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GC–MS analysis of e-cigarette refill solutions: A comparison of flavoring composition between flavor categories

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ABSTRACT

Objectives: Electronic cigarette refill solutions (e-liquids) are available in various flavor descriptions that can be categorized as fruit, tobacco, and more. Flavors increase sensory appeal, thereby stimulating e-cigarette use, and flavoring ingredients can contribute to e-cigarette toxicity. We aim to inform toxicologists, sensory scientists, and regulators by determining flavoring compounds in e-liquids with various flavors, and compare results between flavor categories.
Methods: Gas chromatography - mass spectrometry (GC–MS) was used to identify 79 flavorings in 320 e-liquids, classified in 15 flavor categories. Ten flavorings highly prevalent in e-liquids according to information from manufacturers were quantified. Flavoring prevalence was defined as the number of e-liquids with the flavoring as percentage of the total number of e-liquids. The method was validated in terms of specificity, linearity, repeatability, recovery, and sensitivity.
Results: The mean number of flavorings per e-liquid was 6 ± 4. Flavoring prevalence was highest for vanillin (creamy/vanilla flavor), ethyl butyrate (ethereal/fruity), and cis-3-hexenol (fresh/green). Based on similarities in flavoring prevalence, four clusters of categories were distinguished: (1) fruit, candy, alcohol, beverages; (2) dessert, coffee/tea, nuts, sweets; (3) menthol/mint; and (4) spices, tobacco, and unflavored. Categories from cluster 4 generally had less flavorings per e-liquid than fruit, candy, alcohol, beverages (cluster 1) and dessert (cluster 2) ($p < 0.05$). Flavoring concentrations varied between e-liquids within the categories.
Conclusions: We evaluated flavoring compositions of 320 e-liquids using a simple GC–MS method. Flavoring prevalence was similar within four clusters of typically fresh/sweet, warm/sweet, fresh/cooling, and non-sweet flavor categories. To compare flavoring concentrations between individual flavor categories, additional research is needed.

1. Introduction

Over the past years, the use of electronic cigarettes (e-cigarettes) has been increasing worldwide [1,2]. An important reason for experimentation with e-cigarettes is the variety of available flavors in e-cigarette refill solutions (e-liquids) [3]. Not surprisingly, e-liquid flavors are an important focus for marketeers: we showed that the Dutch e-liquid market (2017) alone already comprised no less than 245 unique flavor names [4].Whereas e-cigarettes may be an appealing, less harmful substitute for tobacco smoking among adults [5], particularly young people who currently do not smoke are attracted to e-liquid flavors and thereby prone to initiation of e-cigarette use [6]. Importantly, e-cigarettes cause users to inhale potentially toxic substances, and are therefore not safe [7]. Additionally, e-cigarettes often contain nicotine and thereby present a substantial risk of nicotine dependence. This makes regulation of e-cigarette flavors, to reduce appeal for those who would otherwise not smoke, currently an important topic of debate [8].

One way to regulate e-liquid flavors is to restrict particular flavoring ingredients (from now on referred to as flavorings). For

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combustible cigarettes and roll-your-own tobacco, the European Commission created a list of 15 priority additives that have no health benefits, but may instead stimulate use of and addiction to an extremely harmful product [9]. Similarly, for e-cigarettes, regulators could focus on flavorings of which inhalation is known to be toxic. Banning these could directly reduce harm from exposure to e-cigarette emissions. Another option is to ban the most popular e-liquid flavorings, thereby reducing overall appeal of the product. This may result in decreased use and thereby decreased exposure to toxic constituents of e-cigarette emissions among vulnerable user groups such as non-smokers and youth.

As the composition of the e-liquid flavor is often not mentioned on the product label, research is needed to identify e-liquid flavorings in order to provide focus for toxicologists, sensory scientists, and regulators. Recently, we published a comprehensive overview of the most prevalent e-liquid flavoring additives across 16,839 e-liquids based on information from manufacturers [10]. However, industry data may not always be present, complete, or correct. In addition, research has shown that e-liquid ingredients can react with one another to form new molecules [11], which may make ingredients lists from manufacturers less suitable for risk assessment of the product. Therefore, additional chemical-analytical research is needed.

Gas chromatographic-mass spectrometric (GC–MS) methods are widely accepted for analysis of volatile compounds such as flavorings in several types of products, such as cheese [12], wine [13], olive oil [14], as well as tobacco [15]. Several studies were performed to determine flavorings and other chemicals in e-cigarette aerosol [16,17], for example to identify harmful and potentially harmful constituents that users are exposed to [18]. As there is a relation between constituents in e-cigarette aerosol generated by vaping machines and constituents in the e-liquid, qualitative and quantitative GC–MS methods have also been used to determine flavorings in e-liquids, e.g. [16,19–23]. However, data on e-liquid flavoring ingredients in relation to their marketed flavor descriptions are limited. Aszyck et al. compared e-liquid flavoring profiles between five different brands for five e-liquid flavors (tobacco, strawberry, cherry, menthol and apple) [22]. Our study provides new insights by comparing flavoring compositions of e-liquids in more than 200 different flavor descriptions across multiple flavor categories, and includes a larger number of e-liquids than any previous chemical-analytical study. Additionally, flavoring concentrations will be compared between flavor categories by quantifying the 10 most prevalent flavorings as reported by manufacturers in our previous study [10]. By identifying common e-liquid flavorings using GC–MS and comparing results between flavor categories, we aim to inform toxicologists, sensory scientists, and regulators regarding attractiveness and toxicity of e-liquids.

