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Effect of Flavoring Chemicals on Free Radical Formation in Electronic Cigarette Aerosols

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Abstract

Background—Flavoring chemicals, or flavorants, have been used in electronic cigarettes (e-cigarettes) since their inception; however, little is known about their toxicological effects. Free radicals present in e-cigarette aerosols have been shown to induce oxidative stress resulting in damage to proliferation, survival, and inflammation pathways in the cell. Aerosols generated from e-liquid solvents alone contain high levels of free radicals but few studies have looked at how these toxins are modulated by flavorants.

Objectives—We investigated the effects of different flavorants on free radical production in e-cigarette aerosols.

Methods—Free radicals generated from 49 commercially available e-liquid flavors were captured and analyzed using electron paramagnetic resonance (EPR). The flavorant composition of each e-liquid was analyzed by gas chromatography mass spectroscopy (GCMS). Radical production was correlated with flavorant abundance. Ten compounds were identified and analyzed for their impact on free radical generation.

Results—Nearly half of the flavors modulated free radical generation. Flavorants with strong correlations included β-damascone, δ-tetradecalactone, γ-decalactone, citral, dipentene, ethyl maltol, ethyl vanillin, ethyl vanillin PG acetal, linalool, and piperonal. Dipentene, ethyl maltol, citral, linalool, and piperonal promoted radical formation in a concentration-dependent manner. Ethyl vanillin inhibited the radical formation in a concentration dependent manner. Free radical production was closely linked with the capacity to oxidize biologically-relevant lipids.

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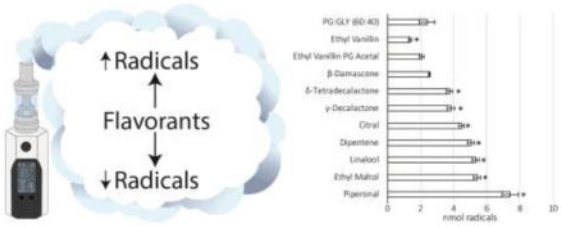
Conclusions—Our results suggest that flavoring agents play an important role in either enhancing or inhibiting the production of free radicals in flavored e-cigarette aerosols. This information is important for developing regulatory strategies aimed at reducing potential harm from e-cigarettes.

Graphical Abstract



Keywords

electronic cigarette; e-cigarettes; e-cig; free radicals; oxidative stress; flavors; flavorants

Introduction

Since their inception, electronic cigarettes (e-cigarettes) have been sold and marketed with flavored e-liquids; however, little is known regarding the products formed by these flavoring additives when heated at the high temperatures found in e-cigarettes. Many of the flavoring chemicals, or flavorants, found in these liquids are “generally recognized as safe” (GRAS) when consumed orally (US FDA 21CFR 182.1320); however, the thermal breakdown of these compounds in e-cigarette aerosols has yet to be fully evaluated, particularly in a toxicological context. In fact, the organization responsible for certifying food-safe flavorings for the FDA, the Flavor Extracts Manufacturers Association (FEMA), has specifically stated that they do not evaluate flavor ingredients for use in e-cigarettes or any other exposures other than ingestion.[1]

The development of many tobacco-related diseases, such as cardiovascular disease, chronic obstructive pulmonary disease (COPD), and cancer are all thought to be influenced or induced by oxidative stress and oxidative damage.[2–5] Oxidative stress can be induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS), of which free radicals are a major constituent.[6] In 2010, the Surgeon General released a report in which it identified free radical induced oxidative stress from tobacco smoke as being a contributor to the development of smoking-related diseases.[7] Free radicals are found in high concentrations in cigarette smoke (>10^{16} molecules/puff).[8–10] Similarly, previous studies done by our lab and others have shown relatively high levels of reactive free radicals in e-cigarette aerosols (>10^{13} molecules/puff) by electron paramagnetic resonance (EPR).[11–14] We found that free radical generation was highly dependent on the propylene glycol content of the e-cigarette liquid. [14] In addition to free radicals, a number of other studies have also found an assortment of other toxic agents in e-cigarette aerosols including nitrosamines, heavy metals, diethylene glycol, and reactive organic compounds such as formaldehyde and acetaldehyde.[15–21]

Flavoring additives represent an important component of tobacco products as they have been shown to directly influence tobacco product preference and use, and have historically been used to attract younger consumers.[22–26] Despite banning characterizing flavors (fruits, candy, etc.) in cigarettes in the 2009 Family Smoking Prevention and Tobacco Control Act, flavorings are still utilized in virtually all other tobacco products, including e-cigarettes.[23, 27] Recent studies, including the Population Assessment of Tobacco and Health (PATH) Study, reported that flavor was the primary reason for using a particular tobacco product among youth and young adults.[28, 29] Nationwide, a survey of young adults reported that their first and usual e-cigarette flavor was something other than tobacco flavored.[29] Preferences for non-tobacco flavored e-cigarettes have also been seen in adults as a recent study found that over 75% of adult users of e-cigarettes preferred flavors other than tobacco for their e-liquids.[30] While the popularity of flavored e-cigarette products continues to grow, the potential harms from these flavoring additives remains largely unknown.

