Analysis of Nicotine Alkaloids and Impurities in Liquids for e-Cigarettes by LC–MS, GC–MS, and ICP-MS

The purpose of this study was the development of various analytical mass spectrometry (MS) methods to investigate the chemical composition of e-liquids used in electronic cigarettes and characterize their quality. Low-quality nicotine (the main active compound), glycerol, propylene glycol (solvents), or flavors could greatly increase the toxicity. The search of alkaloid contaminants of nicotine was performed by liquid chromatography–tandem mass spectrometry (LC–MS-MS) after a study of fragmentation pathways by high-resolution electrospray ionization (ESI)-MS. A fully validated method for quantitation of organic polar impurities such as cotinine, anabasine, myosmine, nornicotine, and *N*-nitroso-nornicotine and nicotine itself was developed using MS coupled to ultrahigh-pressure liquid chromatography (UHPLC). To evaluate organic volatile toxicants, the headspace from e-cigarette refill liquids was sampled by the purge-and-trap method to perform gas chromatography (GC)–MS analysis. Finally, heavy metal residues as inorganic toxicants were determined by inductively coupled plasma (ICP)-MS after simple dilution. A number of cases of contamination by metals (mainly arsenic) were detected.

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The toxicity of e-cigarettes has not been completely clarified. They were introduced in the last 10 years to give smokers a healthier alternative to traditional cigarettes because of reduced burning (1,2). These devices, also known as electronic nicotine delivery systems (ENDS) use a battery to generate a weakly heated aerosol based on polyalcohols, such as propylene glycol and glycerol with a small amount of water, containing pharmaceutical-grade nicotine. Many quantitation methods have been proposed for the study of ENDS components composition (3) and mass spectrometry (MS) techniques play a fundamental role in this context (4,5).

The purpose of this study was the development of various analytical MS methods to investigate the chemical composition of solutions used to fill e-cigarettes (e-liquids or e-juices) and characterize their quality, searching for toxicants. The main active principle of e-liquids is nicotine and ENDS are borderline products between pharmaceuticals and consumer goods (6). First of all, nicotine itself plays a key role in toxicity characterization and regulatory aspects. This alkaloid is generally obtained from tobacco and consequently some authors consider e-cigarettes to be tobacco products. On the other hand, the high concentration of purified nicotine in e-liquids assimilates e-cigarettes to medicinal products and accurate information about active principle quantity, purity, and risk is required. Liquid nicotine toxicity and management has been reviewed (7) and fatal consequences of improper use of e-liquids has been reported (8).

The diffusion of e-cigarettes in quite a different market from the pharmaceutical one claims for analytical methods that are able to individuate low-quality components. In particular, impurities and degradation products could be present or form in e-liquids even before e-cigarette operation and could increase their toxicity. It is not possible to characterize all of these compounds by a single MS technique because of the great chemical heterogeneity: an e-cigarette liquid sample contains polar and volatile organic compounds, metal ions, and more. The impurities that can contaminate the active principle (nicotine) could be its related alkaloids (9) such

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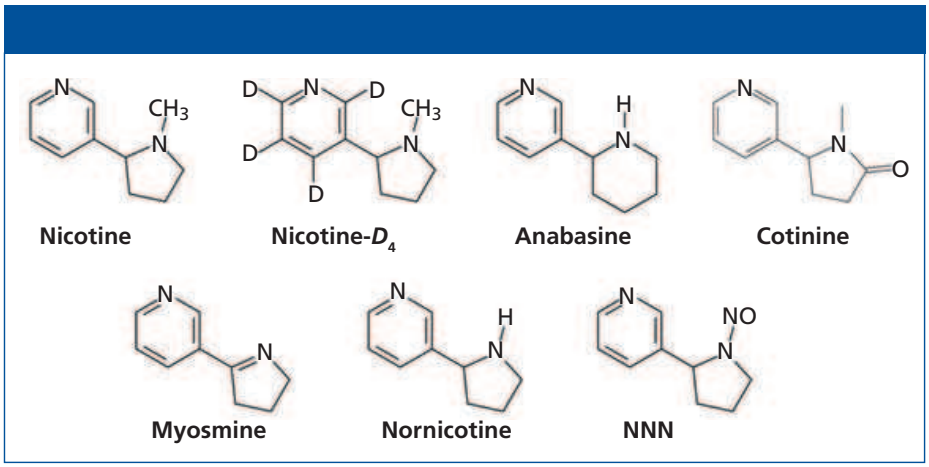

Figure 1: Structures of the studied alkaloids.

