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NMR Analyses of Flavored E-cigarette Liquids Before and After Aerosolization

by

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A thesis submitted in partial fulfillment of the
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in
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Portland State University
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Abstract

Electronic cigarettes (e-cigarettes) are the most commonly used tobacco products among adolescents with ~11 and ~3% of high school and middle school students reporting past 30 day use in 2021 according to the Centers for Disease Control and Prevention (CDC), respectively. Furthermore, greater than ~80% of high school and middle school e-cigarette consumers use flavored e-liquids. Consumers can be exposed to variable toxicant levels upon e-liquid aerosolization, depending on the composition of the e-liquid, type of e-cigarette, e-cigarette settings, and other customizations.

E-liquids are typically composed of a carrier solvent (propylene glycol (PG) and glycerol (GL)), flavorants, and nicotine. PG and GL can degrade thermally upon aerosolization to produce formaldehyde, acetaldehyde, and other harmful and potentially harmful constituents (HPHCs). Flavorants can alter the e-liquid composition before and after aerosolization. Aldehyde flavorants react with PG and GL to form flavorant-PG and -GL acetals that transfer into the aerosol and have unique toxicity profiles. Aerosolized e-liquids with flavorants can contain higher HPHC levels compared to those without. However, there is limited information on the effects of nicotine and other common e-liquid additives on 1) the toxicant levels in aerosolized PG+GL e-liquids with and without flavorants and 2) the kinetics of aldehyde flavorant-PG and -GL acetal formation in e-liquids.

This thesis contains two manuscripts that cover 1) the effects of flavorants and flavorants+nicotine on PG+GL e-liquid degradation and 2) the kinetics of aldehyde flavorant-acetal formation in e-liquids with different solvents and common additives.

Study 1) showed that aerosolized e-liquids with *trans*-cinnamaldehyde, benzyl alcohol, vanillin, benzaldehyde, and a "flavorant mixture" (mixture of the four flavorants) contained increased HPHC levels compared to those without. Flavored e-liquids aerosolized with nicotine decreased HPHC formation for benzyl alcohol, vanillin, benzaldehyde, and a "flavorant mixture", but increased HPHC formation for *trans*-cinnamaldehyde compared to flavored e-liquids without nicotine. The effect of nicotine on flavored e-liquid degradation was complex and requires further study with different flavorants and e-cigarettes.

The following study 2) revealed that *trans*-cinnamaldehyde-, benzaldehyde-, and vanillin-acetals formed at a faster rate and higher yield in GL versus PG. GL formed a 5- and 6-member ring acetal, but PG only formed a 5-member ring acetal. Acetalization was inhibited by water and nicotine (an acetalization product and base, respectively), but catalyzed by benzoic acid in PG e-liquids. Lastly, flavorant-PG acetal formation was delayed in flavored PG e-liquids with nicotine, even when benzoic acid was 2-, 4-, and 10-fold greater than the nicotine concentration. The kinetics of additional aldehyde flavorant-acetal and ketone flavorant-ketal formation should be explored in the future due to their unknown toxicity profiles.

Acknowledgments

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Chapter 1

1. Introduction

1.1. History and Background of Electronic Cigarettes

Electronic cigarettes (e-cigarettes) entered the U.S. market in 2007, and were originally designed to be nicotine cessation devices for smokers, but they have become increasingly popular among adolescent never-smokers.[1,2] Vaping is the act of inhaling aerosolized e-cigarette liquids (e-liquids) from a personal vaporizer. First generation e-cigarettes ("cig-a-likes") were non-customizable, low-powered devices that were prefilled with e-liquids, and mirrored the physical shape of cigarettes (Figure 1, A). Second and third generation e-cigarettes (top-coil and box-mod, respectively; Figure 1, B and C) are higher power devices that allow consumers to customize the refillable e-liquid in the tank, aerosolization temperature, power level, and/or coil composition (depending on the device). Fourth generation devices include pod systems (JUUL™) and disposables (Puff Bar™; Figure 1, D). Pod systems and disposables generally have a sleek flash-drive-like shape, low power output, and high nicotine salt (nicotine + organic acids) concentration e-liquid. Pod systems require the consumer to purchase new pre-filled pods and reuse the e-cigarette, but disposables are not reusable once the e-liquid reservoir is empty.

The harmful and potentially harmful constituents (HPHCs) that e-cigarette consumers are exposed to is still an area that requires further study. This is particularly concerning for adolescent users. The number of adolescents using e-cigarettes significantly increased when JUUL™ entered the U.S. market in 2015 with their e-

cigarettes shaped like USB flash drives containing fruity and sweet e-liquids.[1,3] Russell et al. have shown that ~40 and ~30% of adolescents perceived JUUL™ as less harmful and less addictive than cigarettes as of 2019, respectively.[4]

To combat the youth vaping epidemic, the Food and Drug Administration (FDA) banned the sale of flavored cartridges in 2020, but this excluded tobacco and menthol flavored cartridges, flavored e-liquids for open-tank systems, and flavored disposable e-cigarettes.[5] Following this flavored cartridge ban, the FDA, Center for Disease Control and Prevention (CDC), and others noted that ~1.8 million fewer youth in the U.S. use e-cigarettes in 2020 compared to 2019, but there has been a spike in the use of disposable and menthol-pod e-cigarettes.[6]

Disposable e-cigarettes (i.e. Puff Bar™) have remained prevalent among adolescents despite FDA regulations in July 2020 that could have prohibited their sale.[7,8] Puff Bar™ began selling e-cigarettes with "tobacco-free nicotine" (TFN; (*R*,*S*)-(±)-nicotine) instead of "tobacco-derived nicotine" (TDN; ~99% (*S*)-(-)-nicotine) perhaps to avoid FDA regulations.[9,10] The amount of TFN products (e.g. disposable e-cigarettes, refillable e-liquids, nicotine pouches) on the market increased following Puff Bar's™ success.[11,12] The FDA was eventually granted the power to regulate tobacco products containing synthetic nicotine in 2022, but they have yet to act on any TFN product.[13]

The FDA has attempted to limit adolescents' access to popular flavored e-cigarettes by controlling what is sold on the market, but the industry is usually one step ahead by exploiting loopholes in regulations. High school and middle school students (between the ages of 11-19) have adapted to current regulations by vaping approved cartridge flavors

(without and with flavor add-ons), refillable e-liquids for open-tank systems, disposable e-cigarettes, or black-market flavored cartridges.[14,15] Many adolescents are still inhaling variable levels of nicotine and various toxicants classified as irritants and carcinogens.

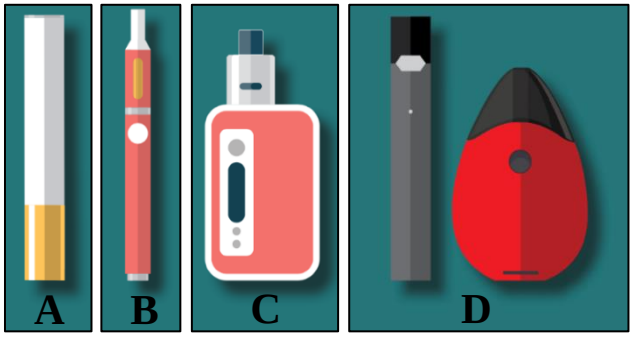


Figure 1. Four generations of e-cigarettes. (A) cig-a-like; (B) top-coil; (C) box-mod; and (D) pod system or disposable.[16]

E-cigarette aerosols are potentially less harmful than cigarette smoke with lower levels of airway inflammatory biomarker expression in mice, less induction of apoptosis in human gingival fibroblasts, and no effect on human epithelial cell barrier [17]. The results from the previous studies were based on the short-term and long-term effects of e-cigarette aerosol and cigarette smoke exposure, respectively. There is limited information on the long-term toxicological effects of e-cigarette aerosols. Further studies are required to adequately compare the long-term health implications of e-cigarettes to cigarettes on humans.

1.2. Chemistry of Toxicant Formation upon E-liquid Aerosolization

Most e-liquids consist of a mixture of propylene glycol (PG) and glycerol (GL), and frequently contain nicotine and various flavorants. The flavorants, PG, and GL have been generally recognized as safe (GRAS) for consumption by the FDA, but are not

necessarily recognized as safe for inhalation.[18] When PG and GL are aerosolized, they can degrade into propanal, acetaldehyde, glycolaldehyde, formaldehyde, acrolein, hemiacetals, and other HPHCs (Figure 2), as has been shown in the literature that will be reviewed in chronological order below. Acrolein is an irritant, formaldehyde and acetaldehyde are probable carcinogens, and hemiacetals are formaldehyde releasing agents (formaldehyde adducts of PG or GL, formed by a reversible reaction; Scheme 1).[19–22]

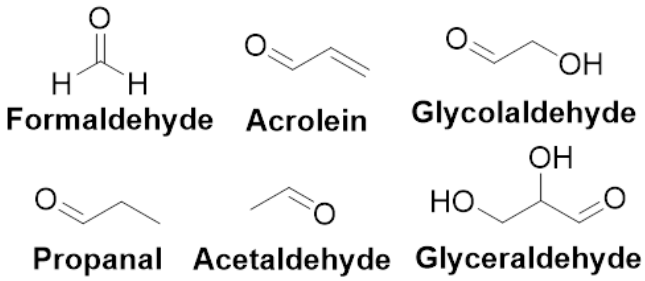
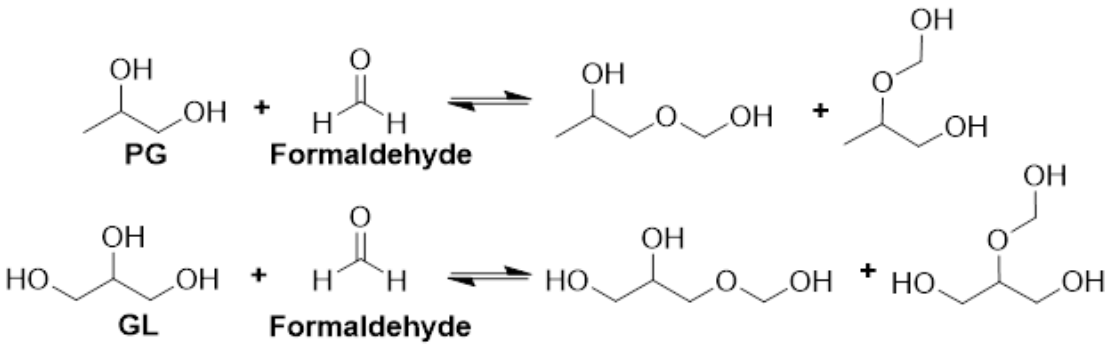


Figure 2. Carbonyls produced from the thermal degradation of propylene glycol (PG) and glycerol (GL).

Formaldehyde was identified in e-cigarette aerosols in trace amounts as early as 2008 by Laugesen[23], and then by Goniewicz et al.[24] in 2012 who identified carbonyl compounds in e-cigarette vapors via high-performance liquid chromatography (HPLC), and compared the results to carbonyls in mainstream smoke. They identified trace amounts of carbonyls in the e-cigarette aerosols, and a 9 to 450-fold increase in carbonyls in cigarette smoke compared to e-cigarette aerosols. Goniewicz et al.'s early research explored the possibilities of e-cigarettes as a harm reduction strategy for cigarette smokers.



Scheme 1. Formation of the formaldehyde hemiacetals from the reaction of propylene glycol (PG) or glycerol (GL) with formaldehyde.

In 2014, Kosmider et al.[25] found that e-liquids aerosolized at a high voltage (4.8 V; within the limits recommended by the manufacturer) and cigarette smoke contained similar formaldehyde levels using high-performance liquid chromatography/diode array detector (HPLC/DAD). One year later, Jensen et al.[19] identified formaldehyde hemiacetals in simulated PG:GL e-liquids aerosolized at 4.8 V using ^{1}H NMR spectroscopy, and found significantly higher levels of total formaldehyde (formaldehyde + formaldehyde hemiacetals) compared to Kosmider et al.'s results. The amounts of total formaldehyde detected by Jensen et al. were likely conservative estimates of the actual amounts present, since only a fraction of the aerosol was captured. Kosmider et al. and Jensen et al.'s studies measured formaldehyde levels in the gas phase (just formaldehyde) and particulate matter (PM; formaldehyde and formaldehyde hemiacetals), respectively.[26]

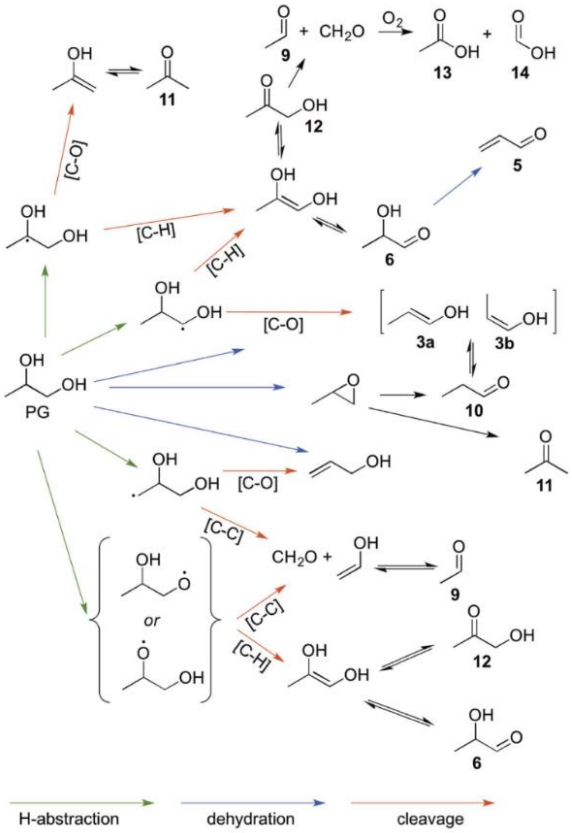
Kosmider et al.[25] and EL-Hellani et al.[27] hypothesized that additional carbonyls (aside from formaldehyde; Figure 1) could form upon e-liquid aerosolization based on pyrolysis studies of PG and GL, but neither study detected many other carbonyls. In 2017, Jensen et al.[28] further studied how PG and GL thermally degrade during e-liquid aerosolization (under the recommended e-cigarette settings), and detected acrolein,

acetaldehyde, propanal, acetone, hydroxyacetone, acetic acid, formic acid, glycidol, allyl alcohol, and other HPHCs – in addition to formaldehyde and formaldehyde hemiacetals – using ^{1}H NMR spectroscopy. Jensen et al. proposed free radical abstraction, free radical addition, and dehydration mechanisms for the formation of the HPHCs (Schemes 2 and 3).[28]

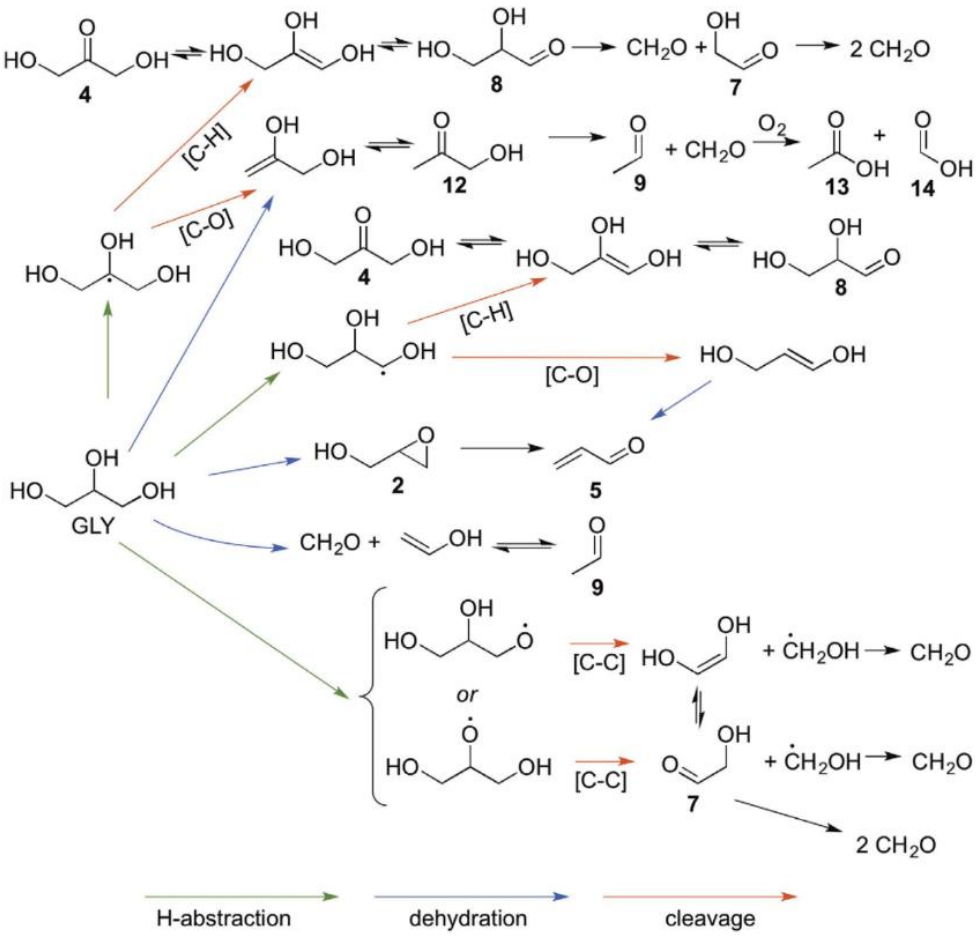
Salamanca et al.[29] repeated Jensen et al.'s[19] study using an equimolar PG:GL e-liquid with the same e-cigarette and settings as used by Jensen et al., and then determined the total formaldehyde levels (formaldehyde+formaldehyde hemiacetal in their study) in aerosols using 2,4-dinitrophenylhydrazine (DNPH) derivatization with HPLC and directly with ^{1}H NMR spectroscopy. They found that formaldehyde hemiacetals made up the majority of the total formaldehyde levels in aerosolized e-liquids as shown by the NMR analysis. However, the total formaldehyde levels were significantly underestimated using derivatization methods. The total formaldehyde levels in aerosols exceeded Occupational Safety and Health Administration (OSHA) workplace limits only with the NMR analysis.[30]

Bitzer et al. examined the effects of temperature, e-liquid composition, and wattage on free radical formation in e-cigarette aerosols using electron paramagnetic spectroscopy and an e-cigarette that had constant-wattage and -temperature control settings.[31] The temperature is unregulated when in constant-wattage mode (e.g. the consumer adjusts the power level and the temperature is inconsistent during aerosolization), but regulated when in constant-temperature mode (e.g. the consumer adjusts the temperature to a value that is not exceeded during aerosolization). E-liquids with mol ratios of PG:GL ranging

from 0:100 to 100:0 were aerosolized at a) 100°, 200°, and 300° C with constant wattage, and b) 10, 25, and 50 W using the constant temperature mode. In each scenario, the 100% PG e-liquid produced 3-fold the level of free radicals compared to aerosolized 100% GL. Increasing the wattage (in constant-wattage mode) significantly increased the number of free radicals produced upon aerosolization versus increasing the temperature (in constant-temperature mode), due to the higher coil temperatures with higher wattages. Although the authors did not determine the identity or quantity of any specific free radicals produced, they demonstrated that PG degradation was the primary source of free radicals.



Scheme 2. The aerobic thermal decomposition of propylene glycol (PG) from Jensen et al.[28] showing the formation of (3) E- and Z-propenol; (5) acrolein; (6) lactaldehyde; (9) acetaldehyde; (10) propanal; (11) acetone; (12) hydroxyacetone; (13) acetic acid; and (14) formic acid.



Scheme 3. The aerobic thermal decomposition of glycerol (GLY) from Jensen et al.[28] showing the formation of (2) glycidol; (4) dihydroxyacetone; (5) acrolein; (7) glycolaldehyde; (8) glyceraldehyde; (9) acetaldehyde; (12) hydroxyacetone; (13) acetic acid; and (14) formic acid.

Bitzer et al.[31] showed that the concentrations of degradation products can vary when e-liquids are aerosolized under different settings with the same e-cigarette. Different types of e-cigarettes can also produce variable concentrations of degradation products. Son et al. compared the e-liquid composition, puff topography, and carbonyl emissions of four generations of e-cigarettes (Figure 1) – from oldest to newest, A) cig-a-like, B) top-coil, C) box-mod, and D) pod system or disposable (JUUL™ or Puff Bar™,

respectively).[28] The e-liquids and aerosols were analyzed with HPLC, and a carbon monoxide analyzer was connected to the vaping apparatus to determine CO concentration. When comparing the four devices, Son et al. found that the top-coil aerosols had the highest concentrations of formaldehyde and CO. For all devices, the emission of carbonyls and CO generally increased linearly with puff duration, and aerosolized flavored e-liquids had higher concentrations of degradation products compared to unflavored e-liquids. Lastly, the aerosols produced from JUUL™ devices contained the lowest concentrations of carbonyls and CO, but the highest concentrations of nicotine. Reilly et al.[33] similarly found that aerosolized e-liquids from JUUL™ devices contained lower levels of carbonyls and free radicals, but delivered higher concentrations of nicotine compared to other e-cigarette brands and cigarettes.

