming consumption of 1 can/day (14 pouches) for the 6, 9, and 12 mg products, the estimated daily dose of nicotine (84, 126, and 168 mg/day, respectively) corresponds to the ICH threshold of 0.5% or 200 μg/day (6 mg nicotine pouches) or 0.2% or 3000 μg/day (9 and 12 mg nicotine pouches) (whichever is lower).

RESULTS AND DISCUSSION

UPLC−MS/MS Analytical Method. Standard 3 from the calibration curve prepared at 0.25 μg/mL of nicotine degradants and impurities was infused into the Waters TQD mass spectrometer to verify precursor/product ion masses. A Q1MS scan mode was used to identify the precursor ion of each compound. Once identified, the cone voltage was optimized for each precursor ion. Then, product ion scan mode was used to find the product ions for each analyte. A scan range from 100 to 340 Da was used to allow for the observation of low-mass product ions as well as the precursor ion. The collision energy was ramped from 5−80 eV to allow for determination of the best confirming product ions for each precursor in terms of sensitivity and unique mass values. Using an MRM scan mode, in conjunction with automated compound optimization mode, the cone voltage and collision energy were optimized for each product ion. The MRM settings and precursor to product ion transitions that were optimized for the determination of nicotine degradants and impurities and their corresponding isotopically labeled internal standards are shown in the Supporting Information (Table S2). The product ion transitions used for quantitation purposes were selected based on both the sensitivity and the presence of a clean background. Nicotine degradants and impurities were separated on a reversed-phase LC column under the described gradient conditions within 6 min. Figure 1 shows the UPLC−MS/MS chromatograms of the product ion signals of the seven nicotine degradants and impurities and their isotopically labeled internal standards in the extracted fortified reference 0 mg nicotine *on! PLUS* nicotine pouches. Retention times of 0.73, 1.4, 2.02, 2.23, 2.21, 2.57, 2.7, and 3.05 min were observed for nicotine-*N*′-oxide, cotinine, nornicotine, anatabine, mysomine, anabasine, and β-nicotyrine, respectively. This elution order is expected based on the polarity of these compounds with nicotine-*N*′-oxide, highest in polarity, and β-nicotyrine, lowest in polarity.

Method Validation. The method was validated according to ICH for establishing documented evidence and providing assurance that the analytical procedure is capable of consistently meeting its predetermined specifications and quality attributes.[29] The validated parameters of the method were calibration, accuracy, precision, LOQ, stability, specificity, and system suitability. The calibration curve was verified to be linear quadratic in the concentration range of 0.025−5.0 μg/mL, demonstrating its suitability for analysis. The coefficient of determination (R^2) was found to be ≥0.999 for all calibration curves over 3 days, and the %RCR for all seven nicotine degradant standards over the 3 days were less than 15%. The accuracy of the method was validated by evaluating the fortification recovery as described in the Materials and Methods section above. Reference (0 mg nicotine) *on! PLUS* Mint, Wintergreen, and Tobacco samples were weighed and fortified with known amounts of nicotine degradants and impurities in triplicate at two different levels prior to extraction. Three replicates of each fortified sample were analyzed to determine accuracy. The measured nicotine degradants and impurities values were divided by the fortified

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Table 2. %RSD from Intermediate Precision Data within 3 days (n = 18)

fortified 0 mg nicotine *on! PLUS* Sample	nicotine-*N*′-oxide % RSD	cotinine % RSD	nornicotine % RSD	anatabine % RSD	myosmine % RSD	anabasine % RSD	*β*-nicotyrine % RSD
mint	3.91	3.62	4.22	4.85	3.80	3.46	6.26
wintergreen	4.05	6.91	4.72	5.00	4.17	6.31	6.82
tobacco	6.07	5.89	6.05	6.39	5.46	6.74	5.13