2. Methods

2.1. E-liquid samples

Commercial e-liquids, all intended to be used as e-cigarette refill solutions, were purchased online from nine different vendors in the Netherlands. Based on the product name and flavor-related descriptors on the vendor's website, the main e-liquid flavor was classified into the following 15 categories of the e-liquid flavor wheel: tobacco, menthol/mint, nuts, spices, coffee/tea, alcohol, other beverages, fruit-berries, fruit-citrus, fruit-tropical, fruit-other, dessert, candy, other sweets, and unflavored [24]. We selected a large variety of specific e-liquid flavors for an optimal representation of each main category. Selection of the specific e-liquid flavors was based on the subcategories identified in our previous analysis of e-liquid flavors on the Dutch market [4]: we aimed to select a maximum of two e-liquids per subcategory (based on availability). Selecting the brand and vendor of an e-liquid flavor was based on availability; when an e-liquid flavor was available from multiple brands or vendors, selection was based on obtaining a large variety in brands rather than a large number of vendors. This resulted in a final sample of 320 e-liquids from 204 different subcategories, that is, with 204 unique flavor names. If an e-liquid was available in multiple nicotine concentrations, a randomized choice was made from one of the following three categories: zero (0 mg/mL), low (1–8 mg/mL), and high (9–18 mg/mL). None of the e-liquids contained nicotine salts. Propylene glycol to vegetable glycerin (PG/VG) ratios as declared are reported; Table 1 shows an overview of the sample characteristics. Samples were stored in their original package at room temperature, and analyzed directly after opening.

Table 1
Characteristics of the 320 e-liquids selected for this study. Marketed flavor descriptions were used to classify the e-liquids in 15 main categories of the e-liquid flavor wheel [42].

Variable		Number of e-liquids (%)	
Main flavor category	Tobacco	32	(10 %)
	Menthol/mint	8	(3 %)
	Nuts	8	(3 %)
	Spices	12	(4 %)
	Coffee/tea	19	(6 %)
	Alcohol	25	(8 %)
	Other beverages	28	(9 %)
	Fruit (berries)	22	(7 %)
	Fruit (citrus)	11	(3 %)
	Fruit (tropical)	22	(7 %)
	Fruit (other	28	(9 %)
	Dessert	61	(19 %)
	Candy	29	(9 %)
	Other sweets	13	(4 %)
	Unflavored	2	(1 %)
Nicotine level (mg/mL)	None (0 mg/mL)	121	(38 %)
	Low (1–8 mg/mL)	107	(33 %)
	High (9–18 mg/mL)	92	(29 %)
VG level	<50 %	75	(23 %)
	= 50 %	91	(28 %)
	>50 %	154	(48 %)

VG = vegetable glycerin Note: percentages may not add to 100 % due to rounding.

2.2. Target flavorings

A targeted approach was used to determine 79 flavorings and nicotine. Selection of target compounds was based on information from manufacturers [10], target lists of previous chemical-analytical studies on e-liquid flavors [19–21], and additives found in flavored tobacco products [15]. We quantified the following 10 flavorings, as these are the most commonly added e-liquid flavorings according to information from manufacturers in the Netherlands [10]: vanillin, ethyl maltol, ethyl butyrate, ethyl acetate, maltol, ethyl vanillin, furaneol, methyl cyclopentenolone, γ-decalactone, and cis-3-hexenol. The 79 target flavorings tested, including their flavor descriptions based on a commercial flavor database [25], are listed in Appendix Table A1.1.

2.3. Other chemicals

Standards of the target compounds in analytical or food grade (purity ≥ 95–99 %) were used to optimize identification accuracy. All flavoring chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands); ethyl acetate was purchased from Alfa Aesar (Kandel, Germany). Ethanol absolute was obtained from Merck (Darmstadt, Germany). Nicotine (purity > 99 %) and the internal standards benzene-d6 (purity 100 %) and n-heptadecane (purity

> 99 %) were obtained from Acros Organics, Sigma-Aldrich, and Merck, respectively.

2.4. Standard solutions and test sample preparation

For qualification of the flavorings and nicotine, the standards were individually dissolved in ethanol (ca. 5 μg/mL). One solution of the internal standards benzene-d6 and n-heptadecane was prepared in ethanol (both 100.0 μg/L). For quantification, nine flavoring standards were dissolved as a mixture in the internal standard solution in 10 different concentrations; furaneol was dissolved separately (see Appendix Table A1.2 for the concentration ranges). All test samples were diluted with the internal standard solution in a 1:100 ratio in duplicate. The standard solution was stored in the refrigerator at 4°C until usage.

2.5. GC–MS conditions

An Agilent 7890B GC system coupled with an Agilent 240 ion trap mass spectrometer was used, equipped with a 7693 auto-sampler and a G4513A injector. Compounds were chromatographically separated using an Inert Cap Aquatic-2 column (60 m ×250 μm i.d., 1.4 μm film thickness; medium polarity), with helium as a carrier gas in a constant flow rate of 1 mL/min. The temperature program was set at 50 °C (hold for 6 min), then ramp to 250 °C with 10 °C/min (hold for 9 min). Total run time was 35 min. The injection volume was 1 μL with a 10:1 split ratio. The injector temperature and temperature of the transfer line were set at 200 °C and 260 °C, respectively. The MS operated in a positive electron impact (EI) mode with an electron energy of 70 eV. The ion source temperature was set at 260 °C. After each test sample, a blank sample containing the ethanol-based internal standard solution (100 μg/L) was included to control for carry-over effects.