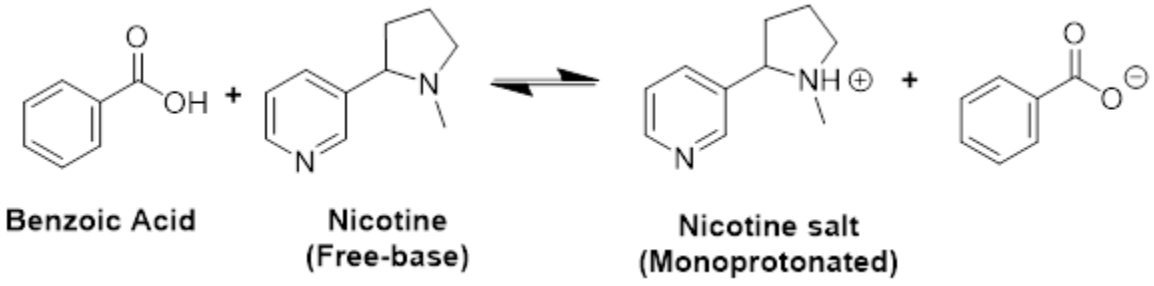
Most of the work described in this section discussed how PG:GL e-liquids can thermally degrade into toxicants upon aerosolization using different e-cigarettes, device settings, and PG:GL mol ratios. It is important for researchers to understand how and what HPHCs are derived from the carrier solvent before determining the effects of additives (e.g. flavorants and nicotine) on degradation levels. The following sections will discuss the chemistry of nicotine and flavorants in e-liquids before and after aerosolization.

1.3. Acid/Base Chemistry of Nicotine

E-liquids contain nicotine with concentrations ranging from 0 to 60 mg/mL in PG:GL.[34] First generation e-cigarettes mostly contained e-liquids with a high free-base

(fb) nicotine fraction ($\alpha_{fb} \approx 1$; nicotine in high pH e-liquids) that was harsh and difficult to inhale for the consumer (20). In 2015, JUUL™ labs was one of the first e-cigarette companies to commercialize their e-liquids with high nicotine concentrations at a lower pH. JUUL™ labs added organic acids (e.g. benzoic acid, levulinic acid, malic acid) to their e-liquids to protonate the nicotine and decrease the α_{fb} to about 0.1, thus making the nicotine more palatable and inhalable in the aerosol.[35] Monoprotonated nicotine (i.e. nicotine salt) can be formed from a reaction with nicotine and benzoic acid that exists at equilibrium (Scheme 4). Nicotine exists in the fb and monoprotonated form in e-liquids since the pH is generally not low enough for considerable amounts of diprotonated nicotine to form.

Fb nicotine is volatile and deposits in the consumer's respiratory tract in both gas phase and as PM, while monoprotonated nicotine is nonvolatile and deposits only as PM.[36] The fb nicotine in the gas phase deposits quickly in the respiratory tract to give the consumer a nicotine "hit." Monoprotonated nicotine can be inhaled more deeply into the lungs, and deposits into lung-blood interfaces by evaporative gas deposition or particle deposition with evaporation – which delays the nicotine delivery to the brain.[26,36]



Scheme 4. The formation of nicotine salts (monoprotonated) from the reaction between benzoic acid and nicotine (free-base).

Altria, previously known as Philip Morris (owner of Marlboro), owns a 35% stake in JUUL™ labs, which continues to use the same strategies as the tobacco industry did to make their products more addictive.[37,38] The decrease in α_{fb} from 1 to 0.1 in e-cigarettes mimicked the decrease in α_{fb} in cigarettes, as discussed by Duell et al. (20). Altering the pH of nicotine with organic acids in tobacco products to create an addiction is an old cigarette industry tactic used on a new product.

In earlier work on traditional cigarettes, Pankow et al. determined the equilibrium α_{fb} values in mainstream cigarette smoke PM using volatility-based measurements.[39] Prior to their study, cigarette smoke was hypothesized to exist at a pH ≈ 5.3 with trivial amounts of fb nicotine in the smoke. Pankow et al. found that the pH of tobacco smoke PM ranged from 6.0-7.8 (α_{fb} = 0.10-0.36), indicating that there was more fb nicotine in smoke PM than previously reported. Internal tobacco documents (posted online by industry in response to a 1998 litigation) from Armitage and Turner that stated the pH of mainstream smoke PM had a pH ≈ 5.3 was based on their analysis using a diluted solutions method (DSM).[40] However, Pankow et al.[40] used a native solution method (NSM) in their study. The α_{fb} determination in smoke PM and aerosols are dependent upon the protonation constants of the acid(s) and nicotine and the ratio of acid to nicotine. Diluting samples with water or organic solvents perturbs the protonation constants. Therefore, NSMs provide a better understanding of the acid/base chemistry of nicotine in tobacco product samples compared to DSMs.

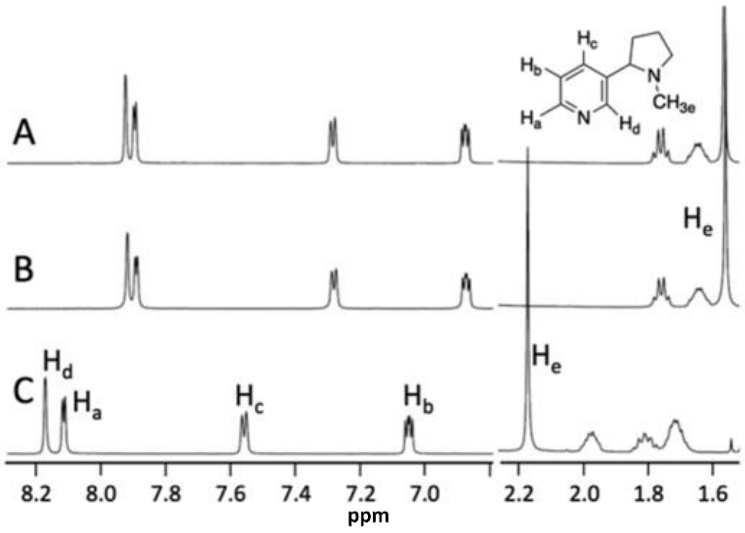


Figure 3. ^{1}H NMR spectra showing the chemical shifts (δ) for nicotine in an equimolar PG:GL e-liquid containing 24 mg/mL nicotine with (A) t-butylamine, (B) no additive, and (C) acetic acid from Duell et al.[41]

Building on this earlier work with conventional cigarette smoke, Duell et al.[41] determined the α_{fb} in e-liquids before and after aerosolization using a NSM with ^{1}H NMR spectroscopy. The chemical shifts (δ) of the aromatic protons on nicotine (H_a-H_d) were identified in an equimolar PG:GL e-liquid with 24 mg/mL nicotine. Then, *t*-butylamine or acetic acid were added to the e-liquid to make the fb and monoprotonated nicotine standards, respectively. The change in chemical shifts ($\Delta\delta = [H_a\text{-}H_d] - [H_e]$) for the fb standard, monoprotonated standard, and commercial e-liquids were determined to calculate α_{fb} for each sample (Figure 3). Duell et al. found that the α_{fb} of the e-liquid was consistent before and after aerosolization, and commercial e-liquids with high amounts of nicotine had low α_{fb} (i.e. JUUL™).

The acid/base chemistry of nicotine in e-liquids influences the palatability and addiction potential of the aerosol inhaled by the consumer. Consumers who vape nicotine

salt e-liquids immediately experience a slight nicotine “hit”, and then inhale the remaining aerosol containing the majority of nicotine, which can unexpectedly make the consumer more dependent on nicotine. However, e-liquids with high concentrations of fb nicotine are immediately harsh and exhaled from the consumers oral cavity.

1.4. The Effects of Flavorants on E-liquid Toxicity Before and During Aerosolization

Confectionary and fruity flavored e-liquids are perceived as less harmful and more attractive to adolescents compared to unflavored e-liquids.[42] Commercial e-liquids frequently contain flavorants — that are GRAS for consumption but not for inhalation – at concentrations that can be cytotoxic and cariogenic.[18,43,44,45] Greater than 100 flavorants have been identified in e-liquids, and most e-liquids contain vanillin, cinnamaldehyde, benzyl alcohol, and menthol at high concentrations.[46,47] Vreeke et al.[48] found that toxicant formation significantly increased in aerosolized equimolar PG:GL e-liquids with triacetin (a flavor enhancer) compared to those without. They identified acetic acid in aerosolized e-liquids with triacetin by NMR spectroscopy, and hypothesized that acetic acid catalyzed the thermal degradation of PG and GL. Khlystov and Samburova[49] similarly found that flavored e-liquids produced significantly higher toxicant levels compared to unflavored e-liquids upon aerosolization using three different e-cigarettes.

Several studies have examined the concentrations and cytotoxicity of flavorants in e-liquids. Tierney et al.[50] determined the concentrations of flavorants in disposable e-cigarettes and refillable e-liquids with gas chromatography-mass spectrometry (GC-MS),

and found that 13 of the 30 products analyzed contained more than 1% by weight (10 mg/mL) flavorants. A quarter of the flavorants identified were aldehydes classified as primary irritants of the respiratory tract. Erythropel et al. showed that aldehyde flavorants (e.g. citral, vanillin, cinnamaldehyde, benzaldehyde) react with PG in e-liquids to form flavorant-PG acetals with yields ranging from 40 – 95%.[51] The flavorant-PG acetal transfer efficiency from the e-liquid to the aerosol was between 50-80%, and half-life up to approximately 36 hours. Jabba et al.[52] later studied the toxicity of flavorant-PG acetals, and found that benzaldehyde- and vanillin-PG acetals increased epithelial cell mortality and were more cytotoxic compared to the parent flavorants, respectively.

Omaiye et al.[53] later analyzed 277 refill commercial e-liquids from four countries, and used a microculture tetrazolium assay (MTT) to analyze the cytotoxicity of menthol and ethyl maltol, which are two of the most common flavorants found in e-liquids. About 85% of the e-liquids contained greater than 1 mg/mL, and 37% contained greater than 10 mg/mL total flavorant concentration. They identified 155 flavorants in the 277 e-liquids, and found menthol, triacetin, cinnamaldehyde, ethyl maltol in greater than 50% of the e-liquids. Nicotine was present in 170 of the 277 e-liquids, and 56% of the nicotine e-liquids contained 2-fold the concentration of flavorants compared to nicotine. Lastly, the MTT revealed that some e-liquids contained 30-, 100-, and 100,000-times the cytotoxic levels for menthol, ethyl maltol, and cinnamaldehyde (based on Behar et al.'s[54] value for cinnamaldehyde). Their findings support the need for regulations on the amounts and types of flavorants added to e-liquids.

High-intensity synthetic sweeteners (also GRAS for consumption but not inhalation) have been added to e-liquids to increase their sweetness.[18] Sucralose, which is ~600 times sweeter than sucrose, can be added to e-liquids or sold separately for consumers to personally add.[55] Sucralose and sucrose have similar structures, but sucralose has three chlorines instead of alcohols.

Duell et al.[56] investigated the effects of sucralose on e-liquid degradation by aerosolizing PG:GL e-liquids with 0, 0.05, 0.075, and 0.10 mol% sucralose, and analyzed the aerosols with NMR spectroscopy, ion chromatography (IC), and GC-MS. They found significantly higher toxicant levels in aerosolized e-liquids with sucralose versus without. The significant increase in degradation levels were hypothesized to be associated with the production of HCl from sucralose degradation. Rahn and Yaylayan[57] determined that sucralose produces 2 mols of HCl, 2 mols of H_2O, and various chloropropanols upon thermal degradation in GL. The presence and absence of sucralose in sucralose-containing e-liquids before and after aerosolization, respectively, was revealed using GC-MS and IC. To further examine the possibility that sucralose degrades when aerosolized, the α_{fb} of sucralose-containing e-liquids were analyzed before and after aerosolization with NMR spectroscopy. The α_{fb} of the e-liquids decreased from 1 to 0.75 upon aerosolization, and equated to ~2 protons being released for every vaped molecule of sucralose, which was consistent with the 2 mols of HCl produced upon sucralose degradation in GL.[57] The presence of sucralose's degradation products and decrease α_{fb} upon aerosolization supported Duell et al.'s hypothesis that sucralose thermally degraded in the aerosolized e-liquid.

The above studies have highlighted how flavorants can alter the toxicity of e-liquids before and during aerosolization. Unvaped e-liquids can contain flavorants that exceed cytotoxic levels in epithelial cells, and react with PG and GL to form adducts (e.g. acetals and ketals) with unique toxicity profiles. Cytotoxic flavorants (and their adducts) in e-liquids can be transferred into the aerosol inhaled by consumers. Aerosolized e-liquids with flavorants have also been shown to increase the toxicant levels compared to without, and produce unique toxicants.

1.5. Introduction to this work

Building on the studies described above, the present work will emphasize the effects of flavorants in e-liquids before and during aerosolization in the presence of nicotine and other common additives using NMR spectroscopy. The following studies discuss a) the effects of common e-liquid flavorants and flavorants+nicotine on toxicant formation upon aerosolization, and b) the kinetics of aldehyde flavorant-acetal formation in e-liquids with different solvents and common additives before aerosolization. The most common and concentrated flavorants identified in commercial e-liquids were chosen for these studies – specifically, *trans*-cinnamaldehyde, benzyl alcohol, vanillin, and benzaldehyde. The common additives included water, nicotine, benzoic acid, and nicotine+benzoic acid mixtures.

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2. The Effects of Common E-liquid Flavorants and Added Nicotine on Toxicant Formation during Vaping Analyzed by ^{1}H NMR Spectroscopy

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2.1. Abstract

A broad variety of e-liquids are used by e-cigarette consumers. Additives to the e-liquid carrier solvents, propylene glycol and glycerol, often include flavorants and nicotine at various concentrations. Flavorants in general have been reported to increase toxicant formation in e-cigarette aerosols, yet there is still much that remains unknown about the effects of flavorants, nicotine, and flavorants + nicotine on harmful and potentially harmful constituents (HPHCs) when aerosolizing e-liquids. Common flavorants benzaldehyde, vanillin, benzyl alcohol, and trans-cinnamaldehyde have been identified as some of the most concentrated flavorants in some commercial e-liquids, yet there is limited information on their effects on HPHC formation. E-liquids containing flavorants + nicotine are also common, but the specific effects of flavorants + nicotine on toxicant formation remain understudied. We used ^{1}H NMR spectroscopy to evaluate HPHCs and herein report that benzaldehyde, vanillin, benzyl alcohol, trans-cinnamaldehyde, and mixtures of these flavorants significantly increased toxicant formation produced during e-liquid aerosolization compared to unflavored e-liquids. However, e-liquids aerosolized with flavorants + nicotine decreased the HPHCs for benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture" but increased the HPHCs for e-liquids containing trans-cinnamaldehyde compared to e-liquids with flavorants and no nicotine. We determined how nicotine affects the production of HPHCs from e-liquids with flavorant + nicotine versus flavorant, herein referred to as the "nicotine degradation factor". Benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture" with nicotine showed lower HPHC levels, having nicotine degradation factors <1 for acetaldehyde, acrolein,

and total formaldehyde. HPHC formation was most inhibited in e-liquids containing vanillin + nicotine, with a degradation factor of ~0.5, while trans-cinnamaldehyde gave more HPHC formation when nicotine was present, with a degradation factor of ~2.5 under the conditions studied. Thus, the effects of flavorant molecules and nicotine are complex and warrant further studies on their impacts in other e-liquid formulations as well as with more devices and heating element types.

2.2. Introduction

Electronic cigarettes (e-cigarettes) continue to be popular in the United States despite a limited understanding of their toxicity. As of 2020, ~20% of high school students reported using e-cigarettes.[1] Despite their prevalence, the potential harmfulness of specific e-cigarettes and components still needs to be assessed. Variables such as device types, coil resistances, device wattages, e-liquid compositions, and vaping patterns can impact the degree to which e-cigarettes may be harmful. Aspects of e-cigarettes that can expose consumers to potential harm include the production of carbonyls during vaping,[2] e-liquid components (e.g., flavorants),[3] and the release of metals mostly from e-cigarette heating coils.[4] Herein, we analyze the impact of individual e-liquid components (i.e. nicotine and common flavorants) on carbonyl production during vaping.

E-liquids typically contain a fluid – made up of propylene glycol (PG) and/or glycerol (GL), nicotine, and flavorants – that can be aerosolized during vaping. Some degradation can occur when vaping the PG and GL solvent, and consequently produce harmful and potentially harmful constituents (HPHCs) as reported by Jensen et al.[5] Li et al.[6] found that aerosolizing different PG:GL mol ratios (i.e. 100:0, 70:30, 50:50, 30:70,

0:100) produced varying levels of carbonyls with high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS). We chose a 50:50 PG:GL mol ratio as the standard for this study. The levels of these components, which generally encompass the majority of the HPHCs, can be compared to assess the effect of a particular chemical on degradation.

The addition of flavorants to e-liquids can produce higher levels of HPHCs as well as novel flavorant toxicants.[7, 8] Furthermore, Gillman et al.[9] and Khlystov & Samburova[10] reported that vaping flavored commercial e-liquids, which contain a mix of flavorants, can increase the formation of aldehydes compared to vaping unflavored e-liquids. Triacetin (a flavor enhancer) was shown by Vreeke et al.[11] to enhance the levels of degradation products. Sweeteners (e.g. sucralose) are also common additives, and sucralose was shown to increase the HPHC aldehyde levels in aerosols, as compared to aerosols from unflavored e-liquids.[12, 13] Thus, the effect of individual flavorants on toxicant formation needs to be assessed further, in particular, for the most common and most concentrated molecules in e-liquids.

Vanillin (vanilla flavor), benzyl alcohol (cherry/fruity/floral flavor), benzaldehyde (cherry/fruity/nutty flavor), and *trans*-cinnamaldehyde (cinnamon flavor) are among the most popular flavorants in commercial e-liquids as reported by Behar et al.[14] *Trans*-cinnamaldehyde is one of the most concerning flavorants analyzed as it is typically present at high concentrations in cinnamon flavored e-liquids and has been linked with cytotoxicity,[15] adverse effects on cardiovascular function during early development of zebrafish embryos,[16] impairment of respiratory immune cell function,[17] disruption of

mitochondrial function and inhibition of bioenergetic processes,[18] and oxidative stress in human osteoblast-like cells.[19] Benzaldehyde is present in many e-liquids, and is especially concentrated in cherry flavored e-liquids, despite being known to cause respiratory tract irritation.[20] Multiple flavorants, including ethyl maltol, ethyl vanillin, and citral, have been found to promote free radical formation during vaping.[21]

Despite the prevalence of nicotine in e-liquids, there is limited information about the effect of nicotine on flavorant and PG+GL degradation. Talih et al.[22] theorized that e-cigarette consumers may be exposed to greater levels of carbonyls when vaping e-liquids with lower nicotine concentrations due to possible self-regulated nicotine dosing (i.e. vaping more overall in order to achieve a particular total nicotine intake). Baker et al.[23] conducted a study that showed that consumers self-regulated ("titrated") their nicotine intake when provided with a lower nicotine e-liquid to achieve a particular total nicotine dose, which was independent of flavorants.

Herein, we used ^{1}H NMR spectroscopy to analyze the aerosols produced by vaping PG+GL e-liquids without and with flavorants and flavorants+nicotine. The HPHC levels in these aerosol samples were compared with those from unflavored e-liquids to determine the effects of these common e-liquid additives individually and together.

2.3. Materials & Methods

2.3.1. Materials

USP grade propylene glycol (PG), USP grade glycerol (GL), benzaldehyde (>99%), and styrene (>99%) were purchased from Sigma-Aldrich (St. Louis, MO). (*S*)-(-

)-nicotine (99%) and vanillin (>99%) were purchased from Alfa Aesar (Ward Hill, MA). Benzyl alcohol (>99%), *trans*-cinnamaldehyde (>98%), and *trans*-cinnamic acid (>99.8%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). "Unicorn Blood" with 6 mg/mL nicotine was purchased online from Fuzion Vapor. The commercial e-liquid "Unicorn Blood" was chosen because it contains nicotine and sucralose (which we have previously shown leads to increased production of carbonyl degradants).[12] The procedure we used to aerosolize the "Unicorn Blood" e-liquid with a refillable tank e-cigarette is given in the caption of Figure S1. Benzene (>99.7%) was purchased from EMD Millipore Corporation (Billerica, MA). Toluene (>99%) was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ). DMSO-d_6 (D 99.9%) and D_2O (D 99.9%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

2.3.2. Methods

2.3.2.1. Vaping Experiments

E-liquid stock containing equimolar quantities of PG and GL was prepared. Aliquots of this stock were then combined with either: 2.5 mg/mL benzaldehyde, 31 mg/mL vanillin, 39 mg/mL benzyl alcohol, 39 mg/mL *trans*-cinnamaldehyde, 155 mg/mL *trans*-cinnamaldehyde or a "flavorant mixture" (0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde). Lastly, aliquots of the PG+GL+flavorant mixtures were combined with 6 mg/mL nicotine. The concentrations of flavorants were selected based upon commercial e-liquid values reported by Behar et al.[14] The chosen nicotine concentration is common and

within the range of observed values (0 – 60 mg/mL) in commercial e-liquids.[24] All ratios were verified by ^{1}H NMR spectroscopy.

E-liquids with flavorants were vaped in the following order: PG+GL, PG+GL+flavorant, PG+GL+flavorant+6 mg/mL nicotine, and then PG+GL. The initial and final aerosolized PG+GL degradation levels were compared to demonstrate that the sequence of vaping experiments did not damage the coil in each series, which would have been shown by significantly increased degradation in the final PG+GL aerosol versus initial. The second PG+GL condition was aerosolized last for every experiment except for one trial with 2.5 mg/mL benzaldehyde, 31 mg/mL vanillin, 39 mg/mL benzyl alcohol, and 155 mg/mL *trans*-cinnamaldehyde as the flavorant. Each experiment was repeated with 3 separate coils of the same type/brand. In addition, a set of e-liquids without flavorants were vaped in the following order: PG+GL, PG+GL+6 mg/mL nicotine, and PG+GL.

