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Article

Stability of Flavoring Chemicals in e-Cigarette Liquids: A Naturalistic Product Aging Study over 24 months

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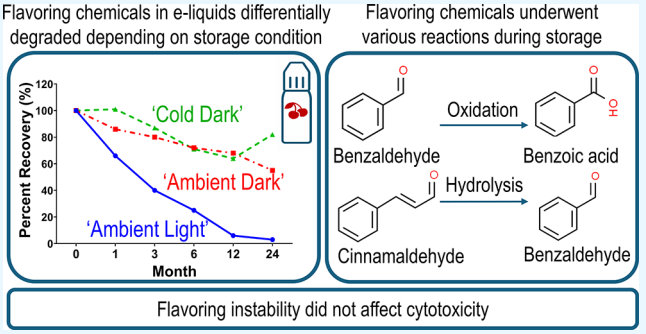
ABSTRACT: Flavoring chemicals commonly used in many consumer products, including pharmaceuticals, foods, and beverages, deteriorate over time. Flavoring chemicals are also common additives in e-cigarette liquid formulations, but their stability in vaping products has not been evaluated. Since e-cigarette liquids are exposed to varying environmental conditions during storage and use, we assessed the stabilities of 20 flavoring chemicals commonly used in vaping products, including benzaldehyde (cherry flavor), menthol (cooling flavor), and vanillin (vanilla flavor). We prepared reference e-cigarette liquids (reference solutions) containing individual flavorings and a mixed liquid with combined flavorings in a 55:45 (v/v) propylene glycol to vegetable glycerin solution. We also purchased 14 commercial e-cigarette liquids. Liquids were stored over 24 months in different temperatures (room and cold) and light exposure conditions (ambient light and dark). Gas chromatography/mass spectrometry techniques were used to measure the concentration of each flavoring chemical at the baseline and after 1, 3, 6, 12, and 24 months. We used a nontargeted approach to identify potential degradation byproducts. Using an air−liquid interface with bronchial epithelial cells and the Neutral Red assay, we also compared the cytotoxicity of selected reference solutions vaped at the baseline and after storage over 24 months. The flavorings in reference solutions stored at ambient temperature and exposed to light were the least stable. Reducing exposure to light and storing reference solutions in cold temperatures delayed the degradation of some flavorings. Tentatively identified byproducts of flavoring degradation found in unstable reference solutions suggested oxidation, hydrolysis, and condensation reactions with solvents. Despite substantial changes in the chemical composition, no significant cytotoxicity differences were detected between fresh and aged reference solutions. Our findings suggest that storing vaping products in dark places and at cold temperatures improves the stability of flavorings in e-cigarette liquids.



INTRODUCTION

Numerous flavoring chemicals have been identified in liquids intended for use with electronic cigarettes (e-cigarettes).[1−4] While flavorings are Generally Recognized as Safe (GRAS) for oral ingestion, concerns about their potential inhalation toxicity have been raised. *In vitro* and *in vivo* studies have identified possible adverse respiratory effects of individual flavorings (including cinnamaldehyde and benzaldehyde) when heated and aerosolized from e-cigarettes.[5−8] Kinetic modeling of frequently used ethyl esters (e.g., ethyl butyrate, which provides a fruity flavor),[9] in e-cigarette liquids[10,11] indicates probable conversion to carboxylic acids, such as butanoic acid. Subsequent degradation of butanoic acid to the highly toxic gas ketene is possible with extreme temperatures experienced during "dry" operating conditions of e-cigarette devices.[12] Sucralose, a commonly used sweetener in e-cigarettes,[13] can produce cytotoxic chloropropanols when aerosolized at elevated temperatures.[14] Additionally, the flavoring triacetin,[15] along with sucralose,[16] can catalyze the thermal degradation of the primary solvents propylene glycol (PG) and vegetable glycerin (VG),[17,18] resulting in significantly greater yields of hazardous carbonyls (e.g., formaldehyde, acetaldehyde, and acrolein) emitted from flavored e-cigarettes.[15,19]

Less focus has been placed on the stability of flavoring chemicals used in e-cigarettes during storage. Studies have shown that flavorings in e-cigarettes may experience chemical changes before being heated and aerosolized. Specifically, harmful hemiacetals are formed from flavoring aldehydes by their reactions with PG and VG.[20] The rate of hemiacetal formation is relatively fast and has been shown for popular flavorings like benzaldehyde, cinnamaldehyde, and vanillin (flavorings commonly used in fruit- and sweet-flavored vaping products).[21,22] Another unstable flavoring is acetoin (com-

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monly found in dessert-flavored vaping products),[23] which can oxidize to form diacetyl in highly alkaline e-cigarette liquids.[24] Prolonged inhalation of high concentrations of diacetyl is known to cause bronchiolitis obliterans ("popcorn lung").[25,26]

Many flavorings are used in pharmaceutical products,[27] foods, and beverages[28,29] that undergo oxidative, photo, and thermal degradation while storing. Pharmaceutical products, regulated by the Center for Drug Evaluation and Research (CDER) of the Food and Drug Administration (FDA) in the US, must meet strict safety standards, including determining expiration dates from stability studies[30] when stored under normal conditions.[31] While the FDA has not specified requirements for inactive ingredients (excipients) such as flavorings in medicinal products, the US Pharmacopeia (USP) Good Manufacturing Practices guidelines recommend similar stability requirements of excipients as those for active pharmaceutical ingredients.[32] In the US, the FDA's Center for Food Safety and Applied Nutrition (CFSAN) regulates flavorings in foods and beverages. The CFSAN can deem flavoring chemicals as GRAS.[33] For unqualified GRAS flavorings, approval of their use in food depends, among other factors, on their stability.[33] While a proposed rule on manufacturing standards for different tobacco products, including e-cigarettes, has been announced by the FDA's Center for Tobacco Products (CTP),[34] critical information on tobacco product safety standards, including packaging and storage conditions that impact flavoring chemical stability, is currently missing. Although some e-cigarette manufacturers include expiration dates on product packaging, these are not based on federally mandated standards for tobacco products.