Qualification of target flavorings and nicotine was performed in full scan mode covering 29-250 *m/z*, a range sufficiently broad to cover the analytes. Qualification was based on the retention times and the MS spectra of the individual standards (i.e., references) listed in Appendix Table A1.1. Figure A1 (appendix) shows chromatograms of a standard mixture and two e-liquid samples. Acceptance criteria for positive identification were: a maximum deviation of ±0.2 min of the expected retention time, a maximum difference of 20 % between the relative intensities of quantifier/qualifier in the e-liquid samples versus the standards, and a match of at least 70 % between the sample and reference spectrum. To verify the presence of flavorings in each e-liquid sample, retention times and the mass spectra were confirmed using those of the standards.

The 10 flavorings of interest were quantified in e-liquids where the respective flavoring was positively identified, based on the quantifier ion (Appendix Table A1.1). Concentrations were reported for flavorings with a signal at least 10 times higher than noise, based on the average signal to noise ratio of two runs. Two internal standards that differed significantly in retention time, ranging from 13 (benzene-d6) to 31 (n-heptadecane) minutes, were selected, thereby spanning the range in which the components of interest eluted. N-heptadecane was used as a back-up in case there would be interference of the peak of the analyte with that of the primary internal standard. This was not the case, hence, only benzene-d6 was used for quantification of the flavorings.

2.6. Method validation procedures

To determine specificity, we compared the retention time - quantifier ion combination between each of the 79 target compounds, nicotine, and the internal standards. A criterium was set that compounds with the same quantifier ion should have a difference in retention time of >0.4 min. This was based on three times the highest absolute standard deviation of the retention times, which was 0.11 min for ethyl maltol.

Linearity of the 10 specific calibration curves was assessed, in duplicate, by dissolving a mixture of 9 flavoring standards in the internal standard solution in 10 different concentrations (~10–100 μg/mL); furaneol was dissolved separately.

To determine repeatability of the retention times and peak areas for the 10 flavorings and the two internal standards, two solutions of PG and VG were made in 30:70 and 70:30 ratio, respectively. A mixture of 9 flavorings (100 μg/mL dissolved in the ethanol-based internal standard solution) was added to these solutions in two different concentrations (20 and 80 μg/mL); furaneol was dissolved separately. Each of the 4 solutions and a blank sample containing only the internal standard solution (100 μg/L) were injected in the GC–MS system 6 times. For each compound, we aimed for a RSD of less than 1% and 10 % for the retention time and peak area, respectively.

To determine recovery, the same solutions of PG/VG (70/30 and 30/70) and flavorings (20 and 80 μg/mL) were used. For each component, results of 6 injections were averaged. The recovery was defined as the determined concentration as percentage of the added concentration of the respective flavoring.

For sensitivity of the 10 flavorings, flavoring standards with a concentration of 10 or 30 μg/mL were used. Limits of detection (LODs) were calculated based on the calibration curve as 3.3 * standard deviation / slope; and limits of quantification (LOQs) as 10 * standard deviation / slope.

2.7. Data analysis

Data processing was performed using the MS workstation (version 7.0.2, Agilent technologies). The statistical software program R (version 3.6.0) and Excel were used to determine flavoring detection frequency and prevalence, the mean number of (unique) flavorings per e-liquid, and median concentrations (including range). Detection frequency is defined as the number of e-liquids in which a flavoring was detected; flavoring prevalence is defined as the number of e-liquids with the flavoring as percentage of the total number of e-liquids (overall or within a category). A heat map (combined with hierarchical clustering) was created to visualize flavoring prevalence. Flavorings and flavor categories were grouped together by similarity in a dendrogram. Clusters of flavor categories were distinguished by cutting off the dendrogram halfway, in order to capture more than 50 % of the variation between the flavor categories. Relative prevalence was used to account for differences in sample size (i.e., number of e-liquids) between the flavor categories. ANOVA and t-tests were used to determine differences in the mean number of flavorings per e-liquid between categories. To correct for multiple testing, Benjamini-Hochberg false discovery rate (FDR) [26] adjusted p-values of < 5% were considered significant. Concentrations of the flavorings in the duplicate runs were averaged for further analyses.

3. Results and discussion

3.1. Method validation

Regarding specificity of the method, none of the flavorings had both the same quantifier ion and a difference in retention time of <0.4 min (see Appendix Table A1.1). However, the mass spectra of decanal and L-menthol overlapped for a large part and the retention time differed only 0.018 min. Therefore, e-liquids that screened positive on either one of those flavorings were manually confirmed. Results for linearity of the method are shown in Appendix Table

A1.2. All coefficient of determination (r^2) values were >0.993. Relative standard deviation (RSD) response factors were <10 %, except for maltol (25 %) and furaneol (22 %). Repeatability of the retention times and peak areas for the 10 flavorings selected for quantification and the two internal standards is shown in Appendix Table A1.3. Repeatability was generally sufficient, except for ethyl maltol, for which the RSD of the peak area reached 27 %. The recovery generally ranged from 92 % to 120 %, but was higher for maltol (up to 207 %) (see Appendix Table A1.4). Finally, the LOD and LOQ for each of the 10 flavorings selected for quantification are shown in Appendix Table A1.5. Quantification limits in the e-liquid samples varied between 0.03 (ethyl acetate and ethyl vanillin) and 0.25 (maltol) mg/mL. In conclusion, validation was considered acceptable, except for ethyl maltol, maltol, and furaneol. Hence, concentrations for these compounds should be considered with appropriate caution.

