Original investigation

Formation of flavorant–propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids

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Abstract

Introduction: “Vaping” electronic cigarettes (e-cigarettes) is increasingly popular with youth, driven by the wide range of available flavors, often created using flavor aldehydes. The objective of this study was to examine whether flavor aldehydes remain stable in e-cigarette liquids or whether they undergo chemical reactions, forming novel chemical species that may cause harm to the user.

Methods: Gas chromatography was used to determine concentrations of flavor aldehydes and reaction products in e-liquids and vapor generated from a commercial e-cigarette. Stability of the detected reaction products in aqueous media was monitored by ultraviolet spectroscopy and nuclear magnetic resonance spectroscopy, and their effects on irritant receptors determined by fluorescent calcium imaging in HEK-293T cells.

Results: Flavor aldehydes including benzaldehyde, cinnamaldehyde, citral, ethylvanillin, and vanillin rapidly reacted with the e-liquid solvent propylene glycol (PG) after mixing, and upward of 40% of flavor aldehyde content was converted to flavor aldehyde PG acetals, which were also detected in commercial e-liquids. Vaping experiments showed carryover rates of 50%–80% of acetals to e-cigarette vapor. Acetals remained stable in physiological aqueous solution, with half-lives above 36 hours, suggesting they persist when inhaled by the user. Acetals activated aldehyde-sensitive TRPA1 irritant receptors and aldehyde-insensitive TRPV1 irritant receptors.

Conclusions: E-liquids are potentially reactive chemical systems in which new compounds can form after mixing of constituents and during storage, as demonstrated here for flavor aldehyde PG acetals, with unexpected toxicological effects. For regulatory purposes, a rigorous process is advised to monitor the potentially changing composition of e-liquids and e-vapors over time, to identify possible health hazards.

Implications: This study demonstrates that e-cigarette liquids can be chemically unstable, with reactions occurring between flavorant and solvent components immediately after mixing at room temperature. The resulting compounds have toxicological properties that differ from either the

flavorants or solvent components. These findings suggest that the reporting of manufacturing ingredients of e-liquids is insufficient for a safety assessment. The establishment of an analytical workflow to detect newly formed compounds in e-liquids and their potential toxicological effects is imperative for regulatory risk analysis.

Introduction

The addition of characterizing flavors to combustible cigarettes is prohibited by regulatory measures in several jurisdictions, including the United States, Canada, and the European Union; however, these restrictions have not been extended to “e-liquids” used in electronic cigarettes (e-cigarettes). Along with the global increase in e-cigarette use,^{1–3} the variety of flavored e-liquids has risen, with currently more than 7000 flavors available.⁴ Among the most popular e-liquids are sweet and fruity flavors, a main driver for the appeal of e-cigarettes to youth.^{5–7} Fragrant aldehydes, such as vanillin and ethylvanillin (vanilla flavor), benzaldehyde (cherry flavor), or cinnamaldehyde (cinnamon flavor), commonly serve as the characteristic flavorants.⁸ E-liquids are prepared by mixing the flavorants and nicotine with different ratios of the solvents propylene glycol (PG) and glycerol (VG for vegetable glycerin), which contain two and three alcohol moieties, respectively.⁹

However, aldehydes and alcohols can undergo chemical reactions resulting in the formation of acetals (Figure 1). An acetalization reaction between an aldehyde (1) and PG (2) generally yields aldehyde PG acetals (3) with two chiral carbons (indicated with *), resulting in the formation of at least three stereoisomers; molecules with different bond orientation and therefore possibly different properties, including toxicological properties.¹⁰ Acetalization reactions are commonly used in synthetic organic chemistry to “protect” reactive aldehyde moieties from reacting further. Consequently, chemical reactions (ie, acetal formation) between e-liquid constituents are possible, suggesting that e-liquids can be unstable environments post-preparation. Indeed, recent analytical studies have reported the presence of flavor aldehyde acetals in e-liquids, in the headspace above e-liquids, and in e-cigarette vapors (e-vapor).^{11–13} In the cases where manufacturers published ingredient lists of e-liquids (available online), only aldehydes, but not acetals, were listed among the flavorants, suggesting that these acetals form after mixing. The published analytical studies did not report the concentrations of the detected flavor aldehyde acetals in the respective e-liquids, and it remains unclear how frequently and how rapidly these compounds form and whether they remain stable during heating and vaporization in e-cigarettes. Beyond flavor aldehyde acetals, the presence of formaldehyde hemiacetals in e-vapors was reported, generated from formaldehyde reacting with a single alcohol moiety on PG or VG.¹⁴ Formaldehyde and other small aldehydes have been shown to form from PG or VG during vaporization.¹⁵ The presence of aldehydes in e-liquids and e-vapor, either as flavorants or chemical reaction products, has raised concerns because of their potential respiratory and cardiovascular toxicological effects. Respiratory sensory irritation is a first sign of potential toxicity of an inhaled chemical exposure, induced by chemical activation of chemosensory receptors in airway-innervating nerves. Respiratory sensory irritants are ranked according to their RD_{50} , the toxicological parameter denoting the exposure concentration causing a 50% drop in respiratory rates in rodents, and used to determine occupational and other exposure limits. Recent studies identified the transient receptor potential (TRP) ion channels, TRPA1 and TRPV1, as the receptors for