To date, only a handful of studies have examined the toxicity of specific flavorants. Specifically, exposure to cinnamaldehyde, 2-methoxycinnamaldehyde, and diacetyl have been shown to cause cytotoxicity at concentrations typically found in e-cigarette liquids.[31–34] The flavorants acetoin and maltol also appear to be potent inducers of inflammation.[35] A wide variety of volatile organic compounds have been identified in both in flavored e-cigarette liquids and their aerosols.[36] More recently, benzene has been shown to form as a result of the thermal decomposition of benzaldehyde, a natural fruit flavorant common in many e-liquid flavors.[37] Benzaldehyde has also been shown to cause respiratory airways irritation in animal exposure studies.[34] Another study found that toxic aldehydes are produced primarily from the decomposition of flavor compounds during vaping. Altogether, these studies suggest that flavor compounds may play an important role in the potential toxicity of e-cigarettes.[38]

While the effects that e-cigarette operating parameters and e-cigarette solvents have only recently been investigated with respect to the delivery of toxins, the effect that e-cigarette flavoring additives have on the generation of these toxic compounds remains largely unknown. Of the studies performed that specifically address this topic of flavorants, only a few compounds have been identified as being harmful.[31, 34, 37] While many of these studies demonstrated cytotoxic effects of various flavorants, there have been no studies that have looked at the effects of flavorants on the generation of free radicals in e-cigarettes. Thus, in this study, we systematically evaluated the free radical generation of forty-nine commercially available, nicotine-free e-liquid flavor concentrates in e-cigarette aerosols. We also identified the individual flavorants found in the e-liquids and evaluated the effects of ten specific flavorants on free radical generation.

Materials and Methods

E-cigarette, coil, and atomizer tank

The e-cigarette used for this study was a Wismec Reuleaux RX200S Mod (MyVaporStore.com) in temperature control mode. Three high amperage Samsung INR18650-25R, 2500mAh, 3.7v batteries were used to power the device. The batteries were recharged after 250 puffs were performed using the device. The heating element used was a

commercially available 0.5 Ω stainless steel coil (SS316) Uwell Crown Coil (MyVaporStore.com). The atomizer tank used had a capacity of 4 mL and was composed of stainless steel and glass (Uwell Crown Tank; MyVaporStore.com).

Reagents

Arachidonic acid (AA), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), cis-5,8,11,14,17-eicosapentaenoic acid (EPA), glycerol (GLY), hexane, phenyl-N-tert-butylnitrone (PBN), propylene glycol (PG), 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO), and tert-benzene, and tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received.

E-cigarette flavor concentrates and flavorants

A commercially available kit containing forty-nine popular nicotine free e-liquid flavor concentrates was purchased from NicVape.com (Spartanburg, SC). Food grade certified flavorants (β-damascone, δ-tetradecalactone, γ-decalactone, citral, dipentene, ethyl maltol, ethyl vanillin, linalool, and piperonal) were purchased from Sigma-Aldrich. Food grade certified ethyl vanillin propylene glycol acetal was obtained from Vigon (East Stroudsburg, PA).

Profiling of Flavorants

E-liquid compositions were analyzed as described previously by gas chromatography-mass spectrometry (GCMS).[39] Using a Gerstel MPS2 multipurpose autosampler (Gerstel GmbH & Co. KG. Mülheim an der Ruhr, Germany), samples (1 μL) were introduced and split 50:1 into an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) with an Agilent 5975C mass selective detector. The GC inlet was a silanized glass straight design inlet liner (78.5 mm long × 6.5 mm o.d. × 0.75 mm i.d.) (Supelco, Bellefonte, PA) and the column was an Agilent J&W VF-35ms capillary column (60 m × 0.25 mm × 0.25 μm) with helium (Airgas) as the carrier gas. The inlet and MS source were both maintained at 280°C. The temperature profile consisted of: injection at 50°C with a 2 min hold, a linear increase of 10°C/min to 240°C, and an isothermal hold at 240°C for 10 min. The MS was set to a scan of 30–300 amu. Chromatogram peaks were analyzed using Mass Hunter Qualitative Analysis B.06.00, software and chemical identities were found by library searching against the NIST11 EI mass spectral database. Quantitation of specific chemicals was done using external standards dissolved in propylene glycol and run using the same method.

