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Analysis of Nicotine and Nicotine-Related Compounds in Electronic Cigarette Liquids and Aerosols by Liquid Chromatography-Tandem Mass Spectrometry *

by

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SUMMARY

The objective of this study was to develop and validate an analytical method for determining nicotine and nicotine related compounds (i.e., nicotine-*N*-oxide, cotinine, nornicotine, anatabine, myosmine, anabasine, and β-nicotyrine) in e-cigarette aerosols and e-liquids. Aerosol collection was achieved using a Cambridge collection pad. The sample preparation consisted of adding deuterated internal standards to the collection pad and extracting with 100 mM ammonium acetate solution using a wrist-action shaker. The filtrate was then analyzed by LC-MS/MS using a Gemini NX C_{18} column (3 µm, 150 × 3 mm) with a mobile phase gradient system consisting of acetonitrile and 10% acetonitrile in 10 mM ammonium bicarbonate (pH = 8.0) and electrospray ionization (ESI) in the positive mode. The e-liquid was analyzed using the same instrumental parameters, but simplifying the sample preparation procedure by adding deuterated internal standards directly to the 100-mg sample. The sample was then extracted with 100 mM ammonium acetate solution, sonicated, and filtered. In this study, the method's accuracy, robustness, and reliability were enhanced by using deuterated analogues of each compound as internal standards and by applying two ion-transition pairs for each compound for the confirmation and quantification. Validation experiments demonstrated good sensitivity, specificity and reproducibility. All the target compound calibrations exhibited satisfactory linearity from 0.050 to 5.0 mg/mL ($r^2 > 0.995$). The average recoveries for e-liquids varied from 85.2% (nicotine-*N*-oxide) to 110% (β-nicotyrine) with recoveries for all compounds exhibiting a coefficient of variation (CV) < 5.0%. Similarly, the average recoveries for e-cigarette aerosols varied from 87.8% (for nicotine-*N*-oxide) to 111% (for myosmine) with all CV < 8.8%. The LOD and LOQ for e-liquids for all target compounds ranged from 0.234 and 0.781 µg/g (cotinine) to 1.66 and 5.48 µg/g (nicotine-*N*-oxide). For e-cigarette aerosols these limits ranged from 0.094 and 0.312 µg/collection (cotinine) to 0.872 and 2.87 µg/collection (nicotine-*N*-oxide). This methodology was used to quantitatively determine if any of the target compounds were present in a variety of sample matrices, including e-cigarette solutions and aerosols, and was successfully applied to stability studies, to monitor changes in the target compound levels which might be caused by e-cigarette formulations, components and the storage conditions. [Beitr. Tabakforsch. Int. 27 (2017) 154–167]

ZUSAMMENFASSUNG

Ziel dieser Studie war die Entwicklung und Validierung einer Analysemethode zur Bestimmung von Nikotin und nikotin-verwandten Verbindungen (d.h. Nikotin-*N*-Oxid, Cotinin, Nornikotin, Anatabin, Myosmin, Anabasin und β-Nicotyrin) in E-Zigaretten-Aerosolen und E-liquids. Die Aerosole wurden mit einem Cambridgefilter aufgefangen. Die Probenvorbereitung umfasste die Zugabe deuterierter interner Standards zum Auffangfilter und die Extraktion mit 100 mM Ammoniumacetat-Lösung mithilfe eines 'Wrist-

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Action-Schüttlers'. Das Filtrat wurde anschließend mittels LC-MS/MS mit einer Gemini NX C_{18}-Säule (3 µm, 150 × 3 mm) unter Verwendung eines Gradientensystems mit mobiler Phase, bestehend aus Acetonitril und 10% Acetonitril in 10 mM Ammoniumbicarbonat (pH = 8,0) und Elektrospray-Ionisierung (ESI) im positiven Modus analysiert. Das E-liquid wurde mit denselben Geräteparametern analysiert; das Probenvorbereitungsverfahren wurde jedoch durch Zugabe deuterierter interner Standards direkt zur 100-mg-Probe vereinfacht. Die Probe wurde danach mit 100 mM Ammoniumacetat-Lösung extrahiert, beschallt und gefiltert. Die Genauigkeit, Belastbarkeit und Zuverlässigkeit der Methode wurde in dieser Studie durch den Einsatz deuterierter Analoga jeder Verbindung als interne Standards und durch Anwendung von zwei Ionenübergangs-Paaren für jede Verbindung zur Bestätigung und Quantifizierung verbessert. Validierungsversuche zeigten eine gute Sensitivität, Spezifität und Reproduzierbarkeit. Alle Zielsubstanz-Kalibrierungen zeigten eine zufriedenstellende Linearität von 0,050 bis 5,0 µg/mL ($r^2 > 0,995$). Die durchschnittlichen Ausbeuten variierten für E-liquids von 85,2% (Nikotin-*N*-Oxid) bis zu 110% (β-Nicotyrin), wobei die Ausbeuten aller Verbindungen einen Variationskoeffizienten (CV) < 5,0% aufwiesen. Auch die durchschnittlichen Ausbeuten für E-Zigaretten-Aerosole variierten von 87,8% (Nikotin-*N*-Oxid) bis 111% (β-Nicotyrin), wobei die Ausbeuten aller Verbindungen einen Variationskoeffizienten (CV) < 8,8% aufwiesen. Die Nachweisgrenze (LOD) und Bestimmungsgrenze (LOQ) für E-liquids lag für alle Zielsubstanzen zwischen 0,234 bzw. 0,781 µg/g (Cotinin) und 1,66 bzw. 5,48 µg/g (Nikotin-*N*-Oxid). Für E-Zigaretten-Aerosole lagen diese Grenzwerte zwischen 0,094 bzw. 0,312 µg/Probe (Cotinin) und 0,872 bzw. 2,87 µg/Probe (Nikotin-*N*-Oxid). Diese Methode wurde eingesetzt, um quantitativ zu bestimmen, ob die Zielsubstanzen in einer Vielfalt von Probenmatrizen, darunter E-Zigaretten-Lösungen und -Aerosole, vorkommen. Sie wurde erfolgreich bei Stabilitätsuntersuchungen angewendet und diente zur Überwachung von Veränderungen in den Konzentrationen der Zielsubstanzen, die durch E-Zigaretten-Formulierungen, -Bestandteile sowie die Lagerungsbedingungen verursacht sein könnten. [Beitr. Tabakforsch. Int. 27 (2017) 154–167]

