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Discrimination of biological and artificial nicotine in e-liquid

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Abstract: As the use of e-liquid cigarettes is rapidly increasing worldwide, it multiplies the potential risk undisclosed to the health of non- and smokers. To reduce the hazard, each country has its own set of regulations for controlling e-liquids. In Korea, the narrow definition of tobacco makes it difficult and have been steadily occurring tax evasion exploiting the difference in natural and artificial nicotine. Therefore, it is very important to distinguish source of nicotine for their regulation. To find biochemical discriminant markers, this study established analysis methods based on high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) and high-performance liquid chromatography coupled with triple Quadrupole mass spectrometry (HPLC-MS/MS) for nicotine enantiomers and tobacco alkaloids targeted using the difference in pathways of nicotine biosynthesis and chemical synthesis. The method was validated by experimenting linearity (R^2 > 0.999), recovery (80.99-108.41 %), accuracy (94.11-109.73 %) and precision (0.04-8.27 %). Then, the results for discrimination of the nicotine obtained from analysis of 65 commercial e-liquid products available in Korean market was evaluated. The method successfully applied to the e-liquids and one sample labelled 'synthetic nicotine' for tax exemption was found to contain a natural nicotine product. This method can be used to determine whether an e-liquid product uses natural or artificial nicotine and monitor non-taxable e-liquid products. The method is more scientific than the existing one, which relies only on field evidence.

Key words: e-liquid, HPLC-DAD, HPLC-MS/MS, nicotine enantiomers, tobacco alkaloids

1. Introduction

Electronic cigarettes of liquid type (e-liquid) are one of the tobacco alternatives developed to assist existing smokers to quit smoking.[1] Electronic cigarette is a device designed to allow users to inhale aerosols generated by heating a solution containing nicotine. An e-liquid consists of propylene glycol (PG), glycerine (VG), flavours, and nicotine extracted from tobacco or prepared artificially.[2]

As the demand for e-liquid cigarettes is rapidly increasing worldwide, the production and import of the refill solution of e-liquid cigarettes is also growing every year.[3] Due to the various types and autonomy

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of e-cigarettes, regulations are needed to guard against ascending nicotine dependence as well as multiplying the potential risks undisclosed to the health of non- and smokers from exposure to hazardous substances.[4] To reduce the hazard, each country has its own set of regulations for controlling e-liquids.

For instance, in Korea, only a product containing nicotine extracted from the tobacco leaf is regulated as tobacco. Due to such a narrow definition of tobacco by law in Korea, the practice of labelling natural nicotine as the artificially synthesised one has been continuously increasing since the last four years.[5] However, owing to the lack of appropriate methods at present, such nicotine products are determined based on circumstantial evidence only. In addition, it is not clear whether US Food and Drug Administration (FDA) will or can regulate e-liquid products containing tobacco-free nicotine (TFN).[6,7] In Korea, the definition makes the regulation of e-liquids difficult and have been steadily occurring tax evasion exploiting the difference in tax between natural and artificial nicotine. Therefore, it is necessary to develop an accurate method for their regulation of distinguishing natural nicotine from the artificially synthesised one.

Nicotine contains a chiral centre at the 2′-position of the methyl pyrrolidine moiety, and thus, it has two enantiomers: (*S*)-nicotine and (*R*)-nicotine.[8] (*S*)-nicotine is the major enantiomer in products containing natural nicotine.[9] In contrast, based on the synthesis method used, artificially synthesised nicotine contains either (*S*)-nicotine or (*R*)-nicotine.[10-12] In addition, naturally and artificially synthesised nicotine samples contain different types of tobacco alkaloids. Nornicotine and anatabine account for 2-3 % of all alkaloids in tobacco followed by anabasine, 0.3 % of total alkaloids.[13] On the other hand, only nornicotine and myosmine can be produced during the artificial synthesis of nicotine.[11,12]

Hellinghausen et al. developed an analysis method of optimal separation conditions for the enantiomers of nicotine and tobacco alkaloids by using the LC-MS systems.[14] Also, the team reported method for investigating nicotine enantiomers and nornicotine using the Jasco CD-2095 circular dichroism chiral detector.[15] The analysis was performed in 3 min and TFN and tobacco-derived nicotine (TDN) were compared based on the ratio of (*S*)-nicotine and (*R*)-nicotine.[15] Zhang *et al*. performed normal-phase high-performance liquid chromatography on the tobacco leaf, cigarette filler, e-liquid, and smokeless tobacco, and reported the presence of *R*-nicotine in 45 kinds of these products.[6] In addition, Tang investigated the effects of trifluoroacetic acid (TFA) using a CHIRALCEL® OJ column for separating (*R*,*S*)-nicotine.[16]

