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# Effective determination of nicotine enantiomers from e-liquids and biological fluids by high performance liquid chromatography (HPLC) using dispersive liquid-liquid microextraction (DLLME)

**Seunghoon Song and Seung-Woon Myung★**

*The Department of Chemistry, Kyonggi University, Suwon 16227, Korea*

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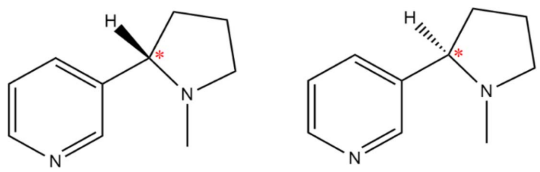
**Abstract:** This study compared the efficacy of chiral GC and chiral HPLC for the analysis of nicotine. To develop a suitable dispersive liquid-liquid microextraction (DLLME) method, the following parameters were optimized: pH, extraction solvent, dispersive solvent, type and quantity of salt, and laboratory temperature. The validation of the method was carried out by the established HPLC method. The LODs were 0.11 μg/mL and 0.17 μg/mL for the (S)- and (R)- enantiomers, respectively. The LOQs were 0.30 μg/mL and 0.44 μg/mL, respectively. The optimal calibration range was between 0.30-18 μg/mL and 0.44-4.40 μg/mL, respectively, and the correlation coefficient ($r^2$) was 0.9978-0.9996. The intra-day accuracy was 79.9-110.6 %, and the intra-day precision was 1.3-12.0 %. The inter-day accuracy was 87.8-108.0 %, and the inter-day precision was 4.0-12.8 %. E-liquid and biological fluids (urine and saliva) were analyzed using the established method.

**Key words:** nicotine enantiomers, DLLME, HPLC, GC/MS, e-liquid, urine, saliva

## 1. Introduction

For many years, people have smoked cigarettes to relieve stress. One of the main ingredients of cigarettes is nicotine, which is an alkaloid-based substance that people frequently encounter in their everyday environments. When nicotine is ingested or inhaled, it can cause several negative outcomes in human health such as cardiovascular, respiratory, central nervous diseases and even cancer.[1] Nicotine dependence and toxicity are also increased.

Nicotine has a chiral central carbon at the 2'-position of the pyrrolidine moiety, and exists as two enantiomers, (R)-(+)-nicotine and (S)-(-)-nicotine (*Fig*. 1). There are several differences between the two enantiomers, including the $LD_{50}$, for which (S)-(-)-nicotine and (R)-(+)-nicotine have values of




(S)-(-)-Nicotine (R)-(+)-Nicotine

*Fig*. 1. Chemical structure of chiral nicotine.

★ Corresponding author
Phone : +82-(0)31-249-9647 Fax : +82-(0)31-249-9647
E-mail : swmyung@kgu.ac.kr

0.38 mg/kg and 2.75 mg/kg, respectively. The oxidative stress levels are also different.[2,3]

These enantiomers require separation because most of their physical properties are the same, except for the direction of polarization and their effects in biological systems.[4] In addition, as the abundance ratio of chiral nicotine serves as a means to distinguish whether the compounds are derived from natural or synthetic origins, separation and quantification of the chiral components are important. (S)-(-)-nicotine is the major component of natural nicotine extracted from tobacco plants, in which (R)-(+)-nicotine has a content of less than 1 %, although the content can vary depending on the origin and type of the tobacco leaves. In the absence of a selective synthesis process, synthetic nicotine is synthesized as a racemic compound in a 1:1 ratio.[5]

The "e-liquid" or "e-cigarette liquid" is the mixture used in vapor products including e-cigarettes. In recent years, e-liquid smoking has gained popularity worldwide among adolescents and females. It is emerging as a major issue with cases of lung disease or death resulting from e-liquid smoking becoming more frequent. While there have been many studies on cigarettes to date, reports on e-liquids are scarce. E-liquid smoking involves putting e-liquid, a solution containing nicotine, into a cartridge, heating it to a high temperature (approximately 350 °C), and inhaling the vapor. E-liquids generally comprise nicotine, purified water, a propylene glycol/glycerin mixture, and flavor additives. On the other hand, the types of nicotine contained herein are also commercially available in liquids containing chemically synthesized nicotine rather than nicotine extracted from tobacco leaves or stems.[6] In cases where the definition of tobacco refers specifically to the tobacco leaf, there may be disputes between the manufacturers of e-liquids and taxation agencies in relation to tobacco tax.

As reports on the toxic effects of synthetic nicotine on the human body are scarce, methods that can detect the origin of nicotine are important. The origin of nicotine (natural or synthetic) can be determined efficiently by extracting, separating, and quantifying the nicotine enantiomers contained in e-liquid and biological samples from smokers.

