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Solid-Phase Microextraction-Based Approach To Determine Free-Base Nicotine in Trapped Mainstream Cigarette Smoke Total Particulate Matter

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Characterizing nicotine delivery from tobacco products is important in the understanding of their addictive potential. Most previous studies report total nicotine and have not differentiated between nicotine in its protonated or free-base form. Rather than simply determining total nicotine, the method described in this paper determines the amount of free-base nicotine associated with trapped mainstream smoke particulate matter generated using a standardized smoking machine protocol. This method quantitatively determines volatile free-base nicotine associated with the particulate phase portion of mainstream cigarette smoke using solid-phase microextraction combined with gas chromatography−mass spectrometry. The headspace above total particulate matter from mainstream cigarette smoke trapped on a Cambridge filter pad (CFP) was analyzed for free-base nicotine in 26 cigarette brands. The selected cigarette brands were chosen to cover a wide range of tar and nicotine deliveries as measured under Federal Trade Commission machine smoking conditions. In the CFP's headspace the free-base nicotine levels ranged from 0.01 to 0.08 mg/cigarette. The measured ranges of free-base nicotine were remarkably similar over the different tar and nicotine delivery categories of full-flavored, light, and ultralight cigarette brands.

KEYWORDS: Free-base nicotine; mainstream cigarette smoke; particulate matter; tobacco; addictive; smoking machine; SPME

INTRODUCTION

Tobacco use remains the leading cause of preventable death in the United States (*1*, *2*). Although thousands of chemical compounds are present in tobacco and cigarette smoke, one of the most important constituents is nicotine because of its physiological properties and addictiveness (*3*, *4*). The acid−base properties of nicotine play a key role in its chemical and physical characteristics. The dibasic nature of nicotine results from the two protonation sites at the pyrrolidine and pyridine nitrogens. In highly acidic aqueous conditions (pH <3), the nicotine molecule exists almost exclusively as a diprotonated species. However, comparably low pH conditions are typically not encountered in tobacco or smoke, and the diprotonated form is relatively unimportant. Aqueous slurries of tobacco filler from conventional cigarettes typically have a neutral or slightly acidic pH (∼5−6), and essentially all nicotine in the filler material exists primarily as a monoprotonated salt with the strong ionic forces minimizing evaporative loss (*5*, *6*).

Historically, researchers have attempted to measure "smoke pH" to estimate the percentage of nicotine present in the free-base form. Cigarette smoke has been characterized as being slightly more alkaline than tobacco filler (*5*, *6*). Changes in acid−base smoke chemistry or, more importantly, shifts in the equilibrium between protonated and free-base nicotine can dramatically affect nicotine's physiological properties because the protonated form is hydrophilic and the free-base form is lipophilic and readily adsorbed across membranes (*7*−*9*), dramatically increasing bioavailability. Even minor changes in the acid−base equilibrium would be expected to dramatically alter the concentrations of the protonated and free-base nicotine conjugates. Therefore, examining nicotine's chemical properties in mainstream smoke helps to address questions regarding the effective delivery and overall bioavailability.

Many previous investigations of mainstream cigarette smoke's acid−base properties used methods that measured values related to the pH of an aqueous sample. This rationale assumed, incorrectly, that smoke was like an aqueous sample with a measurable pH and that the relative ratio of free-base and protonated nicotine could be determined from pH, total nicotine content, and acid−base equilibrium data (*10*). In several earlier studies (*11*−*14*), mainstream cigarette smoke was dissolved in aqueous solvents that were analyzed using a pH electrode. The difficulty with such techniques is that the resulting solution pH depended on how the smoke samples were collected. However, these approaches presumably provided a relative measurement of the smoke's acid−base properties and may have some utility for comparative purposes. To overcome limitations associated

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with such an indirect sampling approach, Sensabaugh and Cundiff (*6*) devised a method, which was refined by Brunnemann and Hoffmann (*5*), using a modified pH electrode placed directly in the mainstream cigarette smoke path during active smoking. Even with this dynamic method that measured "real-time" changes in the pH electrode's potential, it was emphasized that such measurements were best suited to provide relative values for comparing different styles or types of cigarettes.

Fundamental technical difficulties exist with any method attempting to measure cigarette smoke pH. At best they provide limited information on the relative acid−base chemistry. Smoke is a dynamic and continuously evolving stream of aerosol particles and is far from being an aqueous solution with a constant hydronium ion concentration. Therefore, in the normal sense cigarette smoke does not have a pH, at least on the macroscopic scale. However, measuring the pH of cigarette smoke on the microscopic scale of individual aerosol particles may become technically feasible in the future.