Analysis of tobacco alkaloids began in 1962 in an effort to identify them using a pack column. According to Sheng, nicotine, nornicotine, anabasine, anatabine, and 2,3-dipyridine can be extracted from tobacco using dichloromethane and analysed through GC-MS using the pulse injection mode.[13] Cai *et al.* developed an analysis method using megabore capillary GC-FID to analyse seven alkaloids, including anabasine and anatabine, in 30 min with pretreatment.[17] Barhdadi developed a method for analysing eight tobacco alkaloids, including nicotine, in an e-liquid. This process does not involve a separate extraction process, and the analysis was performed after diluting the e-liquid with water only.[18] Flora completed the analysis within 7 min by diluting the e-liquid using aqueous methanol solutions (methanol:water = 7:3) and reported an average limit of detection (LOD) of 0.052 μg/g.[19]

In this study, we developed a high-performance liquid chromatography (HPLC) method using a chiral column to analyse nicotine enantiomers and a UHPLC-MS/MS method to analyse the trace amounts of 11 tobacco alkaloids in an e-liquid. In addition, we applied the established methods to 65 e-liquid products and dried tobacco powder to investigate the differences between natural and artificial nicotine.

2. Experimental

2.1. Chemicals and reagents

E-liquid samples were obtained from Korea, China, USA, and Malaysia either through industrial producers or importers. All these samples declared the source of nicotine as the tobacco leaf, stem, or root; TFN; or simply as ‘synthesised nicotine’. In addition, leaves,

stems, and roots of natural tobacco grown in Korea were individually dried with hot air to remove moisture and then pulverised.

Methanol, ethanol, and hexane were obtained from Merck (Darmstadt, Germany). Water was produced by a Milli-Q IQ 7000 system from Merck. (*R*)-Nicotine was purchased from Merck. (*S*)-Nicotine, *N*-methyl anabasine, and (*R*,*S*)-norcotinine and N'-Nitrosonornicotine-d4(NNN-d4) used as the internal standards for tobacco alkaloids were acquired from Toronto Research Chemicals Inc. (Toronto, Canada). Formic acid (~ 99 %) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Trifluoroacetic acid (TFA) and trimethylamine (TEA) used as the adducts for the analysis of (*R*,*S*)-nicotine, ammonium acetate (reagent grade), and tobacco alkaloids (including (-)-cotinine solution and myosmin) were obtained from Sigma-Aldrich (Darmstadt, Germany). (+/-)-Nornicotine, (*R*,*S*)-anatabine, and *trans*-3′-hydroxy-cotinine were supplied by Cayman Chemical (Michigan, USA). (*S*)-Cotinine-N-oxide, nicotyrine, 2,3′-bipyridyl and anabasine obtained from Santa Cruz Biotechnology (Dallas, United States), United States Pharmacopeia (Maryland, United States), RIEKE Metals (Lincoln, United States), and CSNPharm (Arlington Heights, United States), respectively.

Stock solutions of the (*R*,*S*)-nicotine and tobacco alkaloids were prepared in 1 mg/mL of ethanol and stored in 10 mL amber vials in a freezer. A mixture of tobacco alkaloid standard solutions was prepared by diluting the stock solutions with 25 ng/mL of NNN-d_4 in 10 mM ammonium acetate in water and stored in 4 °C.

2.2. Instrumental conditions

2.2.1. HPLC conditions for the analysis of (R,S)-nicotine

(*R*,*S*)-Nicotine enantiomers were analysed using an Agilent 1200 HPLC-DAD detector (Agilent, USA). The separation was performed using normal-phase liquid chromatography (LC) with a Daicel Chiral OJ-3 column (250 mm × 4.6 mm, 5 μm particle size) at 30 °C and equilibrated with 100 % solvent A (hexane:ethanol:TFA:TEA = 95:5:0.05:0.075, v/v %). First, 10 μL of the sample was injected into the HPLC system. A flowrate of 1 mL/min with a linear gradient was used as follows: 20 % solvent B (hexane: ethanol:TFA:TEA = 80:20:0.05:0.075, v/v %) at 2 min and held for 10 min, 30 % solvent B for 16 min, and then 30-40 % solvent B over the next 20 min. The

