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Analytical and Toxicological Assessment of Chemicals and Elements in Multiple Generations of Electronic Cigarette Products

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Analytical and Toxicological Assessment of Chemicals and Elements in Multiple Generations of Electronic Cigarette Products

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of the requirements for the degree of

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in

Environmental Toxicology

by

Esther E Omaiye

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Dedication

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To everyone who believed in me and encouraged me along the way, Yes! We did it. I desire to become a source of hope that you, too, can do it. I know there will be many more after me.

ABSTRACT OF THE DISSERTATION

Analytical and Toxicological Assessment of Chemicals and Elements in Multiple Generations of Electronic Cigarettes Products

by

Esther E Omaiye

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, June 2022
Dr. Prue Talbot, Chairperson

Electronic cigarettes (e-cigarettes), which remain popular among consumers, have been associated with attractive flavored products, especially among adolescents and young adults. This dissertation aimed to evaluate the chemical composition of e-cigarettes and determine the toxic effects of e-cigarettes and chemicals used in high amounts. First, high-performance liquid chromatography and gas chromatography-mass spectrometry were used to identify and quantify nicotine, solvents, flavor chemicals, and synthetic coolant in over 400 products. Nicotine concentrations varied in one brand of refill fluids purchased worldwide. Concentrations of total flavor chemicals ranged from 0 - 343 mg/ml and were generally greater than nicotine concentrations. Most frequently occurring flavor chemicals (e.g., ethyl maltol, furaneol, benzyl alcohol, ethyl vanillin, corylone, triacetin, menthol, vanillin, and cinnamaldehyde, were categorized as fruity, floral, caramellic, vanilla and minty. These results showed the flavor chemicals in e-cigarettes, which often exceed levels used in edible or household products, are sufficiently high enough to make them attractive to youth and adolescents. Next, we evaluated the cytotoxicity of e-cigarette liquids, aerosols, and authentic chemicals standards using multiple endpoint assays (MTT, NRU, and live-cell imaging) and cell lines. E-liquid and aerosol cytotoxicities ranged from 0.01 - 10%, and 0.2 - 1.8%, respectively. Lower concentrations of pure flavor chemicals and WS-23 than in e-cigarettes significantly affected cells and correlated with toxicity. In some products, the flavor chemical concentrations were 30 (menthol), 100 (ethyl maltol), and 100,000 (cinnamaldehyde) times greater than their cytotoxic concentration. The WS-23 concentration that produced cytotoxic effects was 90 times lower than in an e-cigarette fluid and exceeded levels used in consumer products. Flavor chemicals have profound cytotoxic effects in acute in vitro assays, emphasizing the potential to impact human health negatively with chronic use. Finally, the Margin of Exposure (MOE) was used to calculate the cancer risk of pulegone and the health risk of synthetic coolants and flavor chemicals. Pulegone and synthetic coolant levels in e-cigarettes present a significantly calculated risk for cancer and health hazard, contributing to increased harm to consumers. The work in this dissertation emphasizes the need for continuous monitoring of e-cigarette constituents and the enactment of effective regulation to reduce unwanted toxicological effects.

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Chapter 1: Introduction

Electronic Cigarettes

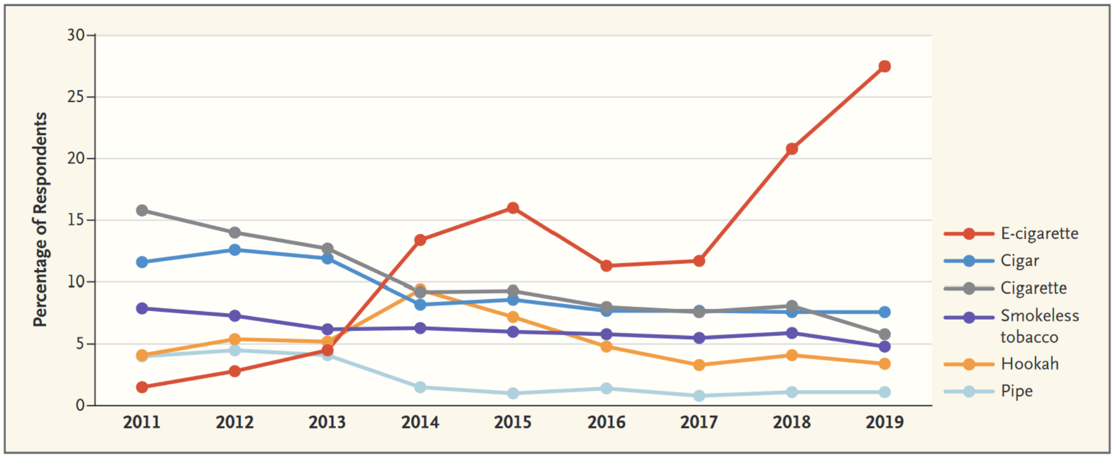
Electronic cigarettes (ECs) are chemical delivery systems that expose consumers to varying concentrations of nicotine, solvents, blends of flavor chemicals, and synthetic cooling agents in an aerosol that directly encounters the lining of the mouth and respiratory system (Trtchounian & Talbot, 2011; Grana, Benowitz & Glantz, 2014; USDHHS, 2016). The earliest EC prototype was invented in the 1960s and received a patent in 1965 (www.casaa.com; Gilbert, 1965). Commercialization began in the early 2000s when the modern EC design as a nicotine delivery system was introduced into the Chinese market. (Hon, 2006). They have continued to evolve and gain popularity worldwide while attracting significant interest from big tobacco companies (Manning, 2013; Esterl, 2014).

Without scientific evidence, manufacturers initially claimed that ECs were harmless and safer alternatives to smoking (Grana, Benowitz & Glantz, 2014; Glantz & Bareham, 2018). US youths and adolescents believe that e ECs cause little to no harm and that EC aerosols are less addictive than cigarettes (Roditis & Halpern-Felsher, 2015; Wang et al., 2018) . A study on perceived risk from exposure to different ECs revealed no difference (McKelvey, Baiocchi & Halpern-Felsher, 2018). National surveys administered between 2012-2017 suggest that knowledge of EC risk perceptions will continue to increase and change as more data on the health effects become available (Huang, Feng, et al., 2019). E-liquids and aerosols generated from ECs contain hundreds of harmful flavor chemicals, toxicants, and carcinogens (Bahl et al., 2012; Etter, Zäther & Svensson, 2013; Behar, Davis, Bahl, et al., 2014; Bekki et al., 2014; Brown et al., 2014; Goniewicz et al., 2014; Davis et al., 2015; Farsalinos et al., 2015;

Jensen et al., 2015; Allen et al., 2016; Behar et al., 2016; EL-Hellani et al., 2016; Khlystov & Samburova, 2016; Tierney et al., 2016; Lee et al., 2017; Aszyk et al., 2018; Behar et al., 2018; Behar, Wang & Talbot, 2018; Czoli et al., 2019; Belushkin et al., 2020; Budzyńska et al., 2020; Yan et al., 2021), that may be detrimental and adversely affect human health (Pisinger & Døssing, 2014; Hua & Talbot, 2016). As ECs remain popular and evolving designs facilitate efficient aerosol delivery and stealth use among consumers, including adolescents and young adults (Kong et al., 2019; Ramamurthi, Chau & Jackler, 2019; Jackson et al., 2020; Yingst, Foulds & Hobkirk, 2021; Dai & Hao, 2022), their constituents have been linked to vaping appeal and enhancement. (Ambrose et al., 2014; Hamberger & Halpern-Felsher, 2020; Kava et al., 2021; Leventhal et al., 2021; Spears et al., 2022). Based on their design and components, ECs can be grouped into 4 generations: 1st generation (cig-a-like), 2nd generation (cartomizers/pens), 3rd generation (mods/tanks), and 4th generation (pods). A hybrid of the 1st and 4th generation classified as disposable pods is currently widely distributed. Disposable 4th generation ECs are readily accessible as newer versions and designs continue to enter circulation more frequently than consumers and researchers can track.

Market Share, Sales, and Demographics

The inverse relationship between declining cigarette smoking (USDHHS, 2012; Arrazola et al., 2015; Jamal, 2017; Glantz & Bareham, 2018) and increasing uptake of ECs, especially among youth and adolescents, has persisted (USDHHS, 2016; NASEM et al., 2018; Wang et al., 2019; Singh et al., 2020; Kavuluru, Han & Hahn, in press). Over 2.4 billion was generated in US sales of EC in 2016, and projections were expected to reach 3.6 billion in 2018 (Statista, 2015). In 2020, JUUL exceeded 60% of sales in the US compared to other brands (Fraga 2022; Statista 2020).



Current Tobacco Product Use among U.S. High School Students, 2011 to 2019.

Current product use is defined as use in the past 30 days. Between 2018 and 2019, there was a change in the mode of survey administration from paper and pencil to electronic tablet. Data are from the National Youth Tobacco Survey and were provided by the Centers for Disease Control and Prevention.

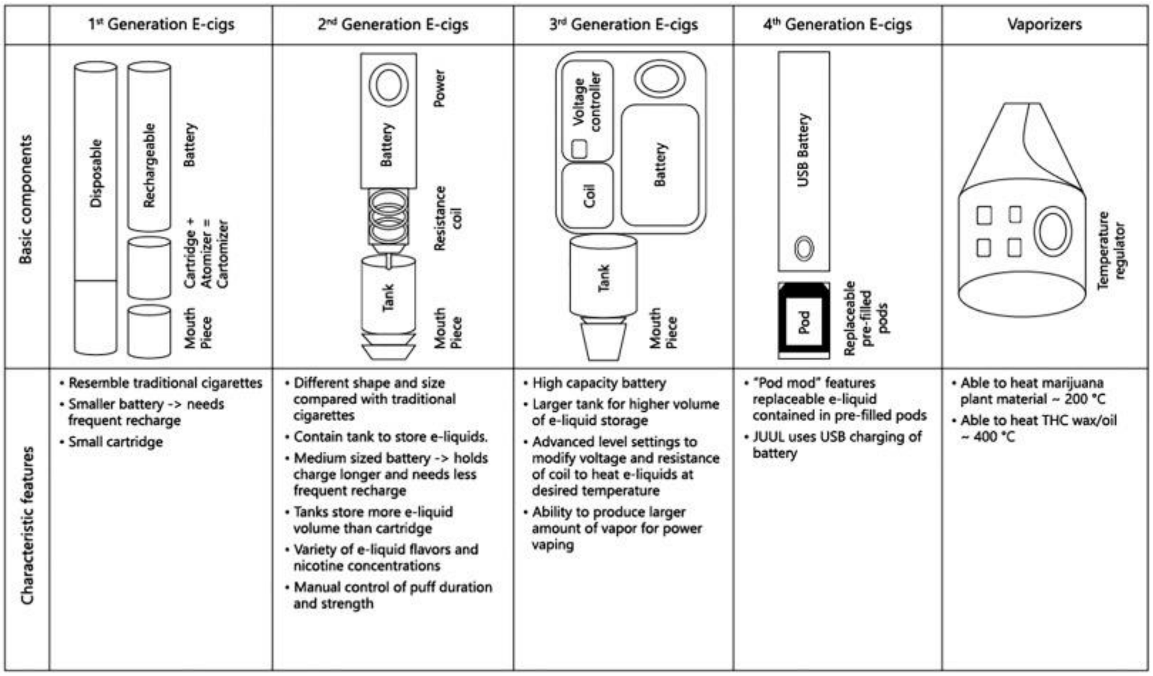
Figure 1.1. Current tobacco product use among U.S high school students, 2011 to 2019.

Retail data analysis from 2014 to 2020 showed an increase in total EC sales per 4-week interval from 7.7 to 17.1 million units (122.2%) and a 294% increase from 2016 to 2019 (Ali, Diaz, et al., 2020). Fluctuations in sales based on device type (cartridge vs. disposables) and flavor (mint vs. tobacco and menthol) were attributable to consumer preferences and the Food and Drug Administration (FDA) enforcement policy prohibiting the sale of flavored prefilled cartridges except for tobacco (Ali, Diaz, et al., 2020; US FDA 2020). The increase in sales between 2016 – 2019 was largely driven by prefilled cartridge-based products, of which JUUL was a major player in sales and dollar amounts (King et al., 2018). Before the FDA enforcement policy limiting access to cartridge-based flavored ECs (US FDA 2020), JUUL voluntarily removed their flavored variants except for menthol and tobacco flavors to reduce use among young consumers amid increased concerns from the public. Since disposables were excluded from the policy, many users switched to Puff Bar, rapidly increasing sales and becoming a dominant household name (Ali, Diaz, et al., 2020; Aubrey, 2020; Miech et al., 2021; Mascarenhas, 2020). Despite concerns over the use of flavor chemicals and additives in ECs, the chemicals used in the ever-changing fluid formulations remain largely unregulated. JUUL™ dominated the EC market in sales between 2018 – 2020 (Ali, Diaz, et al., 2020; Statista, 2020). However, current projections show that disposables, such as Puff Bar, which now comprise a significant share of the EC market, are likely to increase their sales through 2028 (Huang, Duan, et al., 2019). The current EC market is estimated to be worth 17.46 billion, and projections for sales to reach 43.65 billion by 2028 (Vantage Market Research, 2022).

The Evolution of EC Devices

The first commercial ECs aesthetically resembled conventional cigarettes with 2-3 basic components: a battery system, an atomizer (or cartomizer), and a mouthpiece (Trtchounian & Talbot, 2011). ECs have extensively evolved over the last decade, with four generations being recognized based on modifications to external components such as batteries, fluid reservoirs, and internal atomizer components, such as wicks, filaments, and air tubes (NASEM et al., 2018; Williams, Bozhilov & Talbot, 2019; Williams & Talbot, 2019).