3.2. Most common e-liquid flavorings

Of the 79 flavorings, 66 were detected in at least one e-liquid. Eighteen flavorings were identified in more than 10 % of the overall sample of e-liquids (see Table 2), a cut-off that is consistent with our previous study on e-liquid flavorings [10]. The most frequently identified flavorings were vanillin (present in 42 % of the total set), ethyl butyrate (41 %), and cis-3-hexenol (35 %); their flavors are described as respectively creamy/vanilla, ethereal/fruity, and fresh/green [25]. Of the 320 e-liquids, we detected vanillin, ethyl vanillin, or both in 144 e-liquids (45 %). This is comparable to a previous study in which vanillin and/or ethyl vanillin were identified in approximately half of the e-liquid samples [21].

Strikingly, most of the flavoring compounds listed in Table 2 impart a sweet and/or fruity aroma. This finding directly adds to the ongoing debate in the US about why teenagers and young adults who did not previously smoke combustible cigarettes started vaping. Previous research showed that young people typically have a preference for sweet taste [27] and non-smokers are mainly interested in trying sweet e-liquids [28]. This, together with our current and previous findings that sweet and fruity flavoring ingredients dominate [10] and the e-liquid market is generally dominated by sweet flavors [4], may provide directions for regulation of (sweet) e-liquid flavors and/or flavorings in order to reduce e-cigarette appeal among youth non-smokers.

Detection frequencies of the top 18 most frequently identified flavorings in this study were comparable to those found in our previous study (Table 2, final column), in which we analyzed the Dutch e-liquid market (n = 16,839 e-liquids) using information from manufacturers about their e-liquids' flavorings compositions [10]. In addition, other studies using e-liquids from the US [23] and Germany [20] reported the same compounds as most prevalent. It would be interesting to further investigate why these flavorings in particular are so common, and to perform a comparison between e-liquid (flavoring) compositions on an international level.

3.3. Comparison between flavor categories

3.3.1. Prevalence of flavorings within a category

The prevalence of the flavorings in total and within each individual flavor category can be found in Appendix A2. For example, considering the flavorings, vanillin was detected in more than three-quarter of the e-liquids classified as *dessert* (detection frequency 82 %), *other sweets* (77 %), and *nuts* (75 %), and approximately half of the e-liquids classified as *fruit-berries* (50 %), *coffee/tea* (47 %), and *other beverages* (46 %), but not in any of the e-liquids classified as *menthol/mint*, *spices*, and, as expected, *unflavored*. As an example regarding the flavor categories, the most frequently detected flavorings within the *menthol/mint* category were menthol (detected in 75 % of the *menthol/mint* e-liquids), menthyl acetate (63 %), limonene (63 %), and menthone (63 %). All of these compounds are described as having a minty, fresh flavor [25].

Using the data from Appendix A2, we visualized the prevalence of flavorings (rows) in e-liquids from the different flavor categories (columns), including a hierarchical clustering of flavorings and flavor categories by similarity (see Fig. 1). Cutting off the dendrogram halfway resulted in four clusters of flavor categories with similar flavoring prevalence: (1) *fruit*, *candy*, *alcohol*, *beverages*; (2) *dessert*, *coffee/tea*, *nuts*, *sweets*; (3) *menthol/mint*; and (4) *spices*, *tobacco*, and *unflavored*. Compared to other clusters, the first cluster is characterized mostly by a high prevalence of ethyl butyrate (ethereal, fruity flavor), cis-3-hexenol (fresh, green flavor), γ-decalactone (coconut-peach flavor), and isoamyl alcohol (alcoholic, winey-brandy flavor). This explains why this cluster contains the categories that have particularly artificial/fresh sweet and ethereal e-liquid flavors: fresh fruits, artificial sweet candies such as gummy bears, ethereal alcoholic drinks varying from sweet cocktails to liquors, and sweet fresh beverages such as lemonade and cola. The second cluster can be described by a relatively high prevalence of vanillin and ethyl vanillin (both having a creamy, vanilla flavor), ethyl maltol (sweet, fruity-caramellic flavor), and methyl cyclopentenolone (strong, caramellic-maple flavor). This explains the presence of e-liquids with a particularly 'warm' sweet flavor within this cluster: dessert flavors varying from cheesecake to custard, sweet e-liquid flavor descriptions such as vanilla, caramel, and chocolate, coffee flavors varying from cappuccino to caffè mocha, and nut flavors such as peanut with caramel and chocolate. The third cluster contains the *menthol/mint* category and is clearly characterized by a high prevalence of flavorings described as causing a minty, fresh cooling sensation: menthol, menthyl acetate, limonene, and menthone. The fourth cluster includes e-liquids from typically non-sweet categories: *spices*, *tobacco*, and *unflavored*. These can be described by an overall low flavoring prevalence, particularly regarding the flavorings that characterize the other clusters. Compared to other clusters, prevalence of anethole and p-anisaldehyde (both having an anisic herbaceous flavor), eugenol (clove flavor), and trans-cinnamaldehyde (cinnamon flavor) are relatively high, which relates to the nature of particular e-liquids from the *spice* category. Overall, this shows that, as expected, chemical-analytical data on e-liquid flavoring compositions provides information about an e-liquid's flavor label. This is particularly relevant in cases of absent or ambiguous brand names and product descriptions, or when information about e-liquids' flavoring content may not be reliable (e.g. information submitted by manufacturers may not always be complete or correct).