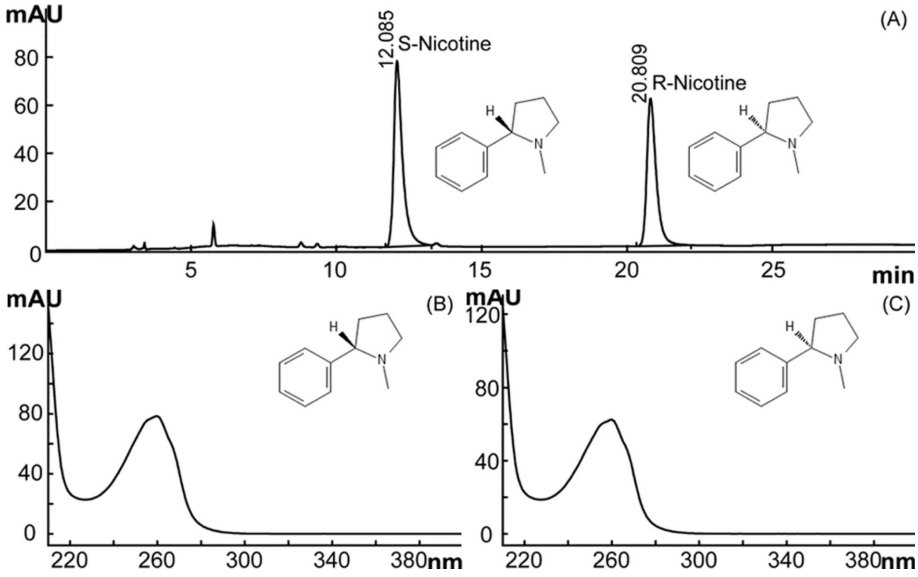

Fig. 1. (A) Chromatogram of R,S-nicotine obtained using the HPLC-DAD method. R-nicotine: 20.809 min, S-nicotine: 12.085 min. UV spectra of (B) S-nicotine and (C) R-nicotine.

Table 1. Results of the validation of the HPLC and UHPLC-MS/MS method for the quantification of R,S-nicotine and tobacco alkaloids

Compound	Calibration curve range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)	Intra/Inter day	RSD (%)	Accuracy (%)	Recovery (%)
R-nicotine	14.48 – 498.33	0.9996	4.78	14.48	Intraday	0.04-0.13	101.59-102.27	91.03-92.22
					Interday	0.92-1.26	101.81-101.30	89.19-90.79
S-nicotine	5.65 – 507.13	0.9999	1.86	5.65	Intraday	0.06-0.43	101.27-101.61	86.41-86.86
					Interday	0.89-1.41	101.17-102.83	84.33-86.54
Compound	Calibration curve range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Intra/Inter day	RSD (%)	Accuracy (%)	Recovery (%)
Anabasine	1.39 – 100	0.9999	0.46	1.39	Intraday	0.32-4.72	100.35-105.29	100.64-103.19
					Interday	4.35-4.90	97.90-101.26	96.79-97.46
Norcotinine	1.51 – 100	0.9998	0.50	1.51	Intraday	0.57-0.65	95.86-98.27	97.12-99.60
					Interday	3.14-5.21	99.31-100.32	98.74-101.78
Anatabine	1.47 – 100	0.9999	0.49	1.47	Intraday	0.05-4.70	94.11-97.40	92.19-97.83
					Interday	1.19-4.10	96.87-97.49	95.87-99.20
Myosmine	1.78 – 100	0.9999	0.59	1.78	Intraday	0.31-1.10	98.80-105.51	92.69-94.32
					Interday	1.27-6.22	98.63-103.35	97.79-99.04
Cotinine	0.81 – 100	0.9999	0.27	0.81	Intraday	2.56-3.06	95.09-102.31	98.91-100.89
					Interday	0.45-3.63	97.08-101.60	98.74-99.81
Nicotyrine	0.77 – 100	0.9999	0.25	0.77	Intraday	1.24-2.36	95.59-99.67	95.76-98.56
					Interday	2.24-3.05	98.39-101.65	97.48-99.95
Cotinine oxide	1.72 – 100	0.9999	0.57	1.72	Intraday	1.29-1.54	95.09-102.31	100.63-104.35
					Interday	1.64-2.73	95.92-100.43	98.28-101.80
Nornicotine	1.74 – 100	0.9999	0.57	1.74	Intraday	1.32-6.18	96.15-99.67	101.95-104.57
					Interday	2.06-2.95	97.24-101.21	98.12-98.76
trans-Hydroxy-cotinine	0.85 – 100	0.9997	0.28	0.85	Intraday	0.71-2.05	95.84-102.60	95.87-100.63
					Interday	2.42-2.88	96.76-103.59	95.91-103.08
Bipyridyl	1.16 – 100	0.9999	0.38	1.16	Intraday	0.78-1.29	95.30-107.57	101.87-108.41
					Interday	2.53-4.04	98.83-102.68	98.73-101.50
Methyl anabasine	2.01-100	0.9995	0.66	2.01	Intraday	2.11-4.47	99.92-109.73	92.75-96.78
					Interday	6.73-8.27	94.18-108.19	80.99-83.22