RESUME

L'étude présentée ici eut pour objet la mise au point et la validation d'une méthode analytique servant à identifier la nicotine et les composés de nicotine (par exemple, le *N*-oxyde de nicotine, la cotinine, la nornicotine, l'anatabine, la myosmine, l'anabasine et la β-nicotyrine) dans les aérosols et les liquides à vapoter contenus dans les cigarettes électroniques. Le prélèvement des aérosols fut réalisé à l'aide d'un tampon de prélèvement Cambridge. La préparation des échantillons inclut l'ajout d'étalons internes deutérés au tampon de prélèvement et une extraction à l'aide de 100 mM de solution d'acétate d'ammonium dans un agitateur oscillant 'wrist-action'. Le filtrat fut ensuite analysé par chromatographie en phase liquide couplée à une spectrométrie de masse en tandem (LC-MS-MS) avec une colonne Gemini NX C_{18} (3 µm, 150 × 3 mm), associée à un suivi du gradient de la phase mobile à l'aide d'une solution d'acétonitrile et de 10% d'acétonitrile dans 10 mM de bicarbonate d'ammonium (pH = 8,0) et par ionisation par électronébuliseur (ESI) en mode positif. Le liquide à vapoter fut analysé à l'aide des mêmes paramètres instrumentaux mais la préparation de l'échantillon fut simplifiée par l'ajout direct d'étalons internes deutérés à l'échantillon de 100 mg. L'échantillon subit ensuite une extraction à l'aide d'une solution de 100 mM d'acétate d'ammonium, une sonication et un filtrage. Dans la présente étude, la précision, la robustesse et la fiabilité de la méthode furent renforcées par l'utilisation, en guise d'étalons internes, d'analogues deutérés pour chaque composé et par l'application de deux paires de transport ionique pour chaque composé en vue d'une confirmation et d'une quantification. Les expériences de validation attestèrent de bons niveaux de sensibilité, spécificité et reproductibilité. Toutes les calibrations des composés ciblés mirent en lumière une linéarité satisfaisante allant de 0,050 à 5,0 mg/mL ($r^2 > 0,995$). Les récupérations moyennes pour les liquides à vapoter varièrent de 85,2% (*N*-oxyde de nicotine) à 110% (β-nicotyrine), sachant que les récupérations pour tous les composés présentèrent un coefficient de variation (CV) inférieur à 5,0%. De la même façon, les récupérations moyennes pour les aérosols pour cigarettes électroniques varièrent de 87,8% (*N*-oxyde de nicotine) à 111% (myosmine) sachant que tous les CV étaient inférieurs à 8,8%. La limite de détection et la limite de quantification pour les liquides à vapoter, tous composés confondus, s'étendirent de 0,234 et 0,781 µg/g (cotinine) à 1,66 et 5,48 µg/g (*N*-oxyde de nicotine). Pour les aérosols utilisés dans les cigarettes électroniques, ces limites s'étendirent de 0,094 et 0,312 µg par prélèvement (cotinine) à 0,872 et 2,87 µg par prélèvement (*N*-oxyde de nicotine). Cette méthodologie fut utilisée afin de déterminer quantitativement si un des composés ciblés était présent dans un éventail de matrices d'échantillon, y compris les solutions et les aérosols pour les cigarettes électroniques et fut appliquée, avec succès, à des études de stabilité afin de suivre ces variations dans la teneur des composés ciblés, qui sont susceptibles d'être imputables aux formulations, aux composés et aux conditions d'entreposage des cigarettes électroniques. [Beitr. Tabakforsch. Int. 27 (2017) 154–167]

1. INTRODUCTION

Electronic cigarettes (e-cigarettes) are growing rapidly in popularity. These devices, also known as electronic nicotine delivery systems (ENDS), generally resemble a conventional cigarette in shape, dimensions, and configurations. The common components for most e-cigarettes include a cartridge for replacement liquid (e-liquid), a heated aerosol generator, a flow sensor, and a battery that provides power to the operating components (1). The primary consumable (e-liquid) typically contains a carrier (e.g., 1,2-propylene glycol or/and glycerol), various kinds of concentrated flavors, a small amount of water and potentially other additives, with variable concentrations of nicotine, which is the physiologically active ingredient in e-liquids (2). Most of the nicotine used in the manufacture of e-liquids comes from the extraction of tobacco. Although

nicotine is the main alkaloid in the extract, related alkaloids such as nornicotine, anatabine, and anabasine may also be found as impurities (3). In addition, several other minor alkaloids such as cotinine, nicotine-*N*-oxide, myosmine, and β-nicotyrine may be present as degradation products as a result of microbial action, flavor oxidation from unstable formulations, exposure to high temperatures, interactions with storage containers, or other issues encountered during manufacture and/or storage of the product (4). These impurities and degradation products (structures shown in Figure 1) can be transferred to the aerosol and subsequently to the user of the device.

There are many methods for the analysis of nicotine and related alkaloids including gas chromatography coupled with flame ionization detection (FID) (6, 7), nitrogen-phosphorus detection (NPD) (8), and mass spectrometry (MS) (9). However, methods for the analysis of nicotine in tobacco smoke and products may not be suitable for e-liquids and aerosols since several of the nicotine related compounds such as nicotine-*N*-oxide, are known to be thermally unstable at temperatures required for GC analysis. Some target compounds such as nornicotine can show severe issues with respect to carryover in the injector (7, 8, 10).

Both of these issues have been solved by analysis of e-liquids and aerosols using HPLC with UV detection (11). However, HPLC-UV methods are prone to error due to the presence of flavors in tobacco extracts, and coloring ingredients that are found in many e-cigarette products (12). First, there are over twenty pyridine-type alkaloids with a characteristic maximum absorption wavelength (ca. 260 nm) reported to be present in tobacco extracts (13, 14). Therefore, an HPLC-UV method cannot differentiate potentially co-eluting compounds. Second, the potentially complicated matrices can also cause baseline fluctuations resulting in difficult and often inaccurate integrations for quantification.

The work presented herein describes a novel liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the simultaneous and unambiguous quantification of nicotine and nicotine-related compounds. The method eliminates matrix interferences arising from other compounds that share the same parent mass but lack the correct transition ion, thus drastically decreasing background interferences and reducing detection limits.

2. EXPERIMENTAL

2.1 Chemicals, reagents and calibration solutions

Nicotine, cotinine, myosmine, anabasine, β-nicotyrine, anatabine, nornicotine, nicotine-*N*-oxide (purity > 95%), and deuterated internal standards nicotine-d_3, cotinine-d_3, myosmine-d_4, anabasine-d_4, β-nicotyrine-d_3, anatabine-d_4, nornicotine-d_4, and nicotine-*N*-oxide-d_3 (purity > 93%) were purchased from TRC, (Toronto, ON, Canada) and used as received. The standards and their deuterated internal standards were dissolved in acetonitrile to prepare primary stock solutions at a concentration of 1 mg/mL (except nicotine-d_3, prepared at 2.5 mg/mL). The primary stock solutions were further diluted with acetonitrile to make secondary stock solutions containing 20 µg/mL for each target compound, 50 µg/mL for the deuterated internal standards, with the exception of nicotine-d_3 at 125 µg/mL. Calibration solutions were made from dilutions of the stock solutions in 10% acetonitrile in an aqueous solution to obtain the following concentrations: 0.050, 0.100, 0.200, 0.500, 1.00, 3.00 and 5.00 µg/mL. Concentrations for nicotine-d_3 and the other deuterated internal standards were 2.50 µg/mL and 1.00 µg/mL in each calibration solution, respectively. Standards, stock solutions, and calibration solutions were stored at 4 °C.