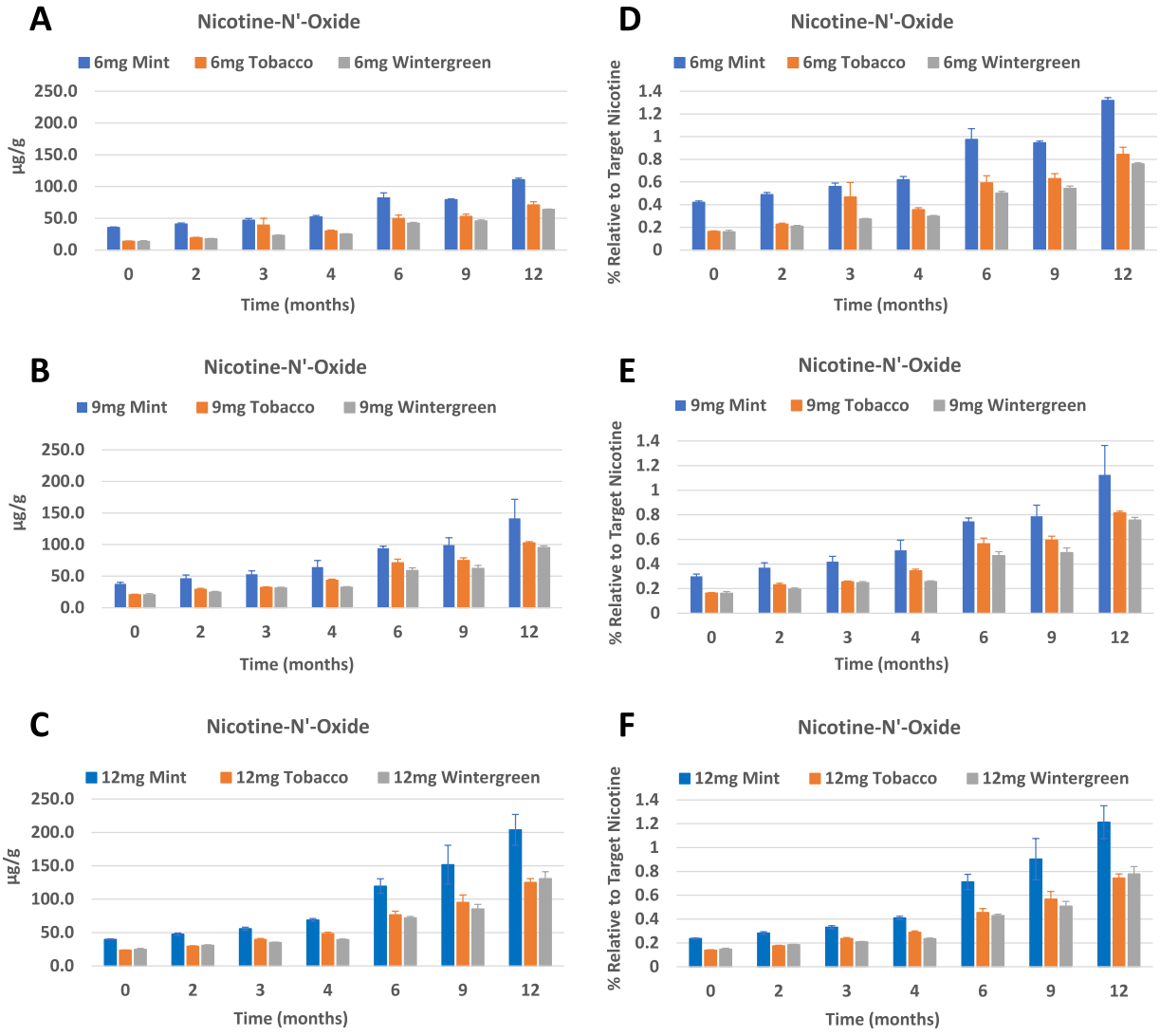

Figure 2. Twelve-month averaged stability data for three lots, each analyzed in triplicate (n = 9), illustrating the concentration (μg/g) of nicotine-*N*′-oxide and percentage relative to target nicotine levels in Mint, Tobacco, and Wintergreen flavors of *on! PLUS* nicotine pouches at 6 mg/pouch (A and D), 9 mg/pouch (B and E), and 12 mg/pouch (C and F) nicotine levels.