irritant aldehydes in airway-innervating nerves, activated by the major tobacco smoke aldehyde, acrolein, and flavor aldehydes such as cinnamaldehyde, benzaldehyde, and vanillin, and eliciting irritation responses, pain, and cardiovascular reflexes increasing stress and inflammation.^{16–19} In vitro tests quantifying the capability of a chemical to activate TRP irritant receptors are currently considered as replacements for the animal studies determining RD_{50} s. It remains to be examined whether aldehyde PG acetals activate the same irritant mechanisms as their parent aldehydes and exhibit toxicological effects of concern.

The aims of this study were to determine whether: (1) aldehyde flavorants and PG yield acetals in e-liquid environments, (2) acetals are carried over into e-vapor, (3) acetals remain stable under physiological conditions, and (4) acetals activate TRP irritant receptors. A workflow is proposed for a comprehensive risk assessment of e-liquids and resulting e-vapors encompassing all compounds a user could be exposed to, including reaction products.

Materials and Methods

Acetal Formation Experiments

One hundred micrograms of benzaldehyde (100 µg, >99%; Sigma-Aldrich, St. Louis, MO), *trans*-cinnamaldehyde (98+%; Alfa Aesar, Haverhill, MA), citral (mixture of the structural isomers neral and geranial; 95%, Fisher Scientific, Waltham, MA) ethylvanillin (99%), and vanillin (99%; both Sigma-Aldrich) were added to plastic vials containing 5 g PG (USP-FCC grade, Waltham, MA), or mixtures of PG/VG (anhydrous; AmericanBio, Natick, MA) at 70/30, 50/50, or 30/70 ratios by weight, to yield an aldehyde concentration of 20 mg/g. The experiments in pure PG were carried out in duplicate (light and dark) storage conditions. Vials were vigorously shaken once per day, and 100 µL samples were drawn, diluted, and injected into a gas chromatograph (GC). Using standard curves, samples were analyzed for their aldehyde and PG acetal content (vanillin PG acetal [97%], ethylvanillin PG acetal [98%, both Bedoukian Research, Danbury, CT], benzaldehyde PG acetal [$>98\%$], *trans*-cinnamaldehyde PG acetal [both Sigma-Aldrich], citral PG acetals synthesized in-house, see [Supplementary Material for details](#)). Results are presented as mole fraction of formed acetal, defined as moles of acetal divided by the combined number of moles of acetal and corresponding aldehyde.

Acetal Content of Commercial E-liquids

Two flavored e-liquids (cherry and vanilla) were purchased with PG/VG ratios of 100/0, 70/30, 50/50, 30/70, and 0/100, containing 12 mg/mL nicotine (AmericaneLiquidStore, Wauwatosa, WI). Samples of all e-liquids were taken, the mass recorded, and diluted for GC injection. Using standard curves, aldehyde, aldehyde PG acetal, and nicotine (99%, Alfa Aesar) concentrations were determined.

Vaping Carryover Experiments

For the vaping experiments, V2 blank cartridges were purchased online and filled with 0.5 g e-liquid and used with the corresponding

79 mm, 4.2 V battery (both VMR Products LLC, Miami, FL). Cartridges were allowed to settle for at least 60 minutes before testing. E-vapors were produced and captured using a custom-built vaping machine described previously, using a series of cold-finger traps chilled with liquid nitrogen.²⁰ The puffing topography was 4-second puffs, 73 ml puff volume, 30-second intervals, for 20 puffs total. The trapped e-vapor was subsequently taken up in methanol (JT Baker, Center Valley, PA) and injected into a GC. Compound e-vapor concentration was calculated as total amount trapped in vapor divided by the mass change of the cartridge after 20 puffs. Carryover percentage was calculated by dividing e-vapor concentration by neat e-liquid concentration.

Acetal Aqueous Stability Testing

To assess the stability of the PG acetals in physiological solution, ultraviolet-visible (UV-VIS) and proton nuclear magnetic resonance (¹H-NMR) spectroscopy were used. The breakdown of vanillin PG acetal (starting concentration 100 µg/mL) to vanillin in Hanks balanced salts solutions buffer (Sigma-Aldrich), adjusted to pH 7.4, was monitored over time by UV-VIS spectroscopy at $\lambda = 347$ nm (Cary-3E; Varian, Palo Alto, CA). Breakdown of the other PG acetals in D₂O (99.9 atom% D; Sigma-Aldrich) was monitored over time by ¹H-NMR spectroscopy, comparing the appearing aldehyde signal at approximately $\delta = 9.6$ ppm to the disappearing acetal doublet between $\delta = 5.6$ – 5.9 ppm. ¹H-NMR studies were carried out at starting concentrations of 150 µg/mL (benzaldehyde- and vanillin PG acetal) and 200 µg/mL (ethylvanillin PG acetal).

