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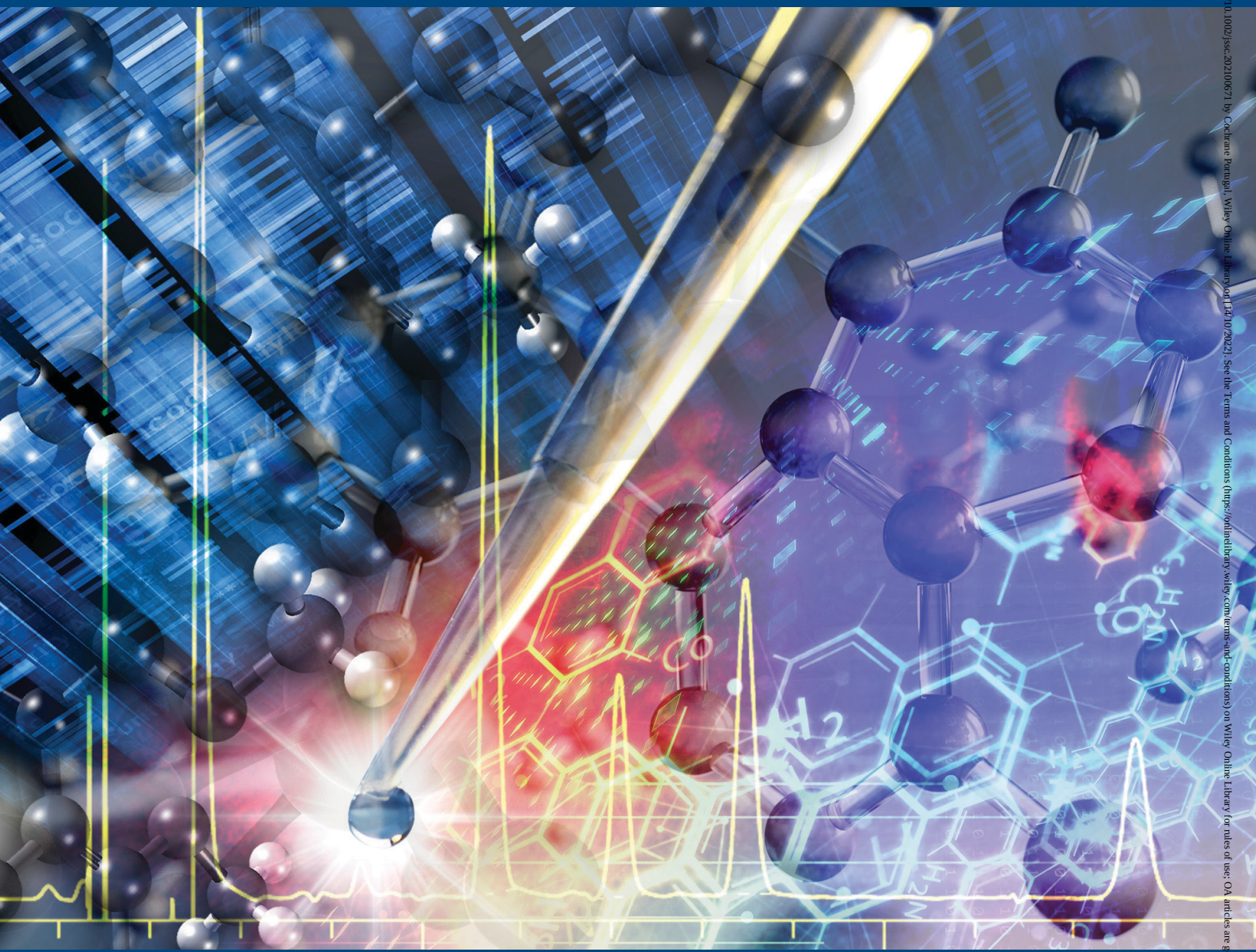

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RESEARCH ARTICLE

Urinary volatomic profile of traditional tobacco smokers and electronic cigarettes users as a strategy to unveil potential health issues

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Many harmful and potentially harmful constituents are present in tobacco products. Cigarette smoke is known to cause different forms of cancer and trigger the development of chronic diseases. In the last decade, electronic cigarettes have emerged as a healthier alternative associated to less harmful effects in comparison to traditional tobacco. However, the lack of standardization of electronic cigarettes products makes it difficult to establish and compare the real effects on health of products from different manufacturers. To better understand the impact of smoking and vaping, the volatomic composition of urine samples from traditional tobacco smokers and electronic cigarette users was established and compared with nonsmokers (control group), using headspace solid-phase microextraction combined with gas chromatography–mass spectrometry. A total of 45 urinary volatile organic metabolites belonging to different chemical families were identified in the urine of the studied groups. Benzene derivatives, terpenes, and aromatics were the chemical families that contributed the most to the urinary profile of smokers. The vapers urinary volatomic pattern was also dominated by terpenes and aromatics, in addition to alcohols. The orthogonal partial least squares-discriminant analysis of the data obtained indicated that the urinary profile of vapers is more closely related to the control group, reinforcing the hypothesis of the lowest harmfulness of electronic cigarettes. Further studies recruiting a higher number of subjects are therefore necessary to consolidate the data obtained.