Our main goal was the development of a robust technique to directly measure the free-base nicotine content in mainstream cigarette smoke using the Federal Trade Commission (FTC) (*15*) machine smoking parameters. This type of an approach has been pioneered by Pankow et al. (*16*) to examine free-base nicotine in mainstream smoke. The free-base nicotine results of Pankow et al. are expressed in terms of α_{fb} (*17*), which for mainstream cigarette smoke can be approximated by

$$\alpha_{fb} = [\text{free-base nicotine}]/[\text{total nicotine}] \quad (1)$$

Using the α_{fb} values an expression for the "effective" mainstream smoke pH, pH_{eff}, can be derived (*18*):

$$pH_{eff} = pK_a + \log[\alpha_{fb}/(1 - \alpha_{fb})] \quad (2)$$

Two of the differences between the current method and Pankow's approach is that we used a commercial smoking machine to collect mainstream smoke particulate on Cambridge filter pads (CFP) (rather than in a gas sampling bag) and sampled the headspace above the CFP enclosed in a serum vial using solid-phase microextraction (SPME) (*19*, *20*) (rather than with a solid sorbent trap). The SPME technique is ideally suited for headspace analysis of free-base nicotine because of the SPME fiber's ability to preconcentrate while sampling volatile components. Typical applications using SPME include analyses of air and water pollution (*21*, *22*), soil (*23*), volatile compounds in biological fluids (*24*), and wine (*25*). For tobacco research, the utility of SPME has been demonstrated for the analysis of various flavor additives (*26*), phenolic compounds in cigarette smoke condensate (*27*), volatile components in tobacco (*28*), and various alkaloids present in tobacco (*29*).

EXPERIMENTAL PROCEDURES

Safety. Personnel involved in weighing, diluting, or otherwise manipulating the compounds used were instructed in the safe handling of chemicals. These instructions included the wearing of personal protection items and proper laboratory practices. All compounds were handled in a fume hood, and personnel used appropriate protective safety glasses, gloves, and laboratory coats.

Materials. Chemical reagents were purchased from several commercial vendors. Nicotine (98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous NH_3 (gas) was from Air Products (Hapeville, GA). The primary labeled internal standard, toluene-d_8, was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). In the preparation of stock solutions, chemical reagents at the microliter level were transferred using positive displacement pipets. To minimize contamination, the glass pipet tips were discarded after each use. Analyte standards were prepared by successive dilutions in methanol after the neat compounds had been weighed to the nearest 0.1 mg. Final dilutions were made in water. Cigarette samples were purchased at various retail outlets in 2001−2002. The unopened cigarette packs were individually sealed in plastic bags and stored at −70 °C until needed.

Nicotine Calibration. Because nicotine is an amphiphilic molecule, that is, part hydrophilic and part hydrophobic, and is readily soluble in water (*30*), we used anhydrous NH_3 gas instead of an aqueous base to convert all of the nicotine to the free-base form for calibration. Triplicate samples were analyzed over the maximum range expected for free-base nicotine. Measurements were obtained at levels of 0.01, 0.1, 0.5, and 1.0 mg of nicotine. We found it difficult to safely add a known quantity of NH_3(g) to the vial. After evaluating several approaches, we obtained acceptable results by placing a blank CFP in a 20-mL vial, spiking with nicotine and the internal standard, and sealing the vial with a septum-lined top. The lower three-fourths of each vial was submerged in liquid N_2 and the air removed by puncturing the septum with a 20-gauge needle attached by tubing to a mechanical low-vacuum pump. The air was replaced with 20 mL of NH_3(g) drawn from a Tedlar storage bag filled with NH_3(g) using a gastight syringe, and the vial was resealed with a new unpunctured septum top.