Gas Chromatography

A GC (GC-2010 Plus; Shimadzu, Kyoto, Japan) was used to quantify aldehydes, PG acetals, and nicotine. Citral and citral PG acetals were quantified as a combination of *cis*- and *trans*-isomers (see [Supplementary Figure 5](#) for an example of a chromatogram). For the acetal formation reaction, 100 µL samples were diluted with 1 mL of methanol (high-performance liquid chromatography [HPLC] grade, JT Baker) containing 1 g/L 1,4-dioxane (99+%, Alfa Aesar) as an internal standard. For the carryover experiments, the complete content of the cold-finger trap was taken up in 1 mL of methanol (JT Baker) containing internal standard. See [Supplementary Material](#) for further GC method details. Calibration curves were prepared to calculate concentrations from peak area ratios of the compounds of interest to the internal standard.

Liquid Chromatography

A liquid chromatography–refractive index (LC–RI) (Shimadzu LC-20 system, Shimadzu, Kyoto, Japan) was used to determine PG and VG contents of commercial e-liquids. Method details are provided in the [Supplementary Material](#). Samples were diluted with deionized water (HPLC grade, JT Baker) and 1,4-dioxane was added as an internal standard. Calibration curves of PG and VG were prepared to calculate concentrations from peak area ratios of compounds to the internal standard.

¹H-NMR Spectroscopy

All ¹H-NMR spectroscopy was carried out on an Agilent DD2 400 MHz NMR spectrometer, using D₂O, methanol (CD₃OD), or chloroform (CDCl₃; all at least 99.8% D, Cambridge Isotope Labs., Andover, MA).

Cell Culture

HEK-293T cells (ATCC, Manassas, VA; Cat# CRL-11268, RRID: CVCL_1926) were cultured in Gibco Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (all Fisher Scientific). Cells were plated on 100 mm tissue culture plastic dishes and grown overnight to 60%–70% confluency. The cells were transiently transfected with either human TRPA1 or TRPV1 plasmid DNA (18 µg plasmid DNA/100 mm dish) for 16–24 hours using Eugene 6 transfection reagent (Promega, Madison, WI) and Gibco Opti-MEM (Fisher Scientific) according to manufacturer's protocols. Cells were then resuspended and plated onto poly-D-lysine-coated (Sigma-Aldrich) 96-well black-walled plates at 50 000 cells per well and allowed to grow for another 16–24 hours before the experiments. Cells were maintained as monolayers at 5% CO₂ and 37°C.

Calcium Influx Imaging

Calcium imaging was performed as described earlier.²¹ Briefly, the fluorescence calcium indicator FLIPR Calcium 6 dye (Molecular Devices, San Jose, CA) was applied to the cells, which were reconstituted in Hanks balanced salts solutions buffered at pH 7.4 using 20 mM HEPES buffer (both Sigma-Aldrich). A FlexStation III benchtop scanning fluorometer chamber (Molecular Devices) at 37°C was used to excite cells at 485 nm and the Ca²⁺ emission signal was recorded at 525 nm in intervals of 1.8 seconds. The change in fluorescence was measured and expressed as $F_{\max} - F_0$, where $F_{\max}$ is the maximum fluorescence and F_0 is the basal fluorescence. The responses were normalized to the known agonists cinnamaldehyde (1 mM; for TRPA1) or capsaicin (3 µM; for TRPV1). A Boltzmann function (3- or 4-parameter logistic fit) was used to generate concentration–response curves and determine the half maximal effective concentration (EC₅₀) values (Prism 7 software; GraphPad, La Jolla, CA). 95% confidence intervals (CI) of the logEC₅₀ values were calculated to display the variability of concentration–response curves. Efficacies (maximal responses) for each of the flavor aldehydes and their corresponding PG acetals were reported as a percentage of the maximum response elicited by saturating concentrations of the full agonists, cinnamaldehyde (1 mM) for TRPA1 and capsaicin (3 µM) for TRPV1 ([Table 1](#)).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software (La Jolla, CA), for one-way analysis of variance tests with Bonferroni post-tests, and Student's *t* tests. A *p* value less than .05 was interpreted as significant, with *p* value less than .05 represented by *, *p* value of less than .01 by **, and *p* value less than .001 by ***.

Results

Acetal Formation and Presence in Laboratory-made and Commercial E-liquids

To investigate the formation kinetics of flavor aldehyde-PG acetals, commonly used flavor aldehydes were mixed with pure PG and sampled over 14 days while stored at room temperature ([Figure 1](#)); aldehydes tested were benzaldehyde, cinnamaldehyde, citral (mixture of the two structural isomers neral and geranial), vanillin, and ethylvanillin, each at 20 mg/g, approximating the concentrations found in commercial e-liquids ([Supplementary Figure 1](#)). The PG acetal formation reactions were monitored in daylight and dark conditions with no significant