KEYWORDS
electronic cigarettes, gas chromatography-mass spectrometry, headspace solid-phase microextraction, tobacco, urinary volatile organic metabolites

Article Related Abbreviations: e-cigarettes, electronic cigarettes; HS, headspace; OPLS-DA, orthogonal partial least squares-discriminant analysis; PAH, polycyclic aromatic hydrocarbon; TSNA, tobacco-specific nitrosamine; uVOM, urinary volatile organic metabolite; VOC, volatile organic compound

1 | INTRODUCTION

Tobacco products and smoking are the leading causes of preventable deaths throughout the world, accounting for approximately 30% of cancer-related deaths [1, 2]. It is well established that cigarette smoking generates more than 60 carcinogens and that some of them are related to the development of different forms of cancer, besides respiratory chronic diseases [3–5]. Nevertheless, carcinogens and toxicants, such as tobacco alkaloids, tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), and volatile organic compounds (VOCs), vary across different tobacco products and brands [4, 6-8]. The impact of cigarette' smoke on health depends on the duration of smoking over the years and on the exposure to free radicals from the components of tobacco smoke, which can lead to increased oxidative stress, inflammation, and DNA damage [3, 5].

Electronic cigarettes (e-cigarettes) have become popular alternatives to traditional tobacco. While traditional cigarettes use combustion to burn tobacco, e-cigarettes use a battery-powered device to heat and aerosolize a liquid, the e-liquid solution, typically containing nicotine and flavoring agents [9–11]. However, the lack of standardization of e-cigarettes products constitutes a great problem to overcome when comparing the health effects of products from different manufacturers. The levels of VOCs in e-cigarette emissions vary with the type of device, the extent of its use, and the conditions applied by the user. Nevertheless, under most conditions, VOCs concentrations in e-cigarettes have been reported to be significantly lower than in traditional tobacco. According to Keith et al. [12] and Lorkiewicz et al. [13], the levels of urinary volatile organic metabolites (uVOMs) of several harmful or potentially harmful substances are reduced by switching from traditional cigarettes to e-cigarettes. Despite this potential advantage, there are still health concerns about e-cigarettes due to their potential cytotoxicity, delivery of carcinogens, such as carbonyls and TSNAs, and the effects on cardiovascular and respiratory functions [14, 15]. In addition, the health effects of e-liquids in human health are still unknown, particularly the effects of nicotine when delivered as an aerosol, the flavoring agents when they enter the bloodstream through the lungs, and the effects of inhaling the humectants propylene glycol and glycerol [11]. Due to their relative newness, there is not enough information about the short- and long-term effects of e-cigarettes use, meaning that it remains unclear how its extended use directly contributes to exposure of our cells to harmful VOCs [12, 13].

uVOMs are end products of the metabolic pathways, and their profile might be a useful platform to monitor the status of different organs and systems and processes in the human body. Thus, the analysis of uVOMs emerged as a powerful tool for the identification of biomarkers in different diseases, including cancers and inflammatory diseases [16–19]. Different analytical procedures, including liquid–liquid extraction, SPE, and dispersive liquid–liquid microextraction, have been traditionally used to extract the volatiles from several matrices [20]. However, these techniques are generally very laborious and time consuming, and use great amounts of samples and organic solvents. In recent years, the progress of different microextraction techniques, with enhanced selectivity, easiness in handling, and improved performance (better extraction yield and efficiency), revolutionized the field of sample processing and handling. SPME, a nonexhaustive extraction technique that involves sampling, extraction, concentration, and sample preparation in a single step [20, 21], is a solventless technique, highly efficient, that does not require any concentration step prior to analysis, preventing the production of interferents [16, 22, 23]. This technique shows increased sensitivity, automation potential, and portability, being a passive sampling approach that does not interfere with the sample when used in headspace (HS) mode. The HS-SPME combination with GC-MS analysis allows reliable and reproducible results in uVOMs analysis [16, 18–20, 22]. Moreover, the availability of extraction materials and basic equilibration mechanisms makes this methodology very selective and with a high performance [22, 23].

In this work, we aimed to establish the volatomic composition of urine samples of e-cigarettes users and traditional tobacco smokers, using HS-SPME combined with GC-MS analysis, as an innovative and useful strategy to evaluate the potential harmfulness of e-cigarettes, based on the identified uVOMs, when compared with traditional tobacco.

2 | MATERIALS AND METHODS

2.1 | Reagents

Sodium chloride (NaCl, 99.5 %) was acquired from Pan-Reac AppliChem ITW Reagents (Barcelona, Spain), and hydrogen chloride (HCl, 37 %) and 3-octanol (internal standard, 99 %) from Sigma-Aldrich (St Louis, MO, USA). Ultrapure water (H_2O) was obtained from a Milli-Q water purification system (Millipore, Bedford, PA, USA). Helium of purity 5.0 (Air Liquide, Algés, Portugal) was used as GC carrier gas. The glass vials, SPME holder for manual sampling, and fibers were purchased from Supelco (Merck KGaA, Darmstadt, Germany). The SPME device included a fused silica fiber coating partially cross-linked with 75 μm carboxen/polydimethylsiloxane (CAR/PDMS). Different pure standards were used to confirm the uVOMs

