Free-Base and Protonated Nicotine in Electronic Cigarette Liquids and Aerosols
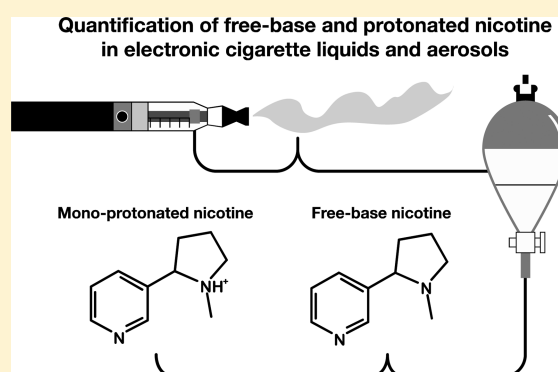
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Ⓢ Supporting Information

ABSTRACT: As with other tobacco aerosols, nicotine delivery from e-cigarettes (ECIG) depends on the total nicotine and its partitioning between free-base (Nic) and protonated (NicH$^+$) forms. Previous studies of ECIG nicotine emissions have generally reported "nicotine yield" without attention to whether the methods employed resulted in quantification of the total nicotine or only one of its forms, making reported results difficult to compare across studies or to evaluate against reported blood exposure. This study reports a convenient solvent extraction method for determining total nicotine and its partitioning in ECIG liquids and aerosols by gas chromatography. Commercial ECIG liquids and aerosols were analyzed, and it was found that most of the nicotine was in the Nic form, with aerosols exhibiting higher Nic fraction than the parent liquids. Apparent pH was found to correlate with nicotine partitioning and can provide a useful indirect measure when chromatography is unavailable. Finally, labeled ECIG liquid nicotine concentration in commercial products was often inconsistent with measured nicotine.

Quantification of free-base and protonated nicotine in electronic cigarette liquids and aerosols

Mono-protonated nicotine   Free-base nicotine

1. INTRODUCTION

Electronic cigarettes (ECIGs) are rapidly gaining popularity around the globe. This phenomenon is likely driven by a number of factors, including the perception that, in comparison to conventional cigarettes, they present a safer method for obtaining nicotine.[1,2] This perception stems from the fact that ECIGs are electrically powered devices that heat and vaporize a nicotine-containing flavored liquid to produce an inhalable aerosol, without involving combustion and presumably much of the exposure to combustion-related toxicants such as CO, PAH (polycyclic aromatic hydrocarbon), and nitric oxide that are characteristic to conventional tobacco products.[3] ECIGs liquids do not contain tobacco but rather a nicotine solution in propylene glycol or glycerol (or a mixture of both) with flavors to make the ECIG attractive.[4,5] In summary, the central concept behind smoking or what is now accepted as "vaping" is volatilization of nicotine into an aerosol of liquid droplets and vapors that will be delivered to the "vaper's" body.[6]

Nicotine has two basic nitrogen groups in its chemical structure (pk_{a_1} = 3.12; pk_{a_2} = 8.02), and thus, it can exist in three forms, namely, free-base (Nic), monoprotonated (NicH$^+$), or diprotonated salt (NicH$_2^+$) depending on the pH of the matrix.[7−9] This intrinsic characteristic is quite important since it affects the bioavailability of nicotine;[10] Nic is thought to be the

only form that diffuses through epithelial tissues in the human body. Thus, all else being equal, an ECIG with a higher proportion of Nic will increase the rate of uptake of nicotine by the user's body.