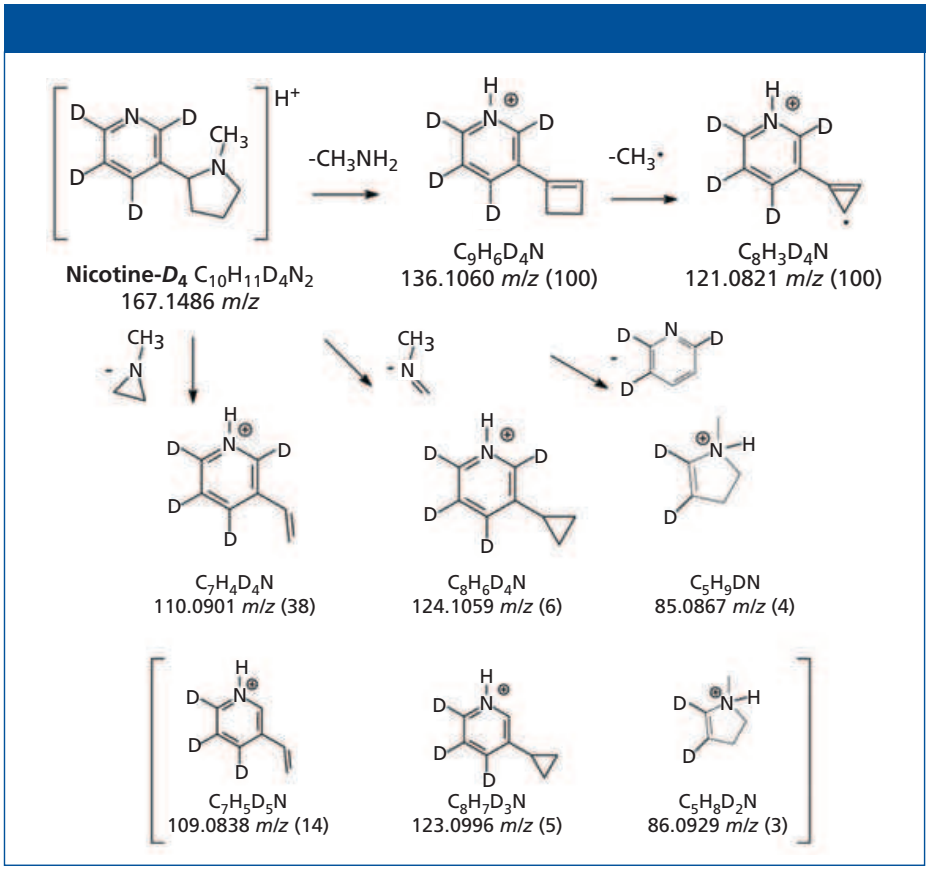

Figure 2: NNN MH^+ $HRMS^n$ proposed fragmentation pathways.

as anabasine, cotinine, myosmine, nornicotine, and its nitrosated derivative (NNN) a well-known carcinogenic compound (10,11). All of these compounds are known to be present in traces of tobacco and other plant parts from the *Solanaceae* family and may vary by different geographical origin (12). The solvents used for e-liquids to facilitate aerosol formation are polyalcohols. But some compounds belonging to this class display high toxicity—for example, diethylene glycol or ethylene glycol (further metabolized to oxalic acid)—and their presence must be avoided. Moreover residual solvents used for nicotine extraction and flavor stock solution could also be present as contaminants. In a similar way, some heavy metal elements could be present as simple pollutants or derive from industrial production and management of flavoring compounds and cosolvents. We developed some quantitative determination methods and applied known procedures to fully characterize as many classes of chemical toxicants as possible by MS. Samples from different producers were acquired in the Italian market.

To evaluate the greatest number of potential contaminants we had to use MS by coupling with different chromatographic and introduction techniques. The search for minor alkaloid and derivatives of nicotine was performed by liquid chromatography–tandem mass spectrometry (LC–MS-MS) after a deep study of fragmentation pathways by high-resolution electrospray ionization (ESI)-MS. To evaluate organic volatile toxicants, the headspace from e-cigarette refill liquids (e-liquids) was sampled by the purge-and-trap method to perform gas chromatography (GC)–MS analysis. Finally, heavy metal residues such as inorganic toxicants were determined by inductively coupled plasma (ICP)-MS after simple dilution.