TABLE 1 Characterization of the smokers (traditional cigarettes, TRAD) and vapers (e-cigarettes, ECIG) recruited for this study

	Smokers				Vapers		
Subjects	**Age**	**Sex**	**Years of use**	**Subjects**	**Age**	**Sex**	**Years of use**
TRAD 1	45	M	28	ECIG 1	47	M	3
TRAD 2	40	M	30	ECIG 2	45	M	2
TRAD 3	44	F	20	ECIG 3	46	F	0.7
TRAD 4	36	F	24	ECIG 4	38	F	3
TRAD 5	44	F	5	ECIG 5	42	M	2.5
TRAD 6	30	M	19	ECIG 6	37	F	1
TRAD 7	50	M	7	ECIG 7	48	M	2
TRAD 8	27	M	25	ECIG 8	31	M	0.16
TRAD 9	40	F	20				–
TRAD 10	41	F	20				–

Abbreviations: ECIG, recruited subject using vaping; F, female; M, male; TRAD, recruited subject using traditional cigarettes. smoking.

identified using the NIST library and were acquired in their maximum purity available from Acros Organics (NJ, USA), MilliporeSigma (Merck KGaA, Darmstadt, Germany), and Panreac AppliChem ITW Reagents, Fisher Scientific (NH, USA).

2.2 | Subjects and samples

This study involved 10 healthy and nonsmokers individuals without any known pathology, who did not take any kind of medication (control group of five male and five female subjects, 22–59 years range), 10 traditional tobacco users (five male and five female subjects, 27–50 years range), and eight e-cigarettes users (five male and three female subjects, 31–48 years range). Historic of tobacco consumption was also recorded (Table 1). All subjects were asked to avoid alcoholic drinks in the days before the sampling was made. A sample of morning urine (after overnight fasting) was provided in a 20-mL sterile PVC container. All participants were fully informed of the objectives of the study and signed the informed consent before the collection of the urine samples. After collection, each sample was individually homogenized, aliquoted in 8-mL glass vials, and stored at −20°C until analysis.

2.3 | Headspace-SPME procedure

Briefly, 4 mL aliquots of urine sample adjusted to pH 1–2 with 500 μL of HCl (5 M) were transferred to an 8-mL sampling glass vial, and 0.8 g of NaCl and 5 μL of 3-octanol (5 ppm, prepared in ultrapure H_2O) were added, with stirring at 800 rpm. The vial was placed in a thermostat bath adjusted to 38 ± 0.1°C and then the SPME fiber was inserted in the HS for 50 min. After sampling, the SPME fiber was withdrawn into the needle, removed from the vial, and inserted in the injector port (250°C) of the GC-MS system for 7 min, for desorption of the analytes. Each sample was analyzed in triplicate. Blanks, corresponding to the analysis of the coating fiber not submitted to any extraction procedure, were run between sets of three analyses, with a desorption time of 10 min.

2.4 | GC-MS analysis

The GC-MS analysis were performed with a Perkin Elmer Clarus SQ 8S GC-MS. The gas chromatograph was equipped with a 60 m × 0.25 mm id × 0.25 μm film thickness, BP-20 (SGE, Dortmund, Germany) fused silica capillary column. After extraction, the SPME coating fiber containing the uVOMs was manually introduced into the GC injection port at 250°C and kept for 7 min for thermal desorption of uVOMs. The oven temperature gradient was set at 45°C for 5 min, followed by a temperature increase up to 150°C for 10 min, at a rate of 2°C min^{-1}, then a hold time of 10 min at 150°C, followed by another ramp until 220°C at a rate of 7°C min^{-1}, and finally resting at 220°C during 10 min, giving a total run time of 87.5 min. The column flow was constant at 1.1 mL min^{-1} using helium as carrier gas. The injection port was operated in splitless mode and held at 250°C. The operating temperatures of the transfer line, quadrupole, and ionization source were 220, 150, and 230°C, respectively, while electron impact mass spectra were recorded at 70 eV ionization voltage and the ionization current was 10 μA. Data acquisition was performed in the scan mode (30 – 300 m/z, 0.2 scans per second). The electron multiplier was set to the autotune procedure. A transfer line heated to 220°C carried the compounds from the GC to the MS. uVOMs were identified by comparison of the obtained mass spectra with the data system library

(NIST, 2014 software, Mass Spectral Search Program v. 2.2; Nist 2014, Gaithersburg, MD), considering a minimum percentage match of 80% between the experimental and the library mass spectra, and with pure standards (Table 2).