To date, most published ECIG studies have reported total nicotine content in ECIG solutions and aerosols without reference to the nicotine form. In addition to the modified NIOSH 2551 method to quantify total nicotine in cartridges, sample preparation methods included steps that converted all the nicotine in a given sample to its free-base or protonated forms by the addition of ammonia, sodium hydroxide, or acidic solutions prior to quantification by gas chromatography (GC),[11−15] without an attempt to measure each fraction separately. Hence, despite its critical importance to systemic nicotine delivery processes,[16] there is no direct information available about the free-base fraction of ECIG nicotine in liquids and aerosols. There are reports of indirect measures of the free-base fraction based on pH measurements.[17] These measures might not give accurate distribution of Nic and NicH$^+$ in the matrix due to the presence of flavors and other additives that may affect the pH measurements.[18]
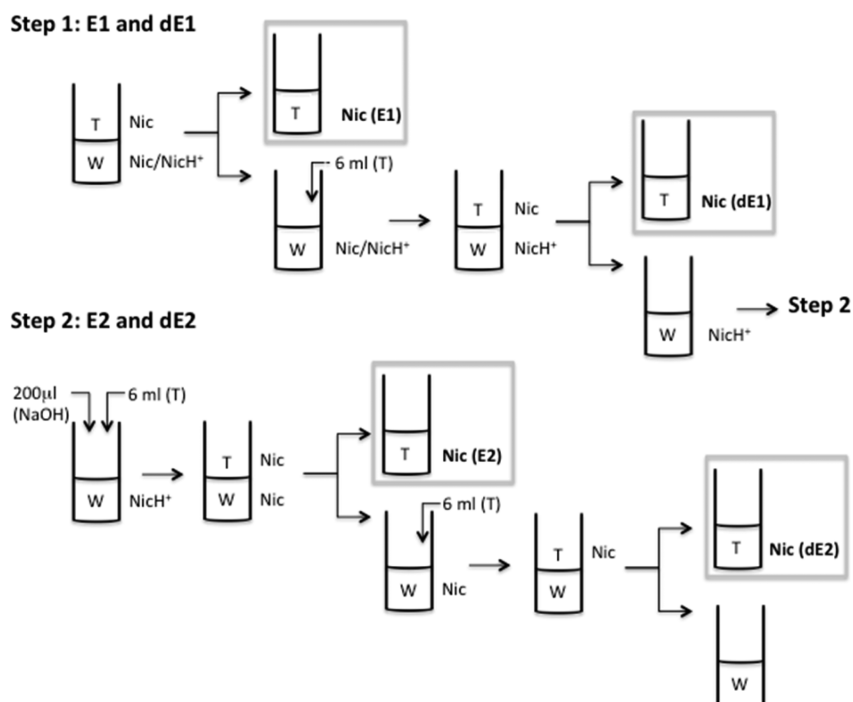
Figure 1. Schematic diagram for the extraction procedure adopted to separate Nic from NicH[+]. "T" stands for toluene and "W" for water. E1, dE1, E2, and dE2 are the toluene extracts that were analyzed by GC-MS.

In this work, Nic and NicH[+] were separated and quantified using a convenient analytical method consisting of a liquid–liquid extraction technique followed by gas chromatography coupled with mass spectrometry (GC-MS) analysis. The extraction technique uses a solvent system consisting of water and toluene and exploits the fact that due to its chemical structure, toluene will extract only Nic from a solution containing both Nic and NicH[+] (detailed discussion of the mechanism of selective extraction at different pHs is presented in the Supporting Information). In this study, we validated the analytical method against laboratory standard solutions (STD) and then deployed it to study a series of ECIG liquids and aerosols to determine the forms of nicotine present in these systems. In addition, we compared measurements of nicotine fractions to theoretical estimates based on pH and pK_a of the pyrrolidine ring. Finally, total nicotine content was compared to labeled nicotine content on commercial products.

2. MATERIALS AND METHODS

The analytical method developed in this study exploits the relative difference in solubility of Nic and NicH[+] in toluene and water, respectively. With a solubility of water in toluene of 3.3×10^{-3} mole fraction,[19,20] 6 mL of toluene absorbs a minimal amount of 3.36 μL from the water phase. The extracted Nic in the toluene phase was then introduced into a gas chromatograph coupled with a mass spectrometer for quantification. NicH[+] remaining in the water phase was transformed into Nic by sodium hydroxide and then extracted by toluene to be analyzed.

2.1. Materials. Prefilled ECIG cartridges of the Vapor for Life (V4L), V2, Green Smoke, Apollo, Bull Smoke, Halo, G6, Bluewater, and Blu brands in various nicotine concentrations were procured from US Internet vendors as were samples of ECIG liquid refill solutions of the brand My Freedom Smoke Do It Yourself (DIY) (100 mg/mL). Selected solutions were smoked (vaped) using a commercial tank based ECIG (VaporZone [Platinum], 2.4 Ohms). HPLC grade toluene (CAS registry number 108-88-3) was obtained from Aldrich. Pure nicotine (CAS registry number 54-11-5) was purchased from Acros Organics.