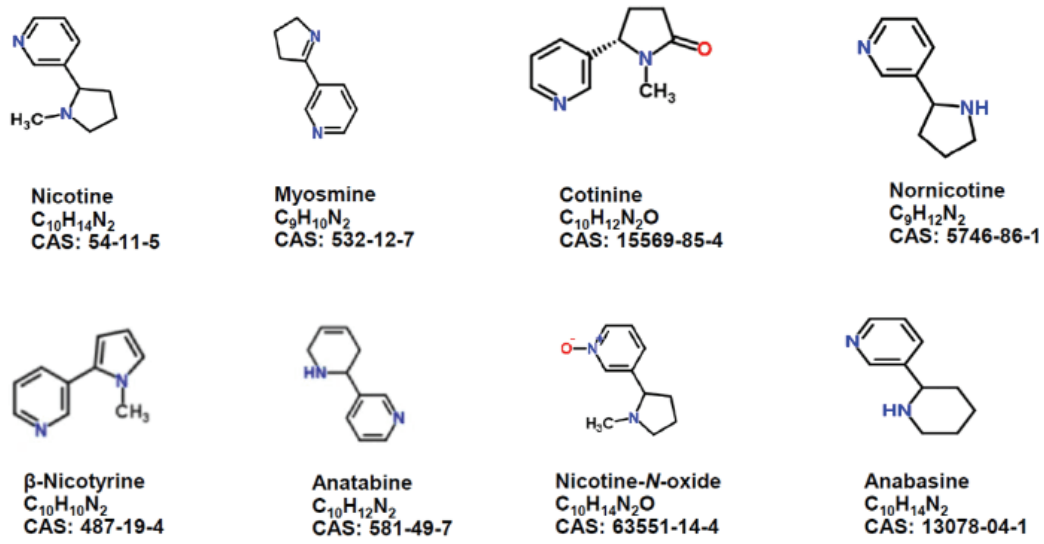

Figure 1. Structures of nicotine and nicotine-related compounds.

Acetonitrile (HPLC grade) was obtained from EMD Chemicals Inc. (VWR International, Mississauga, ON, Canada). Ammonium bicarbonate (HPLC grade) and other chemicals (ammonium hydroxide solution (28–30%), acetic acid, 1,2-propylene glycol and glycerol) were of analytical grade and obtained from Sigma-Aldrich (Oakville, Canada).

2.2 E-liquids and e-cigarette products stability test conditions

The e-liquids and e-cigarette products were maintained at 25 °C/60% relative humidity (RH) and 40 °C/75% RH for various days in two Blinder® climatic chambers. The actual temperature of each chamber was recorded by digital data loggers throughout the study. The samples were pulled, extracted and analyzed at predetermined time intervals.

2.3 E-cigarette aerosol and e-liquid sample preparations

Unless otherwise stated, the e-cigarette aerosol was generated on a smoking machine under the standard smoking conditions recommended by CORESTA Method CRM 81: 55 mL puff volume, 30 s puff interval, 3 s puff duration and rectangular shape puff profile (15). The e-cigarette aerosol was collected using a 44-mM Cambridge filter pad. After puffing, the pad was extracted on a wrist action shaker with a mixture of 38 mL of 100-mM ammonium acetate solution and 2 mL of secondary internal standard solution for 30 min. An aliquot of the extract was syringe filtered (0.22 µm PVDF filter) and then analyzed by LC-MS/MS method.

For e-liquid determinations, a 0.1-g sample was accurately weighed into a glass extraction vial (20 mL) and extracted using an ultrasonic water bath at room temperature with a mixture of 9.5 mL of 100 mM ammonium acetate solution and 0.5 mL of the secondary internal standard solution for 5 min. An aliquot of the extract was syringe-filtered (0.22 µm PVDF filter), and then analyzed by LC/MS/MS.

Laboratory Fortified Matrix (LFM) samples for e-liquid analysis were prepared by fortifying 0.1 g mixture of 1,2-propylene glycol and glycerol (50/50 *v/v*) with known amounts of the target compounds. LFM samples for aerosol analysis were prepared by adding 0.5 g of the mixture of 1,2-propylene glycol and glycerol (50/50 *v/v*) to a fresh collection pad to represent a nominal collection amount and fortifying with a known amount of the target compounds. Laboratory Fortified Blank (LFB) samples for e-liquid analysis were prepared by fortifying an empty 20-mL extraction vial with a known amount of the target compounds. Similarly, the LFB samples for aerosol analysis were prepared by fortifying the filter pads with a known amount of target compounds. Laboratory Reagent Blank (LRB) samples for both e-liquid and aerosol analyses were prepared similarly to LFB samples with no fortification of target compounds. LFMs, LFBs and LRBs were processed through the sample preparation procedure described above.

2.4 Sample analysis

The sample analysis was performed on a Waters ultra-high-pressure liquid chromatography system coupled to a Waters XEVO TQS UPLC-MS/MS system (Waters Corporation, Milford, MA, USA). The Masslynx workstation was used for the system control and data acquisition (Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved by using a Gemini NX C_{18} column (Phenomenex, Torrance, CA, USA) (3 µm, 150 × 3 mm) with SecurityGuard™ cartridge Gemini-NX (4 × 2 mm) with a mobile phase gradient system consisting of acetonitrile (mobile phase A) and 10% acetonitrile in 10 mM ammonium bicarbonate (pH = 8.0) (mobile phase B). The eluent gradient is listed in the Table 1. The injection volume was 10 µL, the flowrate was 0.5 mL/min, and the column temperature was maintained at 30 °C. The sample was analyzed in the positive electrospray ionization (ESI+) mode. The MS conditions were set as follows: 20 L/h flow rate of cone gas, 800 L/h desolvation gas, and 0.15 mL/min collision gas. Source and desolvation temperatures were held at 150 °C and 350 °C, respectively. The capillary voltage was set at 3.00 kV. The detector was used in multiple reaction monitoring (MRM) mode. MRM transitions for target compounds and internal standards, retention times, dwell times, cone voltages, and collision energies are reported in Table 2.

Table 1. HPLC gradient conditions.

