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Article

Multicomponent Analysis of Replacement Liquids of Electronic Cigarettes Using Chromatographic Techniques

Matthaios P. Kavvalakis[1†], Polychronis D. Stivaktakis[1†], Manolis N. Tzatzarakis[1], Dimitrios Kouretas[2], Jyrki Liesivuori[3,4], Athanasios K. Alegakis[1], Dionysios Vynias[1,4] and Aristidis M. Tsatsakis[1*]

[1]Center of Toxicology Science & Research, Division of Morphology, Medical School, University of Crete, Voutes Campus, Heraklion 71003, Crete, Greece, [2]Department of Biochemistry and Biotechnology, University of Thessaly, Larissa 41221, Greece, [3]Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku, Turku, Finland, and [4]TOXPLUS SA, Spin off Company, Agiou Fanouriou 1, Nea Alikarnassos 71601, Heraklion 71003, Crete, Greece

*Author to whom correspondence should be addressed. Forensic Sciences and Toxicology Department, Medical School, University of Crete, P.O. Box 1393, 71003 Heraklion, Crete, Greece. Email: aris@med.uoc.gr

The electronic cigarette (e-cig) is an invention of the past few years and its popularity is rapidly growing all over the world. A rapid multicomponent analytical protocol for the analysis of the replacement liquids (e-liquids) of e-cig was developed using gas (GC) and liquid chromatography (LC)–mass spectrometry (MS). GC–MS-based methods were developed for the determination of the main humectants and polycyclic aromatic hydrocarbons (PAHs). For the determination and quantification of nicotine (NIC) and nitrosamines, appropriate LC–MS-based methods were developed. The approbated methods were applied for the analysis of 263 e-liquid samples obtained from the Greek market. The instruments response was linear; the limits of quantification ranged from 0.003 μg/mL for three PAHs to 1.187 μg/mL for glycerol. The precision was <16% for all analytes, while the mean accuracy ranged from 99.1% for NIC to 106.6% for the flavor 2,5-dimethylpyrazine. The measured concentrations of NIC were correlated with the theoretical concentrations as reported by the manufacturers. An analog relation between the concentration of the glycerol and of propylene glycol was noticed. The frequency of detection of flavors ranged from 30.4% for the methyl cyclopentenolone to 5.3% for 3.4-dimethoxybenzaldehyde. Nitrosamines and PAHs were not detected in any sample. Because a similar analytical protocol was not available from the existing literature so far, our study offers the advantage of complete analytical methods for rapid and simultaneous multicomponent identification.

Introduction

Smoking cigarettes is more than an addictive habit since it is reported as a leading cause of preventable death. According to the U.S. Department of Health and Human Services (USDHHS), more than 480,000 people die every year from smoking-related diseases—more than the annual deaths caused by alcohol, AIDS, traffic accidents, drug abuse, murders and suicide combined (1). Even more people are affected secondhand suffering health problems caused by exposure to the smoking of others (2–5). Nicotine (NIC) is an alkaloid stimulant, highly addictive and travels through the body, affecting the brain and by speeding up the processing rate of the central nervous system. Smokers can quickly become dependent on cigarettes and suffer serious symptoms of withdrawal when they try to quit. Besides NIC, more than 4,000 toxic chemicals (such as tar, nitrosamines, PAHs, etc.) are being absorbed into the bloodstream and then carried throughout the body of the smoker.

The negative effects caused by smoking on human health are arguably one of the most common areas of health science. In recent years, a new alternative smoking habit, the electronic cigarette (e-cig), has been adopted. In 2003, the first modern e-cig was invented and patented by a Chinese pharmacist named Hon Lik (6) and after 1 year this invention was introduced to the market as an alternative NIC delivery device (7). E-cig is a battery-operated device and simulates the operation of a conventional cigarette. The e-cig device is composed of a cartridge for replacement liquid (e-liquid), an evaporator and a battery that provides power to the evaporator. The e-liquid of the e-cig typically contains concentrated flavors [i.e., methyl cyclopentenolone (FL1), ethyl maltol (FL2), 2,5-dimethylpyrazine (FL3), ethyl vanillin (FL4), 3,4-dimethoxybenzaldehyde (FL5)], humectants [i.e., propylene glycol (PG), glycerol (G)] and variable concentration of NIC (8).

In contrast to the conventional cigarette, the research on the electronic cigarette is quite limited. However, chemical analysis of e-cig vapor has shown that many toxicants and carcinogens (i.e., nitrosamines) that are present in cigarette smoke are also detectable, generally though at lower levels in various e-cig products (8–13). The overall estimation for the e-cig is that it is less harmful than conventional because it contains less chemicals and carcinogens. Due to lack of standardization in the manufacture and quality control of e-cig and e-liquid refill products, there is considerable variation in performance among different e-cig brands as well as within the same brand (14–16).

The ever growing use of the e-cig creates a necessary imperative, the need for further investigation of its impact on human health. The World Health Organization (WHO) stated that the efficacy of e-cig in aiding smoking cessation has not been demonstrated scientifically and recommended that 'consumers should be strongly advised not to use e-cig until a reputable national regulatory body has found them safe and effective' (17). In combination with these concerns of the international scientific community concerning e-cig and the obligation for quality control of e-liquid products by a reputable regulatory body, the need of a fully validated analytical method for the multicomponent determination in these products was emerged.

In this study liquid and gas chromatography (GC)–mass spectrometry (MS) techniques were performed to determine the composition of several e-liquid products for e-cig devices. Specifically, the concentrations of NIC, PG, G and the main flavor ingredients (FL1–FL5) were calculated, while the presence of potentially harmful chemical compounds such as

†Authors with equal contribution.

polycyclic aromatic hydrocarbons (PAHs), nitrosamines, diethylene glycol (DG) and linalool (L) was also investigated. The purpose of this study was to develop a complete and fully validated analytical method for the rapid and simultaneous multicomponent identification and quality control of e-liquid products. Similar analytical protocol is not available from the existing literature so far.