Hexadecane (CAS registry number 544-76-3) procured from Sigma-Aldrich was used as internal standard (IS). Glass fiber and quartz were purchased from Pall Corporation and Whatman International Ltd., respectively.

We note that the nicotine content for 9 of the 17 e-liquids studied were labeled with units "mg", without clear indication whether this corresponded to the mass of nicotine in an entire cartridge, or the mass of nicotine per mL of liquid, or both (i.e., that a given cartridge contained 1 mL of liquid). Following common practice among ECIG users[21,22] and based on communication with one of the manufacturers,[23] we interpreted for the purposes of this study the unit "mg" to mean mg/mL of ECIG liquid, and "%" to indicate mg/g, where unity specific gravity is assumed (i.e., 1.2% was taken to mean 12 mg/mL).
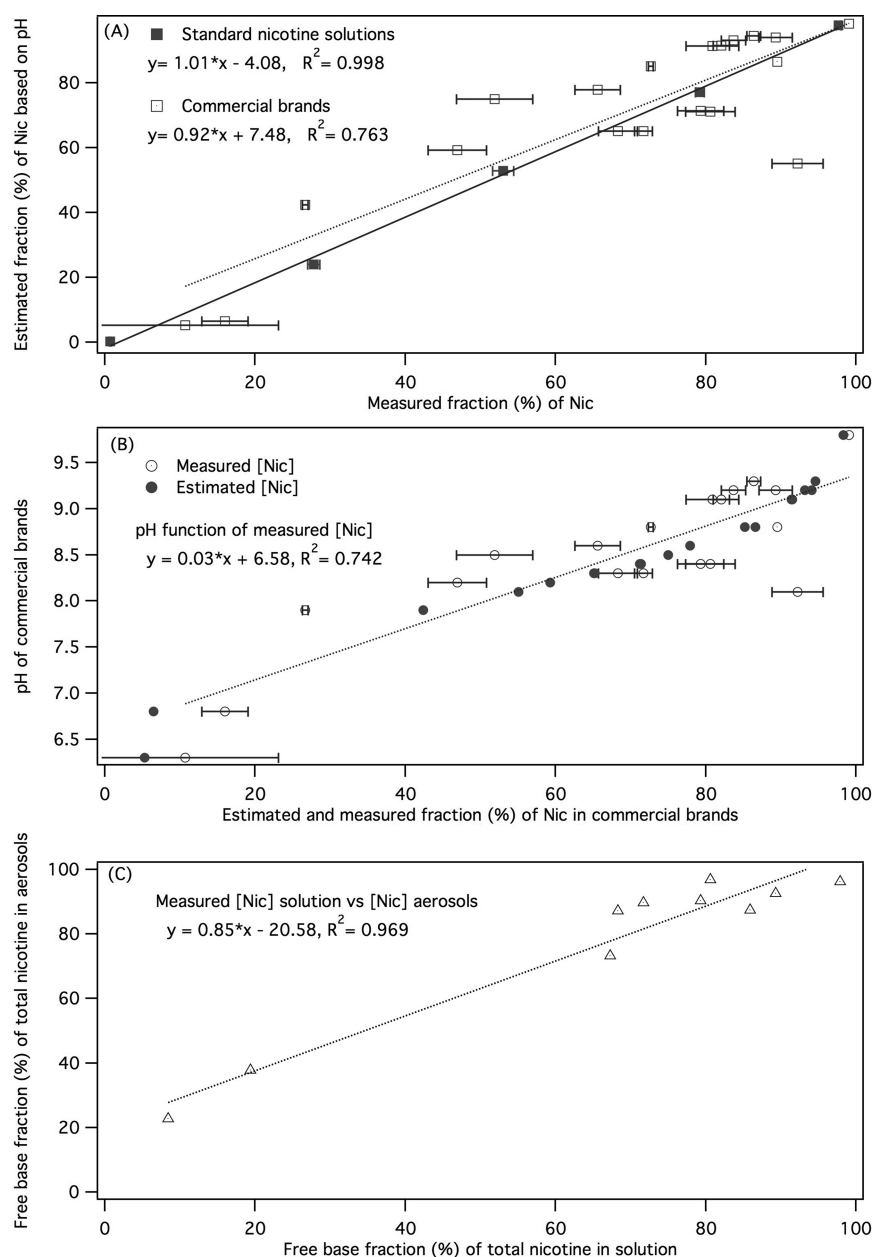
2.2. pH Measurements. The pH of the prefilled cartridges and DIY liquids and tank solutions was measured using a Starter 3100 OHAUS pH-meter. Prior to measurement, a volume of each commercial solution was added to deionized water in order to prepare a final nicotine concentration of 600 ppm in a final volume of 6 mL. It is noted that because the nicotine concentration varied across commercial ECIG refill liquids, the volume of refill liquid required to attain a final aqueous solution concentration of 600 ppm also varied across samples.