3.3.2. Flavorings detected in any e-liquid within a category

From the 79 target flavorings, zero flavorings were identified in any e-liquid from the *unflavored* category. Apart from that, the number of target flavorings (79 in total) detected in any (at least one) e-liquid within a category ranged from 15 for the *spices* category to 52 for the *dessert* category (see Table 3). The number of flavorings detected in more than 10 % of the e-liquids within a category ranged from 7 for *tobacco* to 34 for *fruit-other* (Table 3, final column). For most categories, this number was much lower than the number of target flavorings detected in any e-liquid. Thus, part of the flavorings are relatively common for a category, but various flavorings are added to only a few e-liquids within that category. These flavorings are probably used to define a particular e-liquid flavor (i.e., a subcategory of the flavor wheel) or a particular brand.

Table 2
The top 18 most frequently identified flavorings in a sample of 320 e-liquids using GC–MS. Prevalence is reported as the number of e-liquids containing the respective flavoring as percentage of the total number of e-liquids. Flavor descriptions were retrieved from a commercial flavor database [44].

	Flavoring ingredient	Prevalence (% of total e-liquids)	Flavor description	Prevalence across 16,839 e-liquids reported in the EU-CEG system*
1	Vanillin	42 %	Sweet, powerful, creamy, vanilla-like	35 %
2	Ethyl butyrate	41 %	Ethereal, fruity with buttery-pineapple-banana, ripe fruit & juicy notes	28 %
3	Cis-3-hexenol	35 %	Strong, fresh, green, grassy	18 %
4	Benzyl alcohol	32 %	Faint, sweet, almond fruity, somewhat chemical	14 %
5	Ethyl maltol	31 %	Sweet, fruity-caramellic, cotton candy	32 %
6	Ethyl vanillin	25 %	Intense, sweet, creamy, vanilla-like	19 %
7	γ-Decalactone	23 %	Coconut-peach	18 %
8	Methyl cyclopentenolone	23 %	Very strong, caramellic-maple, lovage	18 %
9	Ethyl methyl butyrate	22 %	Strong, green, fruity, apple with strawberry notes	16 %
10	Isoamyl alcohol	20 %	Breathtaking, alcoholic odor; in dilution a winey-brandy taste	4 %
11	γ-Nonalactone	19 %	Strong, fatty, coconut odor and taste	10 %
12	Menthol	18 %	Strong trigeminal cooling sensation with a slight mint note	12 %
13	Isoamyl isovalerate	16 %	Fruity, green-apple, pineapple, tropical, mango, apricot, cognac	11 %
14	Ethyl propionate	15 %	Strong, ethereal, fruity, rum-like	11 %
15	Linalool	15 %	Sweet floral-woody with slight citrus notes	15 %
16	γ-Octalactone	13 %	Sweet-coumarinic, coconut-like odor and taste	7 %
17	Cis-3-hexenyl acetate	12 %	Strong, fruity-grassy-green odor with banana notes	9 %
18	Maltol	11 %	Sweet, fruity, berry, strawberry, caramellic	23 %

EU-CEG = European Common Entry Gate * In a previous study, we analyzed information from manufacturers in the EU-CEG system about flavoring compositions of 16,839 e-liquids reported to be marketed in the Netherlands in 2017 [29].

Table 3
The sum of unique target flavorings (79 in total) detected in any (i.e., at least one) e-liquid within a category, and in more than 10 % of the e-liquids within a category.

Flavor category	Sum of unique flavorings (n = 79)	Sum of unique flavorings in >10 % of the e-liquids (n = 79)
Dessert	52	22
Candy	49	25
Fruit (other)	48	34
Other beverages	45	31
Alcohol	44	20
Fruit (berries)	35	20
Coffee/tea	33	23
Fruit (tropical)	32	19
Tobacco	29	7
Fruit (citrus)	25	9
Nuts	23	23
Other sweets	22	13
Menthol/mint	16	16
Spices	15	10
Unflavored	0	0

3.4. Mean number of flavorings per e-liquid

In total, 1969 flavorings were detected in 320 e-liquids, of which two e-liquids were marketed as *unflavored*. In 14 e-liquids that were marketed as having a flavor (4% of total sample) and therefore expected to contain flavorings, zero of the target flavorings were detected. As we used a targeted approach rather than an open screening approach, flavorings outside the target list were not identified, and thus, unknown compounds may be present.

The mean number (± standard deviation) of flavorings per individual e-liquid was 6 ± 4 for the overall sample. The mean number of flavorings per e-liquid was zero within the *unflavored* category, and further ranged from 3 ± 3 for e-liquids classified as *tobacco* to 8 ± 4 for both *dessert* and *fruit-other* (see Fig. 2).