Devices used, setup, collection methods including the sample puff protocol, and NMR parameters were detailed previously.[25-27] The power button was pressed one second prior to the start of each puff, and followed the CORESTA puff protocol.[25] All samples were collected using a Kangertech Subtank Mini (equipped with a 1.2 Ω coil) attached to a KBOX Mini (Kangertech; Shenzen, China) using 22 watts.

New coils were conditioned with 10 puffs at 26 watts prior to first time use per previous methods.[26] Ten or 20 "wicking puffs" at 22 watts were done using each new e-liquid condition prior to sample collection. Samples (3 puffs/sample) were generated using 22 watts, and collected as described elsewhere.[5, 12, 28] When the e-liquid was

changed during an experiment, the tank was emptied of e-liquid and dried using lint-free tissues prior to refilling the tank with the new e-liquid. Between experiments, coils were washed with methanol and dried using a vacuum oven at room temperature. All aerosolized samples were evaluated by ^{1}H NMR spectroscopy within 1 hour of collection. The aerosol and e-liquid composition samples were prepared in DMSO-d_6, then analyzed using a 600 MHz Bruker AVANCE III NMR spectrometer using either 16 or 64 scans, a 30° observation pulse, and a 3 s relaxation delay at 25 °C.

2.3.2.2. Identification of Degradation Products Derived from Flavorants

To identify substances unambiguously, vaped PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine was independently spiked with toluene, styrene, and benzaldehyde; PG+GL+155 mg/mL *trans*-cinnamaldehyde was spiked with cinnamic acid; and PG+GL+2.5 mg/mL benzaldehyde+6 mg/mL nicotine was spiked with benzene (data not shown) to identify if the spiked substance was formed upon aerosolization. The amount of each degradation product in the aerosol samples was determined by comparing the integrations from the spiked and original samples.

2.4. Results & Discussion

2.4.1. Percent Aerosol Collected

The percent of the aerosol collected in the sample vial (%-collected) was calculated for each sample by dividing the absolute value of the change in the collected vial mass by the absolute value of the change in e-cigarette tank mass and multiplying by

100 to generate a percent. Values were then averaged for each condition and the standard deviation (SD) was calculated. The average % aerosol collected ± SD from the samples generated in each experiment are shown in Tables 1 and S1.

Table 1. The average % aerosol collected for trials 1-3 for each vaping experiment.

		Average % aerosol collected ± standard deviation				
		PG+GL	PG+GL +flavorant	PG+GL+flavorant +6 mg/mL nicotine	PG+GL	Overall
Benzaldehyde (2.5 mg/mL)	*Trial 1*	46 ± 3	59 ± 8	52 ± 2	62 ± 14	55 ± 7
	Trial 2	52 ± 5	52 ± 6	47 ± 6	44 ± 3	49 ± 4
	Trial 3	62 ± 18	50 ± 19	53 ± 10	NA[a]	55 ± 6
Vanillin (31 mg/mL)	*Trial 1*	49 ± 2	47 ± 11	58 ± 4	48 ± 6	50 ± 5
	Trial 2	61 ± 10	57 ± 6	60 ± 5	53 ± 3	58 ± 3
	Trial 3	60 ± 19	32 ± 4	46 ± 3	NA[a]	46 ± 14
Benzyl alcohol (39 mg/mL)	*Trial 1*	48 ± 6	53 ± 3	50 ± 4	46 ± 3	49 ± 3
	Trial 2	45 ± 12	52 ± 3	56 ± 5	47 ± 5	40 ± 5
	Trial 3	43 ± 6	40 ± 15	37 ± 1	NA[a]	40 ± 3
***Trans*-cinnamaldehyde (39 mg/mL)**	*Trial 1*	59 ± 2	57 ± 8	49 ± 4	60 ± 4	56 ± 5
	Trial 2	60 ± 1	42 ± 6	44 ± 6	56 ± 5	50 ± 9
	Trial 3	64 ± 3	55 ± 3	39 ± 7	49 ± 17	52 ± 10
***Trans*-cinnamaldehyde (155 mg/mL)**	*Trial 1*	76 ± 14	45 ± 30	20 ± 2	56 ± 15	49 ± 23
	Trial 2	82 ± 33	32 ± 17	9 ± 1	51 ± 15	44 ± 31
	Trial 3	48 ± 4	11 ± 2	8 ± 1	NA[a]	23 ± 22
"Flavorant mixture"[b]	*Trial 1*	41 ± 7	59 ± 2	62 ± 3	71 ± 4	58 ± 13
	Trial 2	56 ± 14	55 ± 3	52 ± 10	52 ± 4	54 ± 2
	Trial 3	64 ± 5	55 ± 3	57 ± 3	39 ± 8	54 ± 11

[a] Not able to be analyzed; PG+GL was not aerosolized again after the *+6 mg/mL nicotine* addition.
[b] Flavorant mixture = 0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde.

The average % aerosol collected was similar for most of the flavorants when comparing trials 1-3 (each trial represents a different coil). However, there was a notable decrease in the average % aerosol collected when vaping PG+GL e-liquids containing 155 mg/mL *trans*-cinnamaldehyde compared to the initial aerosolized PG+GL for each

trial (Table 1). The average % aerosol collected decreased less when e-liquids contained 39 mg/mL *trans*-cinnamaldehyde instead of 155 mg/mL *trans*-cinnamaldehyde (Table 1). This is similar to the report by Duell et al.,[5, 12, 28] stating that the addition of sucralose (a flavorant enhancer) to e-liquids also can alter the % aerosol collected compared to the PG+GL only conditions. Aldehydes can polymerize, form hemiacetals, and/or form acetals in the PG+GL mixture, which could alter the particulate matter (PM) and gas phase fractions in the aerosol, thereby causing variations in the % aerosol collected.[29, 30]

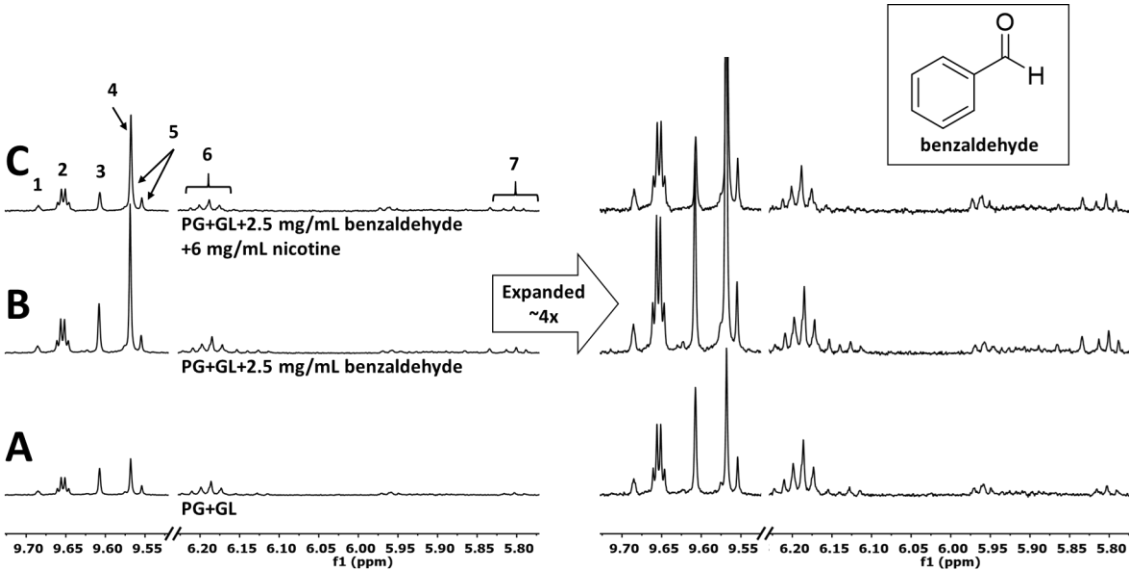

Figure 1. The ^{1}H NMR spectra for aerosolized (A) propylene glycol+glycerol (PG+GL), (B) PG+GL+2.5 mg/mL benzaldehyde, and (C) PG+GL+2.5 mg/mL benzaldehyde+6 mg/mL nicotine show the enhancing effects of benzaldehyde and inhibitory effects of nicotine on the degradation levels relative to the PG+GL. The intensities were normalized to the PG methyl resonance at ~1.05 ppm. The samples (3 puffs each) were aerosolized at 22 W using a 1.2 Ω coil and the CORESTA puff method. 1 = propanal; 2 = acetaldehyde; 3 = glycolaldehyde; 4 = formaldehyde; 5 = acrolein; 6 = formaldehyde hemiacetals; 7 = 5.8 ppm multiple addition product (MAP).

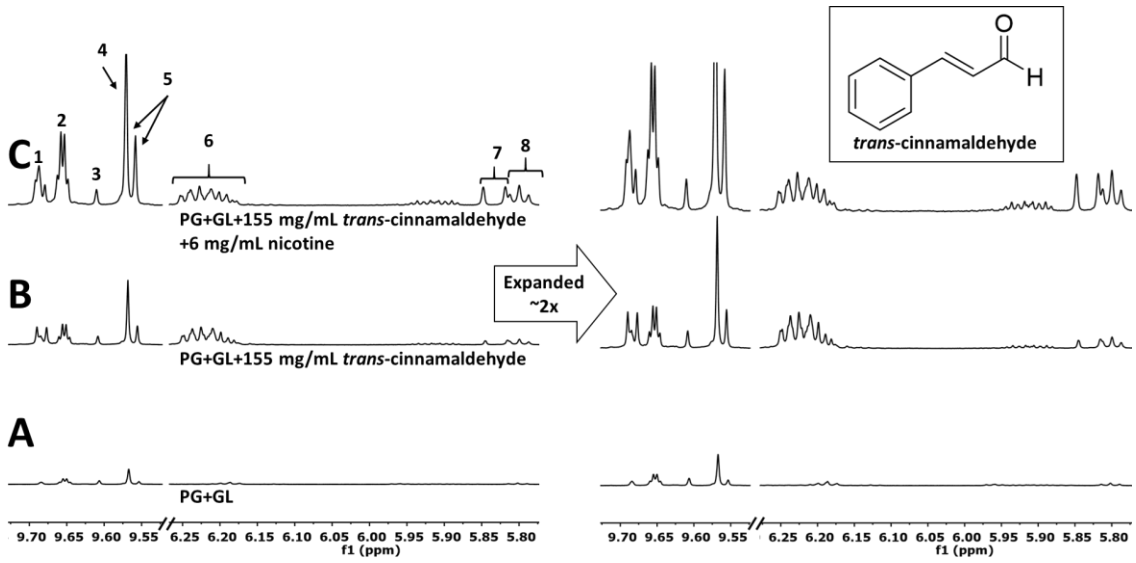

Figure 2. The [1]H NMR spectra for the aerosolized (A) propylene glycol+glycerol (PG+GL), (B) PG+GL+155 mg/mL trans-cinnamaldehyde, and (C) PG+GL+155 mg/mL trans-cinnamaldehyde+6 mg/mL nicotine illustrate the enhancing effects of trans-cinnamaldehyde and nicotine on the levels of degradation relative to PG+GL. The samples (3 puffs each) were generated at 22 W using a 1.2 Ω coil and the CORESTA puff method. The intensities were normalized to the PG methyl resonance at ~1.05 ppm. 1 = propanal; 2 = acetaldehyde; 3 = glycolaldehyde; 4 = formaldehyde; 5 = acrolein; 6 = trans-cinnamaldehyde-acetal peaks overlapped the formaldehyde hemiacetals; 7 = styrene; 8 = 5.8 ppm multiple addition product (MAP).

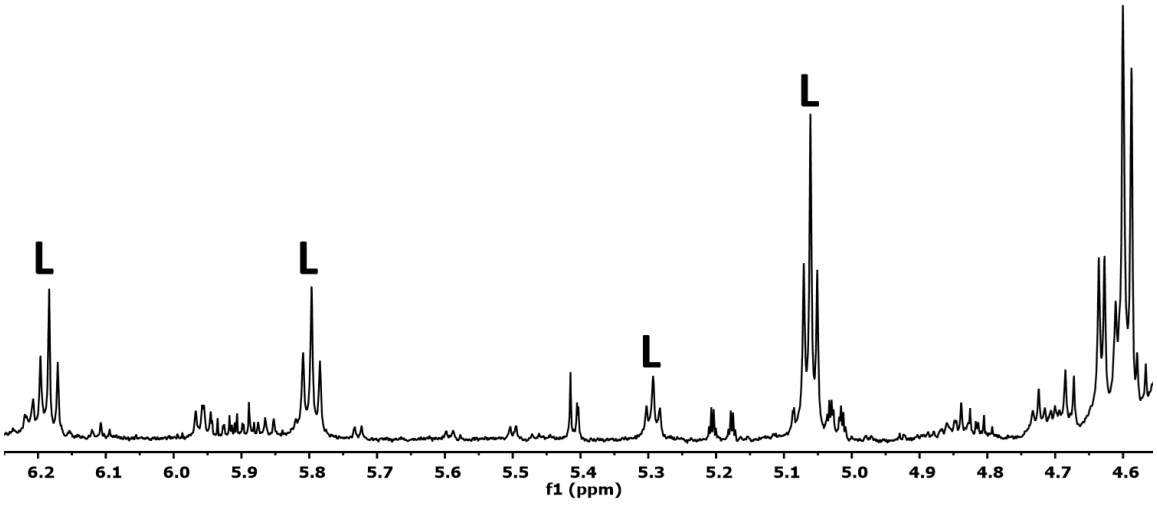

Figure 3. The [1]H NMR spectrum for vaped pure glycerol in DMSO-d_6. These peaks labeled "L" are labile (in the context of e-liquids, hemiacetal $–CH_2–OH$ resonances that are coupled to upfield doublets (See Figure S2), which become singlets when the $–CH_2–OH$ is exchanged to form $–CH_2–OD$, as discussed in the text. The triplet in the 6.2 ppm region has already been identified as hemiacetals from propylene glycol and glycerol.[5, 28]

2.4.2. Flavorants and Flavorant+Nicotine Effects on Degradation

The levels of propanal, acetaldehyde, glycolaldehyde, acrolein, formaldehyde, formaldehyde hemiacetal (6.2 ppm), total multiple formaldehyde-addition products (sum of 5.8+5.3+5.1 ppm MAPs), and total formaldehyde (sum of formaldehyde+formaldehyde hemiacetal+total MAPs) were determined in aerosol samples by integrating their respective peaks relative to the 3-proton PG methyl peak (divided by 3 to represent 1 proton) in the ^{1}H NMR spectra (Figures 1 and 2). While mass spectrometry (MS) methods may be more generally available in labs working on e-cigarettes, NMR spectroscopy may allow detection and quantitation of species that are not directly amenable to MS. For example, Salamanca et al.[31] compared the total formaldehyde levels (formaldehyde+formaldehyde hemiacetals in their study) in aerosolized equimolar PG+GL e-liquids using 2,4-dinitrophenylhydrazine (DNPH) derivatization by HPLC with direct analysis of aerosols by NMR spectroscopy. They found that formaldehyde hemiacetals detected by NMR make up a considerable fraction of the total formaldehyde levels produced upon e-liquid aerosolization. However, the total formaldehyde levels were significantly underestimated using derivatization.[31, 32]

The MAP peaks are from formaldehyde releasing agents, similar to the formaldehyde hemiacetals identified by Jensen et al.,[28] that are formed by the addition of formaldehyde to glycerol, and exhibit triplets at 5.8, 5.3, and 5.1 ppm (Figures 3 and S1-S2). The 5.8 (Figure S1) and 5.3 ppm peaks are found when vaping GL, but not when vaping PG (with no GL). The peak at ~5.1 ppm appears to correspond to a product (again hemiacetal-like) from either solvent. Both disappear when D_2O is added, consistent with

hemiacetal –CH_2–O**H** resonances. Because the 6.2 ppm region is from the single addition products,[5, 28] we provisionally assign these 5.8, 5.3, and 5.1 ppm to MAP resonances, from both PG and GL (at 5.1 ppm) and from GL (at 5.3 and 5.8 ppm). Consistent with these assignments, the homonuclear correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) from an aerosolized "Unicorn Blood" e-liquid sample shows connectivities to upfield doublets that become singlets when the hemiacetal –CH_2–O**H** is exchanged by D_2O to form –CH_2–O**D** (Figure S2). An aerosolized "Unicorn Blood" sample was collected and analyzed since the sucralose-containing commercial e-liquid has been shown to produce high levels of HPHCs (including MAPs).[12] The high concentration of MAPs made the connectivities easier to observe on the COSY and TOCSY. We were unable to determine the integration of the formaldehyde hemiacetals, and consequently the total formaldehyde, for e-liquids containing *trans*-cinnamaldehyde due to peak overlap from the PG- and GL-*trans*-cinnamaldehyde acetals (Figure 1).

The % values, relative to the remaining PG peak, for the degradation products are shown in Table S3. The effects of additives on HPHC formation in aerosolized PG+GL e-liquids were evaluated by comparing the degradation levels of acetaldehyde, acrolein, and total formaldehyde for e-liquids without (set to 1) versus with flavorant and flavorant+nicotine (Tables 2-4). The concentrations of flavorants used were chosen based on the upper limit of values observed in commercial e-liquids.[13] Vaping e-liquids with the addition of each flavorant resulted in increased amounts of acetaldehyde, acrolein, and total formaldehyde relative to PG+GL (Tables 2-4). We also compared the HPHCs in aerosols produced from the initial and final PG+GL only e-liquids to assess coil changes

that may have occurred during the vaping process. Individual flavorants in e-liquids could thermally degrade to contribute to the levels of HPHC formation. *Trans*-cinnamaldehyde (an α,β-unsaturated aldehyde) could undergo nucleophilic attack at the β–carbon to produce acrolein similar to *trans*-2-hexenal.[33] However, specific degradation of flavorants will be limited by the amount of flavorant present, which is typically a small relative to PG and GL.

There was a decrease in HPHCs for e-liquids containing benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture" when aerosolized with 6 mg/mL nicotine versus without nicotine (Tables 2-4). The basicity of nicotine would decrease the HPHCs in aerosols from e-liquids with benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture" if the primary thermal degradation mechanism is acid-catalyzed. For example, sucralose and triacetin can thermally degrade into hydrochloric acid and acetic acid that were shown to enhance degradation levels, respectively.[11, 12] However, the degradation levels increased in aerosolized *trans*-cinnamaldehyde-containing PG+GL e-liquids (39 and 155 mg/mL *trans*-cinnamaldehyde) with 6 mg/mL nicotine versus without (Tables 2-4). *Trans*-cinnamaldehyde can initially be oxidized to produce acids that promote the neutralization of nicotine and promote degradation during aerosolization. Friedman et al.[34] showed that *trans*-cinnamaldehyde in food products and essential oils can be oxidized with heat to produce benzaldehyde and glyoxal. Yu et al.[35] used gas chromatography–mass spectrometry (GC-MS) to identify oxidation products from *trans*-cinnamaldehyde, finding acetaldehyde, benzaldehyde, benzoic acid, and cinnamic acid as some of the main oxidation products.

The effect of nicotine on degradation in aerosolized e-liquids with flavorants was determined by dividing the degradation levels of "PG+GL+flavorant+6 mg/mL nicotine" by "PG+GL+flavorant" in Tables 2-4. The average values (± SD) for the "nicotine degradation factors" are shown in Table 5. The nicotine degradation factors for acetaldehyde, acrolein, and total formaldehyde were similar for each flavorant (Table 5). E-liquids with benzaldehyde, vanillin, benzyl alcohol, and the "flavorant mixture" had nicotine degradation factors less than 1 (where 1 = no observed effect), thereby inhibiting HPHC formation (Table 5). Vanillin was the flavorant that generated toxicants that were most inhibited by a nicotine degradation factor of ~0.5 (Table 5). E-liquids with the greatest promoted toxicant formation contained 39 and 155 mg/mL *trans*-cinnamaldehyde, and had nicotine degradation factors of 2.3 and 2.9, respectively (Table 5).

The interactions of nicotine with the e-cigarette solvents, flavorants, and metal coil could further alter toxicant formation upon aerosolization. Son et al.[36] found that hydroxyl radical levels were slightly higher in aerosolized GL and PG+GL e-liquids when the nicotine concentration was higher; aerosolized PG e-liquids had higher hydroxyl radical levels when the nicotine concentration was lower. Bhagwat et al.[37] observed an increase in lipid peroxidation products when rat brain tissues were exposed to chronic levels of nicotine (1.6 mg/kg/day) daily for a 10 day period, indicating that nicotine had oxidative properties. However, Linert et al.[38] found that nicotine could be an antioxidant with its ability to bind Fe^{2+} and reduce transferrin-mediated Fe uptake in rat

brain tissue. The role of nicotine as a pro-oxidant or antioxidant in flavored and unflavored e-liquids during aerosolization is unknown and requires further study.

The effect of 6 mg/mL nicotine on toxicant formation was determined by aerosolizing e-liquids containing PG+GL, followed by PG+GL+6 mg/mL nicotine, and PG+GL (to compare the final and initial degradation levels). The average % aerosol collected for each trial is shown in Table S1, and the HPHC levels produced upon aerosolization are shown in Table S3. The degradation levels for acetaldehyde, acrolein, and total formaldehyde were similar in aerosolized e-liquids with and without nicotine (Table S2). The average nicotine degradation factor (degradation levels of "PG+GL+6 mg/mL nicotine" divided by the average initial "PG+GL") was 1, which indicates nicotine had no effect on the HPHCs formed upon aerosolization (Table 5).