Table 1. Overview of Compounds Tested: Compound and CAS Number, Boiling Point (bp), Observed Carryover from E-liquid to E-vapor, Calculated Half-lives Based on First-order Kinetics for Propylene Glycol (PG) Acetals in Two Different Aqueous Media, and EC₅₀ (Including 95% Confidence Interval [CI]) and Efficacy Compared to the Known Antagonists for Human TRPA1 and TRPV1 Ion Channels (1 mM Cinnamaldehyde and 3 μ M Capsaicin, Respectively). Statistical Analysis Indicated for Differences Between Parent Aldehyde and Corresponding Acetal, for EC₅₀s and Efficacies, Respectively Parent Aldehyde and Corresponding Acetal (ns Nonsignificant; *, $p < .05$; **, $p < .01$; ***, $p < .001$)

Compound (CAS no.)	bp (°C)	Observed carryover range to e-vapor (%)	Half-life in D ₂ O (HBSS buffer) (h)	Human TRPA1		Human TRPV1	
				EC ₅₀ (95% CI)	Efficacy (to cinnamaldehyde)	EC ₅₀ (95% CI)	Efficacy (to capsaicin)
Benzaldehyde (100-52-7) ^a	178	51%–71% ^c	—	0.30 mM ^{***} (0.23 to 0.39)	91% ^{ns}	> 12 mM	36% [*]
Benzaldehyde PG acetal (2568-25-4) ^a	230–232	47%–80% ^c	44 h	0.88 mM ^{***} (0.70 to 1.2)	93% ^{ns}	2.4 mM (1.7 to 4.2)	47% [*]
Ethylvanillin (121-32-4) ^a	295	62%–71% ^d	—	0.17 mM ^{***} (0.13 to 0.22)	74% ^{***}	> 5.6 mM	21% ^{***}
Ethylvanillin PG acetal (68527-76-4) ^b	346–347	61%–71% ^d	70 h	0.69 mM ^{***} (0.57 to 0.81)	90% ^{***}	3.5 mM (3.3 to 3.7)	40% ^{***}
Vanillin (121-33-5) ^a	285	63%–71% ^d	—	0.18 mM ^{**} (0.12 to 0.28)	63% ^{***}	> 3 mM	9% ^{***}
Vanillin PG acetal (68527-74-2) ^b	353	59%–65% ^d	34–39 h (48 h)	0.91 mM ^{**} (0.76 to 1.1)	93% ^{***}	1.1 mM (0.92 to 1.3)	56% ^{***}
Nicotine (54-11-5) ^a	247	52%–66% ^c	—	—	—	—	—

^aSource: Sigma-Aldrich; ^bSource: Bedoukian Research, Danbury, CT; ^ce-liquid PG/VG range 100/0 to 5/95; ^de-liquid PG/VG range 100/0 to 40/60. HBSS = Hanks balanced salts solutions; VG = glycerol.

Figure 1. Top: general aldehyde propylene glycol (PG) acetal formation reaction involving an aldehyde **1** and PG **2**, to form the PG acetal **3**. The reversible reaction yields compound **3** with two stereocenters indicated by *; middle: reaction kinetics of formation of aldehyde PG acetals from aldehydes dissolved in PG at a concentration of 20 µg/g of aldehyde, displayed as mole fraction of PG acetal against time. Citral PG acetals quantified as combined *cis*- and *trans*-isomers. Mean of *n* = 2 experiments in daylight and dark storage shown; error bars indicate the measured range; inset: formation of cinnamaldehyde PG acetal in mixtures of PG and glycerol; bottom: mole fractions of PG acetal to aldehyde in commercial cherry- and vanilla-flavored e-liquids as a function of PG content, for benzaldehyde, ethylvanillin, and vanillin.

difference observed. Acetal mole fractions of the combined aldehyde and acetal content were measured by GC-flame ionization detector, and conversion rates and equilibrium concentrations were determined (Figure 1): cinnamaldehyde and citral conversions plateaued within the first day, at 92% and 88%, respectively. A small decline in PG acetal

mole fractions was observed subsequently. Benzaldehyde PG acetal formed more slowly, reaching equilibrium around day 5 and exceeding 95% mole fraction. Ethylvanillin and vanillin were converted to PG acetals more slowly, reaching equilibrium after approximately 7 days, at ca. 50% and 40% mole fractions, respectively.

The effect of varying PG/VG ratios, which are commonly offered in commercial e-liquids, on the formation of cinnamaldehyde PG acetal was also evaluated (Figure 1 inset). When the PG/VG ratio was decreased, lower equilibrium concentrations of cinnamaldehyde PG acetal were observed. These findings demonstrate that the rate and amount of PG acetal formed varies for different aldehydes and depends on the ratio of PG/VG present.