The anatomy of an EC includes a battery, an atomizing unit (containing a filament), a fluid component (reservoir or cotton sheet/wick soaked in the flavored/nicotine-containing fluids), and an air tube between the atomizer and the mouthpiece. These components work together to produce an aerosol by heating a liquid containing nicotine, solvents, and flavor chemicals (NASEM et al., 2018; Williams, Bozhilov & Talbot, 2019; Williams & Talbot, 2019).



	1st Generation E-cigs	2nd Generation E-cigs	3rd Generation E-cigs	4th Generation E-cigs	Vaporizers
Characteristic features	• Resemble traditional cigarettes • Smaller battery -> needs frequent recharge • Small cartridge	• Different shape and size compared with traditional cigarettes • Contain tank to store e-liquids. • Medium sized battery -> holds charge longer and needs less frequent recharge • Tanks store more e-liquid volume than cartridge • Variety of e-liquid flavors and nicotine concentrations • Manual control of puff duration and strength	• High capacity battery • Larger tank for higher volume of e-liquid storage • Advanced level settings to modify voltage and resistance of coil to heat e-liquids at desired temperature • Ability to produce larger amount of vapor for power vaping	• "Pod mod" features replaceable e-liquid contained in pre-filled pods • JUUL uses USB charging of battery	• Able to heat marijuana plant material ~ 200 °C • Able to heat THC wax/oil ~ 400 °C

Figure 1.2. Schematics of e-cigarette generations. For more information on electronic delivery systems for nicotine or cannabis, e-liquids, and practices in altering devices to change delivery see the "E-Cigarette, or Vaping, Products Visual Dictionary" freely available at https://www.cdc.gov/tobacco/basic_information/e-cigarettes/pdfs/ecigarette-or-vaping-products-visualdictionary-508.pdf.

First-generation ECs or cig-a-likes/cartomizers (e.g., NJOY, Mark Ten, and Green Smoke) have a close resemblance to tobacco cigarettes in length and width and were available in three styles differentiated by the number of external pieces (1 to 3) that made up the device (Trtchounian & Talbot, 2011; Grana, Benowitz & Glantz, 2014; Williams & Talbot, 2019). Their atomizers contained solder joints that connected components, polyfil fibers for liquid absorption, and microprocessors that controlled puff duration (Williams et al., 2013; Williams, Bozhilov & Talbot, 2019; Williams & Talbot, 2019). Second-generation or clearomizers (e.g., Ego C Twist) have larger atomizers and tanks (fluid reservoirs) compared to cig-a-likes with internal components (solder joints, polyfil fibers, and microprocessors) removed in some models (e.g., Vuse) or replaced with more stable components. Larger fluid reservoirs and batteries were introduced in the third generation (mods) with alterations or complete removal of some atomizer components, including thick wires, fibers, and sheaths (Williams, Bozhilov & Talbot, 2019). The mod-style ECs are more user-friendly and give the ability to control and change power settings as desired (Talih et al., in press).

Figure 1.3 Fourth generation E-cigarette devices from multiple brands.

The fourth-generation pods, e.g., JUUL, were recently introduced with low-powered batteries similar to the first generations (Bowen et al., 2019; www.phixvapor.com). The fluid reservoirs in the fourth generation have capacities that cut through the first to third generations ranging from 0.7 – to 3 mL. Additionally, fourth-generation ECs can be prefilled (closed-system) characteristic of first- and second-generation devices, refillable (open-system) characteristic of the second and third-generation, or disposable. The disposable fourth-generation devices such as Puff Bar are generally regarded as a “hybrid” since they appear and deliver aerosols in some way that resembles a merger between first and fourth-generation ECs.

EC Fluid Formulations

EC device evolution has been accompanied by changes in the fluid formulation in terms of nicotine, flavor chemical, and synthetic cooling agents. E-liquid formulations originally contained free-based nicotine and a blend of characterizing flavor chemicals such as menthol and cinnamaldehyde. The liquids of fourth-generation ECs are popular among young consumers (Kann et al., 2018; Cullen, Gentzke, et al., 2019; Cullen, Liu, et al., 2019; Gentzke, 2019; Huang, Duan, et al., 2019; Leventhal et al., 2019; Wang et al., 2020, 2021; Park-Lee et al., 2021) and contain very high concentrations of synthetic coolants, salt-based nicotine, and flavor chemicals (Leventhal et al., 2019, 2022; Erythropel, Anastas, et al., 2021; Jabba et al., 2022). The appealing designs, increased flavorings, "icy-ness," ease of use, and aesthetics have contributed to their attraction and popularity among younger users (Kong et al., 2019; Jackson et al., 2020; Leventhal et al., 2021; Yingst, Foulds & Hobkirk, 2021; Dai & Hao, 2022). Despite these changes, the major components of EC liquid or refill fluids remain solvents, nicotine, flavor chemicals, and synthetic coolants.

Nicotine

Nicotine is an addictive chemical with neurological and pharmacological effects that facilitates behavioral re-enforcement upon exposure and absorption into the bloodstream. Earlier e-liquids were made with freebase nicotine with high alkaline pH and associated with bitterness, harshness, and respiratory tract irritation (Pankow, 2001; DeVito & Krishnan-Sarin, 2018). The nicotine concentrations in earlier EC products and DIY nicotine products ranged from 0 to 100 mg/mL in fluid and aerosol samples purchased worldwide, and product concentration labels were often inaccurate (Trehy et al., 2011; Goniewicz et al., 2013; Behar, Davis, Wang, et al., 2014; Cameron et al., 2014; Hahn et al., 2014; Davis et al., 2015; Kim et al., 2015; Zain et al., 2019). Recently, e-liquids used in ECs like JUUL and Puff Bar contain nicotine coupled with an acid (benzoic, acetic, lactic, benzoic, levulinic, salicylic, malic, and tartaric acid,) which alters the liquid pH by protonation to produce a nicotine salt solution. The resulting aerosol has a low pH that is less harsh and easier to inhale by consumers, thereby increasing the possibility of addiction by novice users (Chen, 1975; Pankow et al., 2017; Duell, Pankow & Peyton, 2018; Harvanko et al., 2020). The concentrations of nicotine in the fourth-generation prefilled cartridge and disposable devices range between ~40 - 60 mg/mL) (Pankow et al., 2017; Goniewicz et al., 2019; Talih et al., in press). In a randomized clinical trial of adults in an outpatient facility in Southern California, salt-based nicotine products were rated significantly higher for appeal, smoothness, and sweetness and lower for bitterness and harshness (Leventhal et al., 2021). The widespread distribution and use of nicotine-containing EC products presents a public health problem that could increase nicotine addiction, cause poisoning, and lead to other unwanted health effects and death (Benowitz, 2009; Dwyer, McQuown & Leslie, 2009; Cantrell, 2014; Pisinger & Døssing, 2014; Benowitz & Burbank, 2016; Hua & Talbot, 2016; Eltorai, Choi & Eltorai, 2019; Winer, 2016; Kim & Baum, 2015; Mohney, 2014).

Flavor Chemical Identification and Quantification

While flavor chemicals in ECs are generally regarded as safe (GRAS) for ingestion and use in food products, the Flavor and Extracts Manufacturers' Association (FEMA) has not evaluated their use in inhalation products (Burdock & Fenaroli, 2010; Hallagan, 2014). To limit exposures and protect food and flavor processing workers against work-related adverse health effects, including lung injuries, the National Institute for Occupational Safety and Health has published exposure guidelines for use in manufacturing plants (NIOSH 2004;2011). Flavor chemicals similar to those used in foods, cosmetics, and medicines are major ingredients in ECs and have been identified, quantified, and classified in e-liquids and aerosols (Behar, Davis, Wang, et al., 2014;

Brown et al., 2014; Hahn et al., 2014; Hutzler et al., 2014; Farsalinos et al., 2015; Allen et al., 2016; Behar et al., 2016, 2018; Tierney et al., 2016; Gerloff et al., 2017; Aszyk et al., 2018; Bitzer et al., 2018; Czoli et al., 2019; Budzyńska et al., 2020; Krüsemann et al., 2020; Erythropel, Garcia Torres, et al., 2021). Their concentrations in ECs frequently exceed those recommended for use in ingestible or dermal products (Behar et al., 2016, 2018; Tierney et al., 2016) and induce an array of toxic responses that may contribute to adverse health effects after prolonged exposures.

Results of analysis performed using analytical methods for identification and quantification of flavor chemicals in ECs varied based on the samples of interest, flavor categories of products, manufacturer/brand, place of purchase, method of aerosol generation, the generation of the device, and type of fluid (free base vs. salt-based nicotine).

While concentrations vary between types and flavors of e-liquids and place of purchase, some flavor chemicals appear frequently and usually in dominant concentrations (> 1 mg/mL) in e-liquids. Menthol, ethyl maltol, benzyl alcohol, triacetin, and vanillin have been identified as dominant and frequently occurring at high concentrations in multiple EC libraries studied (Tierney et al., 2016; Behar et al., 2018)

Menthol has been historically permitted for use in combustible cigarettes by the Family Smoking Prevention and Tobacco Control Act of 2009 (US Congress 2009). It is widely used in tobacco products (Giovino et al., 2004; Ai et al., 2018), sometimes appearing frequently and at high concentrations in ECs that are not explicitly labeled “mint” or “menthol.”(Behar et al., 2016, 2018) The cooling effects of menthol and its appealing minty flavor may make smoking uptake and initiation easier among novice users (Klausner, 2011; Villanti et al., 2021). The concentrations of menthol in ECs exceed levels in combustible cigarettes and other consumer goods and have been quantified at 84 mg/mL in an EC refill fluid.(Behar et al., 2018).

Ethyl maltol, vanillin, benzyl alcohol, and triacetin are dominant and frequently occurring flavor chemicals in multiple EC samples that impart fruity-caramellic, vanilla, sweet-almond, and fruity flavors (Behar et al., 2018; Czoli et al., 2019). Ethyl maltol concentrations in ECs range from < 1 – 61.2 mg/mL (Behar et al., 2018; Bitzer et al., 2018; Khachatoorian et al., 2022), which exceeds the maximum levels of 0.015% recommended for edible products (Oser & Ford, 1977; Opdyke, 1975). Vanillin has been identified in multiple studies evaluating chemical constituents of EC fluids and aerosols, and measured concentrations have been up to 31 mg/mL (Gerloff et al., 2017; Behar et al., 2018; Czoli et al., 2019; Krüsemann et al., 2020; Erythropel, Garcia Torres, et al., 2021; Khachatoorian et al., 2022).

Cinnamaldehyde imparts cinnamon flavor in edible products and as a fragrance in cosmetics (J T Gowder, 2014; Behar et al., 2016, 2018). and was frequently used in earlier formulations of e-liquids. Although its usage in e-liquids has significantly decreased over time, concentration in foods and cosmetics applied to the skin should not exceed 1% (Burdock & Fenaroli, 2010; J T Gowder, 2014). However, concentrations in ECs were found to range from <0.01% to 15.5 % (Behar et al., 2018). A convenience sample of cinnamon-flavored refill fluids and DIY flavorings was analyzed with HPLC and GCMS, cinnamaldehyde, 2-methoxycinnamaldehyde, dipropylene glycol, and vanillin were identified with concentrations ranging from 0.0005 to 0.3M (Behar, Davis, Wang, et al., 2014).

Diacetyl, 2,3- pentanedione, acetoin, and acetyl propionyl were identified in 45 - 92% of e-liquids and aerosol samples in fruit, candy, and cocktail flavors selected to represent flavors appealing to young consumers from a leading brand (Farsalinos et al., 2015; Allen et al., 2016). Concentrations of all three chemicals ranged from <LOQ – 529 ug/EC, with acetoin having the highest concentrations (Allen et al., 2016).

Synthetic Coolants

Non-menthol synthetic cooling agents such as WS-3 (N-ethyl-p-menthane-3-carboxamide) and WS-23 (2-isopropyl-N,2,3-trimethylbutyramide) were among the agents originally developed by Wilkinson Sword Ltd for use in cosmetics and edibles (Leffingwell & Rowsell, 2014). WS-23 and WS-3 have been used in tobacco cigarettes since 1974 and quantified in refill fluids purchased as early as 2012 and more recently in salt-based nicotine products such as JUUL and Puff Bar at concentrations that exceed recommended ingestion limits (Erythropel, Anastas, et al., 2021; Jabba et al., 2022). WS-3 and WS-23 are considered safe for ingestion by FEMA and are used extensively in consumer products, including breath fresheners, confectionaries, and cosmetics (Adams et al., 1996; Leffingwell, 2009; Marnett, et al., 2013; Smith et al., 1996). Even though they impart little to no flavor, they create a cooling and relaxing sensation by activating transient receptor potential channels on cell membranes (Behrendt et al., 2004). Discussion by DIY EC consumers suggests that these agents are widely used as they are added to EC fluids and flavoring to create icy-hybrid formulations. (https://www.reddit.com/r/DIY_eJuice/comments/aangb4/ws23_expertise/; https://www.reddit.com/r/DIY_eJuice/comments/9uhdny/ws3_vs_ws23;

Aldehyde

Flavor chemicals and solvents used in EC fluids produce carbonyl compounds and toxic aldehydes upon aerosolization/evaporation or thermal decomposition (Bekki et al., 2014; Goniewicz et al., 2014; Kosmider et al., 2014, 2016; Jensen et al., 2015; EL-Hellani et al., 2016; Geiss, Bianchi & Barrero-Moreno, 2016; Khlystov & Samburova, 2016; Uchiyama et al., 2016, 2020; Klager et al., 2017; Wang et al., 2017; Reilly et al., 2019; Ebersole et al., 2020; Chen, Canchola & Lin, 2021; Talih et al., in press). The production and concentrations of 7 aldehydes during thermal decomposition varied between EC brands, were largely dependent on the presence of flavor chemicals in the e-liquid and ranged from 0.04µg of glyoxal per puff in unflavored products to 49.5 µg of formaldehyde per puff in watermelon flavored product (Khlystov & Samburova, 2016). At high voltage, levels of formaldehyde in EC vapors are comparable to levels in tobacco smoke, and increasing device voltage results in an exponential increase in formaldehyde, acetaldehyde, and acetone concentrations (Kosmider et al., 2014).