We analyzed the composition of e-liquids containing benzaldehyde, vanillin, and *trans*-cinnamaldehyde over time and observed that the composition changed as determined by ^{1}H NMR. The e-liquids with aldehyde flavorants formed acetals with PG- and GL, as indicated by the new peaks in the aged *trans*-cinnamaldehyde e-liquids (Figure S3). Erythropel et al.[29] reported that *trans*-cinnamaldehyde, benzaldehyde, and vanillin form and reach equilibrium with PG-acetal conversions up to ~92% in 1 day, ~95% in 5 days, and ~40% in 7 days, respectively. We did not observe a difference in the HPHCs produced from aerosolized e-liquids with aldehyde flavorants before and after they reached equilibrium with their respective PG-acetals, which is consistent with the values reported by Erythropel et al.[29] The PG-flavorant acetals had a similar effect as the parent flavorant on HPHCs produced upon e-liquid aerosolization under our conditions.

Similar to what was reported by Erythropel et al.,[39] we noticed that the PG- and GL-flavorant acetals carried over into the aerosols. The differences in degradation levels from each trial with flavorants were more likely associated with the quality of the coil used in each experiment[12] instead of acetal versus aldehyde presence in the e-liquid.

By the time consumers purchase e-liquids flavored with aldehydes, the PG- and GL-flavorant acetals have likely reached equilibrium. The PG- and GL-flavorant acetals can have different toxicological properties than the individual solvents and flavorants. Jabba et al.[40] reported that PG-flavorant acetals were cytotoxic to pulmonary epithelial cells and hindered mitochondrial function generally more than the parent flavorants. According to the results reported herein, consumers can also be exposed to higher levels of carbonyls when vaping flavored e-liquids compared to unflavored e-liquids,[7, 8, 10] although consumers who vape flavored e-liquids with nicotine can be exposed to higher or lower amounts of carbonyls compared to flavored e-liquids without nicotine, depending on the specific flavorants. El-Hellani et al.[41] and Reilly et al.[42] inferred that nicotine did not affect carbonyl and oxidant production, but under our conditions we found that nicotine can promote, inhibit, or have no effect on HPHC formation, depending on the conditions including the identities of the flavorants.

Table 2. The levels of the degradation product acetaldehyde, normalized to the amount formed by aerosolization of equimolar PG+GL, followed by sequential addition of the indicated flavorants, then 6 mg/mL nicotine.

		Acetaldehyde (normalized relative to that formed by PG+GL only) ± standard deviation					
		Benzaldehyde (2.5 mg/mL)	Vanillin (31 mg/mL)	Benzyl alcohol (39 mg/mL)	*Trans*-cinnamaldehyde (39 mg/mL)	*Trans*-cinnamaldehyde (155 mg/mL)	"Flavorant mixture"[a]
Trial 1	*PG+GL*	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.2
	+Flavorant	1.6 ± 0.2	2.1 ± 0.2	6.8 ± 0.6	62.6 ± 2.6	48.6 ± 12.4	17.2 ± 0.5
	+6 mg/mL Nicotine	1.3 ± 0.1	0.9 ± 0.2	4.9 ± 0.4	196.8 ± 4.6	164.8 ± 25.5	13.5 ± 1.0
Trial 2	*PG+GL*	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.7	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
	+Flavorant	1.6 ± 0.2	2.8 ± 0.4	9.1 ± 0.7	128.1 ± 16.8	4.3 ± 0.2	20.8 ± 2.1
	+6 mg/mL Nicotine	1.1 ± 0.1	0.9 ± 0.2	7.4 ± 0.4	227.6 ± 10.8	14.9 ± 0.7	14.9 ± 1.8
Trial 3	*PG+GL*	1.0 ± 0.3[b]	1.0 ± 0.2[b]	1.0 ± 0.4[b]	1.0 ± 0.6	1.0 ± 0.1[b]	1.0 ± 0.7
	+Flavorant	1.7 ± 0.1	2.0 ± 0.5	39.3 ± 2.9	267.3 ± 7.0	5.5 ± 0.5	8.8 ± 0.7
	+6 mg/mL Nicotine	1.1 ± 0.1	1.1 ± 0.2	28.4 ± 4.2	503.6 ± 21.2	9.5 ± 1.9	3.6 ± 0.5

[a] Flavorant mixture = 0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde.

[b] PG+GL was not aerosolized again after the *+6 mg/mL nicotine* addition.

Table 3. The levels of the degradation product acrolein, normalized to the amount formed by aerosolization of equimolar PG+GL, followed by sequential addition of the indicated flavorants, then 6 mg/mL nicotine.

		Acrolein (normalized relative to that formed by PG+GL only) ± standard deviation					
		Benzaldehyde (2.5 mg/mL)	Vanillin (31 mg/mL)	Benzyl alcohol (39 mg/mL)	*Trans*-cinnamaldehyde (39 mg/mL)	*Trans*-cinnamaldehyde (155 mg/mL)	"Flavorant mixture"[a]
Trial 1	*PG+GL*	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.6
	+Flavorant	1.5 ± 0.2	3.6 ± 0.3	6.4 ± 0.5	28.7 ± 0.5	66.1 ± 13.7	12.5 ± 0.3
	+6 mg/mL Nicotine	1.3 ± 0.1	1.4 ± 0.2	4.8 ± 0.2	97.2 ± 3.9	212.1 ± 32.6	8.4 ± 0.9
Trial 2	*PG+GL*	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.5	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.3
	+Flavorant	1.5 ± 0.3	4.3 ± 0.9	7.6 ± 0.6	145.4 ± 9.9	8.8 ± 1.2	24.1 ± 2.7
	+6 mg/mL Nicotine	1.2 ± 0.1	1.2 ± 0.5	6.4 ± 0.3	243.8 ± 21.3	31.3 ± 1.6	15.3 ± 3.0
Trial 3	*PG+GL*	1.0 ± 0.3[b]	1.0 ± 0.3[b]	1.0 ± 0.3[b]	1.0 ± 0.1	1.0 ± 0.2[b]	1.0 ± 0.7
	+Flavorant	1.6 ± 0.1	2.1 ± 0.6	36.7 ± 2.1	296.0 ± 19.1	8.6 ± 1.0	26.2 ± 3.2
	+6 mg/mL Nicotine	1.3 ± 0.1	1.4 ± 0.2	27.6 ± 4.8	541.7 ± 19.2	17.0 ± 3.8	9.1 ± 1.4

[a] Flavorant mixture = 0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde.

[b] PG+GL was not aerosolized again after the *+6 mg/mL nicotine* addition.

Table 4. The levels of the degradation product total formaldehyde[a], normalized to the amount formed by aerosolization of equimolar PG+GL, followed by sequential addition of the indicated flavorants, then 6 mg/mL nicotine.

		Total formaldehyde[a] (normalized relative to that formed by PG+GL only) ± standard deviation					
		Benzaldehyde (2.5 mg/mL)	Vanillin (31 mg/mL)	Benzyl alcohol (39 mg/mL)	*Trans*-cinnamaldehyde (39 mg/mL)	*Trans*-cinnamaldehyde (155 mg/mL)	"Flavorant mixture"[b]
Trial 1	*PG+GL*	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	NA[d]	NA[d]	NA[d]
	+Flavorant	1.6 ± 0.2	1.7 ± 0.4	3.2 ± 0.3	NA[d]	NA[d]	NA[d]
	+6 mg/mL Nicotine	1.2 ± 0.1	0.9 ± 0.1	2.7 ± 0.1	NA[d]	NA[d]	NA[d]
Trial 2	*PG+GL*	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.3	NA[d]	NA[d]	NA[d]
	+Flavorant	1.6 ± 0.1	1.7 ± 0.2	6.4 ± 0.6	NA[d]	NA[d]	NA[d]
	+6 mg/mL Nicotine	0.8 ± 0.1	0.5 ± 0.2	5.4 ± 0.4	NA[d]	NA[d]	NA[d]
Trial 3	*PG+GL*	1.0 ± 0.2[c]	1.0 ± 0.3[c]	1.0 ± 0.1[c]	NA[d]	NA[cd]	NA[d]
	+Flavorant	1.6 ± 0.1	1.6 ± 0.2	22.6 ± 1.1	NA[d]	NA[d]	NA[d]
	+6 mg/mL Nicotine	0.8 ± 0.2	0.9 ± 0.2	18.5 ± 2.8	NA[d]	NA[d]	NA[d]

[a] Formaldehyde+formaldehyde hemiacetal+total MAPs; total MAPs = 5.8+5.3+5.1 ppm multiple addition products.
[b] Flavorant mixture = 0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde.
[c] PG+GL was not aerosolized again after the *+6 mg/mL nicotine* addition.
[d] Not able to be analyzed due to peak overlap.

Table 5. The average nicotine degradation factors (levels of degradation of *+6 mg/mL nicotine*/*+flavorant*) for acetaldehyde, acrolein, and total formaldehyde[a]

	Average nicotine degradation factor (+6 mg/mL nicotine/+Flavorant) ± standard deviation						
	No flavorant [b]	Benzaldehyde (2.5 mg/mL)	Vanillin (31 mg/mL)	Benzyl alcohol (39 mg/mL)	*Trans*-cinnamaldehyde (39 mg/mL)	*Trans*-cinnamaldehyde (155 mg/mL)	"Flavorant mixture"[c]
Acetaldehyde	1.0 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	2.3 ± 0.8	2.9 ± 1.0	0.6 ± 0.2
Acrolein	1.0 ± 0.1	0.8 ± 0.1	0.5 ± 0.2	0.8 ± 0.1	2.3 ± 0.9	2.9 ± 0.8	0.6 ± 0.2
Total formaldehyde[a]	1.0 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	NA[d]	NA[d]	NA[d]

[a] Formaldehyde+formaldehyde hemiacetal+total MAPs; total MAPs = 5.8+5.3+5.1 ppm multiple addition products.
[b] The nicotine degradation factor was calculated by dividing the *+6 mg/mL nicotine* by *PG+GL*.
[c] Flavorant mixture = 0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL *trans*-cinnamaldehyde.
[d] Not able to be analyzed due to peak overlap.

2.4.3. Toxicological Implications of Degradation Products Derived from Flavorants

Aerosolized PG+GL e-liquids containing *trans*-cinnamaldehyde were individually spiked with benzaldehyde, *trans*-cinnamic acid, toluene, and styrene in order to confirm the identities of the unknown peaks in the ^{1}H NMR spectra. Also, benzene was identified as a degradation product in aerosolized e-liquids containing benzaldehyde with and without nicotine which is consistent with what was reported by Pankow et al.[43] As noted above, Yu et al.[35] identified benzaldehyde and *trans*-cinnamic acid as oxidation products of *trans*-cinnamaldehyde, and Li et al.[44] identified styrene and toluene as pyrolysis products of *trans*-cinnamaldehyde.

The presence of benzaldehyde, *trans*-cinnamic acid, toluene, and styrene in aerosolized *trans*-cinnamaldehyde-containing PG+GL e-liquids (39 and 155 mg/mL *trans*-cinnamaldehyde) with and without 6 mg/mL nicotine was identified based on NMR chemical shifts and peak splitting. Benzaldehyde, toluene, and styrene were individually spiked into NMR samples containing aerosolized e-liquids with 155 mg/mL *trans*-cinnamaldehyde and 6 mg/mL nicotine (Figures S4-S6). *Trans*-cinnamic acid was spiked into NMR samples containing aerosolized e-liquids with 155 mg/mL *trans*-cinnamaldehyde (Figure S7). The benzaldehyde, toluene, and styrene resonances were not present in the previous aerosolized PG+GL or unvaped *trans*-cinnamaldehyde-containing e-liquid samples indicating that they were formed during aerosolization (Figures S4-S6). The *trans*-cinnamic acid peaks were not observed in the aerosolized PG+GL, but were observed in the unvaped *trans*-cinnamaldehyde-containing e-liquid, and then formed ~2x more during aerosolization (Figure S7). We estimated that 1×10^{-4}, 3×10^{-4}, 0.05, and 0.02 mg/puff benzaldehyde, *trans*-cinnamic acid, toluene, and styrene were formed in each aerosol, respectively, under our conditions (Figures S4-S7).

The Environmental Protection Agency's (EPA) Integrated Risk Information System (IRIS) determined that the no-observed-adverse-effect level (NOAEL)for toluene was 46 mg/m^3 per day (1.9 mg/m^3 per 1 h) for human subjects.[45] The physiological daily inhalation rate (PDIR) of 17.48 m^3/day (0.73 m^3/h) for 23-30 yr old males was used to estimate the breath volume.[46] The IRIS limit per hour for toluene would be 1.40 mg/h based on the chosen inhalation rate. The e-cigarette used in this study produced 0.05 mg/puff toluene at 22 W at a flow rate of 18.3 mL/s. Kośmider et al.[47] found that the

average number of puffs per day for 24 adult e-cigarette consumers was 156 puffs/day (~7 puffs/h). At 7 puffs/h the rate of toluene inhalation would be 0.35 mg/h which does not exceed the IRIS limit, and does not account for any aerosol exhaled.

The EPA determined that the Acute Exposure Guideline Level (AEGL-1) for nondisabling effects of styrene inhalation in the central nervous system of humans was 85 mg/m^3 per hour.[48] Using the PDIR for 23-30 yr old males of 17.48 m^3/day (0.73 m^3/h) yields an AEGL-1 limit of 62.1 mg/h.[46] If 156 puffs/day[47] (7 puffs/h) were inhaled using the e-cigarette and e-liquid in this study at 22 W, a flow rate of 18.3 mL/s, and 0.02 mg/puff the consumer would inhale styrene at a rate of 0.14 mg/h. Under our conditions the levels of styrene inhaled do not exceed the AEGL-1 limit (also assuming no aerosol is exhaled). The consumer could be exposed to higher concentrations of toluene and styrene by vaping with a higher power setting (>22 W)[41] and/or having a higher concentration of *trans*-cinnamaldehyde (>155 mg/mL) in the e-liquid.[42] Inhaling any styrene and/or toluene is concerning due to classifications as a Group 2A probable human carcinogen and nervous system depressant, respectively.[45, 49]

Yu et al.[35] found that the oxidation of *trans*-cinnamaldehyde to benzaldehyde formed more readily at higher temperatures and involved oxidative cleavage; however, the oxidation of *trans*-cinnamaldehyde to *trans*-cinnamic acid was less dependent on temperature than the formation of benzaldehyde. The *trans*-cinnamaldehyde in e-liquids underwent partial oxidation during storage at room temperature resulting in *trans*-cinnamic acid formation (Figure S7). Li et al. reported that toluene and styrene were produced upon the pyrolysis of *trans*-cinnamaldehyde,[38] and proposed seven possible

pathways for styrene to form, many of which begin with the H radical addition to or abstraction from *trans*-cinnamaldehyde (Figures S4 and S5). Toluene and styrene were previously identified as degradation products from e-cigarettes through GC-MS analysis by others, but conversion to toluene or styrene from cinnamaldehyde was not reported.[50] The presence of benzaldehyde, *trans*-cinnamic acid, styrene, and toluene in aerosolized e-liquids with *trans*-cinnamaldehyde show that *trans*-cinnamaldehyde underwent oxidation and free radical cleavage during thermal degradation.

The presence of benzene was determined in the aerosolized benzaldehyde-containing PG+GL e-liquids based on the observed chemical shift (7.37 ppm) and peak shape consistent with that reported for benzene by Pankow et al.[43] The peak was not observed in the unvaped e-liquid, nor vaped samples of PG+GL. We calculated approximately 4×10^{-4} mg/puff of benzene in the aerosolized e-liquid with benzaldehyde. Benzene is carcinogenic to humans, and there is no safe level of exposure via inhalation according to the World Health Organization (WHO).[51] Pankow et al. identified benzene as a degradation product of various e-liquid mixtures (including benzaldehyde-containing e-liquids) upon vaporization, and Namysl et al. identified benzene as a pyrolysis product of benzaldehyde.[43, 52]

2.5. Conclusions

We found that benzaldehyde, vanillin, benzyl alcohol, and *trans*-cinnamaldehyde can enhance PG and GL degradation during vaping, consistent with other reports, including that e-liquids that contain greater concentrations of flavorants produce more

HPHCs (as measured by carbonyl production).[7, 10, 53] We also found that nicotine inhibited the levels of HPHC formation in the presence of benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture" when aerosolized, as compared to flavored e-liquids without nicotine. However, nicotine enhanced the levels of degradation when added to e-liquids with low and high concentrations of *trans*-cinnamaldehyde (39 and 155 mg/mL, respectively), as compared to the same e-liquids without nicotine. The effects of other common flavorants with nicotine should also be explored since there is widespread use of many different flavorants and combinations thereof,[54, 55] and because concentrations of nicotine in e-liquids can vary by brand and local regulations.[54]

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2.6.3. Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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2.6.6. Abbreviations

E-cigarette, electronic cigarette; e-liquid, electronic cigarette liquid; PG, propylene glycol; GL, glycerol; NMR, nuclear magnetic resonance; HPHC, harmful and potentially harmful constituents; PM, particulate matter; HPLC-HRMS, high performance liquid chromatography-high resolution mass spectrometry; MAP, multiple addition formaldehyde hemiacetal product; MS, mass spectrometry; DNPH 2,4-dinitrophenylhydrazine; GC-MS, gas chromatography−mass spectrometry; IRIS, Integrated Risk Information System; NOAEL, no-observed-adverse-effect level; PDIR,

physiological daily inhalation rate; EPA, Environmental Protection Agency; AEGL, acute exposure guideline levels; COSY, homonuclear correlation spectroscopy; TOCSY, total correlation spectroscopy.

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2.8. Appendix A: Supporting Information

2.8.1. Materials & Methods and Additional NMR Data for the Multiple Addition Product (MAP)

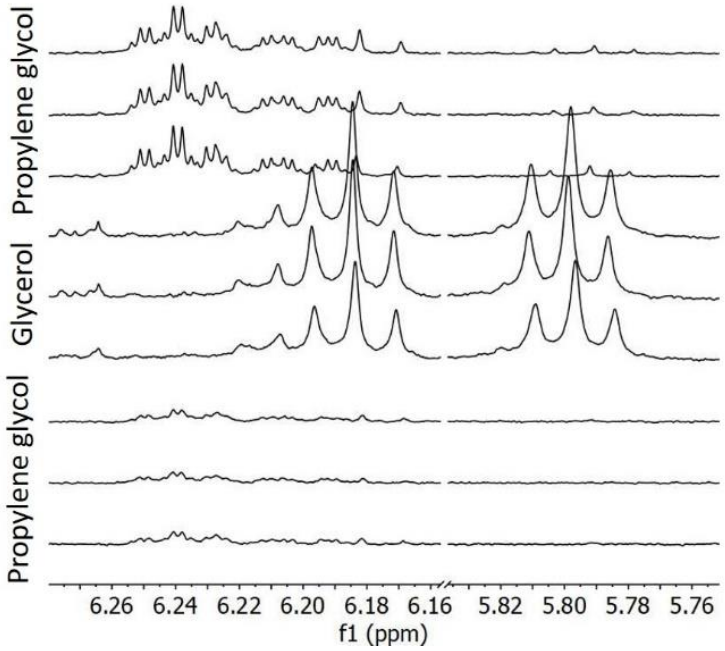

Figure 4. Spectra depict the single formaldehyde addition products (6.24 and 6.19 ppm) and the theorized multiple formaldehyde addition product (5.80 ppm MAP) by 1H NMR in either vaped propylene glycol (PG) or glycerol (GL) aerosol samples, respectively. Samples/spectra were generated in order from the bottom to the top. Three samples were collected for each condition and each sample contained 3 puffs. A Kangertech subtank mini was filled with PG, conditioned per methods described in the main manuscript, and vaped at 26 watts (bottom 3 spectra). The tank was emptied, cleaned with ethanol and lint-free tissues, and filled with GL. Wicking puffs were generated per methods described herein. Three vaped GL samples were collected. The tank was emptied, cleaned, and again filled with PG. Three samples were generated. The same 1.2 Ω coil was used for all samples. The single formaldehyde addition product was previously identified by Jensen et al.,1, 2 and can form from the addition of formaldehyde to either propylene glycol or glycerol. The 5.8 ppm MAP (shown in this figure) and 5.3 ppm MAP (not shown in this figure) only appears in the presence of glycerol and is theorized to form from the multiple addition of formaldehyde to glycerol, based on similarities to the spectra presented by Jensen et al. and 2D NMR experiments we conducted (Figure S2); these 2D experiments indicated connectivity of the 5.8 ppm triplet to at 4.6 doublet and another peak (splitting was not able to be observed due to peak overlap) at 3.4 ppm.