2.5 | Statistical analysis

Results were expressed as mean ± SD of the independent experiments. Differences among samples were estimated by analysis of variance (ANOVA) followed by Tukey's "honest significant difference" test. The statistical significance level was set to p-values < 0.05. The data matrix was subjected to orthogonal partial least squares-discriminant analysis (OPLS-DA) to check for the clustering pattern between the recruited group. The VIP scores

generated from the OPLS-DA model were indicative of the uVOMs that were most influential (VIP score > 1.0) in discriminating the subjects by groups. The R^2 and Q^2 values from the OPLS-DA model were employed to evaluate the quality and reliability of the mathematical model generated, in which the R^2 value indicates the goodness of t and the Q^2 value represents the predictability of the model. To visualize the clustering of the two groups under this

study, hierarchical cluster analysis was carried out. All statistical analyses were performed using Statistica software and MetaboAnalyst 5.0 [24]. Sample-specific normalization was applied, followed by log transformation and auto-scaling.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of the urinary volatomic profiles of the recruited subjects

To understand the impact of smoking in the volatomic composition of urine, samples from traditional tobacco smokers and e-cigarette users was compared with healthy subjects (control group), as described in Section 2.2. Overall, urine samples from 28 subjects were analyzed by HS-SPME/GC-MS to identify the uVOMs that compose each group and the differences among them. Different chromatographic profiles were obtained for the control group, traditional cigarette smokers, and vapers (e-cigarette users). A total of 45 uVOMs were identified in the urine of the studied groups (Table 2), from which 34, 41, and 36 in the control, traditional cigarette smokers, and vaper users, respectively (Figure 1). Not surprisingly, a higher number of uVOMs was identified in the urine samples of traditional cigarette smokers, which is certainly associated with the high number of VOCs that is generated during the cigarette burning. Part of these VOCs is inhaled and transferred to the blood stream in the lung and them metabolized and eliminated in the urine. In contrast, the number of uVOMs identified in the control and vapers users is not very different (34 vs. 36, Table 2).

Regarding the type of uVOMs identified, the most predominant, in terms of accumulated mean peak areas, were the terpenic, aromatic, sulfur, and furanic compounds. However, there are appreciable differences between groups, particularly the high abundance of benzenic derivatives in traditional tobacco smokers and alcohols in vapers users. Again, benzenes derivatives are certainly associated with the tobacco smoke inhalation, while the alcohols can be associated with the e-liquids used during vaping (Figure 1). Such differential abundance reflects the composition of cigarette smoke and e-liquids previously reported [25].

The human urinary profile changes over time because of bacterial activity, metabolism, pH variations, or decomposition of urine constituents, and it is affected by different factors, such as health status, dietary habits, physical stress, and environmental exposure [26]. In contrast, exposition to environmental contaminations and diet constitute two important exogenous sources for many of the VOMs found in our organism. Most of these exogenous VOMs will be further metabolized inside our organism. Moreover, many VOMs can be both produced endogenously, or result from external exposure or contamination, making very difficult to discriminate their origin and evaluate potential up- or downregulation of specific biochemical pathways.

Our results reflect a strong influence of dietary habits in the urinary volatomic profile of the subjects recruited (Table S1). Terpenes, for instance, result from the consumption of foods and beverages [27–29]. Norisoprenoids derive from the enzymatic degradation of carotenoid-rich foods and have an important impact on the aroma of fruits, such as grapes, apples, and mango [30–33, 35]. Sulfur compounds, such as dimethyl disulfide and dimethyl trisulfide that are often detected in human urine [22, 26, 36, 37], can also result from the diet, since dimethyl disulfide and dimethyl trisulfide are present in many foods (vegetables, cheese, fish, meat) and beverages (coffee, wine, beer, milk) [26, 38, 39]. The primary source of furanic compounds is the thermal degradation and rearrangement of carbohydrates, such as glucose, lactose, and fructose. Jarred and canned food, dry products (snacks, bread crust), and roasted coffee beans also show significant levels of furanic compounds [34, 40–42]. However, the heterocyclic compound furan and its derivative 3-methylfuran, which are classified as carcinogens [43], can be also originated from cigarette smoke [44]. Ketones can be originated from the decarboxylation of oxo-acids [45], as well as,

TABLE 2 Identification, chemical family, possible origin, and mean relative peak area of the identified volatile organic metabolites in the control group, in smokers and vapers ($n = 3$; RSD < 20%)