To evaluate whether aldehyde PG acetals are present in commercial e-liquids, and whether their formation depends on PG content, a range of commercial vanilla (vanillin and ethylvanillin) and cherry (benzaldehyde)-flavored e-liquids were examined (Figure 1 and Supplementary Figure 1). The vanilla e-liquids tested in this study were purchased with PG contents of 0%, 30%, 50%, 70%, and 100% labeled as such by the vendor, however, LC-IR revealed that the actual PG contents were 60%, 70%, 80%, 85%, and 100%,

Figure 2. Carryover rates of benzaldehyde and benzaldehyde propylene glycol (PG) acetal from e-liquid to vapor generated by a first-generation e-cigarette, as a function of PG content of the neat e-liquid with the residual made up by glycerol (VG). Error bars represent the SD of at least $n = 3$ separate experiments (filled symbols), or the range when $n = 2$ (non-filled symbols). Statistical significance (*, $p < .05$) found only between benzaldehyde PG acetal 5% and 100%. Carryover results for vanillin, ethylvanillin, and nicotine are shown in Supplementary Figure 2.

respectively. For all three compounds shown in Figure 1, a trend toward increasing mole fraction of the respective acetals with increasing PG content of the e-liquid was observed, which means a higher proportion of flavor aldehyde was converted to the corresponding acetal. This effect was more pronounced for benzaldehyde PG acetal, especially at low PG content. These data demonstrate that the amounts of PG acetals present in commercial e-liquids are proportional to the ratio of PG/VG used to prepare the e-liquid. Further, they support the earlier finding that benzaldehyde reacts more readily with PG than vanillin or ethyl vanillin.

Compound Delivery to E-cigarette Vapor

During vaping, flavor aldehydes are aerosolized and deposited in the airways along with other constituents. To determine whether aldehyde PG acetals are also carried over into e-vapor, the concentrations of benzaldehyde, vanillin, ethylvanillin, their respective PG acetals, and nicotine were measured in cold-trapped e-vapor (Figure 2 and Supplementary Figure 2). Carryover rates for aldehydes and their corresponding acetals were very similar, amounting to approximately 50%–80% of their concentration in neat e-liquid (Table 1). Carryover rates did not vary statistically significantly with change in PG content, except for benzaldehyde at 5% and 100% PG content (Figure 2). Nicotine e-vapor carryover was also determined at rates close to 60% (Supplementary Figure 2) that is similar to rates reported in previous studies,^{22,23} thereby validating the used methodology for quantifying carryover. Taken together, these results suggest that similar to flavor aldehydes, a significant proportion of the aldehyde PG acetal will reach the airways during vaping.

Acetal Aqueous Stability

For aldehyde PG acetals to exert biological effects, acetals need to persist on deposition in the respiratory system. To determine whether aldehyde PG acetals undergo hydrolysis in physiological solution, UV-VIS and ¹H-NMR spectroscopy were used. First, the hydrolysis of vanillin PG acetal to vanillin was monitored by UV-VIS spectroscopy in physiological Hanks balanced salts solutions buffer at pH 7.4, resembling the pH of airway lining fluid of healthy humans.²⁴ A series of UV-VIS measurements followed the hydrolysis of vanillin PG acetal to pure vanillin by the growth of an absorption peak at 347 nm (Supplementary Figure 3). This technique is used to determine vanillin concentrations in foods,²⁵ yet it is pH-dependent,²⁶

Figure 3. Effects of vanillin and vanillin propylene glycol (PG) acetal on human sensory irritant receptors, TRPA1 and TRPV1, measured by fluorescent calcium imaging in HEK-293T cells. Responses were normalized to maximal cinnamaldehyde (for TRPA1) or capsaicin (for TRPV1) responses. Each point represents mean values of 15–21 independent measures from 4 experiments, and error bars show standard error of the mean. Results for benzaldehyde, ethylvanillin, and their corresponding PG acetals are shown in Supplementary Figure 4.

warranting the use of a buffered solution. Using first-order kinetics, the half-life of vanillin PG acetal under these conditions was determined to be approximately 48 hours (Table 1). Due to the absence of characteristic UV-VIS absorption peaks for the other aldehyde PG acetals, their hydrolysis was monitored by $^1\text{H-NMR}$ in unbuffered D_2O . Vanillin PG acetal was used as control for comparison of methods and buffer effects, with a series of $^1\text{H-NMR}$ spectra shown in Supplementary Figure 3 indicating the disappearance of vanillin PG acetal (doublet peak at $\delta = 5.6\text{--}5.8$) because of its conversion to vanillin (singlet peak $\delta = 9.6$) over time. Similar series of spectra were recorded for the PG acetals of benzaldehyde and ethylvanillin. After peak integration, the half-lives of these three compounds in D_2O were calculated as 34–39 hours (vanillin PG acetal), 44 hours (benzaldehyde PG acetal), and 70 hours (ethylvanillin PG acetal) based on first-order kinetics (Table 1.) A similar attempt to determine the aqueous stability of cinnamaldehyde PG acetal failed because of the very low solubility of the compound in D_2O . All measured half-lives are in the order of days, suggesting that the tested acetals are not instantly hydrolyzed once they reach the aqueous environment of the airway surfaces, warranting investigations of their potential biological effects.

Acetal Irritation Potential

To compare the effects of flavor aldehydes and their corresponding PG acetals on TRP irritant receptors, HEK-293T cells were used for heterologous expression of human TRPA1 and TRPV1 ion channels and fluorometric measurements of TRP-mediated Ca^{2+} influx.