Experimental

Chemicals and Materials

E-liquid samples were obtained from four different Italian industrial producers or importers (the products were manufactured in Italy, China, Poland, and Germany and are nonspecifically labeled as brands A, B, C, and D); all of them were solutions based on polyethylene glycol (60%), glycerol (30%), and water (10%) with various flavoring and five different nicotine declared concentrations (0, 9, 11, 16, and 18 mg/mL). LC–MS-grade acetonitrile was purchased from VWR (VWR International). Extrapure formic acid was obtained from Fluka (Sigma-Adrich). EPA/8260B (13) standard mix, nicotine, nicotine-D_4, cotinine, anabasine, myosmine, nornicotine, *N*-nitrosonornicotine, chlorobenzene-D_5, 1,4-dichlorobenzene-D_4, fluorobenzene, methanol, trichloroacetic acid, heptafluorobutanoic acid, and ammonia were obtained from Sigma-Aldrich at a purity of ≥99%. High performance liquid chromatography (HPLC)-grade water was obtained from a MilliQ Academic water purification system (Millipore).

Sample Preparation

Samples for LC–MS analysis were prepared by diluting e-liquids in 95:5 (v/v) 5 mM heptafluorobutanoic acid and acetonitrile. Nicotine-D_4 was added in methanolic solution to reach a final concentration of 100 ng/mL. For GC–MS, 50-µL e-liquid samples were diluted in 40 mL of water in a 40-mL gastight vial, adding the internal standard mixture (chlorobenzene-D_5, 1,4-dichlorobenzene-D_4, fluorobenzene at 0.5 ng/mL).

Samples for ICP-MS analysis were prepared by simple dilution in ultrapure water. Then 100 µL of e-liquid was diluted to 10 mL to minimize matrix interferences.

High-Resolution Mass Spectrometry

A LTQ Orbitrap hybrid mass spectrometer (Thermo Scientific), equipped with an ESI ion source, was used. The syringe pump effluent was delivered to the ion source at 10 µL/min, using nitrogen as both sheath and auxiliary gas. The source voltage was set to 4.5 kV. The heated capillary temperature was maintained at 275 °C. The acquisition method used had previously been optimized in the tuning sections for the parent compound (capillary, magnetic lenses, and collimating octapoles voltages) to achieve maximum sensitivity. The main tuning parameters adopted for the ESI source were 13 V for capillary voltage and 50 V for the tube lens. Full-scan spectra were acquired in the 50–700 *m/z* range. MS^n spectra were acquired in the range between ion trap cut-off and precursor ion *m/z* values. Mass resolution was set to 30,000. Mass accuracy of recorded ions (versus calculated) was ±0.001 u (without internal calibration).

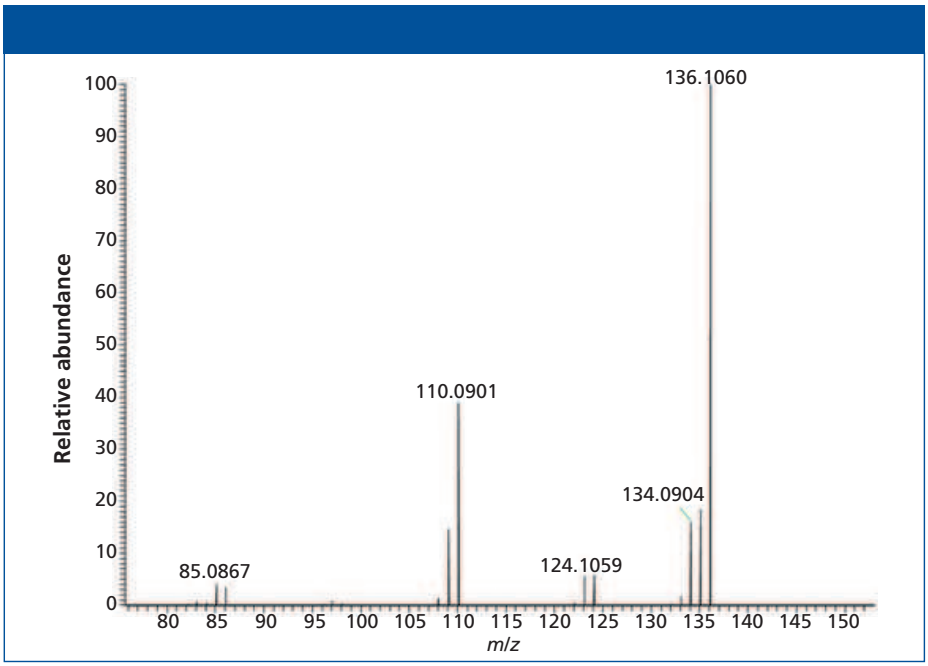

Figure 3: High resolution MS^2 spectrum of nicotine-D_4.

