ARTICLE

Colorimetric assay for the rapid determination of free-base nicotine in e-liquid

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Nicotine exists in e-liquids primarily as the monoprotonated form and free-base form, the former is absorbed by the smoker relatively slowly and the latter is considered the bioavailable form of nicotine. Nowadays e-liquids manufacturers tend to increase nicotine in smoke aerosols, upto a content comparable to conventional cigarettes. Organic acids are added to suppress nicotine in free-base from, because the quick absorption of free-base nicotine (FBN) by the upper respiratory tract produces more bitterness and harshness to smokers. Although several methods have been developed to access FBN in conventional cigarettes or electronic cigarettes, spectrometric methods have rarely been reported. A water-solubility indicator Alizarin Red S (ARS) was introduced for the measurement of free-base nicotine. Since ARS exhibits lower acidity than organic acids, it does not compete for the tertiary amine with organic acids, but can only interact with FBN. The ARS turns from pale yellow to pink once it has been deprotonated by nicotine, and the binding constant between ARS and nicotine was determined to be 1.08×10^6 M^{-1}. A linear calibration curve $A = 0.0056\ c + 0.3309$ with $r^2 = 0.9984$ as a function of FBN was constructed, and applied for the evaluation of FBN in prepared e-liquid samples, with RMSE 1.12 mg/g for the 20 mg/g liquids, and 1.37 mg/g for the 50 mg/g liquids. The evaluation of FBN in commercial e-liquids agreed well with published e-liquid values. It is believed that the convenient method herein developed will be useful for manufacturers to balance the strength and harshness levels of nicotine in e-liquids.

1 Introduction

Electronic cigarette aerosolizes a nicotine-containing solution known as e-liquid without combustion or smoke. This nicotine delivery system is gaining rapid acceptance as it is argued though not proven to be safer than conventional cigarettes.[1,2] A CDC survey demonstrated that in 2017 2.8% of U.S. adults were current e-cigarette users and in 2018 more than 3.6 million U.S. middle and high school students used e-cigarettes.[3,4] Nicotine has three forms (Scheme 1): diprotonated, monoprotonated and unprotonated/free-base. The free-base form is lipophilic and absorbed through the skin and mucous membranes more quickly than its protonated forms,[5] which produce bitterness and greater harshness sometimes described by smokers as 'throat hit'.[6] Before 2015 most e-liquids were in the 2% nicotine range, but nowadays e-liquids with nicotine content higher than 5% have been introduced by manufacturers, with the aim of offering comparable amounts of nicotine to conventional cigarettes.[7] Accompanied with the dramatic increased use of nicotine in e-liquids, organic acids are often added to commercial e-liquids to generate so-called 'nicotine salt', since controlling the form of nicotine will improve palatability especially when administrating e-liquids with high nicotine level.[8]

Scheme 1 Nicotine in three forms

The tertiary nitrogen on the pyrrole fragment is much more electron-rich than that on the pyridine, and easier to be protonated (pK_1=3.12 and pK_2 = 8.02 for the diprotonated form and monoprotonated form respectively, 25 ℃).[9] Smoke aerosol contains primarily free-base and monoprotonated forms because conditions in the aerosol particulate matters (PM) are not considered to be sufficiently acidic to generate significant diprotonated nicotine.[10] The partitioning of nicotine between its forms is similar in smoke aerosol to its e-liquid,[11] thus free-base nicotine (FBN) levels could be estimated from pH measurement.[12-14] FBN in e-liquids could also be determined by liquid-liquid extraction,[11] however the multi-step operation might perturbate nicotine partitioning between the organic and aqueous phases. Headspace solid-phase microextraction (HS-SPME) combined with GC/MS methods have been developed to distinguish volatile FBN from its protonated form by Waston *et.al*.[15] Although this method is simple, reproducible and well suited to the determination of free-base nicotine in cigarette

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smoke, it is highly dependent on the extraction temperature and the water content of the smoke PM.[16] Recently Duell *et.al* reported the direct measurement of the FBN fraction in e-liquid using proton nuclear magnetic resonance (^{1}H-NMR).[17] Basically the difference between chemical shifts $\Delta\delta$ of protons on the methyl- group and protons on the pyridinyl group were acquired using ^{1}H-NMR, and the FBN fraction could be calculated as the chemical shifts of methyl- protons are low-field shifted dramatically upon protonation of the nitrogen. This method is claimed to be the most direct way to look into the protonation of nicotine since no treatment is required prior to instrumental measurement.