Time (min)	Mobile phase B (%)	Flow rate (mL/min)	Curve
0.00	90	0.5	Initial
8.00	75	0.5	6
15.0	40	0.5	6
17.0	90	0.5	6
18.0	90	0.5	6

3. RESULTS AND DISCUSSION

3.1 The effect of mobile phase pH on the chromatographic profiles of target compounds

As shown in Figure 1, the target compounds generally consist of pyridine and pyrrolidine structures. Structurally similar compounds can have the same major mass spectral fragments with common precursor and/or product ions. For example, nicotine and anabasine have identical precursors (as shown in Table 2) which can produce the same fragment ions. In these cases, differentiation must rely on both the mass resolution capability of the mass spectrometer and the chromatographic resolution. Moreover, the target compounds are generally weak basic compounds, but have a rather wide range of polarities (log P from 0.04 of cotinine to 1.00 of β-nicotyrine) and pKa values (pKa from 4.63 of nicotine-*N*-oxide to 9.86 of anabasine) (16). The retention times of myosmine, β-nicotyrine, cotinine, and nicotine-*N*-oxide show little change as the pH of the mobile phase was increased from 6.52 to 10.6 as shown in Table 3. It was most likely that these compounds existed in a fixed form in these pH ranges. On the contrary, the retention times of nornicotine, anatabine, anabasine and nicotine were significantly changed when the pH of the mobile phase was decreased below 7.68. At lower pH, it was postulated that the target compounds were protonated and

Table 2. MRM parameters for the detection of target compounds.

Compound	Retention time (min)	Precursor ion (m/z)	Fragment ion (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (V)
Nicotine-*N*-oxide	1.41	179	132	0.017	32	22
		179	130*	0.017	32	16
Nicotine-*N*-oxide-d_3	1.41	182	132	0.017	26	18
		182	130*	0.017	26	26
Cotinine	2.37	177	80	0.017	34	22
		177	98*	0.017	34	20
Cotinine-d_3	2.38	180	80	0.017	26	20
		180	101*	0.017	26	22
Nornicotine	2.97	149	117	0.017	14	20
		149	149*	0.017	14	20
Nornicotine-d_4	2.99	153	121	0.017	20	20
		153	153*	0.017	20	20
Anatabine	3.82	161	144	0.017	22	14
		161	161*	0.017	20	14
Anatabine-d_4	3.85	165	111	0.017	28	16
		165	165*	0.017	28	16
Myosmine	4.11	147	117	0.017	40	22
		147	147*	0.017	40	20
Myosmine-d_4	4.18	151	81	0.017	38	28
		151	151*	0.017	30	10
Anabasine	4.14	163	94	0.017	28	18
		163	163*	0.017	24	10
Anabasine-d_4	4.20	167	96	0.017	24	10
		167	167*	0.017	24	10
Nicotine	5.52	163	132	0.017	34	14
		163	130*	0.017	10	18
Nicotine-d_3	5.54	166	117	0.017	22	24
		166	130*	0.017	22	16
β-Nicotyrine	9.91	159	144	0.017	34	20
		159	159*	0.017	25	10
β-Nicotyrine-d_3	9.97	162	135	0.017	38	20
		162	162*	0.017	38	20

* Indicates secondary MRM transitions used for compound verification.

Table 3. Analyte characteristics and retention times of target compounds with mobile phases at various pHs [a].

Compound	pKa	logP	RT time (min)					
			pH = 6.52	pH = 7.68	pH = 9.00	pH = 9.33	pH = 10.0	pH = 10.6
Nicotine-*N*-oxide	4.63	NA [b]	1.07	1.06	1.04	1.04	0.99	0.95
Cotinine	4.72	0.04	2.94	2.88	2.78	2.75	2.73	2.81
Myosmine	7.81	0.70	5.55	5.51	5.39	5.34	5.22	5.41
β-Nicotyrine	NA [b]	1.00	11.5	11.5	11.3	11.3	11.2	11.3
Nicotine	9.13	0.93	3.04	5.98	7.14	7.24	7.25	7.43
Nornicotine	9.83	0.50	1.30	2.16	2.82	3.93	4.41	4.58
Anatabine	8.77	0.93	2.01	3.97	4.99	5.09	5.08	5.26
Anabasine	9.86	0.96	1.87	3.42	5.3	5.85	6.45	6.69

[a] For this study, the LC gradient program was set as time / % mobile phase-B (with various pHs): 0.00 / 100.0, 10.0 / 80.0, 20.0 / 10.0, 21.0 / 100.

[b] NA: Not available

could not be strongly retained on the reversed phase stationary phase. Under this low pH mobile phase condition, some target compounds showed tailing peak shapes and coeluted. When the pH of the mobile was made higher than 7.68, nornicotine, anatabine, anabasine and nicotine were in their neutral molecular forms resulting in longer and more reproducible retention times regardless of fluctuations in the pH of the mobile phase. However, further experiments showed that using a higher pH mobile phase could result in a severe decrease in the sensitivity of some target compounds, especially for that of nornicotine. Based on these experiments, an optimal pH value of 8.0 for mobile phase was selected, which was a compromise between peak shape, separation, and sensitivity of all target compounds.

3.2 Evaluation of matrix effects

In order to have a good balance of flavor intensity, throat irritancy, and vapor density, the majority of e-liquids contain a solvent mixture (which works as a carrier) with different proportions of glycerol (most commonly of vegetable origin, VG) and 1,2-propylene glycol (PG), rather than either alone (17). However, this kind of solvent mixture has been reported to have matrix effects on the signals of the analytes by interfering with the ionization process and causing a reduction of the accuracy (18). Given that the composition of e-cigarette liquids changes from product to product, the applicability of this method should be evaluated over a wide range of e-liquid compositions. Therefore, three representative mixtures with various ratios of PG to VG (90/10 *v/v*, 50/50 *v/v*, and 10/90 *v/v*) were used. Various amounts (150, 300 and 500 mg) of the mixture were also used to assess their potential impact on the analysis. The results were summarized in Table 4. The average recoveries for all target compounds were within 88.2–112%, demonstrating that the matrix of the mixture of PG and VG with different amounts has no significant effect on final results. The use of isotopically labelled internal standards appears to effectively account for matrix effects.

3.3 Calibration curves and limits of detection (LOD) and quantitation (LOQ)

A set of calibration curves with range of concentrations as described in section 2.1 was constructed by linear regression of the peak area ratios of analytes to internal standards versus the nominal analyte concentrations with a 1/x weighting factor. All target compounds showed good linearity with correlation coefficients (R^2) greater than 0.996. The accuracies for most of the calibration points evaluated by the deviation of the nominal concentration were less than 15% except at the lowest standards, for which the maximum acceptable deviation was less than 20%.

Sensitivity was evaluated by analyzing the lowest calibration standard a minimum of 10 times as an unknown over a span of several days. The LODs and LOQs were calculated as three times and ten times the standard deviation of these determinations, respectively. The results are summarized in Table 5 and show a wide range of sensitivity according to the target compound.

Table 4. Recoveries of target compounds from sample matrices with various PG/VG ratios.