RT (min)	Metabolites	Formula	CAS No.	Chemical family	Possible origin	Mean relative peak area		
						CTRL	TRAD	ECIG
4.67	Methanethiol[a]	CH_4S	74-93-1	S	End (syst, bact)	4.49	6.89	3.68
5.51	Furan[b]	C_4H_4O	110-00-9	F	End/Exo (diet)	1.31	1.07	1.27
6.60	3-Methylfuran[b]	C_5H_6O	930-27-8	F	Exo (diet)	0.40	0.58	0.30
8.69	2,5-Dimethylfuran	C_6H_8O	625-86-5	F	Exo (diet)	0.36	0.93	0.63
11.65	2-Ethyl-5-methylfuran[b]	$C_7H_{10}O$	1703-52-2	F	End/Exo (diet)	0.79	0.29	0.26
12.42	3-Hexanone[b]	$C_6H_{12}O$	589-38-8	CC	Exo (env)	0.30	0.16	–
12.71	2,3,5-Trimethylfuran	$C_7H_{10}O$	10504-04-8	F	Exo (diet)	1.54	0.88	0.25
13.40	Dimethyl disulfide[b]	$C_2H_6S_2$	624-92-0	S	End/Exo (mic, diet)	1.25	1.50	2.12
15.19	2,2,6-Trimethyl-6-vinyltetrahydropyran	$C_{10}H_{18}O$	7392-19-0	M	End/Exo (diet)	2.56	0.29	0.43
16.12	4-Heptanone[b]	$C_7H_{14}O$	123-19-3	CC	End	–	5.18	3.75
18.73	Isoterpinolene	$C_{10}H_{16}$	586-63-0	T	Exo (diet)	0.48	0.54	0.80
19.29	1,4-Cineole[b]	$C_{10}H_{18}O$	470-67-7	T	Exo (diet)	–	0.77	0.61
22.89	γ-Terpinene[b]	$C_{10}H_{16}$	99-85-4	T	Exo (diet)	–	1.80	2.12
23.28	2,2-Dimethyl-5-(1 -methyl-1-propenyl)-tetrahydrofuran	$C_{10}H_{18}O$	7416-35-5	F	Unk	1.27	0.48	0.90
24.78	o-Cymene[b]	$C_{10}H_{14}$	527-84-4	T	End/Exo (diet)	4.93	7.66	6.85
27.70	2-Methoxythiophene[b]	C_5H_6OS	16839-97-7	S	Unk	0.04	0.11	0.09
29.29	1,2,3-Trimethylbenzene[b]	C_9H_{12}	526-73-8	B	Exo (env)	–	0.10	0.08
31.85	Dimethyl trisulfide[b]	$C_2H_6S_3$	3658-80-8	S	End (mic)/Exo (diet)	0.46	0.54	0.49
32.44	2-Methyl-5-(methylthio)furan[b]	C_6H_8OS	13678-59-6	F	Exo (diet)	0.25	0.38	0.43
35.83	1-Methyl-3-(prop-1-en-2-yl) benzene	$C_{10}H_{12}$	1124-20-5	B	Unk	–	3.89	–
35.85	3,7-Dimethyl-3-octanol[b]	$C_{10}H_{22}O$	78-69-3	T	Unk	2.61	1.11	0.57
35.89	p-Cymenene[b]	$C_{10}H_{12}$	1195-32-0	T	End/Exo (diet)	9.06	11.57	6.86
35.93	3,4-Dimethylstyrene[b]	$C_{10}H_{12}$	27831-13-6	B	Unk	1.07	4.33	–
36.28	Linalool oxide[b] (isomer)	$C_{10}H_{18}O_2$	5989-33-3	T	Exo (diet)	0.32	0.38	0.46
38.08	Dihydromyrcenol	$C_{10}H_{20}O$	18479-58-8	T	Exo (diet)	0.99	2.38	1.73
39.42	2-Ethyl-1-hexanol[b]	$C_8H_{18}O$	104-76-7	Al	End/Exo (diet)	1.20	1.04	1.17
41.14	Benzaldehyde[b]	C_7H_6O	100-52-7	CC	Exo (env, diet)	–	0.24	1.07
41.59	1,1,4a-Trimethyl-3,4,4a,5,6,7-hexahydro-1H-napththalen-2-one	$C_{13}H_{20}O$	4668-61-5	Ar	Unk	4.64	2.46	2.69
42.51	Theaspirane[b]	$C_{13}H_{22}O$	36431-72-8	N	Exo (diet)	1.73	0.65	0.42

(Continues)

TABLE 2 (Continued)

RT (min)	Metabolites	Formula	CAS No.	Chemical family	Possible origin	Mean relative peak area		
						CTRL	TRAD	ECIG
44.56	1-Terpinenol[b]	$C_{10}H_{18}O$	586-82-3	T	Exo (diet)	–	0.29	–
46.08	Terpien-4-ol	$C_{10}H_{18}O$	20126-76-5	T	Exo (diet)	–	–	0.29
47.95	5-Methyl-5-isopropyl-3-heptyne-2,6-dione	$C_{11}H_{16}O_2$	63922-44-1	CC	Unk	0.48	–	–
48.01	3-Acetyl-2,5-dimethyl furan	$C_8H_{10}O_2$	10599-70-9	F	Exo (diet)	0.09	0.07	–
48.50	Menthol[b] (isomer)	$C_{10}H_{20}O$	15356-70-4	T	Exo (diet)	0.42	0.38	10.76
51.64	α-Terpineol[b]	$C_{10}H_{18}O$	98-55-5	T	End/Exo (diet)	1.68	1.01	1.44
52.03	4,7-Dimethyl-1-benzofuran	$C_{10}H_{10}O$	28715-26-6	F	Unk	0.94	0.95	4.77
52.77	Phellandral	$C_{10}H_{16}O$	21391-98-0	T	Exo (env)	0.65	–	–
52.81	2-Hydroxymethyl-1,3-dioxolane	$C_4H_8O_3$	5694-68-8	M	Unk	–	0.07	–
53.45	Carvone[b]	$C_{10}H_{14}O$	99-49-0	T	Exo (diet)	1.19	1.88	–
54.17	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)[b]	$C_{13}H_{16}$	30364-38-6	N	Exo (diet)	3.81	1.10	1.38
54.24	1,3,5-Trimethyl-2-(1,3-butadienyl) benzene	$C_{13}H_{16}$	5732-00-3	B	Unk	3.30	0.56	–
58.48	β-Damascenone[b]	$C_{13}H_{18}O$	23726-93-4	N	Exo (diet)	0.31	0.36	0.36
64.67	α-Calacorene[b]	$C_{15}H_{20}$	21391-99-1	T	End/Exo (diet)	0.83	1.37	2.10
72.07	Phenol[b]	C_6H_6O	108-95-2	Ph	End/Exo (env)	–	0.16	0.20
79.82	p-Cresol	C_7H_8O	106-44-5	Ph	End/Exo (mic)	0.90	0.75	3.26