There are several methods for extracting analytes from biological samples. Dispersive liquid–liquid microextraction (DLLME) is an advanced liquid-liquid extraction (LLE) method, which uses aqueous samples, dispersion solvents, and extraction solvents.[7,8] The quantity of extraction solvent used can range from several tens to several hundred microliters, and the extraction and analysis time is relatively short compared to other LLE methods.[9]

The nicotine assay was established many years ago. After nicotine is extracted using an extraction method such as LLE or solid-phase extraction (SPE), analysis by high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS),[10-18] gas chromatography (GC), or gas chromatography/mass spectrometry (GC/MS) is necessary.[19-24] However, few studies have analyzed enantiomers of nicotines in biological samples such as urine and saliva.

In this study, an effective method for the pretreatment of chiral nicotine from e-liquid and biological samples (urine and saliva) using DLLME was established and optimized, and nicotine enantiomers separation using chiral columns of HPLC and GC were compared.

## 2. Experimental

### 2.1. Reagents and materials

The (S)-(-)-nicotine and (R)-(+)-nicotine standards were purchased from Sigma-Aldrich (St Louis, USA) and Carbosynth (Berkshire, UK), respectively. A 1000 μg/mL stock solution was prepared by dissolving the standard in methanol and storing it in a dark place. The stock solution was diluted to an appropriate concentration with methanol when necessary.

Hydrochloric acid and potassium hydroxide were purchased from Samchun (Gyeonggi, South Korea) and anhydrous sodium sulfate was purchased from FUJIFILM Wako (Osaka, Japan). Sodium chloride and magnesium sulfate were purchased from DAEJUNG (Gyeonggi, South Korea) and TCI (Tokyo, Japan), respectively.

Diethyl ether, n-hexane, methanol, ethanol, acetone,

and acetonitrile were purchased from Duksan (Gyeonggi, South Korea). Purified water with a specific resistance of 18.2 MΩ·cm was obtained from a Synergy UV system (Millipore S.A.S, Molsheim, France).

### 2.2. Real sample analysis

Quantitative analysis of nicotine isomers in urine and saliva from e-liquid smokers and in e-liquid sold in South Korea was performed using the established analytical methods.

### 2.3. Apparatus and instrumentation

For the separation analysis, a 1050 series HPLC system from Agilent (Palo Alto, CA, USA) was used, along with an Agilent 1100 diode array detector (DAD). The chiral column used was an OD-H column (4.6 × 250 mm, 5 μm) manufactured by Daicel (Tokyo, Japan). Chromatographic separation was achieved with gradient elution using n-hexane and ethanol. Gradient elution began with a flow of 96.5 % n-hexane for 6 min, followed by a linear decrease to 80 % over 20 min. This was maintained for 30 min. The flow rate of the mobile phase was 1.3 mL/min, the sample injection volume was 10 μL, and the wavelength of the detector was 262 nm (*Table* 1).

The GC-MS system was comprised of an Agilent 6890N GC and an Agilent 5973N MS from Agilent (Palo Alto, CA, USA). The column was a CHIRALDEX G-TA (20 m × 0.25 mm, 0.12 μm) from Supelco (Pennsylvania, USA). The column temperature was programmed to remain at 40 °C for 20 min, before increasing from 40 °C to 70 °C at 0.3 °C/min, holding at 70 °C for 10 min, increasing from 70 °C to 110 °C at 2.0 °C/min, holding at 110 °C for 5 min, and finally increasing to 150 °C at 2.5 °C/min. The injection port and mass transfer line temperatures were both set to 260 °C (*Table* 2).

*Table* 1. HPLC-UV conditions for analysis of chiral nicotine

| Parameters | Conditions | |
|---|---|---|
| Column | CHIRALCEL OD-H (4.6 × 250 mm, 5 μm) | |
| Time (min) | n-Hexane | Ethanol |
| 0 | 96.5 | 3.5 |
| 5 | 96.5 | 3.5 |
| 20 | 80 | 20 |
| 30 | 80 | 20 |
| Flow Rate | 1.3 mL/min | |
| Injection volume | 10 μL | |
| Wavelength | 262 nm | |

*Table* 2. GC/MS conditions for analysis of chiral nicotine

| Parameter | Conditions |
|---|---|
| GC/MS | Agilent 6890N (GC), Agilent 5973N (MS) |
| Column | CHIRALDEX G-TA (20 m × 0.25 mm, 0.12 μm) |
| Carrier Gas | He, 1.0 mL/min |
| Injection Mode | Split mode (30:1) |
| Injector temp. | 260 °C |
| Injection volume | 2 μL |
| Transfer line temp. | 260 °C |
| Ionization mode | EI (electron ionization) 70 eV |
| Mass Spectrometer | Quadrupole |

An MF80 centrifuge from Hanil (Seoul, South Korea) and a TurboVap LV nitrogen concentrator from Caliper Lifescience (Seattle, USA) were used.

### 2.4. Sample preparation

To analyze commercial e-liquid, 200 μL of e-liquid was diluted 50 times with methanol to achieve the volume of 5 mL, using an aqueous solution with pH 10.