Indeed, ethyl vanillin PG acetal, vanillin PG acetal, and benzaldehyde PG acetal robustly activated Ca^{2+} influx through TRPA1, with all three flavor aldehyde acetals as efficacious as the full TRPA1 agonist, cinnamaldehyde (>90% of maximum cinnamaldehyde response; Figures 3 and Supplementary Figure 4; Table 1). Further, all tested PG acetals activated TRPA1 with higher EC_{50} values compared to their corresponding aldehydes, and interestingly, high concentrations of vanillin PG acetal and ethylvanillin PG acetal (at 3 mM and 10 mM, respectively) were more efficacious at activating TRPA1 than equivalent concentrations of their corresponding parent aldehydes (Table 1; Figure 3 and Supplementary Figure 4). The flavor aldehyde PG acetals also had surprising effects on TRPV1, the vanilloid receptor: While benzaldehyde, ethylvanillin, and vanillin weakly activated TRPV1 only at high concentrations (>3 mM) close to their solubility limits, their corresponding PG acetals robustly activated TRPV1 at lower concentrations and with higher efficacies (Table 1, Figure 3 and Supplementary Figure 4). This effect on TRPV1 was most conspicuous when comparing vanillin and vanillin PG acetal (Table 1, Figure 3). Cinnamaldehyde PG acetal robustly activated TRPA1 and significantly activated TRPV1 at a nominal concentration of 500 μM ; however, a dose-response relation could not be established because of limited solubility of the compound.

Discussion

In this study, a reaction between e-liquid constituents of aldehydic flavorants and PG to form aldehyde PG acetals is described. These compounds form in situ and are shown to be present in commercial e-liquids. Carryover experiments reveal that quantities of 50%–80% of the initial acetal concentration in the neat e-liquid are transferred to the e-vapor generated by a first-generation e-cigarette. The relative stability of these acetals to hydrolysis at physiological pH is demonstrated, implying that they persist after inhalation by the e-cigarette user. Finally, this study shows that the tested acetals

activate respiratory irritant receptors with broader selectivities and higher potencies than their aldehyde precursors, suggesting that they may act as stronger airway irritants in e-cigarette users.

To pursue this study, several e-liquids with various PG/VG ratios were purchased online, however, their chemical analysis revealed that their contents were not accurately reflected by the composition on the label. This was evidenced by the measured differences in the component concentrations reported by the vendor versus those measured in the purchased e-liquid products (eg, PG/VG ratios in vanilla e-liquids). Several recent studies reported incorrectly labeled nicotine contents of e-liquids, including substantial amounts of nicotine detected in e-liquids labeled as “nicotine-free”, purchased in several different countries.^{27–29} In light of these reports, the deviating PG/VG ratios measured in this study add to the concerns about inaccurate mixing, labeling and resulting unexpected toxicities of commercial e-liquids. Rapid regulatory action is needed to address the ongoing failure of some e-liquid manufacturers to provide labeling consistent with product contents to the public.^{29–31}

Acetal Formation and Presence in Laboratory-made and Commercial E-liquids

The reaction between aldehyde flavorants and PG to form an acetal occurs at conditions common during the mixing and storage of e-liquids by vendors and users alike. PG acetals formed from all of the tested aldehydic flavorants, including benzaldehyde, cinnamaldehyde, citral (mixture of the structural isomers neral and geranial), vanillin, and ethylvanillin, when mixed with PG (Figure 1). As mentioned in the Introduction section, aldehyde PG acetals contain two chiral carbons, and the two diastereomers can be distinguished in the NMR spectra (Supplementary Figure 3) and GC chromatograms (Supplementary Figure 5). It is interesting to note that the acetal formation kinetics differ for the tested aldehydes, which may be related to how quickly these molecules are solubilized by PG. Vanillin and ethylvanillin are both solid at room temperature, and while they partially dissolved in PG on initial mixing, dissolution was not complete until after 24 hours (observed visually). The slight decline in the concentrations of the PG acetals of cinnamaldehyde and citral at later times suggests that further reactions may occur in e-liquids: both compounds are α,β -unsaturated aldehydes, known to be relatively reactive.³²

Further experiments investigated how the amount of acetal formed can vary as a function of the PG/VG ratio. When the PG/VG ratio is decreased, the equilibrium concentration of the corresponding PG acetal was reduced (Figure 1 inset). Besides PG, VG can also form acetals with aldehydes,⁸ yet because VG contains three alcohol moieties, a multitude of optical and structural isomers of VG acetals can form. At least four of such isomers are shown in a GC-MS chromatogram for cinnamaldehyde VG acetal (Supplementary Figure 6) and vanillin VG acetal (Supplementary Figure 7). Since no standard for cinnamaldehyde VG acetal nor vanillin VG acetal was available, they were not quantified.