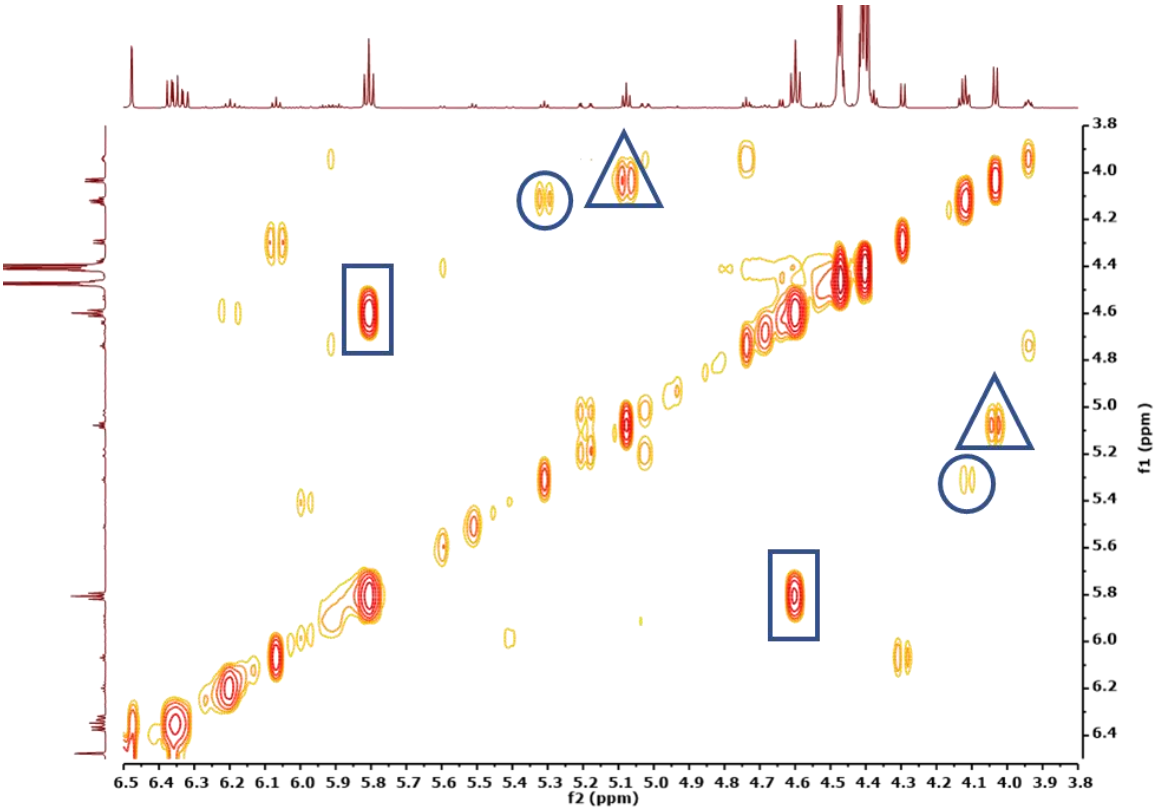

Figure 5. TOCSY of a vaped "Unicorn Blood" e-liquid sample containing the 5.8, 5.3, and 5.1 ppm MAPs (multiple addition products) showing connectivity between the MAP peaks at 5.8 (t) and 4.6 ppm (d); 5.3 ppm (t) and 4.1 ppm (d); and 5.1 ppm (t) and 4.0 ppm (d). The 5.8 (t), 5.3 (t), and 5.1 (t) ppm peaks disappeared when D2O was added to the sample. The 4.6 (d), 4.1 (d), and 4.0 (d) ppm peaks became singlets when the hemiacetal –CH2–OH is exchanged by D2O to form –CH¬2–OD. These resonances show TOCSY/COSY connectivities to the downfield triplets and are designated by boxes, circles, and triangles, respectively.

2.8.2. Additional Information Regarding the Aerosolization of E-liquids with Nicotine

Table 6. The average % aerosol collected from the experiments with and without added nicotine

		Average % aerosol collected ± standard deviation			
		PG+GL	PG+GL+6 mg/mL nicotine	PG+GL	Overall
Nicotine (6 mg/mL)	*Trial 1*	62 ± 12	69 ± 4	60 ± 6	63 ± 5
	Trial 2	41 ± 6	52 ± 9	53 ± 16	49 ± 7
	Trial 3	58 ± 1	58 ± 0	49 ± 3	55 ± 5

Table 7. The degradation levels of acetaldehyde, acrolein, and total formaldehyde[α] normalized relative to PG+GL from aerosolization of equimolar PG+GL, followed by sequential addition of 6 mg/mL nicotine

		Amount of degradation (normalized relative to PG+GL) ± standard deviation		
		Acetaldehyde	Acrolein	Total formaldehyde[α]
Trial 1	*PG+GL*	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
	+6 mg/mL Nicotine	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
Trial 2	*PG+GL*	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
	+6 mg/mL Nicotine	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
Trial 3	*PG+GL*	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
	+6 mg/mL Nicotine	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1

[α] Formaldehyde+formaldehyde hemiacetal+total MAP; total MAPs = the 5.8+5.3+5.1 ppm multiple addition products.

2.8.3. Additional Data for the Levels of Degradation from Each Experiment

Table 8. The levels of degradation for each degradation product normalized relative to PG+GL from aerosolization of equimolar PG+GL, followed by sequential addition of flavorant, then 6 mg/mL nicotine. This table can be found in the supplemental information document available online.

2.8.4. ^{1}H NMR of Aged E-liquids with *Trans*-cinnamaldehyde

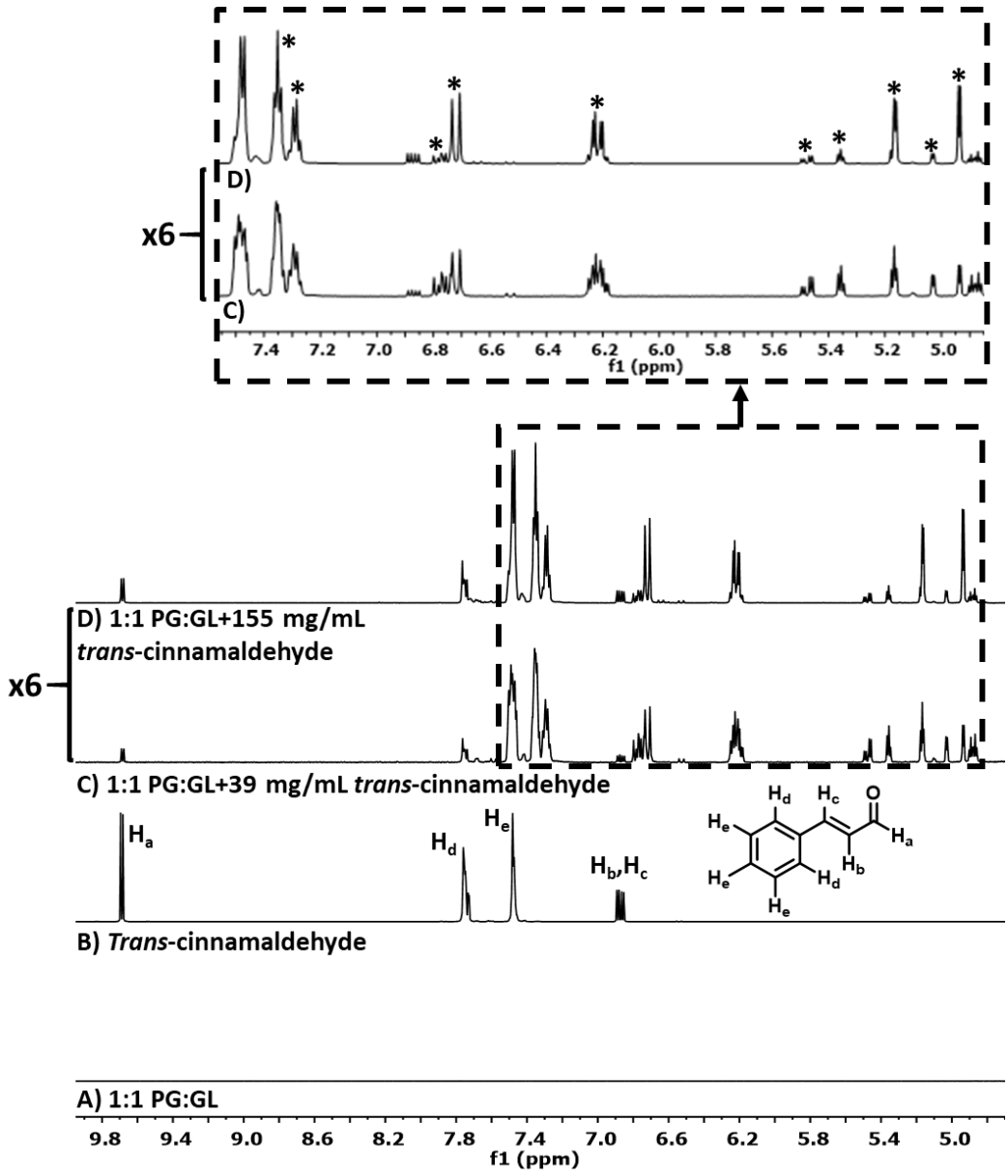

Figure 6. ^{1}H NMR spectra for the unvaped (A) equimolar PG:GL, (B) *trans*-cinnamaldehyde standard, (C) PG:GL+39 mg/mL *trans*-cinnamaldehyde (~2 weeks old), and (D) PG:GL+155 mg/mL *trans*-cinnamaldehyde (~2 months old) in DMSO-d_6. The (D) PG:GL+155 mg/mL *trans*-cinnamaldehyde was

formulated first, followed by the (C) PG:GL+39 mg/mL *trans*-cinnamaldehyde ~6 weeks later, and then ~2 weeks later we analyzed both (D) and (C) with ^{1}H NMR spectroscopy. E-liquids in spectra (C) and (D) reached equilibrium sometime within their incubation time. Spectra A, C, and D were normalized to the PG methyl peak at ~1.05 ppm, and spectrum C was expanded by x6. The peaks with an "*" above them are not from PG, GL, or *trans*-cinnamaldehyde, and are from PG- and GL-*trans*-cinnamaldehyde acetals. The region between 5.0 and 7.4 ppm for spectra C and D show many of the peaks associated with the PG- and GL-*trans*-cinnamaldehyde acetals.

2.8.5. ^{1}H NMR of Spiked Aerosol Samples

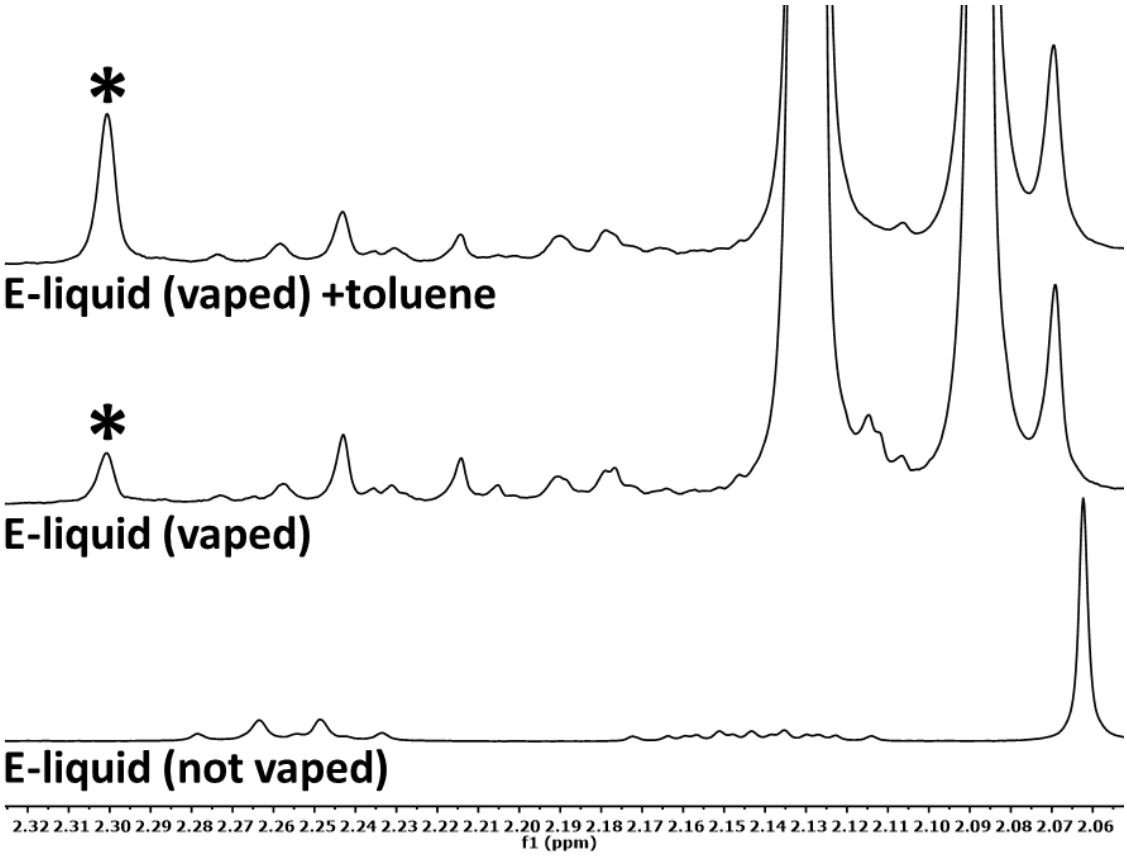

Figure 7. A vaped e-liquid (PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine) sample was spiked with toluene which resulted in an increase for the unknown peak at ~2.30 ppm (shown with an "*" above the peak) in the ^{1}H NMR spectra. This suggests that toluene was formed during aerosolization and is a degradation product of the PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine e-liquid.

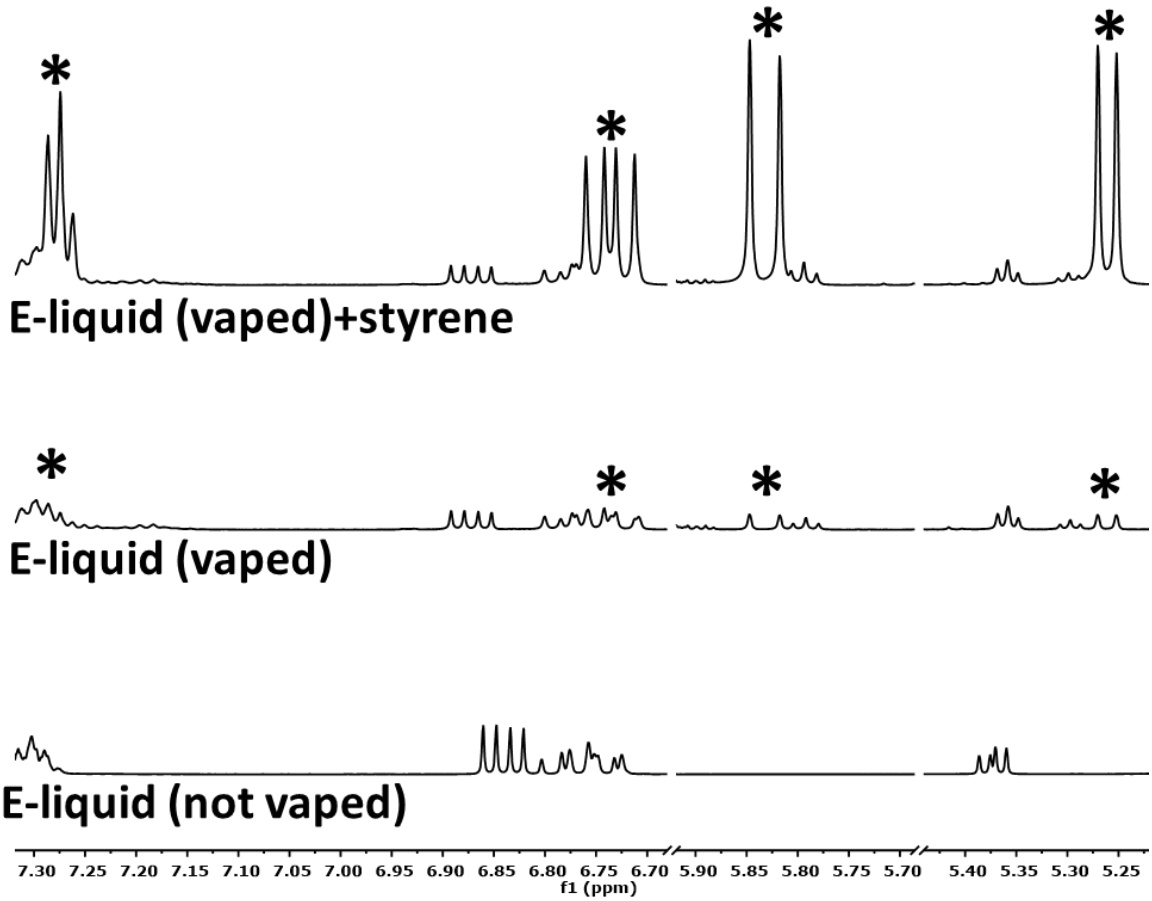

Figure 8. The peaks at 5.25, 5.83, 6.70-6.75 and 7.30 ppm (shown with an "*" above the peaks) in the ^{1}H NMR spectra increased when styrene was added to a vaped e-liquid sample containing PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine. This suggests that styrene was formed during aerosolization and is a degradation product of the PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine e-liquid.

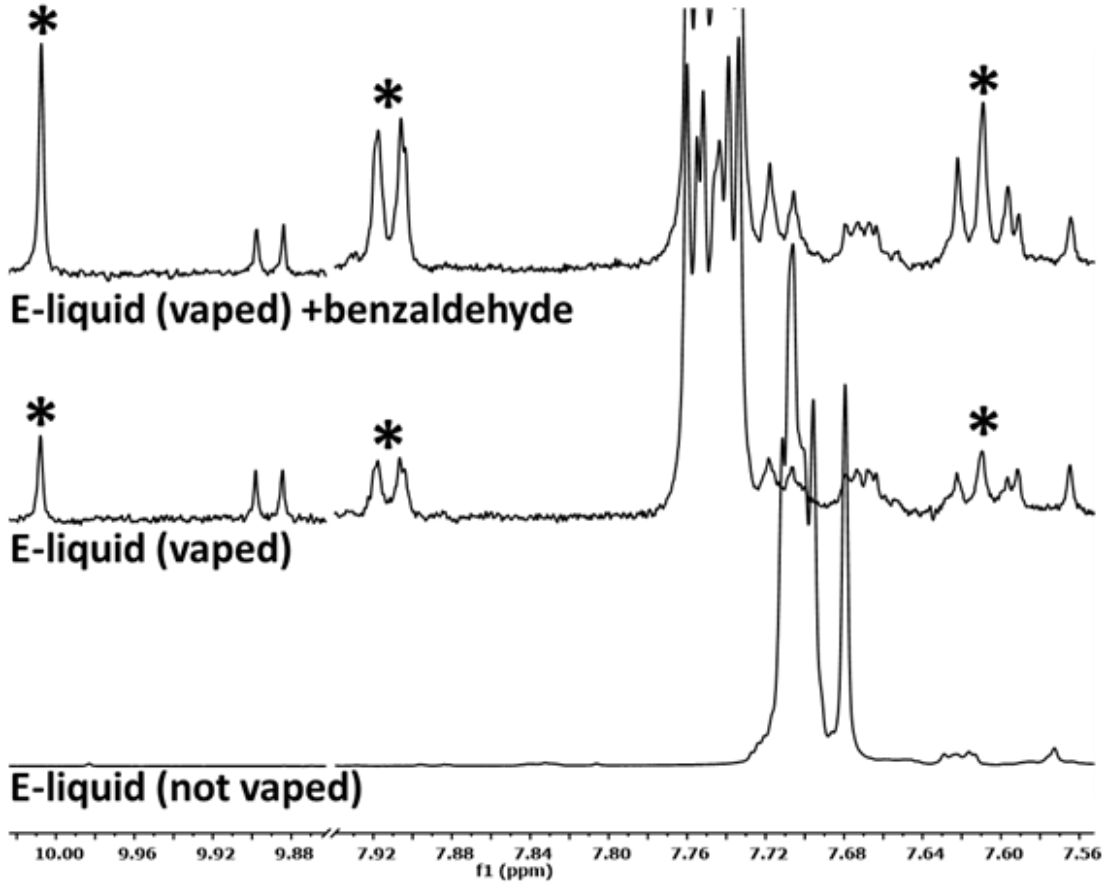

Figure 9. Benzaldehyde was added to a vaped e-liquid composed of PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine which resulted in an increase of the peaks at 7.61, 7.91, and 10.01 ppm (shown with an "*" above the peaks) in the ^{1}H NMR spectra. This suggests that benzaldehyde was formed during aerosolization and is a degradation product of the PG+GL+155 mg/mL *trans*-cinnamaldehyde+6 mg/mL nicotine e-liquid.

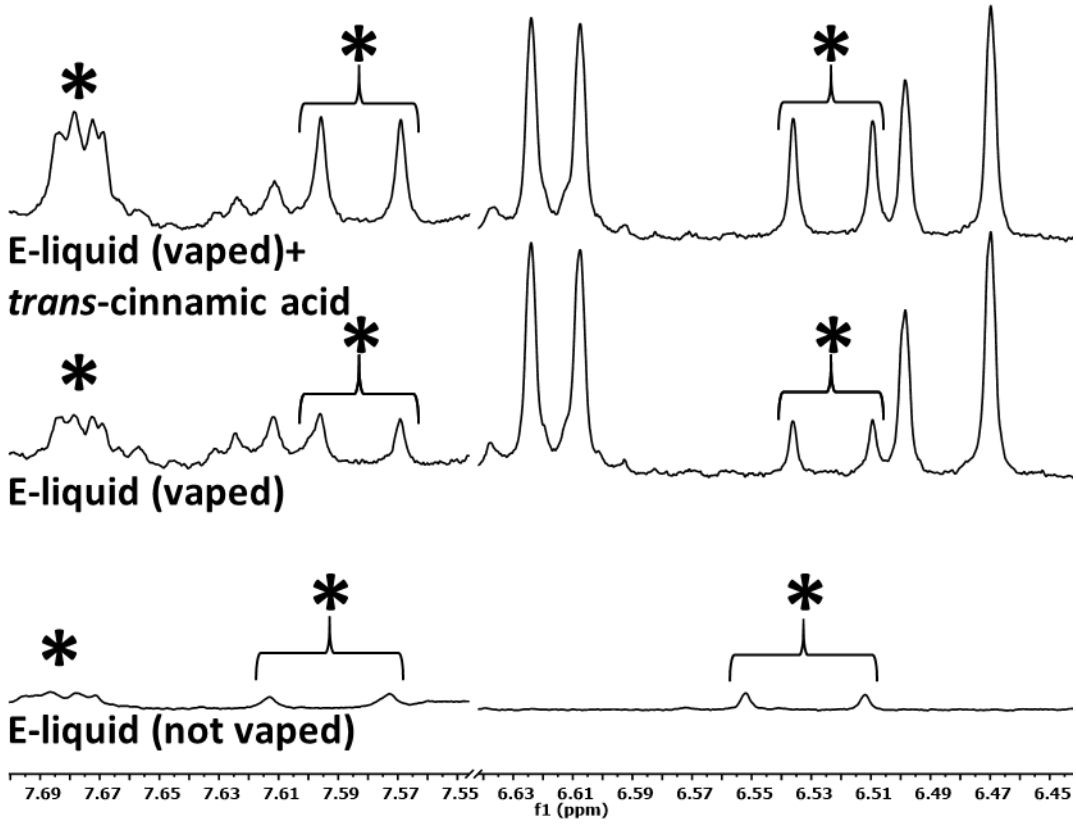

Figure 10. There was an increase in the unknown peaks at 6.52, 7.58, and 7.68 ppm (shown with an "*" above the peaks) in the ^{1}H NMR spectra when *trans*-cinnamic acid was added to a vaped e-liquid containing PG+GL+155 mg/mL *trans*-cinnamaldehyde. The J-coupling for the doublets at 6.52 and 7.58 ppm were confirmed to be similar values. This suggests that *trans*-cinnamic acid was formed during aerosolization and is a degradation product of the PG+GL+155 mg/mL *trans*-cinnamaldehyde e-liquid.