The primary aim of our study was to measure the stability of 20 commonly used flavoring chemicals in reference (lab-made) solutions and commercial e-cigarette liquids when stored for prolonged times in naturalistic conditions reasonably expected by consumers of these products. We selected ambient temperatures, which reflect the user's tendency to have their product nearby continuously. We also evaluated the impact of light exposure since liquids can be manufactured and sold in clear bottles (e.g., e-cigarette liquids) or light-proof packaging (e.g., disposable devices). We also evaluated the effect of low storage temperatures on flavoring stability. Using a nontargeted approach, we tentatively identified several possible byproducts for select flavorings that demonstrated the most instability. Finally, we assessed whether the degradation of select flavorings in e-cigarette liquids affects the cytotoxicity of those products.

EXPERIMENTAL PROCEDURES

Chemicals. The following flavoring chemicals were analyzed in the study: 2,3,5-trimethylpyrazine, acetoin, benzaldehyde, benzyl alcohol, butanoic acid, cinnamaldehyde, limonene, ethyl maltol, ethyl salicylate, ethyl vanillin, eucalyptol, eugenol, furaneol, isovanillin, maltol, menthol, methyl salicylate, pulegone, triacetin, and vanillin, based on their frequent use across e-cigarette liquid flavors.[23] Neat standards for each flavoring chemical were purchased from vendors previously reported.[23] Neat nicotine, PG, and VG were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Haverhill, MA). LCMS-grade methanol and HPLC-grade dichloromethane (Fisher Scientific, Waltham, MA) were used to dilute the liquids for chemical analyses.

Preparation of Reference Solutions. Reference solutions (n = 20) containing each flavoring chemical independently at 1 mg/mL concentration with 40 mg/mL nicotine were prepared by adding each neat chemical to a 55:45 (v/v) PG/VG solution. A separate reference solution ("mix") containing all flavoring chemicals (1 mg/mL each) and nicotine (40 mg/mL) was also prepared into a 55:45 (v/v) PG/VG solution. We also prepared a separate "nicotine-only control solution" (without flavorings) containing 40 mg/mL of nicotine in 55:45 (v/v) PG/VG and a "solvent control solution" (without flavorings and nicotine) containing 55:45 (v/v) PG/VG-only. Solutions were gently mixed for 1 h using a vertical multifunction rotator (Grant Instruments, Shepreth, United Kingdom). Each solution was aliquoted into three separate clear 15 mL polypropylene tubes (n = 69 total) to store under the three study conditions described below.

Commercial E-Cigarette Liquids. *MyBlu* pod-style cartridges (n = 14), containing approximately 1.5 mL of liquid each, were purchased online from Fontem US Inc. (Imperial Brands plc, Bristol, United Kingdom) in November 2019 and included "Blueberry, 2.4% (nicotine concentration)", "Cherry, 2.4%", "Citra Zing, 4.0%", "Fresh Mint, 3.6%", "Green Apple, 2.0%", "Honeymoon, 4.0%", "Mango Apricot, 2.0%", "Melon Time, 4.0%", "Menthol, 2.4%", "Mint-Sation, 3.6%", "Neon Dream, 4.0%", "Tobacco, 3.6%", "Tobacco Chill, 4.0%", and "Vanilla, 2.4%" flavors. A manufactured date was not provided, so it was unknown how long liquids were stored in ambient conditions before they were purchased. However, all products were sold in opaque packaging (Figure S1). The liquid was extracted from each cartridge using centrifugation and transferred into separate clear 1.5 mL polypropylene Eppendorf microcentrifuge tubes (n = 42 total) to store under the three study conditions described below. Due to a transfer error from the cartridge, a limited volume of "Mint-Sation, 3.6%" remained for some analyses after 1 month, as noted in the results. All liquids were stored at 4.0 ± 2.0 °C and in dark conditions until the start of the study (2 days after preparing reference solutions).

Storage Conditions and Sampling Intervals. Aliquots of reference solutions (10 mL, each) and commercial liquids (1.5 mL, each) were placed in three storage conditions: 1) "ambient light" with room temperatures and light exposure; 2) "ambient dark" with room temperatures but without light exposure; and 3) "cold dark", where liquids were stored at 4.0 ± 2.0 °C without light exposure. Temperature, light intensity, and relative humidity values were recorded in 1 h increments from each condition using HOBO data loggers (Onset Computer Corporation, Bourne, MA) (Supporting Results). Temperatures remained stable (±20%) across time points in each condition, while light intensity was lower (−28%) in the first month at "ambient light" conditions compared to the average intensity across 24 months. Light intensity was stable (±20%) in both "ambient dark" and "cold dark" across 24 months. While relative humidity fluctuated (more than ±20%) among several time points in the "ambient light" and "ambient dark" conditions, solutions were sealed throughout the study and presumably had minimal impact.