2.3. Liquid–Liquid Extraction (LLE). Samples were extracted from standard nicotine solutions, ECIG cartridges (or tanks), and filters collecting ECIG aerosols described in section 2.7 below. To extract samples from ECIG cartridges, the polyester filler was removed from each prefilled cartridge, transferred into a 1 mL plastic syringe, and then pressed into a glass vial. As with the pH measurements, a calculated volume of e-liquid is dissolved in 6 mL of water in order to prepare a final nicotine concentration of 600 ppm. Then 6 mL of toluene was added (Figure 1), and the mixture was shaken for 30 min. The mixture was then allowed to separate into toluene and aqueous phases. The toluene phase (denoted as "E1" in Figure 1), which is capable of dissolving only Nic, was removed from the sample and then introduced into a GC-MS for quantification against hexadecane internal standard (IS) added prior to injection. To verify that all Nic has been extracted, the extraction step was repeated by adding 6 mL of toluene and repeating the above steps. This resulted in a second toluene sample (denoted "dE1"). After separating toluene from water as before, 200 μL of NaOH solution (1N) was added to the aqueous layer to convert NicH[+] in solution into Nic.

Table 1. % Nic Recovery at Different Stages of the Extraction (E1, dE1, E2, and dE2) for Known Concentrations of Standard Solutions[a]

sample	pH	% E1	% dE1	total Nic	% E2	% dE2	total NicH$^+$	measured Nic/NicH$^+$ (%)	theoretical Nic/NicH$^+$ (%)
STD(1)	9.7	90.0	7.7	97.7	2.3	...	2.3	97.7/2.3 ± 0.3	97.8/2.2
STD(2)	8.5	69.3	9.9	79.2	20.8	...	20.8	79.2/20.8 ± 0.6	77.2/22.8
STD(3)	8.1	47.2	5.8	53.0	44.8	2.2	47.0	53.0/47.0 ± 1.4	52.9/47.1
STD(4)	7.5	22.3	5.5	27.8	65.9	6.2	72.2	27.8/72.2 ± 0.8	24.0/76.0
STD(5)	5.2	0.3	0.4	0.7	91.6	7.7	99.3	0.7/99.3 ± 0.1	0.2/99.8

[a]Each number is an average of triplicate samples, and the deviation from the mean is shown by the standard deviation. STD stands for standard solutions.

Figure 2. Correlations among (A) estimated versus measured free base nicotine in commercial and standard solutions, (B) pH and estimated or measured free base nicotine in commercial solutions, and (C) measured free base proportion of total nicotine in solution versus measured free base proportion of total nicotine in the aerosol phase for commercial and standard nicotine solutions.

Six milliliters of toluene was then added to the sample, and the previously described steps used to generate "E1" (and "dE1") were repeated to generate "E2" (and "dE2") samples. Thus, "E1" (and "dE1") contains Nic that was originally in the free-base form, while "E2" (and "dE2") contains Nic that was in the protonated form in the original sample.

2.4. Gas Chromatorgraphy–Mass Spectrometry (GC-MS) Conditions. The GC-MS analysis was performed on Thermo-Finnigan Trace GC-2000 Polaris QMS equipped with an AS 3000 II autosampler.