proportion of solvent B was increased to 90 % at 24 min and 100 % at 28 min and held for 1 min, followed by 0 % solvent B at 31 min. The total run time was 31 min. The diode array detector (DAD) range was set from 210 to 400 nm, and a wavelength of 258 nm was detected (*Fig.* 1 and supplementary *Table* 1).

2.2.2. LC-MS/MS conditions for the analysis of tobacco alkaloids

The Agilent 1290 infinity ultra-high-performance liquid chromatography system (Agilent, USA) coupled with the AB Sciex Triple Quad 5500 plus mass spectrometer (SCIEX) was used for the analysis of tobacco alkaloids. The Agilent ZORBAX Eclipse Plus C18 Narrow Bore C18 column (150 mm × 2.1 mm, 3.5 μm particle size) was equilibrated with 95 % solvent A (10 mM ammonium acetate in water) and used to perform the separation of tobacco alkaloids in the LC system at 30 °C. A 3-μL sample was injected into the UHPLC system. A flowrate of 0.3 mL/min with a gradient elution was used as follows: 5 % solvent B (0.05 % formic acid in methanol) for 1 min, 5-15 % solvent B for 2 min, and 15-25 % solvent B for 6 min, followed by up to 35 % solvent B at 7.5 min and 90 % solvent B at 10 min held for 1 min. The proportion of B solvent was

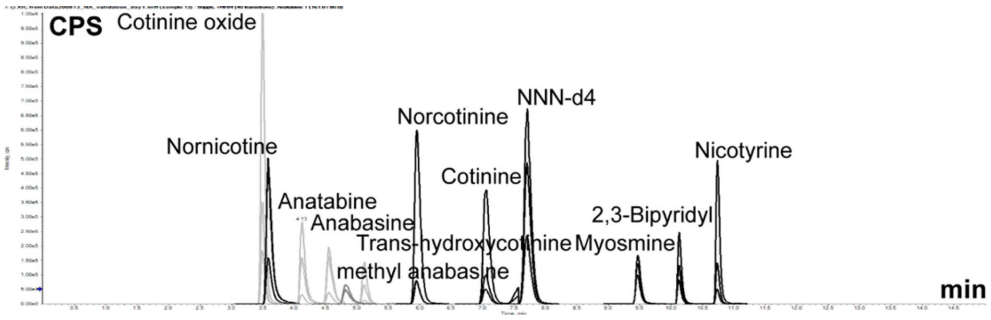

Fig. 2. Extract ion chromatograms (XICs) of tobacco alkaloids obtained using the UHPLC-MS/MS method. Peaks: anabasine (4.55 min), norcotinine (5.96 min), anatabine (4.13 min), myosmine (9.42 min), cotinine (7.06 min), methyl anabasine (4.80 min), nicotyrine (10.71 min), cotinine oxide (3.51 min), nornicotine (3.56 min), trans-hydroxycotinine (5.10 min), and bipyridyl (10.11 min), NNN-d_4 (7.71 min).

increased to 100 % at 13 min and then decreased to 5 % within 0.1 min and held for 1.9 min. The total run time was 15 min. For mass detection, electrospray ionisation of the positive mode was used and compound-dependent parameters were optimised from the limit of quantification to 10 ng/mL. The optimised values were set as follows: curtain gas (CUR), 35 psi; collision gas (CAD), 9 psi; ion spray (IS), 5500 V; temperature, 500 °C; ion source gas 1 (GS1, nitrogen), 50 psi; ion source gas 2 (GS2, nitrogen), 50 psi; entrance potential (EP), 10 V; collision cell exit potential (CXP), 10 V. Nitrogen was used as the collision gas. For each analyte, three ion transition pairs were used under scheduled multiple reaction monitoring (see *Fig.* 2 and Supplementary *Table* 2).