E-cigarette apparatus

The e-cigarette setup used here was similar to that used in our previous study.[14] In brief, the e-cigarette's fire button was activated by a 12 V relay timer switch (SainSmart; Amazon.com) and a second relay switch was connected to a 12 VDC solenoid valve (RioRand; Amazon.com). Upstream and downstream ends of the solenoid valve were connected to of the solenoid valve were connected an impinger and a flow meter respectively. The flow meter was connected to the house vacuum and adjusted to a flow rate of 500 mL/min. A diagram of this setup is shown in Supplemental Figure 1.

Solvent components, temperature, and wattage factors

To investigate the thermal degradation of flavors, e-liquid flavor concentrates were diluted, per the manufacturer's instructions, to 20% in a mixture of PG and GLY for a final PG:GLY ratio of 60:40 (v:v). To investigate the thermal degradation of individual flavorants, each flavorant was dissolved in a PG:GLY mixture (60:40, PG:GLY) at similar concentrations to those found in the e-liquid flavors. All experiments were conducted using the e-cigarette device in constant temperature mode set at 225°C and 50W.

Generation of e-cigarettes aerosols

Aerosols were generated under normal laboratory conditions using puffing parameters previously used to resemble typical usage.[11, 40] Puffs were simulated using the following puffing topography: puff duration, 5 seconds; interpuff interval, 30 seconds; flow rate, 500 mL/min; and number of puffs, 40.

Spin trapping of free radicals in e-cigarette aerosols

Free radicals in e-cigarette aerosols were trapped and quantified as previously reported.[14] In brief, e-cigarette aerosols were passed through a 25 mL impinger containing 6 mL of 0.05 M PBN in hexane. The nitrone spin trap, PBN, has been used extensively for the detection of radical species in cigarette smoke.[10, 11, 41] Hexane was evaporated and the remaining residue was reconstituted in 500 μL of tert-benzene. High purity quartz EPR tubes were filled with 200 μL of the reconstituted tert-benzene solution and deoxygenated using a freeze-pump-thaw technique with a Schlenk line.[42] Samples were deoxygenated as described previously.[11] In brief, the samples received three freeze-pump-thaw argon cycles before being blanketed with gaseous argon.

EPR measurements

The spectra derived from PBN radical adducts was measured using a Bruker eScan R spectrometer (Bruker-Biospin, Billerica, MA) operating in X-band. The EPR parameters were as follows: microwave frequency, 9.7 GHz; modulation frequency, 86.0 kHz; microwave power, 6.00 mW; scan range, 60G; modulation amplitude, 2.04 G; sweep time, 5.24 s; time constant, 10.24 ms; and conversion time, 10.24 ms. All measurements were carried out at room temperature (22 ± 1°C). The spin quantification of the radical signals obtained was performed in MatLab. Each spectrum was processed automatically to produce a double integral. In the process, point-based-spline baseline correction was applied to the absorption data (first integral) prior calculation of the second integral. Conversion factors from double integral values to spin concentrations we obtained from the known concentrations of a stable radical standard, TEMPO.[43]

Lipid peroxidation analysis

Lipid peroxidation studies were performed as previously reported.[14] In brief, 6 mL of 50 μg/mL AA, DHA, and EPA in 0.1 M TRIS-HCl (pH 7.4) was added to an impinger. Flavorants were then vaped as they had been done for the EPR measurements with the aerosol passing through the impinger containing the AA, DHA, and EPA. The impinger solution was then analyzed for secondary lipid oxidation products using a thiobarbituric acid

reactive substances (TBARS) assay kit (Cayman, Ann Arbor, MI). The samples were also analyzed for 8-isoprostane formation using an 8-isoprostane enzyme-linked immunosorbent assay (ELISA) (Eagle Biosciences, Inc., Nashua, NH). Both TBARS and 8-isoprostane levels were compared to controls that received the same puffing treatment but in the absence of the e-cigarette to remove the effect of bubbling as a variable.

Data analysis

All measurements were done in triplicate and free radical concentrations were compared to the flavor-free PG:GLY (60:40) base using a one-way ANOVA and Dunnett's multiple comparison for the flavor samples via GraphPad Prism (San Diego, CA). Comparison of the free radical concentrations for the individual flavorants were done using a one-way ANOVA and Tukey's multiple comparison to compare the effects of different concentrations and the base (60:40). Comparisons for the TBARS and 8-isoprostane measurements were done using a one-way ANOVA and Dunnett's multiple comparison to compare all samples to the base (60:40). Significant differences were identified at the $p<0.05$ level. Pearson's correlation values for each chemical's peak area and the flavor's free radical content were determined using the SciPy statistical packages for Python.[44, 45]