PG/VG (*v/v*)	Amount (mg)	Nicotine			Cotinine			Anabasine			β-Nicotyrine		
		Recovery	Average	CV (%)	Recovery	Average	CV (%)	Recovery	Average	CV (%)	Recovery	Average	CV (%)
90/100	150	109	107	1.9	108	108	1.4	106	103	3.9	109	111	1.4
	300	107			110			104			112		
	500	105			107			98.2			111		
50/50	150	106	106	0.5	106	107	1.1	101	98.3	2.5	113	112	1.4
	300	105			108			97.7			112		
	500	106			106			96.2			110		
10/90	150	110	108	2.4	107	107	0.5	95.9	96.3	1.2	111	111	0.9
	300	109			107			95.5			110		
	500	105			106			97.6			112		

PG/VG (*v/v*)	Amount (mg)	Anatabine			Nornicotine			Nicotine-*N*-oxide			Myosmine		
		Recovery	Average	CV (%)	Recovery	Average	CV (%)	Recovery	Average	CV (%)	Recovery	Average	CV (%)
90/100	150	106	110	3.3	97.6	97.0	0.8	87.7	88.2	1.8	106	107	0.9
	300	111			96.2			86.9			107		
	500	113			97.3			90.0			108		
50/50	150	109	110	0.5	96.5	98.0	1.4	86.6	88.5	2.1	112	110	3.7
	300	110			99.1			88.5			105		
	500	110			98.4			90.3			112		
10/90	150	110	109	1.6	97.3	97.1	0.2	89.4	89.8	0.4	106	106	3.3
	300	110			96.9			90.1			102		
	500	107			97.1			89.8			109		

Table 5. Calibration data, LOD and LOQ for nicotine and nicotine-related compounds in e-liquids and aerosols.

Compound	Linear range (µg/mL)		E-liquid		Aerosol	
		R^2	LOD (µg/g)	LOQ (µg/g)	LOD (µg/collection)	LOQ (µg/collection)
Nicotine	0.048 – 4.80	0.998	0.760	2.530	0.304	1.010
Cotinine	0.052 – 5.15	0.999	0.234	0.781	0.094	0.312
Anabasine	0.052 – 5.15	0.999	0.718	2.390	0.287	0.953
β-Nicotyrine	0.053 – 5.23	0.999	0.476	1.590	0.190	0.635
Anatabine	0.051 – 5.10	0.998	0.595	1.980	0.238	0.793
Nornicotine	0.052 – 5.20	0.999	0.595	1.990	0.238	0.794
Nicotine-*N*-oxide	0.062 – 6.20	0.998	1.660	5.480	0.872	2.870
Myosmine	0.052 – 5.15	0.996	1.110	3.710	0.445	1.480

The LODs and LOQs for e-liquids analysis ranged from 0.234 and 0.781 µg/g (cotinine) to 1.66 and 5.48 µg/g (nicotine-*N*-oxide). In the case of e-cigarette aerosols, these ranged from 0.094 and 0.312 µg/collection (cotinine) to 0.872 and 2.87 µg/collection (nicotine-*N*-oxide).

3.4 Performance of the method

In the absence of generally accepted reference materials for e-liquids and aerosols, method accuracy was evaluated by fortifying simulated e-liquid solutions and aerosol matrix samples (LFMs) prepared according to the protocol described in section 2.3. As shown in Table 6, the average recoveries for all target compounds were within 85.2–110% with coefficient of variation (CV) ≤ 8.3% for e-liquids and within 87.8–111% recovery with CV≤ 8.9% for aerosols. To evaluate the extent of potential compounds loss, the recoveries of the target compound from LFB were also tested. The average recoveries for target compounds (also in Table 6) were within 85.7–110% with CV≤ 4.4% for e-liquids and within 83.2–113% with CV≤ 3.6% for aerosols. LRBs were prepared as per section 2.3 and analyzed to determine the presence of target compounds in glassware or reagents. As presented in Table 6, traces of nicotine-*N*-oxide and nornicotine were detected in both e-liquids and aerosols samples but were below the LOQs, while all other target compounds were not detected. This result is an indication of the ubiquitous existence of nicotine-related compounds, which requires careful culling of contaminated reagents or glassware before the implementation of this method.

3.5 Determination of nicotine and nicotine-related compounds in e-liquids

The validated method was used to determine the target compounds in e-liquid samples. Figure 2 shows the MRM chromatograms of the target compounds and internal standards in the samples. All target compounds demonstrated satisfactory peak shapes and separations. As shown in Table 7, anabasine and β-nicotyrine were unmeasurable while the yields of other target compounds ranged from 4.83 µg/g (nornicotine) to 8350 µg/g (nicotine) with the CV less than 11.9%. The presence of nicotine-*N*-oxide, cotinine, nornicotine, anatabine, and myosmine was 0.11%, 0.37%, 0.06%, 0.27%, and 0.07% of the nicotine content, respectively. This indicated that the content of each studied compound was lower than the identification threshold of 0.5% (5 mg/g) proposed by the ICH Guideline Q3B (R2) and the USP for a single impurity (19, 20). The total amount of target compounds was 0.87% of the nicotine content which was lower than 1.0% (10 mg/g) suggested by ICH guidelines (19).

Table 6. Summary of Laboratory Fortified Matrix (LFM), Laboratory Fortified Blank (LFB), and Laboratory Reagent Blank (LRB) for e-liquid and aerosol.

Compound	LFM				LFB				LRB	
	Refill solution		Aerosol		Refill solution		Aerosol		Refill solution	Aerosol
	% (n = 3)	CV (%)	% (n = 3)	CV (%)	% (n = 3)	CV (%)	% (n = 3)	CV (%)	µg/g (n = 5)	µg/collection (n = 5)
Nicotine	106.0	2.6	111.0	5.5	104.0	2.7	113.0	1.8	ND	ND
Cotinine	102.0	3.5	103.0	8.9	105.0	0.6	104.0	1.0	ND	ND
Anabasine	95.1	3.4	92.2	2.4	99.1	4.4	92.6	1.6	ND	ND
β-Nicotyrine	110.0	1.9	111.0	1.1	110.0	1.5	108.0	1.5	ND	ND
Anatabine	103.0	8.3	106.0	6.0	97.8	1.2	98.7	3.6	ND	ND
Nornicotine	97.9	1.2	101.0	2.3	101.0	2.4	96.9	1.7	< LOQ	< LOQ
Nicotine-*N*–oxide	85.2	1.9	87.8	5.2	85.7	2.5	83.2	0.8	< LOQ	< LOQ
Myosmine	105.0	2.9	111.0	3.0	108.0	1.2	107.0	1.8	ND	ND