2.3. Sample preparation

2.3.1. Sample preparation for the analysis of (R,S)-nicotine.

First, 0.1 g of the e-liquid or tobacco powder and 1 mL of 5 % sodium hydroxide were mixed by vortex in a 15-mL falcon tube and kept at room temperature for 1 min. Next, 5 mL of hexane was added to the mixture, which was then agitated by vortex for 2 min and left to stand for 30 min. The supernatant was transferred to a 2 mL auto sampler vial for the analysis.

2.3.2. Sample preparation for the analysis of tobacco alkaloids

Here, 0.01 g of the e-liquid was weighed in a 20 mL volumetric flask and filled with 25 ng/mL of NNN-d_4 in 10 mM ammonium acetate aqueous solution. Next, 2 mL of this solution was filtered and transferred to a 2 mL auto sampler vial for the analysis. Then, 0.5 g of tobacco powder and 10 mL of 70 % methanol (25 ng/mL NNN-d_4) were placed in a 15 mL falcon tube and vortex-mixed for 1 min and sonicated for 30 min. The mixture was centrifuged at 4,000 rpm and the supernatant was filtered using a 0.2 μm syringe filter. The solution was then transferred to a 2 mL auto sampler vial for analysis after diluting it with 25 ng/mL of NNN-d_4 in 10 mM ammonium acetate.

2.4. Method validation

The HPLC method was validated for the selectivity, linearity, LOD, limit of quantification (LOQ), recovery, accuracy, and precision in propylene PG:VG = 1:1 (w/w) solution, as shown in *Tabl*e 1. The specific UV spectra of (*R*,*S*)-nicotine completely separated from each other and no peaks were observed at the retention time in the blank PG,VG solution. The linearity was obtained with a correlation coefficient (R^2) of >0.999, in concentration ranges of 5.65-507.13 and 14.48-498.33 μg/mL for (*S*)- and (*R*)-nicotine, respectively. The LOD and LOQ were estimated through the following relations using five calibration curves:

3.3 × (standard deviation of y-intercepts) / (slope of calibration curves) for LOD

10.0 × (standard deviation of y-intercepts) / (slope of calibration curves) for LOQ

Table 2. Data for 65 e-liquid samples and dried tobacco powder using the established method

Number	Labelled	Results (n=3)			
		Nicotine (mg/g)		Tobacco alkaloid (μg/g)	
		S-nicotine	R-nicotine	Anabasine	Anatabine
1	Natural nicotine	8.55±0.05	-	53.61±1.5	187.63±3.4
2	Salt nicotine	8.76±0.04	-	9.20±0.5	9.89±0.2
3	Stem nicotine	10.65±0.00	-	34.57±0.3	73.37±1.3
4	Natural nicotine	8.45±0.01	-	3.58±0.1	-
5	Stem nicotine	8.53±0.07	-	13.12±0.9	44.1±0.9
6	Stem salt nicotine	29.54±0.13	-	-	-
7	Stem nicotine	7.32±0.04	-	22.13±1.4	52.35±4.9
8	Stem nicotine	7.46±0.04	-	3.42±0.2	-
9	Natural stem nicotine	7.44±0.14	-	10.96±0.2	-
10	Salt nicotine	8.52±0.06	-	6.37±0.4	5.72±0.1
11	nicotine	8.07±0.10	-	24.46±0.5	48.15±0.7
12	Natural stem nicotine	7.28±0.08	-	42.91±1.5	69.34±1.9
13	Stem nicotine	8.65±0.04	-	30.01±1.0	89.61±2.1
14	Natural stem nicotine	7.07±0.01	-	9.50±0.3	12.87±0.5
15	Stem nicotine	7.69±0.02	-	16.84±0.3	34.34±0.6
16	Stem nicotine	9.18±0.02	-	-	-
17	Stem nicotine	8.93±0.04	-	32.65±0.5	67.33±3.0
18	Stem nicotine	7.61±0.11	-	11.59±0.3	10.56±0.1
19	Stem nicotine	9.36±0.10	-	NQ	-
20	Stem nicotine	9.15±0.07	-	-	-
21	Stem nicotine	9.66±0.02	-	26.08±0.5	53.01±1.4
22	Salt nicotine	8.37±0.02	-	-	-
23	Stem nicotine	12.20±0.13	-	51.96±0.6	98.88±2.3
24	Stem nicotine	8.04±0.10	-	27.03±0.5	36.11±0.7
25	Stem salt nicotine	7.66±0.03	-	-	-
26	Nicotine	2.73±0.02	-	3.50±0.2	6.99±0.1
27	Nicotine	7.84±0.06	-	-	-
28	Nicotine USP/EP	2.29±0.05	-	3.88±0.1	6.05±0.2
29	Nicotine	7.75±0.07	-	10.88±0.2	7.66±0.4
30	Stem/synthesis nicotine	8.39±0.08	-	24.36±0.8	33.79±0.4
31	Stem, root/synthesis nicotine	8.15±0.10	-	-	-
32	Natural nicotine	8.74±0.03	-	39.61±1.4	75.59±1.6
33	Snicotine	8.59±0.10	-	19.58±0.2	26.64±0.9
34	Nicotine (54-11-2)	8.40±0.01	-	11.73±0.4	17.81±0.9
35	Nicotine	9.01±0.02	-	-	-
36	Refined nicotine	11.10±0.06	-	23.68±0.3	27.38±0.6
37	Stem, root/synthesis nicotine	8.70±0.21	-	22.71±1.1	24.86±1.0
38	Snicotine	8.54±0.16	-	37.52±0.3	107.56±2.6
39	Refined nicotine	9.60±0.04	-	39.69±0.8	60.81±1.0
40	USP Nicotine	9.38±0.08	-	-	-
41	Nicotine	9.92±0.09	-	-	-
42	Stem nicotine	9.29±0.07	-	-	-
43	Snicotine	8.99±0.07	-	20.20±1.2	28.00±1.9
44	S-Nicotine	8.67±0.10	-	9.39±1.3	18.81±2.3
45	Nicotine (USP)	9.68±0.05	-	52.82±0.8	107.57±1.6