Experimental

Materials

Methanol [liquid chromatography–mass spectrometry (LC–MS) grade], ammonium acetate (98%), L (>97%), PG (>99.5%), G (>99%), DG (>99%), methyl cyclopentenolone (>98%) (FL1), ethyl maltol (>99%) (FL2), 2,5-dimethylpyrazine (98%) (FL3), ethyl vanillin (>98%) (FL4), 3,4-dimethoxybenzaldehyde (99%) (FL5), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA, synthesis grade), pyridine (99.8%), NIC (99%) and ketamine (>98%, used as external standard) were all purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (LC–MS grade) was purchased from Fisher Chemicals (Leicestershire, UK). Formic acid (>98%) was purchased from Riedel de-Haen (Seelze, Germany). For the PAHs (EPA 525 mix A) analysis, multicomponent mixtures were purchased from Supelco (Bellefonte, USA). The nitrosamines: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada) while *N*-nitrosoanatabine (NAT) and *N*-nitrosoanabasine (NAB) were purchased from Fluka (Steinheim, Germany). Ultrapure water was obtained by a Direct-Q 3UV water purification system (Merck, Germany).

Purchase of e-liquid for e-cig

E-liquid for e-cig devices were purchased from several companies in various regions in Greece. The selection of samples was performed according to market criteria. E-liquids with the highest demand in the Greek market were selected. A total of 263 samples were collected during the period of 2011–2013. Samples were analyzed within 15 days of purchase after storage in a dark place at room temperature. Samples included in this study were produced by 13 different companies. A total of 70 samples (26.6%) were non-colored (transparent), while 90 samples (34.2%) were yellow. Other colors recorded were golden (10.6%), orange (8.7%) and brown (8.0%) and shades of red (11.9%).

Preparation of samples

For PG, G, L and DG analysis

For the determination of the main humectants ingredients of e-liquids, PG and G as well as for the detection of L and DG a derivatization process with MSTFA followed. In 5 mg of each sample, 0.1 mL MSTFA and 0.1 mL pyridine were added. Each solution was incubated in ambient temperature for 30 min with intermediate mechanical shaking (every 10 min). Then solutions were properly diluted in methanol (to provide a final concentration range from 0 to 500 ppm), 10 μg of ketamine was also added (as an external standard) and then GC–MS analysis was initiated (Figure 1a).

For PAHs analysis

For the detection of PAHs, 5 mg of each sample were diluted in acetonitrile and 10 μg ketamine were added as an external

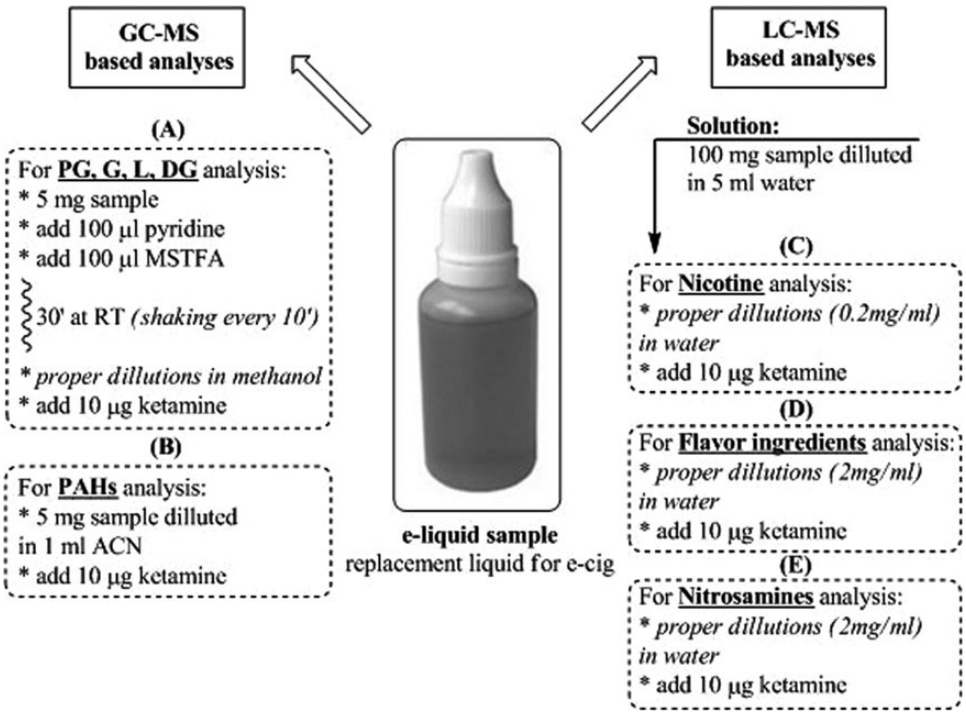

Figure 1. Scheme of the samples preparation for the analyses of e-liquids.

standard (final volume 1 mL per sample) and analyzed by GC–MS. A total of 13 PAHs was investigated in each sample: acenaphthylene (PAH1), fluorene (PAH2), phenathrene (PAH3), anthracene (PAH4), pyrene (PAH5), benzo-(a)-anthracene (PAH6), chrysene (PAH7), benzo-(k)-fluoranthene (PAH8), benzo-(a)-fluoranthene (PAH9), benzo-(a)-pyrene (PAH10), benzo-(g,h,i)-perylene (PAH11), dibenzo-(a,h)-anthracene (PAH12) and indeno-(1,2,3-cd)-pyrene (PAH13) (Figure 1b).