ND: not detected; LOQ: Limit of quantitation

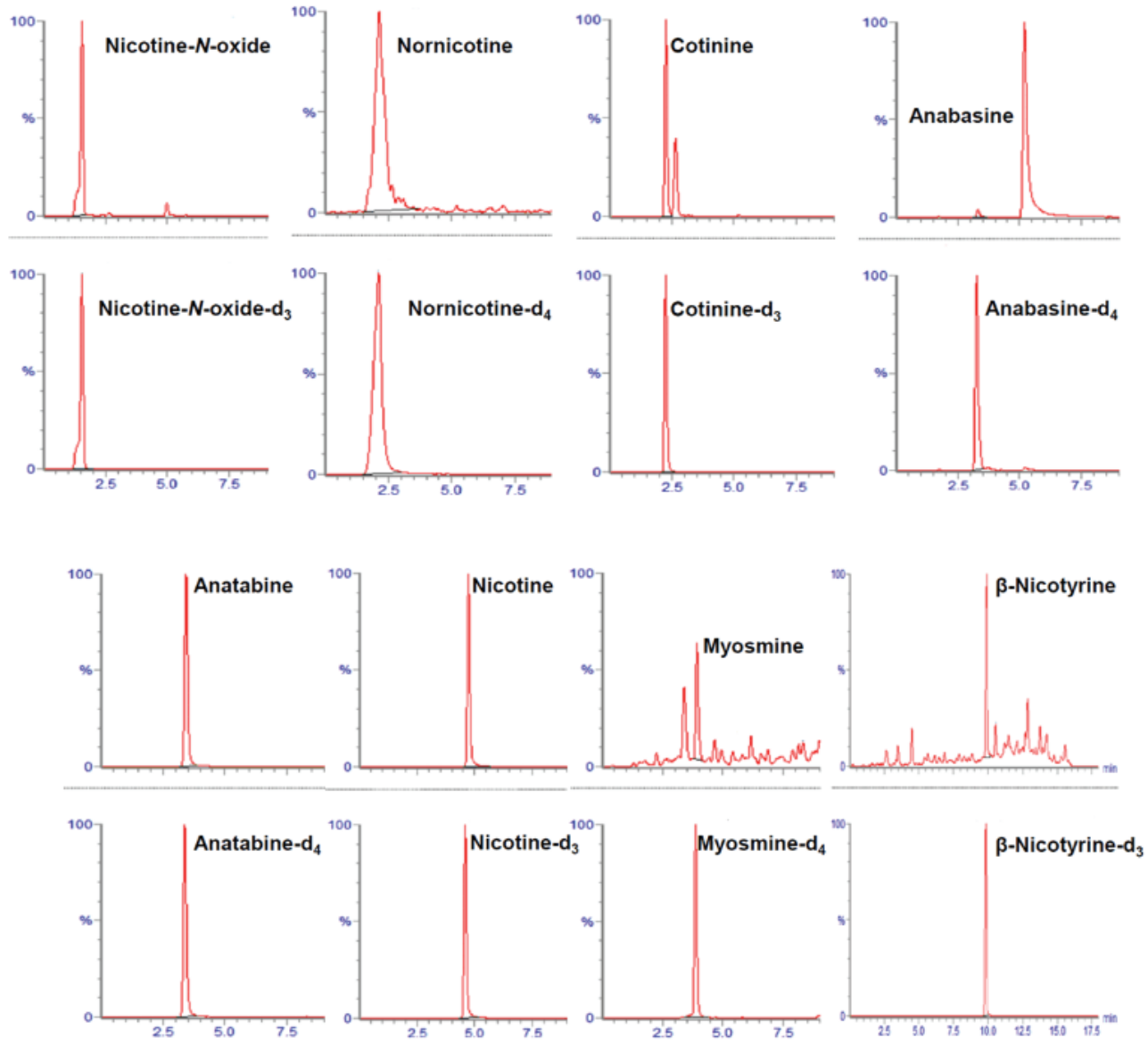

Figure 2. MRM chromatograms of nicotine and nicotine-related compounds.

Table 7. Summary of e-liquid sample analytical results.

Compound	Mean (n = 3) (μg/g)	Standard deviation (μg/g)	RSD (%)	Ratio to nicotine (%)
Nicotine-*N*–oxide	9.06	0.164	1.81	0.11
Cotinine	30.8	0.181	0.59	0.37
Nornicotine	4.83	0.148	3.05	0.06
Anatabine	22.7	0.845	3.72	0.27
Myosmine	6.24	0.742	11.9	0.07
Anabasine	< LOD	NA	NA	NA
Nicotine	8350	173	2.10	100
β-Nicotyrine	< LOD	NA	NA	NA

NA: not applicable

3.6 Determination of nicotine-related compounds in e-cigarette aerosols

The performance characteristics of one type of e-cigarette was evaluated by determining the yields of selected nicotine related compounds. The puffing regimes used to generate the test aerosols (puff volume (mL) / interval (s) / duration (s) / puff numbers) were (A) 55/30/3/50, (B) 80/30/4/50, respectively. Both were puffed under rectangular shape puff profiles with outputs of power 5 and 20 W alternately. The yields of nicotine related compounds (μg per collection) are shown in Table 8. Interestingly, unlike smoking combustible cigarettes where puff volume has a strong effect on the overall yields (21), no significant change in the yields of nicotine-related compounds between those two puff regimes was observed under the same applied power (5 and 20 W). Some studies have proven that

Table 8. Yields of selected nicotine-related compounds under various puffing conditions.

Sample ID	Puffing regime puff volume (mL) / interval (sec) / duration (sec) /puff number	Applied power (W)	Nornicotine (n = 7) (μg / collection)	Anatabine (n = 7) (μg / collection)	Anabasine (n = 7) (μg / collection)	Myosmine (n = 7) (μg / collection)
1	(A) 55/30/3/50	5	0.91 ± 0.27	6.04 ± 1.96	2.07 ± 0.60	< LOQ
2		20	2.85 ± 0.38	11.7 ± 3.28	4.10 ± 0.97	56.6 ± 16.1
3	(B) 80/30/4/50	5	1.33 ± 0.30	11.4 ± 3.40	3.37 ± 0.91	1.02 ± 0.57
4		20	3.81 ± 0.87	12.1 ± 3.79	4.10 ± 1.34	70.5 ± 8.74

during vaping both e-cigarette puff volume and puff flow rate have little to no effect on the overall yield (21, 22). Instead, puff duration is the major factor because aerosol yield will increase with increased puff duration. However, in this experiment, a one-second difference (3 s *vs* 4 s) in duration did not bring a significant effect in the total amount of aerosols between both puffing regimes. Therefore, there were no statistically significant differences in the yields of selected compounds. On the other hand, the applied power played an important role for the yields of specific nicotine-related compounds, especially for myosmine. When the applied powers for cartridges were increased from 5 to 20 W, the yields of myosmine were dramatically increased (as shown in Table 8). A possible explanation for this is that when the 5-W output power was applied, the energy supplied by the battery was probably primarily consumed on the evaporation of the e-liquid mixture and less for further heating up of the generator (23). Accordingly, the detectable levels of nicotine-related compounds in the aerosols might originate directly from the evaporative transfer of minor impurities present in the e-liquids. Conversely, the higher power output (at 20 W) might heat the aerosol generator to a temperature high enough to trigger the thermal breakdown of nicotine to myosmine, which is one of the main thermal degradation products of nicotine (24). Nicotine was fragmented slowly at low temperatures but was reported to be dramatically decomposed once the heating temperature was beyond 500 °C (24). Consequently, under two puffing regimes, the levels of myosmine were both increased sharply from less than LOQ to 56.6 μg per collection and from 1.02 to 70.5 μg per collection, respectively, while the yields of nornicotine, anatabine, and anabasine were only slightly increased. These experimental results suggest maintaining the accuracy of the applied power in an e-cigarette device is an important design feature that must be considered to safeguard public health.