amounts to determine % recovery. All fortification levels and matrix types had calculated nicotine degradants and impurities recovery values within 92 to 114% (Table 1). Instrument precision was ≤7% relative standard deviation (%RSD) based on six injections each of standards and fortified sample extracts. Repeatability was ≤10%RSD based on six replicate analyses performed on the same day for each sample type (see Supporting Information, Table S3). Intermediate precision was ≤7%RSD by analyzing six replicate samples over the course of 3 days (n = 18 replicates) (Table 2). All the samples were free of matrix interferences showing adequate specificity and the ability of the method to quantify the seven nicotine degradants and impurities in the presence of components that are present in the sample matrix (Figure 1). The LOQ of the method was based on the lowest calibration standard (0.025 μg/mL), as it produces a peak with a signal-to-noise ratio exceeding 10. Finally, the system suitability was used to confirm that the sensitivity and reproducibility of the system were adequate for the analysis to be performed. System suitability evaluation was performed by injecting the lowest standard three times to produce a signal-to-noise ratio greater than 10 and a peak response %RSD less than 10%.

Stability Studies for Nicotine Degradants and Impurities Determination in *on! PLUS* Pouches. The stability studies were conducted on nine *on! PLUS* nicotine pouch products that include three flavors (Mint, Wintergreen, and Tobacco) and three nicotine levels (6 mg, 9 mg, and 12 mg/pouch). The purpose of the study was to evaluate the

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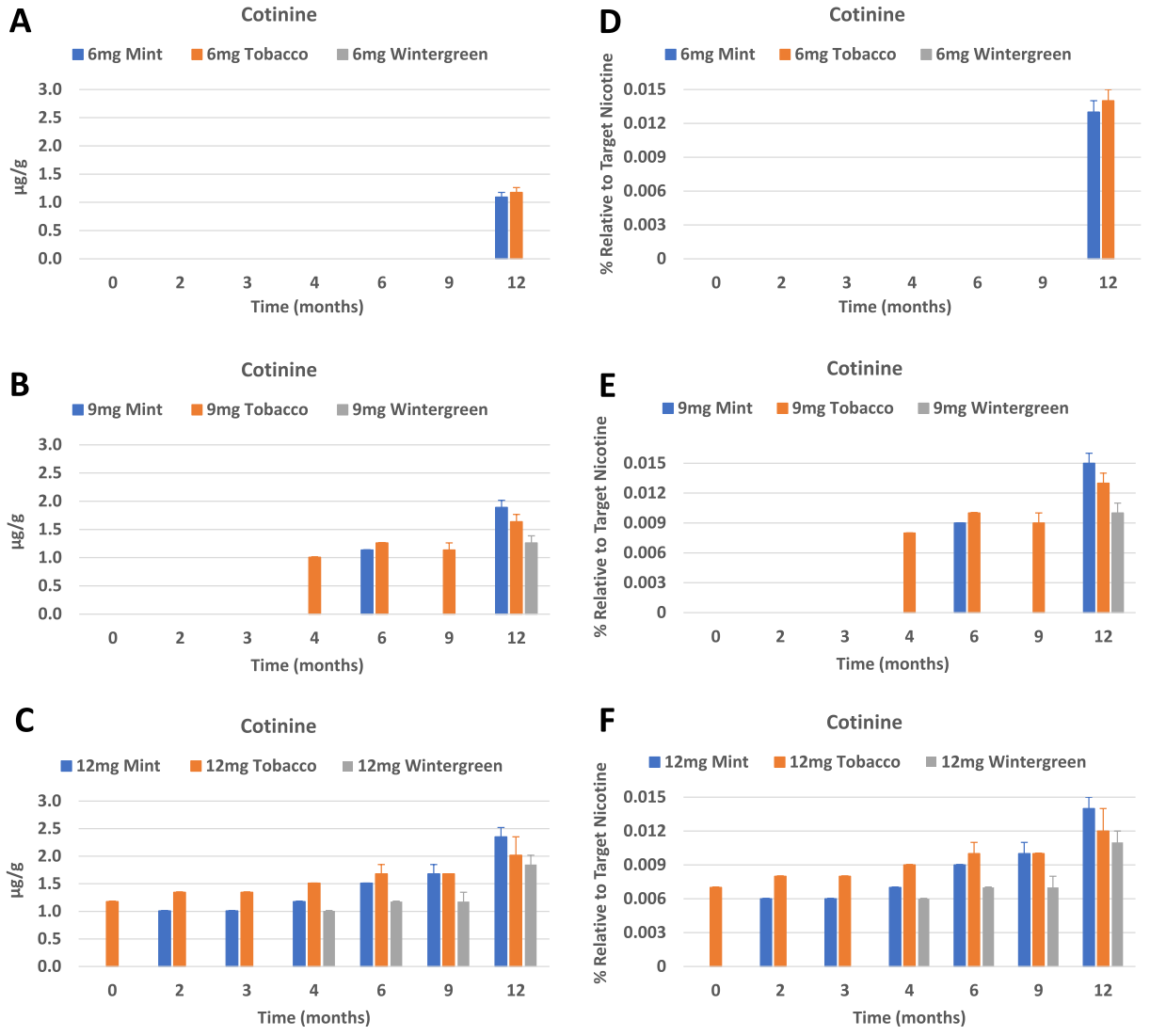