Standardized Machine Smoking. The current method was applied to mainstream cigarette smoke collected on CFPs from 26 brands of cigarettes under FTC (*16*) conditions using a 60 s puff interval, 2 s puff duration, and 35 mL puff volume. Free-base nicotine was quantitatively determined using headspace SPME (*19*, *20*) combined with gas chromatography−mass spectrometry (GC-MS). The mainstream smoke of selected brands of cigarettes was collected using a revised version of a modified FTC protocol (*31*) analogous to that used by Labstat International Inc. (Ontario, Canada) (*32*). For each brand, a minimum of five cigarettes from different packs were smoked in parallel with collection of the total particulate matter onto CFPs using an automated Filtrona smoking machine (Filtrona Instruments and Automation Ltd., Milton Keynes, U.K.). The cigarettes were smoked to a butt length of 23 mm or the length of the filter overwrap plus 3 mm, whichever was longer. Before smoking, the cigarettes and CFPs were conditioned at 22 °C and 60% relative humidity for at least 24 h. The percentage filter ventilation for each brand was determined using a Filtrona QTM 5 from the average of five cigarette measurements. The smoking machine was calibrated to take one puff of 2 s duration and 35 mL volume every minute and to maintain an average air flow velocity over the cigarettes of 200 ± 30 mm/s. Temperature, humidity, puff volume, and air flow inside the smoking machine were checked daily.

CFP Sample Preparation. After smoking, the CFP was removed from the filter holder assembly and placed in a 20 mL serum vial along with a 50 μL aliquot from a 1 ng/μL stock solution of the toluene-d_8 internal standard, and the vial was crimped shut. Toluene-d_8 was selected as the internal standard for nicotine because of its nonpolar nature and pH-independent analytical signal. The SPME response of toluene-d_8 was consistent when spiked on a series of CFPs that had been saturated with 2 mL of aqueous buffer solutions ranging from pH 4 to 12. Use of an isotopically labeled nicotine analogue as an internal standard for the smoke samples was not possible because its analytical response depends on the acid−base mainstream smoke chemistry associated with the brand. To evaluate the utility of toluene-d_8 as the internal standard, a series of measurements of the analytical responses for free-base nicotine and toluene-d_8 were made as a function of TPM deposited on a CFP from a brand of denicotinized cigarette (Murty Pharmaceuticals, Inc., Lexington, KY). After triplicate smoking runs with 2, 4, 6, 8,and 10 puffs, 0.1 mg of nicotine and toluene-d_8 were spiked on the CFPs. As the TPM levels increase with puff count, the responses for the free-base nicotine and the toluene-d_8 both decreased. However, the free-base nicotine relative response factors showed no dependence on the TPM levels. Therefore, we concluded that the internal standard choice of toluene-d_8 was satisfactory.

SPME/GC-MS. A Leap CTC CombiPAL autosampler (Carrboro, NC) mounted on an Agilent 6890 gas chromatograph (Palo Alto, CA) automated the SPME headspace analysis. A 75-μm Carboxen/PDMS fiber (Supelco, Bellefonte, PA) was used for the headspace sampling.

After a 1 min exposure in the headspace above the CFP sample, the fiber was introduced into the heated inlet of a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA). The injection inlet, which was operated in splitless mode and maintained at 285 °C, used a narrow-bore (75 μm) inlet liner. The chromatograph was equipped with a 30 m J&W Scientific (Folsom, CA) DB-624 column with a 1.8 μm film thickness. A constant flow of 3.0 mL/min was maintained through the column using helium as the carrier gas. The following temperature program was used: hold at 50 °C for 2 min, 30 °C/min ramp to 210 °C, and hold at 210 °C for 5 min. The total run time was 12.33 min.

An Agilent model 5973 mass spectrometer was used for data acquisition. Instrument tuning and mass calibration were checked daily using perfluorotributylamine (PFTBA). Full-scan mass spectra were acquired over a mass range of 29−200 amu at a rate of 4.19 scans/s. All mass spectral results were manually evaluated for proper integration limits, correct baseline determination, interferences, and confirmatory masses. After the reconstructed ion chromatogram had been checked, the tabulated peak area data were exported to a spreadsheet program for further analysis.

RESULTS AND DISCUSSION

Like the conventional determination of total nicotine in mainstream smoke (*33*), we focused our attention on nicotine associated with the particulate portion of the smoke aerosol trapped on a CFP. All nicotine measured using the SPME headspace technique was attributed to the gaseous portion of free-base form because the protonated form has substantially lower volatility. Free-base nicotine in smoke might be expected to remain in the vapor-phase portion of mainstream smoke and pass through the CFP. To investigate this possibility, the gaseous materials that passed through the CFP were collected in Tedlar gas sampling bags during smoking. Subsequent analysis of this vapor-phase portion using SPME/GC-MS detected no nicotine in the gas sampling bags, although many other chemicals were readily detected at nanogram to milligram levels.