Extrapolation of results generated using a high voltage device (5V) shows that vaping 3mL per day would expose a user to 14.4 mg of formaldehyde which is about 5 times higher than the risk of exposure from cigarettes (Jensen et al., 2015). Formaldehyde, acetaldehyde, methylglyoxal, propionaldehyde, acrolein, and benzaldehyde were some aldehydes detected in propylene glycol, vegetable glycine, and flavored e-liquids emissions (range = 85– 22,717 ng/puff). (Chen, Canchola & Lin, 2021). Sub-ohm vaping using devices with coil resistance < 0.5 Ω increases the levels of carbonyls when compared to regular vaping with >1 Ω to 4.5 Ω resistance coils (Noël et al., 2020). In a study evaluating multiple disposable devices, including Puff Bar and JUUL, total carbonyls were device and flavor-dependent. The highest concentrations of carbonyls were quantified in Berry and Mango flavored disposables, and the lowest concentrations were in JUUL tobacco flavor pods, with a significant difference between the disposables and JUUL (Talih et al., in press). The unstable nature of aldehyde-containing EC fluids may lead to the formation of acetals and several other reaction products even at room temperature, which may influence the toxicity of the parent chemicals (Erythropel et al., 2019; Jabba et al., 2020).

Invitro Toxicity of ECs and Flavor Chemicals

The toxicity of ECs has been investigated and linked to the presence of flavor chemicals (Bahl et al., 2012; Behar, Davis, Wang, et al., 2014; Behar et al., 2016; Leigh et al., 2016; Clapp et al., 2017, 2019; Gerloff et al., 2017), particularly at high concentration (Behar et al., 2018; Fetterman et al., 2018) that significantly correlates with cytotoxicity (Bahl et al., 2012).

In vitro experimental models exposed to e-liquids, their aerosols, and individual constituents show increased or elevated levels of oxidative stress, inflammatory responses, cellular senescence, impairment of membrane potentials, the release of LDH activity, altered cellular morphology, impaired pulmonary defenses, mucin production, and loss of transepithelial resistance (Cervellati et al., 2014; Lerner et al., 2015; Palpant et al., 2015; Scheffler, Dieken, Krischenowski & Aufderheide, 2015; Scheffler, Dieken, Krischenowski, Förster, et al., 2015; Sussan et al., 2015; Sherwood & Boitano, 2016; Rowell et al., 2017; Muthumalage et al., 2018; Sassano et al., 2018; Hickman, Herrera & Jaspers, 2019; Lucas et al., 2020; Nair et al., 2020; Pinkston et al., 2020; Sundar et al., 2016).

The response of cells to chemicals in e-liquids and aerosols is largely dependent on the fluid composition, device type and operating parameters, cell type, and specific endpoints being investigated. A cytotoxicity screening study of e-liquids with varying concentrations of flavor chemicals revealed that mouse neural and human embryonic stem cells were generally more sensitive than human pulmonary fibroblasts and human lung epithelial cells (Bahl et al., 2012; Behar et al., 2018; Behar, Wang & Talbot, 2018).

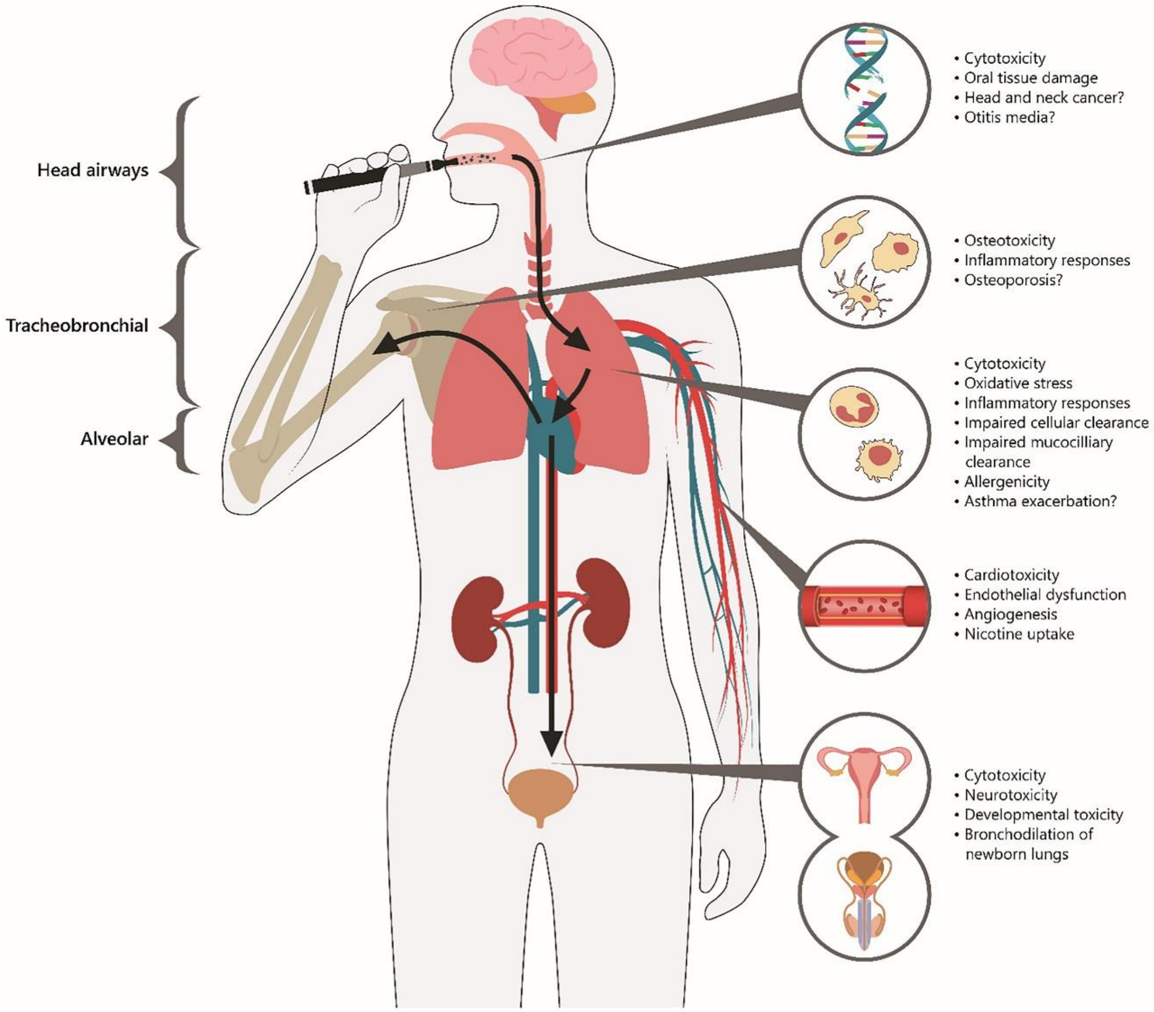


Figure 1.4. Aerosolized flavored e-liquids and target organs/systems within the body including known toxic responses and potential adverse health effects.

Menthol and menthol-flavored ECs alter a host of biological responses in vitro, including induction of cytotoxicity, calcium influx, mitochondrial hyperfusion, increased levels of superoxide production, and inflammatory biomarkers (e.g., IL-8, Il-6, COX-2, and RAGE), impairment of nitric oxide production, and decrease cell viability and mitochondrial function (Lee et al., 2018; Muthumalage et al., 2018; Sassano et al., 2018; Zahedi et al., 2018; Nair et al., 2020; Sundar et al., 2016).

Ethyl maltol is a dominant flavor chemical in most e-liquids and aerosols. In cell-free systems, it modulates free radical production in a dose-dependent manner (Bitzer et al., 2018; Muthumalage et al., 2018). A screening of flavorings used in ECs was the most cytotoxic to human bronchial epithelial cells at the highest concentrations tested (Sherwood & Boitano, 2016). Aerosolized e-liquids containing ethyl maltol were more cytotoxic to A549 cells and increased cytosolic calcium in multiple cell types (Otręba et al., 2018; Rowell et al., 2020). Maltol, an analog of ethyl maltol, is cytotoxic, induces IL-8 and IL-6 release, and impairs barrier function in human respiratory cells (Gerloff et al., 2017).

Cinnamaldehyde is highly cytotoxic, impairs barrier function, decreases the phagocytic activity of macrophages and cellular mitochondrial function by altering ATP production, and depolymerizes microtubule function, resulting in impairments of cellular dynamics, such as growth and motility (Behar et al., 2016, 2018; Clapp et al., 2017, 2019). A concentration-dependent generation of ROS was observed in cell-free experiments performed with Cinnamon flavored e-liquids and authentic standard flavor chemicals (Fetterman et al., 2018). Toxic concentrations of cinnamaldehyde were quantified in a broad spectrum of non “cinnamon” refill fluids suggesting “fruit,” “berry,” “coffee,” “tobacco,” and “sweet” flavors based on their label names (Behar, Davis, Wang, et al., 2014; Behar et al., 2016).

In vivo Toxicity ECs and Flavor Chemicals

Compared to in vitro systems, fewer studies have evaluated the effects of ECs and flavor chemicals in vivo. Due to their genetic and physiological similarities to humans, mouse models are commonly used in vivo systems to evaluate potential human exposure to a toxicant. Circulatory, respiratory, and cardiovascular endpoints have been measured in mice exposed to ECs and flavorings (Stefaniak et al., 2021). Exposure to aerosols of tobacco, menthol, vanilla, fruity, dessert, and ice-flavored e-liquids, increased the acrolein metabolite, 3-HPMA, and total nicotine excretion in the urine was observed in mice exposed to the menthol flavor product compared to tobacco (Conklin et al., 2018; Chapman et al., 2019). A cinnamon-flavored product increased airway hyperresponsiveness, while a licorice-flavored product slightly stimulated airway inflammation (Chapman et al., 2019). Investigation of skin exposures revealed increased mucin production and pro-inflammatory responses in submerged culture of epithelial cells (Go et al., 2020).

Observations from exposure to investigate the respiratory effects of solvents only and solvents with vanilla flavoring indicate that vanilla flavoring can increase tidal lung volume. In contrast, increased counts of immune cells were observed in the treatment with or without flavoring (Szafran et al., 2020). No differences were observed in rats exposed to flavored and unflavored aerosol. Lower levels of BALF protein, ALP, and LDH were observed at higher doses in the treatment group compared to the control (Werley et al., 2016). Another study evaluating the effects of ECs and measuring cardiovascular endpoints in rats revealed impaired endothelial function and increased soluble lung collagen in both flavored and unflavored exposures (Rao, Liu & Springer, 2020).

In a human study, flavored e-liquid's effects on the circulatory system depended on the flavor (St.Helen et al., 2017). Compared to tobacco flavors, inhalation of strawberry flavored aerosol was associated with significantly higher nicotine intake and plasma nicotine concentrations (*ad libitum*) and increased heart rate. In another menthol and unflavored e-liquids study containing equal concentrations of solvents and nicotine, higher uptake and maximum plasma concentrations of nicotine were observed after using the unflavored product and not menthol flavor (Walele et al., 2016).

Risk Assessment of EC Constituents Using the Margin of Exposure Analysis

Expert groups and regulatory agencies recommend the margin of exposure approach to access the risk of carcinogens and chemicals (EFSA, 2005, 2007; Alexander et al., 2012; FDA 2018). It is dimensionless and incorporates a reference point (usually a No Observed Adverse Effect Level or a BenchMark Dose) based on animal dose-response data (e.g., the incidence of tumor formation) and an estimated human daily intake of a chemical (EFSA, 2005; Barlow et al., 2006; Benford, Leblanc & Setzer, 2010). It is an important risk assessment tool for assessing the health risk of different chemicals and prioritizing risk management. For carcinogens and non-carcinogens, MOE values below 10,000 and 100 indicate a high risk for humans, respectively (EFSA, 2005, 2007; Alexander et al., 2012; EFSA Scientific Committee, 2012; Hahn et al., 2014). MOE has been historically used to assess the risk of exposure to genotoxic and carcinogenic substances in foods and beverages (Berg, Restani, et al., 2011; Berg, Serra-Majem, et al., 2011; Monakhova, Jendral & Lachenmeier, 2012; van den Berg et al., 2014).

The MOE approach for risk assessment has been applied to chemicals in ECs using a threshold of 100 to indicate a high risk for exposure (Hahn et al., 2014; Jabba & Jordt, 2019; Jabba et al., 2022). Of seven compounds for which MOEs were calculated, only nicotine was significantly below the threshold of 100 in all cases (MOE = 0.1). Ethylene glycol and 1,2-propanediol concentrations in about 50% of the products produced a MOE that was below 100 (Hahn et al., 2014). A more recent study evaluated synthetic cooling agents in US marketed refill fluids and e-liquids from disposables and assessed the risk of exposure to WS-3 and WS-23 (Jabba et al., 2022). MOEs (2 – 28) for WS-3 and WS-23 were below the safety thresholds if 1 – 15 mL of refill fluids were consumed per day. For Puff Bar disposable, exposure was significantly higher if ½ - 2 devices were consumed per day (MOEs = 3 – 88) (Jabba et al., 2022).