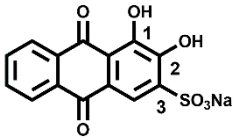


Figure 1 Structure of Alizarin Red S

The water-soluble indicator Alizarin Red S (ARS, Figure 1) had been widely applied in food, environmental and biological analysis.[18-22] In this work ARS was introduced to interact with nicotine and underwent a color change from pale yellow to pink upon deprotonation of its catecholic hydroxyl group. The phenol group at position **2** showed stronger dissociation constant (pK_1 = 6.57)[23-25] than phenol (pK = 9.99) due to the electron-withdrawing property of the adjacent anthraquinonic carbonyls, but weaker acidity than normal organic acids (pK = 3~4)[9]. Once nicotine is protonated by organic acids, ARS is not sufficiently competitive thus can only react with FBN, and quantification of FBN can be achieved by monitoring absorption spectrometry. Based on this scenario a handy colorimetric assay has been developed to determine the FBN in e-liquids. GC-MS instrumentation was used to validate the total nicotine content in the e-liquids.

2 Experimental

Reagents and samples

Alizarin Red S (95+%) was obtained from Matrix Scientific. Benzoic acid (99.0%), malic acid (99.0%), citric acid (98.0%) were obtained from TCI. Ethanol (HPLC), glycerine (99%), propylene glycol (99.5%) and isopropanol (99.5%) were purchased from JKChemical. Nicotine (99.5%) was obtained from Hubei Heno Biological Engineering Co., LTD for e-liquid preparation. Nicotine (Analytical Standard) was purchased from Alfa Aesar for analytical purpose. Heptadecane (Analytical Standard) was purchased from Aladdin Chemicals as GC internal standard. Deionized water used in this study was produced using a laboratory ultrapure water system (Millipore Milli-Q Integral 15), and the resistivity was as high as 18.2 Ω. Tobacco extract was provided by Fujian Tobacco company.

E-liquid samples were purchased from an online retailer and stored in their original container until analysed. Samples in cartridge form were uncapped, and the contained liquids were collected in a vial. Refillable samples were used as provided. Laboratory e-liquids were prepared by dissolving the corresponding amount of nicotine, organic acids and tobacco extracts in 1:1 glycerine-propylene glycol.

Apparatus and instrumentation

All UV-Vis spectra were recorded on a PerkinElmer Lambda 35 spectrometer. The slit width was set at 1 nm. All measurements took place in a quartz cuvette with path length 1.0 cm.

Nicotine GC analysis was performed using an Agilent 7890A GC system, which injects 1.0 µL of sample per vial for analysis. The injector was maintained at 250 ℃ with a helium flow rate of 1.5 mL/min for 3 min. Injections were made with a split ratio of 20:1. The chromatographic separation was accomplished using a DB-WAX capillary column (30 m x 250 µm x 0.25 µm, J&W Scientific) with helium as the carrier gas. The GC ramp conditions were as follows: 160 ℃ for 4.5 min; ramp at 30 ℃ /min to 200 ℃, for 1.5 min. The detector temperature was set at 250 ℃.

Sample preparation and analysis procedures

Nicotine titration. 0.0342 g ARS was accurately weighed and dissolved in 500.0 mL deionized water to produce a 200 µmol L^{-1} ARS stock solution. 0.0405 g nicotine was accurately weighed and dissolved in 25.0 mL ethanol to give a 10 mmol L^{-1} nicotine solution. Appropriate amounts of nicotine were injected to 2 mL of the ARS stock solution, and the UV-Vis spectra was recorded to follow the nicotine-ARS reaction.