Aliquots (90 μL) of liquids were sampled for chemical analyses at the baseline (0 months) and after intervals of 1, 3, 6, 12, and 24 months at each storage condition. During each sampling time point, liquids were gently rotated for 1 h before taking separate aliquots for independent analyses of flavoring concentrations, pH, and qualitative nontargeted identification of byproduct formation, following the methods described

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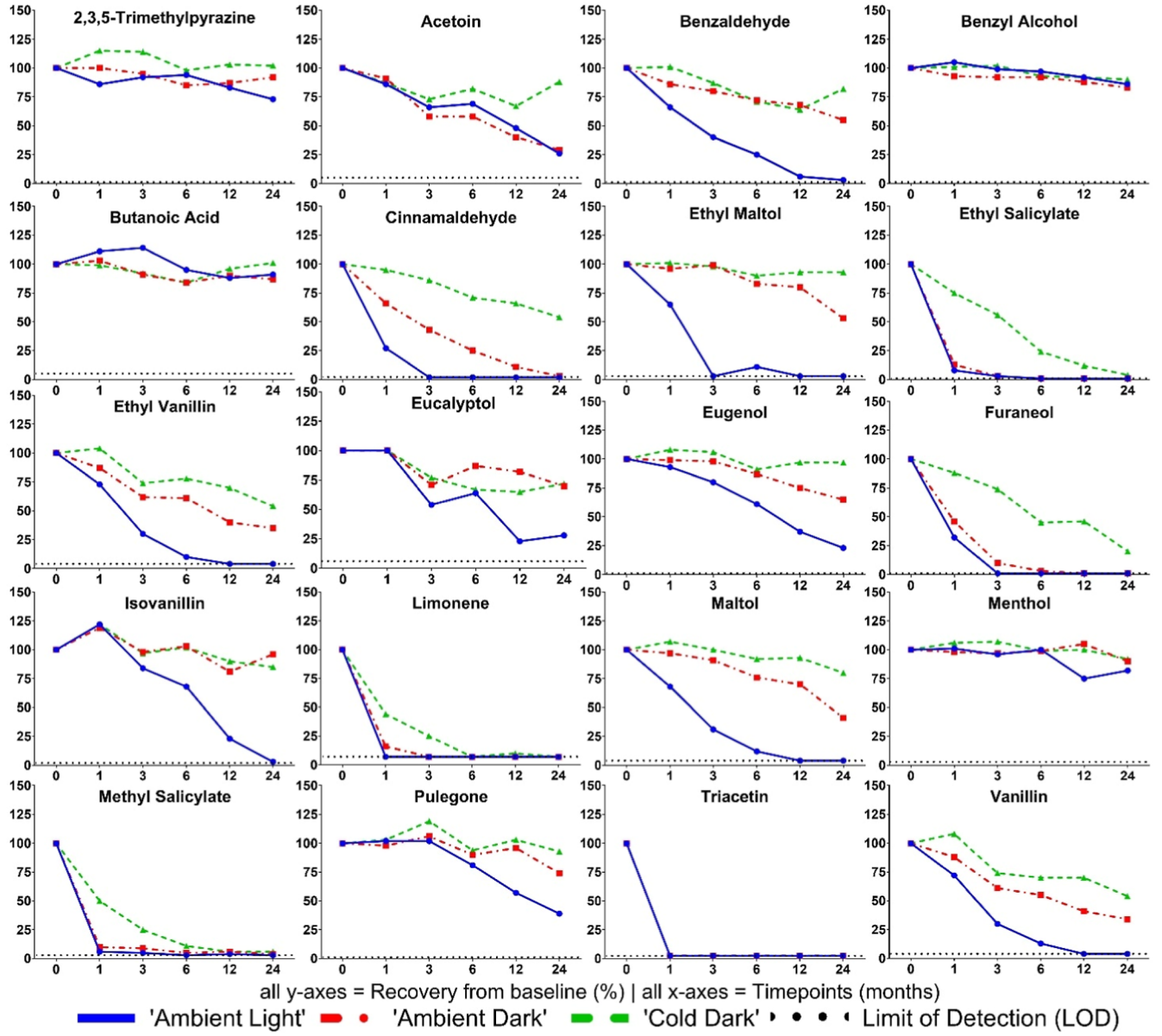

Figure 1. Comparison of flavoring concentration recovery at 1, 3, 6, 12, and 24 months compared to the baseline (0 months) in individual reference solutions stored in "ambient light" (blue), "ambient dark" (red), and "cold dark" (green) conditions. Recoveries below the LOD are reported as the LOD value. Due to a calibration error at the baseline for eucalyptol, the reported measurement at the baseline reflects the 1 month recovery. The triacetin reference solution was reprepared to confirm substantial concentration loss at the baseline."

below. Chemical and physical changes observed in reference solutions were also compared to those in commercial liquids.

Sample Analysis. At each time point for all three conditions, a single replicate of each reference solution (n = 69) and *MyBlu* liquids (n = 42) was prepared and analyzed for flavoring concentrations following validated procedures previously reported.[23] Briefly, 30 μL of each liquid was added to 3 mL of LCMS-grade methanol with internal standards (1 mg/mL), and the mixture was vortexed for 5 min. Aliquots were measured once using an Agilent 7890B/7250 GC/Q-TOF (Agilent Technologies, Santa Clara, CA) equipped with a PAL RSI 120 autosampler (CTC Analytics, Zwingen, Switzerland) and an Agilent DB-624 UI column (30 m, 250 μm I.D., 1.4 μm film thickness). Up to 75 injections were performed bracketed by calibration curves. Calibration curves ranging from 0.02 to 10.0 mg/mL and quality control samples (QCs) ranging from 0.03 to 7.0 mg/mL in 55:45 (v/v) PG/VG were freshly prepared at each sampling time. Calibration and QC samples were prepared for analysis using the same procedure as reference solutions and *MyBlu* liquids. QCs were included in each sample bracket to verify that sample preparation and analysis were conducted consistently at each time point. When necessary, additional dilutions were performed to calculate flavoring concentrations within the upper limit of quantitation as established by calibration curves previously reported.[23] Flavoring chemicals were included in data analysis if concentrations recovered above the limit of detection (LOD) (Table S1). Solvent blanks were prepared by dissolving 55:45 (v/v) PG/VG in methanol (1:100), and they were included in each sample bracket to confirm no additional contamination.