Pulegone, a constituent of mint oil and a known carcinogen, which the FDA bans as a food additive, has been identified at high levels in flavored e-liquids (Lisko et al., 2015; Jabba & Jordt, 2019)USFDA 2018; NTP 2011). MOEs calculated for pulegone in EC fluids generated values below the 10,000 thresholds if 5 – 20 mL of fluids were used per day to account for light users (5mL), moderate users (10 mL), and heavy users (20mL) (Jabba & Jordt, 2019).

Health Effects of EC Exposure

While COVID infections increased exponentially worldwide, the USA witnessed increasing cases of an outbreak of electronic cigarettes and vaping-associated illnesses (EVALI) or vaping-associated pulmonary injuries (VAPI) in all 50 states, which led to thousands of hospitalizations or deaths (Boland & Aesif, 2019; Butt et al., 2019; Christiani, 2019; Henry, Kanne & Kligerman, 2019; Ali, Khan, et al., 2020; Blount et al., 2020; Choe et al., 2020; Furlow, 2020; Henry et al., 2020; Layden et al., 2020; Schaffer et al., 2021; Rai et al., 2022). Most patients and product sampling to identify the cause of the illnesses reported the presence of tetrahydrocannabinol (THC) in poor quality or counterfeit/black-market ECs or vaping products obtained from informal sources (Furlow, 2020).

Further product sampling and fluid samples collected from the patient lungs strongly linked the outbreak to vitamin E acetate, and further recommendations involved avoiding vaping products until the causes of EVALI are determined. (Furlow, 2020)

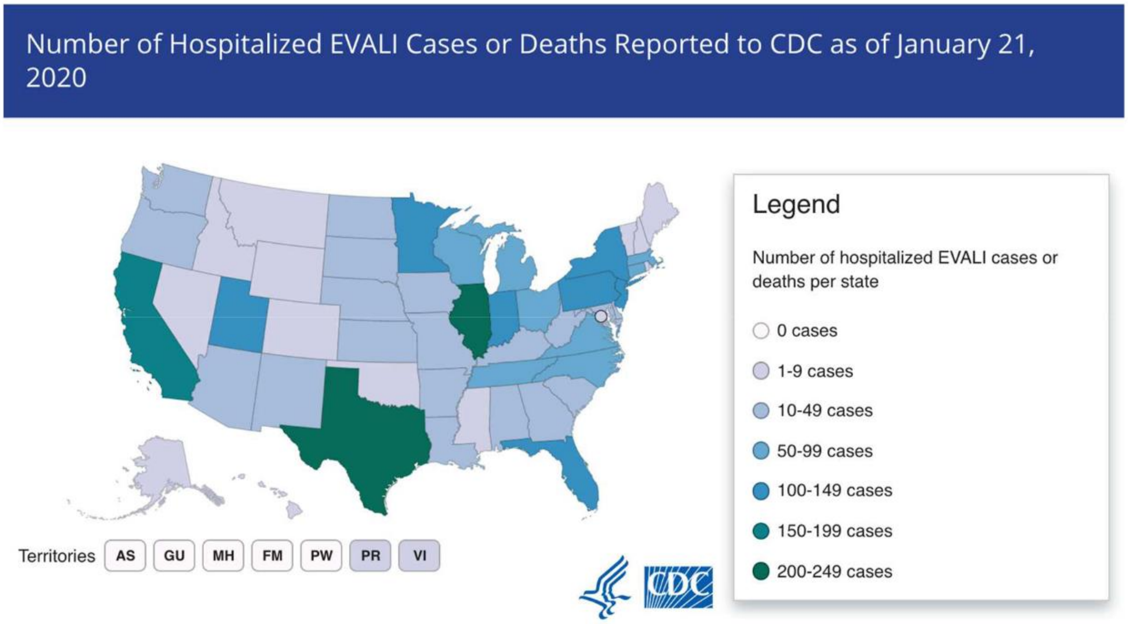


Figure 1.5. Number of hospitalized EVALI cases or deaths reported to CDC as of H=January 21, 2020.

While not easily quantified or linked, acute and chronic health effects, including nicotine addiction, toxicity, and respiratory and cardiovascular symptoms, have been associated with ECs (Gilley & Beno, 2020; Hamberger & Halpern-Felsher, 2020; Bonner et al., 2021; Stefaniak et al., 2021). Adverse health effects, including inflammatory lung diseases, such as bronchiolitis obliterans, acute eosinophilic pneumonia, digestive diseases, increased airway resistance, impaired mucocilliary clearance, and cough reflex have been reported and associated with EC (Kreiss et al., 2002; Egilman & Schilling, 2012; Vardavas et al., 2012; Hua, Alfi & Talbot, 2013; Pisinger & Døssing, 2014; Dicpinigaitis et al., 2016; Hua & Talbot, 2016; Kumral et al., 2016; Skotsimara et al., 2019; Hua et al., 2020). The observed health effects have been linked to EC aerosol particles, harmful metals, tobacco-specific nitrosamines, and toxic carbonyl-containing degradation products. (Pisinger & Døssing, 2014) Survey studies on children sub-acutely exposed to ECs have shown bronchitis and increased asthma symptoms (McConnel et al., 2017). Respiratory effects, including inflammation, oxidative stress, decreased lung function, and suppressed protective and immune responses reported in humans are like those observed in animal models (Garcia-Arcos et al., 2016; Chun et al., 2017; Larcombe et al., 2017; Chapman et al., 2019; Skotsimara et al., 2019; Wills et al., 2019; Marczylo, 2020; Marshall et al., 2020; Miyashita & Foley, 2020).

Purpose of the dissertation

This dissertation aimed to evaluate the constituents of ECs, determine the cellular effects of EC liquids and aerosols, and assess the potential health effects associated with exposure to EC constituents. Specifically: (1) nicotine concentrations were analyzed in one brand of EC refill fluids purchased worldwide to understand the role of sales location in EC composition. (2) Flavor chemical and synthetic coolant composition were evaluated, and concentrations were measured in multiple libraries of ECs to provide useful data and improve our understanding of EC constituents. (3) Multiple end-point cellular assays were performed to determine the cytotoxicity of ECs liquids and aerosols and authentic standards of chemicals individually and in mixtures. (4) Cancer and safety risk assessment of chemicals in ECs was performed using the margin of exposure calculations.

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Chapter 2

Counterfeit Electronic Cigarette Products with Mislabeled Nicotine Concentrations

Omaiye et al. 2017.

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ABSTRACT

Objectives: We compared nicotine concentrations in one brand of refill fluids that were purchased in 4 countries and labeled 0 mg of nicotine/mL. We then identified counterfeit e-cigarette products from these countries. **Methods:** Overall, 125 e-cigarette refill fluids were purchased in Nigeria, the United States (US), England, and China. Nicotine concentrations were measured using high performance liquid chromatography and compared to labeled concentrations. Refill fluids were examined to identify physical differences and grouped into authentic and counterfeit products. **Results:** Whereas nicotine was in 51.7% (15/29) of the Nigerian, 3.7% (1/27) of the Chinese, and 1.6% (1/61) of the American refill fluids (range = 0.4 - 20.4 mg/mL), 8 British products did not contain nicotine. Products from China, the US, and Nigeria with trace amounts of nicotine (0.4 to 0.6 mg /mL) were authentic; however, all products from Nigeria with more than 3.7 mg/mL were counterfeit. **Conclusions:** We introduce 2 novel issues in the e-cigarette industry, the production of counterfeit refill fluids under a brandjacked label and inclusion of nicotine in 81.3% of the counterfeit products labeled 0 mg/mL. This study emphasizes the need for better control and monitoring of nicotine containing products and sales outlets.

INTRODUCTION

Nicotine is readily available in electronic cigarette (e-cigarette) refill fluids that are sold worldwide. Nicotine concentrations in these products range from 0 to over 100 mg/mL, and product concentration labels are often inaccurate. [1-3] Even do-it-yourself flavoring products used to create these fluids sometimes contain nicotine. [4] (Davis, Razo, et al., 2015) The widespread distribution and use of nicotine containing e-cigarette products presents a new public health problem that could increase nicotine addiction, cause poisoning, and lead to other unwanted health effects. [5-11]

The purpose of this study was to evaluate e-cigarette refill fluids produced by one manufacturer and sold worldwide. Specifically, we quantified nicotine in products labeled 0 mg/mL, evaluated products to determine authenticity, and identified counterfeit zero nicotine refill fluids that contained nicotine.

MATERIALS AND METHODS

Sample Collection and Assessment

Between March 2015 and May 2016, 125 of LiQua e-cigarette refill fluids (Ritchy Group Limited) were purchased in Nigeria (29 refill fluids, 7 flavors, purchased over the counter in an Abuja department store and at an online store in Lagos), the United States (61 refill fluids, 50 flavors, purchased over the Internet from Kansas and California), England (8 refill fluids, in 8 flavors, purchased over the Internet from Northamptonshire), and China (27 refill fluids, 25 flavors, purchased over the Internet from Xiamen and Guangdong). These countries were chosen to represent different: (1) global regions, (2) levels of economic development, and (3) levels of consumer product regulation and quality. Labeled nicotine concentration for all 125 products was “0 mg/mL”, which was interpreted as zero nicotine. Ritchy Group Limited is a Russian company with production plants in China and Italy and contact centers in Moscow, Kansas, the Czech Republic, and China that distributes products to over 85 countries (www.ritchy.com). Ritchy was chosen because of its broad global distribution of refill fluids which enabled comparison of products purchased in the 4 countries. When possible, products with the same flavors were purchased in multiple countries.

Each product was assigned an inventory number, photographed, and stored at 4ºC. All products were received sealed and undamaged and were analyzed within 1 month of receipt. All products came in individual boxes, except those from Guangdong (China). Coloration of each fluid was compared visually.

Nicotine Concentration Quantification

High performance liquid chromatography (HPLC) was used to quantify nicotine in each sample using a method described previously. (Davis et al., 2014) The limit of quantification for nicotine was 10 µg/ml with a limit of detection of 50 ng/ml.

Authentication of E-cigarette Refill Fluids

Counterfeit products were defined as those that were not manufactured by Ritchy but were sold under the Ritchy label. The Quick Response (QR) barcode, European Article Number (EAN) barcode, and guidelines from consumer websites were used to determine if refill fluids were authentic or counterfeit.[13,14] Products were examined for the presence of QR codes as recommended by personnel at Ritchy. QR codes on refill fluids have 5 sets of 4-digit numbers printed on white stickers that were located on the bottom or the caps of refill fluid boxes or bottles. These codes were either inputted or scanned into the verification section (www.ritchy.com/check) on the Ritchy website, which recognizes numbers that belong to authentic Ritchy products and those not generated by Ritchy.

The globally used ***13-digit*** EAN barcode, which ***identifies*** items for sale at retail establishments, was also used for authentication. This barcode consists of: (1) the GS1 prefix which identifies the country where the product was manufactured or the member organization to which the manufacturer is registered, (2) the unique manufacturer's identification code assigned by the GS1 office, (3) the item or product code which is selected by the manufacturer, and (4) the check digit which proves that the manufacturer has thoroughly inspected the item. EAN barcodes appeared on stickers pasted or printed directly on each refill fluid box or bottle and were scanned using ICONIT software. The user is directed to an Internet site that: (1) identifies the product as a Ritchy product, (2) fails to identify the product, or (3) identifies an incorrect Ritchy product indicating the barcode had been hijacked. A second line of EAN identification was performed using a government-supported online database (*www.gepir.gs1.org*) that provides information on the company, products, and illegal EAN numbers.

Further guidelines from e-cigarette websites and forums were also used to identify counterfeit LiQua products. [13,14] These criteria included the quality of printing on boxes and bottles, which is inferior on counterfeit products, the appearance of identical product images on the Ritchy website, and the packaging of the product in a box at the time of receipt, which is characteristic of authentic Ritchy products. "Product Name on Database" was not available for the LiQua Q and LiQua HP products and some premium LiQua flavors.

RESULTS

Nicotine Concentrations in Zero Nicotine Products

Nicotine was quantified in 125 LiQua e-cigarette refill fluids labeled 0 mg (Table 1, Figures 1A-D, and Supplementary Table 1). 108 samples contained no nicotine (Table

1 and Supplementary Table 1). Figures 1A and B show Nigerian products that contained nicotine peaks is indicated by the red arrow at 8 min in Bright Tobacco flavor (A) and Menthol flavor (B). Figures C and D show that the same flavors purchased in the USA contained no nicotine, as indicated by the black arrows. Samples of Two-Apples from the USA, China, and Lagos contained trace amounts of nicotine (range = 0.4 to 0.6 mg/mL), probably due to contamination or carry over during manufacturing. In contrast, all LiQua Bright Tobacco, MB, and Menthol flavors purchased in Abuja (N = 13) contained 3.7 - 20.4 mg/mL of nicotine (Table 1). Nicotine concentrations varied within the same flavor purchased at separate times, e.g., the first set of MB fluids contained 20.4 mg/mL of nicotine (product #1, Table 1), while the second (products #2 - #4) and third (products #5 - #7) sets contained 12.3 and 14.6 mg/mL, respectively.