Abbreviations: Al, alcohol; Ar, aromatic; B, benzene derivatives; Bact, bacterial; CC, carbonyl compound; CTRL, control group; Diet, dietary; ECIG, electronic cigarettes; Endo, endogenous; Env, environmental; Exo, exogenous; F, furanic compound; M, miscellaneous; Mic, microbial; N, norisoprenoid; Ph, phenolic compound; S, sulfur-containing compound; Syst, systemic; T, terpene; TRAD, traditional cigarettes; Unk, unknown origin; "–", not identified.

[a]Acquired to Acros Organics in the maximum quality available.

[b]Confirmed with analytical standard acquired to Sigma in the maximum quality available.

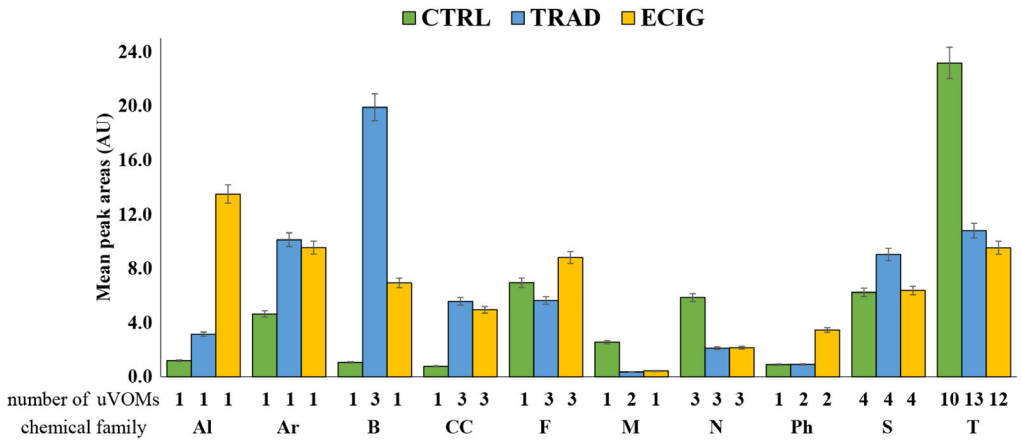

FIGURE 1 Chemical families and respective number of urinary volatile organic metabolites (uVOMs) identified in control (CTRL, $n = 10$), traditional cigarette smokers (TRAD, $n = 10$), and vapers groups (ECIG, $n = 8$). Legend: Al: alcohol; Ar: aromatic; B: benzene derivative; CC: carbonyl compound; F: furanic compound; M: miscellaneous; N: norisoprenoid; Ph: phenolic compound; S: sulfur-containing compound; T: terpene

from exogenous sources, like beverages (beer, wine, rum, whisky, coffee, tea), foods (vegetables, fruit, cheese, milk, meat, bread), and flavoring ingredients [38, 46]. The origin of urine 4-heptanone, however, is still unknown, but it is most probably from an exogenous origin, as a product of β-oxidation of 2-ethylhexanoic acid [22, 26, 36, 45]. The aldehydes found in urine are produced during lipid peroxidation of lipid alkoxyl radicals and are common constituents of natural flavors of foods and beverages [38, 46]. Nevertheless, aldehydes can be formed during smoking from precursors of polysaccharides, pectins, proteins, and possibly, triglycerides present in tobacco [47]. They constitute a group of reactive compounds responsible for damaging the respiratory tract [12, 26]. Phenol, detected in urine samples of smokers and vapers (Table 2), can be produced through the metabolism by mixed-function oxidases [9, 44, 48, 49]. *p*-Cresol is an end-product of protein breakdown and one of the metabolites of the amino acids tyrosine and phenylalanine, and a constituent of tobacco smoke [50–52]. According to the Food and Drug Administration [43], *p*-cresol and phenol are classified as carcinogenic and toxicants for the respiratory and cardiovascular tract, respectively. *p*-Cresol was found in all studied groups, and the highest abundance was seen in the vapers group. Phenol was not identified in the control group and was also more abundant in the vapers group (Table 2). Even though these potentially harmful compounds can be originated from tobacco products, a higher number of subjects would be needed to understand the impact of these compounds on consumer's health. As referred above, benzene derivatives, terpenes, and aromatics were the chemical families that contributed the most to the volatomic profile of smokers (Figure 1). Benzene derivatives can be originated from industrial (synthesis of chemicals, production of rubber, lubricants, pesticides, and dyes) or natural processes (volcanic eruptions, fires). Automobile service stations, industrial emissions, and tobacco smoke are the major sources of benzene exposure [53]. Besides benzene derivatives, aromatic compounds can also result from the incomplete combustion of the organic matter (e.g., sugars and cellulose) of the cigarette [49, 54]. Nicotine, one of the biomarkers used for assessing the exposure to cigarette smoke, was not detected in any sample. This result lies in the fact that nicotine has a short half-life ($t_{1/2}$ ~2 h) and a variable metabolic rate, and the urine samples used in this study correspond to the first morning urine after overnight fasting [55]. E-cigarettes do not achieve true combustion temperatures and do not emit many VOCs and PAHs in measurable quantities as in traditional tobacco cigarettes [55]. Other reports have showed that e-cigarette users have significantly lower levels of PAHs, VOCs, and TSNAs biomarkers, when compared with cigarette smokers [56]. However, toxic and carcinogenic compounds, as well as biomarkers of their metabolized by-products, have been observed in the urine of e-cigarette users [57]. Alcohols, terpenes, and aromatic compounds were the families that contributed the most to the volatomic profile of vapers (Figure 1). The higher levels of these families are very likely to be related to the composition of the e-liquids chosen, which are composed by propylene glycol, glycerol, nicotine, and optional flavoring compounds [25]. The flavoring solutions are mainly composed of aromatic chemicals to give an intense flavor and aroma. However, the lack of standardization of e-cigarettes products makes it difficult to compare the health effects of products from different manufacturers.