Job's Plot experiment. Achieved by combining different volumes *i.e.* 0, 80, 160, 240, 320, 400, 480, 560, 640, 720 and 800 µL of nicotine (5.0 mmol L^{-1}) with 800, 720, 640, 560, 480, 400, 320, 240, 160, 80 and 0 µL of ARS (5.0 mmol L^{-1}) respectively into a 10.0 mL volumetric flask, and water was added to produce a total concentration of nicotine and ARS of 400 µmol L^{-1}. The absorption at 518 nm was plotted against the mole fraction of nicotine.

Free-Base Nicotine measurement. 100 mg e-liquid was dissolved in 1 mL ethanol to produce a sample solution. 10 µL of the sample solution was injected into 2 mL of the ARS stock solution, and the UV-Vis spectra was recorded.

Total nicotine measurement. 200 mg e-liquid was weighed and dissolved in 10 mL isopropanol, spiked with heptadecane internal standard of 1.3 mmol L^{-1}. The resultant solution was subject to GC analysis directly. Nicotine standard solutions spiked with heptadecane internal standard were also prepared to generate a calibration curve for nicotine analysis.

All samples were analyzed in duplicate unless otherwise mentioned.

3 Results and discussion

3.1 Spectroscopic properties

ARS bears two hydroxyl group in positions **1** and **2**, and a sulfonic group in position **3** on the catechol fragment, ensuring its high solubility in water. As shown in Figure 2, in the visible region ARS displays an absorption maxima at 423 nm, which can be ascribed to the neutral state of the catechol fragment. Upon addition of nicotine, the solution turned from pale yellow to

pink instantly. A new absorption peak appeared at *c.a.*518 nm, accompanied by a decreased absorption at 423 nm, with an isobestic point at 451 nm. This transition is in accordance with the transformation of the catechol fragment from neutral state to the mono-anion state in weak basic solution.[23] After benzoic acid was added to the solution, the acid-base equilibria is reestablished, as a result a yellowish solution is observed indicating that the ARS returned to its original state. The solution was stable for 15 min under ambient conditions (Figure S1).

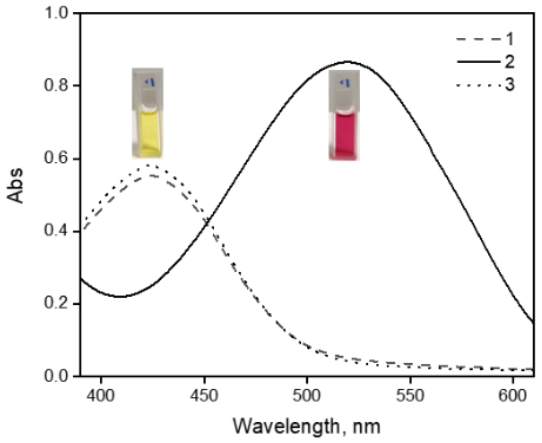


Figure 2 UV-Vis spectra of Alizarin Red S in the presence of nicotine and organic acid in water, 1.ARS, 2.ARS + Nicotine, 3.ARS + Nicotine + Benzoic acid.

3.2 Stoichiometry and mechanism

Detailed experiments have been carried out to configure the interaction of nicotine and ARS (Figure 3). Gradual addition of nicotine to 100 µmol L^{-1} ARS solution, results in the absorption at 518 nm increasing almost linearly and reaching a plateau when the content of nicotine was above 100 µmol L^{-1}. Following the absorption at 518 nm and fitting the data to a 1:1 binding algorithm[26] a binding constant of 1.08 x 10^6 L mol^{-1} was obtained (Figure S2). The Job's plot also revealed 1:1 binding stoichiometry between nicotine and ARS (Figure 4).