Although previous studies detected flavor aldehyde acetals in commercial e-liquids, these were not quantified.^{11,12} In this study, substantial amounts of flavor aldehyde PG acetals were detected in the analyzed commercial e-liquids, with 15%–30% of the aldehyde converted to its corresponding PG acetal, and increasing acetal concentrations at higher PG contents. It is noteworthy that the mole fractions of acetals in the commercial e-liquids did not reach the same equilibrium levels as in the experimental mixtures of only PG and the aldehyde in question (Figure 1). This can be explained by the higher complexity of the commercial e-liquids that contain additional ingredients, including water and other compounds that

can compete with PG to react with the aldehydes, or influence the reaction equilibrium.⁵ However, the same order of mole fractions is found in both the experimental and commercial mixtures: benzaldehyde PG acetal > ethylvanillin PG acetal > vanillin PG acetal. This study also showed that the tested e-liquids contained lower amounts of flavor aldehydes as the ratio of PG/VG in the e-liquid decreased (Supplementary Figure 1). In this case, this likely means that the “flavor base” was mixed in pure PG, and the appropriate amount of VG was added to create the final product.

Flavor aldehyde PG acetals, including the compounds detected in this study (Table 1), are produced commercially as flavorants and scents for food, perfumes, cosmetics, and body care products. It is, therefore, possible that the acetals were added directly to the commercial e-liquids. However, acetals have never been listed in the e-liquid ingredient lists in the few cases where these were revealed, and are less economical than their corresponding aldehydes. They are prepared from the parent aldehyde, suggesting that the acetals would generally be more costly, without a requisite gain in aroma as acetals generally resemble the odor of the parent aldehyde, but are less pronounced.³² The odor is particularly important as the olfactory system (smell) is the dominating system with regards to a user's flavor perception of e-vapor.

Acetal Carryover to Vapor in E-cigarettes

The fate of flavor aldehydes during heating and vaporization in e-cigarettes, and their resulting toxicities, has been the subject of intense controversy. All PG acetals discussed earlier exhibit higher boiling points than their corresponding aldehydes (Table 1), possibly impacting vaporization. Heating may also affect the stability of acetals.

A substantial carryover of intact acetals from commercial e-liquids into the e-vapor was observed, higher than 50% in all cases, with no correlation between boiling points and carryover ratios (Table 1; Figure 2 and Supplementary Figure 2). No major breakdown products of the acetals could be detected in the e-vapor. Aldehydes and nicotine, while having divergent boiling points, were carried over at similar rates. Among the studied compounds, benzaldehyde has the lowest boiling point (178°C), yet its carryover rate was in the same range as for most other compounds, including those with very high boiling points, such as vanillin PG acetal (bp: 353°C; Table 1). To the best of our knowledge, only a few flavorant molecules with boiling points higher than 300°C are used in e-liquids,¹² and boiling points of acetals would not be expected to be much higher than those of vanillin PG acetal. This suggests that the most important parameter for compound carryover from e-liquid to e-vapor is its solubility in the solvents PG and VG rather than a compound's boiling point. Therefore, most flavorants, including solvent adducts such as acetals, are likely to reach the e-vapor in appreciable amounts.

Carryover of benzaldehyde PG acetal increased significantly with higher PG content of the e-liquid, with similar trends observed for the other PG acetals (Figure 2 and Supplementary Figure 2). Previous studies reported similar observations for nicotine carryover at varying PG/VG ratios.³³ It should be noted that the first-generation devices used in this study are not typically recommended for use with e-liquids with high VG contents because of their limited power, and this is likely to impact carryover rates, especially at low PG/VG ratios. Newer generation devices are much more powerful and are likely more effective in delivering flavorants as well as the described acetals to the e-vapor, as has already been described for nicotine.³⁴

Acetal Stability

The formation of acetals from an aldehyde and PG is reversible, with hydrolysis favored at acidic pH in aqueous solutions³⁵ (Figure 1). The airway lining fluid in which e-vapor is deposited is slightly alkaline,^{17,24} therefore, acetal hydrolysis is expected to occur at slower rates. Indeed, the half-lives of the acetals measured in aqueous solution exceeded 36 hours, sufficient for substantial amounts of acetals to be maintained in physiological fluids, potentially exerting direct effects on the airway lining fluids and underlying structures.

Recruitment of Additional Respiratory Irritant Receptors by Flavor Aldehyde PG Acetals

Exposures to aldehydes cause respiratory irritation through activation of irritant receptors in sensory nerve endings in the airway lining. TRPA1 is the major aldehyde-activated receptor, responding during exposures to acrolein in tobacco smoke, formaldehyde, and flavor aldehydes such as cinnamaldehyde and benzaldehyde.^{16,17,36} The vanilloid receptor, TRPV1, may also contribute to flavorant irritant effects as it is activated by vanillin-related compounds such as capsaicin, however, TRPV1 responds poorly to free aldehydes.³⁷ The present study strongly suggests that flavor aldehyde PG acetals are delivered to the airways of e-cigarette users, however, except benzaldehyde PG acetal,³⁸ it is unknown whether these compounds also activate irritant receptors. Our *in vitro* studies clearly demonstrate that the acetals activate human TRPA1 irritant receptors with increased efficacies at the highest concentrations (Figure 3 and Supplementary Figure 4). Also, higher concentrations of vanillin and ethyl vanillin seem to be desensitizing TRPA1 and attenuating the ion-channel activation, whereas their corresponding PG acetals do not demonstrate ion-channel desensitization or attenuation of response. Although vanillin, ethylvanillin, and benzaldehyde had only negligible effects on TRPV1, their corresponding PG acetals robustly activated this receptor (Figure 3 and Supplementary Figure 4; Table 1). These observations suggest that the tested flavorant aldehyde PG acetals may produce stronger sensory irritation effects than the free aldehydes alone, by recruiting additional sensory irritant receptors such as TRPV1. It remains to be investigated whether these effects occur *in vivo*, for example, by examining the respiratory irritation response in rodents to e-vapors containing PG acetals, and by measuring inflammatory parameters.^{39,40}