For NIC analysis

For the determination of NIC, an amount of 100 mg of each sample was diluted in 5 mL ultrapure water. Further dilutions of the samples were done in order to achieve a sample content of 0.2 mg per 1 mL and 10 μg ketamine were added before analyzing by LC–MS. All necessary dilutions were done using ultrapure water (Figure 1c).

For main flavor ingredients analysis

Five flavor ingredients (FL1–FL5) were investigated and quantified in each sample. An amount of 100 mg of each e-liquid sample was added in 5 mL ultrapure water. Further dilutions were done in order to achieve a sample content of 2 mg per 1 mL and 10 μg ketamine were added before analyzing by LC–MS. All necessary dilutions were done using ultrapure water (Figure 1d).

For nitrosamines analysis

For the detection of four nitrosamine compounds (NNAL, NNK, NAT and NAB), 100 mg of each sample were diluted in 5 mL ultrapure water. Further dilutions were done in order to achieve a sample content of 2 mg per 1 mL and 10 μg ketamine were added before analyzing by LC–MS. All necessary dilutions were done using ultrapure water (Figure 1e).

Instrumental conditions

GC–MS system

The GC–MS technique was used for the determination and quantification of PG and G as well as for the detection of L, DG and PAHs as aforementioned. Electron ionization mass spectrometric analysis was performed on a GC-MS QP-2010 Shimadzu system (Shimadzu, Japan) equipped with a DB-5 (30 m × 0.25 mm, 0.25 μm) capillary column (Agilent Technologies, USA) for PG, G, L and DG analysis and with a SLB-5 ms (30 m × 0.25 mm, 0.25 μm) capillary column (Supelco, USA) for PAHs analysis. Pure helium (99.999%) with a column flow of 1 mL/min was used as a carrier gas. One microliter of each solution was injected into the system in the splitless mode and analyzed under the following conditions: the column temperature was initially held at 55°C for 2 min and raised to 320°C at 20°C/min (for PG, G, L and DG analysis), while the temperature was initially held at 120°C for 3 min, raised to 310°C at 5°C/min where held for 1 min and finally raised to 325°C at 10°C/min where held for 1 min (for PAHs analysis). The injector temperature was 230°C. The interface temperature was set at 310°C. The ion source temperature was 220°C.

An auto-tune of the mass spectrometer using perfluorotributylamine (PFTBA, tuning standard) was performed before the analysis of every batch of samples. Quantitative analysis was achieved in selected ion monitoring (SIM) mode with a scan time of 0.2 s, using one target ion for quantification and two qualifier ions for the confirmation of each compound. The target ions (m/z) and the retention time for each analyte are presented in Table I. Data acquisition and processing was performed by using the GC-MS Solution software (Shimadzu, version 3.40.307).

Table I
Retention Times (Min), Target (Italics) and Qualifier m/z Ions, Linearity (r^2) and Limits of Quantification (LOQ, μg/mL) Presented for Each Analyte

Analyte		Rt (min)	m/z	r^2	LOQ
GC–MS analysis					
Propylene glycol	PG	4.64	*117*, 147	0.998	0.198
Linalool	L	6.63	*143*, 93	0.997	0.139
Diethylene glycol	DG	6.70	*66*, 191	0.995	0.149
Glycerol	G	6.95	*205*, 131	0.990	1.187
Acenaphthylene	PAH1	10.55	*152*, 76	0.997	0.005
Fluorene	PAH2	13.53	*166*, 82	0.996	0.010
Phenanthrene	PAH3	17.87	*178*, 76	0.997	0.005
Anthracene	PAH4	18.10	*178*, 76	0.997	0.006
Pyrene	PAH5	24.48	*202*, 101	0.997	0.003
Benzo-(a)-anthracene	PAH6	30.21	*228*, 114	0.997	0.003
Chrysene	PAH7	30.35	*228*, 114	0.998	0.003
Benzo-(k)-fluoranthene	PAH8	34.99	*252*, 126	0.998	0.006
Benzo-(a)-fluoranthene	PAH9	35.09	*252*, 126	0.997	0.006
Benzo-(a)-pyrene	PAH10	36.29	*252*, 126	0.997	0.006
Benzo-(g,h,i)-perylene	PAH11	40.43	*276*, 138	0.997	0.014
Dibenzo-(a,h)-anthracene	PAH12	40.55	*276*, 138	0.998	0.021
Indeno-(1,2,3-cd)-pyrene	PAH13	41.31	*276*, 138	0.995	0.022
LC–MS analysis					
Nicotine	NIC	4.98	*84*, 162	0.999	0.073
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol	NNAL	10.91	*210*, 251	0.994	0.009
4′-(Nitrosomethylamino)-1-(3-pyridyl)-1-butanone	NNK	12.08	*208*, 249	0.996	0.012
N-nitrosoanatabine	NAT	13.36	*190*	0.995	0.009
N-nitrosoanabasine	NAB	13.75	*192*	0.997	0.053
Methyl cyclopentenolone	FL1	11.38	*113*, 145	0.999	0.121
Ethyl maltol	FL2	16.05	*141*, 173	0.992	0.135
2,5-Dimethylpyrazine	FL3	17.30	*109*, 141	0.995	0.010
Ethyl vanillin	FL4	18.63	*167*, 199	0.997	0.014
3,4-Dimethoxybenzaldehyde	FL5	19.11	*167*, 199	0.999	0.032

LC–MS system

For the determination and quantification of NIC, flavors (FL1–FL5) and nitrosamine compounds an LC–MS technique was performed. LC was carried out using a Shimadzu Prominence LC system consisting of a binary LC pump, a vacuum degasser, an auto-sampler and a column oven (Shimadzu, Japan). A gradient program of two mobile phases was selected for the analysis of the aforementioned compounds as presented in Table II. Total mobile phase pumped at 0.5 or 0.6 mL/min through a GraceSmart RP 18 5 μm (250 mm × 4.6 mm, 5 μm) column (Grace, Belgium) thermostated at 30–45°C. An aliquot of 10 μL of each sample was injected in the mobile phase flow for separation and analysis.