We found significant differences in the mean number of flavorings per e-liquid between categories (p = 3.72E-11). The mean number of flavorings per e-liquid within the *alcohol*, *other beverages*, *fruit-berries*, *fruit-tropical*, *fruit-other*, *dessert*, and *candy* categories were significantly higher than within the *tobacco*, *spices*, and *unflavored* categories (FDR < 0.05). This is consistent with the low number of flavorings per e-liquid as a potential reason for hierarchical clustering of the *tobacco*, *spices*, and *unflavored* categories based on flavoring prevalence (see section 3.3.1). The mean number of flavorings per e-liquid for *dessert* and *fruit-other* was also significantly higher than for the plausibly related *other sweets* and *fruit-citrus* categories (FDR < 0.05). This can be explained by the type of flavors within these categories: the *other sweets* and *fruit-citrus* categories contains e-liquids with a relatively simple flavor such as vanilla, caramel, or lemon; flavors that could be created with only a few flavorings (e.g., vanillin for vanilla flavor and limonene for citrus flavor). In contrast, the *dessert* and *fruit-other* categories contain many e-liquids with multiple flavor descriptors, for example respectively blueberry cheesecake and a pomegranate-flavored e-liquid with hints of anise, violet, and mint. Thus, our results show that e-liquids with a combined flavor description contain more different flavorings than e-liquids described as simply having one flavor. See Appendix A3 for other significant differences and p-values.

3.5. Flavorings with a low detection frequency

Some flavorings that manufacturers reported to have added to more than 10 % of the e-liquids on the Dutch market (see previous study [10]) were identified in this study with a frequency of less than 5% or not at all: furaneol (identified in 2.5 % of the e-liquids included in this study), benzyl butyrate (0%), benzaldehyde (0%), δ-decalactone (0%). The difference in detection frequency between both studies may be assigned to the different study aims, and thereby the selection of e-liquid flavors. That is, in the current study, we selected only a maximum of two e-liquids per flavor subcategory in order to obtain an optimal representation of each main category, while our previous study included the complete set of e-liquids and their flavors as reported to be marketed in the Netherlands, thereby containing much more e-liquids within some flavor subcategories.

Other reasons why some flavorings that manufacturers reported to have added were not at all detected in the current study may be that their concentrations were below our limit of detection, or due to the chemically unstable character of the e-liquid. For example,