LC–MS

A Nexera LC-30AD (Shimadzu) ultrahigh-pressure liquid chromatography (UHPLC) instrument equipped with a 100 mm × 2.1 mm, 1.7-µm d_p Kinetex C18 column (Phenomenex) was used to carry out the chromatography analysis. The eluents were acetonitrile (mobile-phase A) and 2.5 mM

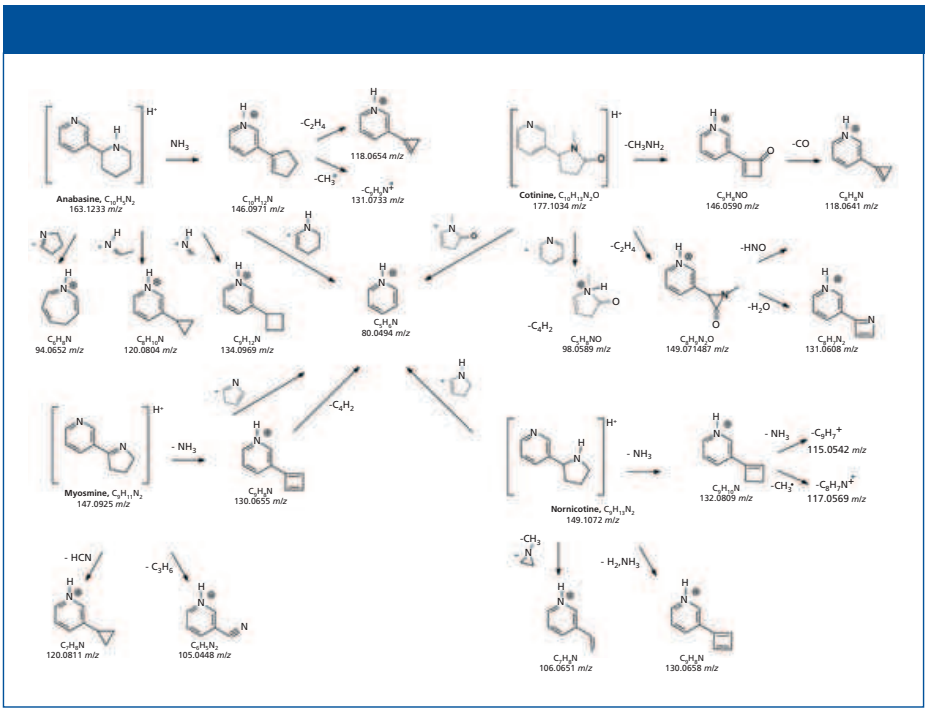

Figure 4: Anabasine, myosmine, and nornicotine MH^+ high-resolution MS^n proposed fragmentation pathways, evidencing common fragmentations and isobaric losses with different elemental composition.

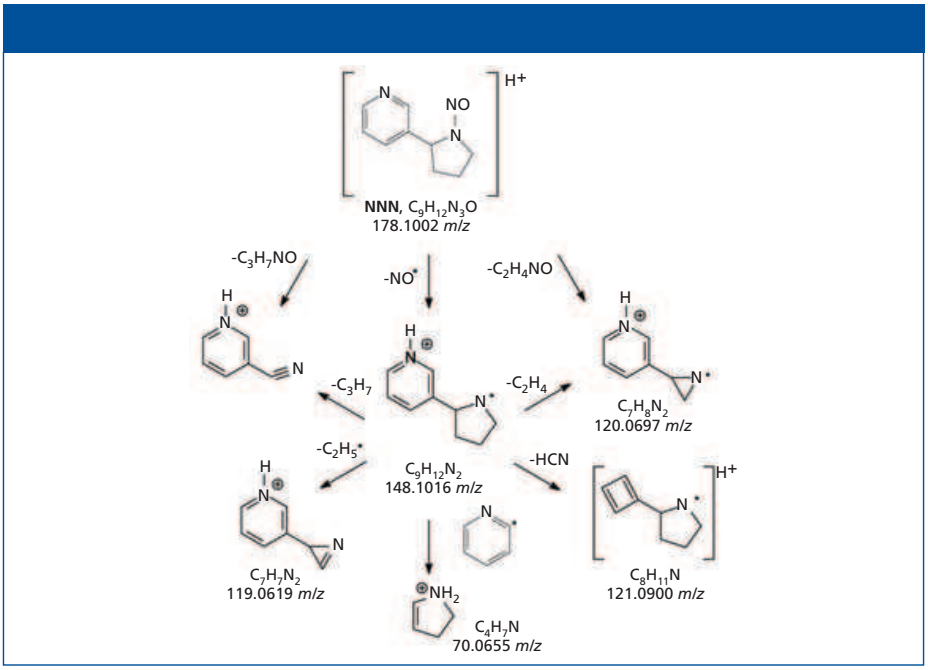

Figure 5: NNN MH^+ high-resolution MS^n proposed fragmentation pathways.