Table 2. Continued

Number	Labelled	Results (n=3)			
		Nicotine (mg/g)		Tobacco alkaloid (μg/g)	
		S-nicotine	R-nicotine	Anabasine	Anatabine
46	TFN	4.32±0.07	4.84±0.06	-	-
47	TFN	4.74±0.12	4.85±0.05	-	-
48	TFN	3.32±0.04	3.71±0.04	-	-
49	TFN	3.59±0.02	3.98±0.06	-	-
50	Synthesis nicotine	5.75±0.07	-	10.13±0.2	6.4±0.2
51	TFN	4.07±0.05	4.59±0.04	-	-
52	TFN	5.59±0.07	6.26±0.06	-	-
53	TFN	4.21±0.01	4.76±0.03	-	-
54	TFN	3.96±0.02	4.46±0.02	-	-
55	TFN	3.93±0.02	4.42±0.02	-	-
56	TFN	4.09±0.01	4.61±0.01	-	-
57	TFN	4.26±0.12	4.76±0.06	-	-
*58	Leaf nicotine	6.47±0.06	-	28.02±3.9	36.83±3.9
*59	Leaf nicotine	6.41±0.04	-	3.01±0.4	NQ
*60	Leaf nicotine	7.34±0.49	-	30.00±3.1	53.41±4.9
*61	Leaf nicotine	6.45±0.04	-	-	-
*62	Leaf nicotine	6.58±0.03	-	-	-
*63	Leaf nicotine	6.52±0.04	-	-	-
*64	Leaf nicotine	6.23±0.05	-	-	-
*65	Leaf nicotine	6.33±0.11	-	-	-
Leaf	Dried powder (tobacco leaf)	5.18±0.34	-	76.27±9.21	382.36±39.86
Stem	Dried powder (tobacco stem)	0.51±0.00	-	15.12±2.49	102.66±15.03
Root	Dried powder (tobacco root)	0.67±0.05	-	59.64±5.24	263.52±18.88

*: the products which pay tax of tobacco in Korea

The LODs and LOQs for (*S*)-nicotine were determined as 1.86 and 5.65 μg/mL, respectively, and those for (*R*)-nicotine were 4.78 and 14.48 μg/mL, respectively. The recovery, accuracy, and precision of the method were evaluated by spiking known amounts of the (*R*,*S*)-nicotine mixture on a blank PG-VG sample (PG:VG = 1:1 (w/w)); all the parameters were replicated three times in three consecutive days. The analytes were added at three concentrations: 100, 300, and 500 μg/mL. The recovery was calculated by dividing the area of the spiked blank sample before extraction on the column by that determined after extraction. The accuracy was determined by dividing the value of the experimentally determined concentration by the nominal concentration. The relative standard deviations of the calculated concentrations with a calibration curve were used as the precision. The recoveries were in the range of 84.33-92.22 % for (*R, S*)-nicotine. The intraday accuracy and precision were 101.17-103.30 % and 0.04-1.41 %, respectively for (*R, S*)-nicotine.