Results

Flavor effects on radical production

The impact of adding flavoring agents to PG:GLY base on radical production was examined with 49 different flavors. Overall, radical production varied widely across the different flavored e-liquids tested (Figure 1). Nearly 43% of the flavors resulted in significant increases in radical production as compared to the base PG:GLY (60:40) mixture. Significant increases ($p<0.05$) in radical production were observed for Lemon (46%), Blue Raz (49%), Grape (56%), Coffee (58%), Root Beer (61%), Sweet Tea (65%), Kiwi (66%), Real Honey (67%), Pear (70%), Tootie Frootie Cereal (71%), Raspberry (72%), Dark Raz (74%), Bubblegum (76%), Ripe Strawberry (80%), Real Watermelon (94%), Rainbow Candy (102%), Subtle Cinnamon (105%), Butterscotch (106%), Cotton Candy (114%), Vanilla Custard (122%). Interestingly, significant reductions in radical production below baseline were observed as a result of adding Vanilla flavoring. Direct interactions with PBN, or autoxidation, with the flavors were measured and accounted for less than 7% of the radicals seen in the vaped samples. (Supplemental Figure 2)

A six-line spectrum is typically associated with PBN adducts, the overmodulation of our spectra results in a broader three-line spectra. (Figure 1) Overmodulation can cause peak broadening which distorts the PBN's characteristic six-line spectra. This overmodulation was done intentionally in order to reliably quantitate and detect low level concentrations of radicals. The six-line spectrum can be seen with more concentrated samples at lower modulation amplitudes. (Supplemental Figure 6)

Flavor concentrate chemical composition, correlation, and concentration

Among the forty-nine flavor concentrates analyzed by GCMS, nearly 300 unique chemicals were identified. Of these 10% were found in 5 or more flavor concentrates and 3% in 10 or

more concentrates. Ethyl vanillin PG acetal and ethyl maltol were the two most common flavoring additives occurring in more than 45% of flavors. In order to identify specific flavorants that might impact free radical production, relative abundance of the different flavorants in each e-liquid concentrate was correlated with radical production. The 10 most highly correlated flavor compounds (positively or negatively) were chosen for further analysis. (Table 1) Ethyl vanillin PG acetal, ethyl vanillin, β-damascone, and δ-tetradecalactone were chosen as they exhibited negative correlations suggesting they may inhibit radical production. Conversely, γ-decalactone, neral, ethyl maltol, piperonal, d-limonene, and linalool were chosen as they exhibited the strongest positive correlations suggesting they contribute to increased radical production. The concentrations of these flavorants in their e-liquid flavor concentrates were then determined using GCMS from standards and used as a range for further testing. (Table 1)

Flavorant effects on radical production

The flavorants selected above were tested individually by adding to PG:GLY (60:40) mixtures within the concentration ranges found in the commercially obtained flavors and then diluted to 20% with additional PG:GLY (60:40) in order to match the final concentration ranges of the commercial e-liquids. The results show that free radical generation can either be increased or decreased as a result of flavorant additions. (Figure 2) The addition of ethyl vanillin significantly decreased radical production by 42% from baseline at the 0.8 mg/mL concentration. While ethyl vanillin PG acetal showed a slight decrease (14%) in radical production at 20 mg/mL, the effect was not significant. β-Damascone also showed a small but not significant decrease. δ-tetradecalactone at 2 mg/mL showed a significant increase (56%) in radical production. Linalool, at a concentration 4 mg/mL, significantly increased radical production (122%) as compared to the baseline and was significantly higher than the 2 mg/mL concentration. Dipentene, a mixture of isomers including d-limonene, also produced significant 62–112% increases in radical production at both the 8 mg/mL and 16 mg/mL concentrations and both values were significantly higher than baseline and significantly different from one another. The addition of 0.8 mg/mL and 1.6 mg/mL piperonal resulted in significant 94% and 210% increases in radical production, respectively, as compared to the baseline and were significantly different from each other. Radical production also increased significantly with increasing concentrations of ethyl maltol (97% at 6 mg/mL and 127% at 12 mg/mL and citral, a mixture of both neral and geranial, in a concentration dependent fashion (26% at 2 mg/mL and 88% at 4 mg/mL). Higher concentrations of γ-decalactone (2 mg/mL) significantly increased radical production by 61% above baseline. Direct interactions with PBN, or autoxidation, with the flavorants were measured and accounted for less than 6% of the radicals seen in the vaped samples. (Supplemental Figure 3)

Flavorant effects on lipid peroxidation

The flavorants were then used to examine their potential for modulating lipid peroxidation products using malondialdehyde (MDA) formation as a marker. (Figure 3) Linalool (4 mg/mL), piperonal (1.6 mg/mL), and citral (4 mg/mL) all resulted in significant increases (257%, 197%, and 205% respectively) of lipid peroxidation products. The addition of ethyl vanillin (0.8 mg/mL) reduced lipid peroxidation by 60% from baseline; however, the

decrease was not significant. Ethyl vanillin PG acetal (20 mg/mL) showed a mild decrease (2%) but this change was not significant. β-Damascone (0.4 mg/mL), dipentene (16 mg/mL), ethyl maltol (12 mg/mL), γ-decalactone (2 mg/mL), and δ-tetradecalactone (2 mg/mL) all showed mild increases in lipid peroxidation (96%, 139%, 106%, 24%, and 75% respectively); however, the changes were not significant. The effects of direct interactions with the un-vaped flavorants on TBARS formation were measured and accounted for less than 7% of the TBARS formation seen in the vaped samples. (Supplemental Figure 4)