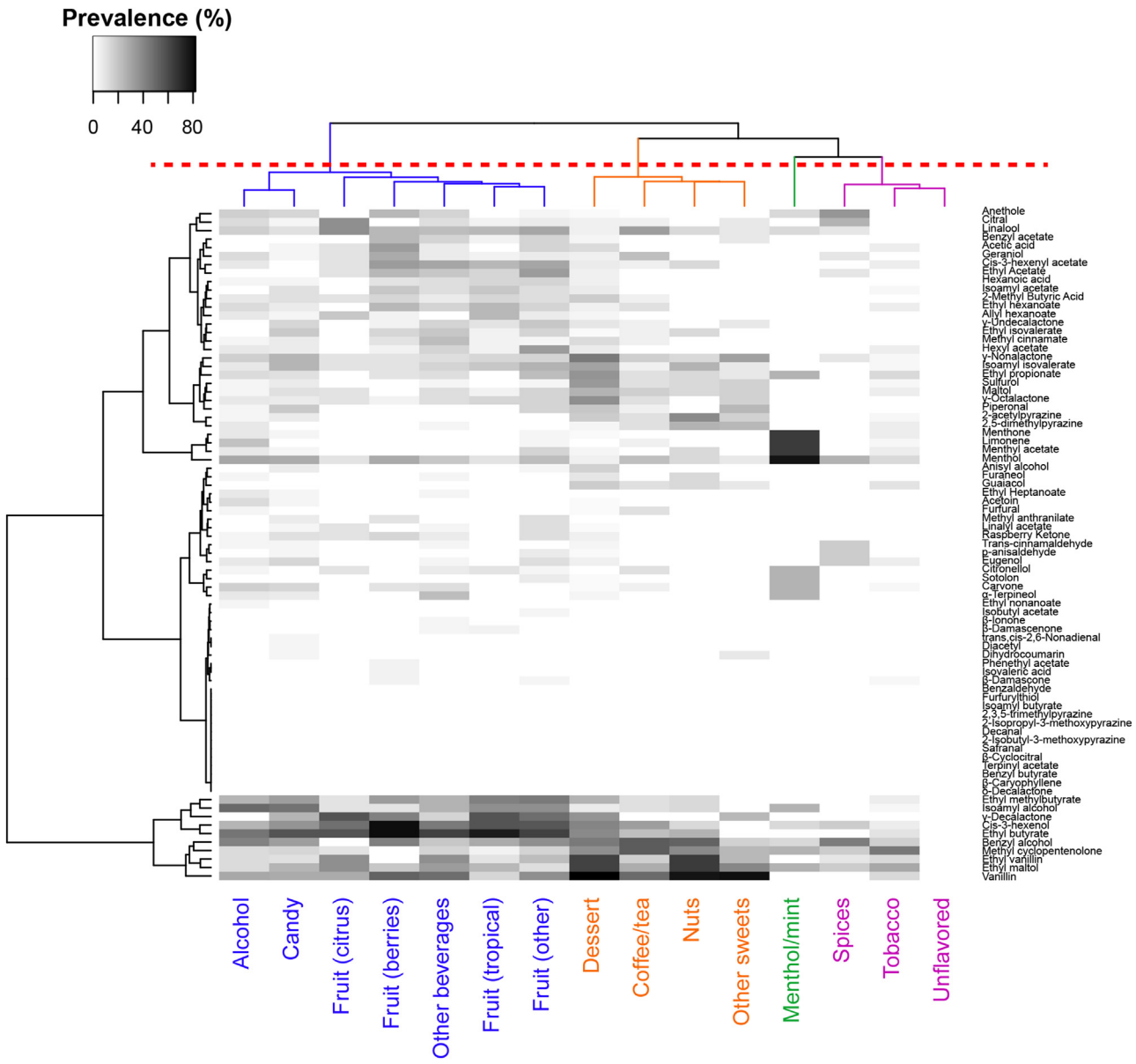

Fig. 1. Visualization of the prevalence of 79 target flavorings (rows) in e-liquids, for each of the 15 flavor categories (columns). Relative prevalence is expressed on a scale from black to white, which indicates high to low prevalence (%), respectively. Flavorings and flavor categories are hierarchically clustered (organized as a dendrogram) based on similar data. Cutting off the dendrogram halfway (dotted red line) distinguishes four clusters of flavor categories (highlighted in blue, orange, green, and purple) that represent groups of similar flavoring prevalence data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

benzaldehyde and other flavoring aldehydes have been shown to rapidly react with the e-liquid solvent propylene glycol (PG), which causes almost half of the aldehyde content to be converted into flavor aldehyde PG acetals [11]. Thus, even though manufacturers reported to add flavoring aldehydes to e-liquids, chemical analysis of the neat e-liquid may show distinct chemical profiles due to a changed composition during storage. Flavoring aldehyde profiles may also differ between the neat e-liquid and the e-cigarette aerosol [16], as aldehydes are formed during the aerosolization process by pyrolysis of PG and VG [29]. More chemical-analytical research is needed to obtain insight in the part of flavoring aldehydes, as well as other potentially reactive compounds, that is added, converted, and formed, when chemically analyzing the e-liquid itself compared to e-cigarette aerosol. Also, not only regarding the aldehyde content, further research is needed to chemically assess the complete composition of e-cigarette aerosol and the associated health risks. Although this type of research provides insight in what users are actually exposed to, it will be extremely time-consuming.

3.6. Flavoring concentrations

The median concentrations and ranges of the 10 flavorings quantified, stratified by flavor category, are shown in Appendix A4. Within the flavor categories, concentrations of some flavorings varied substantially. For example, within the dessert category, vanillin concentrations (n = 50 data points) ranged from 0.4–13.5 mg/mL (see Table A4.1), ethyl vanillin concentrations (37 data points) ranged from 0.2–12.8 mg/mL (Table A4.2), and ethyl maltol concentrations (28 data points) ranged from <0.1–17.3 mg/mL (Table A4.4). No statistical comparisons of flavoring concentrations between categories could be performed, as the

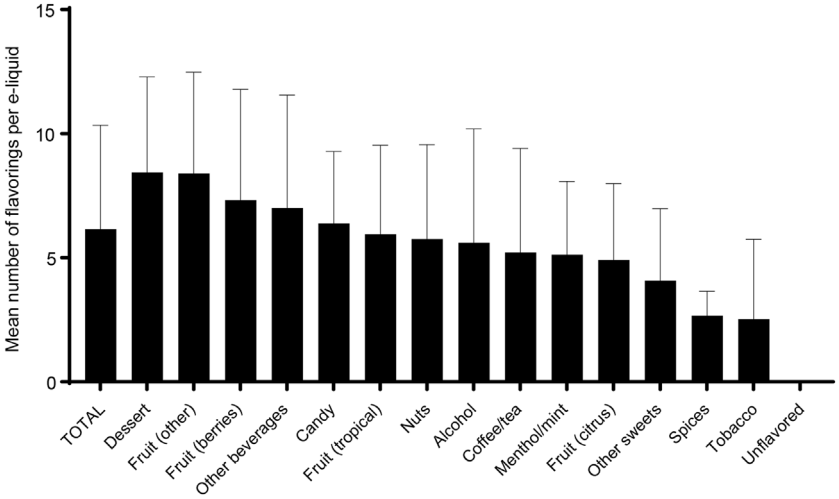

Fig. 2. Mean (±SD) number of flavorings per e-liquid for the total dataset (n = 320 e-liquids) and for each individual flavor category. The mean number of flavorings per e-liquids was significantly lower for the *unflavored* category compared to all other categories, except for *menthol/mint* and *nuts*. The mean number of flavorings per e-liquids was significantly lower for both *tobacco* and *spice* compared to *alcohol, other beverages, fruit* (berries, tropical, and other), *dessert*, and *candy*. See Appendix A3 for p-values.

number of data points within a category was often too low or sometimes even zero. In order to determine whether manufacturers create different e-liquid flavors by varying flavoring concentrations besides adding distinct flavorings, further research is needed. For example, flavoring concentrations could be statistically compared between flavor categories, which requires a large number of data points (i.e., sufficient e-liquids containing the respective flavoring to perform such analyses). Also, as most e-liquids contain multiple flavorings, future research is needed not only to investigate the relation between concentrations and perception of individual flavorings, but also the interaction between the flavoring mixtures, and how differences in concentrations may influence overall flavor perception [30].