2.8.6. Appendix A References

(1) Jensen, R. P.; Luo, W.; Pankow, J. F.; Strongin, R. M.; Peyton, D. H. Hidden formaldehyde in e-cigarette aerosols. *N Engl J Med* **2015**, *372* (4), 392-394. DOI: 10.1056/NEJMc1413069.
(2) Jensen, R. P.; Strongin, R. M.; Peyton, D. H. Solvent Chemistry in the Electronic Cigarette Reaction Vessel. *Sci Rep* **2017**, *7*, 42549. DOI: 10.1038/srep42549.

3. The Kinetics of Aldehyde Flavorant-Acetal Formation in E-liquids with Different E-cigarette Solvents and Common Additives Studied by ^{1}H NMR Spectroscopy

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Link to source: https://pubs.acs.org/10.1021/acs.chemrestox.2c00159

3.1. Abstract

Flavorants, nicotine, and organic acids are common additives found in the e-liquid carrier solvent, propylene glycol (PG) and/or glycerol (GL), at various concentrations. Some of the most concentrated and prevalent flavorants in e-liquids include *trans*-cinnamaldehyde, vanillin, and benzaldehyde. Aldehyde flavorants have been shown to react with PG and GL to form flavorant-PG and -GL acetals that have unique toxicity properties in e-liquids before aerosolization. However, there is still much that remains unknown about the effects of different e-cigarette solvents, water, nicotine, and organic acids on the rate of acetalization in e-liquids. We used ^{1}H NMR spectroscopy to determine the first-order initial rate constant, half-life, and % acetal formed at equilibrium for flavorant-acetal formation in simulated e-liquids. Herein we report that acetalization generally occurs at a faster rate and produces greater yields in e-liquids with higher ratios of GL (relative to PG). *Trans*-cinnamaldehyde acetals formed the fastest in 100% PG simulated e-liquids, followed by benzaldehyde, and vanillin based on their half-lives and rate constants. The acetal yield was greatest for benzaldehyde in PG e-liquids, followed by *trans*-cinnamaldehyde, and vanillin. Acetalization in PG e-liquids containing aldehyde flavorants was inhibited by water and nicotine, but catalyzed by benzoic acid. Flavorant-PG acetal formation was generally delayed in the presence of nicotine, even if benzoic acid was present at 2-, 4-, or 10-fold the nicotine concentration, as compared to the PG e-liquids with 2.5 mg/mL flavorant. Thus, commercial e-liquids with aldehyde flavorants containing a higher GL ratio (relative to PG), little water, no nicotine, nicotine with excess organic acids, or organic acids without nicotine would

undergo acetalization the fastest and with the highest yield. Many commercial e-liquids must therefore contain significant amounts of flavorant acetals.

3.2. Introduction

Electronic cigarettes (e-cigarettes) have become increasingly popular since their introduction to the United States market in 2007.[1] In 2021, ~3 and ~11% of adolescents in middle and high school reported e-cigarette use, respectively.[2] Flavorants and nicotine are frequently added to the e-liquid carrier solvent (propylene glycol (PG) and/or glycerol (GL)) and can aerosolize and degrade during vaping. Disposable e-cigarettes (i.e. Puff Bar™) replaced JUUL™ as the most popular e-cigarette device among adolescents after the Food and Drug Administration (FDA) prohibited the sale of prefilled e-cigarette cartridges in any flavor except tobacco or menthol in 2020.[3] Fix et al. has reported how regulations concerning flavorant and nicotine concentrations in e-liquids vary by region, and that the concentration of nicotine listed on the packaging can be inaccurate.[4] Flavored e-liquids with and without nicotine are widely accessible to consumers despite the limited information on the potential harmfulness of flavorants before and after e-liquid aerosolization.

Behar et al. identified some of the most common flavorants in commercial e-liquids as benzaldehyde (cherry flavor), vanillin (creamy, vanilla flavor), and *trans*-cinnamaldehyde (cinnamon flavor).[5] Aerosolized commercial e-liquids with *trans*-cinnamaldehyde can be cytotoxic,[6] increase respiratory infection by disrupting mitochondrial function and bioenergetic processes,[7] promote oxidative stress on osteoblast-like cells,[8] and impair respiratory immune cell function.[9] Commercial e-liquids

that contain benzaldehyde and vanillin have been linked to the impairment of phagocytosis[10] and hepatotoxicity[11] upon aerosolization, respectively. The physiological effects of inhaling aerosolized e-liquids with flavorants require further assessment to minimize the consumers exposure to harmful and potentially harmful chemicals (HPHCs).

PG and GL can thermally degrade during aerosolization to produce propanal, acetaldehyde, glycolaldehyde, formaldehyde, acrolein, formaldehyde hemiacetals (formaldehyde adducts of PG or GL, formed by a reversible reaction), and other HPHCs.[12] Khlystov and Samburova[13] found that aerosols produced from flavored commercial e-liquids contain increased levels of toxic aldehydes compared to aerosolized unflavored e-liquids. The addition of sweeteners (e.g. sucralose) and flavorant enhancers (e.g. triacetin) to e-liquids can increase the degradation levels compared to e-liquids that are unsweetened and unflavored upon aerosolization as shown by Duell et al.[14] and Vreeke et al.[15], respectively. The type of e-cigarette device,[16] heating element,[17] e-liquid composition,[18] and use patterns the consumer employs[16] can enhance the formation of HPHCs upon aerosolization.

Popular disposable e-cigarette brands (e.g. Puff Bar™, SEA™, Ezzy Oval™) mimic aspects of JUUL, but do not have a microcontroller to regulate electrical power to the heating coil, and consequently can emit higher levels of carbonyls and metals compared to JUUL™.[19] Noël et al.[20] showed that aerosolized butter flavored e-liquids produced under sub-ohm conditions (< 1 Ω; increased wattage) leading to higher temperature, contained higher levels of carbonyls and nicotine compared to supra-ohm (>

1 Ω; decreased wattage) at presumably lower temperature conditions using the same e-cigarette with different atomizers. Yogeswaran and Rahman found that disposable e-cigarettes containing tobacco-derived nicotine generated more reactive oxygen species upon aerosolization than disposable e-cigarettes with tobacco-free nicotine.[18] Further studies are necessary to understand how flavorants, nicotine, and organic acids react in e-liquids before and after aerosolization under different conditions.

Aldehyde flavorants can react with PG and GL to form flavorant-PG and -GL acetals in e-liquids before aerosolization. Erythropel et al.[21, 22] observed that greater than 40% of vanillin, ethylvanillin, benzaldehyde, citral, and *trans*-cinnamaldehyde were converted to flavorant acetals in PG e-liquids. The aerosol transfer efficiency of the flavorant-PG acetals from the e-liquids to the aerosols ranged from 50 to 80%. PG-flavorant acetals have similar scents but different toxicological properties compared to the parent flavorant.[23] Jabba et al.[24] showed that benzaldehyde- and vanillin-PG acetals can increase respiratory epithelial cell mortality and be more cytotoxic than their parent flavorants, respectively. The kinetics of acetal formation in e-liquids with water, nicotine, organic acids, and mixtures of nicotine and benzoic acid are unknown. Herein, we used ^{1}H NMR spectroscopy to analyze the rate and yield of aldehyde flavorant-acetal formation in PG, GL, and equimolar PG+GL e-liquids, as well as PG e-liquids with water, nicotine, benzoic acid, and mixtures of nicotine and benzoic acid.

3.3. Materials & Methods

3.3.1. Materials

USP grade propylene glycol (PG), USP grade glycerol (GL), benzoic acid (>99.5%), and benzaldehyde (>99%) were purchased from Sigma-Aldrich (St. Louis, MO). (*S*)-(-)-nicotine (99%) and vanillin (>99%) were obtained from Alfa Aesar (Haverhill, MA). *Trans*-cinnamaldehyde (>98%) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Benzaldehyde-PG acetal (>95%) and *trans*-cinnamaldehyde-PG acetal (*trans*-4-methyl-2-(2-phenylvinyl)-1,3-dioxolane) were purchased from Sigma-Aldrich (St. Louis, MO). Vanillin-PG acetal was purchased from Carbosynth Ltd. (Compton, UK). 1,3,5-Trimethoxy benzene (TMB) was purchased from Oakwood Chemical (Estill, SC). DMSO-d_6 (D 99.9%), $CDCl_3$ (D 99.8%), and D_2O (D 99.9%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

3.3.2. Methods

The compositions of the simulated equimolar PG+GL, pure GL, and pure PG e-liquids used for the studies comparing the rates and yields of acetal formation in different solvents are shown in Table 1. The internal standard, TMB, was first added to the PG+GL, PG, and GL by heating and stirring the mixture. Lastly, the flavorant was added to each e-liquid, and stirred for ~5 min. The concentrations of flavorants were within the range observed in commercial e-liquids, based upon values from the literature.[5] PG e-liquids containing 10 mg/mL flavorant without the internal standard TMB were formulated, and used as control experiments to demonstrate that TMB had no effect on

acetal formation (Table 1). All ratios were verified using ^{1}H NMR spectroscopy prior to the addition of flavorant (Table 1).

Once the flavorant was dissolved in the e-liquid, aliquots of each sample were placed in NMR tubes pre-charged with 0.5 mL DMSO-d_6 at various time points during the monitored reaction period. Each sample was evaluated by ^{1}H NMR spectroscopy, using a Bruker AVANCE III NMR spectrometer using a 30° observation pulse with 16 scans and a 3 s relaxation delay at 25 °C, shortly after generation. The first-order initial rate constant, half-life, and % acetal formed at equilibrium for each e-liquid were determined by integrating the aldehyde flavorant peaks relative to TMB in each sample.

Table 1. Composition of the simulated e-liquids used to study the kinetics of flavorant-acetal formation

	E-cigarette Solvent	Internal standard, TMB[α] (mg/mL)	Flavorant (mg/mL)	Nicotine (mg/mL)	Benzoic acid (mg/mL)	Water (% by wt)
Trans-cinnamaldehyde	PG	—	10.0	—	—	—
	PG+GL	1.0	10.0	—	—	—
	GL	1.0	10.0	—	—	—
	PG	1.0	10.0	—	—	—
	PG	1.0	2.5	—	—	20.0
				1.6	—	—
				6.3	—	—
				—	4.8	—
				6.3	4.8	—
				3.1	4.8	—
				3.1	9.3	—
				3.1	23.0	—
Vanillin	PG	—	10.0	—	—	—
	PG+GL	3.1	31.0	—	—	—
	GL	3.1	31.0	—	—	—
	PG	3.1	31.0	—	—	—
	PG	1.0	2.5	—	—	—
				5.4	—	—
				—	4.0	—
				5.4	4.0	—
				2.7	4.0	—
				2.7	8.1	—
				2.7	20.0	—

Table 1. Continued

	E-cigarette Solvent	Internal standard, TMB[α] (mg/mL)	Flavorant (mg/mL)	Nicotine (mg/mL)	Benzoic acid (mg/mL)	Water (% by wt)
Benzaldehyde	PG	—	10.0	—	—	—
	PG+GL	1.0	2.5	—	—	—
	GL	1.0	2.5	—	—	—
	PG	1.0	2.5	—	—	—
				—	11.0	—
				7.0	11.0	—
				7.0	21.0	—
				7.0	51.0	—

[α]TMB = 1,3,5-trimethoxybenzene

3.3.3. Experiment details

3.3.3.1. Effects of Water, Nicotine, Benzoic acid, and Nicotine+Benzoic Acid on PG-Flavorant Acetal Formation

The % flavorant-acetal formed at equilibrium, first-order initial rate constant, and half-life were determined in PG e-liquids containing 2.5 mg/mL flavorant with water, nicotine, benzoic acid, and nicotine+benzoic acid (at different mol ratios). PG e-liquids with 2.5 mg/mL flavorant were chosen as the standard for these experiments with common e-liquid additives. The composition of the simulated flavored e-liquids with additives are shown in Table 1. First, the additives were mixed into PG, and followed by

the addition of flavorant to each e-liquid. The reaction time began after the flavorant was mixed into the e-liquid (after ~5 min).

Some simulated PG e-liquids with flavorants and additives were placed in an oven set at 100° C for 24 h, and then reheated for another 24 h to determine their acetal yield (Tables 2-4). Aliquots of the heated e-liquids were analyzed before the first 24 h in the oven and then after each 24 h period (to verify their compositions were similar) by ^{1}H NMR spectroscopy. We compared the ^{1}H NMR spectra before, after 24 h, and after 48 h of heat – knowing the peak assignments for PG, GL, flavorants, additives, and flavorant-acetals – to ensure that other degradants did not form (e.g. presence of unknown peaks in heated e-liquids).

3.3.3.2. Analysis of Commercial E-liquids

Commercial e-liquids (>~5 yrs old, based on their purchase date) containing vanillin, ethyl vanillin, or *trans*-cinnamaldehyde were analyzed by ^{1}H NMR spectroscopy as above, to determine if any flavorant-PG or -GL acetals formed overtime. The total age of the commercial e-liquids, including the time they sat on store shelves, was unknown. The brand designation, flavor, and composition of the commercial e-liquids studied are shown in Table 5. The original flavorant and nicotine concentrations in the e-liquids were determined using gas chromatography-mass spectrometry (GC-MS) when purchased.

The e-liquids were stored in a freezer when not in use. Similar to section 2.3.1, the commercial e-liquids were placed in an oven at 100° C for 24 h, and then reheated for another 24 h to simulate naturally aged e-liquids (Table 5). Aliquots of the heated e-

liquids were analyzed before the first 24 h in the oven and then after each 24 h period (to verify that their compositions were similar) by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of the unheated and heated e-liquids were compared, with the aldehyde flavorant and acetal peaks known, to confirm that degradants did not form (e.g. the presence of unknown peaks in the heated e-liquids).

3.4. Results

3.4.1. Rate of Flavorant-PG and -GL Acetal Formation

The first-order initial rate constant and half-life for the formation of *trans*-cinnamaldehyde-, vanillin-, and benzaldehyde-PG and -GL acetals in simulated e-liquids were determined by analyzing aliquots of the samples overtime with ^{1}H NMR spectroscopy. The kinetics were based on the consumption of the flavorant concentration in the e-liquid, assuming the rate constant was pseudo-first-order due to excess PG and GL (relative to the initial flavorant concentration). The initial rates were for the early time data points which gave a linear fit to the first-order rate equation. The flavorant and flavorant-PG and -GL acetal peaks – identified following the procedure in the supplemental information – were integrated relative to the internal standard (1,3,5-trimethoxy benzene; TMB) peak in each ^{1}H NMR spectrum (Figure S1 – S3). We compared the ^{1}H NMR spectra from the flavored e-liquids with versus without TMB (at different points in time), and found that TMB did not interact with acetal formation based on a) their similar acetal yields and b) the absence of any unknown peaks in e-liquids

with TMB (besides the known resonances of TMB). The reaction time was taken as the point when the flavorant dissolved in the e-liquid (after ~5 min of mixing time).

Behar et al.[5] detected 155, 31, and 2.5 mg/mL as the highest concentrations of *trans*-cinnamaldehyde, vanillin, and benzaldehyde in commercial e-liquids, respectively. The concentration of *trans*-cinnamaldehyde chosen for this study was ~15x less than the maximum determined by Behar et al. because conversion to the acetal was nearly instantaneous at 155 mg/mL (Table 1). The first-order initial rate constant and half-life were measurable at the maximum concentrations previously detected in commercial e-liquids for vanillin (31 mg/mL) and benzaldehyde (2.5 mg/mL; Table 1).

Acetal formation[25] typically includes an acid catalyst (not included in Figure 1) that protonates the carbonyl-oxygen, making the carbonyl carbon more partially positive (e.g. an electrophile), and then the alcohol moiety (e.g. a nucleophile) can attack the carbonyl-carbon (Figure 1). Next, the acid-catalyst is regenerated with formation of a hemiacetal intermediate. The acid will protonate the -OH group on the hemiacetal, and water is eliminated as a product. Then, the alcohol moiety attacks the carbonyl-carbon on the reactive *O*-alkylated intermediate, and the acid is regenerated by removing a proton from the acetal. Acetal formation is possible without an acid catalyst, but the rate of formation is much slower.

The *trans*-cinnamaldehyde-, vanillin-, and benzaldehyde acetals formed ~2, ~12, and ~35x faster in GL than their respective flavorant acetals in PG (Tables 2- 4). The rate of flavorant-acetal formation was higher in GL than PG, in part because GL forms two acetals (5- and 6-member rings),[26] but PG forms one acetal (a 5-member ring[27]; Figures

S2 and S3). There are additional effects to consider such as relative nucleophilicity and more. The formation of flavorant-acetals was slowest in PG compared to GL and PG+GL for the flavorants used in this study (Tables 2 - 4). *Trans*-cinnamaldehyde-acetals formed at a slightly faster rate in PG+GL versus GL, with half-lives of 3.0 and 3.4 h, respectively (Table 2). However, the rates of vanillin and benzaldehyde acetal formation were faster in GL than PG+GL (Tables 3 and 4).

The acetal yield was lower with PG as the e-cigarette solvent, compared to GL for *trans*-cinnamaldehyde and vanillin (Tables 2 and 3). However, the acetal yield was >99% for benzaldehyde in PG, GL, and PG+GL (Table 4). The percentage of *trans*-cinnamaldehyde- and vanillin-GL acetals formed were 5% and 24% greater than the PG-acetals at equilibrium in GL and PG e-liquids, respectively (Tables 2 and 3). The first-order initial rate constants, half-lives, and acetal yield at equilibrium for flavorant acetals formed in pure GL generally form at a faster rate and give a higher final yield as compared to the acetals formed in pure PG for the flavorants used in this study.

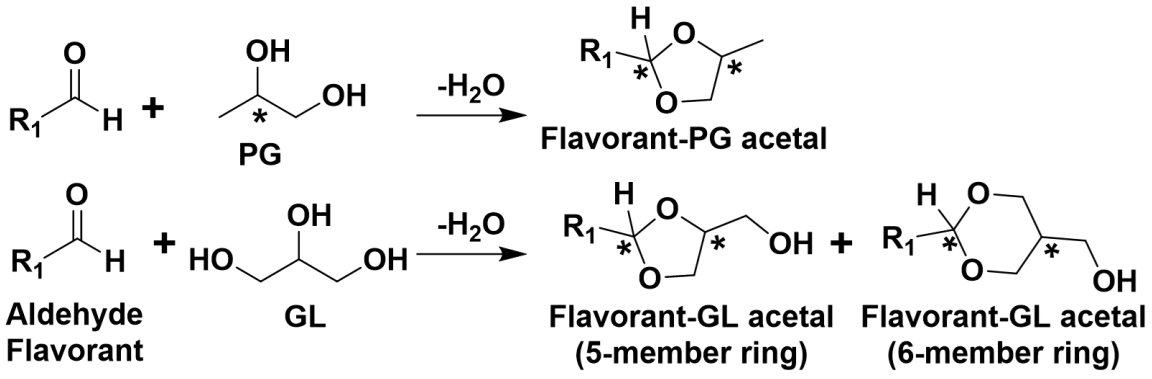

Figure 1. The general reactions for the formation of the flavorant-propylene glycol (PG) and –glycerol (GL) acetals. First, a) the carbonyl-oxygen would be protonated (typically by an acid catalyst), then b) the alcohol (e.g. PG or GL) would attack the carbonyl-carbon to form a hemiacetal, and finally c) steps a) and b) are repeated once more to form the cyclic acetal. The "*" on each molecule indicates a stereocenter.