The HPLC-MS/MS method for tobacco alkaloids was validated using the same procedures as those for (*R*,*S*)-nicotine in three concentrations: about 2.5, 50.0, 100.0 ng/mL. Tobacco alkaloids had individual MRM transitions, except for anabasine, norcotinine, cotinine, and methyl anabasine, which were distinguished by retention times and sMRM. The linearity was obtained with $R^2 > 0.999$ for the alkaloids in the range of 1-100 ng/mL. The LODs and LOQs calculated using the same method as that used for (*R*,*S*)-nicotine were 0.27-0.66 and 0.81-2.01 ng/mL, respectively. The recoveries of tobacco alkaloids showed from 80.99 to 108.41 %, indicating 94.11-109.73 %

of accuracy and 8.27 % of precision. The accuracy and precision values of the LC-MS/MS method are listed in *Table* 1.

2.5. Application of the method

65 e-liquid products labelled as 'containing nicotine solution' extracted from the tobacco leaf, stem or TFN (synthesised nicotine) were collected (*Table* 2) and applied to test the established method for determination of (*R*,*S*)-nicotine and tobacco alkaloid contents. Also, to check the composition of a natural tobacco plant(or the assumption for natural nicotine in this study), dried tobacco powder was analysed. Each sample was analysed in triplicate.

3. Results and Discussion

3.1. Optimization of conditions for (R,S)-nicotine analysis and extraction

For separating the nicotine enantiomers using a chiral column, the ratio of the solvent to the additive was adjusted. A comparison of the ratios of hexane and ethanol in the order of 95:5, 90:10, and 85:15, v/v % by using the isocratic elution method, in which the proportion of ethanol is gradually increased, confirmed the separation of the (*R*)- and (*S*)-nicotine enantiomers using the hexane:ethanol = 85:15, v/v %. However, a wide peak width and peak tailing were observed, which were compensated by using TFA and TEA as additives. As the proportion of TFA in the mobile phase increased, the distance between the peaks of (*R*)- and (*S*)-nicotine also increased. In contrast, the peak tailing decreased as the proportion of TEA increased. Hence, the ratio of hexane: ethanol: TFA:TEA = 90:10:0.075:0.0375, v/v % was selected for the separation. When the above analysis method was applied to the e-liquid sample, the interference of the sample matrix occurred at the (*R*,*S*)-nicotine peak. In addition, PG and VG, the main components of the liquid e-cigarette filling solution, remained in the normal-phase column, causing adverse effects when the number of theoretical plates in the column is reduced during repeated analysis. To resolve these problems, liquid–liquid extraction (LLE) was performed using hexane for removing PG and VG and the isocratic elution method was replaced with the gradient elution method, giving changes in the composition of hexane and ethanol within 80-95 % of hexane to separate the substances that interfere with the analytes. Based on the results obtained, the final optimised mobile phase composition is (A) hexane:ethanol:TFA:TEA = 95:5: 0.05:0.075, v/v % and (B) hexane:ethanol:TFA:TEA = 80:20: 0.05:0.075, v/v %.

3.2. Application and discrimination

The 11 kinds of e-liquids were detected both (*S*)-nicotine and (*R*)-nicotine as concentrations of the ranges 3.32-5.59 and 3.71-6.26 mg/g, respectively. In addition, the sample were indicated as TFN. Also, all e-liquid products labelled TFN were not detected both anabasine and anatabine and can be investigated following the same assumption. Cotinine oxide, *trans*-hydroxycotinine were not detected in any of the e-liquid samples. One sample marked as 'synthesised nicotine' which was considered as a product providing false information about the source of synthetic nicotine, because the sample was detected both anabasine and anatabine as well as purely (*S*)-nicotine form. This sample, suspected to contain natural nicotine but claiming to have synthetic nicotine, was identified because it contained both anabasine and anatabine, which are found only in natural nicotine.