A mass spectrometer (LCMS-2010 EV Shimadzu), in conjunction with an atmospheric pressure chemical ionization (APCI) interface with a single quadrupole mass filter, was used to detect and quantify the analytes in column effluent. Interface, curved desolvation system (CDL) and heat block temperatures were 400, 200 and 200°C, respectively. The detector voltage was 1.5 kV and the nebulizing gas flow 2.5 L/min. Drying gas was set at 0.02 MPa (Table II). Ion signals were acquired in time SIM mode with ions and retention time presented in Table I. The MS operating conditions were tuned according to the manufacturer procedure. Data acquisition and processing were performed using LC–MS Solution software (Shimadzu, version 3.40.307).

Table II
Setup Information for the LC–MS System Per Analyte Group

Analyte	Mobile phases	LC program				MS interface
		Minimum	*B* concentration (%)	Total flow (mL/min)	Oven Temperature (°C)	
Nicotine	A: Formic acid 0.1%	0.01	10	0.6	45	APCI (+)
	B: Methanol	10.00	80			Interface temperature: 400°C
		10.01	10			CDL temp: 200°C
		13.00	10			Heat block: 200°C
		13.00	Stop			Detector: 1.5 kV
Nitrosamines	A: Ammonium acetate 10 mM (pH 5.22)	3.00	10	0.5	45	Nebulizing gas: 2.5 L/min
	B: Acetonitrile	30.00	95			Drying gas: 0.02 MPa
		30.01	10			
		34.00	10			
		34.00	Stop			
Main flavor ingredients	A: Formic acid 0.1%	3.00	5	0.6	30	
	B: Methanol	30.00	95			
		30.01	5			
		34.00	5			
		34.00	Stop			

Calibration and quantification

Stock solutions of PG, G, L and DG at the concentration of 1 mg/mL were prepared in methanol. Working solutions of each analyte were prepared before each batch analysis of samples by dilutions in methanol and by following the same derivatization process as described before ('Preparation of samples' section). The concentrations of PG and G were 0, 31.25, 62.5, 125, 250 and 500 μg/mL, while for L and DG were 0, 2.5, 5, 10 and 20 μg/mL. Stock mix solution of 13 PAHs described before ('Preparation of samples' section) at the concentration of 1 mg/mL was prepared in acetonitrile. Working solutions were prepared by dilutions in acetonitrile at the concentrations of 0, 0.5, 1, 2.5 and 5 μg/mL.

Stock solution of NIC, flavor (FLs) and nitrozamines at the concentration of 1 mg/mL was prepared in methanol. Working solutions were prepared by dilutions in methanol at the concentrations of 0, 0.5, 1, 2.5, 5 and 10 μg/mL for NIC and FLs and of 0, 0.1, 0.25, 0.5 and 1 μg/mL for nitrozamines. All above working solutions were stored at −20°C.

Statistical analysis

Continuous variables were expressed in the form of mean ± standard deviation (SD) and min–max values, while discrete as counts and percentages. Percentiles and quartiles were also applied to recode continuous variables. Associations were examined using Pearsons and Spearman's rho (continuous variables) and Pearson's χ^2 (discrete variables). A series of plots like scatterplots and bar-charts were produced using IBM SPSS Statistics 20.0 or EXCEL 2007 for Windows. A level of significance was set at 0.05.

Results and discussion

Method validation

The developed methodology for the multicomponent detection and quantification in e-liquid products of e-cig are comprised of fast and one-step (dilution) treatment of samples, combining two chromatographic approaches. During methods development several conditions and parameters were optimized for the most efficient analytes detection and quantification.

Table III
Optimization of Sufficient Aliquot for MSTFA Derivatization Agent Results

Examined level (μg/mL)	Added MSTFA (μL)	(Analyte)/(ES) area ratio			
		PG	G	L	DG
62.5	50	73.2	11.0	5.9	10.5
	100	80.5	25.5	10.3	24.6
	150	76.5	24.9	8.5	23.2
250	50	293.5	43.7	21.6	43.0
	100	320.1	102.5	43.2	97.5
	150	304.5	99.8	35.2	91.8

Table IV
The Mean Accuracy (%) and the Mean Inter-days Precision (% RSD) Calculated for the Most Commonly Detected Compounds in Replacement e-Liquid Samples

Analyte	% Accuracy[a]	Inter-days precision[a,b,c]
GC–MS analysis		
Propylene glycol (PG)	105.1 ± 13.9	8.7
Glycerol (G)	102.8 ± 12.8	8.9
LC–MS analysis		
Nicotine (NIC)	99.1 ± 4.9	3.6
Methyl cyclopentenolone (FL1)	105.8 ± 10.9	8.8
Ethyl maltol (FL2)	101.7 ± 9.8	8.9
2,5-Dimethylpyrazine (FL3)	106.6 ± 18.1	16.0
Ethyl vanillin (FL4)	101.3 ± 10.3	7.9
3,4-Dimethoxybenzaldehyde (FL5)	105.7 ± 11.4	8.2

[a] $n = 6$.
[b] % RSD.
[c] Mean values for the tested concentrations (62.5, 125 and 250 μg/mL for PG and G and 2.5, 5 and 10 μg/mL for NIC and FLs).

Optimization of PG, G, L and DG derivatization parameters

For the optimization of derivatization parameters of PG, G, L and DG the proper aliquot of the derivatization reagent MSTFA was tested in two different concentrations (62.5 and 250 μg/mL). The examined aliquots were 50, 100 and 150 μL. After following the procedure described before for the derivatization of analytes, each sample was analyzed by GC–MS ('Preparation of samples' section). The results were compared and found that adding 100 μL of MSTFA for 30 min at room temperature provides the better results and so this was selected as the sufficient amount of derivatization reagent. The results for both examined concentrations are presented in Table III.