Table 2. pH Measurements, Total Nicotine, and Percentages of Nic in Several e-Liquid of Commercial Cartridges and Tanks[a]

sample (flavor)	nicotine label (mg/mL)	measured total nicotine (mg/mL)	pH	% Nic estimated	% Nic measured
DIY(2) Myfreedom smoke (unflavored)	100	97.7	9.8	98.3	99.1
DIY Myfreedom smoke (caramel capuccino)	100	97.7	8.8	86.6	89.5
V4L (tobacco)	18	7.8 ± 0.1	6.3 ± 0.7	5.3 ± 6.5	10.7 ± 12.4
V4L (strawberry)	18	14.3 ± 0.9	8.1 ± 0.3	55.1 ± 14.9	92.2 ± 3.4
V2 (red)	12	7.4 ± 0.5	9.1 ± 0.1	91.5 ± 0.9	82.1 ± 1.1
V2 (green menthol)	12	8.3 ± 0.2	8.8 ± 0.0	85.2 ± 0.9	72.7 ± 0.3
Green Smoke (red label)	24	19.5 ± 1.1	9.1 ± 0.1	91.4 ± 1.0	80.9 ± 3.5
Green Smoke (menthol ice)	24	20.1 ± 0.4	9.2 ± 0.1	93.2 ± 0.8	83.7 ± 1.6
Apollo (tobacco black)	Med	7.6 ± 0.6	8.5 ± 0.1	75.0 ± 6.6	51.9 ± 5.1
Apollo (tobacco)	12	8.1 ± 0.5	8.3 ± 0.0	65.1 ± 0.4	68.3 ± 2.6
Apollo (banana cream)	12	9.3 ± 0.4	8.3 ± 0.0	65.2 ± 1.0	71.7 ± 1.2
Apollo (blueberry kona coffee)	12	9.7 ± 0.6	8.4 ± 0.0	71.4 ± 0.2	79.3 ± 3.1
Blu (classic tobacco black)	9−12	8.8 ± 0.4	7.9 ± 0.1	42.4 ± 2.7	26.7 ± 0.4
Blu (tobacco)	13−16	13.2 ± 0.2	6.8 ± 0.1	6.5 ± 1.6	16.0 ± 3.1
Bluewater (punch lights)	8	7.7 ± 0.4	9.2 ± 0.1	94. ± 1.0	89.3 ± 2.2
Bull smoke (Turkish tobacco)	18	10.8 ± 0.5	8.6 ± 0.1	77.9 ± 3.0	65.6 ± 3.0
Bull smoke (menthol breeze)	18	15.0 ± 0.5	9.3 ± 0.0	94.6 ± 0.1	86.4 ± 0.9
Halo (Tribeca)	18	14.0 ± 0.9	8.4 ± 0.0	71.2 ± 0.6	80.6 ± 3.3
G6 (Tribeca)	18	7.4 ± 0.2	8.2 ± 0.0	59.3 ± 1.4	46.9 ± 3.9

[a]Standard deviation (±STDEV) is calculated based on the analysis of triplicate sample.

Separation was achieved with a RTx-5MS (30 m × 0.25 mm × 0.25 μm film thickness) fused silica capillary column purchased from Restek. Splitless injection mode of 1 μL was utilized. The mobile phase was helium gas with 1 mL/min flow rate. The injector temperature was set at 250 °C. The oven temperature program was 70 °C for 2 min, 20 °C/min to 230 °C, and held for 1 min. The total run time was 11 min, and solvent delay time was 4 min. Quantification was done in the ion current mode (m/z = 84 for nicotine and 57 for IS).

2.5. Analytical Characteristics of the LLE Method. A standard calibration curve was prepared by extracting a range of concentrations (50−1000 ppm) (diluted 50 times before injection onto the GC) of laboratory-prepared standard nicotine solutions following the afore-mentioned method. The linearity of the method was assessed by linear regression, and the correlation coefficient (R^2) was found to be greater than 0.997. The accuracy of the method was measured by spiking blank filters with Nic standard solutions of pH 9.7 at a concentration of 500 ppm, and the recovery was found to be 101.0 ± 3.2%. Two triplicate measurements, which were completed by two different operators, were used to determine the recovery. The yields of % RSD were <4%. Furthermore, the precision of the method was examined by quantifying six replicate samples at three different concentrations spanning the whole calibration curve. These samples were subjected to the entire extraction procedure, and the relative standard deviation (% RSD) at each concentration was found to be ≤6%.