Physical Properties of E-cigarette Refill Fluids

Within LiQua flavor groups, color varied with country, e.g., Bright Tobacco purchased in Abuja was coral to light orange but clear in other countries (Table 1, Supplementary Table 1, and Figure 1E). The color of LiQua MB flavors purchased in Abuja at separate times varied from coral to orange (Figure 1F). This color variation in counterfeit products is suggestive of inconsistencies during manufacture. Watermelon flavored products purchased in Abuja were clear and identical to those purchased in the USA (Table 1; Supplementary Table 1).

Labeling on Abuja products was fuzzy and of inferior quality compared to products from other countries which were of superior quality. Watermelon flavored fluids from Abuja were in blue boxes without a QR code for authentication (Figures 1G and 1I), while the Kansas sample was in a green box with a QR code (Figures 1H and 1J) and was identical to the image on the Ritchy website. Bright Tobacco labels from Abuja were printed on a tan background, while labels from other countries were on white backgrounds that were identical to images on the Ritchy website. The MB flavor had no semblance to product images on *www.ritchy.com* but existed only on websites discussing “Fake LiQua e-juices”. Samples from Guangdong China were not received with boxes; therefore, the semblance and quality of packaging could not be evaluated. All products from Abuja had identical lot/batch numbers unlike products from other countries, which had different lot/batch numbers for each sample. Only the “variety pack of ten”, purchased from Santa Clara (California) and Xiamen had the same production lot/batch numbers on the fluids as well as on the variety pack box.

Identification of Counterfeit Products

Refill fluids were examined to determine if they were counterfeit (Table 1) or authentic (Supplementary Table 1) using the QR code, EAN barcode, and differences in physical properties of the products. Using QR codes, products from the USA (except for one), England, Lagos, and China (Xiamen) were verified to be authentic. Products from Abuja had no QR codes on their boxes and products from Guangdong (China) were received without boxes and therefore their authenticity could not be verified (Table 1).

Additional information on counterfeit products was obtained using the EAN barcode (Table 1). Counterfeit Nigerian products were registered to: (1) Ritchy Group LTD but were linked to the incorrect product, e.g., the 10 ml Bright Tobacco code identified it as a 30 ml Energy Drink; (2) Spoilt LTD, a different company, identified by the barcode as an “illegal number” (e.g., Watermelon); or (3) no company, meaning matching documents were unavailable (e.g., Menthol flavors) and it could not be verified (Table 1). All flavors from other locations had barcodes and were identical to flavors found on *www.ritchy.com.*

Labeling and Warning Symbols

All boxes had a skull and cross bones, over 18, and X (harmful) symbols (Figures 1K and 1L); however, only the counterfeit samples had the Société Générale de Surveillance (SGS) insignia and the ecotoxic symbol (Figures 1L and 1M). SGS is a worldwide organization that inspects, verifies, tests, and certifies that imported goods have been checked and meet quality control standards (*www.sgs.com*). Similar health warnings were reported on the bottles or boxes of all refill fluids.[15] Only LiQua HP flavors stated that a user should "contact a poison center or seek medical assistance if you feel ill after use".

Association between Nicotine and Counterfeit Refill Fluids

The above criteria were used to determine that 16/125 refill fluids labeled 0 mg were counterfeit products sold under a brandjacked label. 81.25% (13/16) of the counterfeit products contained nicotine (3.7 – 20.4 mg/mL). The 3 counterfeit flavors with nicotine were MB, Menthol, and Bright Tobacco. 18.75% (3/16) of Watermelon flavored LiQua, purchased in Abuja, were also counterfeit but did not contain nicotine.

DISCUSSION

This paper introduces novel issues in tobacco control and global health, the production of counterfeit e-cigarette refill fluids and the inclusion of nicotine in counterfeit products labeled 0 mg. The identification of nicotine in e-cigarette products that should be nicotine free is a health concern for several reasons. First, zero nicotine users with access to counterfeit products could develop an unwanted addiction that may be difficult to break. Secondly, a growing number of pregnant women use nicotine-free refill fluids[16] and could unwittingly expose their fetuses/newborns to a neuroteratogen[5]. Thirdly, refill fluids containing nicotine have caused numerous poisonings, often in children; (AAPCC, 2016; Hua & Talbot, 2016a) this potential danger is not apparent from the mislabeled counterfeit products. Finally, some e-cigarette users gradually decrease nicotine usage with e-cigarettes.[18] If these users purchase counterfeit products that contain nicotine, they would be unsuccessful in weaning themselves off nicotine.

Refill fluid users can identify counterfeit products using the criteria presented in this paper. Counterfeit fluids purchased in Abuja were ₦500.00 NGN in contrast to authentic products purchased from recommended LiQua distributors in Lagos for ₦1500.00 NGN. Although counterfeit products with nicotine were only purchased in Abuja, these products are readily distributable to other countries, and we had no difficulty bringing them into the USA. In addition, the counterfeit products varied in color within flavors, suggesting inconsistencies in their manufacture.

Unlike earlier generations, the authentic products in this study were generally labeled with safety warnings and reasonably accurate nicotine concentrations. LiQua Q flavors purchased in California carried the Proposition 65 warning stating the product contains substances that may cause cancer or produce reproductive/developmental problems.[20] However, only LiQua HP flavors contained warnings such as: not recommended for non-smokers, contact with skin maybe toxic, keep out of reach of children and pets, and contact a poison center if you feel ill after use. The SGS logo implies products have undergone supervision and quality control from acquisition of raw materials through manufacturing to final production and distribution. Users of refill fluids should be skeptical of this logo as it appeared only on counterfeit products.

Counterfeit products have been a problem in the conventional tobacco cigarette industry[21]. Our study demonstrates for the first that the problem of counterfeit products extends to the e-cigarette retail market. However, because our study is limited to products from one company and 4 countries, future studies will be needed to determine the breadth of counterfeit e-cigarette sales.

Conclusions

This is the first report that counterfeit e-cigarette products with inaccurate nicotine labeling and invalid quality control certification logos are being produced under a brandjacked label. Users of these products would be exposed to nicotine without their knowledge, which could lead to unwanted nicotine induced health effects, as recently summarized by the Surgeon General.[12] In addition, the counterfeit products varied in color within flavors, suggesting inconsistencies in their manufacture. These data will be useful in establishing regulatory policies for e-cigarettes.

IMPLICATIONS FOR TOBACCO REGULATION

This paper introduces a new issue in the emerging e-cigarette industry, the inclusion of nicotine in counterfeit products labeled 0 mg/mL. Nicotine has also been reported in some DIY e-cigarette flavor products that should be nicotine free[4]. Mislabeled counterfeit and DIY e-cigarette products containing nicotine are a public health concern that could be addressed by agencies involved in the regulation of tobacco products. These findings emphasize the need for education of e-cigarette users to the existence of zero nicotine products that contain nicotine and for identification and confiscation of counterfeit products.

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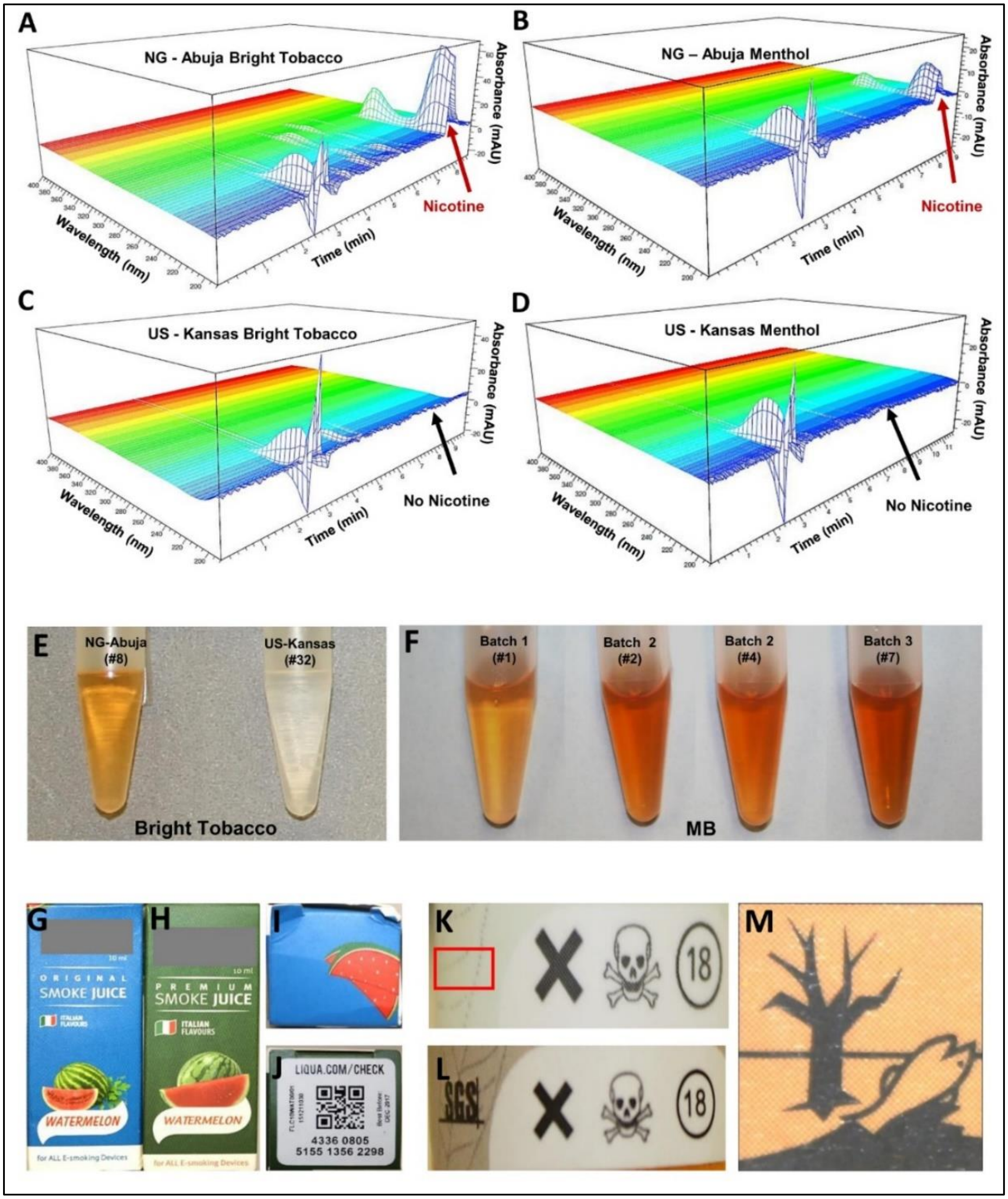
A
NG - Abuja Bright Tobacco
Absorbance (mAU)
Wavelength (nm)
Time (min)
Nicotine
B
NG – Abuja Menthol
Absorbance (mAU)
Wavelength (nm)
Time (min)
Nicotine
C
US - Kansas Bright Tobacco
Absorbance (mAU)
Wavelength (nm)
Time (min)
No Nicotine
D
US - Kansas Menthol
Absorbance (mAU)
Wavelength (nm)
Time (min)
No Nicotine
E
NG-Abuja (#8)
US-Kansas (#32)
Bright Tobacco
F
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Batch 2 (#2)
Batch 2 (#4)
Batch 3 (#7)
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ITALIAN FLAVOURS
WATERMELON
for ALL E-smoking Devices
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PREMIUM SMOKE JUICE
ITALIAN FLAVOURS
WATERMELON
for ALL E-smoking Devices
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J
LIQUA.COM/CHECK
4336 0805
5155 1356 2298
K
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L
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Figure 2.1: Comparison of 0 mg E-cigarette Refill Fluids and Identification of Counterfeit Products. (A-D) Three-dimensional HPLC chromatograms showing presence or absence of nicotine in e-cigarette products labeled 0 mg of nicotine. X axis = time (minutes), Y-axis = absorbance (mAU), and Z-axis = wavelength (nm). (E-F) Color variations between identical refill fluids for Bright Tobacco Nigeria vs. USA (E) and MB flavors (F). (G-J) Differences in packaging between Watermelon from Nigeria (G) without a QR code (I) and USA (H) with a QR code for authentication (J). (K -L) Warning labels and certification logos on Bright Tobacco refill fluid boxes purchased in the USA (K) without the SGS logo (red box) and in Nigeria (L) with the SGS logo. The ecotoxic symbol (M) was present on only the counterfeit LiQua refill fluids.