The PG/VG ratio and nicotine concentration of all reference liquids and commercial products were confirmed at the

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baseline only following previously published procedures.[35] Recoveries are reported in Supporting Results.

Assessment of the Stability of Flavoring Chemicals. The percent recovery of each flavoring chemical after 1, 3, 6, 12, and 24 months was calculated by comparing the measured concentrations at each time point to the concentration measured at the baseline. Thus, the measurement at the baseline represents 100%. However, calibration error (e.g., $r^2 < 0.985$) was observed for eucalyptol (impacting both the individual and "mix" reference solutions) and limonene (impacting the "mix" solution only) at baseline. Therefore, where appropriate, the one-month sampling point was used as the baseline for these two flavorings. Our study's primary outcome was the stability of each flavoring chemical between storage conditions. pH measurements (see Supporting Information, page S4) and images taken at baseline captured using a Sony Cyber-shot DSC-W800 digital camera (Sony, Tokyo, Japan) compared to 24 months served to provide secondary confirmation of physicochemical changes to liquids observed between conditions (see Supporting Images, pages S2−S11).

Identification of Potential Degradation Byproducts of Flavoring Chemicals. We also used a nontargeted approach (see Supporting Information, page S4) to detect new chemicals not present in the baseline sample (0 month). We narrowed our analysis to the condition and flavoring(s) that demonstrated the most substantial instability (concentration loss of at least 50% by 24 months). Chemicals were excluded if they were: 1) present only at one time point, including 0 month; and 2) present only in nonconsecutive time points (e.g., 3 month and 12 month). Therefore, these newly detected chemicals were suspected to be byproducts of unstable flavorings, where concentration decreased during storage. Because our method was developed to measure concentrations of parent flavoring chemicals and not their derivatives, we could not measure the concentrations of newly generated chemicals. However, we assessed whether the abundance of those newly identified chemicals increased over time using heat maps generated by Mass Profiler Professional Software (v14.9.1, Agilent Technologies, Santa Clara, CA), where mean abundance was log2 transformed and compared (baselined) to 0 months.

After identifying potential degradation byproducts, we hypothesized possible reactionary processes (e.g., oxidation) to explain the observed instability among flavorings. This was done by comparing similarities in the molecular structure of the parent flavoring compound to potential degradation byproducts. We report structural comparisons and changes in abundance among these suspect byproducts over 24 months.

Cytotoxicity Testing of the Reference Solutions with Degraded Flavorings. Ethyl maltol, furaneol, maltol, and triacetin reference solutions were evaluated for cytotoxicity since these chemicals are frequently added to commercial e-cigarette liquids and degraded substantially (more than 50%) after 24 months when stored in "ambient light" conditions. Aliquots of each reference solution of ethyl maltol, furaneol, maltol, and triacetin after 24 months of storage in the "ambient light" ($n = 4$), along with aliquots of freshly prepared reference solutions identical to the baseline ($n = 4$), and the 'nicotine-only control solution' were added to separate 1.5 mL CE4 e-cigarette tanks. As described previously, NCI-H292 bronchial epithelial cells were exposed to an aerosol generated from each tank using an air−liquid interface.[36] Briefly, a smoking machine (LX-1, single-port piston, Borgwaldt, Hamburg, Germany) with the following puffing parameters: 55 mL puff volume, 2 s duration, 30 s puff frequency,[37] and 55 total puffs per reference solution was used to generate aerosols from an EGO puff-activated e-cigarette. After exposure, cell viability was assessed using the Neutral Red assay.[36] Results for each flavoring tested ($n = 5$) were compared against the 'nicotine-only control solution' using Mann−Whitney nonparametric U-tests to determine the percent viability between the baseline (0 months) and 24 months for each flavoring using GraphPad Prism (v10.2.2).

RESULTS

Most flavoring concentrations were within ±20% of the expected concentration (1 mg/mL) in individual reference solutions at the baseline (0 months), aside from ethyl salicylate, methyl salicylate, and triacetin, which recovered below the expected concentrations (74, 59, and 6.6%, respectively). The triacetin reference solution was reprepared, and the initial results were confirmed. Due to differences between the reference solution and calibration preparations, recoveries of ethyl vanillin, isovanillin, and vanillin exceed 1 mg/mL by over 20%.