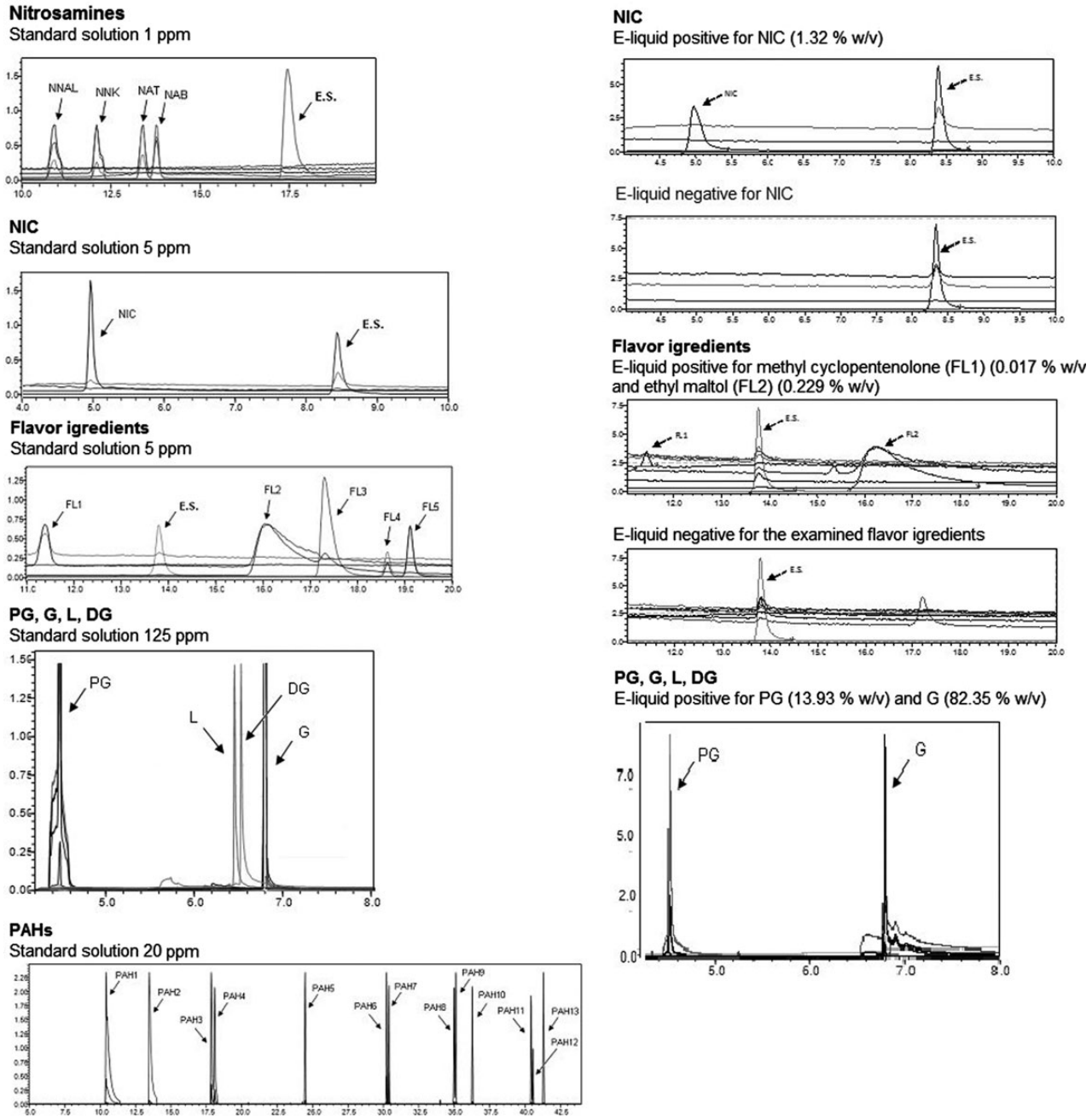

Figure 2. Typical chromatograms of standard solutions for each analyte group (a) and chromatograms of positive e-liquids to nicotine, PG, G and flavors (b).

Linearity

The internal standard method was used for analytes quantification. The instrument response was linear in the concentration range between 31.25 and 500 μg/mL for PG and G, from 2.5 to 20 μg/mL for L and DG, from 0.5 to 5 μg/mL for PAHs, from 0.5 to 10 μg for NIC and investigated flavor ingredients and from 0.1 to 1 μg/mL for nitrosamines, with $r^2 > 0.99$ in all cases (Table I).

Limits of quantification

The limit of quantification (LOQ) of the method was determined as the concentration of analyte at which the signal-to-noise ratio

Table V
Differences Between the Mean Theoretical and the Mean Measured Concentration of Nicotine Per Company

Company	*N*	Nicotine	Mean concentration (% w/v)	± SD	*P* value
1	20	T	1.53	0.59	0.140
		M	1.62	0.72	
2	25	T	1.30	0.57	0.229
		M	1.24	0.50	
3	30	T	1.58	0.57	0.030
		M	1.48	0.57	
4	55	T	1.13	0.69	<0.001
		M	1.01	0.63	
5	55	T	0.94	0.76	<0.001
		M	0.78	0.65	
6	18	T	1.05	0.83	0.462
		M	0.98	0.82	
7–13	60	T	1.02	0.69	0.023
		M	0.97	0.65	
Total	263	T	1.16	0.71	<0.001
		M	1.07	0.69	

T, theoretical concentrations; M, measured concentrations; SD, standard deviation; *N*, number of samples.