Table 2.1 Counterfeit and Suspected Counterfeit E-cigarette Refill Fluids[a]

Flavor	[c][Q] (mg/mL)	Coloration of Fluid	[d]QR Status	[e]EAN Status	[f]Company Name	Product Name on Database
MB	20.4 ± 0.3	Coral	NC	IC	RGHK	Variety (0mg)
MB	12.3 ± 0.2	Orange	NC	IC	RGHK	Variety (0mg)
MB	12.4 ± 0.2	Orange	NC	IC	RGHK	Variety (0mg)
MB	12.3 ± 0.1	Coral	NC	IC	RGHK	Variety (0mg)
MB	14.9 ± 0.4	Deep Orange	NC	IC	RGHK	Variety (0mg)
MB	15.5 ± 0.4	Deep Orange	NC	IC	RGHK	Variety (0mg)
MB	13.6 ± 0.6	Deep Orange	NC	IC	RGHK	Variety (0mg)
Bright Tob.	13.6 ± 0.2	Coral	NC	IC	RGHK	10bottles 30ml Energy Drink (18mg)
Bright Tob.	12.9 ± 0.5	Light Orange	NC	IC	RGHK	10bottles 30ml Energy Drink (18mg)
Menthol	9.2 ± 0.0	Clear (translucent)	NC	IC	RC: 13	Illegal Number
Menthol	3.7 ± 0.0	Clear	NC	IC	RC: 13	Illegal Number
Menthol	4.2 ± 0.1	Clear (translucent)	NC	IC	RC: 13	Illegal Number
Menthol	4.1 ± 0.0	Clear (translucent)	NC	IC	RC: 13	Illegal Number
Watermelon	ND	Clear	NC	IC	SLHK	No record found
Watermelon	ND	Clear	NC	IC	SLHK	No record found
Watermelon	ND	Clear	NC	IC	SLHK	No record found
Two Apples	0.4 ± 0.0	Yellow	NB	NB	N/A	N/A
Cola	ND	Clear	NB	NB	N/A	N/A
Peach	ND	Clear	NB	NB	N/A	N/A
Licorice	ND	Clear w/ yellow tint	NB	NB	N/A	N/A
Brownie	ND	Clear	NB	NB	N/A	N/A
Berry Mix	ND	Clear	NB	NB	N/A	N/A
Cheesecake	ND	Clear	NB	NB	N/A	N/A
Ry4 Tob.	ND	Pale yellow	NB	NB	N/A	N/A
Bright Tob.	ND	Pale yellow	NB	NB	N/A	N/A
Virginia Tob.	ND	Clear w/ yellow tint	NB	NB	N/A	N/A
Traditional Tob.	ND	Pale yellow	NB	NB	N/A	N/A
Mild Kretek Tob.	ND	Clear w/ yellow tint	NB	NB	N/A	N/A
Red Oriental Tob.	ND	Clear w/ yellow tint	NB	NB	N/A	N/A
Golden Oriental Tob.	ND	Clear w/ yellow tint	NB	NB	N/A	N/A

American Blend Tob.	ND	Clear	NB	NB	N/A	N/A
Goldenrod Oriental Tob.	ND	Clear w/ yellow tint	NB	NB	N/A	N/A
Vermillion Oriental Tob.	ND	Yellow Orange	NB	NB	N/A	N/A

[a] *#1 – 16 were verified to be counterfeit using all criteria. Packaging for #17-33 were not available and were suspected to be counterfeit. Supplementary Table 1 contains all authentic products*

[b] *Country of Origin = Locations of product purchase (NG-AJ = Nigeria, Abuja; CN-GD = China, Guangdong)*

[c] *[Q] = Quantified nicotine concentration (± standard deviation) using HPLC (ND = Not Detected)*

[d] *QR Status = Availability and Verification of Manufacturer's Quick Response Code (NC/NB = No Code/No Box = Unverified)*

[e] *EAN Status = Availability and Verification of Company and Product Information using the European Article Number barcode (IC = Incorrect; NB = None)*

[f] *Company Name = Name of manufacturer to which product EAN barcode is linked; RGHK = Ritchy Group Ltd HK; SLHK = Spoilt Ltd HK; RC:13 = Illegal/None; N/A = Not Available*

Chapter 3

High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations

Omaiye et al., 2019

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ABSTRACT

Whereas JUUL electronic cigarettes (ECs) have captured the majority of the EC market, with a large fraction of their sales going to adolescents, little is known about their cytotoxicity and potential effects on health. The purpose of this study was to determine flavor chemical and nicotine concentrations in the eight currently marketed prefilled JUUL EC cartridges ("pods") and to evaluate the cytotoxicity of the different variants (e.g., "Cool Mint" and "Crème Brulee") using in vitro assays. Nicotine and flavor chemicals were analyzed using gas chromatography−mass spectrometry in pod fluid before and after vaping and in the corresponding aerosols. 59 flavor chemicals were identified in JUUL pod fluids, and 3 were >1 mg/mL. Duplicate pods were similar in flavor chemical composition and concentration. Nicotine concentrations (average 60.9 mg/mL) were significantly higher than those of any EC products we have previously analyzed. The transfer efficiency of individual flavor chemicals that were >1 mg/mL and nicotine from the pod fluid into aerosols was generally 35−80%. All pod fluids were cytotoxic at a 1:10 dilution (10%) in the MTT and neutral red uptake assays when tested with BEAS-2B lung epithelial cells. Most aerosols were cytotoxic in these assays at concentrations between 0.2 and 1.8%. The cytotoxicity of collected aerosol materials was highly correlated with nicotine and ethyl maltol concentrations and moderately to weakly correlated with total flavor chemical concentration and menthol concentration. Our study demonstrates that (1) some JUUL flavor pods have sufficiently high concentrations of flavor chemicals that may make them attractive to youth and (2) the concentrations of nicotine and some flavor chemicals (e.g., ethyl maltol) are high enough to be cytotoxic in acute in vitro assays, emphasizing the need to determine if JUUL products will lead to adverse health effects with chronic use.

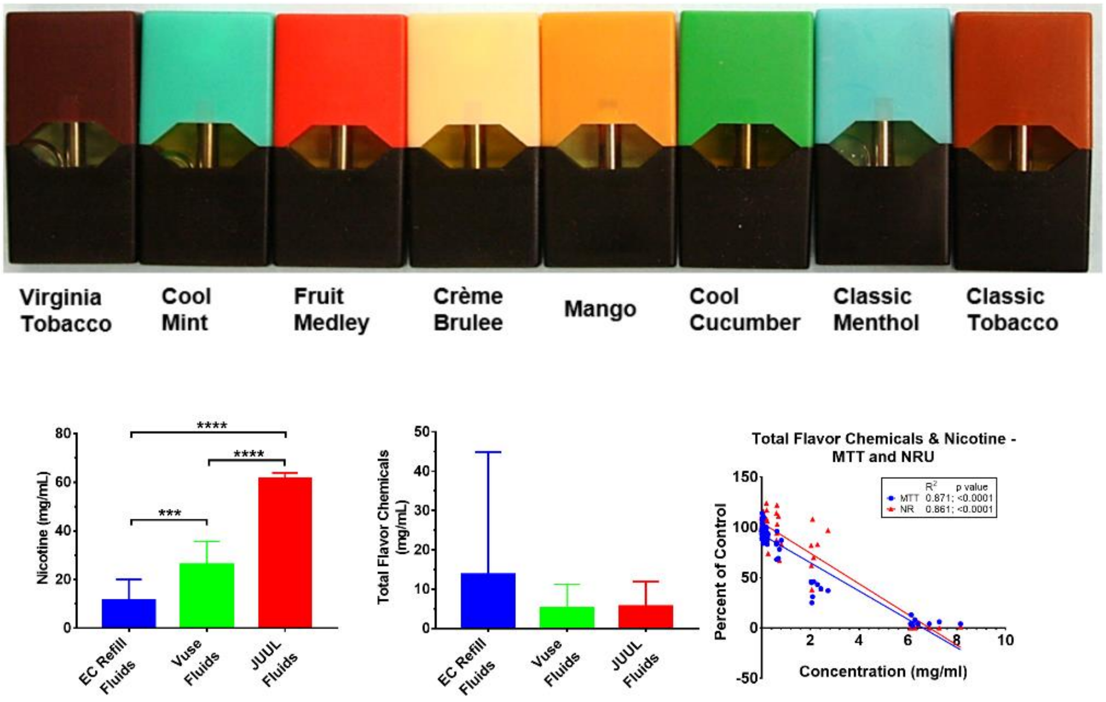

Figure 3.1 Graphical abstract

INTRODUCTION

While cigarette smoking is declining in many countries, youth and adult use of e-cigarettes (ECs) has increased.[1-3] and EC sales are estimated to reach 3.6 billion dollars in 2018.[4] To appeal to consumers and improve nicotine delivery, ECs have evolved since their introduction into world markets about 10 years ago. Although original models looked similar to tobacco cigarettes and were often termed “cig-a-likes”,[5] some highly evolved models have large tanks and batteries with features that allow power control by the user.[6]

The JUUL brand is one of the newer entries into the EC market and is more similar to the “cig-a-like” products than to recently available tank/box mod styles.[7] JUUL has spurred the development of many competing single pod style atomizers designed to be used with refill fluids containing dissolved nicotine salts.[8,9] In June 2018, in the US, it was estimated that about 68% of current EC sales are JUUL products.[10] Middle and high school students, as well as young adults, make up a large fraction of JUUL consumers.[11] This demographic may be attracted to JUUL in part because of its appealing compact design, which resembles a USB drive, and its ability to create relatively small clouds of aerosol making its use indoors and in schools difficult to detect.[12] Unlike many other EC fluids, JUUL products contain high concentrations of nicotine and sufficient acid to protonate most of the nicotine; lower free-base nicotine levels have been associated with increased palatability on inhalation.[13-15]

The JUUL system utilizes pre-filled EC fluid “pods”, originally sold exclusively by JUUL, but now offered by third parties. JUUL currently sells eight flavors of pods, which can be characterized as minty (“Cool Mint” and “Classic Menthol”), fruity (“Mango”, “Fruit Medley” and “Cool Cucumber”), sweet (“Crème Brulee”), and tobacco (“Classic Tobacco” and “Virginia Tobacco”). In spite of their sudden surge in popularity, relatively little has been reported on the chemicals delivered by JUUL products. We have previously shown that many other EC refill fluids contain very high concentrations of flavor chemicals[16,17] and that these concentrations are cytotoxic when tested in vitro with lung cells.[17-20]

The purposes of this study were to: (1) quantify nicotine concentrations in the eight flavor versions offered by JUUL and compare to those in other EC products, (2) identify and quantify the flavor chemicals in the eight flavor pods and compare to those in other EC products, (3) determine the transfer efficiency of nicotine and flavor chemicals into aerosols, and (4) test these products for cytotoxicity *in vitro* using human lung cells.

MATERIALS AND METHODS

Purchase of JUUL Products

The five original flavors of JUUL pods and three “limited edition” flavors were purchased online from the manufacturer’s USA website. These were “Cool Mint”, “Crème Brulee”, “Mango”, “Fruit Medley”, “Virginia Tobacco”, “Cool Cucumber”, “Classic Menthol” and “Classic Tobacco” (see Supporting Information, S1). Products were inventoried and stored at room temperature until used. Manufacturer’s label information stated that each JUUL Pod flavor contained 0.7 mL of flavored fluid at 5% nicotine.

Acquisition and Sampling of EC Refill Fluids

Nicotine concentrations (>1 mg/mL) of 66 EC refill fluids were obtained from previously published data. [27,29] In addition, 103 bottles of EC refill fluids were purchased from product lines offered by manufacturers in Nigeria and the USA (see Supporting Information, S2). Products were inventoried and stored at room temperature until analyzed.

Aerosol Production and Capture Using an Impinger Method

Each JUUL Pod was pre-conditioned by taking 3 puffs prior to weighing the pods and making aerosol solutions. Aerosol generated from pod fluids was bubbled through and captured in either isopropyl alcohol for flavor chemical and nicotine analysis or basal cell culture medium for cytotoxicity evaluation. During method development, we determined that about 96% of the flavor chemicals in the aerosol was captured in the two impingers. The aerosol materials captured in a fluid will be referred to as "aerosol" in the remainder of the paper. Aerosols produced from different pod flavors were collected at room temperature in two tandem 125 mL impingers, each containing 25 mL of isopropanol or basal cell culture medium. A JUUL EC (battery and pre-filled pod) connected to a Cole-Parmer Masterflex L/S peristaltic pump was puffed using a 4.3 s puff duration,[21] interpuff interval of 60 s, and an air flow rate of 10 – 13 mL/s. To reduce the likelihood of "dry puffing", only ¾ of the pod fluid was vaped. The pods were weighed before and after aerosol production to collect at least 15 mg for GC/MS analysis. Aerosol solutions were stored at −20 °C until shipped to Portland State University for analysis.

For the MTT assay, 6 total puff equivalents or TPEs (1 TPE = 1 puff/milliliter of culture medium) aerosol solutions were prepared in BEAS-2B basal medium, and supplements were added after aerosol production. The complete medium was passed through a 0.2 µm filter, and aliquots were stored at -80 ^{o} C until testing. Aerosols were tested at 0.02, 0.06, 0.2, 0.6, 2 and 6 TPE. To convert from TPE to percentage of the concentration of the pod fluid, the pod weight difference before and after aerosol collection was used to obtain the mg of fluid consumed. The weight (grams) of fluid consumed/puff of aerosol was calculated, and the density of the pod fluid was determined. Then the grams/puff were converted to milliliters using the density values. Finally, the percent for concentrations used in the aerosol cytotoxicity assays was determined according to the equation: $(Np \times Vp)/Vm$ where Np is the number of puffs, Vp is the volume of 1 puff, and Vm is the volume of the medium.

Identification and Quantification of Flavor Chemicals in JUUL EC Pod Fluids and Aerosols

The pre-filled pod fluid obtained prior to aerosolization of the JUUL pod is referred to as "unvaped fluid". The fluid left in the pod after the aerosol has been collected is referred to as "vaped fluid". Unvaped fluids, vaped fluids and aerosols were analyzed using GC/MS. For each unvaped and vaped sample, 50 µL were dissolved in 0.95 mL of isopropyl alcohol (IPA) (Fisher Scientific, Fair Lawn, NJ). All diluted samples were shipped overnight on ice to Portland State University and analyzed using GC/MS on the day they were received. A 20 µL aliquot of internal standard solution (2000 ng/µL of 1,2,3-trichlorobenzene dissolved in IPA) was added to each diluted sample before analysis. Using internal standard-based calibration procedures described elsewhere,[22] analyses for 178 flavor-related target analytes were performed with an Agilent 5975C GC/MS system (Santa Clara, CA). A Restek Rxi-624Sil MS column (Bellefonte, PA) was used (30 m long, 0.25 mm id, and 1.4 µm film thickness). A 1.0 µL aliquot of diluted sample was injected into the GC with a 10:1 split. The injector temperature was 235 °C. The GC temperature program for analyses was: 40 °C hold for 2 min; 10 °C/min to 100 °C; then 12 °C/min to 280 °C and hold for 8 min at 280 °C, then 10 °C/min to 230 °C. The MS was operated in electron impact ionization mode at 70 eV in positive ion mode. The ion source temperature was 220 °C and the quadrapole temperature was 150 °C. The scan range was 34 to 400 amu. Each of the 178 target analytes was quantitated using authentic standard material and an internal standard compound normalized multipoint calibration.