Figure 3. Twelve-month averaged stability data for three lots, each analyzed in triplicate (n = 9), illustrating the concentration (μg/g) of cotinine and percentage relative to target nicotine levels in Mint, Tobacco, and Wintergreen flavors of *on! PLUS* nicotine pouches at 6 mg/pouch (A and D), 9 mg/pouch (B and E), and 12 mg/pouch (C and F) nicotine levels.

seven nicotine degradants and impurities over the shelf life of these products. The studies focused on quantifying the nicotine degradants and impurities in the nicotine pouch products. The products were stored under ICH long-term storage conditions, which involve a temperature of 25 ± 2 °C and a relative humidity of 60 ± 5%. After 12 months of storage, it was observed that the total of seven nicotine degradants and impurities remained at levels ∼ 1.4% of the target nicotine level. During the 12 months of storage, four nicotine degradants and impurities (anabasine, β-nicotyrine, anatabine, and nornicotine) were either not detected or below the LOQ. The only observed nicotine degradants and impurities in the nicotine pouches were nicotine-N'-oxide, cotinine, and myosmine, as shown in Figures 2, 3, and 4. The presence of nicotine-N'-oxide was observed in all samples, with slightly higher levels in Mint samples compared to Wintergreen and Tobacco samples. The weight percentage of nicotine-N'-oxide relative to the nicotine target ranged from 0.14 to 0.42% for products analyzed at T = 0 month (Figure 2). After a storage period of 12 months, the weight percentage of nicotine-N'-oxide relative to the nicotine target increased to a range of 0.75 to 1.32% for all nine products. At T = 0, cotinine was only detected in the 12 mg Tobacco variant. Over the course of the 12-month stability study, cotinine levels in all samples remained very low and only reached 0.015% relative to the nicotine target in the 9 mg Mint product (Figure 3). It is worth noting that cotinine was never detected in the 6 mg *on! PLUS* Wintergreen over the 12-month stability study. However, it started appearing at the 12-month period in Wintergreen 9 mg, Mint 6 mg, and Tobacco 6 mg *on! PLUS*. Similarly, mysomine levels were found to be very low in all products across all flavor variants and nicotine levels, ranging from 0.01 to 0.055% relative to the nicotine target (Figure 4), throughout the 12-month stability studies. It is worth noting that while nicotine degradants and impurities are commonly found in tobacco products like cigarette filler and traditional moist smokeless tobacco products,[14] the levels of these degradants and impurities in tobacco-derived nicotine pouch products are very low. This observation is supported by a recently published work by Avagyan et al., where the authors characterized the nicotine degradants and impurities in various tobacco products, including smokeless tobacco, cigars, and nicotine