After obtaining consent from the smoker (27-year-old male) for the collection of urine and saliva, samples were collected 1 h after smoking an e-cigarette. The sample collection procedures of biological fluids (urine and saliva) were carried out in compliance with relevant laws and institutional guidelines.[25] The informed consent was obtained for experimentation with participant. After abstaining from smoking for 7 days to ensure no nicotine remained in his body, the subject smoked a regular cigarette, and a sample was collected in the same manner as before.[26]

In the case of the urine sample, 5 mL of urine was adjusted to pH 10. The saliva was centrifuged at 4000 rpm for 10 min to precipitate the protein component. A 2 mL aliquot of the supernatant was adjusted to pH 10 and was then diluted to 5 mL with

purified water for analysis.[27,28]

A mixed solution of 250 μL diethyl ether (extraction solvent) and 250 μL of acetone (dispersion solvent) was added to each sample, along with 1.5 g of sodium sulfate, and the mixture was shaken for 15 s.

The mixed sample was centrifuged at 4000 rpm for 10 min, and then 50 μL of the supernatant, which contained the extract, was transferred to a new conical tube. After the extraction solvent was evaporated for 5 min at 60 °C using a nitrogen concentrator, the sample was re-dissolved in the same quantity of ethanol, transferred to the insert tube, and injected into the HPLC and GC/MS.

## 3. Results and Discussion

### 3.1. Comparison of GC and HPLC methods

Nicotine enantiomers were analyzed using chiral GC column and chiral HPLC column and the performances of both systems were compared.

The retention time of the two isomers using the GC/MS system was considered too long. In addition, since the peaks were not completely separated, quantitative analysis was difficult (*Fig*. 2). However, the HPLC system allowed complete separation of the two enantiomers within 7 min, and the total analysis time was short. Thus, HPLC was selected for the quantitative analysis of nicotine enantiomers (*Fig*. 3).

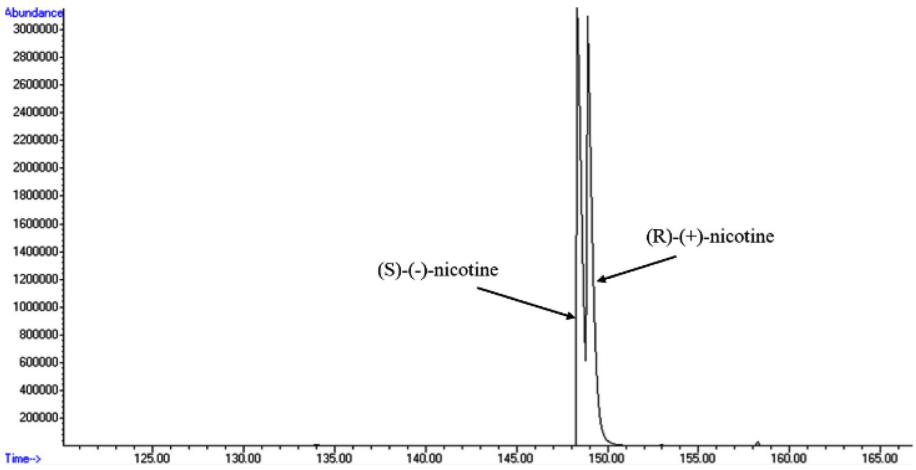



*Fig*. 2. Separation of nicotine enantiomers standard using GC/MS.

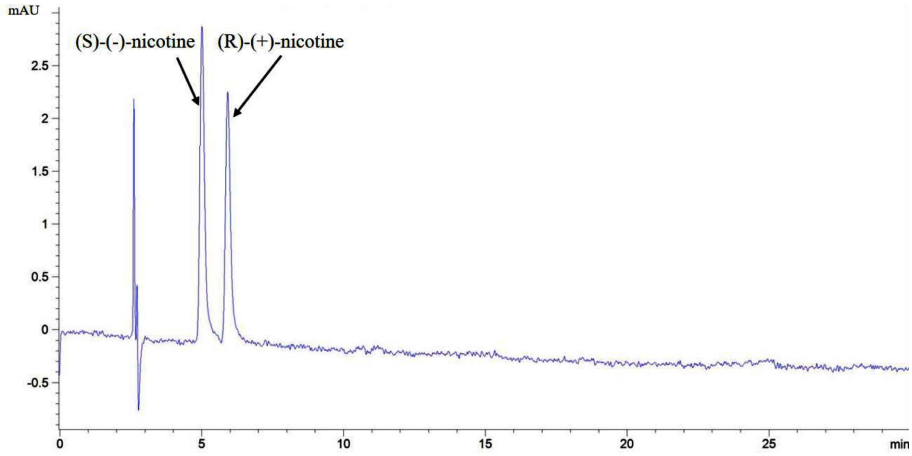



*Fig*. 3. Separation of nicotine enantiomers standard using HPLC-UV.

### 3.2. Parameter optimization

The DLLME experimental procedure was as follows. After the aqueous sample solution was placed in a falcon tube, a small quantity of extraction solvent and dispersion solvent were quickly added to form fine droplets, and the increase in the contact surface area between the extraction solvent and the solution caused instantaneous extraction. Thereafter, when the sample was centrifuged, layer separation occurred between the extraction solvent and the aqueous solution in the form of small droplets. The extraction solvent was taken up by a syringe and analyzed by HPLC (*Fig*. 4).