heptafluorobutanoic acid (mobile-phase B) in the following gradient conditions: 5–12% A in 7 min, 12–100% A in 1 min and re-equilibration. The injection volume was 5 µL, the flow rate was 500 µL/min, and the column was maintained at the temperature of 30 °C. A QTrap-5500 (Sciex) instrument, equipped with a Turbo Ion Spray source, was used to analyze samples. The source parameters were as follows: curtain gas, 25 (arbitrary units); gas 1, 20 (arbitrary units); gas 2, 30 (arbitrary units); temperature, 400 °C; ion spray voltage, 3500 V; declustering potential, 200 (arbitrary units); and entrance potential, 11 (arbitrary units). The detector was used in multiple reaction monitoring (MRM) mode, and the transitions for each alkaloid are reported in Table I.

GC–MS

To detect and quantify volatile organic compounds (VOCs) a Varian Saturn 3900 system (Agilent) was used. The GC was equipped with a Tekmar purge-and-trap concentrator (operating on a 25-mL aqueous sample from gas-tight vials) and a Varian 1177 injector. For GC separation we used a 30 m × 0.25 mm Varian VF624 column in a temperature interval between 35 °C and 250 °C. The injector temperature was 170 °C and injection was done in splitless mode. Helium gas at 1.2 mL/min was used as carrier. The MS analyzer was a Varian Saturn 2100 ion-trap system with an EI source, and full-scan spectra were acquired in the 45–400 *m/z* range.

ICP-MS

Elemental ICP-MS determination was performed on an Agilent 7700 instrument with a quadrupole analyzer. Argon at 15 mL/min was used for plasma formation and at 1 L/min as the nebulizing gas. The RF power was 1.55 kW, and the RF matching was set at 1.80 V. Samples were introduced using a peristaltic flow at 250 µL/min.

The analytical quantitative determination, after building a calibration curve over seven concentration levels, involved boron, chromium, nickel, cobalt, copper, silver, arsenic, manganese, cadmium, antimony, barium, aluminum, iron, and zinc. Yttrium, iridium, terbium, and scandium were used as internal standards.

Results and Discussion

Nicotine-Related Alkaloids: High-Resolution MS-MS Study

Nicotine and related alkaloids subjected to the study are reported in Figure 1. The structures based on high-resolution MS^n of the studied ions were hypothesized to clarify fragmentation pathways with the aim to optimize the selection of product ions for the following quantitative analysis by UHPLC–MS. The fragmentation of nicotine and some related alkaloids was described (14), but their spectra share several ions and it was not simple to determinate some molecules selectively (especially if they are isobars such as nicotine and anabasine). At first we investigated the fragmentation pathways of the compounds generally analyzed in biological samples, nicotine and its main metabolite cotinine. To evidence the mechanism of fragmentation, high-resolution MS^n of tetradeutero nicotine (nicotine-D_4) is shown in Figure 2. Figure 3 displays its high-resolution MS^2 spectrum. The chief fragmentation pathway proceeds with elimination of methylamine without

Table I: UHPLC–MS-MS parameters

Analyte	*m/z* Q1	*m/z* Q3	Dwell Time (ms)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)	Retention Time ± SD (min) N=30
Cotinine	177	98 40	25 25	125 125	8 10	21 45	13 13	1.88 ± 0.02
NNN	178	148 120	310 310	90 90	10 10	12 22	8 10	3.11 ± 0.04
Nornicotine	149	130 80	20 20	170 170	10 10	21 28	13 12	3.62 ± 0.07
Myosmine	147	130 118	1500 1500	233 233	10 10	27 33	11 16	3.66 ± 0.25
Nicotine	163	132 106	60 60	160 160	10 10	18 18	17 17	3.81 ± 0.07
Nicotine-D_4	167	136 110	60 60	188 188	10 10	20 20	8 8	3.83 ± 0.02
Anabasine	163	146 120	15 15	207 207	10 10	17 21	15 12	5.44 ± 0.02

involvement of pyridine deuterium atoms. A particular MS^3 fragmentation based on methyl radical elimination was observed. Because this behavior does not agree with the one reported in literature (14) (loss of hydrogen) we also checked undeuterated nicotine to confirm the methyl loss.