Flavorant effects on 8-isoprostane formation

The flavorants' biological impact on the oxidation of AA and subsequent formation of its oxidation product, 8-isoprostane, were then examined. (Figure 4) δ-Tetradecalactone (2 mg/mL), linalool (4 mg/mL), dipentene (16 mg/mL), citral (4 mg/mL) and γ-decalactone (2 mg/mL) resulted in significant increases in 8-isoprostane formation (60%, 39%, 111%, 62% and 78%, respectively). Ethyl vanillin PG acetal (20 mg/mL), piperonal (1.6 mg/mL), ethyl maltol (12 mg/mL), and β-damascone (0.4 mg/mL) showed a mild increases in 8-isprostatane formation (25%, 38%, 29%, and 5%, respectively), but these changes were not significant. The effects of direct interactions with the un-vaped flavorants on 8-isoprostane formation were measured and accounted for less than 12% of the 8-isoprostane formation seen in the vaped samples. (Supplemental Figure 5)

Discussion

In our study, we found that flavorants in e-liquids can have a direct impact on the formation of highly reactive free radicals. Overall, aerosols from 49 different flavored e-liquids showed nearly an 8-fold range in radical production. Since conditions such as temperature and PG:GLY ratios were held constant, the differences in radical production could be directly attributed to the flavorants. As flavor descriptors are largely subjective, and the chemical composition of e-liquids is proprietary, we took the approach of identifying the specific flavorants in each flavored e-liquid by GCMS. Using these data, we performed correlational analyses between the relative abundance of each flavorant in and their corresponding free radical output. Using this approach, we selected 10 specific flavorants that had the strongest correlation (positive or negative) in terms of radical production for further investigation. It should be noted that future studies should address whether other flavorants also contribute to radical production.

Of the 10 flavorants examined, 7 (ethyl vanillin, δ-tetradecalactone, linalool, dipentene, piperonal, ethyl maltol, citral, and γ-decalactone) were found to significantly modulate the generation of radicals in e-cigarette aerosols. Three of the highest radical producing flavorants (linalool, dipentene, and citral) are non-phenolic terpenes which are found in a number of different plants. The addition of each of these compounds yielded a concentration-dependent increase in radical production, suggesting that they contribute to radical formation. Many of these non-phenolic terpenes are used heavily in the fragrance, food, and beverage industries to add floral and citrus aromas and flavors.[46] Several studies have found that some of these compounds can undergo autoxidation to form hydroperoxides, which have been linked to allergic contact dermatitis in some people.[47–49] Focusing

specifically at the hydroperoxides formed from linalool and d-limonene, one study found that that these hydroperoxides can rapidly degrade into a number of different allyloxyl and carbon-centered radicals all of which reacted readily with histidine amino acids *in vitro*.[48] These studies seem to explain a possible route for the increase in radical generation observed in our studies and suggest that the addition of non-phenolic terpene flavor compounds should be investigated further.

Ethyl maltol, a common flavoring ingredient used to impart sweet and caramel-like aromas to fragrances, foods, and beverages, also produced a concentration dependent increase in radicals.[46] Ethyl maltol and maltol have both been shown to interact with iron or copper to produce a number of different hydroxypyranone complexes that can promote the generation of radicals.[50, 51] These complexes by themselves have also been shown to be toxic in animal models.[52] Trace amounts of iron or copper may be present in the e-liquid solvent, wick, or coil and in turn be responsible for the formation of these complexes and their subsequent radical generation. This is also of concern as these compounds may react with biologically available iron or copper. A recent study looking at inflammatory responses in lung cells found that maltol increased inflammatory cytokines iterleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) production and decreased barrier function in human bronchial epithelial (Beas2B) and human lung fibroblasts (HFL-1) cell lines.[35]

Lactones, such as δ-tetradecalactone and γ-decalactone, are a group of flavorants composed of intramolecular hydroxy fatty acid esters and used to impart fruity and creamy notes to fragrances, foods and beverages.[46] Radical production from some lactones has been reported and specific radical products have been identified.[53] Unfortunately, radicals formed by γ-decalactone or δ-tetradecalactone have still yet to be investigated.

The addition of piperonal also increased radical generation in the e-cigarette aerosols in a concentration dependent manner. Piperonal is an aromatic aldehyde that is used to add cherry and vanilla like properties to fragrances, foods, and beverages.[46] Despite the increase in radical generation in the aerosols we observed, there has been little research done looking at the oxidative properties of piperonal.