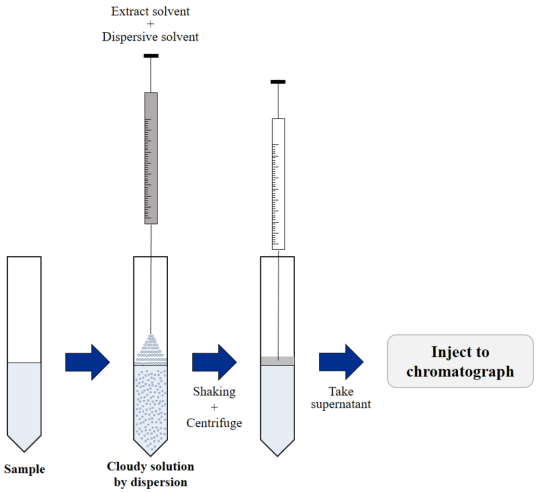



*Fig*. 4. A scheme of dispersive liquid-liquid microextraction (DLLME) procedure.

Sample preparation was carried out at 28 °C. To develop the DLLME method for pretreatment, the following parameters were optimized: pH, types, and quantities of extraction and dispersion solvents, the type and quantity of salt, and temperature. The effects of the parameters were studied by the "one variable at a time" method. As the two enantiomers share almost identical physical properties, the optimization experiments were performed using only (S)-(-)-nicotine.

### 3.3. Effect of sample pH

To investigate the optimum pH for the extraction of nicotine from an aqueous solution, pH 2, 7, 9, 10, and 11 were controlled using 0.1 M HCl and 0.5 M KOH. After fixing the quantity of extraction and dispersion solvent at 500 μL and the quantity of sodium sulfate at 1.0 g, an extraction experiment was performed and the peak areas from the chromatograms were compared to establish the optimum conditions. As a result, nicotine could not be extracted under acidic and neutral conditions, and the extraction efficiency was highest with pH 10 (*Fig*. 5).

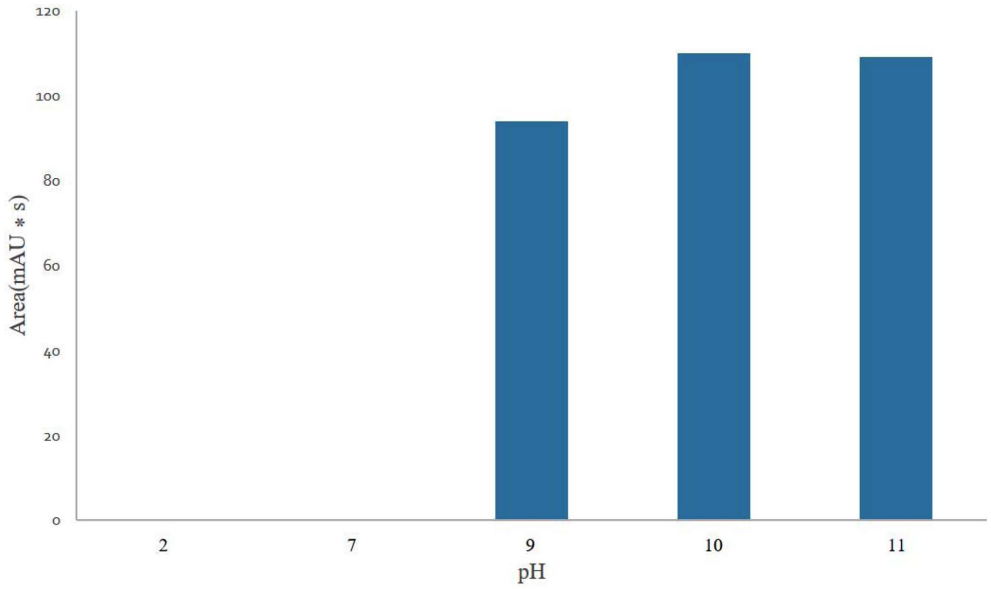



*Fig*. 5. Effect of pH on peak area of (S)-(-)-nicotine for optimization.

This is because nicotine is a basic compound, so it exists in a neutral form at a high pH, so that the extraction efficiency for an organic solvent is high.

### 3.4. Effect of the combined extraction and dispersion solvents

Diethyl ether and n-hexane, which are less dense than water, were used as extraction solvents, and methanol, ethanol, acetone, and acetonitrile, which are hydrophilic organic solvents, were tested as potential dispersion solvents. In all combinations except for diethyl ether-methanol, diethyl ether-acetone, n-hexane-ethanol, and n-hexane-acetone, two solvents did not mix, and further experiments were not possible because the solvent was not completely volatilized.