Stability of Flavoring Chemicals by Storage Conditions. Results from reference solutions of individual flavoring chemicals revealed that 11 out of 20 (55%) flavorings experienced at least 50% loss after 6 months in the "ambient light" storage condition, while similar losses were observed in 16 (80%) flavorings after 24 months (Figure 1). By comparison, 6 (30%) and 10 (50%) flavorings experienced 50% loss after 6 and 24 months, respectively, in the "ambient dark" condition. Only 4 (20%) flavorings incurred a 50% loss after 6 months in the "cold dark" condition, while 5 (25%) flavorings experienced a similar loss after 24 months. Similar trends were observed among the "mix" solution, where the greatest number of flavorings with 50% loss at 6 and 12 months were observed among the "ambient light" condition (10 and 15 flavorings, respectively; Figure S2). In the commercial *MyBlu* products, "ambient light" conditions also facilitated the greatest frequency of concentration loss after 6 and 24 months (20 and 72.9% of detected flavorings, respectively). By comparison, 8.6% and 25.7% of flavorings detected at baseline experienced a 50% loss at 6 and 24 months, respectively, in "ambient dark". Among "cold dark" conditions, 50% losses were not observed for any flavoring after 6 months, whereas such loss occurred in only 4.3% of flavorings after 24 months (Table S2).

Among the "ambient light" conditions, which facilitated the most instability, the most rapid losses were observed with cinnamaldehyde, ethyl- and methyl- salicylate, furaneol, limonene, and triacetin. For each reference solution, more than 50% of the initial concentration was lost after 1 month. These flavorings were also rapidly lost in the "mix" reference solution, apart from ethyl- and methyl-salicylate, where a 50% loss was observed after 3 months (Figure 1). Furaneol instability was similar among commercial *MyBlu* liquids in "ambient light" conditions. Alternatively, improved stability of triacetin was observed, where a 50% loss occurred between 6 months (e.g., "Tobacco") and 24 months ("Mint-Sation", Table S2).

Four flavorings (2,3,5-trimethylpyrazine, benzyl alcohol, butanoic acid, and menthol) maintained at least 50% of the initial concentration across 24 months in all conditions in

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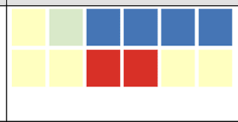
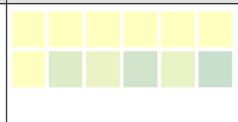
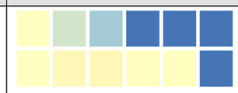
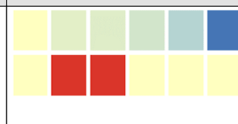
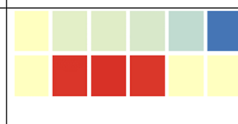
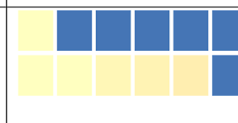
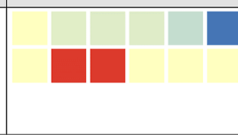
Table 1. Predicted Reaction Pathways and Byproduct Formation in Liquids Stored in Room Temperature and Light-Exposed Condition ("Ambient Light")

Parent Compound	Byproduct	Change in Peak Response Compared to Baseline[1] (Peak Abundance: Decreasing / Baseline / Increasing) 0 1 3 6 12 24 (Months)	
Oxidation			
Benzaldehyde	Benzoic Acid (65-85-0)		Benzaldehyde Benzoic Acid
Degradation (Temperature Induced)			
Furaneol	Hydroxyacetone (116-09-6)		Furaneol Hydroxyacetone
Fragmentation			
Triacetin	1-Monoacetin (106-61-6)		Triacetin[2] 1-Monoacetin
Hydrolysis			
Cinnamaldehyde	Benzaldehyde (100-52-7)		Cinnamaldehyde Benzaldehyde
Condensation (Solvent)			
Ethyl Vanillin	Ethyl Vanillin propylene glycol (PG) acetal (68527-76-4)		Ethyl Vanillin Ethyl Vanillin PG acetal
Vanillin	Vanillin propylene glycol (PG) acetal (68527-74-2)		Vanillin Vanillin PG acetal
Methyl Salicylate	2-Methoxyethyl 2-hydroxybenzoate (26735-04-6)		Methyl Salicylate 2-Methoxyethyl 2-hydroxybenzoate
Condensation (Other)			
Benzaldehyde	Cinnamaldehyde (104-55-2)		Benzaldehyde Cinnamaldehyde

[1]Raw peak abundances are log2 transformed; values within each compound at each time point are baselined to the log2 abundance at 0 months. The red squares indicate increased peak response relative to 0 months, while the blue squares indicate reduced peak response relative to 0 months. Changes in abundance are only comparable within each chemical. [2]Triacetin was not detected at any time point.

individual and the "mix" reference solutions (Figures 1 and S2). These same flavorings remained stable across storage conditions in the commercial products, except for butanoic acid in *MyBlu* "Vanilla" after 24 months stored in 'ambient dark' conditions and 2,3,5-trimethylpyrazine in four *MyBlu* flavors ("Cherry", "Menthol", "Tobacco", and "Tobacco Chill") stored at "ambient light" conditions (Table S2).

Changes in pH of Flavored Liquids Stored in Different Conditions. The average pH among the individual reference solutions at baseline was 9.7 ± 0.2 and varied from 9.4 (butanoic acid) to 10.0 (limonene). After 24 months, average pH values were 8.2 ± 0.12, 9.1 ± 0.2, and 9.1 ± 0.2 when stored in "ambient light", "ambient dark", and "cold dark" conditions, respectively. All 20 reference solutions stored in the "ambient light" condition became more acidic over the

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study than liquids stored at "ambient dark" and "cold dark" conditions (Figure S3A). This same pattern was observed in the "mix" reference solution. The pH of the "nicotine-only control solution" was 9.8 at the baseline and 8.4 ("ambient light"), 9.3 ("ambient dark"), and 9.4 ("cold dark") after 24 months (Figure S3B). The "solvent control solution" became more acidic over time when stored in the "ambient light" condition (5.9 vs 3.4). Interestingly, the alkalinity increased when stored in "ambient dark" (5.9 vs 7.5) and "cold dark" (5.9 vs 7.3) conditions after 24 months.