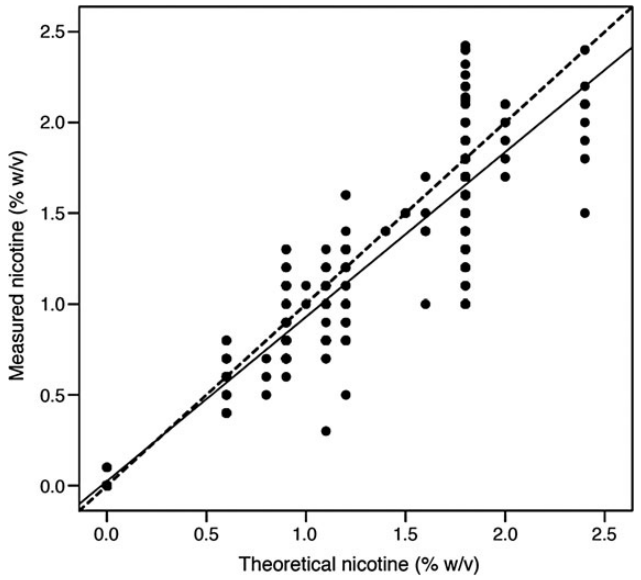

Figure 3. The mean theoretical and the mean measured nicotine concentrations for each sample showed a statistically significant ($r = 0.940$, $P < 0.001$) correlation.

of the quantification ion was at least 10. LOQ values ranged from 0.003 μg/mL (for three PAH compounds) to 1.187 μg/mL (for G) (Table I).

Method precision and accuracy

The precision (interday) and the accuracy of the method were calculated for the most commonly detected compounds in replacement e-liquid samples. Interday precision was measured and expressed as % relative standard deviation (% RSD) of instrument response for replicate measurements ($n = 6$) of calibration samples in three different concentrations for each analyte (62.5, 125 and 250 μg/mL for PG and G, 2.5, 5 and 10 μg/mL for NIC and the main flavor ingredients). Precision was calculated <16% in all cases. Accuracy was also determined for the same concentrations of each analyte ($n = 6$) (>99.1% for all cases). The mean accuracy and precision values for each analyte are presented in Table IV.

Samples analysis

E-liquids of e-cig have complicated matrix properties because they are made of a mixture of various flavors in PG and/or G. The developed methods were applied to e-liquid samples collected from the Greek market and no interfering peak was observed in the chromatograms close to the retention times of the analytes. Figure 2 shows the LC and GC chromatograms of standard solutions analyzed for each target compound.

We analyzed the concentrations of all components in 263 e-liquids produced by 13 companies as aforementioned. The theoretical NIC concentration, as reported by the manufacturer, and the measured concentration of NIC are shown in Table V. The differences between the mean theoretical and the mean measured concentration of NIC were also examined per company. It can be seen that five of six of the companies provided measured NIC concentrations lower than the theoretical ones (Table V). Generally, theoretical and measured NIC concentrations for each sample showed a statistically significant ($r = 0.940$, $P < 0.001$) correlation (Figure 3). The most frequent NIC concentrations were 1.8% w/v (33.8% of total samples). Additionally, 0.9 and 1.2% w/v of NIC were detected in 12.9 and 8.4% of the samples, respectively. A percentage of 19.0% did not contain NIC and this was according to the manufacturer statement. Furthermore, colored and transparent samples were noticed to have different concentrations of NIC ($x^2 = 73.214$, df = 2, $P < 0.001$). Colored samples provide higher concentrations of NIC

Table VI
Mean Found Concentration (% w/v) (± SD) and Detection Frequency Rates (%) for the Examined Flavor Ingredients Per Company and Total

Company	Methyl cyclopentenolone		Ethyl maltol		2,5-Dimethylpyrazine		Ethyl vanillin		3,4-Dimethoxybenzaldehyde	
	%	Mean (± SD)	%	Mean (± SD)	%	Mean (± SD)	%	Mean (± SD)	%	Mean (± SD)
1	27.3	0.156 (±0.227)	21.8	0.411 (±538)	5.5	0.009 (±0.012)	18.2	0.327 (±812)	1.8	0.012 (−)
2	38.2	0.117 (±0.140)	34.5	0.150 (±0.357)	10.9	0.012 (±0.010)	16.4	0.051 (±0.105)	7.3	0.043 (±0.075)
3	36.7	0.172 (±0.216)	26.7	0.413 (±0.494)	ND	–	60.0	0.011 (±0.032)	ND	–
4	12.0	0.043 (±0.059)	12.0	0.007 (±0.009)	ND	–	4.0	0.046 (±0.000)	ND	–
5	45.0	0.039 (±0.025)	30.0	0.062 (±0.082)	20.0	0.001 (±0.000)	5.0	0.003 (±0.000)	15.0	0.003 (±0.001)
6	50.0	0.032 (±0.033)	66.7	0.020 (±0.025)	5.6	0.001 (±0.000)	ND	–	5.6	0.005 (±0.000)
7–13	20.0	0.061 (±0.088)	30.0	0.460 (±0.635)	5.0	0.007 (±0.006)	16.7	0.066 (±0.129)	8.3	0.005 (±0.003)
Total	30.4	0.103 (±0.155)	29.7	0.256 (±0.464)	6.5	0.007 (±0.008)	18.6	0.095 (±0.379)	5.3	0.016 (±0.040)

SD, standard deviation; ND, no detection or below LOD.