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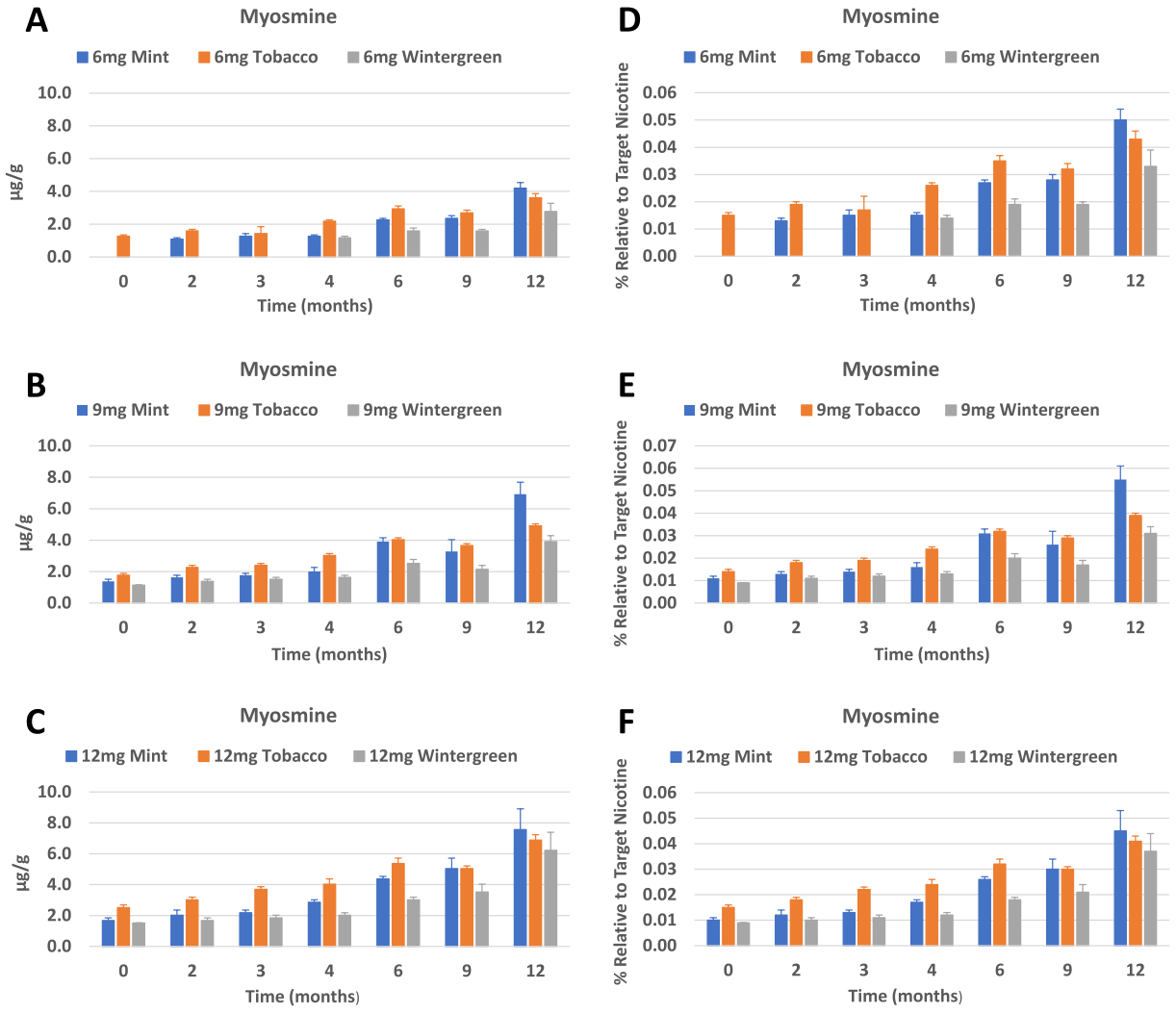

Figure 4. Twelve-month averaged stability data for three lots, each analyzed in triplicate ($n = 9$), illustrating the concentration (μg/g) of myosmine and percentage relative to target nicotine levels in Mint, Tobacco, and Wintergreen flavors of *on! PLUS* nicotine pouches at 6 mg/pouch (A and D), 9 mg/pouch (B and E), and 12 mg/pouch (C and F) nicotine levels.