The method showed optimal extraction efficiency when the pH = 10, the quantity of sodium sulfate was 1.0 g, the combined extraction and dispersion solvent volume was 500 μL, diethyl ether was used as the extraction solvent, and acetone was used as the dispersion solvent (*Fig*. 6).

### 3.5. Effect of volume ratio of dispersion solvent and extraction solvent

When the volume of the dispersion solvent was too small compared to the volume of the extraction solvent, the extraction solvent was not dispersed in the form of fine droplets in the aqueous sample solution. In contrast, when the volume of the dispersion solvent was high, the extraction solvent was partially dissolved in the aqueous sample solution, which inhibited extraction.

To determine the optimal ratio of the extraction solvent (diethyl ether) and the dispersion solvent (acetone), the following ratios were tested: 2:1, 1:1, 1:2, and 1:3. After comparing the peak areas from the HPLC results, 1:1 was found to be the optimal ratio for extraction (*Fig*. 7).

### 3.6. Effect of total volume of dispersion and extraction solvents

After the extraction solvent:dispersion solvent ratio was fixed at 1:1, the combined volume of extraction solvent and dispersion solvent was optimized. The extraction efficiency was tested by comparing the following total volumes: 500, 600, 800, and 1000 μL.

The extraction efficiency was found to be highest when the total volume was 500 μL (*Fig*. 8). If the volume is smaller than this, there is a concentration effect, but if the volume is too small, the subsequent

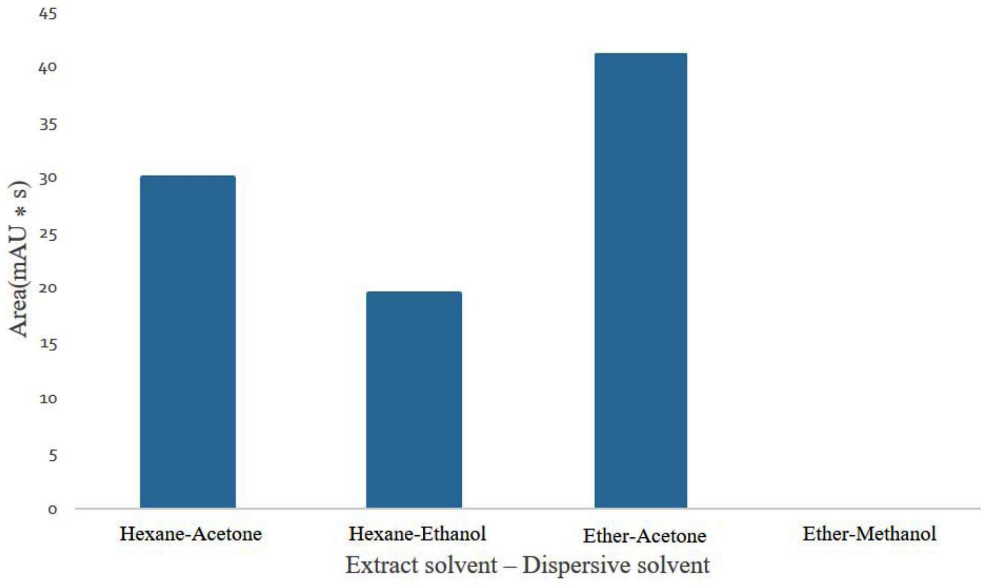



*Fig*. 6. Type of extract and dispersive solvent for optimization.

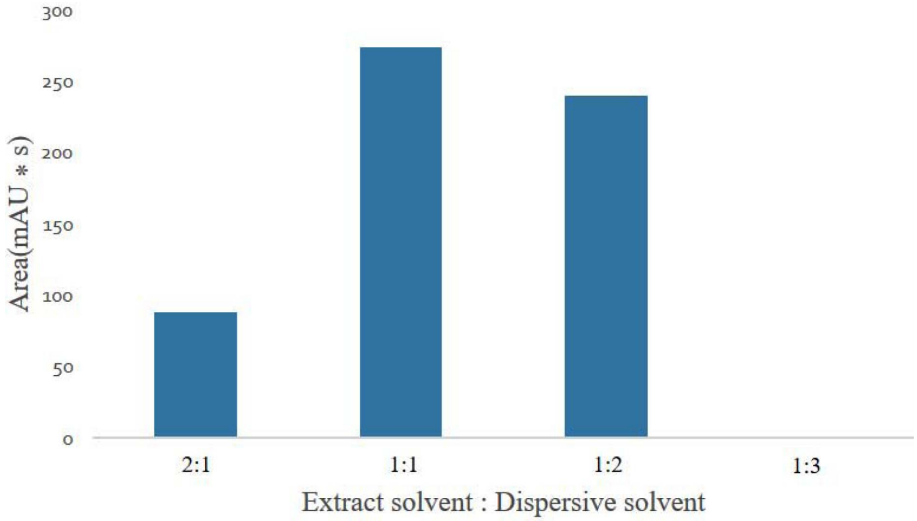



*Fig.* 7. Ratio of extract and dispersive solvent for optimization.