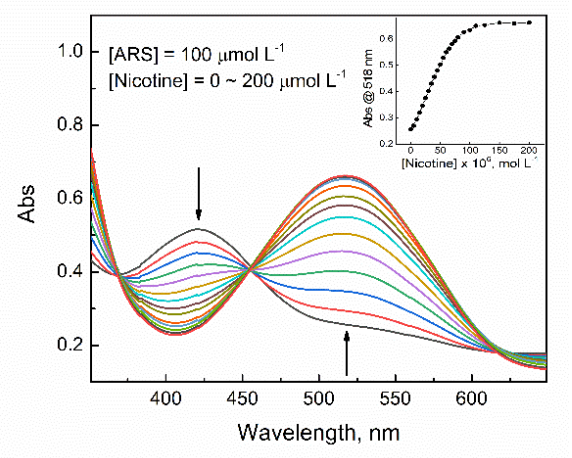


Figure 3 UV-Vis spectra of ARS in the presence of nicotine over 0 to 200 µmol L^{-1} ranges. Inset shows the absorbance at 518 nm as a function of nicotine concentration. [ARS]=100 µmol L^{-1}.

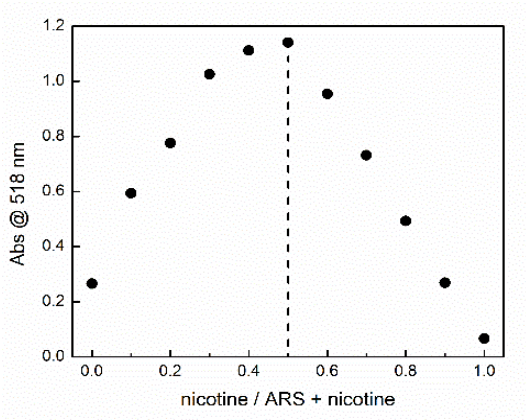


Figure 4 Job's plot of ARS and nicotine. The total of concentration of ARS and nicotine was kept constant at 400 µmol L^{-1} in water. The absorption was measured at 518 nm.

Incremental addition of benzoic acid (monotopic), malic acid (ditopic) and citric acid (tritopic) respectively to the ARS-nicotine complex solution, was performed to investigate their interaction towards nicotine. As shown in Figure 5, in the presence of excess ARS, benzoic acid sensitively reduced the solution absorbance at 518 nm as expected, indicative of liberation of ARS from the complex since benzoic acid is more affinitive to nicotine. Malic acid and citric acid showed similar but more significant effects than benzoic acid as they bear two and three acidic carboxyl groups respectively. The gradual dissociation constants p*K*s for malic acid are 3.40 and 5.11, while those for citric acid are 3.13, 4.76 and 6.40,[9] as such only 1:2 acid-nicotine binding occurs effectively as the third carboxyl group of citric acid is much weaker than the other two. Thus, it can be assumed that when organic acids and ARS coexist in an aqueous solution, a competition for the tertiary amine on nicotine exists. However, the nicotine preferably interacts with organic acids as they are significantly more acidic, leaving ARS to be deprotonated only by FBN (Scheme 2).