pouch products. The study found that the levels of degradants and impurities were higher in smokeless tobacco and cigars but very low or not detected, especially for anatabine, anabasine, and β-nicotyrine, in nicotine pouch products.[14] As mentioned previously, the stability of nicotine in these pouches can be influenced by various factors, including product characteristics such as pH, temperature, humidity, etc. The pH of *on! PLUS* was measured to be 8.4−8.6 for all studied products and remained consistent throughout the 12-month stability study. This basic pH range, which is higher than the pK_a value of nicotine (8.02), ensures that a significant proportion of nicotine remains in its unprotonated form, thereby facilitating efficient absorption through the buccal mucosa. Given the stability of the pH over the 12-month period, we do not believe that any pH changes could have contributed to the formation of nicotine degradants in the products. However, the basic pH environment could have influenced the hydrolysis of nicotine, potentially leading to the formation and slight increase of nicotine-N'-oxide, cotinine, and myosmine over-time. Understanding the impact of pH on the formation of these degradation products is critical for product formulation, quality control, and regulatory considerations. In our case, maintaining a stable pH for *on! PLUS* products is essential to better manage the chemical pathways that lead to the formation of these degradants, thereby enhancing the overall stability and safety of the product.

Toxicological Assessment. In the USP nicotine monograph, a limit of 0.3% relative to nicotine target is listed for each of nornicotine, anatabine, anabasine, cotinine, nicotine-N'-oxide, myosmine, and β-nicotyrine, indicating that this level is considered tolerable for minimizing health risks from these compounds.[19] However, the toxicological weight of evidence supports that five (5) of the seven (7) nicotine degradants and impurities do not pose health risks greater than nicotine and therefore do not increase the health risks of the oral tobacco product.

The available toxicological data and USP benchmarks suggest that the presence of nornicotine, anabasine, β-nicotyrine, cotinine, and nicotine-N'-oxide within oral nicotine products would not be expected to cause significant risks to consumers over and above that associated with nicotine itself. Nornicotine, anabasine, cotinine, β-nicotyrine, and nicotine-N'-oxide are all known in vivo metabolites of nicotine.[30−37] The ICH Q3B(R2) guidance states that "Degradation products that are also significant metabolites present in animal and/or human studies are generally considered qualified".[28] In

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addition, available toxicological data show that nornicotine, anabasine, cotinine, β-nicotyrine, and nicotine-N′-oxide have comparable genotoxicity and acute toxicity to nicotine.[38−47]

Further, nornicotine, anabasine, and β-nicotyrine were either not detected or below the limit of quantitation in *on! PLUS* nicotine pouches over 12 months of storage. While cotinine was detected, it never exceeded 0.015% relative to the nicotine target which is approximately 20 times below the USP benchmark of 0.3%. Levels of nicotine-N′-oxide were highest among any of the nicotine degradants and impurities and reached a maximum of 1.32% relative to nicotine. Although the level of nicotine-N′-oxide exceeds the 0.3% threshold listed by the USP monograph, this level in nicotine pouches does not pose a toxicological concern greater than nicotine itself. For instance, the only difference in the chemical structures of nicotine and nicotine-N′-oxide is the additional presence of an oxide anion bonded to the pyrrolidine nitrogen, imparting a charge (N^+-O^-) across the bond. Nicotine-N′-oxide has shown a much lower acute toxicity than nicotine for both the oral route, LD_{50} values of 195 and 3.24 mg/kg body weight (bw) for the N-oxide and nicotine, respectively,[42] and for the intraperitoneal route, LD_{50} values of 615 and 9.5 mg/kg bw for the N-oxide and nicotine, respectively.[47] These results indicate a lower acute toxicity concern for the N-oxide, compared to nicotine, suggesting that N-oxidation represents a detoxification step in nicotine metabolism. An OECD expert assessment of a group of 15 N-oxides reported that these chemicals are extensively metabolized and readily excreted after ingestion.[47] Rat studies indicate that acute oral toxicity is moderate-to-low, and no evidence of carcinogenic activity was seen in three long-term studies in rats or mice by dietary, drinking water, or dermal routes. Five Ames tests found no evidence of any ability to induce bacterial mutations, and four in vivo genotoxicity studies (mouse and hamster micronucleus, hamster chromosome aberration and mouse dominant lethal mutation assays) found no evidence of a genotoxic potential.[48] On this basis, and considering the existing toxicity data on nicotine and its N-oxide, the single difference in chemical structure would not be expected to introduce a novel health hazard.