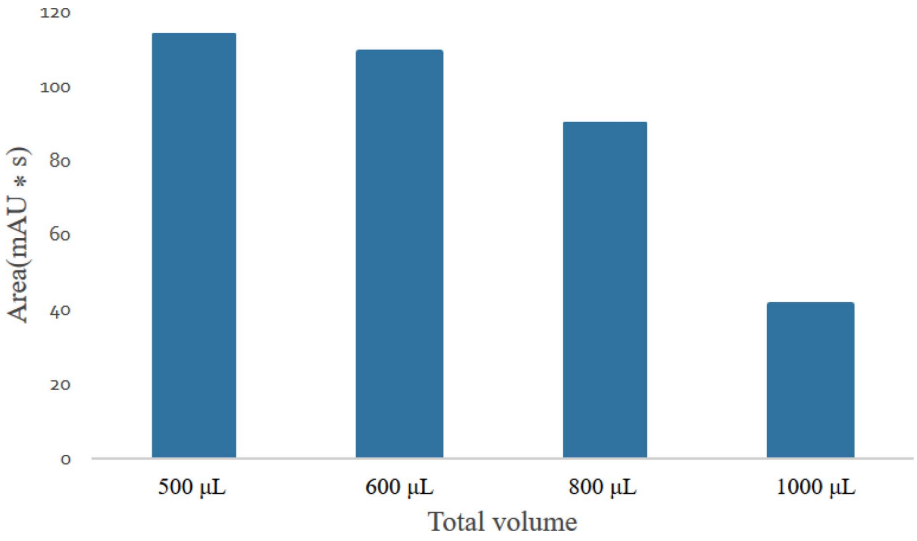



*Fig.* 8. Volume of extract and dispersive solvent for optimization (1:1).

experimental process is difficult.

### 3.7. Effect of salting-out

When salt is added to an aqueous sample solution, as the ionic strength increases, the analytes contained in the aqueous solution are effectively extracted, while the solubility of the organic solvent in the aqueous solution layer is reduced. In order to maximize the salting-out effect, the type and quantity of salt were optimized.

After fixing the quantity of salt to 1 g using NaCl, $Na_2SO_4$, and $MgSO_4$, the best extraction efficiency was achieved using $Na_2SO_4$, and layer separation was not achieved with $MgSO_4$.

The following quantities of sodium sulfate were tested: 0 g, 1.0 g, 1.5 g, and 2.0 g, and the results demonstrated that layer separation did not occur with no salt, and the highest efficiency was achieved with 1.5 g.

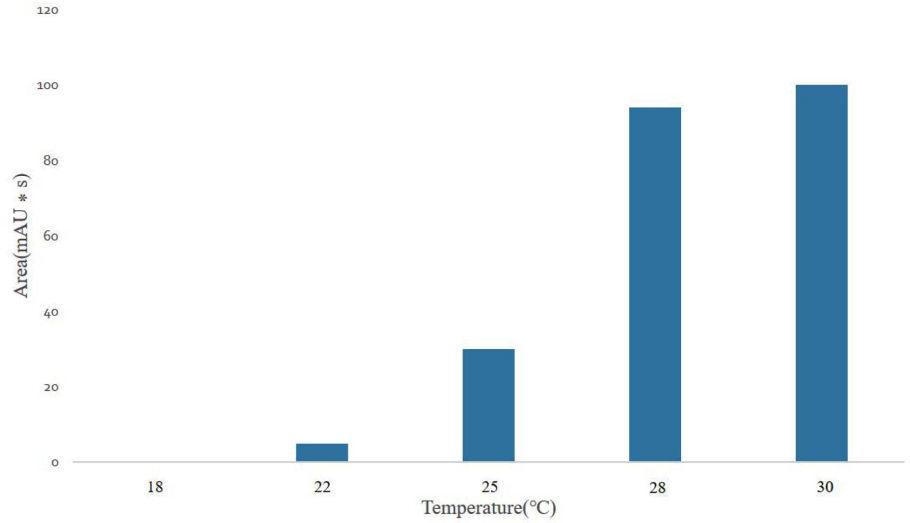



*Fig*. 9. Effects of temperature for optimization.

### 3.8. Effect of laboratory temperature

To investigate the temperature of experimental environment at which the added salt in tube was completely dissolved in the aqueous sample solution, experiments were conducted at temperatures of 18 °C, 22 °C, 25 °C, 28 °C, and 30 °C.

Layer separation between the extraction solvent and the aqueous solution did not occur at 18 °C, and the extraction efficiency increased as the temperature increased. However, at 30 °C, the viscosity of diethyl ether was reduced, resulting in significant error when collecting the supernatant. Therefore, 28 °C was established as the optimal laboratory temperature (*Fig*. 9).

### 3.9. Method Validation

Method validation was performed for several parameters to ensure the reliability of the quantitative results determined using the established method by the HPLC.