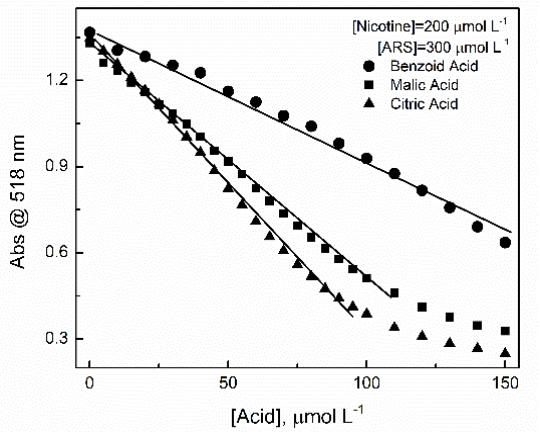
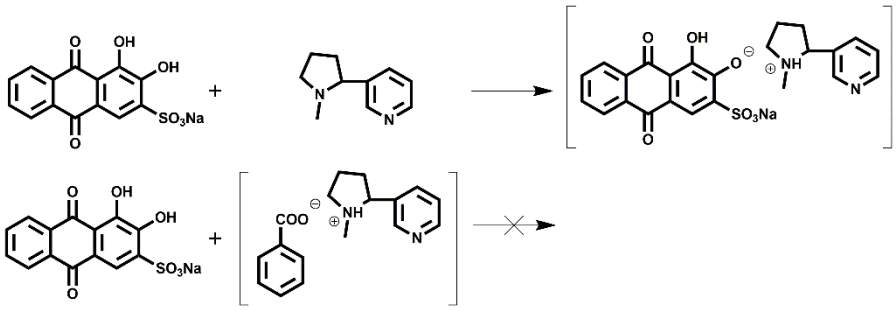


Figure 5 Absorbance at 518 nm of Nicotine-ARS solution as a function of concentration of organic acids. [Nicotine] = 200 µmol L^{-1}, [ARS] = 300 µmol L^{-1}, ●Benzoic Acid, ■Malic Acid, ▲Citric Acid.



Scheme 2 proposed interaction of ARS and nicotine in the absence and presence of benzoic acid.

Table 1 Predicted and determined free-base nicotine in 28 laboratory prepared e-liquids

E-liquid	Total nicotine, mg/g	Organic acid	Nicotine-Acid Ratio	Tobacco Extract	Predicted FBN, mg/g	Determined FBN, mg/g	%Difference from prediction	%FBN fraction
1	20	/	/	/	20.0	21.0	5.0%	100
2	20	Benzoic acid	5:1	/	16.0	16.7	4.4%	83.5
3	20	Benzoic acid	5:2	/	12.0	12.3	2.5%	61.5
4	20	Benzoic acid	5:3	/	8.0	7.6	-5.0%	38.0
5	20	Benzoic acid	5:4	/	4.0	3.5	-12.5%	17.5
6	20	Benzoic acid	5:5	/	0	1.1	NA	5.5
7	20	Malic acid	10:1	/	16.0	16.5	3.1%	82.5
8	20	Malic acid	10:2	/	12.0	13.1	9.2%	65.5
9	20	Malic acid	10:3	/	8.0	8.8	10.0%	44.0
10	20	Malic acid	10:4	/	4.0	5.6	40.0%	28.0
11	20	Malic acid	10:5	/	0	3.2	NA	16.0
12	20	Citric acid	10:1	/	16.0	16.6	3.8%	83.0
13	20	Citric acid	10:2	/	12.0	11.6	-3.3%	58.0
14	20	Citric acid	10:3	/	8.0	7.3	-8.8%	36.5
15	20	Citric acid	10:4	/	4.0	3.9	-2.5%	19.5
16	20	Citric acid	10:5	/	0	1.3	NA	6.5
17	50	Benzoic acid	25:15	/	20.0	21.5	7.5%	43.0
18	50	Benzoic acid	25:17	/	16.0	17.1	6.9%	34.2
19	50	Benzoic acid	25:19	/	12.0	12.8	6.7%	25.6
20	50	Benzoic acid	25:21	/	8.0	8.6	7.5%	17.2
21	50	Benzoic acid	25:23	/	4.0	4.7	17.5%	9.4
22	50	Benzoic acid	25:25	/	0	2.5	NA	5.0
23	20	Benzoic acid	/	5%	20.0	18.5	-7.5%	92.5
24	20	Benzoic acid	5:1	5%	16.0	15.1	-5.6%	75.5
25	20	Benzoic acid	5:2	5%	12.0	11.3	-5.8%	56.5
26	20	Benzoic acid	5:3	5%	8.0	7.5	-6.3%	37.5
27	20	Benzoic acid	5:4	5%	4.0	2.7	-32.5%	13.5
28	20	Benzoic acid	5:5	5%	0	<LOD	NA	NA

Note: 1.E-liquids were prepared using 1:1 propylene glycol and glycerin as solvent, 2.Predicted values were calculated based on the stoichiometry of nicotine and effective carboxyl groups, 3. FBN fractions were calculated with measured FBN over total nicotine, 4.NA = not applicable, 5.LOD = limit of detection.