The average pH among *MyBlu* liquids at the baseline was 5.7 ± 2.3, compared to an average of 4.3 ± 0.3 ("ambient light"), 6.1 ± 1.9 ("ambient dark"), and 6.0 ± 2.0 ("cold dark") after 24 months. Increased acidity was observed in all flavors when stored in the "ambient light" condition, as compared to "ambient dark" and "cold dark" conditions after 24 months (Figure S4).

Color Changes of Flavored Liquids Stored in Different Conditions. Notably visible darkening of most reference liquids was observed over 24 months when stored in "ambient light" conditions (Supporting Images pages S2−S7). Among reference solutions stored in the "ambient dark" conditions, several solutions, including acetoin, cinnamaldehyde, ethyl maltol, ethyl vanillin, eugenol, furaneol, maltol, and vanillin, became visibly darker compared to the baseline. The "cold dark" condition yielded less darkening among reference solutions compared to the baseline, except for furaneol. A similar darkening trend between conditions was observed in the "nicotine-only control solution", whereas the "solvent control solution" did not darken in any condition. Darkening of all 14 *MyBlu* liquids was observed when stored in the "ambient light" condition. "Citra Zing", "Melon Time", "Tobacco", and "Tobacco Chill" also darkened in the "ambient dark" conditions. In contrast, liquids stored in the "cold dark" condition demonstrated the least amount of darkening after 24 months (Supporting Images, pages S8−S11).

Degradation Byproducts of Flavoring Chemicals and Their Potential Degradation Pathways. Table 1 shows the most probable byproducts, based on structural similarities to parent flavorings, tentatively identified in individual reference liquids of flavoring chemicals that demonstrated substantial loss from the baseline (more than 50%) over 24 months under "ambient light" storage conditions. Table 1 also shows the potential degradation mechanisms of each flavoring. For example, benzoic acid identified in the benzaldehyde reference solution after 1 and 3 months indicates probable oxidation. The presence of 1-monoacetin in the triacetin reference solution suggests fragmentation by eliminating two acetone molecules. Cinnamaldehyde likely underwent hydrolysis, as indicated by the presence of benzaldehyde in the reference solution. Condensation reactions between the PG solvent and ethyl vanillin and vanillin were also observed, resulting in their corresponding acetals. Likewise, possible condensation reactions between methyl salicylate and the solvents may have formed 2-methoxyethyl 2-hydroxybenzoate. Interestingly, we detected cinnamaldehyde in the benzaldehyde-only reference solution, suggesting a possible condensation reaction. While other chemicals were identified, as reported in Figure S5, these are less likely to have formed from reactions with flavoring chemicals given structural divergence from the parent molecule. For example, cotinine (identified in all reference solutions except the "solvent control") is likely a reactionary byproduct of nicotine (Figure S5).

Among *MyBlu* liquids, we tentatively detected several of the same suspected byproducts, as identified in the individual reference solutions. For example, benzoic acid was detected in "Cherry" at 3, 6, and 12 months, and this flavor also contained benzaldehyde at baseline (data not shown). Likewise, we detected hydroxacetone in "Melon Time", "Mint Sation", and "Tobacco Chill" after at least 3 months (data not shown). These flavors also contained furaneol. In nearly all time points, 1-monacetin was identified in flavors containing triacetin (data not shown). Similarly, the PG acetals of ethyl vanillin and vanillin were detected in each flavor containing ethyl vanillin or vanillin except "Citra Zing" and "Green Apple" in nearly every time point (data not shown).

Changes in the Cytotoxicity of Reference Solutions after 24 Months. We did not identify significant differences in the cytotoxicity of the aged liquid compared to reference solutions at the baseline (Figure 2) for the four flavorings tested. Further, none of the flavorings exhibited significantly different cytotoxicity from the 'nicotine-only control solution' (all $p > 0.05$).

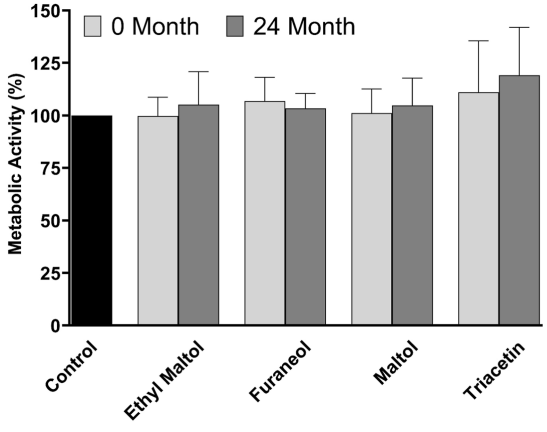

Figure 2. Metabolic activity between reference solutions at the baseline (0 months) and 24 month-aged liquids stored in the "ambient light" conditions. Control refers to the "nicotine-only control solution", which contained 55:45 (v/v) PG/VG and 40 mg/mL nicotine. Within flavorings, no significant differences were observed (all $p > 0.05$). Error bars represent standard deviation. All $n = 5$.