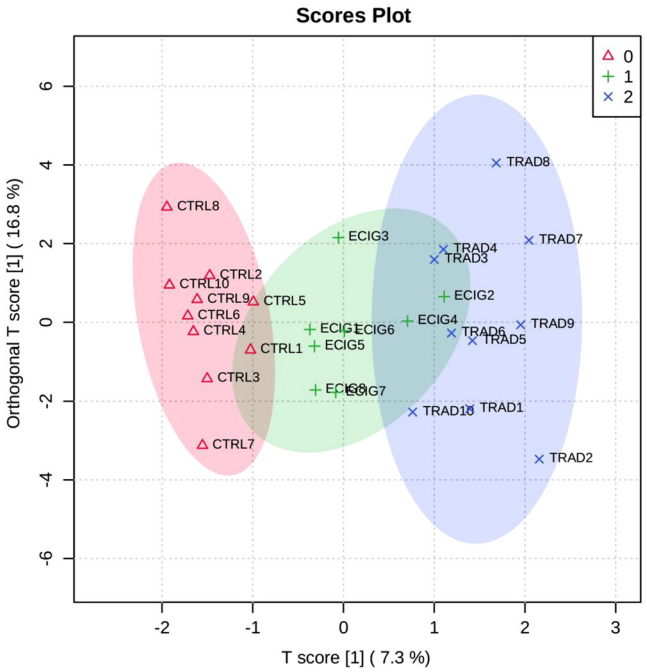

FIGURE 2 Separation of the control group, vapers, and smokers based on orthogonal partial least squares-discriminant analysis (OPLS-DA) scores plot. Legend: 0—CRTL, control; 1—ECIG, vapers; 2—TRAD, traditional cigarette smokers

3.2 | Definition of discriminative urinary volatile organic metabolite profiles for the selected groups

The urine volatomic data obtained was subjected to advanced statistical analysis. Intrinsic clustering and possible outliers were studied through orthogonal partial least squares-discriminant analysis (OPLS-DA) of the GC-MS chromatograms obtained using MetaboAnalyst 5.0 (http://www.metaboanalyst.ca) [20]. The resulting OPLS-DA analysis showed two separate groups—control and traditional smokers (Figure 2). In the vapers group, it was possible to see correlations to both control and traditional tobacco groups, where subjects ECIG2 and ECIG4 showed a urinary profile similar to the traditional tobacco group. These subjects have some common features (Table 3). Both have been consuming e-cigarettes for 2 years, they use the same amount of nicotine (0.9 mg), and the same proportion of propylene glycol and glycerine. However, the flavor of the e-liquid was different. In our previous study [57], we showed that the vaping behavior, including the device used and time of vaporization, has an important role in the delivery of chemicals to the users. Since these subjects did not use the same model of vaping device nor the same e-liquid, it was not possible to compare the vaping conditions. Interestingly, subjects ECIG2 and ECIG4 were the ones who consumed the lower amount of nicotine and the liquids they use did not include tobacco. The comparison of the content of uVOMs, particularly benzene derivates, did not show a correlation between these subjects and the traditional cigarette smokers, thus they are closely related probably due to metabolic reasons.

TABLE 3 Characterization of the e-liquid composition used by the vapers recruited for this study

	E-liquid composition		
Subjects	% of PG/VG	Nicotine (mg)	Flavor
1	40/60	6	Tobacco, caramel, vanilla
2	30/70	0.9	Menthol
3	30/70	–	Fruits
4	30/70	0.9	Peach
5	30/70	3	Fruits
6	50/50	3	Tobacco, nuts
7	50/50	5	Tobacco
8	30/70	1.5	Strawberry, mango, lime,

Abbreviations: PG, propylene glycol; VG, vegetable glycerine.