3.7 Stability of nicotine in e-cigarette products under various storage conditions

Pharmaceutical products are required to be comprehensively evaluated in rigorous stability studies to provide proof on how the quality, safety, and effectiveness of the drug products change with time under the influence of a variety of environmental factors such as temperature, humidity, and light (25, 26). The stability studies, including real-time and accelerated tests, have been used to investigate the potential degradation products and impurities that originate from exposure and interaction with excipients, a specific container / closure system, and to ensure that the products retain their fitness for use up to the end of their expiration dates. Nicotine in e-cigarette products is one of the key components which satisfies the vaper's physiological craving (27). However, the stability of nicotine can be affected by formulations, package materials, as well as the storage, shipment and handling conditions (4). Therefore, the quantitation of the changes of nicotine-related compounds can be used as one of the stability-indicating measures to determine the shelf life of e-cigarette products before they are formally introduced into the market. The effect of temperature and humidity excursion on the shelf life of e-cigarette products was demonstrated under International Council for Harmonization (ICH) long-term storage conditions (real time: 25 ± 2 °C, 60 ± 5% RH and accelerated conditions: 40 ± 2 °C and 75 ± 5% RH) (26). Figure 3 shows the results of nicotine-related degradations for one brand of e-cigarette products, under both storage conditions. Anatabine, anabasine and β-nicotyrine were unmeasurable after four months of storage. This suggests that aforementioned degradations were not easily formed or the rates of degradation from other precursors were slow using this kind of formulation. Interestingly, the yields of cotinine and myosmine under both storage conditions were similar, which implies that the degradation might be irrelevant at the studied conditions. On the contrary, the yields of nicotine-*N*-oxide and nornicotine were temperature- and humidity-dependent and the yields of nicotine-*N*-oxide and nornicotine under 40 °C / 75% RH were about two times higher than those under 25 °C / 60% RH condition.

3.7 Stability of nicotine in highly flavored e-cigarette products

For current e-cigarette products, the flavor is reported to be an important ingredient and has been found to be an attractive factor to e-cigarette adopters, as well as potentially aiding with smoking abstinence (28, 29). However, it has been well known that many flavors such as mint, vanilla and fruit flavors have the potential to degrade nicotine by oxidation reactions (4). In some instances, instability problems due to the specific flavors are so acute to result in the e-cigarette products becoming unfit for use within quite a short period of time. This method was used to study the stability of nicotine of one e-cigarette product with highly flavored formulation stored under two different conditions.

As shown in Figure 4, after two months, anatabine, anabasine and β-nicotyrine were immeasurable while the yields of cotinine, nornicotine and myosmine, which are

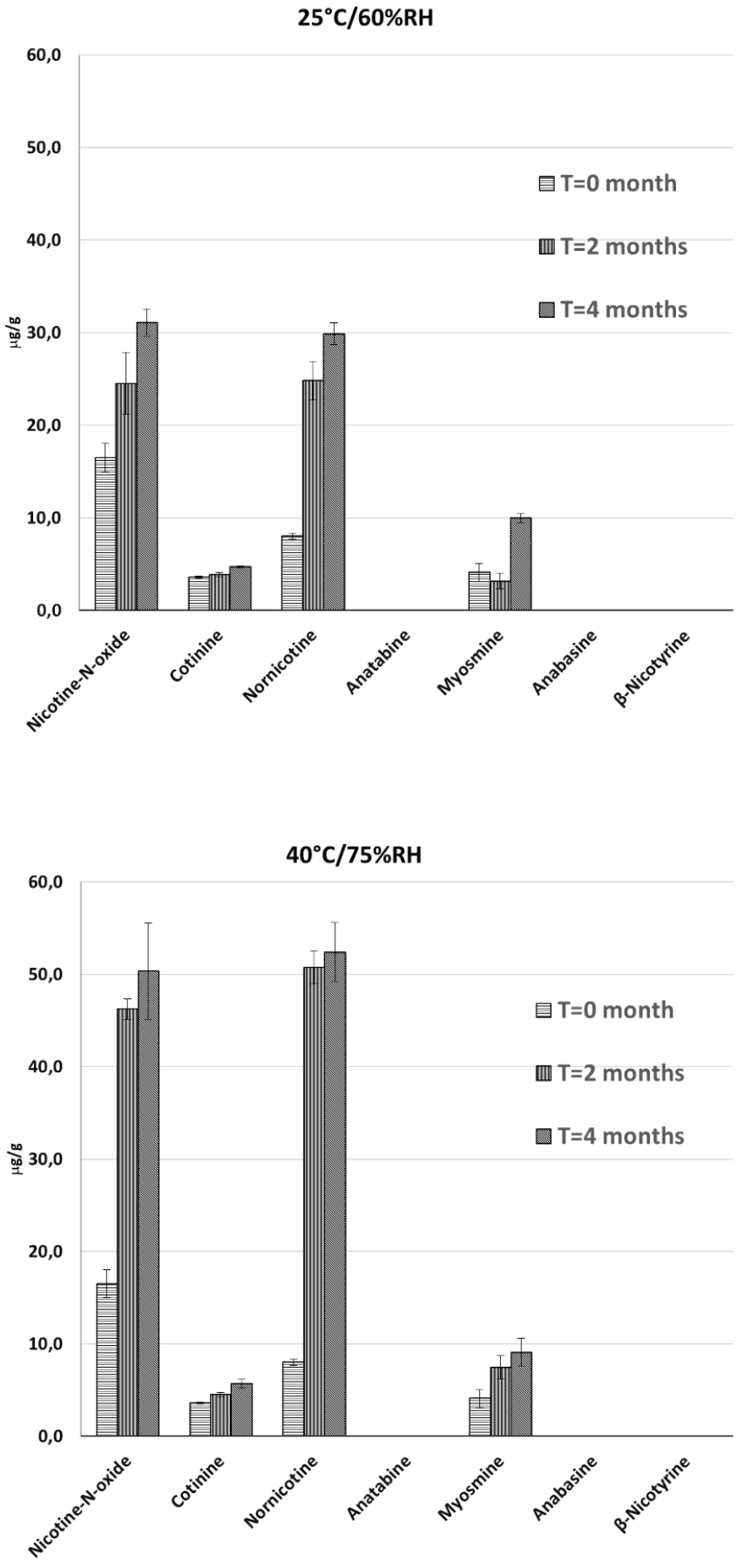

Figure 3. Changes in yields of nicotine-related compounds as function of time under various storage conditions.