The aldehydes and acetals used in this study are listed as GRAS (generally regarded as safe) by the US Food and Drug Administration and the World Health Organization, as are several other PG acetals,^{41–43} but not furfural PG acetal.⁴³ Yet, GRAS status is based purely on ingestion safety when used as food additives, and cutaneous safety when used as scents. Currently, no data exist that would support inhalational safety of flavor aldehyde PG acetals. Since TRPA1 and TRPV1 contribute to chronic pulmonary conditions such as asthma, the formation of more strongly irritating compounds in e-liquids is of concern.^{39,44–47} Indeed, recent epidemiological and toxicological studies detected links between asthma frequency and e-cigarette use in adolescents and reported that vaporized e-liquids containing the same flavor aldehydes as tested in this study induce inflammation in human respiratory epithelia.⁴⁸ The current study warrants further investigation into possible toxic effects of inhaling flavorant PG acetals by means of an e-cigarette, and one of the outcomes may be that GRAS status is not sufficient to deem certain flavor additives as safe for inhalation by an e-cigarette.

Figure 4. Proposed comprehensive testing scheme for e-liquid regulation: beyond e-liquid ingredients reported by the supplier, the final e-liquid and vapor generated from it should be characterized, all compounds therein identified, and quantified. Using this final list of compounds an e-cigarette user would be exposed to, a hazard and risk assessment needs to be carried out to approve or disapprove an e-liquid, based on known inhalation toxicity values. If such values do not yet exist, they need to be determined, including measurements as presented in this article: aqueous stability mimicking the environment in the airways, irritation potential, and various toxicity endpoints regarding inhalation toxicity.

Implications

This study demonstrates the potential chemical instability of e-liquids: acetals can be formed between flavor aldehydes and the solvents PG and VG post-formulation, such as during mixing, shipping, and storage of e-liquids. This reaction starts almost immediately and continues over days, leading to significant acetal generation even though water is not removed actively from the reaction. Given the frequency at which aldehydes are used in e-liquids (eg, 11 different aldehydes found in 28 e-liquids,¹² and a total of 126 aldehydes on the FEMA GRAS list⁸), and that at least two PG- and four VG-acetal stereoisomers (and structural isomers of the latter) are formed during the acetalization reaction, a large number of distinct acetal compounds could potentially be present in e-liquids, each possibly with different reaction kinetics, aqueous stability, and most importantly, potentially different physiological and toxicological effects. It should be noted that in this study, the potential to activate irritant receptors was not separately tested for optical isomers (enantiomers or diastereomers), but it is well known that these can have very different physiological effects.^{10,49}

To comprehensively assess the risk of e-liquids, it is important to consider the complete life cycle of the e-liquid constituents from mixing through shipping and storage, heating, and oxidation in

e-cigarette devices, stability in biological environments (eg, mouth, airways, and lungs) to toxicity potential for a variety of endpoints (Figure 4). Without this comprehensive framework, important classes of compounds, such as the PG acetals studied here, could be unintentionally neglected if they are not directly added and disclosed by e-liquid manufacturers. To fully assess the risk potential of e-liquid use for regulatory purposes, it is imperative that the compounds that a user will actually be exposed to are reported and evaluated, and not only the initial ingredients combined during manufacturing.⁵⁰ When selecting compounds to screen for potential toxicity concerns, the chemical instability of the given e-liquid, which will depend on the reactivity of the individual constituents, needs to be taken into account. These can undergo a multitude of reactions given the complex mixture environment of e-liquids, making the prediction of equilibrium concentrations of reaction products, including PG or VG acetals, extremely challenging. In addition, a comprehensive risk assessment should consider the potential reaction products that can result from heating and oxidation in the e-cigarette, requiring characterization and quantification of the e-vapor constituents for toxicity concerns.⁵⁰ Finally, the biological stability of compounds present in the e-vapor should be considered, including interactions with pulmonary surfactants, as reactions may occur that change

the composition of the e-vapor and could result in new chemical products with unknown toxicities. This suggests that ingredient disclosure of e-liquids by suppliers may be insufficient to inform a comprehensive risk assessment of e-liquids and vaping more generally.

Supplementary Material

Supplementary data are available at *Nicotine and Tobacco Research* online.

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Declaration of Interests

None declared.

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