In the dendrogram analysis (Figure 3A), controls 2 and 8 showed a different profile than the remaining subjects of this group. Control 2 is daily exposed to cigarette emissions due to his job, and for that reason can be more closely related to the smokers' group, while control 8 is a very rare consumer of e-cigarettes. However, during the sampling time, both individuals were not exposed to any of these products for a few days, meaning that some compounds remain in the metabolism for some time. All e-cigarette subjects recruited for this study were previously smokers, but the dendrogram analysis did not show any correlation regarding the years of use (Table 1). The heatmap (Figure 3B) shows the clustering result between the uVOMs and the groups studied. The uVOMs 2,5-dimethylfuran, o-cymene, γ-terpinene, 2-methoxythiophene, and phenol showed a higher correlation between the groups of vapers and smokers. Most of these metabolites can be related to the composition of the e-liquids and cigarettes, respectively. Inside each class, the e-cigarettes users showed the higher correlation. The highest variance inside the traditional cigarette group can be due to differences in the number of cigarettes consumed per day and to the type of tobacco used. All the different parts of a cigarette, including the tobacco itself, filter, paper, and smoke, contribute with different chemicals to the user and all these factors also depend on the smoking behavior [57].

4 | CONCLUDING REMARKS

In this study, a HS-SPME/GC-MS methodology was applied to investigate the volatomic composition of urine

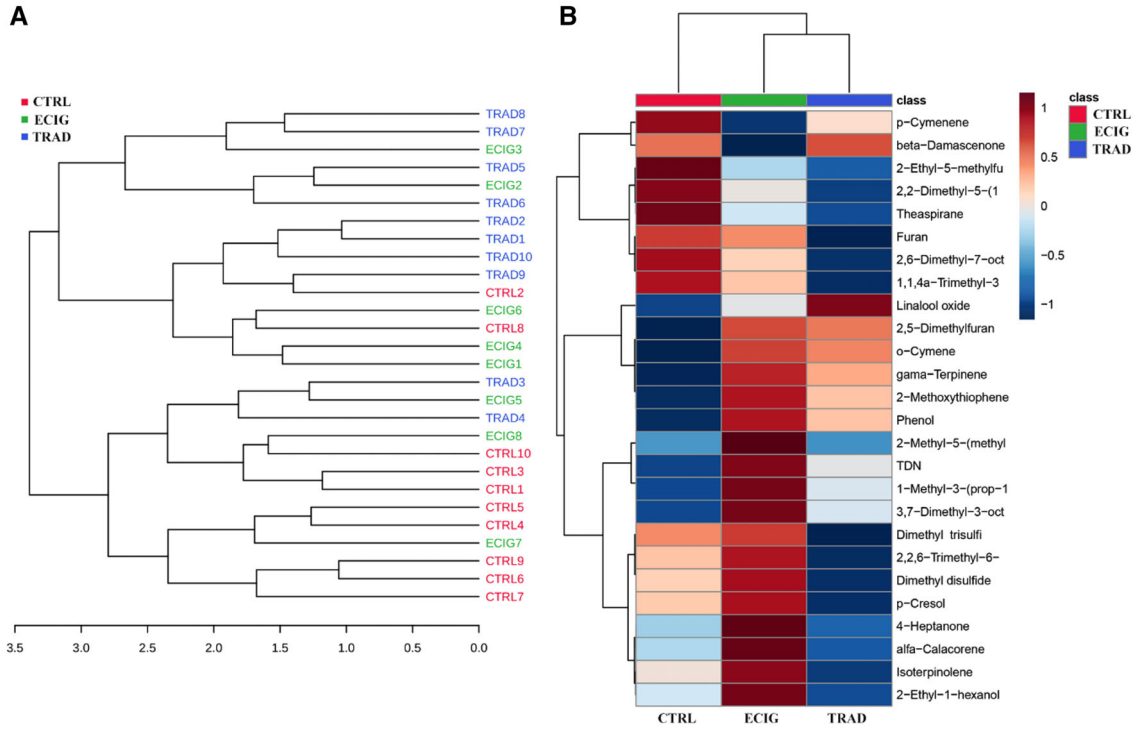

FIGURE 3 Hierarchical cluster analysis of CTRL (control), ECIG (vapers), and TRAD (traditional cigarette smokers) groups: (A) Dendrogram analysis of the volatomic data, using Pearson's correlation and complete linkage. (B) Heatmap illustrating the group averages of the urinary volatile organic metabolites (uVOMs) identified. Columns correspond to each sample group, while rows correspond to the mean normalized peak area of each discriminant uVOM coloured from minimum (−1, dark blue) to maximum (1, dark red)

samples from traditional tobacco smokers and e-cigarette users taking nonsmokers as a control group. Overall, 45 uVOMs were detected and identified in the urine of the studied groups. Benzene derivatives, terpenes, and aromatics were the chemical families that contributed the most to the urinary profile of smokers. In contrast, alcohols, terpenes, and aromatic compounds predominate in the vapers group. The lower presence of benzenes in the urine of vapers suggests such products may cause less harm to human bogy. However, the lack of standardization of e-cigarettes products makes it difficult to compare the real health effects of products from different manufacturers. Moreover, there are still many concerns about the long-term effects and potential cytotoxicity on cardiovascular and respiratory functions, particularly among young users. The effects of nicotine, flavorings, and the humectants propylene glycol and glycerol, for instance, are still not known. The results of this preliminary study point that the vapers urinary profile is closely related to the control group. Despite this, a higher number of subjects must be recruited to consolidate the data obtained.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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