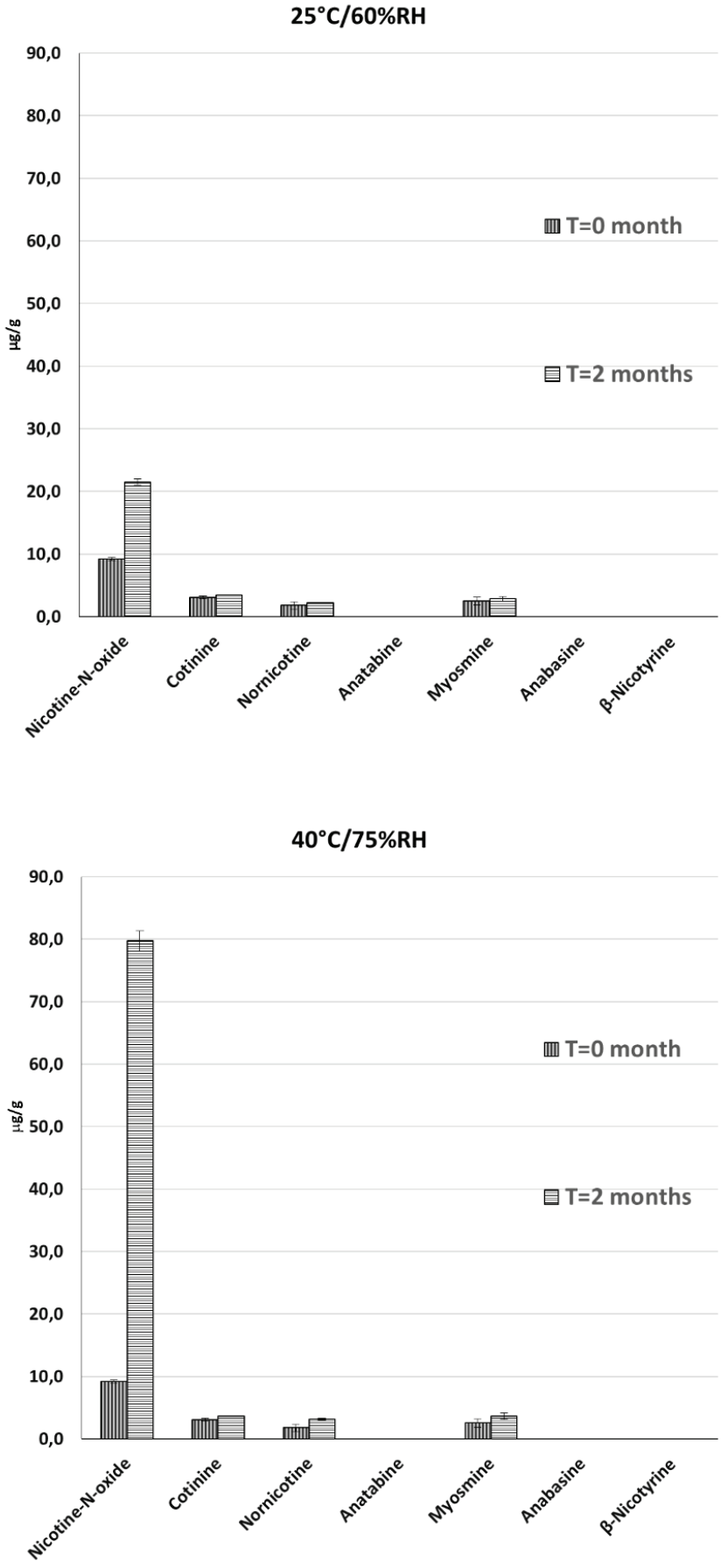

Figure 4. Changes in yields of nicotine-related compounds as function of time in highly flavored e-liquid under various storage conditions.

usually indicators of the thermal decomposition of nicotine (30–32), did not show noticeable changes. However, the yields of nicotine-*N*-oxide, which is the primary oxidation product of nicotine (25), increased quickly, especially under 40 °C / RH 75% storage condition. This experiment suggests that quantifying the yields of nicotine degradation products is an effective way to discern an unstable formulation. However, it is important to note that when nicotine is exposed to air, oxidation may occur simultaneously which also results in the generation of minor alkaloids (33–35), and therefore, the yields of nicotine-*N*-oxide might be the superimposed results due to air exposure and flavor oxidation. Nevertheless, the changing levels of nicotine oxidation products such as nicotine-*N*-oxide can act as an important indicator for decisions on appropriate flavor ingredient in the final formulation of the e-cigarette product.

4. CONCLUSIONS

A new liquid chromatography-tandem mass spectrometry-based method was successfully developed and validated for the determination of nicotine and nicotine-related compounds in e-cigarette liquids and aerosols. The sample preparation procedures, both based on the extraction with 100 mM ammonium acetate solution, were simple and effective. The method's accuracy, robustness, and reliability were enhanced by using deuterated analogues of each compound as internal standards and by applying two ion-transition pairs for each compound for confirmation and quantification. Chromatographic separation parameters were optimized to achieve the best compromise between the peak shape, separation, and the sensitivity of all target compounds. Validation experiments demonstrated satisfactory sensitivity, specificity and reproducibility. The newly developed method was applied to analyze nicotine and nicotine-related compounds in actual e-cigarette liquids and aerosols. The results presented here show that the analysis of nicotine and nicotine-related compounds can be an excellent quality-indicator to evaluate the performance of the e-cigarette device with various battery power settings, and to investigate the stability of nicotine in e-liquids with flavoring formulations stored under various conditions. The present method has a wide range of adaptability and can be potentially extended into other studies such as e-cigarette photodegradation stress tests.

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Appendix: Abbreviations and definition of terms.

Term	Definition
CV	Coefficient of variation
ENDS	Electronic nicotine delivery systems
FID	Flame ionization detection
GC	Gas chromatography
HPLC	High performance liquid chromatography
kV	Kilovolt
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LFB	Laboratory Fortified Blank
LFM	Laboratory Fortified Matrix
LRB	Laboratory Reagent Blank
LOD	Limit of detection
LOQ	Limit of quantitation
MS	Mass spectrometry
MRM mode	Multiple reaction monitoring mode
NPD	Nitrogen-phosphorus detection
PG	Propylene glycol
PVDF	Polyvinylidene difluoride
RH	Relative humidity
RSD	Relative standard deviation
VG	Vegetable glycerol