Cell Culture

Human bronchial epithelial cells (BEAS-2B) obtained from the American Type Culture Collection (ATCC) were cultured in Airway Epithelial Cell Basal Medium from ATCC

(Manassas, VA) supplemented with 1.25 mL of human serum albumin, linoleic acid and lecithin (HLL supplement), 15 mL of L-glutamine, 2 mL of extract P, and 5.0 mL airway epithelial cell supplement from ATCC (Manassas, VA). Nunc T-25 tissue culture flasks were coated overnight with basal medium, collagen, bovine serum albumin and fibronectin prior to culturing and passaging cells. At 90% confluency, cells were harvested using Dulbecco's phosphate buffered saline (DPBS) for washing and incubated with 2 mL of 0.25% trypsin EDTA/DPBS and poly-vinyl-pyrrolidone for 3 mins at 37ºC to allow detachment. Cells were cultured in T-25 flasks at 75,000 cells/flask, and the medium was replaced every other day. For the in vitro assays, cells were plated at 8,000 – 10,000 cells/well in pre-coated 96-well plates and allowed to attach overnight prior to a 24-hour treatment.

Cell Viability and Cytotoxicity Assays

The toxicities of unvaped and vaped pod fluids and their resulting aerosol fluids were determined using three assays. Treatments were performed over 3-fold dilutions with the highest concentration being 10% for the fluids and 6 TPE solutions for the aerosols, which ranged from 1.3 to 3%. Serial dilutions in culture medium were arranged in 96-well plates with negative controls placed next to the highest and lowest concentration to check for a vapor effect.[18] Cells were exposed for 24 hours before performing the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Neutral Red Uptake, (NRU) and Lactate Dehydrogenase (LDH) assays.

The MTT cytotoxicity assay measures mitochondrial reductases which convert the water soluble MTT salt to a formazan that accumulates in healthy cells. Post 24-hours of treatment, 20 µL of MTT (Sigma-Aldrich, St Louis, MO) dissolved in 5 mg/mL of DPBS (Fisher Scientific, Chino, CA) were added to each well and incubated for 2 hrs at 37ºC.

Solutions were removed, and 100 µL of dimethyl sulfoxide (DMSO) (Fisher, Chino, CA) were added to each well and gently mixed on a shaker. The absorbance of control and treated wells was read against a DMSO blank at 570 nm using an Epoch micro-plate reader (Biotek, Winooski, VT). Each chemical was tested in three independent experiments.

The NRU assay measures the uptake of neutral red dye, which accumulates within the lysosomes of healthy living cells. A working solution of 4 µg of neutral red stock (4 mg NR/mL of PBS without Ca^2+ and Mg^2+) per mL of cell culture medium was prepared and incubated at 37^{o}C overnight to dissolve the neutral red. Following exposure of cells to treatments, all medium was removed, and cells were incubated with 150 µL of neutral red solution for 2 hours. Cells were washed with PBS and 150 µL of lysis buffer (50% EtOH/ 49% deionized H_2O/ 1% acetic acid) were added to each well and gently mixed to achieve complete dissolution. The absorbance of control and treated wells at 540 nm was recorded using an Epoch micro-plate reader (Biotek, Winooski, VT).

The LDH leakage assay measures the activity of lactate dehydrogenase released into the culture medium and is an indicator of cell death or cytotoxicity due to plasma membrane damage. Reagents and solutions were prepared using an in-house recipe developed by OPS Diagnostics (Sigma-Aldrich, St Louis, MO). 200 mM TRIS, pH 8 (22.2 g Tris-HCl and10.6 g Tris-base and 50 mM of lithium lactate (19.6 mg/mL) were prepared in water. Tetrazolium salt (INT) was dissolved in DMSO (33 mg/mL), phenazine methosulphate (PMS) was dissolved in water (9 mg/mL), and β-nicotinamide adenine dinucleotide (NAD) sodium salt was dissolved in water (3.7 mg/mL). All three reagents (INT, PMS and NAD) were used to make the INT/PMS/NAD solution. 50 µL of all reagents were added to 96-well plates followed by 50 µL of culture medium obtained from both treated and control cells. The absorbance of all wells was measured at 490 nm using an Epoch micro-plate reader (Biotek, Winooski, VT).

Statistical Analyses

All cytotoxicity assays were carried out using three independent experiments each with different passages of cells, and each experiment had triplicate points. Data were statistically analyzed with a one-way analysis of variance (ANOVA), and each concentration was compared to the untreated control with Dunnett's post hoc test using Prism software (GraphPad, San Diego). For the nicotine concentration data, means were analyzed using an ANOVA followed by Bonferroni's post hoc test.

RESULTS

Identification of Flavor Chemicals in JUUL Pods

Fifty-nine of 178 flavor chemicals on our target list were identified and quantified in duplicates of the eight JUUL flavor pods (Figure 1). The duplicate data were generated using fluids from two different unvaped pods analyzed at different times. The total concentration of flavor chemicals in each product appears above each column. Abbreviations of JUUL pod names are on the *x*-axis, and safety classifications based on existing oral rat LD_{50} data[23] are on the *y*-axis. Within each safety classification, the chemicals are ranked from the most to least potent. Rat oral toxicity data were used for ranking because they were available for most chemicals in the heat map, while inhalation LD_{50} data were seldom available for rats or humans. Forty-three of the 59 chemicals had concentrations >0.01 mg/mL, 13 were >0.1 mg/mL, and 3 (menthol, vanillin and ethyl maltol) were >1.0 mg/mL. The highest concentrations of menthol, vanillin and ethyl maltol in unvaped pod fluids were 15, 6.9 and 1.8 mg/mL, respectively. Duplicate pods were generally similar to each other, however, "Fruit Medley-1" contained five times the total flavor chemical concentration as its duplicate pod. The “Fruit Medley” sample at 0.3 mg/mL was similar to the “Classic Tobacco” and “Virginia Tobacco” samples, which were all lower than 0.5 mg/mL.

Nicotine and Total Flavor Chemical Concentrations in EC Products

JUUL pods contain solvents, flavor chemicals, and varying concentrations of nicotine. The nicotine concentrations in 66 refill fluids from previous studies, [27, 29] 103 EC refill fluids, 5 Vuse cartomizer fluids, and 8 JUUL pod fluids in the current study (Figure 2a) were evaluated. Nicotine concentrations in the EC fluids fell into one of three groups: (1) most products had 1.6 – 34.4 mg/mL (blue dots), (2) Vuse products had 18.9 – 38.8 mg/mL (green dots), and (3) JUUL had 59.2 – 66.7 mg/mL (red dots) (Figure 2a). The average concentration of nicotine was significantly higher in JUUL than in the other two groups (Figure 2b).

The total concentration of flavor chemicals was compared in 182 EC products (169 refill fluids, five Vuse cartomizer fluids and eight JUUL pod fluids) (Figure 2c). Concentrations in refill fluids were highly variable and ranged from 0.1 to 362.3 mg/mL. In contrast, concentrations in cartomizers and pods were similar and generally lower than in refill fluids. Vuse cartomizers had total flavor chemical concentrations ranging from 0.7 to 15.7 mg/mL, while JUUL pods ranged from 0.2 to 15.6 mg/mL.

Concentrations of Total Flavor Chemicals and Nicotine in JUUL Fluids and Aerosols

The total concentration of flavor chemicals in unvaped pod fluids, vaped fluids, and aerosols ranged between 0.1 – 16.7, 0.1 – 14.7, and 0.1 – 9.1 mg/mL, respectively (Figure 3a). Transfer from the fluid to the aerosol was variable, but in general was over 50% efficient. Only fluids from “Cool Mint” and “Classic Menthol” pods had total flavor chemical concentrations >10 mg/mL. “Crème Brulee”, “Mango”, “Cool Cucumber” and “Fruit

Medley" had total flavor chemical concentrations between 0.3 and 8.1 mg/mL, while the two tobacco flavors had negligible concentrations.

In JUUL products, nicotine concentrations averaged 60.9 mg/mL, 63.5 mg/mL and 41.2 mg/mL in unvaped, vaped, and aerosol samples, respectively (Figure 3b). Transfer efficiently for nicotine to the aerosol was between 56 – 75%.

Individual Flavor Chemicals and Transfer Efficiency

In comparison with other EC refill fluids that we have analyzed,[17] JUUL uses a small number of different flavor chemicals in their pods (Figure 4). Five of eight products had 1-2 flavor chemicals (menthol, vanillin or ethyl maltol) >1 mg/mL, and these were generally present in about equal concentrations in both unvaped and vaped fluids. Menthol was the major flavor chemical in four of the flavor pods ("Cool Mint", "Classic Menthol", "Cool Cucumber" and "Fruit Medley"), although its concentration varied with "Classic Menthol" having the highest concentration (14.9 mg/mL) and "Fruit Medley" the lowest (0.7 mg/mL). Vanillin and ethyl maltol were the major flavor chemicals in "Crème Brulee" and "Mango", respectively. "Classic Tobacco" had low levels of benzyl alcohol, while flavor chemicals were negligible in "Virginia Tobacco". These major flavor chemicals in each product generally transferred well to the aerosol with transfer efficiencies ranging from 39 to 62%.

Cytotoxicity of JUUL Pod Fluids and Aerosols

Cytotoxicities of both fluids and aerosols were evaluated with BEAS-2B cells using the MTT, NRU, and LDH assays. Products were considered cytotoxic if they produced an effect that was 30 % less than the untreated control (referred to as the IC_{70}) in accordance with ISO protocol # 10993-5:2009(E) international standard.[24] JUUL pod fluids were cytotoxic in both the MTT and NRU assays for all pod flavors (Figures 5a-b and 5d-e). Generally, IC_{70}s and IC_{50}s were reached at fluid concentrations between 1-10% (Table 1), and all products produced a maximum effect at 10% (Figures 5a-b and 5d-e). Cytotoxicity was also observed in the MTT and NRU assays when cells were tested with JUUL pod aerosols (Figures 5c and 5f). The highest aerosol concentration of 6TPE, when converted to percentage concentration of pod fluid, ranged from 1.3% to 3.0% (Figure 5c and f). In the MTT assay, IC_{70}s for aerosols varied with different pod flavors and generally were reached between concentrations of 0.31% to a 1.8% (Table 1), which was considerably lower than observed with the fluids. In the NRU assay, IC_{70}s were reached for five of the eight JUUL flavor pods (Table 2 and Figure 5 d and e). Aerosols from three flavors pods ("Classic Menthol", "Classic Tobacco", and "Virginia Tobacco") did not produce a significant effect. As seen in the MTT assay, aerosols were more toxic than the fluids in the NRU assay (Figures 5 a-f and Table 1 and 2).

With JUUL pod fluids and aerosols, little effect was seen in the LDH assay (Figures 5g-i), indicating that in general, fluids and aerosol treatments did not cause rupture of BEAS-2B plasma membranes.

Correlation between Nicotine Concentration, Flavor Chemical Concentration, and Toxicity

Since some flavor chemicals can cause cytotoxicity, especially at concentration >1 mg/mL,[17] linear regression analyses were performed to parse out the relative contribution of nicotine, total flavor chemicals, and individual flavor chemicals to the cytotoxicity observed with JUUL pod fluids and aerosols (Figures 6 and 7). For unvaped JUUL fluids, there was a high correlation between cytotoxicity (percent of untreated control) and the concentration of nicotine plus total flavor chemicals in both the MTT ($R^2 = 0.871$; $p<0.0001$) and NRU ($R^2 = 0.861$; $p<0.0001$) assays (Figure 6a). When nicotine and flavor chemical concentrations were analyzed separately (Figures 6b and 6c), the correlation coefficient for nicotine concentrations alone versus cytotoxicity ($R^2 = 0.879$ for MTT) was almost equivalent to that of nicotine and flavor chemicals concentrations combined ($R^2 = 0.871$ for MTT). In contrast, total flavor chemical concentration alone (without nicotine) was only moderately/weakly correlated to cytotoxicity ($R^2 = 0.379$ for MTT and 0.383 for NRU), nevertheless the correlation was significant ($p<0.0001$ for both MTT and NRU). The correlation between cytotoxicity and the concentrations of individual flavor chemicals found at concentrations >1 mg/mL was moderate for ethyl maltol and weak for menthol and vanillin (Figures 6d-f); nevertheless, all correlations were statistically significant (Figures 6d-f). A similar pattern of linear correlation and statistical significance was observed with vaped fluids in both the MTT and neutral assays (see Supporting Information, S2).

For JUUL aerosols, correlations between cytotoxicity and total chemicals (nicotine plus flavor chemicals) (Figure 7a), nicotine alone (Figure 7b), and ethyl maltol (Figure 7d) were strong ($R^2 > 0.75$, except for two NRU R^2s which were > 0.45) and significant (all $p<0.0001$) (Figures 7a-b, and 7d). Flavor chemicals alone (Figure 7c) and menthol (Figure 7e) were weakly correlated to cytotoxicity (R^2 ranged from 0.099 to 0.361), while R^2 for vanillin was weak and not significant ($p>0.05$) (Figure 7f).