3.3 Method validation

Based on the scenario, our assay employed an ARS solution of 200 μmol L^{-1} to generate a calibration curve A = 0.0056 c + 0.3309 with r^2= 0.9984, where c in μmol L^{-1} is the concentration of nicotine in test solution, and A is the absorbance at 518 nm wavelength, covering the content of FBN range from 0 to 32.4 mg/g (Figure S3). The limit of detection (LOD) of this assay is 0.69 mg/g in e-liquid, which was calculated by using equation LOD = $3S_d/S$, where S_d is the standard deviation of the blank measurement and S is the slope of calibration curve.

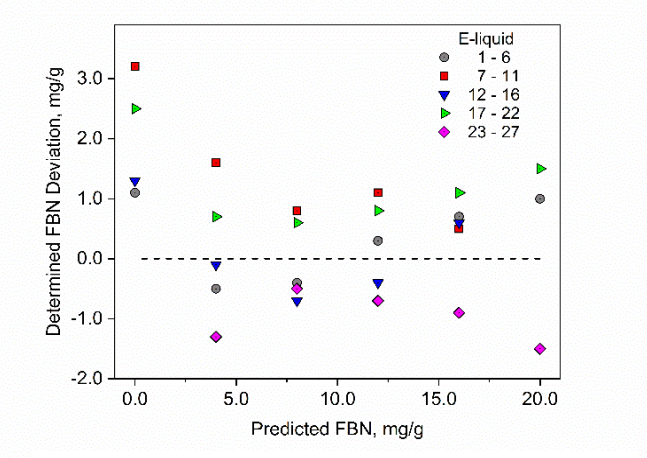


Figure 6 Deviation of determined FBN in e-liquids 1-27 from predicted values

Laboratory prepared e-liquids with varying formulation of nicotine, organic acids and tobacco extract have been used to validate the above method. Before spectrometric measurement, 100 mg of an e-liquid was dissolved 1 mL of ethanol, followed by transferring 10 μL of the ethanol solution into 2 mL of ARS solution. As shown in Table 1 and Figure 6, generally the determination of FBN in e-liquids with benzoate counter anion agreed well with the prediction, except for a few samples with low FBN concentration. For e-liquids 17-22, containing up to 50 mg/g total nicotine, higher FBN values were determined, but the differences from predicted were less than 17.5%. Since tobacco normally contains various organic acids,[27] lower contents of FBN were expected in e-liquids 23-28. With malic acid and citric acid, deviation could be observed in e-liquids 7-16, where the highest difference from predicted was 40.0% for malic acid. Since malic acid is less acidic than citric acid, more nicotine was left in the solution as free-base from, therefore the predicted free-base value may not correlate with the stoichiometry of nicotine and carboxyl groups. The deviation for the laboratory prepared e-liquids gave root mean squared error (RMSE) of 1.12 mg/g for the 20 mg/g liquids, and 1.37 mg/g for the 50 mg/g liquids, demonstrating the potential of this method to accurately evaluate FBN in e-liquids.