An interesting finding from our study was that certain flavorants may actually inhibit the formation of radicals. Ethyl vanillin PG acetal and ethyl vanillin, both used in fragrances, foods, and beverages to impart a vanilla characteristic, showed similar inhibitions of radicals. While the decrease in radicals by ethyl vanillin PG acetal was not significant, decreases observed with higher concentrations of the unacetalated ethyl vanillin were significant. This differential effect may indicate that the aldehyde group present in ethyl vanillin but not in ethyl vanillin PG acetal may play a role in its antioxidant potential. A recent study found that ethyl vanillin and vanillin both can act as a strong antioxidants *in vitro* and *in vivo* further suggesting a role in radical inhibition.[54] This study also suggests the importance of the aldehyde group found on both ethyl vanillin and vanillin as the antioxidant properties were not observed with vanillyl alcohol or vanillic acid, both of which lack the aldehyde group.[54] The radical inhibition effects of ethyl vanillin suggest its possible use an additive in e-liquids reduce free radical production during aerosol formation.

Further tests will be needed to determine if there are any toxic compounds formed during the aerosolizing process of e-liquids.

While no significant differences were seen compared to the base for β-damascone, a flavorant used to add floral aromas to fragrances, foods, and beverages, the trend suggests a decrease in radical formation. β-damascone is known to be a nucleophilic Michael addition acceptor capable of scavenging radicals by reacting with nucleophiles and has been found to decrease inflammatory cytokine gene expressions of interleukin-10 (IL-10) and TNF-α in human colon epithelial cells (T84).[55]

In order to assess the potential biological impact of free radicals produced from e-cigarettes, we assessed their reactivity with biologically relevant lipids *in vitro*. Free radicals are highly reactive with polyunsaturated fatty acids resulting in the initiation of lipid peroxidation.[56] Thiobarbituric acid-reactive substances (TBARS) are a well-established byproduct of the reaction of free radicals with biologically-relevant unsaturated lipids such as AA, DHA, and EPA. Hence, TBARS have long been used as a marker of oxidative stress in blood and tissues and, in a recent study, elevated levels of TBARS were found in the lung homogenate of mice exposed to e-cigarette aerosols[12] Free radicals are also responsible for promoting the oxidation of AA to form 8-isoprostane, a well-characterized marker of oxidative stress.[57] The finding of increased levels of lung TBARS and plasma 8-isoprostane *in vivo* provide evidence that the e-cigarette-derived radicals may have a biological impact.[58] Here we show that direct exposure of e-cigarette aerosols to biologically relevant lipids resulted in the production of both TBARS and 8-isoprostane. Further, we observed TBARS and 8-isoprostane were elevated with addition of flavorants which enhance free radical production including linalool, piperonal, and citral for TBARS and δ-tetradecalactone, linalool, dipentene, citral, and γ-decalactone for 8-isoprostane. Conversely, addition of ethyl vanillin, which decreased free radical production, also decreased both TBARS and 8-isoprostane formation. These findings suggest a tight linkage between free radical exposure and the oxidation of AA, DHA, and EPA, highlighting the potential toxicological importance of e-cigarette-derived free radicals

Overall, our results demonstrate that flavorants can modulate radical production in e-cigarette aerosols. While our study did not look into all of the over 300 chemicals found within the 49 flavors tested or in the other flavored e-liquids on the market, we did find significant correlations between certain common flavorants and their effects on radical generation. Specifically, we found that linalool, dipentene, piperonal, ethyl maltol and citral, all appear to promote radical production in a concentration dependent manner suggesting these flavorant additions may pose more oxidative harm to the e-cigarette user. Conversely, we also found that ethyl vanillin and, to a lesser extent, β-damascone appear to block radical production in a concentration dependent manner suggesting that adding these flavorants may in fact reduce oxidative harm for the consumer. More research will be needed to further investigate these flavorants and the products they form when heated and aerosolized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AA	arachidonic acid
COPD	chronic obstructive pulmonary disease
DHA	cis-4,7,10,13,16,19-docosahexaenoic acid
ELISA	enzyme-linked immunosorbent assay
EPA	cis-5,8,11,14,17-eicosapentaenoic acid
EPR	electron paramagnetic resonance
GLY	glycerol
PBN	phenyl-N-tert-butylnitrone
PG	propylene glycol
RNS	reactive nitrogen species
ROS	reactive oxygen species
TBARS	thiobarbituric acid reactive substances
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxyl
TRIS-HCl	tris(hydroxymethyl)aminomethane hydrochloride

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Highlights

- Flavoring chemicals can modulate the production of free radicals in e-cigarettes
- Citral, dipentene, ethyl maltol, linalool, and piperonal all promote radical formation
- Ethyl vanillin dose-dependently inhibits radical delivery
- Flavorants can modulate lipid peroxidation induced by e-cigarette aerosols