To serve as a further check on the possibility that some of the volatile nicotine remained in the vapor phase after passing through the CFP and was possibly lost to the walls of the tubing or collection bag, a custom CFP holder was constructed with a septum injection port located ~4 mm behind the pad. A Carboxen/PDMS SPME fiber was inserted through this port directly in the path of the vapor passing through the CFP during the smoking run. Subsequent GC-MS analysis confirmed that nicotine breakthrough was below our detection limit (4 μg). Regardless of whether some free-base nicotine passes through the CFP, the acid/base characteristics of the total particulate matter on the CFP in the sealed vial will re-establish equilibrium between the free-base and protonated nicotine. At equilibrium, the ratio of free-base and protonated nicotine will reflect the intrinsic characteristics of the smoke particulate matter trapped on the pad.

Using this technique, free-base nicotine in the headspace above the pads was measured for 26 top-selling cigarette brands selected from the four major U.S. manufacturers: Brown & Williamson, Lorillard, Philip Morris, and RJ Reynolds (**Table 1**). The free-base nicotine deliveries from the 26 brands of cigarettes when subdivided into three tar delivery categories spanned remarkably similar ranges (**Figure 1**). These delivery categories were arbitrarily assigned on the basis of FTC tar levels: (a) full-flavor (≥14 mg of tar per cigarette), (b) light (9−13 mg of tar per cigarette), and (c) ultralight (1−6 mg tar per cigarette) (*16*). When possible, we selected both soft and hard packs of each brand. For a given brand, no statistically significant differences were observed in free-base nicotine levels between cigarettes from soft or hard packs of the same brand. The CFPs were analyzed in random order some 30−180 min postsmoking, and no significant aging effects in terms of the free-base nicotine levels were observed over this time interval.

Table 1. Data Obtained for Various Brands of Cigarettes, Organized by FTC Tar Level (Full Flavor, Light, and Ultralight)

brand sorted by tar category[a]	FTC nicotine[b] (mg/cig[c])	FTC tar[b] (mg/cig)	filter ventilation (%)
full flavor			
A	0.9	15	1.6
B	1.0	14	20.8
C	1.0	16	2.2
D	1.2	16	23.7
E	1.1	15	15.1
F	1.1	15	19.9
G	0.8	16	6.0
H	1.1	15	14.0
I	1.3	17	2.1
J	1.0	14	18.3
K	1.2	17	2.0
light			
L	*c*	*c*	16.4
M	0.8	13	12.6
N	0.8	12	22.3
O	0.9	12	22.5
P	0.7	9	21.5
Q	*c*	*c*	18.6
R	0.8	11	16.1
S	0.8	10	26.6
T	0.7	9	31.6
ultralight			
U	0.1	1	82.0
V	0.4	5	43.5
W	0.5	5	52.3
X	0.5	6	46.7
Y	0.5	5	49.7
Z	0.4	4	68.4

[a] Full flavor, >14 mg of tar/cigarette; light, 9–13 mg of tar/cigarette; ultralight, 1–6 mg of tar/cigarette. [b] *FTC Report of Tar, Nicotine, and Carbon Monoxide of the Smoke of 1294 Varieties of Domestic Cigarettes for the Year 1998*, issued 2000 (protonated nicotine equated to the difference between FTC and free-base nicotine amounts). [c] Published values not available.

Pankow (*18*) published a detailed treatment of the gas/particle partitioning of nicotine and other volatile constituents in tobacco smoke and concluded that up to 40% or more of the nicotine present in mainstream smoke could be in the free-base form for some brands. Our results indicate that the average percentage of free-base nicotine in mainstream smoke was 4.5% for the selected full-flavor cigarettes, 6.8% for lights cigarettes, and 14.7% for ultralight cigarettes. However, the percentages of free-base nicotine in the mainstream smoke for ultralight cigarettes ranged from 9 to 29%, supporting Pankow's assertion that a significant fraction of the nicotine could be present in the volatile free-base form (*18*). Recent work from Pankow et al. (*16*) reported the free-base nicotine content (α_{fb} values) for 12 cigarette brands as a function of the first three puffs and the remaining eight or so puffs. Although their method employed a different analytical approach, we find excellent agreement between the two methods. They report their finding in terms of α_{fb} as a function of puffs, so we normalized these values against the puff count [$3\alpha_{fb}$(first three puffs) + $8\alpha_{fb}$(remaining eight puffs)/11] to obtain a per cigarette α_{fb} comparable to the values we obtained when the entire cigarette was smoked. Comparison of these normalized values with our α_{fb} values showed good agreement (**Figure 2**).