2.6. Validation of the LLE Method Using pH-Manipulated STD Nicotine Solutions. It has been reported that toluene is an effective agent for nicotine extraction in the liquid−liquid equilibrium of the nicotine/water/toluene system[20] and that, in comparison to other common solvents, toluene has the lowest water solubility.[24] Using the reported solubility of water in toluene of 3.3×10^{-3} mole fraction,[19] we calculated that less than 0.1% by volume of water can be dissolved in the toluene extract, resulting in a similarly negligible bias error in the determination of NicH$^+$. A high percent total Nic recovery of 92.1% (% Nic$_{E1}$ = ((Nic$_{E1}$)/(Nic$_{total}$)) = 92.1%) was obtained when the extraction method was tested on standard solutions of known concentrations as shown in Table 1. Furthermore, in order to ensure that we only extracted Nic with toluene, the extraction method was tested on acidified samples (STD(5), pH 5.2). More than 99% recovery of total nicotine was found in the form of NicH$^+$ in E2 and dE2, and less than 1% Nic was recovered from E1 and dE1 (for more details on the mechanism of selective extraction at different pHs, check Supporting Information).

For external validation, five standard solutions at pHs ranging between 5.2 and 9.7 were prepared and tested (Table 1). Starting with

an original pH nicotine solution of 9.7, the STD solutions were prepared by adding the appropriate amount of acetic acid in order to reach the sought pHs. Measurements were then compared to the theoretical estimations using the Henderson−Hasselbalch equation (pH = pK_a + log[Nic]/[NicH$^+$]). It was found that at 7 < pH < 9 both forms of nicotine (Nic and NicH$^+$) were present in solution and that at pH > 9 or <5, Nic or NicH$^+$ predominates, respectively. Measurements were in excellent agreement with the estimated Nic/NicH$^+$ ratios with an R^2 > 0.998 and a slope of 1.01 as shown in Figure 2A.

2.7. Aerosol Generation and Sampling. A custom-designed digital puff production machine[25] was used to generate ECIG aerosols from the cartridges.[26] Puff topography (puff duration, interpuff interval, and flow rate) was selected to represent an experienced e-cigarette user (4 s puff duration and 10 s interpuff duration) with a puff velocity of 1 l/min.[26−29] The ECIG was powered using 3.3 V regulated DC power supply representing an average of the most commonly applied voltages (2.6−4.1 V). Total particulate matter was collected by drawing the ECIG aerosol generated in 15 puffs through a filter trap located at the ECIG mouthpiece outlet. Immediately after the 15th puff, each filter was immersed in 6 mL of water and shaken for 30 min, and then the same liquid−liquid extraction steps described above were carried out.

To investigate potential biases due to the interaction of nicotine with different filter materials, a sample of STD solution with pH of 8 was placed on quartz and glass fiber filters and then extracted. The Nic/NicH$^+$ ratio was found on the quartz filter to be 49.7/50.3, which is consistent with the predicted partitioning described above. In contrast, the solution extracted from the glass fiber filter condition exhibited a 100/0 ratio, clearly indicating that the glass fiber filter constructed from a basic borosilicate glass had converted all NicH$^+$ to Nic. Quartz filters were therefore used for the remainder of the study. We note that puff profile was not affected by the choice of filter material because the puff generating machine automatically compensates for varying flow resistance within and across puffs.[25]

Five analytical standard and five commercial ECIG liquids were aerosolized. The analytical standard solutions (labeled "STD6-STD10") were prepared by adding different amounts of pure and concentrated acetic acid to propylene glycol solutions of nicotine with different Nic/NicH$^+$ ratios each at a concentration of 8417 μg/mL.

3. RESULTS AND DISCUSSION

3.1. Quantification of Nic and NicH$^+$ in DIY, Cartridges, and Tank Solutions. pH measurements of different prefilled ECIG cartridges and e-liquid tanks showed a range of 6.3 < pH <

9.3 (Table 2). Similar results were recently reported by Stepanov and Fujioka.[30] The range of reported pH values suggested and confirmed that e-liquid solutions contain both Nic and NicH+.

As shown in Figure 2, measured free-base fractions in the commercial brands were correlated with estimated values only when the values for V4L (strawberry) were removed ($R^2 = 0.87$; $p < 0.05$). If added, R^2 becomes 0.76 but with a $p < 0.3$ (Figure 2A). A more significant correlation ($R^2 = 0.998$, $p < 0.001$; Figure 2A) was observed between measured and estimated Nic in laboratory-prepared analytical solutions. The correlation between measured free-base fractions in the commercial brands and pH was found to be $R^2 = 0.74$ and $p < 0.001$ in Figure 2B, indicating that pH can be used as an indicative measure for nicotine partitioning but lacks precision. The relative scatter between estimated and measured Nic fractions may be due to the fact that the Henderson–Hasselbalch equation does not account for the effects of glycols and flavor additives on the pH of the commercial solutions.