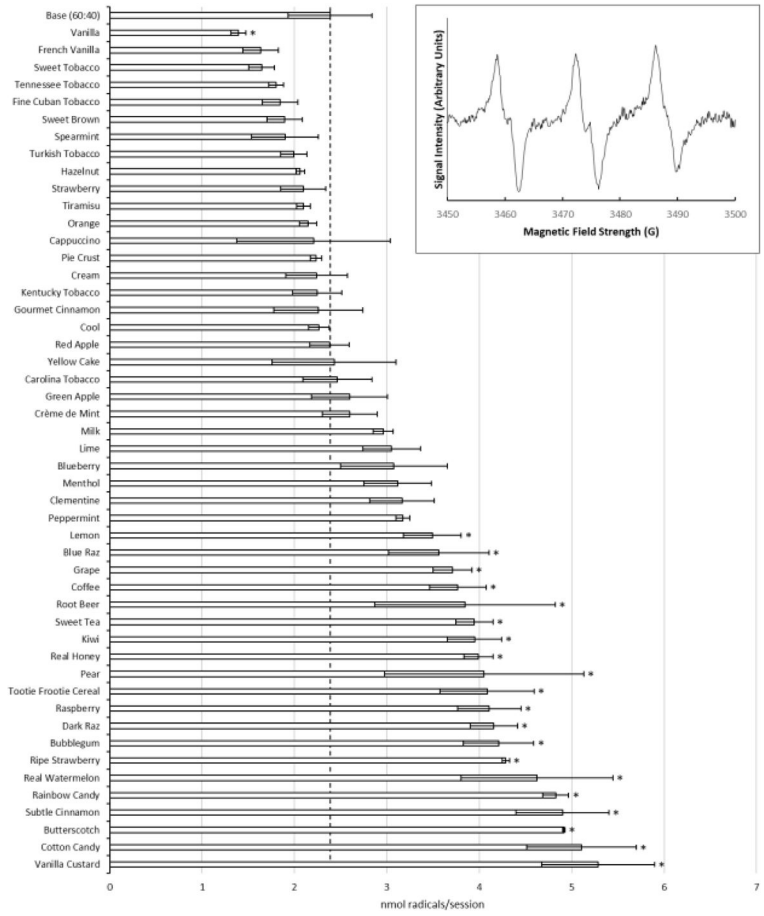

Figure 1.

The effects of different commercially available e-liquid flavor concentrates on radical production. Asterisks (*) represent a statistical difference ($p<0.05$) from the base PG:GLY (60:40) mixture. Inset graph is a representative spectra showing the base PG:GLY (60:40).

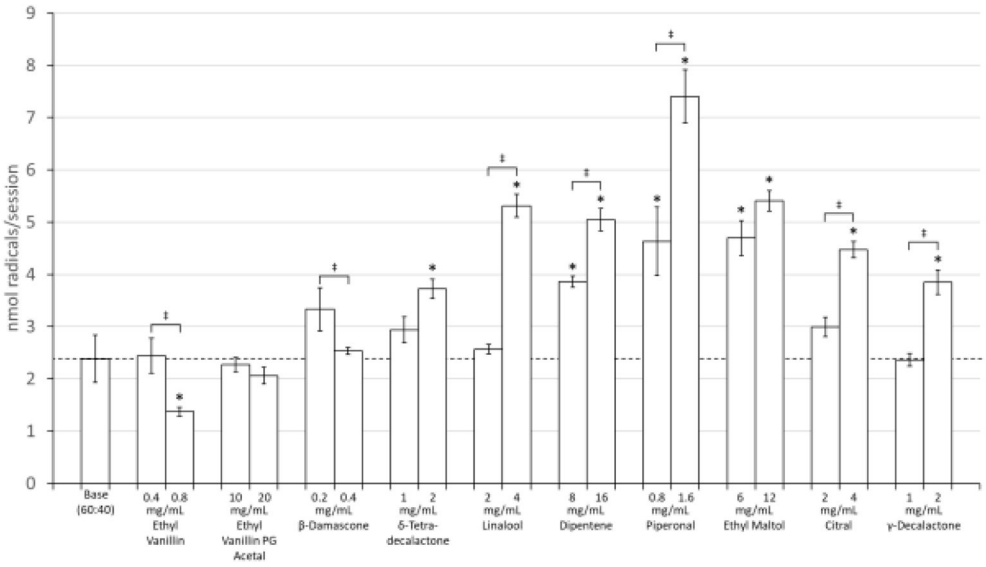

Figure 2.

Concentration dependent flavorant effects on radical production. Asterisks (*) represent a statistical difference ($p<0.05$) from the base PG:GLY (60:40) mixture. ‡ represents a statistical difference ($p<0.05$) between the chemical's concentrations.