As a result, the presence of nornicotine, anabasine, β-nicotyrine, cotinine, and nicotine-N′-oxide do not have a significant impact on the quality, safety, or efficacy of oral nicotine products made with pharmaceutical grade nicotine. Monitoring of these nicotine degradants and impurities is considered low priority from a toxicological perspective, because any potential toxicity will be limited by the concentration of nicotine used since the quantity of reactants will always be equal to or less than the parent compound.

Some potential toxicity concerns exist for myosmine and anatabine, since both compounds have been observed to exert a level of toxicity greater than that associated with nicotine itself. Below, we describe the toxicological rationale for continued stability testing of myosmine and anatabine against appropriate qualification thresholds.

Myosmine produced evidence of mutagenicity in mammalian cells in vitro and DNA damage in human cells in vitro.[49,50] Myosmine also demonstrated the ability to form DNA adducts in rats in vivo.[51] Notably, myosmine was found to be nonmutagenic to bacteria in an Ames test and did not induce sister chromatid exchanges (SCEs) in Chinese Hamster Ovary (CHO) cells.[38,41] Nicotine was similarly nonmutagenic and nongenotoxic in equivalent assays.[45] Myosmine, in the presence of dietary nitrite and at pH values occurring in the stomach, has been shown to be nitrosatable to N′-nitrosonornicotine (NNN).[51,52] NNN is a nitrosamine and known human carcinogen thought to have significant involvement in the mechanism of lung, esophageal, oral and pancreatic cancers caused by tobacco product use.[53,54] Nitrosation generally requires acidic conditions and a source of nitrite.[55] As such, a certain level of endogenous nitrosamines are formed in the human gastrointestinal tract, where amines in dietary protein can react with nitrite. It is estimated that normal consumers of meat might produce 244 μg of endogenous nitroso- (−N−N═O) compound/day.[56] Based on a formula weight of 44 g mol^{-1} for the −N−N═O functional group, this correlates to 5.55 μmol/day. Applying the molecular weight of 177.2 g mol^{-1} for NNN, if the source amine were nicotine or one of its degradants and impurities, this endogenous −N−N═O production equates to 982.7 μg/day as NNN. Considering a maximum nicotine intake of 100 mg/day (or 300 μg/day of myosmine based on the 0.3% USP benchmark) and a worst-case assumption of 100% nitrosation, approximately 363.6 μg/day of NNN could be formed from mysomine.[2] However, there are at least two factors that would act to limit the amount of NNN that could be formed. First, additional sources of amine for nitrosation reactions exist from typical diets. Around 1−10 mg/day of secondary amines are consumed as part of a "typical European diet".[57] Second, there is a limited amount of nitrite present in the stomach. In the average population, dietary nitrite intake is approximately 0−20 mg/day although nitrites can also be produced endogenously through the oxidation of nitric acid and/or the reduction of nitrate by oral or gastrointestinal bacteria.[58,59] These two factors combined indicate that there would be sufficient competition from more easily nitrosatable amine sources for the limited nitrite in the stomach to make 100% conversion of these nicotine degradants and impurities extremely unlikely. Shephard et al. suggest that the "[carcinogenic] risk posed by in vivo nitrosation of primary and secondary amines is probably negligibly small."[57]

In consideration of these factors, myosmine is considered tolerable if the detected levels are below the relevant thresholds in the USP monograph (0.3%) and the ICH Q3B(R2) guidance over the oral nicotine product shelf life. If myosmine remains below these appropriate thresholds, then it is not considered to increase the overall toxicity of the products. Throughout the 12-month *on! PLUS* stability studies, mysomine levels were below these thresholds and never exceeded 0.06% relative to nicotine. To mitigate potential toxicological risks, monitoring mysomine against appropriate qualification thresholds should be part of shelf life evaluations for nicotine pouch products.