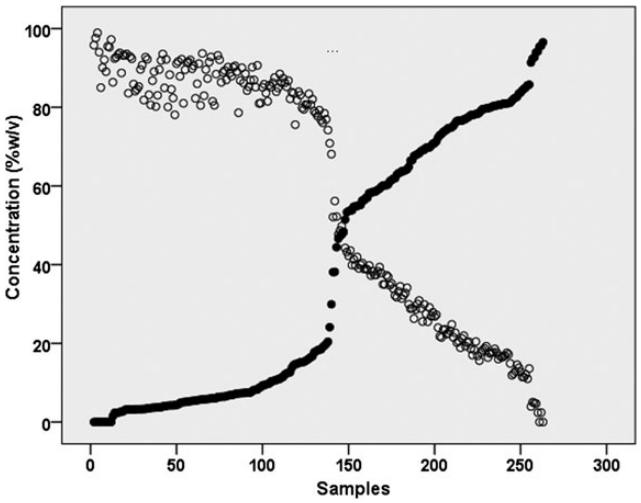

Figure 4. Correlation between PG and G levels, expressed in % w/w, found in the examined e-liquid sample.

Table VII
Mean Found Concentration (% w/v) (± SD), Positive Samples (*N*) and Detection Frequency Rates (%) for the Main Humectants PG and G, L and DG

Analyte	Detection, *N* (%)	Mean concentration (% w/v)	± SD
PG	251 (95.4)	37.11	32.89
G	260 (98.9)	58.15	31.57
L	21 (8.0)	0.031	0.292
DG	0 (0)	–	–

PG, propylene glycol; G, glycerol; L, linalool; SD, standard deviation; *N*, number of samples.

(180 samples, 93.3%) compared with only 13 samples (6.7%) without NIC detected.

Concerning the main flavor ingredient analysis, results revealed fluctuated detection rates from 5.3% (for 3,4-dimethoxybenzaldehyde) to 30.4% (for methyl cyclopentanolone) in all companies. More specifically, the ingredients methyl cyclopentanolone (from 12.0 to 50.0%) and ethyl maltol (12.0 to 66.7%) were detected in higher rates in all companies and ethyl vanillin was detected in an impressive 60.0% of the samples of company 3 (Table VI). The mean concentrations found for these flavor ingredients ranged from 0.001% w/v (for 2,5-dimethylpyrazine, company 5) to 0.460% w/v (for ethyl maltol, companies 7–13) (Table VI). For companies 3 and 4, there was no detection of 2,5-dimethylpyrazine and 3,4-dimethoxybenzaldehyde in any sample, as well as in company 6 that there was no detection of ethyl vanillin. All these fluctuations in detection frequency rates and the detected concentrations provide the differentiation of the taste/odor of the final product per company.

Although the variation of the PG and G found concentrations in e-liquid samples is obvious, the results indicated that the relations of these two main humectants are analog to each other for all examined samples (Figure 4). Because the mean values presented in Table VII for these two humectants are not quite representative for the summary of the samples, we further report

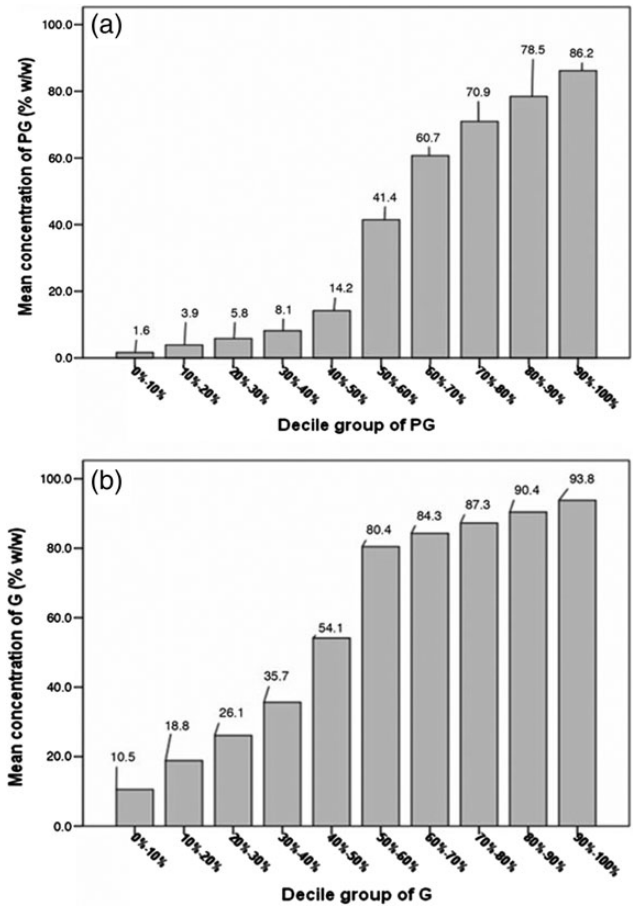

Figure 5. Mean concentrations of PG (a) and G (b) per deciles groups.

the mean concentration expressed in % w/w for each deciles group for both PG and G (Figure 5). For L, the detection rates were low (8%) with adequate low mean concentration 0.031 ± 0.292% w/v (Table VII). Furthermore, the harmful chemicals DG, PAHs and nitrosamines were not detected in any sample.

Discussion and conclusion

In a study published by Hutzler *et al.* (18), a wide spectrum of flavors and allergens as an additive in liquid and vapors of e-cig purchased in Germany were reported. Some of them (e.g., cinnamic aldehyde and coumarin) are prohibited in Germany while many others (e.g., eugenol and benzyl alcohol) are regulated by the European Cosmetics Directive. Totally, 141 volatile flavors were detected in 28 e-cig liquid samples, with vanillin, ethyl maltol, ethyl vanillin, menthol and 3-methyl-1,2-cyclopentanedione to be the additives with the higher frequencies of detection. More specific, the authors of the aforementioned study report 21.4% (6/28) positive samples for 3-methyl-1,2-cyclopentanedione, 57.1% (16/28) for ethyl maltol and 50% (14/28) for ethyl vanillin which are in accordance to those detected in this study 30.4, 29.7 and 18.6%, respectively, in a total of 263 e-liquid products.