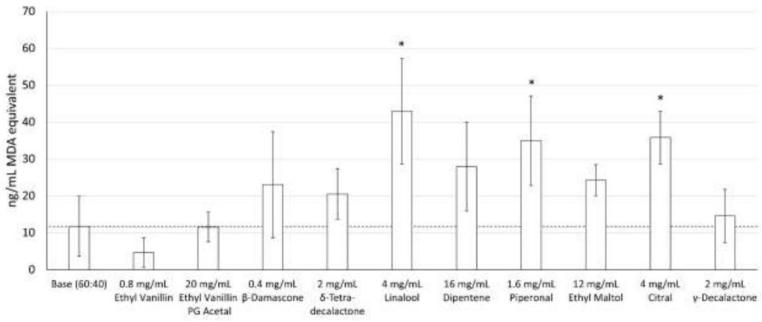

Figure 3.

Flavor chemical effects on lipid (AA, DHA, and EPA) peroxidation. Asterisks (*) represent a statistical difference ($p<0.05$) from the base PG:GLY (60:40) mixture.

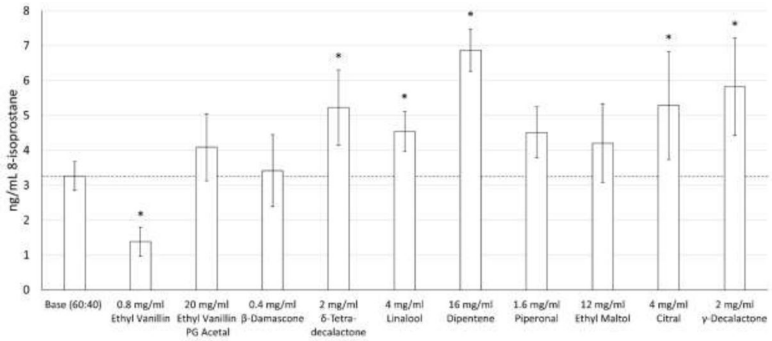

Figure 4.

Flavor chemical effects on AA oxidation to 8-isoprostane. Asterisks (*) represent a statistical difference ($p<0.05$) from the base PG:GLY (60:40) mixture.

Table 1

Flavorants identified in the e-liquids.

Chemical	n	Pearson's [a] Correlation	p Value	Flavors Containing	Concentration [b] Ranges (mg/mL)
Ethyl vanillin PG acetal	50	−0.2243	0.1173	Blue Raz, Bubblegum, Butterscotch, Cream, French Vanilla, Hazelnut, Raspberry, Root Beer, Sweet Tobacco, Vanilla, Vanilla Custard	0.42 – 113.12
Ethyl vanillin	50	−0.0943	0.5146	Blue Raz, Bubblegum, Cream, Hazelnut, Sweet Tea	0.62 – 3.98
β-Damascone	50	−0.1781	0.2159	Kentucky Tobacco, Sweet Tea, Sweet Tobacco	0.02 – 1.50
δ-Tetradecalactone	50	−0.1795	0.2123	Cream, Milk, Tootie Frootie Cereal, Vanilla, Vanilla Custard	0.99 – 9.30
Linalool	50	0.1769	0.2190	Blue Raz, Bubblegum, Clementine, Lime, Orange, Pear, Rainbow Candy, Raspberry, Subtle Cinnamon, Sweet Tea, Tootie Frootie Cereal	4.78 – 22.80
D-Limonene	50	0.2359	0.0990	Bubblegum, Clementine, Kiwi, Lemon, Lime, Orange, Rainbow Candy, Real Watermelon, Root Beer, Spearmint, Tootie Frootie Cereal	0.16 – 76.52
Piperonal	50	0.3336	0.0179	Butterscotch, Cream, French Vanilla, Raspberry, Real Honey, Sweet Tea, Vanilla, Vanilla Custard, Yellow Cake	0.01 – 7.52
Ethyl maltol	50	0.4235	0.0022	Blue Raz, Cotton Candy, French Vanilla, Grape, Kiwi, Raspberry, Real Honey, Real Watermelon, Ripe Strawberry, Root Beer, Strawberry, Sweet Tobacco, Tennessee Tobacco, Vanilla Custard, Yellow Cake	1.19 – 61.23
Neral	50	0.2857	0.0443	Bubblegum, Lime, Rainbow Candy, Tootie Frootie Cereal	3.89 – 21.83
γ-Decalactone	50	0.3284	0.0199	Blue Raz, Butterscotch, Kiwi, Pear, Rainbow Candy, Raspberry, Ripe Strawberry, Tootie Frootie Cereal	0.44 – 8.16

[a] The Pearson's correlation values compare the area under the curve for each peak to the free radical concentration for that flavor.

[b] The concentration ranges are those found in the undiluted e-liquid flavor concentrates.