Secondary fragmentation pathways, because of the loss of methylaziridine, methylamine, and pyridine showed evidence of a significant grade of hydrogen scrambling (Figure 2).

The most intense high-resolution MS^n pathways of other nicotine-related alkaloids are reported in Figure 4. It is noteworthy that some apparently common ions are in fact isobaric by nominal mass, but own a different elemental composition only resolved by the use of high-resolution analyzers. For all of the investigated compounds, the pyridine ring is less prone to fragment and the elimination of small molecules containing nitrogen of pyrrole moiety represent the favorite way of fragmentation. The only circumstance where homolytic bond breaking is significant is the case of the nitroso derivative NNN. The scheme of

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Table II: UHPLC–MS-MS validation parameters

Analyte	Selectivity %	Linearity %	Intra- and Interday Accuracy (Total Overall BIAS%)	Intra- and Interday Precision (Total RSD% of Accuracy%)	Stability (24 h and 3 Cycles Freeze/Thaw)	LLOQ (ng/mL)	Calibration Curve (Mean Values)
Cotinine	2	6.0 ± 3.0	7 8	6 8	98 103	10	$y = 0.0284x - 0.0742$
NNN	3	4.7 ± 2.1	7 11	7 12	97 99	0.5	$y = 0.1140x - 0.0768$
Nornicotine	5	9.3 ± 6.4	10 11	6 15	90 95	50	$y = 0.0090x - 0.0121$
Myosmine	14	6.7 ± 3.5	9 12	3 13	89 89	20	$y = 0.0046x - 0.0131$
Nicotine	17	10 ± 6.2	8 10	2 8	85 87	10	$y = 0.0278x - 0.0615$
Anabasine	3	10 ± 5.6	15 9	8 9	98 98	20	$y = 0.0045x - 0.0023$

Table III: Selected nicotine UHPLC–MS-MS quantitation

Brand, Different Flavors and Nicotine Content	Measured Nicotine (mg/mL)
A, Tobacco, nicotine 9 mg/ml	8.18 ± 0.52
A, Mint, nicotine 9 mg/mL	9.40 ± 0.61
A, Cigar, nicotine 9 mg/mL	8.70 ± 0.94
A, Sweet, nicotine 18 mg/mL	13.26 ± 1.11
A, Menthol, nicotine 9 mg/mL	8.18 ± 0.72
A, Mint, zero nicotine	0.015 ± 0.0006
A, Tobacco, zero nicotine	0.012 ± 0.0005
A, Menthol, zero nicotine	0.011± 0.0005
Brand A, medium content verified: 91%	
B, Tobacco 1, nicotine 18 mg/mL	3.73 ± 0.21
B, Tobacco 2, nicotine 18 mg/mL	4.48 ± 0.38
B, Mint, nicotine 18 mg/mL	9.6 ± 0.77
B, Tobacco 1, nicotine 18 mg/mL	12.6 ± 1.41
B, Caramel, nicotine 9 mg/mL	8.48 ± 0.68
Brand B, medium content verified: 52%	

high-resolution MS^n spectra generation of NNN is shown in Figure 5.

UHPLC–MS-MS Analytical Method Validation

Knowledge of mass fragmentation behavior allowed us to develop a liquid chromatographic method that was able to distinguish and selectively evaluate nicotine and related alkaloids in a typical e-liquid matrix (60% ethylene glycol, 30% glycerol, 10% water). Precursor and product ions were chosen to guarantee minimum interferences. MRM parameters and retention times are reported in Table I. For each alkaloid, the following validation parameters were evaluated in accordance with the United States Food and Drug Administration (FDA) guidelines (15): the selectivity (versus matrix and for each analyte versus the standard mixture of the other six molecules, including internal standard), the linearity of calibration curve, the intra and inter day accuracy and precision, the lower limit of quantification (LLOQ), and the short term stability (up to 24 h and after three cycles of freeze and thaw) of the analyzed samples. The calibration curves were obtained in a range of 10–750 ng/mL, using 10-, 50-, 100-, 500-, and 750-ng/mL combined standards solutions. All of these parameters are reported in Table II and are in good agreement with the FDA guideline limits. Definitions and details for calculation are described in a previously reported validation protocol (16). Linearity parameters were fully observed; selectivity was <20% in all of the cases, intrarun repeatability was between 3% and 14%; inter-run repeatability was between 5% and 20%; precision (relative standard deviation [RSD]% of accuracy%) was <13%. A room temperature stability of 24 h was ascertained for the studied analytical solutions. LLOQ values were in the 0.5–20 ng/mL range and recovery for all analytes was 98%.