#### 3.9.1. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD concentrations were predicted based on past experience to be 0.4 μg/mL (for (S)-(-)-nicotine) and 0.5 μg/mL (for (R)-(+)-nicotine). Seven spiked samples at these concentrations were prepared in purified water and analyzed using the established sample method to obtain the standard deviation (σ). In addition, a one-point calibration curve was prepared by analyzing spiked samples at concentrations of 2 μg/mL and 2.5 μg/mL. The theoretical LOD (3σ/m) and LOQ (10 σ/m) were calculated using the slope (m) and standard deviation (σ) obtained from this calibration curve.

The LOD was found to be 0.11 μg/mL and 0.17 μg/mL for (S)-(-)-nicotine and (R)-(+)-nicotine, respectively, and the LOQs were 0.30 μg/mL and 0.44 μg/mL, respectively. The LOD was S/N > 3, and the LOQ was a concentration satisfying S/N > 3 and the relative standard deviation (RSD) was > 15%.

*Table* 3. Working range, linear equation, and correlation coefficient for analysis of chiral nicotine

| Compounds | Working range (μg/mL) | Linear equation | $r^2$ |
|---|---|---|---|
| (S)-(-)-nicotine | 0.30–18 | y = 56.921x + 24.604 | 0.9996 |
| (R)-(+)-nicotine | 0.44–4.4 | y = 66.809x + 2.6145 | 0.9978 |

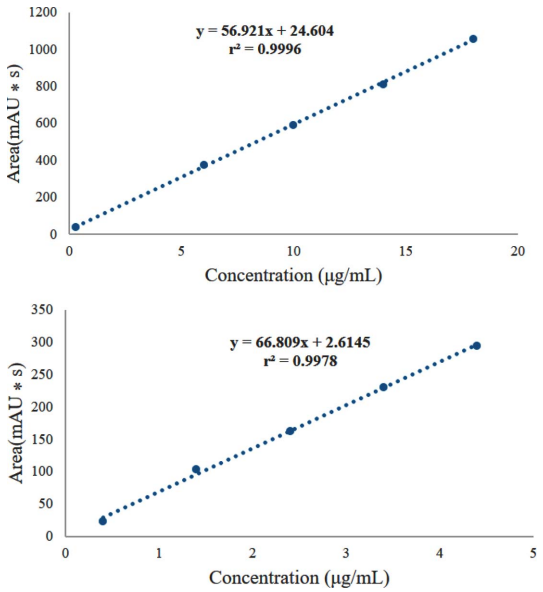



*Fig*. 10. Calibration curves (upper layer: (S)-nicotine, lower layer: (R)-nicotine) of nicotine enantiomers.

### 3.9.2. Calibration curves

For (S)-(-)-nicotine and (R)-(+)-nicotine, the calibration curves were prepared in the ranges of 0.30-18 μg/mL and 0.44-4.4 μg/mL, respectively. The correlation coefficients ($r^2$) were 0.9978 and 0.9996, indicating good linearity (*Fig*. 10 and *Table* 3).

### 3.9.3. Accuracy and precision

The accuracy was expressed as a relative recovery, which was calculated by dividing the experimental value by the theoretical value and multiplying by 100. The precision was expressed as the relative standard deviation (RSD).

The intra-day accuracy and precision were 79.9-110.6 % and 1.3-12.0 %, respectively, and the inter-day accuracy and precision over 3 days were 87.8-108.0 % and 4.0-12.8 %, respectively. These results indicate that the method has good accuracy and precision and is suitable for quantitative analysis (*Table* 4).

## 3.10. Assay of real samples

(S)-(+)-nicotine was detected in all e-liquids. However, in products other than tobacco-free nicotine (TFN), (R)-(+)-nicotine was detected at a lower concentration than the LOQ. (*Table* 5).

After smoking stem nicotine, (S)-(-)-nicotine was present in both the urine and saliva samples at levels of 0.34 μg/mL and 0.62 μg/mL, respectively. However, (R)-(+)-nicotine was not detected. After smoking TFN, (S)-(-)-nicotine and (R)-(+)-nicotine were detected at levels of 0.32 μg/mL and 0.46 μg/mL in urine, and 0.39 μg/mL and 0.67 μg/mL in saliva, respectively (*Fig*. 11).

Thus, we have demonstrated that by analyzing the ratio of nicotine isomers contained in the smoker's urine or saliva, it is possible to determine the type of e-liquid smoked.

*Table* 4. Intra-day and inter-day accuracy and precision (n=3)

| Compounds | Concentration (μg/mL) | Intra-day | | Inter-day | |
|---|---|---|---|---|---|
| | | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) |
| (S)-(-)-nicotine | 0.30 | 79.2 | 5.9 | 93.3 | 10.7 |
| | 6.0 | 109.3 | 8.9 | 102.8 | 4.3 |
| | 10 | 101.2 | 1.3 | 99.2 | 6.1 |
| | 14 | 98 | 11.5 | 98.9 | 7.2 |
| | 18 | 102.2 | 10.3 | 100.6 | 4.1 |
| (R)-(+)-nicotine | 0.44 | 79.9 | 10.5 | 87.8 | 10.5 |
| | 1.4 | 110.6 | 4.0 | 108.0 | 4.0 |
| | 2.4 | 100.6 | 12.0 | 100.1 | 12.8 |
| | 3.4 | 100.7 | 11.3 | 100.1 | 5.6 |
| | 4.4 | 97.1 | 10.3 | 99.3 | 4.1 |