3.7. Identification of nicotine

The nicotine content as stated on the e-liquid package varied from 0–18 mg/mL. We identified nicotine in 16 e-liquids that were marketed as nicotine-free (5 % of total sample). In 3 e-liquids that were marketed as having 6 mg/mL nicotine, nicotine was not identified. This is in line with a previous study showing nicotine labelling discrepancies [20], and supports our hypothesis that information from manufacturers may not always be correct, thereby confirming the importance of chemical-analytical measurements.

3.8. Limitations

A few limitations of this study should be noted. Firstly, it is hard to separate stereoisomers, but many flavorings are chiral and the odor of two isomers may differ (e.g., R-carvone has a spearmint odor and S-carvone has a spicy, caraway odor). Furthermore, the validation experiment did not include flavorings with close structural similarities to our target flavorings (e.g., alpha- and beta-damascone, which differ only in the position of a double bond). Therefore, conclusive identification of these types of flavorings in e-liquids would require additional analyses. Secondly, only positively identified flavorings were confirmed using standards, and quantification of the 10 flavorings selected was only performed in e-liquids where the respective flavoring was positively identified. This means that false negatives may have remained, which were not included in the quantification analysis and may have caused an underestimation of flavoring prevalence. Thirdly, analyzing the standards of maltol, ethyl maltol, and furaneol using GC–MS resulted in tailing peaks, explaining the suboptimal recovery and relatively high RSD response factors for these compounds. Although validation results for the other compounds were considered acceptable, determined concentrations of maltol, ethyl maltol, and furaneol should be treated with appropriate caution. Peaks of PG and VG also tended to be tailed and their spectra may show some overlap with spectra of the target flavorings. Although identification of the flavorings was based on the reference spectra of individual standards, PG and VG may have affected our quantification results. To minimize the carryover effect between samples, a blanc sample with ethanol was run between each test sample.

It should also be noted that classification of the e-liquids into the main categories of the flavor wheel was based on the e-liquids' flavor as a whole or the first flavor descriptor mentioned [24]; other flavor descriptors were not taken into account in this study. Including more than one flavor descriptor would result in some e-liquids being classified in multiple flavor categories. Although this would be a more accurate approach to flavor classification, it is not possible, within an e-liquid, to separate the flavorings that contribute to the e-liquid's main flavor category from the flavorings that contribute to the secondary flavor descriptors.

Further, the resulting clusters of the flavor categories were based on the prevalence of the flavorings tested in this study. Selecting different target compounds or cutting off the dendrogram at a higher or lower level may result in different clusters of flavor categories. However, as the prevalence results were comparable to those reported by the industry and found in previous chemical-analytical studies, we believe that the majority of flavorings important for distinguishing between e-liquid flavor categories were covered in our analysis.

Finally, we quantified the flavorings that were most prevalent according to information from manufacturers as analyzed in our previous study. However, these compounds were not necessarily most prevalent according to our current GC–MS results. As some of the 10 flavorings selected for quantification were not detected in each flavor category or only in a few e-liquids, groups were too small to statistically compare concentrations between the 15 flavor categories.

4. Conclusions

This study used a simple and pragmatic GC–MS method to identify and quantify target flavorings in a large sample of e-liquids with

different marketed flavor descriptions. Vanillin (creamy/vanilla flavor), ethyl butyrate (ethereal/fruity flavor), and cis-3-hexenol (fresh/green flavor) were most frequently detected in the overall dataset. Based on similarities in flavoring prevalence, four clusters of flavor categories could be distinguished: (1) fruit, candy, alcohol, beverages; (2) dessert, coffee/tea, nuts, sweets; (3) menthol/mint; and (4) spices, tobacco, and unflavored. These clusters can be characterized by the presence or absence of particular flavorings, and by the mean number of flavorings per e-liquid. This shows that, as expected, chemical-analytical data on e-liquid flavoring compositions provides information about an e-liquid's flavor label, and that e-liquids from some flavor categories are more similar in terms of flavoring compositions than others. This information could be used, for example, when product descriptions are absent or ambiguous, or when regulators have no access to information from manufacturers about e-liquids' flavoring content. In addition, our study showed that flavoring concentrations varied within the overall dataset. Additional research is needed to compare flavoring concentrations between the individual flavor categories in order to investigate whether manufacturers create different types of e-liquid flavors by varying flavoring concentrations besides adding distinct compounds. In conclusion, our comparison of flavoring compositions between e-liquid flavor categories may provide focus to regulators, sensory scientists, and toxicologists in their efforts to respectively decrease e-liquid appeal for particularly youth, and to further investigate e-liquid appeal and the potentially harmful effects of inhaling particular e-liquid constituents such as flavorings.

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Erna J.Z. Krüsemann: Conceptualization, Methodology, Formal analysis, Writing - original draft, Visualization, Project administration. **Jeroen L.A. Pennings:** Formal analysis, Writing - review & editing, Visualization. **Johannes W.J.M. Cremers:** Conceptualization, Methodology, Investigation. **Frank Bakker:** Methodology, Validation, Investigation. **Sanne Boesveldt:** Writing - review & editing, Supervision. **Reinskje Talhout:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113364.

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