Table 2. Experimental details for e-liquids containing *trans*-cinnamaldehyde including the % acetal formed at equilibrium, initial rate constant, and half-life determined under the various conditions

E-cigarette solvent	*Trans*-cinnamaldehyde (mg/mL)	Nicotine:*trans*-cinnamaldehyde mol ratio	Benzoic acid:*trans*-cinnamaldehyde mol ratio	Water (% by wt)	% acetal formed at equilibrium	Initial rate constant (h^{-1})	Half-Life (h)
PG+GL$^{\alpha}$	*10*	—	—	—	94	23.0 x 10^{-2}	3.0
PG$^{\alpha}$	*10*	—	—	—	91	11.0 x 10^{-2}	6.6
GL$^{\alpha}$	*10*	—	—	—	96	20.0 x 10^{-2}	3.4
PG$^{\alpha}$	*2.5*	—	—	—	90	5.2 x 10^{-2}	14.0
	2.5	—	—	*20*	NR$^{\theta}$	NR$^{\theta}$	NR$^{\theta}$
	2.5	*0.5*$^{\beta}$	—	—	NR$^{\theta}$	NR$^{\theta}$	NR$^{\theta}$
	2.5	*2*$^{\gamma}$	—	—	NR$^{\theta}$	NR$^{\theta}$	NR$^{\theta}$
	2.5	—	*2*$^{\delta}$	—	54	22.0 x 10^{-2}	3.2
	2.5	*2*$^{\gamma}$	*2*$^{\delta}$	—	87$^{\iota}$	0.8 x 10^{-3}	880.0
	2.5	*1*$^{\varepsilon}$	*2*$^{\delta}$	—	88	1.0 x 10^{-3}	690.0
	2.5	*1*$^{\varepsilon}$	*4*$^{\zeta}$	—	91	1.8 x 10^{-3}	370.0
	2.5	*1*$^{\varepsilon}$	*10*$^{\eta}$	—	90	8.4 x 10^{-3}	82.0

$^{\alpha}$ + 1.0 mg/mL 1,3,5-Trimethoxy benzene (TMB)
$^{\beta}$ 1.6 mg/mL nicotine
$^{\gamma}$ 6.3 mg/mL nicotine
$^{\delta}$ 4.8 mg/mL benzoic acid
$^{\varepsilon}$ 3.1 mg/mL nicotine
$^{\zeta}$ 9.3 mg/mL benzoic acid
$^{\eta}$ 23.0 mg/mL benzoic acid
$^{\theta}$ NR = no reaction
$^{\iota}$ Forced to equilibrium by heating at 100° C for 24 h then verified by reheating for another 24 h

Table 3. Experimental details for e-liquids containing vanillin including the % acetal formed at equilibrium, initial rate constant, and half-life determined under the various conditions

E-cigarette solvent	Vanillin (mg/mL)	Nicotine:vanillin mol ratio	Benzoic acid:vanillin mol ratio	% acetal formed at equilibrium	Initial rate constant (h^{-1})	Half-Life (h)
PG+GL[α]	*31*	—	—	81	1.3×10^{-2}	52.0
PG[α]	*31*	—	—	63	0.3×10^{-2}	240.0
GL[α]	*31*	—	—	87	3.7×10^{-2}	19.0
PG[β]	*2.5*	—	—	61[ι]	0.4×10^{-3}	1700.0
	2.5	*2*[γ]	—	NR[θ]	NR[θ]	NR[θ]
	2.5	—	*2*[δ]	73	18.0×10^{-2}	3.8
	2.5	*2*[γ]	*2*[δ]	71[ι]	0.3×10^{-3}	2100.0
	2.5	*1*[ε]	*2*[δ]	79	1.2×10^{-3}	590.0
	2.5	*1*[ε]	*4*[ζ]	80	1.8×10^{-3}	390.0
	2.5	*1*[ε]	*10*[η]	77	4.1×10^{-3}	170.0

[α] + 3.1 mg/mL 1,3,5-Trimethoxy benzene (TMB)
[β] + 1.0 mg/mL TMB
[γ] 5.4 mg/mL nicotine
[δ] 4.0 mg/mL benzoic acid
[ε] 2.7 mg/mL nicotine
[ζ] 8.1 mg/mL benzoic acid
[η] 20.0 mg/mL benzoic acid
[θ] NR = no reaction
[ι] Forced to equilibrium by heating at 100° C for 24 h then verified by reheating for another 24 h

Table 4. Experimental details for e-liquids containing benzaldehyde including the % acetal formed at equilibrium, initial rate constant, and half-life determined under the various conditions

E-cigarette solvent	Benzaldehyde (mg/mL)	Nicotine: benzaldehyde mol ratio	Benzoic acid: benzaldehyde mol ratio	% acetal formed at equilibrium	Initial rate constant (h^{-1})	Half-Life (h)
PG+GL$^{\alpha}$	*2.5*	—	—	>99	0.3×10^{-2}	240.0
PG$^{\alpha}$	*2.5*	—	—	>99	0.1×10^{-2}	580.0
GL$^{\alpha}$	*2.5*	—	—	>99	4.2×10^{-2}	16.0
PG$^{\alpha}$	*2.5*	—	*2*$^{\beta}$	>99	5.4×10^{-2}	13.0
	2.5	*1*$^{\gamma}$	*2*$^{\beta}$	>99$^{\zeta}$	2.7×10^{-4}	2600.0
	2.5	*1*$^{\gamma}$	*4*$^{\delta}$	>99$^{\zeta}$	3.6×10^{-4}	1900.0
	2.5	*1*$^{\gamma}$	*10*$^{\varepsilon}$	>99$^{\zeta}$	7.9×10^{-4}	880.0

$^{\alpha}$ + 1.0 mg/mL 1,3,5-Trimethoxy benzene (TMB)
$^{\beta}$ 11.0 mg/mL benzoic acid
$^{\gamma}$ 7.0 mg/mL nicotine
$^{\delta}$ 21.0 mg/mL benzoic acid
$^{\varepsilon}$ 51.0 mg/mL benzoic acid
$^{\zeta}$ Forced to equilibrium by heating at 100° C for 24 h then verified by reheating for another 24 h

3.4.2. Rate of Flavorant-PG Acetal Formation with the Common Additives Water, Nicotine, and Benzoic Acid

PG e-liquids containing 2.5 mg/mL flavorant without versus with water, nicotine, benzoic acid, and nicotine+benzoic acid (at varying mol ratios) were compared to determine the additives' effects on the rate constant, half-life, and % acetal formed at equilibrium (Table 1). The addition of 20% water (by wt) to the PG e-liquid with 2.5 mg/mL *trans*-cinnamaldehyde inhibited acetal formation (Table 2). Water is a product of acetal formation, and the addition of excess water to the e-liquid shifted the equilibrium towards the reactants (i.e. parent flavorant + PG; Figure 1). Roldán et al.[28] increased the yield of solketal (the ketal product of acetone and glycerol) by using a zeolite membrane batch reactor to remove water from the reaction environment. Half and twice the amount

of nicotine relative to *trans*-cinnamaldehyde (by mol), and twice the amount of nicotine relative to vanillin (by mol) also inhibited acetal formation (Tables 2 and 3).

Twice the amount of benzoic acid relative to *trans*-cinnamaldehyde, vanillin, and benzaldehyde (by mol) decreased the half-life by ~4.2, ~458.6, and ~45.2x compared to the PG e-liquids with 2.5 mg/mL flavorant, respectively (Tables 2 - 4). The % acetal formed at equilibrium decreased by 46% for *trans*-cinnamaldehyde, increased by 10% for vanillin, and was unchanged for benzaldehyde in PG e-liquids containing 2.5 mg/mL flavorant with benzoic acid compared to without (Tables 2 - 4). Acetalization in e-liquids was inhibited by nicotine (a base) and catalyzed by benzoic acid. The behavior of aldehyde flavorants in e-liquids with nicotine and benzoic acid was consistent with the acid-catalyzed acetal formation[26, 29] (Figure 1; also see Organic Chemistry textbooks).[25]

Nicotine can exist in the free-base (harsh upon inhalation), monoprotonated (pK_a = 8.0 in water; more palatable than free-base upon inhalation), or diprotonated (pK_a = 3.1 in water) form in e-liquids depending on their acid/base conditions.[30] Duell et al.[31] have also shown that e-cigarette manufactures (i.e. Puff Bar) have recently been using synthetically created tobacco-free nicotine (often (*R*,*S*)-(±)-nicotine) instead of tobacco-derived nicotine ((*S*)-(−)-nicotine) perhaps to avoid FDA regulations. E-liquid manufacturers frequently add organic acids (e.g. benzoic acid, levulinic acid, and malic acid) to protonate nicotine, thus decreasing the harshness and increasing the inhalability of the aerosol.[32] Simulated PG e-liquids with 2.5 mg/mL flavorant and a 1:2, 1:4, and 1:10 nicotine:benzoic acid mol ratio (relative to each flavorant) were formulated to determine the effects of nicotine and benzoic acid mixtures on acetal formation. Two

additional PG e-liquids with 2.5 mg/mL *trans*-cinnamaldehyde and vanillin were mixed with a 2:2 mol ratio of nicotine to benzoic acid (relative to each flavorant).

Flavorant-PG acetals formed ~6.1 and ~1.5x slower when in the presence of nicotine, even if benzoic acid was present at 10-fold the nicotine concentration compared to the PG e-liquids with 2.5 mg/mL flavorant for *trans*-cinnamaldehyde and benzaldehyde, respectively (Tables 2 and 4). Vanillin-PG acetals formed ~2.9x faster in the presence of nicotine when benzoic acid had a concentration 2-fold greater than nicotine compared to the PG e-liquid with 2.5 mg/mL vanillin (Table 3). The acetal yields were similar in the PG e-liquids containing 2.5 mg/mL flavorant with and without mixtures of nicotine and benzoic acid for *trans*-cinnamaldehyde and benzaldehyde (Tables 2 and 4). However, the acetal yield increased by 10-19% in PG e-liquids containing 2.5 mg/mL vanillin with mixtures of nicotine and benzoic acid versus without (Table 3).

3.4.3. Analysis of Commercial E-liquids

Most commercial e-liquid selected in this study had a greater total flavorant concentration (considering only vanillin+ethyl vanillin+*trans*-cinnamaldehyde) than nicotine by mol. Flavorant-PG and -GL acetals were present in every e-liquid in Table 5. Two of the seven commercial e-liquids evaluated, “Winters Bite” and “Aries,” did not contain nicotine. “Winters Bite” and “Aries” (containing vanillin and ethyl vanillin without nicotine) had a greater % flavorant converted into total PG- and GL-flavorant

acetals compared to “Taurus” and “Snow White’s Demise” (containing vanillin and/or ethyl vanillin with nicotine; Table 5).

“Dragons Breath” contained ~3.3x more *trans*-cinnamaldehyde than nicotine, yet had the lowest % of flavorant-PG and -GL acetals (Table 5). Nicotine appeared to inhibit acetal formation in “Taurus,” “Snow White’s Demise,” and “Dragons Breath.” Commercial e-liquids may be complex and contain other additives that might inhibit acetal formation, but we compared simulated e-liquids with and without nicotine and found that nicotine inhibited acetal formation (Tables 2 and 3). However, “Snake eyes” and “Snake oil” contained nicotine, and had the highest total acetal yield among the e-liquids. “Snake eyes” and “Snake oil” had a higher ratio of GL than PG (1.6:1.0 GL:PG mol ratio), and contained organic acids (an unknown amount) to protonate nicotine,[30] both of which could increase the total % of acetals formed over time.

Commercial e-liquids with and without nicotine frequently contain a wide range of flavorants at varying concentrations to create the desired flavor.[33] The effects of flavorant mixtures on aldehyde flavorant-PG and -GL acetal formation in e-liquids without and with nicotine and organic acids requires further study. We showed that nicotine generally delays individual *trans*-cinnamaldehyde-, vanillin-, and benzaldehyde-acetal formation in simulated PG e-liquids, even if benzoic acid was present at 10-fold the nicotine concentration. We also found that the rate of total flavorant-PG and -GL acetal formation increased as the ratio of GL increased (relative to PG) in e-liquids. Commercial e-liquids containing aldehyde flavorants with little water, without nicotine,

with organic acid(s), or a higher ratio of GL than PG would generally form acetals at a faster rate compared to the opposite of these e-liquid conditions.

Table 5. The % aldehyde flavorant converted into PG- and GL-flavorant acetals in commercial e-liquids with and without nicotine

Commercial e-liquid		Initial nicotine and flavorant concentrations				
Brand	Flavor	Nicotine (mg/mL)	Vanillin (mg/mL)	Ethyl vanillin (mg/mL)	*Trans*-cinnamaldehyde (mg/mL)	% flavorant converted into total PG- and GL-flavorant acetals
Twelve Vapor	Taurus	3	3	6	ND[α]	6
	Aries	ND[α]	13	5	ND[α]	14
Seduce Juice	Snake Eyes	6	11	9	ND[α]	22
	Snake Oil	12	7	11	ND[α]	20
The Mad Alchemist	Snow White's Demise	11	7	ND[α]	ND[α]	11
	Winters Bite	ND[α]	9	20	ND[α]	17
	Dragons Breath	12	ND	ND[α]	39	4

[α] ND = Not Detected

3.5. Discussion

Erythropel et al.[22] determined the % acetal formed at equilibrium and half-life of 21 mg/mL *trans*-cinnamaldehyde, vanillin, and benzaldehyde in PG over a 2-week period via gas chromatography-flame ionization detector (GC-FID). They found that 92, 40, and 95% of *trans*-cinnamaldehyde, vanillin, and benzaldehyde were converted into acetals in PG in <1, ~7, and ~5 days, respectively. We found that acetal formation was the fastest in

PG with *trans*-cinnamaldehyde, followed by benzaldehyde, and vanillin by comparing the half-lives for PG e-liquids with 2.5 mg/mL flavorant (Table 2 - 4). *Trans*-cinnamaldehyde (an α,β-unsaturated aldehyde) was the most reactive flavorant used in this study with two electrophilic sites at the β– and carbonyl-carbon which are available for nucleophilic attack. Benzaldehyde and vanillin are simpler aldehydes having one electrophilic site at the carbonyl-carbon. The acetal yield was highest for benzaldehyde, followed by *trans*-cinnamaldehyde, and vanillin amongst the PG e-liquids with 2.5 mg/mL flavorant. The order of the half-lives from fastest to slowest and acetal yield from most to least for flavorants in this study were thus consistent with Erythropel et al.'s results.

Agirre et al.[34] and Nanda et al.[35] showed the rate and yield of GL-ketal and -acetal formation increased as the GL:ketone and aldehyde mol ratio increased (for either reactant), respectively. There was excess PG and GL relative to aldehyde flavorants in e-liquids, yet we still observed an increased reaction rate with a slight change in acetal yield for PG e-liquids with 31 versus 2.5 mg/mL vanillin and 10 versus 2.5 mg/mL *trans*-cinnamaldehyde (Tables 2 and 3). Yu et al.[36] found that *trans*-cinnamaldehyde can be oxidized to form 3.6% (by wt) *trans*-cinnamic acid at temperatures as low as 35° C. Our samples were stored at room temperature, but we observed ~1.5-fold more *trans*-cinnamic acid in the PG e-liquid with 10 versus 2.5 mg/mL *trans*-cinnamaldehyde.

Most e-liquids are aerosolized by metal coils (e.g. Kanthal = FeCrAl alloy, nichrome = NiCr, nickel = Ni, stainless steel = FeNiCr) in the tank or cartridge of an e-cigarette, and remain in the reservoir until most of the e-liquid is consumed. Olmedo et

al.[37] have shown that metals from the coils can be transferred into the aerosol, and then leach into the remaining e-liquid in the reservoir post-aerosolization; although they did not specify the metals could be oxides or salts. Saliba et al.[38] showed that different metal coil materials can affect the thermal degradation of PG via the surface chemistry during aerosolization. Subaramanian et al.,[39] da Silva & Teixeira,[40] and Dhakshinamoorthy et al.[41] have catalyzed the acetalization of various aldehydes and alcohols with a Ni^{II}-complex, transition metal salts (i.e. $FeCl_3$, $NiCl_2$, and $CuCl_2$), and metal organic frameworks (containing Fe, Cu, and Al) respectively. E-liquids in contact with the metal coil could contain metals that catalyze the formation of aldehyde flavorant-PG and -GL acetals before, during, and after aerosolization.[24] The rates of acetal formation in e-liquids with aldehyde flavorants in the original container versus in the e-cigarette reservoir (before and after aerosolization) requires further study.

PG and GL can thermally degrade into formaldehyde, acetaldehyde, and other toxicants upon aerosolization.[42] Formaldehyde can react with PG and GL in the aerosol to form formaldehyde-PG and -GL hemiacetals that can release formaldehyde before or after particle deposition in the respiratory tract.[43] Formaldehyde-PG and -GL hemiacetals can be converted into chemically stable cyclic acetals under acidic conditions.[44] Duell et al.[14] observed acetaldehyde and formaldehyde cyclic acetals in acidic aerosols produced from e-liquids with sucralose (i.e. sucralose can thermally degrade upon aerosolization to form HCl) by GC-MS. Acetalization can occur before and after e-liquid aerosolization.

3.6. Conclusions

In this study we used ^{1}H NMR spectroscopy to determine the rate and yield of aldehyde flavorant-acetal formation in PG, GL, and PG+GL e-liquids, and then PG e-liquids with water, nicotine, benzoic acid, and mixtures of nicotine+benzoic acid. Acetalization occurred at a faster rate and produced a higher yield in e-liquids with GL compared to PG. Acetal formation in PG e-liquids with flavorants were inhibited by nicotine and water (i.e. a base and an acetalization product, respectively), and catalyzed by benzoic acid. PG e-liquids containing nicotine and flavorants with 2, 4, and 10x more benzoic acid than nicotine (by mol) generally formed acetals at a slower rate compared to e-liquids without nicotine and benzoic acid. Many of the flavorant-PG and -GL acetal peaks were assigned in their ^{1}H NMR spectra to identify the acetals in e-liquids. Flavorant acetals have unique toxicity profiles and can be more harmful than the parent flavorant.[22, 24] The rate and yield of additional flavorant-acetal and -ketal formation in e-liquids with and without common additives and the impact on other reactions should also be explored to inform consumers and regulators about the HPHCs in e-liquids before and after aerosolization.

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3.7.5. Abbreviations

E-cigarette, electronic cigarette; e-liquid, electronic cigarette liquid; PG, propylene glycol; GL, glycerol; NMR, nuclear magnetic resonance; TMB, 1,3,5-trimethoxy benzene; HPHC, harmful and potentially harmful constituents; GC-MS, gas chromatography–mass spectrometry; GC-FID, gas chromatography-flame ionization detector.

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3.9. Appendix B: Supporting Information

3.9.1. Materials & Methods for Assigning PG- and GL-Flavorant Acetal Peaks

PG-vanillin, -*trans*-cinnamaldehyde, and -benzaldehyde acetal standards were purchased, and the peaks were identified by ^{1}H NMR spectroscopy (2D COSY, HSQC, TOCSY), and identifying labile protons by adding a drop of D_2O, then mixing. GL-flavorant acetal standards were not available for purchase, but some peaks were identifiable by ^{1}H NMR spectroscopy by a similar strategy of using 2D COSY, HSQC, TOCSY, as well as identifying labile protons by adding a drop of D_2O, then mixing, using the GL e-liquids with flavorants after acetal equilibrium was reached.

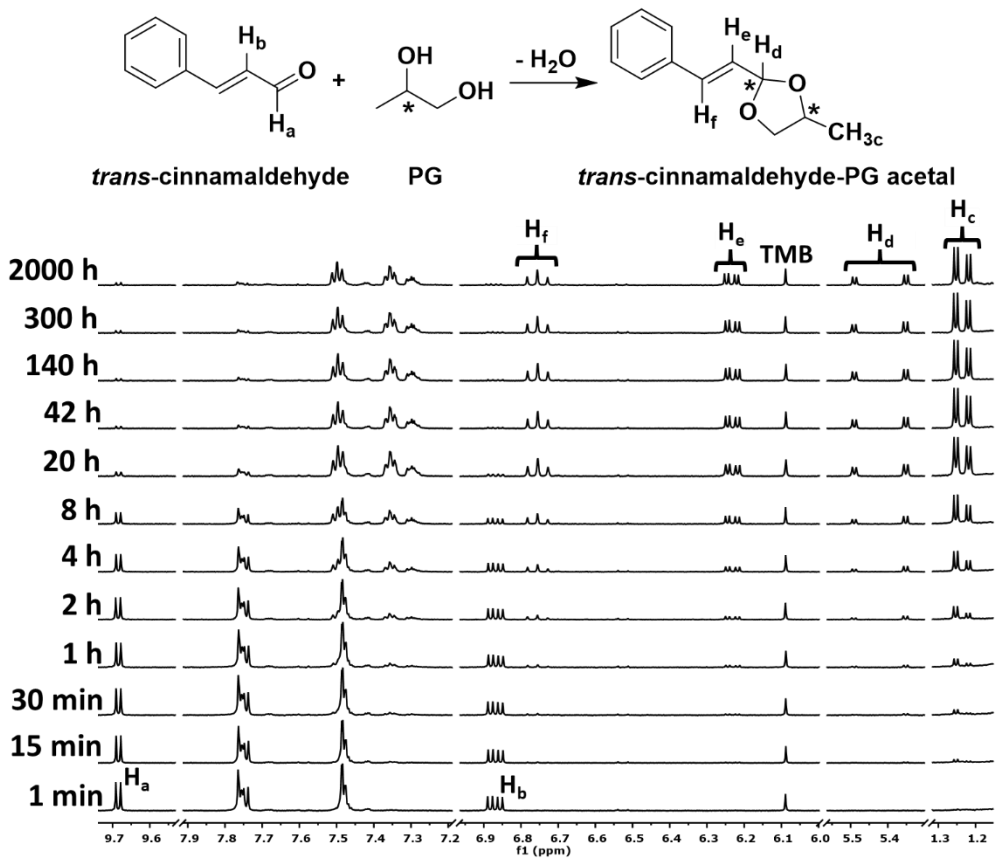

Figure 2. The ^{1}H NMR spectra collected from a propylene glycol (PG) e-liquid with 10 mg/mL *trans*-cinnamaldehyde overtime showing the decrease in *trans*-cinnamaldehyde peaks (H_a and H_b) and increase in *trans*-cinnamaldehyde-PG acetal peaks (H_c – H_f) normalized to 1,3,5-trimethoxybenzene (TMB). The peaks associated with the *trans*-cinnamaldehyde-PG acetal protons (H_c- H_f) are doubled since the acetal is a diastereomer. Undesignated peaks are from aromatic protons. The "* on each molecule indicate a stereocenter.