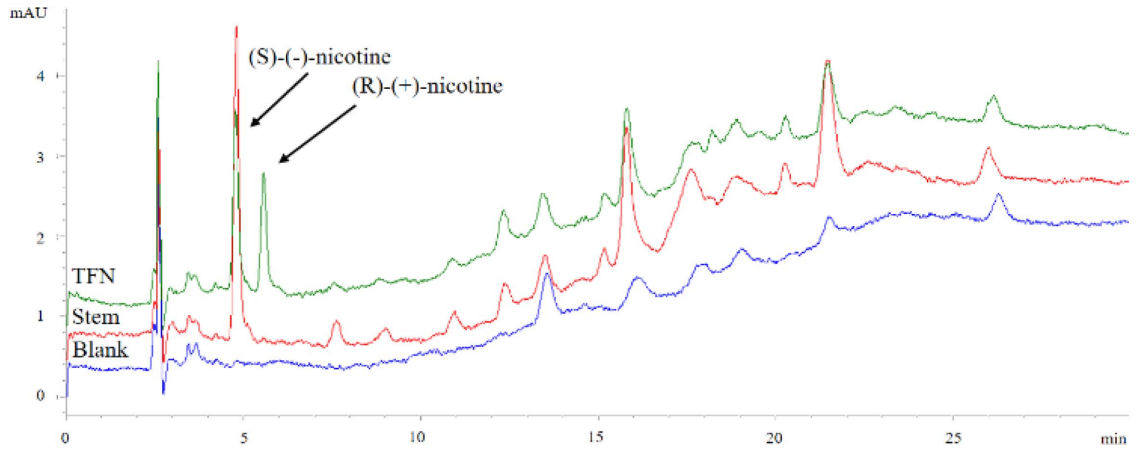



*Fig.* 11. Comparison of chromatograms from urine samples after smoking different types of e-liquid and cigarettes.

*Table* 5. Chiral nicotine in e-Liquid

| Sample | (S)-nicotine (mg/mL) | (R)-nicotine (mg/mL) |
|---|---|---|
| Salt 1 | 11.37 | <LOQ |
| Salt 2 | 11.33 | <LOQ |
| Salt 3 | 11.23 | <LOQ |
| Stem 1 | 10.11 | <LOQ |
| Stem 2 | 10.07 | <LOQ |
| Stem 3 | 12.15 | <LOQ |
| Natural 1 | 9.21 | <LOQ |
| Natural 2 | 8.17 | <LOQ |
| Natural 3 | 11.46 | <LOQ |
| TFN 1* | 6.30 | 4.95 |
| TFN 2 | 5.49 | 4.17 |
| TFN 3 | 6.79 | 5.44 |

*TFN = tobacco-free nicotine

## 4. Conclusions

This study compared the efficacy of two systems, chiral GC/MS and chiral HPLC-UV, for the analysis of nicotine enantiomers. The HPLC system was found to be suitable for the analysis of nicotine enantiomers.

A sample preparation method for aqueous samples containing nicotine was established using DLLME. Various parameters were optimized, including the pH (10), the extraction solvent (diethyl ether), and the dispersion solvent (acetone). The optimized extraction solvent:dispersion solvent ratio was 1:1, the combined extraction and dispersion solvent volume was 500 μL, and the quantity of salt (sodium sulfate) was 1.5 g.

The LODs of (S)-(-)-nicotine and (R)-(+)-nicotine were 0.11 μg/mL, 0.17 μg/mL, respectively, and the LOQ was 0.30 μg/mL and 0.44 μg/mL, respectively. The intra-day accuracy and precision were 79.9-110.6 % and 1.3-12.0 %, respectively and the inter-day accuracy and precision were 87.8-108.0 % and 4.0-12.8 %, respectively. The correlation coefficient ($r^2$) of the calibration curve was 0.9978-0.9996.

In summary, (S)-(-)-nicotine and (R)-(+)-nicotine were detected in all samples. However, in all samples except for TFN, the content of (R)-(+)-nicotine was much lower than that of (S)-(-)-nicotine.

In TFNs, which are thought to contain synthetic nicotine, the (S)- and (R)- forms were found to be present in similar proportions. The content ratio of (S)-(-)-nicotine and (R)-(+)-nicotine in the urine and saliva samples differed depending on the type of e-liquid smoked.

This method, which measures chiral nicotine using DLLME, is a more efficient analysis method than the classical LLE method, as it uses lesser organic solvent and has a shorter analysis time. It can also measure μg/mL concentrations, which means it can be applied to e-liquid and biological samples.

## Acknowledgements

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**Authors' Position**

Seunghoon Song : Graduate Student
Seung-Woon Myung : Professor