Filter ventilation has a strong influence on total nicotine deliveries (*33*) and was observed to have a strong impact on the free-base nicotine deliveries. Our results demonstrate that the relative percentage of free-base nicotine increased with filter ventilation levels (**Figure 3**). This behavior was also previously

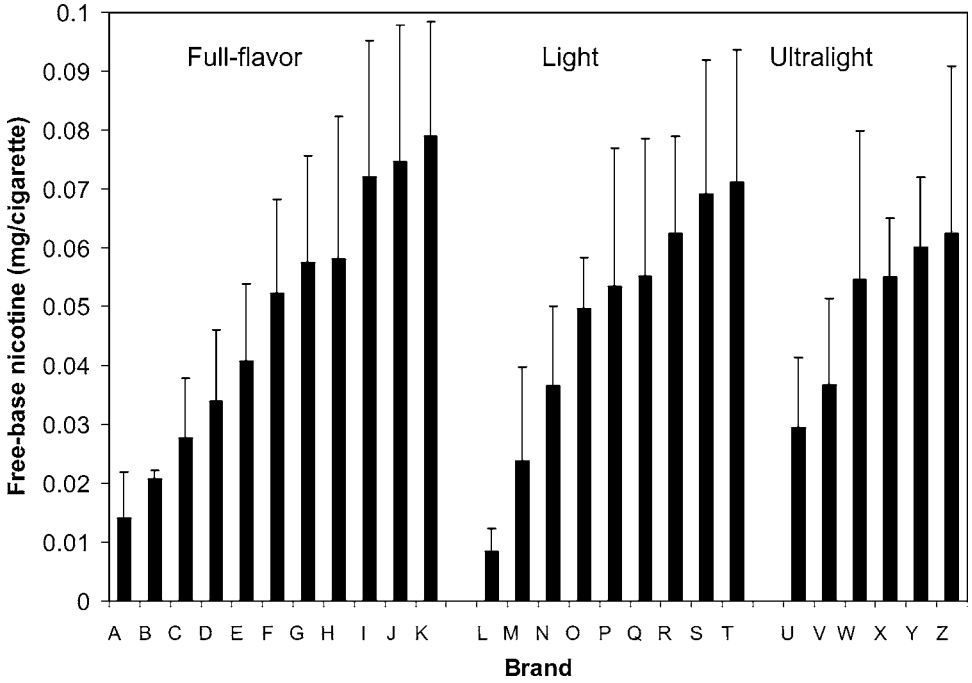

Figure 1. Amount of free-base nicotine (mg/cigarette) measured in the mainstream smoke of 26 brands of cigarettes, grouped by FTC tar level: full flavor (F), ≥14 mg of tar/cigarette; light [L and M (medium)], 9–13 mg tar/cigarette; ultralight (U), 1–6 mg tar/cigarette.

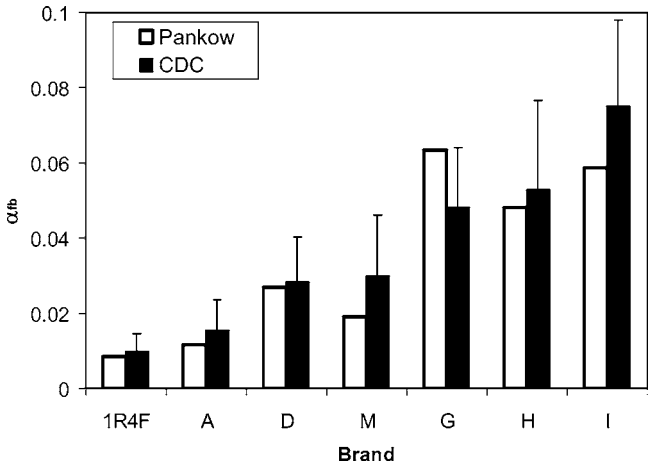

Figure 2. Comparison between the ratio of free-base to total nicotine (α_{fb}) measured free-base nicotine levels in this study with data determined independently by Pankow et al. (*16*).