On the other hand, the 53 kinds of e-liquid samples marked as containing 'natural nicotine extracted from tobacco' and 3 tobacco powder samples was only detected (*S*)-nicotine in the range 0.51-29.54 mg/g. However, the concentration of tobacco alkaloids and their distributions were different in each the e-liquid sample. In case, the 53 kinds of e-liquid samples, norcotinine was not entirely detected and anabasine, anatabine, myosmine, cotinine, nicotyrine, nornicotine, bipyridyl and methyl anabasine were detected in the range varying from 'not quantifiable' (NQ) to 187.6 μg/g or not detected. Unlike the assumption for natural nicotine, 37 of the 53 samples labelled as containing natural nicotine showed anabasine and/or anatabine. The 16 samples did not show particular difference of qualitative tobacco alkaloids in comparison with

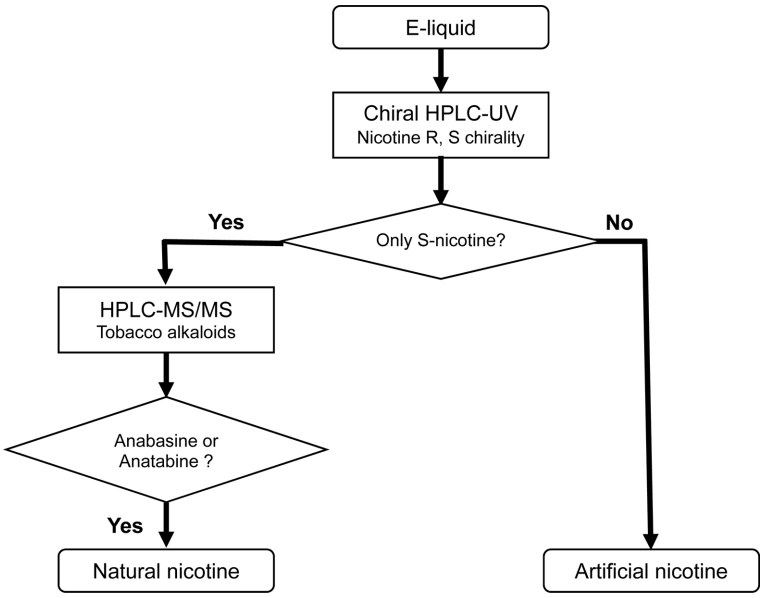

Fig. 3. Discriminative algorithm for determining the nicotine source in e-liquid products.

other samples including TFN and tobacco powder. However, among these 16 samples, 5 samples pay tobacco tax in Korea because their manufacturers have declared that their products contain the nicotine solution extracted from the tobacco leaf. In the dried tobacco leaf, stem, and root samples, anabasine, anatabine, myosmine, cotinine, nicotyrine, nornicotine, bipyridyl detected in the range varying from 3.62 to 382.36 μg/g and were investigated to confirm the possible tobacco alkaloids present.

3.3. Discriminative algorithm of nicotine source

The presence of natural and artificial nicotine in e-liquid products can be determined through the following (*Fig.* 3); (1) Natural nicotine if only (*S*)-nicotine was detected in the samples, otherwise artificial nicotine. We assumed that it is not economical to use artificially synthesised (*S*)-nicotine in the e-liquid. (2) Natural nicotine if either anabasine or anatabine was detected, otherwise artificial nicotine, because it was assumed that synthetic nicotine may not contain both anabasine and anatabine since they had a piperidinyl functional group contrary to the chemical structure of nicotine.

4. Conclusions

We introduce a method that distinguishes natural and artificially synthesised nicotine in e-liquid samples. The method involves the use of a chiral column to analyse nicotine enantiomers and a narrow-bore C18 column to analyse tobacco alkaloids. The established method was applied to 65 commercial e-liquid products obtained from the Korean market. The (R,S)-nicotine discrimination method was successfully used to determine the presence of natural and artificial nicotine in commercial e-liquid products, but tobacco alkaloids in natural nicotine did not match the model of assumption. Nevertheless, we found that one sample labelled ‘artificially synthesised nicotine’ for tax exemption purposes possibly contained a natural nicotine product.

We regard the absence of anabasine or anatabine in 16 e-liquids samples containing natural nicotine to be due to differences in the contents and amounts of tobacco alkaloids among tobaccos, and the various procedures used to produce e-liquids. Therefore, a legal and institutional supplement, data, including raw materials and product-manufacturing processes from importers and sellers need to be reviewed in

order to determine the source of nicotine more accurately in the future. The reason why anabasine or anatabine were not detected in e-liquid samples containing natural nicotine, and other markers used to distinguish natural and artificially synthesised nicotine will be studied in future work.

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