As it is mentioned in the study of Goniewicz *et al.* (9), the vapor of e-cig contains potentially toxic compounds (such as acrolein, toluene, nitrosamines, heavy metals) but the concentrations of these compound are 9- to 450-fold lower than those in smoke from conventional cigarettes. Moreover, the content of

N-nitrosonornicotine (NNN) ranged from 0.8 to 4.3 ng while the content of NNK ranged from 1.1 to 28.3 ng per one e-cig (150 puffs). Furthermore, these nitrosamines were found in the vapor of e-liquids and not in the e-liquid itself which is in agreement with the results of another study (19), where no nitrosamines were detected in the e-liquid product.

In this study, we have developed and validated an analytical method by using both GC–MS and LC–MS techniques after relatively simple sample preparation for simultaneous determination of the components in e-liquid of e-cig. Excellent precision and accuracy were achieved with the use of ketamine as an external standard. The LOQs of all analytes were relatively low, the accuracy was in range of 99.1–106.6% and precision of the method was <16% for all analytes. Thus, the proposed method could be suitable for routine multicomponent analysis in e-liquid of e-cig.

The main humectants, the main flavor ingredients and NIC were identified and quantified in 263 e-liquid products of 13 companies, available in the Greek market. The theoretical NIC concentration, as reported by the manufacturer, and the measured concentrations of NIC were strongly correlated. Moreover, no PAHs, nitrosamines or DG were detected in any sample.

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References

1. USDHHS. The health consequences of smoking—50 years of progress. A Report of the Surgeon General. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2014 (accessed Jul 9, 2014).
2. Flouris, A., Metsios, G., Carrillo, A., Jamurtas, A., Terzi, I., Tzatzarakis, M. *et al.* (2010) Effects of passive smoking on active and resting humans 0, 1, and 3 h following exposure. *Toxicology Letters*, **196**, S87–S88.
3. Tzatzarakis, M.N., Tsitoglou, K.I., Chorti, M.S., Poulianiti, K.P., Jamurtas, A.Z., Koutedakis, Y. *et al.* (2013) Acute and short term impact of active and passive tobacco and electronic cigarette smoking on inflammatory markers. *Toxicology Letters*, **221**, S86.
4. Tabara, Y., Takahashi, Y., Setoh, K., Muro, S., Kawaguchi, T., Terao, C. *et al.* (2013) Increased aortic wave reflection and smaller pulse pressure amplification in smokers and passive smokers confirmed by urinary cotinine levels: the Nagahama Study. *International Journal of Cardiology*, **168**, 2673–2677.
5. Lu, L., Mackay, D.F., Pell, J.P. (2013) Association between level of exposure to secondhand smoke and peripheral arterial disease: Cross-sectional study of 5686 never smokers. *Atherosclerosis*, **229**, 273–276.
6. Pauly, J., Li, Q., Barry, M.B. (2007) Tobacco-free electronic cigarettes and cigars deliver nicotine and generate concern. *Tobacco Control*, **16**, 357.
7. Gardiner, P.S. (2013) The vapor this time? In University of California Office of the President (ed.), Tobacco Related Disease Research Program (TRDRP). UCSF Health Sciences West 301, San Francisco, CA, pp. 1–19.
8. Schaller, K., Rupper, L., Kahnert, S., Bethke, C., Nair, U., Potschke-Langer, M. *Electronic Cigarettes—An Overview, Res Series Tobacco Prevention and Tobacco Control*; German Cancer Research Center (DFKZ): Heidelberg, Germany, pp. 1–59, 2013.
9. Goniewicz, M.L., Knysak, J., Gawron, M., Gawron, M., Kosmider, L., Sobczak, A. *et al.* (2014) Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tobacco Control*, **23**, 133–139.
10. Kim, H.J., Shin, H.S. (2013) Determination of tobacco-specific nitrosamines in replacement liquids of electronic cigarettes by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, **1291**, 48–55.
11. McAuley, T.R., Hopke, P.K., Zhao, J., Babaian, S. (2012) Comparison of the effects of e-cigarette vapor and cigarette smoke on indoor air quality. *Inhalation Toxicology*, **24**, 850–857.
12. Westenberger, B. (2009) Evaluation of e-cigarettes, FDA Report, Food and Drug Administration (FDA).
13. Williams, M., Villarreal, A., Bozhilov, K., Lin, S., Talbot, P. (2013) Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. *PLoS ONE*, **8**, e57987.
14. Goniewicz, M.L., Kuma, T., Gawron, M., Knysak, J., Kosmider, L. (2013) Nicotine levels in electronic cigarettes. *Nicotine and Tobacco Research*, **15**, 158–166.
15. Goniewicz, M.L., Hajek, P., McRobbie, H. (2014) Nicotine content of electronic cigarettes, its release in vapour and its consistency across batches: regulatory implications. *Addiction*, **109**, 500–507.
16. Trtchounian, A., Williams, M., Talbot, P. (2010) Conventional and electronic cigarettes (e-cigarettes) have different smoking characteristics. *Nicotine Tobacco Research*, **12**, 905–912.
17. World Health Organization (WHO) (2013) Questions and answers on electronic cigarettes or electronic nicotine delivery systems (ENDS), Tobacco Free Initiative (TFI); Statement. http://www.who.int/tobacco/communications/statements/eletronic_cigarettes/en/ (accessed Jul 17, 2014).
18. Hutzler, C., Paschke, M., Kruschinski, S., Henkler, F., Hahn, J., Luch, A. (2014) Chemical hazards present in liquids and vapors of electronic cigarettes. *Archives of Toxicology*, **88**, 1295–1308.
19. Alliance Technologies LLC. (2009) Characterization of regal cartridges for electronic cigarettes—phase II. http://truthaboutecigs.com/science/9.pdf (accessed Sep 19, 2014).