3.9.2. Results & Discussion for Flavorant-PG and -GL Acetal ^{1}H NMR Peaks Assigned

The 5-member PG-, 5-member GL-, and 6-member GL-flavorant acetal rings that form have two stereocenters, as shown in Figures S2 and S3. The two peaks associated to each proton on the 5-member PG-acetals (H_a – H_p), 5-member GL-acetals (H_a – H_d; H_i – H_k; H_o; H_p), and 6-member GL-acetals (H_e – H_h; H_l – H_n; H_q; H_r) represent each diastereomer (Figures S2 and S3). The signals for the labile protons on the flavorant-PG and -GL acetals were also doubled. We observed that the signals for H_k from the vanillin-

PG acetal, and H_a, H_e, H_i, H_k, H_l, H_n, H_o, and H_q from the flavorant-GL acetals decreased upon addition of a small amount of D_2O (Figures S2 and S3).

Pawar et al.,[1] Armylisas et al.,[2] and Oger et al.[3] studied the selectivity for the production of 5- and 6-member ring acetals and ketals from the acetalization of GL with aldehydes and ketones using different catalysts. The diastereomers associated with each peak from H_p (5-member ring) and H_r (6-member ring) of the benzaldehyde-GL acetals identified in the ^{1}H NMR spectra ($CDCl_3$ solvent) were consistent in the three studies (Figure S3). Oger et al. included *trans*-cinnamaldehyde in their study, and identified the peaks associated with the diastereomers for H_b (5-member ring) and H_f (6-member ring) from the *trans*-cinnamaldehyde-GL acetals on the ^{1}H NMR spectrum ($CDCl_3$ solvent) (Figure S3). We also collected ^{1}H NMR spectra for GL e-liquids with benzaldehyde and *trans*-cinnamaldehyde in $CDCl_3$ (not shown). The two peaks for H_b (5-member ring), H_f (6-member ring), H_p (5-member ring), and H_r (6-member ring) were observed at the same chemical shift values of others (Figure S3).[1-3]

Wang et al.[4] synthesized vanillin-GL acetals under mild acidic conditions, and identified the signals for H_j (5-member ring) and H_m (6-member ring) from the vanillin-GL acetals in the ^{1}H NMR spectrum (DMSO-d_6 solvent). We observed the signals for H_j (5-member ring) and H_m (6-member ring) at the same ppm values as Wang et al. (Figure S3). We determined the diastereomeric ratio of each flavorant-PG and -GL acetal in this study. The diastereomeric ratio for each flavorant-PG acetal standard was 0.8:1.0 ± 0.1, and was similar to the diastereomeric ratio for flavorant-acetals in PG e-liquids when acetal formation reached equilibrium (Figure S2). The diastereomeric ratio for the 5-

member GL acetals (H_b, H_j, and H_p) were similar for each flavorant in GL e-liquids (at equilibrium) with a ratio of 0.9:1.0 ± 0.1 (Figure S3). The 6-member flavorant-GL acetals (H_f, H_m, and H_r) had diastereomeric ratios of 1.0:0.5 ± 0.1 for each flavorant in GL e-liquids (at equilibrium) (Figure S3). The diastereomeric ratio for flavorant-PG and -GL acetals would change if a catalyst was added to the e-liquid.[5]

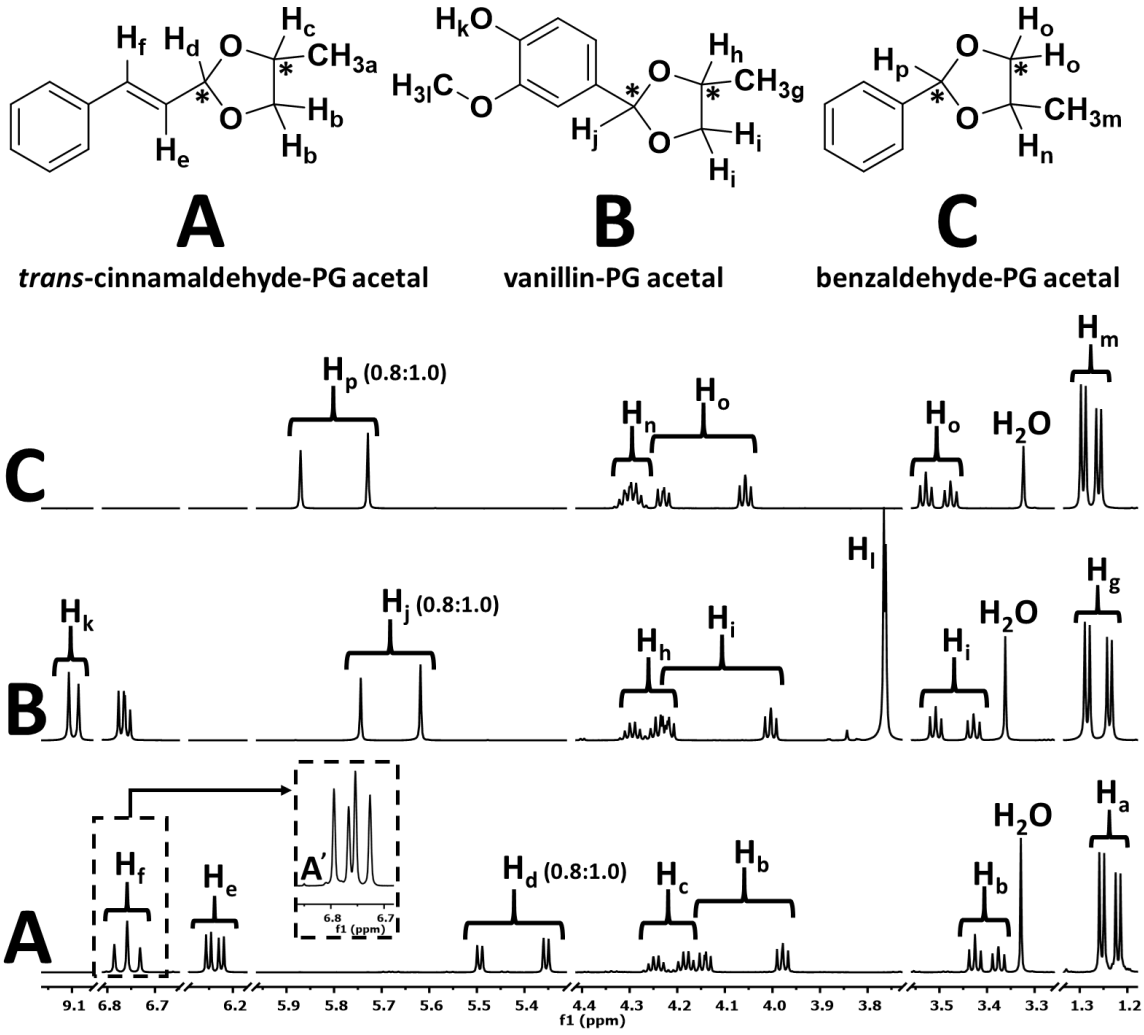

Figure 3. The ^{1}H NMR spectra for the (A) *trans*-cinnamaldehyde-propylene glycol (PG) acetal, (B) vanillin-PG acetal, and (C) benzaldehyde-PG acetal in DMSO-d_6 with proton assignments. A 600 and 400 MHz NMR spectrometer were used to collect spectra A-C and A', respectively. The "*" on each flavorant-PG acetal indicates a stereocenter. The peaks from each proton (H_a- H_p) are doubled since the flavorant-PG acetals are diastereomers. The assignment for H_f resembles a triplet on A, but is two doublets as shown on A'. Undesignated peaks on the spectra are from aromatic protons. The integrations for the two peaks associated with H_d, H_j, and H_p are from each acetal diastereomer, and represent molar equivalents.

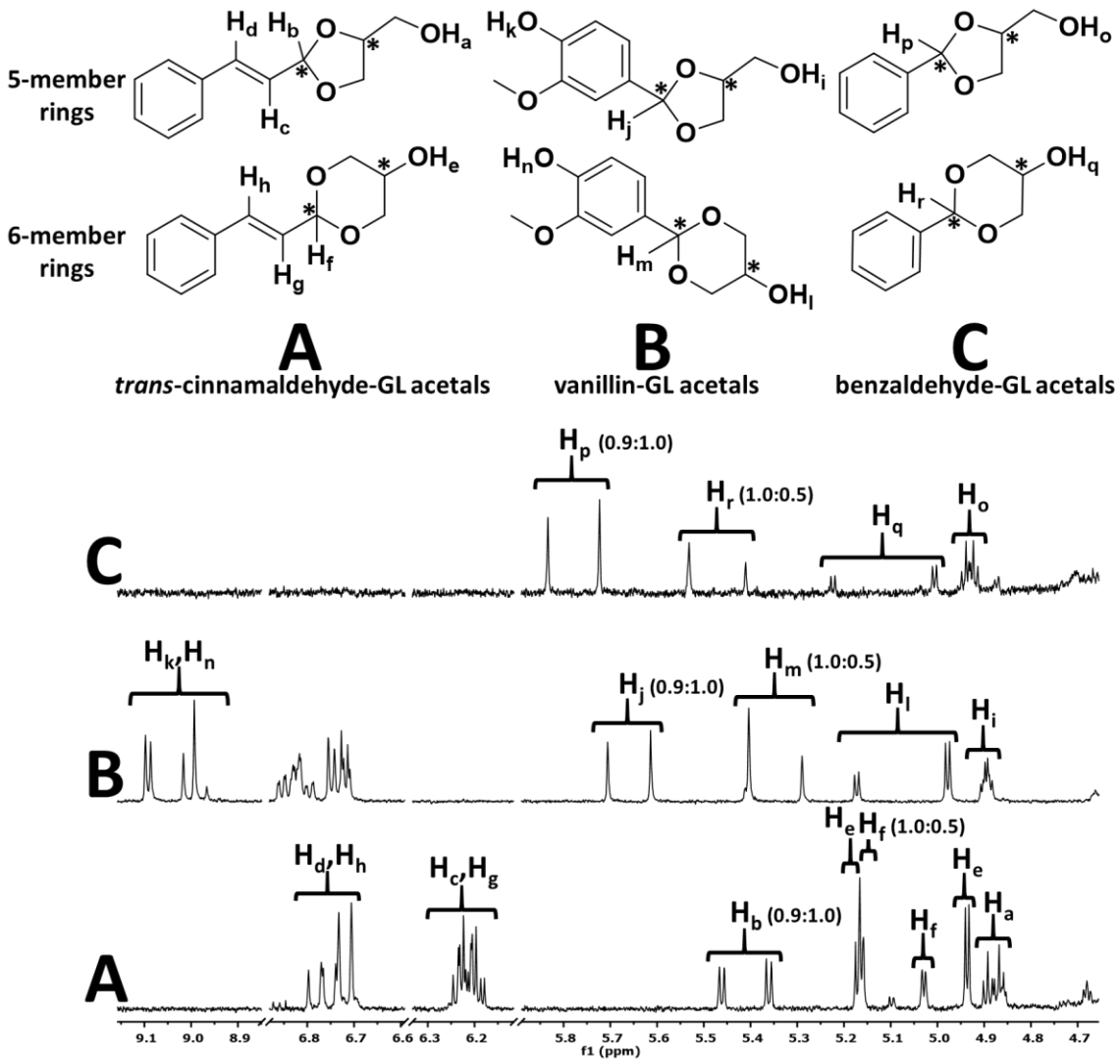

Figure 4. The ^{1}H NMR spectra for glycerol (GL) e-liquids with (A) *trans*-cinnamaldehyde, (B) vanillin, and (C) benzaldehyde in DMSO-d_6 showing some of the peaks associated with the 5- and 6-member ring flavorant-GL acetals. The "*" on each flavorant-GL acetal indicates a stereocenter. The peaks from each proton (H_a- H_r) are doubled since the flavorant-GL acetals are diastereomers. Undesignated peaks on the ^{1}H NMR spectra are from the parent flavorant or aromatic protons. The integrations for the two peaks associated with H_b, H_f, H_j, H_m, H_p, and H_r are from each acetal diastereomer, and represent molar equivalents.

3.9.3. Appendix B References

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4. Conclusions

The two e-cigarette chemistry manuscripts[1,2] in this thesis discuss how some flavorants affect the composition of e-liquids with and without common additives before and during aerosolization. The purpose of determining the effects of common e-liquid flavorants and added nicotine on toxicant formation (as measured by carbonyl production) during vaping was to show how individual additives and mixtures of additives alter the aerosol toxicant profile. The study of the kinetics of aldehyde flavorant formation in e-liquids with different solvents and common additives aimed to illustrate how individual and mixtures of additives alter the rate constant, half-life, and yield of acetalization before aerosolization. The results from these studies show that some flavorant and additive mixtures in e-liquids (i.e. *trans-cinnamaldehyde*) are more toxic than others (i.e. vanillin). These results should be valuable to regulators and consumers for the purpose of harm reduction.

4.1. The Effects of Common E-liquid Flavorants and Added Nicotine on Toxicant Formation during Vaping Analyzed by ^{1}H NMR Spectroscopy[1]

The effects of flavorants and flavorants+nicotine on toxicant formation upon e-liquid aerosolization were explored using ^{1}H NMR spectroscopy. The samples were aerosolized with a Kangertech Subtank Mini (a box mod e-cigarette; equipped with a 1.2 Ω coil) attached to a KBOX Mini set at 22 W, and followed the CORESTA puff protocol, which were detailed previously by Duell et al.[3,4] An equimolar PG+GL e-liquid was aerosolized, followed by an e-liquid with flavorant, and then an e-liquid with flavorant+6

mg/mL nicotine. The selected flavorants and their concentrations – based on the most common and concentrated flavorants from Behar et al.[5] – were 2.5 mg/mL benzaldehyde, 31 mg/mL vanillin, 39 mg/mL benzyl alcohol, 39 and 155 mg/mL *trans*-cinnamaldehyde, and a "flavorant mixture" (0.025 mg/mL benzaldehyde; 7.75 mg/mL vanillin; 9.75 mg/mL benzyl alcohol; 39 mg/mL trans-cinnamaldehyde). In the absence of nicotine, toxicant levels increased in aerosolized e-liquids with flavorants compared to those without flavorants. However, when nicotine was present, the toxicant levels decreased in aerosolized flavored e-liquids containing benzaldehyde, vanillin, benzyl alcohol, and "flavorant mixture," but increased in e-liquids containing *trans*-cinnamaldehyde compared to those without.

The effects of nicotine on HPHC levels were interpreted by comparing aerosolized flavored e-liquids with nicotine versus without nicotine, herein referred to as the "nicotine degradation factor." A nicotine degradation factor <1, =1, and >1 meant degradation levels were inhibited, not affected, and enhanced, respectively. The degradation factors were <1 for benzaldehyde, vanillin, benzyl alcohol, and a "flavorant mixture," but >1 for *trans*-cinnamaldehyde (for both 39 and 155 mg/mL flavorant). Flavored e-liquids containing nicotine with vanillin had the lowest degradation factor at ~0.5, and *trans*-cinnamaldehyde had the highest degradation factor at ~2.5.

The effects of nicotine on degradation levels were also determined by aerosolizing an equimolar PG+GL e-liquid, followed by an e-liquid with 6 mg/mL nicotine. The nicotine degradation factor was determined by comparing the aerosolized e-liquid with nicotine compared to without. The HPHC levels were similar in e-liquids with

and without nicotine, and the degradation factor was ~1. These results indicated that nicotine alone had no effect on toxicant levels.

The effects of nicotine on toxicant levels in flavored e-liquids is complex, and dependent on the individual flavorant. Nicotine could inhibit thermal degradation if the primary degradation mechanism of the flavored e-liquid is acid catalyzed. Known oxidation products of *trans*-cinnamaldehyde, including *trans*-cinnamic acid, benzaldehyde, styrene, and toluene, were identified in aerosolized *trans*-cinnamaldehyde-containing e-liquids. *Trans*-cinnamaldehyde can produce acids upon oxidation to promote the neutralization of nicotine and enhance degradation levels (with excess acids). Similarly, triacetin[6] and sucralose[3] degrade into acetic acid and HCl during aerosolization which results in enhanced carbonyl levels, respectively. Flavorants that produce acids upon aerosolization have the potential to increase degradation levels in e-liquids (with and without nicotine) by catalyzing reactions or neutralizing basic species compared to flavorants that generate nonacidic species.

4.2. The Kinetics of Aldehyde Flavorant-Acetal Formation in E-liquids with Different E-cigarette Solvents and Common Additives Studied by ^{1}H NMR Spectroscopy[2]

The kinetics of aldehyde-flavorant acetal formation in e-liquids were determined using ^{1}H NMR spectroscopy. The e-liquid solvents included pure PG, pure GL, and equimolar PG+GL. The common additives water, nicotine, benzoic acid, and nicotine+benzoic acid were only added to pure PG e-liquids. E-liquids were formulated in the laboratory with their additives (e.g. water, nicotine, benzoic acid, nicotine+benzoic

acid) and an internal standard (1,3,5-trimethoxybenzene; TMB) prior to the addition of flavorants. The acetalization reaction was monitored ~5 minutes after the flavorants were added to the e-liquid. Aliquots of each e-liquid sample were collected in NMR tubes pre-filled with DMSO-d_6 and analyzed over time. PG, GL, and PG+GL e-liquids contained 2.5 mg/mL benzaldehyde (+1 mg/mL TMB), 31 mg/mL vanillin (+3.1 mg/mL TMB), and 10 mg/mL *trans*-cinnamaldehyde (+1 mg/mL TMB). The concentrations were again based on the maximum values of flavorants determined in commercial e-liquids from Behar et al.[5] PG e-liquids contained 2.5 mg/mL flavorant (+1 mg/mL TMB) with water, nicotine, benzoic acid, and nicotine+benzoic acid. Commercial e-liquids with high concentrations of aldehyde flavorants were also analyzed for acetal formation.

In this study, it was found that acetalization occurs at a faster rate and produces a higher yield in e-liquids with a higher ratio of GL than PG. *Trans*-cinnamaldehyde acetals formed the fastest in PG e-liquids, followed by benzaldehyde, and vanillin according their half-lives and first order rate constants. However, benzaldehyde produced the highest acetal yield in PG e-liquids, followed by *trans*-cinnamaldehyde, and vanillin. Additionally, acetal formation was inhibited by water and nicotine, but promoted by benzoic acid in PG e-liquids. The presence of nicotine in flavored PG e-liquids generally delayed the rate of acetal formation, even if benzoic acid was present at 2-, 4-, and 10-fold the nicotine concentration, compared to the e-liquid with 2.5 mg/mL flavorant. The commercial e-liquids without nicotine typically contained a higher percentage of acetals compared to those without. The rates and cytotoxicity of flavorant acetals in PG e-liquids

have been studied by others[7–9], but there was limited information about how different solvents and additives affect the rate of acetal formation prior to the present study.

4.3. Overall Conclusions

The two manuscripts in this document detail how flavorants with and without additives chemically alter the e-liquid composition before and after aerosolization. Understanding the toxicological implications of e-cigarette flavorants, additives, and flavorant+additive mixtures can impact how regulatory agencies (e.g. the FDA) create harm reduction policies. For example, aerosolized flavored e-liquids with nicotine versus without were more toxic in the presence of *trans*-cinnamaldehyde compared to benzaldehyde, vanillin, benzyl alcohol, and "flavorant mixture." Before aerosolization, flavored e-liquids with acids formed a new molecule with a unique toxicant profile (i.e. aldehyde flavorant-acetals) at a faster rate compare to without. Regulators can use this information to predict the effects of flavorants on toxicant formation in the presence of additives, design similar experiments including other flavorants, and inform policies limiting what manufacturers are legally allowed to put in e-liquids.

In the future, e-liquids containing other α,β-unsaturated aldehyde flavorants with and without nicotine should be explored. Chen et al.[10] have previously shown that the carbonyl- and β-carbon on *trans*-2-hexenal (an α,β-unsaturated aldehyde) are more susceptible to nucleophilic attack than simple carbonyls (e.g. benzaldehyde, acetaldehyde). In the e-liquids used in the present study, nicotine was held constant at 6 mg/mL, but commercial e-liquids can contain nicotine at concentrations between 0 – 60

mg/mL.[11] Increasing the nicotine concentration in the flavored e-liquids up to 60 mg/mL should be explored in the future. Furthermore, the effects of monoprotonated nicotine on toxicant formation should be studied in aerosolized e-liquids with and without flavorants due the popularity of nicotine salts.

Many of the popular flavorants identified in commercial e-liquids are chemically classified as aldehydes and ketones. Ketone flavorants, similar to aldehydes, can react with PG and GL to form PG- and GL-flavorant ketals. Future studies of acetal and ketal formation in e-liquids should examine other acid additives, especially diprotic and triprotic acids (e.g. salicylic and malic acids, respectively).[12] Furthermore, studying the kinetics of acetal and ketal formation in the presence of other flavorants would be valuable since commercial e-liquids typically contain flavorants mixtures.[13,14]

4.4. References

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