The available toxicological information on anatabine is extremely limited, but some indirect evidence of DNA-damaging ability exists. On one hand, neither anatabine nor nicotine caused an increase in SCEs in the presence of S9, and neither were mutagenic in an Ames test.[38,41] In the absence of S9, anatabine caused a dose-dependent increase in SCEs in CHO cells (with only S9), while nicotine was found to give a slight increase in SCEs, and was regarded as only "weakly positive".[38] It is worth noting that the OECD guideline for testing SCE induction was withdrawn several years ago, suggesting that some experts do not consider SCE induction to reflect genotoxicity potential. Nonetheless, Riebe et al. reported anatabine to be the "most effective" tobacco alkaloid of those tested (including nicotine) in inducing DNA damage

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in a repair-deficient bacterium.[38,41] No follow-up mammalian gene mutation data were identified. However, anatabine occurs naturally in tobacco and the USP monograph indicates regulatory acceptance at up to 0.3% of nicotine, presumably at any point in the shelf life of a product.

In consideration of these factors, anatabine is deemed tolerable if the detected levels are below the relevant thresholds in the USP monograph (0.3%) and the ICH Q3B(R2) guidance over the oral nicotine product shelf life. If anatabine remains below these appropriate thresholds, then it is not considered to increase the overall toxicity of the products. Notably, anatabine was either not detected or below the LOQ throughout the 12-month *on! PLUS* stability studies. To mitigate potential toxicological risks, monitoring anatabine against appropriate qualification thresholds should be part of shelf life evaluations for nicotine pouch products.

CONCLUSIONS

In this study, we developed a reliable UPLC−MS/MS method for the quantitation of seven nicotine degradants and impurities in *on! PLUS* nicotine pouches. The method demonstrated excellent linearity, precision, and accuracy. Stability studies for *on! PLUS* nicotine pouches did not show quantifiable amounts of anabasine, β-nicotyrine, anatabine, and nornicotine. Myosmine, cotinine, and nicotine-*N*′-oxide remained consistently below 0.055, 0.015, and 1.32% of the target nicotine level, respectively, over the 12-month stability. These data, combined with the toxicological weight of evidence, indicate that five out of the seven nicotine degradants and impurities (specifically nornicotine, anabasine, β-nicotyrine, cotinine, and nicotine-*N*′-oxide) do not pose greater health risks than nicotine itself. Consequently, the presence of these degradants and impurities in oral tobacco products, such as nicotine pouches, does not increase the overall health risk associated with their use. However, monitoring myosmine and anatabine is recommended during shelf life evaluations for nicotine pouches.

ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c00263.

Detailed procedures for the preparation of calibration working standards, including serial dilutions of stock solutions; the retention times and multiple reaction monitoring (MRM) settings used for the quantitative analysis of nicotine degradants and impurities; %RSD values from repeatability data within the same day (n = 6); and calibration curves for the quantitation of Nicotine-*N*′-Oxide, Cotinine, Nornicotine, Anatabine, Myosmine, Anabasine, and β-Nicotyrine, along with their respective regression analyses and correlation coefficients (PDF)

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Author Contributions

F.A. led the research, wrote the main manuscript text, prepared figures, and reviewed the manuscript, V.H. wrote the toxicological assessment in the main manuscript text, K.W. reviewed the manuscript, E.S. conducted the stability study, and V.L. developed and validated the method.

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Notes

The authors declare no competing financial interest.

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ADDITIONAL NOTES

[1] *on! PLUS* is a trademark of Helix Innovations LLC.

[2] NNN levels were calculated by multiplying the amount of myosmine (μg/day) by the ratio of molecular weights (NNN/mysomine). E.g., for myosmine, 300 μg/day myosmine × (177.2 g mol^{-1} NNN/146.2 g mol^{-1} myosmine) = 363.6 μg NNN/day.

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