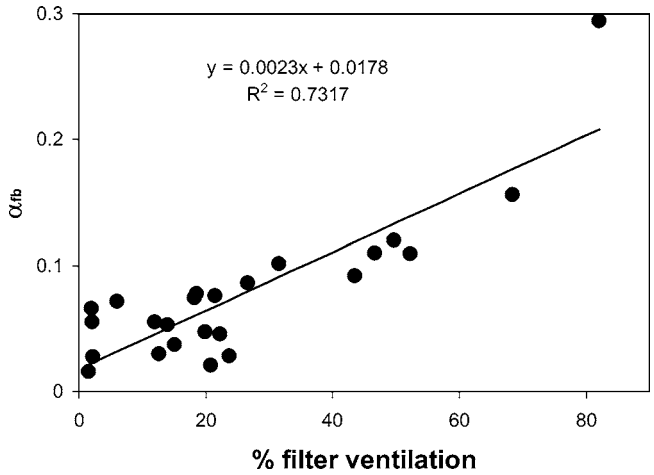

Figure 3. Relationship between the ratio of free-base to total nicotine (α_{fb}) and the percent filter ventilation for 26 brands of cigarettes.

observed in the denuder work by Lewis et al. (*34*). The consequence of increased filter ventilation may seem counterintuitive given that air dilution of mainstream smoke from filter vent holes reduces the deliveries of both tar and nicotine under machine smoking conditions, but in general we found that the higher filter ventilation levels increase the relative free-base nicotine levels in mainstream smoke. Lower tar and nicotine delivery cigarettes, as categorized using the FTC smoking protocol, may have a significant portion of their total nicotine present as the free-base form. Our findings show that low-delivery brands, that is, lights and ultralights, can produce a relatively broad range of free-base nicotine levels.

The effect of filter ventilation on free-base nicotine deliveries could be partially related to the phenomenon known as "off-gassing" (*4*, *35*). Essentially, as the filter ventilation level increases, the smoke aerosol is increasingly diluted, slowing the coalescence of the aqueous particles and resulting in increased surface area and surface residence times for evaporation. Mainstream smoke air dilution also contributes to a more dispersed deposition of TPM on the CFP. This could reduce coalescence of trapped particles and tend to maximize the particulate surface area, enhancing the evaporation of volatile species from the CFP. Regardless of the mechanism involved, we observed a relation between free-base nicotine delivery and the percentage of filter ventilation.

To further investigate the effect of filter ventilation on free-base nicotine delivery, mainstream smoke from 1R5F Kentucky reference cigarettes with unblocked, partially blocked, or totally blocked filter ventilation holes was analyzed. The 1R5F cigarettes are designed to have a contemporary American blend with low tar and nicotine deliveries due in part to the incorporation of a relatively high amount of filter ventilation (~70%). A machine-smoked 1R5F had the largest ratio of free-base to total nicotine of the cigarettes examined. However, if the ventilation holes were obstructed with tape during smoking, the relative response of total nicotine increased while the free-base nicotine decreased. These experimental results provide support that changes in the amount of filter ventilation alter the relative distribution of nicotine between the free-base and

protonated form. A direct consequence of this behavior is that a smoker can alter the amount of filter ventilation through physical obstruction of the vent holes by either their fingers or lips and significantly influence the delivery of both free-base and total nicotine on a puff-by-puff basis.

In conclusion, we have developed a methodology for directly assessing the amount of free-base nicotine in trapped mainstream cigarette smoke particulate. However, because we obtained these values from a smoking machine using a standardized smoking protocol, they do not account for individualized human smoking variations under real-world conditions. Deliveries measured for cigarettes under machine smoking conditions are best used to estimate exposure ranges and for comparative purposes. Many factors will contribute to the smoke delivery achieved by an individual smoker. Cigarette smoke is a complex mixture of chemicals that have a wide range of acid−base properties; there are likely to be chemical constituents in the filler or smoke that contribute to the ratio between free-base and protonated nicotine. In addition to chemicals in cigarette smoke, physical characteristics of the cigarette influence the amount of free-base nicotine. This influence is evident from the observed relationship between the level of filter ventilation and the measured ratio of free-base nicotine to total nicotine. Other physical characteristics of the cigarette also may influence the distribution of nicotine protonation states. Therefore, we emphasize the need for comprehensive studies on the effects of chemicals naturally present in tobacco, additives, physical properties, and real-world smoking conditions to fully investigate the nature of nicotine delivery in cigarette smoke. Such studies will allow a more accurate assessment of nicotine dosage and delivery thresholds to add to our understanding of and deal with the consequences of nicotine addiction and dependence.

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