DISCUSSION

Our study revealed that most flavoring chemicals in e-cigarette liquids are the least stable when exposed to ambient temperatures and light. All but four flavoring chemicals in individual reference solutions experienced at least 50% concentration loss by the end of the study in the "ambient light" condition. Importantly, ambient temperatures with light exposure also led to more instability of flavorings identified in *MyBlu* commercial products compared to storage at each ambient and cold temperature without light exposure. Nearly three-quarters (72.9%) of the flavoring chemicals detected in the commercial products at the baseline experienced a 50% loss by the end of the study when stored in "ambient light" conditions, whereas only 25.7 and 4.3% demonstrated similar instability in "ambient dark" and "cold dark" conditions.

Notably, some flavorings, including cinnamaldehyde, ethyl salicylate, furaneol, limonene, methyl salicylate, and triacetin, showed greater instability since their degradation occurred

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within one month after formulation. Cinnamaldehyde appeared to be differentially affected by light and temperature, where 50% loss occurred after 3 months in "ambient dark" conditions and 24 months in "cold dark" conditions. Ethyl salicylate and furaneol were most impacted by temperature, as both chemicals demonstrated at least 50% loss by one month in the "ambient light" and "ambient dark" but not in "cold dark" conditions. The instability of furaneol at room temperature, regardless of light exposure, was observed in reference solutions and commercial *MyBlu* products. This is consistent with the reported instability of furaneol at higher temperatures.[38] Other chemicals, such as maltol and ethyl maltol, were significantly impacted by light exposure, consistent with the reported degradation of maltol when exposed to light.[39] Methyl salicylate and triacetin were highly unstable when stored under all three conditions, suggesting that reactions with the solvent may be more strongly favored. Interestingly, we observed a near-complete loss of triacetin immediately upon preparing the reference solution. A second prepared reference solution confirmed our original results. However, the delayed loss of methyl salicylate in the "mix" reference solution in all three conditions suggests the presence of other chemicals may impede this reaction. Likewise, triacetin stability was improved among eight *MyBlu* flavors in all three conditions, likely because additives such as benzoic acid, which act as preservatives in cosmetic products,[40] may have inhibited degradation reactions.

Evidence of byproduct formation in individual reference solutions indicates several potential mechanisms leading to degradation of flavoring chemicals. We hypothesized the hydrolysis of cinnamaldehyde to benzaldehyde, which has been reported previously in cosmetic and pharmaceutical products.[27] We identified benzoic acid through a possible oxidation mechanism in the "Cherry" flavored *MyBlu* liquid at 6 months, which contained benzaldehyde at the baseline. Importantly, benzoic acid is a known precursor to toxic benzene formation.[41] While e-cigarette manufacturers commonly add benzoic acid to form nicotine salts,[42] the pH of the "Cherry" flavored solution (>8.0) indicates salts were not used in this formulation. Interestingly, we detected cinnamaldehyde in the aged benzaldehyde reference solution, which can be generated by condensing benzaldehyde and acetaldehyde.[43] While acetaldehyde could not be measured using our methodology, possible thermally induced degradation of VG into glycidol,[44] which rapidly and favorably[45] decomposes into acetaldehyde,[44] could explain these results. We tentatively identified glycidol in several reference solutions across time points, supporting probable VG degradation. The formation of cinnamaldehyde from benzaldehyde has implications for increased cytotoxicity associated with cherry and sweet-flavored liquids.[46] However, we did not identify cinnamaldehyde in the "Cherry" *MyBlu* liquid (which contained benzaldehyde).

Interestingly, we did not detect the benzaldehyde acetal, as previously reported;[21] however, the corresponding acetals of condensation between the solvents and ethyl vanillin and vanillin[21] were identified in our reference solutions and commercial *MyBlu* liquids. While prior findings suggest acetal formations between esters and the solvents are unlikely,[47] we identified possible solvent condensation reactions with methyl salicylate to form 2-methoxyethyl 2-hydroxybenzoate. A similar reaction is suspected to account for the observed concentration loss of ethyl salicylate.

Hydroxyacetone, detected in the furaneol reference solution, is a known thermally induced furaneol degradation byproduct,[38] possibly from the opening of the furan ring.[27] Alternatively, hydroxyacetone can be generated as a byproduct of PG degradation.[48,49] This chemical was tentatively identified in 16 reference solutions and the solvent control solution by 6 months in the "ambient light" condition. However, among reference solutions stored in "ambient dark" at 6 months, hydroxyacetone was detected only in the furaneol reference solution, further supporting the probable pathway of furaneol instability in e-cigarette liquids. We also identified 1-monoacetin in the triacetin reference solution, possibly generated with the loss of 2 acetone molecules from triacetin. While this has not been previously reported, monoacetin, diacetin, and triacetin are each formed from the acetylation of glycerol.[50] In heated e-cigarette liquids, pyrolysis of triacetin forms acetic acid,[15] although we could not measure this compound owing to limitations in our instrument parameters.

Although we detected several potential byproducts of flavoring degradation in aged e-liquid solutions, this study was not designed to elucidate the reaction mechanisms of individual flavoring chemicals. Our identifications of byproducts are tentative, given the nontargeted approach, and we could not measure changes in their concentrations over time. However, changes in abundance provide evidence of the reported reactions. For example, the abundance of benzoic acid at 3 months exceeded that identified at 0 month in the "benzaldehyde" reference standard, which remained stable up to 12 months before decreasing to a level below 0 month. Nonetheless, other reaction mechanisms may be favored where alternative byproducts are generated that were not detected with our methods. Further, the role of nicotine in flavoring stability is still being determined. Nevertheless, these findings provide the necessary groundwork for future studies to confirm the suspected reactions and their byproducts.