The developed method is fast (the total analysis time was 16 min including re-equilibration), reliable, and, after proper dilution of e-liquid samples, allows highly sensitive determination of nitrogenous impurities of nicotine.

UHPLC–MS-MS Analytical Results

Using the developed UHPLC–MS-MS method, we analyzed e-liquid samples from different producers to evaluate nicotine title and search for the presence of impurities. The nicotine concentration in examined samples strongly disagrees from the declared quantity (in some samples nicotine is only 20% with respect to the stated quantity: see selected results in Table III). This could be a big issue for the so-called vapers and underlines the requirement of more-strict standards in quality control of e-liquid manufacturing. We did not find significant presence of any of the searched impurities over the limit of 0.3% compared to nicotine. This is in agreement with the declared use of pharmaceutical-grade active principle by the producers. Nicotine itself has been rather identified in samples where instead it should have been absent, probably because of mismanagement of the production and packaging facilities.

GC–MS Analytical Results

We searched for toxic glycols and semi-vol-

Table IV: Potentially toxic VOCs quantitation by GC–MS

Brand, Different Flavors and Nicotine Content	Benzene (µg/g)	Toluene (µg/g)	Styrene (µg/g)	Alkylbenzenes (µg/g)	Ethanol (%)
A, Kiwi, nicotine 11 mg/mL	10.6	<0.05	<0.05	20.2	1.8
A, Tobacco, nicotine 18 mg/mL	29.3	0.17	<0.05	3.4	1.8
A, Tobacco 2, nicotine 11 mg/mL	17.6	0.31	<0.05	0.40	2.0
A, Tobacco, zero nicotine	0.42	<0.05	<0.05	<0.05	2.5
A, Tobacco 2, zero nicotine	0.27	0.08	<0.05	<0.05	1.9
A, Green, nicotine 18 mg/mL	13.5	0.29	<0.05	0.51	0.70
A, Sweet, nicotine 11 mg/mL	3.3	0.11	<0.05	42.3	3.6
B, Tobacco 3, nicotine 16 mg/mL	0.21	0.38	<0.05	1.36	<0.01
B, Tobacco 4, nicotine 16 mg/mL	0.23	0.42	<0.05	0.78	<0.01
B, Orange, nicotine 16 mg/mL	<0.05	0.12	<0.05	20.2	1.2
B, Peach, nicotine 6 mg/mL	<0.05	<0.05	<0.05	1,6	2.5
B, Mint, nicotine 11 mg/mL	<0.05	1.1	<0.05	23.8	2.0
B, Beer, nicotine 11 mg/mL	<0.05	0.12	<0.05	3.6	0.31
C, Sweet, nicotine 6 mg/mL	<0.05	<0.05	0.05	0.18	3.6
C, Tobacco, nicotine 18 mg/mL	<0.05	<0.05	6.87	0.08	<0.01
C, Cola, zero nicotine	<0.05	<0.05	0.11	13.5	<0.01

Table V: Heavy metals quantitation by ICP-MS

Brand, Nicotine Content	Metal Quantity (ng/mL)													
	B	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Ag	Cd	Sb	Ba
A1, nicotine 9 mg/ml	460	40.8	129	1.26	50.8	0.44	3.37	3.43	47.1	64.3	<LOQ	<LOQ	0.40	6.38
A2, nicotine 9 mg/mL	77.6	60.4	138	4.83	179	0.17	6.49	5.49	33.3	26.8	< LOQ	< LOQ	0.55	3.94
B1, nicotine 18 mg/mL	35.4	23.6	140	0.85	55.7	0.24	1.97	2.33	11.4	17.3	< LOQ	< LOQ	2.46	4.42
B2, nicotine 18 mg/mL	31.8	53.4	124	3.49	110	0.27	3.08	6.48	41.3	10.6	< LOQ	< LOQ	2.42	6.74
C1, nicotine 16 mg/mL	51.5	36.8	129	0.97	33.1	0.03	1.63	3.60	6.13	7.17	< LOQ	< LOQ	5.72	1.93
C2, nicotine 16 mg/mL	59.7	82.0	144	0.77	53.1	0.04	1.67	3.60	0.66	8.39	< LOQ	< LOQ	5.47	1.68
D1, zero nicotine	350	274	374	53.6	100	49.5	264	60.2	122	52.7	53.6	63.6	3.19	53.5
D2, nicotine 11 mg/mL	201	191	277	62.6	256	24.5	131	31.2	4173	492	4.36	32.0	0.25	35.8
D3, nicotine 16 mg/mL	865	399	413	436	338	58.9	308	130	438	35880	31.9	78.7	0.77	89.4
D4, nicotine 9 mg/mL	459	338	465	70.8	134	65.1	349	72.7	135	117	59.6	83.8	0.79	75.1
D5, nicotine 18 mg/mL	331	265	357	50.4	89.8	46.9	247	51.2	75.6	139	26.0	60.0	0.64	64.7
D6, nicotine 16 mg/mL	360	273	375	61.1	152	50.5	269	68.6	122	57.8	53.6	66.5	53.5	56.0

atile organic components of added flavors by direct injection of methanol solutions of e-liquids (data not shown), but the raw materials used were of good quality and no contamination was detected.