Table 2 also shows a wide range of discrepancies (1–67%) between labeled and measured total nicotine (Nic + NicH+) concentrations. These discrepancies may result from poor quality control at the manufacturing facility, from variations in label definitions across brands, or both. As mentioned in the Materials and Methods section, nicotine content labels are commonly ambiguous as to the meaning of the reported numbers. The discrepancies shown in Table 2 underscore the need for the standardization of nicotine labeling and product conformance standards, as some users may attempt to regulate nicotine intake based on otherwise misleading user labels.

3.2. Quantification of Nic and NicH+ in ECIG Aerosols. For both analytical standard and commercial solutions, free-base nicotine fraction in the sampled aerosols was found to be highly correlated to that in the parent liquid ($R^2 = 0.94$; $p < 0.001$) (Figure 2C). This finding indicates that differences in nicotine partitioning across ECIG liquids carry over to the inhaled aerosol and may influence nicotine delivery (Table 3). To a significant,

Table 3. Comparison of the Percentage of Nic in e-Liquid and Its Corresponding Aerosol for Standard and Commercial Samples

sample (flavor)	nicotine label	liquid % Nic	aerosols % Nic
STD nicotine (6)[a]	8.4	98.0 ± 0.3	96.2 ± 0.3
STD nicotine (7)[a]	8.4	85.9 ± 0.8	87.4 ± 0.3
STD nicotine (8)[a]	8.4	67.3 ± 2.4	73.2 ± 0.3
STD nicotine (9)[a]	8.4	19.4 ± 0.9	37.8 ± 0.3
STD nicotine (10)[a]	8.4	8.4 ± 0.4	22.7 ± 0.3
Apollo (tobacco)	12	68.3 ± 2.6	87.2 ± 1.8
Apollo (banana cream)	12	71.7 ± 1.2	89.7 ± 0.6
Apollo (blueberry kona coffee)	12	79.3 ± 3.1	90.3 ± 0.4
Bluewater (punch lights)	8	89.3 ± 2.2	92.6 ± 1.8
Halo (Tribeca)	18	80.6 ± 3.3	96.9 ± 1.3

[a]STD Nicotine: standard nicotine solutions. Standard deviation (±STDEV) is calculated based on the analysis of triplicate samples.

although lesser extent, the Nic fraction was also correlated to the pH of the liquid solution ($R^2 = 0.81$; $p < 0.001$). Aerosols derived from analytical standard and commercial solutions are enriched with Nic. This finding is consistent with the notion that Nic is the only form that can be volatilized upon heating.[18] We speculate that the presence of NicH+ in the aerosol likely derives from the recombination of Nic with H+ in the PG/VG matrix of the aerosol droplets as they condense downstream of the ECIG heater. In summary, the Nic fraction in ECIG aerosols depends on that of the liquid, which in turn is related to the liquid's pH. This finding suggests that partitioning in the parent liquid determines that in the ECIG aerosol.

4. CONCLUSIONS

Free-base and protonated nicotine fractions in ECIG liquids and aerosols can be conveniently and precisely analyzed using the liquid–liquid extraction (LLE) method presented here. Using this method, it is reported that nicotine partitioning varies considerably across commercial ECIG liquids and that these differences can persist when the liquids are vaped. To the extent that the nicotine form can affect nicotine delivery to the bloodstream, these findings suggest that ECIG liquids of a given total nicotine concentration may result in different nicotine uptake efficiencies when vaped. Future studies should empirically investigate this possibility in order to better inform product regulation.

■ ASSOCIATED CONTENT

S Supporting Information

Mechanism of selective extraction of nicotine by toluene at different pHs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00107.

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Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Food and Drug Administration.
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

ECIG, electronic cigarette; Nic, free-base nicotine; NicH+, protonated nicotine; PAH, polycyclic aromatic hydrocarbons; NIOSH, National Institute for Occupational Safety and Health; DIY, do it yourself solution; STD, standard solution; IS, internal standard; LLE, liquid–liquid extraction; STDEV, standard deviation

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