Most liquids became notably more acidic when exposed to ambient temperature and light throughout the study. Similar changes were observed among the "nicotine-only control solution" and "solvent control solution", and most commercial *MyBlu* liquids after 24 months. Likewise, reference solutions and *MyBlu* liquids were visibly darker when stored under this condition. The "nicotine-only control solution" was also visibly darker, but the "solvent control" did not darken in any condition. The changes in pH and coloration were possibly driven by nicotine degradation rather than the flavorings alone. While we did not measure nicotine concentration in this study beyond the baseline, it reportedly can be unstable in e-cigarette liquids under ambient conditions,[49,51] with notable color change, suggesting possible nicotine oxidation.[52]

Findings from this study have implications for the FDA's proposed rule on "Requirements for Tobacco Manufacturing Practice",[34] which includes e-cigarette liquids. As part of the proposed rule, products that do not meet specifications defined by manufacturers in their Master Manufacturing Record are deemed nonconforming.[34] Unstable flavorings identified in this study, such as ethyl maltol, furaneol, and triacetin, were present in at least 25% of e-cigarette liquids among 215 commercial products we previously surveyed.[23] These products would likely be deemed nonconforming after implementing the proposed rule. Further, products that differ from their initial applications (e.g., Premarket Tobacco Applications and PMTAs) are considered adulterated by the Food and Drug Cosmetic Act (FD&C) under Section 902(6).[34] Several e-

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cigarette liquids that have received marketing granted orders through the PMTA process use flavorings, such as ethyl maltol and ethyl vanillin,[53] to create the tobacco flavor. Thus, e-cigarettes with a "tobacco" flavor may also be deemed nonconforming over time due to flavoring degradation.

The rule proposed by the FDA further requires manufacturers to "ensure tobacco products are handled and stored under appropriate conditions to prevent non-conforming products as well as mix-ups, deterioration, contamination, adulteration, and misbranding of tobacco products".[34] Our study suggests appropriate storage should, at minimum, include light-proof packaging and, ideally, low temperatures throughout the product's lifecycle. Results from our study support recommendations to include requirements in the rule for manufacturer-labeled expiration dates based on stability studies and labeled storage conditions for consumers.

Our findings from the *in vitro* testing suggest degradation of common flavoring chemicals in e-cigarette liquids such as ethyl maltol, furaneol, maltol, or triacetin does not appear to increase the cytotoxicity of the inhaled flavored aerosols emitted from e-cigarettes. However, this experiment does not purport to capture effects under realistic conditions when these chemicals are combined with other e-cigarette ingredients. E-cigarette liquids are a complex matrix with an estimated average content of 17 ingredients per liquid.[11] Alternative *in vitro* and *in vivo* toxicity assays to assess health effects may reveal more harmful outcomes associated with deteriorated liquids.[20] Our study evaluated only 20 of the hundreds[54] of commercially available flavorings used in e-cigarette liquids. Understanding interactions between these flavoring chemicals in varying concentrations and formulations with each other, nicotine, and PG/VG when in contact with components of the storage containers (e.g., plastics, metals, and porous materials in disposable devices) when exposed to different environmental conditions remains challenging. However, our data strongly suggest that the degradation of flavorings was best mitigated when stored in cold (4 °C) and dark conditions. Importantly, this observation applies to all brands and e-cigarette liquid formulations.

Limitations. We note several limitations of the study. First, reference solutions were prepared only once and analyzed only once per sampling event. Since chemical stability was the primary interest of this study, we aimed to eliminate additional time variables and potential reactions with extraction solvents. For this reason, we were limited by analytical time, which proceeded within 2 days of each sampling event. Further, the degree to which high temperatures of the instrument method affected chemical stability and byproduct detection is unknown. However, this should have remained the same as the observed differences between the storage conditions. We also experienced poor instrument accuracy of eucalyptol and limonene when measured at the baseline. Since we adjusted baseline recovery to one month, one-month stability was not reported. The time between when *MyBlu* liquids were manufactured and when we received the liquids for testing could not be determined. Although cartridges were stored in dark packaging, some degradation of the flavorings may have occurred before our measurements.

CONCLUSIONS

Several common flavoring chemicals rapidly degrade in e-cigarette liquids stored at ambient temperature and light, generating byproducts that could increase respiratory health risks to users. Furthermore, stability improves when e-cigarette liquids are stored at 4 °C in the dark. Our findings suggest it is necessary that good manufacturing guidelines recommend light-proof packaging throughout e-cigarette liquid manufacturing, storage, and use. This would encourage manufacturers to redesign bottles and devices to protect liquids from light exposure. Further, instructions for proper storage and expiration dates on product labels are necessary to inform and protect consumers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01266.

Additional tables, figures, and method descriptions (DOCX)

Storage temperature, humidity, and light intensity within conditions over 24 months, and PG/VG ratio and nicotine content at the baseline (XLSX)

Supporting Images: Color changes between baseline and 24 months among reference solutions and *MyBlu* liquids (PDF)

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†M.K.P. and A.D.M. are cofirst authors. M.K.P., N.J.L., and M.L.G. contributed to the conception of the work. M.K.P., A.D.M., and N.J.L. performed experiments and data analysis. The manuscript was written with contributions from all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): MLG received a research grant from Pfizer and served on the Scientific Advisory Board to Johnson & Johnson. He has also consulted with the US Food and Drug Administration, World Health Organization, and Campaign

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for Tobacco-Free Kids on the toxicity of tobacco products and tobacco control policies. MLG is also a Member of the IASLC Tobacco Control and Smoking Cessation Committee and the AACR Tobacco Product and Cancer Subcommittee. Other authors declared no competing interests.

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