By analyzing the VOC fraction, we could conversely observe significant organic solvent contamination in a number of cases. We operate in agreement with Environmental Protection Agency (EPA) Method 8260 (13), using the water-diluted e-liquid solution as a model of vaped aerosol dispersion in the oral cavity. Table IV lists some findings that are noteworthy from the toxicological point of view. Carbonyls were not reported in this article because they could form by thermal degradation of glycols and will be the subject of a future publication. Benzene, styrene, alkylbenzenes (ethylbenzene, *n*-propylbenzene, isopropylbenzene, *sec*- and *tert*-butylbenzene, 1,2,4- and 1,3,5-trimethylbenzene, isopropyltoluene, *o*-, *m*- and *p*-xylene), and ethanol were detected. These impurities could be linked to low-quality nicotine extraction solvents used. This was confirmed by one circumstance (a set of similar products imported from a single producer, Table IV, brand A) where the benzene quantity is directly related to the presence of nicotine.

ICP-MS Analytical Results

We quantified 14 different metal and semimetal elements on diluted water solution of various e-liquid samples by ICP-MS. Normally, heavy metals are not significant pollutants of e-liquids and their presence is generally acknowledged not to be higher than 0.2 mg/L (19). Since reference values for risk management are referred to drinking water, these data seem to suggest that metals are of little or no concern in evaluat-

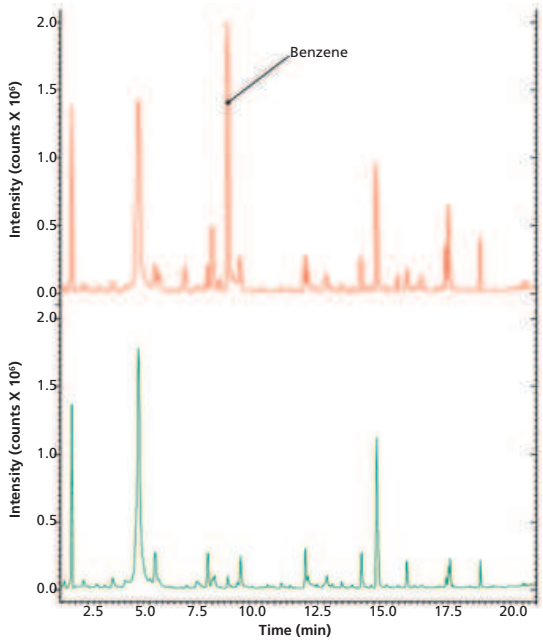

Figure 6: VOCs GC–MS chromatogram showing evidence of benzene contamination.

ing e-cigarette sources of toxicity. A selection of results is reported in Table V. All of the data are in agreement with previous reports with the exception of strong contaminated "outlier" samples such as e-juice D3. In particular, arsenic high concentration, even if classifiable as spot contamination, drives the attention on the importance of quality controls to avoid unexpected harming or toxic effects.

Conclusions

In our work, fragmentation pathways of nicotine-related impurities were elucidated using high-resolution mass spectrometry. With the information we obtained, a selective UHPLC–MS-MS multianalyte method was developed for the quantitative determination of nicotine and related alkaloids in the classical e-cigarette refill liquid matrix. The method was completely and successfully validated following FDA guidelines. We analyzed several e-liquids from different producers in Italy, China, Poland, and Germany. The nicotine concentration in the analyzed samples did not result in compliant declared values. We found differences between declared and actual concentrations ranging from -70% to +20%. This has been observed by other authors too (17,18), indicating that it is a common problem in the e-cigarette market. No sample contained nitrosamines at levels above the limit of detection nor any common nicotine impurities above 0.3% of nicotine itself. By analyzing the VOC fraction and metals we could observe significant contamination by benzene, styrene ethanol, and even arsenic in a number of circumstances. Finally, this study highlights the fundamental role of different MS analyzers in the characterization of sources of potential toxicity by chemically heterogeneous compounds in a atypical matrix.

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