3.4 Commercial e-liquid test

E-liquids of various brands were subject to evaluation of free-base nicotine (Table 2) and the FBN fraction agreed well with the published values.[10-12] It should be noted that although color had been observed, all e-liquids were transparent and two thousand times dilution was performed prior to spectrometric measurement, so it was supposed that the visible absorption would not interfere with the intrinsic chromophores from e-liquids. For e-liquids announced use of nicotine salt, FBN was suppressed to less than 5% with success. Exceptions were found for Vype and Langsen, with FBN fraction 13.4% and 13.7%, maybe due to the manufacturer's consideration to offer sufficient strength and reasonable harshness. For e-liquids not using nicotine salt, nicotine was protonated by the acids introduced from flavor constituents, so the FBN was less than the total nicotine to some extent. Discrepancies between the labeled nicotine content and measured total nicotine were also observed in the samples tested. For example, the VP (Fresh Mango) and VP (Hazelnut Coffee) were labeled as nicotine 40 mg/g, whilst the measured values were 28.1 mg/g and 27.8 mg/g, respectively. This might be attributed to the poor quality control at the manufacturing facility, but does mislead users as some users attempt to regulate nicotine intake based on the label values.

Table 2 Total nicotine and free-base nicotine in 15 commercial e-liquids

E-liquid	Region	Color	Nicotine salt	Label Nicotine	Total Nicotine, mg/g	FBN, mg/g	%FBN fraction
Juul (Cool Mint)	USA	Pale yellow	Yes	5%	48.4	<LOD	NA
Juul (Virginia Tobacco)	USA	Pale yellow	Yes	5%	48.7	1.2	2.4%
Juul (Crème Brulee)	USA	Golden yellow	Yes	5%	50.5	1.9	3.8%
Juul (Mango)	USA	Pale yellow	Yes	5%	50.6	<LOD	NA
iQOS-VEEV (Mellow Tobacco)	EU	Colorless	No	18 mg/mL	16.5	15.1	91.5%
iQOS-VEEV (Red Berry Fushion)	EU	Pale red	No	11 mg/mL	10.1	8.9	88.2%
Vype (Wild Berries)	UK	Light brown	Yes	18 mg/mL	13.4	1.8	13.4%
Apollo (Classic Tobacco)	USA	Brown red	No	6 mg/mL	5.2	2.4	46.6%
Relx (Tropical Fruity)	China	Dark yellow	Yes	50 mg/g	50.3	<LOD	NA
Relx (Chinese Tobacco)	China	Golden yellow	Yes	50 mg/g	50.3	1.4	2.7%
VP (Fresh Mango)	China	Golden yellow	Yes	40 mg/g	28.1	1.6	5.5%
VP (Hazelnut Coffee)	China	Brown	Yes	40 mg/g	27.8	0.9	3.4%
Ovale (Milan 7)	China	Pale yellow	No	3 mg/mL	3.1	1.8	57.4%
Ovale (Milan 5)	China	Pale yellow	No	6 mg/mL	5.0	2.8	54.9%
LANGSEN (Icy LeeChee)	China	Pale yellow	Yes	35 mg/g	32.3	4.4	13.7%

Note: 1. Total nicotine was determined by GC method, 2.FBN fractions were calculated with measured FBN over measured total nicotine, 3. NA = not applicable, 4. LOD = limit of detection.

Conclusions

Free-base nicotine in electronic cigarette liquids can be conveniently and precisely determined using a spectrometric method. The introduced water-soluble indicator Alizarin Red S interacts with unprotonated nicotine. Monitoring free-base nicotine by following the absorption change at long wavelength generated a calibration curve $A = 0.0056\,c + 0.3309$ ($r^2 = 0.9984$, c in μmol L^{-1} nicotine in test solution, A the absorbance at 518 nm). Although the inclusion of water might impact on the acid-base reaction, after calibration the determined FBN contents were consistent with prediction and the FBN fraction agreed well with the published e-liquid values. The method could be applied successfully for the assessment of commercial e-liquids. With the prevalence of nicotine salt in electronic cigarettes, it is believed that this analytical method will allow manufacturers to improve palatability enabling higher concentration without undue bitterness. In addition, given the increased interest in using e-liquids to deliver therapeutic agents, our method will facilitate determination of the free-base concentration of the target drug.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The acknowledgements come at the end of an article after the conclusions and before the notes and references.

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