DISCUSSION

While the health complications associated with EC use are appearing in case reports and the infodemiological literature,[25,26] to date no health reports have been made for consumers of JUUL products. Nicotine concentrations were higher in JUUL pod fluids than in any of the 174 EC refill and cartomizer fluids that we have examined previously [27,29] (Figure 2a). Concentration-response curves for the JUUL fluids were remarkably similar among the flavor pods and reached a maximum effect in the MTT and NRU assays at a 10% concentration for all samples. Aerosols were more cytotoxic than fluids and reached a maximum response at concentrations between 0.2 and 1.8%%. Cytotoxicity of aerosols was strongly correlated with total chemical concentrations, nicotine concentration, and ethyl maltol concentration, which was 1.81 mg/mL in one JUUL product. While we have previously reported that the concentrations of some flavor chemicals in some EC products are high enough to be cytotoxic,[19,20] JUUL pods are the only EC product that we have studied in which cytotoxicity can be attributed to the concentrations of both nicotine and a flavor chemical (ethyl maltol).

Only 1-2 flavor chemicals were present at concentrations >1 mg/mL in each JUUL product, similar to some refill fluids from other manufacturers that contained 1-4 flavor chemicals/product at 1 mg/mL or greater.[17] In general, the concentrations of individual flavor chemicals in JUUL products were relatively low compared to other cartomizer style EC and refill fluids.[16,17] Two exceptions were JUUL "Cool Mint" and "Classic Menthol", which both had menthol concentrations >10 mg/mL. Others have reported that the minty flavors may be the most popular of the JUUL products,[3] which could be due to a stronger flavor imparted by their high concentrations of menthol or the effects of menthol on nicotine metabolism.[28] In contrast to the minty products, the two JUUL tobacco-flavored pods had very low concentrations of flavor chemicals. It is possible that the high concentration of nicotine and acid in JUUL pods imparts some flavor features to the aerosol making the use of additional chemicals unnecessary in the "Classic Tobacco" and "Virginia Tobacco" pods or that the predominant aroma molecules for those flavor profiles were not included in the GC/MS target compounds. The low levels of flavor chemicals in most JUUL pods may reduce their odor, which would facilitate "stealth" use, a desirable feature among middle and high school students who vape in class or in rest rooms.[12]

The flavor chemicals that were present in JUUL pods at very low concentrations are likely co-constituents of the major flavor chemicals (i.e., menthol, vanillin and ethyl maltol) or may in some cases be added to impart subtle flavor accents. With respect to manufacturing practices, duplicate pods and packages were identical and contained similar flavor chemicals. However, during aerosol production, pods did not perform uniformly on the smoking machine, some pods produced low density aerosols, and some pods did not work at all. This inconsistency in puff production may also account for the relatively low transfer efficiencies seen with some pods.

Nicotine concentrations in the JUUL products were significantly higher than in any other EC cartomizers and refill fluids our laboratory has evaluated (total 174).[27,29] The average nicotine concentration in JUUL pods in our study (60.9 mg/mL) agrees well with our earlier report 61.6 mg/mL.[14] Other laboratories have reported similar values (56.2 mg/mL,[30] 75.6 mg/mL,[31] and 69 mg/ml [32]). The variation between labs may be due to differences in the analytical technologies used. A single JUUL pod contained more nicotine (56 - 66 mg) than a pack of cigarettes (2 mg/stick * 20 sticks = 40 mg/pack). The high concentrations of nicotine in JUUL EC is coupled to a high concentration of benzoic acid, which protonates nicotine making it less harsh when inhaled by users.[14,15] The combination of the high nicotine concentration and its protonation by benzoic acid likely facilitates JUUL use and subsequent addiction, especially of adolescent or naïve consumers of JUUL products. Concern about the potential for addiction to JUUL products is compounded by the report that only 37% of the past 30-day consumers were aware that JUUL products always contain nicotine.[33]

In contrast to nicotine, total flavor chemical concentrations were not unusually high in JUUL pods and were found over a relatively narrow range of concentrations (15.7

mg/mL being the highest). Currently marketed refill fluids, in contrast, have a much wider range of total flavor chemical concentrations with the highest we have detected being 362.3 mg/mL. Moreover, the high concentrations of flavor chemicals are cytotoxic when tested in vitro.[17] In this study, only one flavor chemical (ethyl maltol) was correlated with cytotoxicity, as discussed below.

JUUL fluids and aerosols produced no significant effects in the LDH assay**.** Since this assay measures the release of LDH, a cytoplasmic enzyme, it is probable that treatment did not lyse cells or cause significant damage to the plasma membrane. In contrast, all pod fluids and most aerosols produced a cytotoxic response at a 10% concentration in the MTT and NRU assays. Our linear regression analysis showed that the nicotine and ethyl maltol concentrations in JUUL aerosols were high enough to account for most of the cytotoxicity observed with the MTT and NRU. Since nicotine concentrations were similar in all JUUL products and since cytotoxicity can be attributed mainly to nicotine, the concentration-response curves for JUUL fluids were all similar. In some prior work with other EC products that had lower nicotine concentrations, cytotoxicity was correlated with the flavor chemical concentration, not nicotine.[17,18,34] Ethyl maltol concentration, which was also strongly correlated with aerosol cytotoxicity, was highest in the Mango pods (1.57 mg/mL), which were more potent than “Crème Brulee” and “Virginia Tobacco” (Figures 5c, and 5f), which both had lower concentrations of ethyl maltol (0.65 mg/mL and 0.03mg/mL, respectively) (Figure 1).

In the NRU assay, the “Classic Menthol” and “Classic Tobacco” aerosol did not inhibit uptake relative to the control. This could be because the concentrations of the aerosol did not reach 10%, as they did with fluids. In addition, these were the only flavors that contained caffeine (Figure 1), which is a stimulant. The caffeine concentrations in

"Classic Menthol" and "Classic Tobacco" aerosols were 0.037mM and 0.090 mM, respectively. These concentrations are similar to those reported to provide protection to cells in other models[35] and may explain our results with "Classic Menthol" and "Classic Tobacco" aerosol.

In summary, the current popularity of JUUL products has raised two major concerns for the FDA. The first is the likelihood that JUUL use, which is widespread among middle school and high school students, will addict a new generation of adolescents to nicotine. The second is that these adolescents will eventually migrate to tobacco products that may be more dangerous, such as conventional cigarettes. Our data clearly identify a third concern related to the high nicotine concentration in JUUL products, i.e., the potential for high levels of nicotine, as well as flavor chemicals such as ethyl maltol, to damage or even kill cells at the concentrations used in JUUL pods. Our exposures were acute and produced a maximal cytotoxic response that was strongly correlated with nicotine and ethyl maltol concentrations. It will be important in future work to determine if JUUL products, and other products containing nicotine salts, have adverse effects on consumers and if such effects lead to health problems with chronic use. In the meantime, the FDA could limit nicotine and flavor chemical concentrations in EC products.

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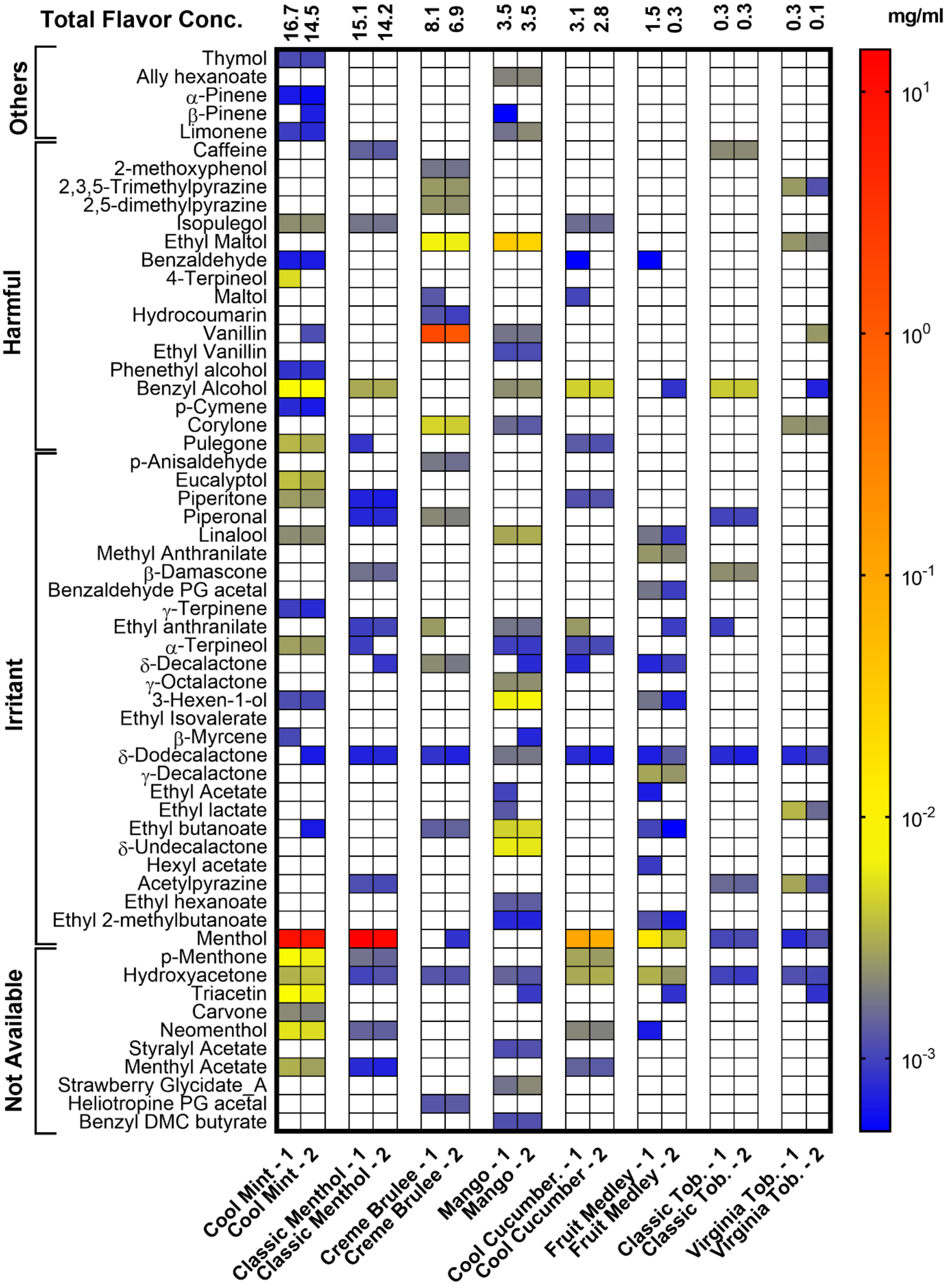
Total Flavor Conc.
16.7
14.5
15.1
14.2
8.1
6.9
3.5
3.5
3.1
2.8
1.5
0.3
0.3
0.3
0.3
0.1
mg/ml
Others
Harmful
Irritant
Not Available
Thymol
Ally hexanoate
α-Pinene
β-Pinene
Limonene
Caffeine
2-methoxyphenol
2,3,5-Trimethylpyrazine
2,5-dimethylpyrazine
Isopulegol
Ethyl Maltol
Benzaldehyde
4-Terpineol
Maltol
Hydrocoumarin
Vanillin
Ethyl Vanillin
Phenethyl alcohol
Benzyl Alcohol
p-Cymene
Corylone
Pulegone
p-Anisaldehyde
Eucalyptol
Piperitone
Piperonal
Linalool
Methyl Anthranilate
β-Damascone
Benzaldehyde PG acetal
γ-Terpinene
Ethyl anthranilate
α-Terpineol
δ-Decalactone
γ-Octalactone
3-Hexen-1-ol
Ethyl Isovalerate
β-Myrcene
δ-Dodecalactone
γ-Decalactone
Ethyl Acetate
Ethyl lactate
Ethyl butanoate
δ-Undecalactone
Hexyl acetate
Acetylpyrazine
Ethyl hexanoate
Ethyl 2-methylbutanoate
Menthol
p-Menthone
Hydroxyacetone
Triacetin
Carvone
Neomenthol
Styralyl Acetate
Menthyl Acetate
Strawberry Glycidate_A
Heliotropine PG acetal
Benzyl DMC butyrate
Cool Mint - 1
Cool Mint - 2
Classic Menthol - 1
Classic Menthol - 2
Creme Brulee - 1
Creme Brulee - 2
Mango - 1
Mango - 2
Cool Cucumber - 1
Cool Cucumber - 2
Fruit Medley - 1
Fruit Medley - 2
Classic Tob. - 1
Classic Tob. - 2
Virginia Tob. - 1
Virginia Tob. - 2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}

Figure 3.2: Heat map of flavor chemicals in eight duplicate JUUL pod fluids. Chemicals are ordered on the *y*-axis according to their toxicity (Others, Harmful, Irritant) based on LC_{50} data from rat oral exposures, and within each class, they are ranked from most to least toxic. The “Others” category on the *y*-axis represents chemicals that are corrosive, toxic, harmful, irritants as well as dangerous to the environment. JUUL products (*x*-axis) are ordered according to the total weight (mg/mL) of the flavor chemicals in each product with the highest concentration at the left. The total flavor chemical concentration (mg/mL) is indicated at the top of each column. The color gradient on the right shows the concentrations of the flavor chemicals in the heat map. Three chemicals (vanillin, ethyl maltol, and menthol) in the orange to red color gradient were ≥1 mg/mL in at least one product. JUUL pod code: Classic Tob. = “Classic Tobacco”; Virginia Tob. = “Virginia Tobacco”. The numbers 1 and 2 with the JUUL pod codes designate the first and second pod tested.

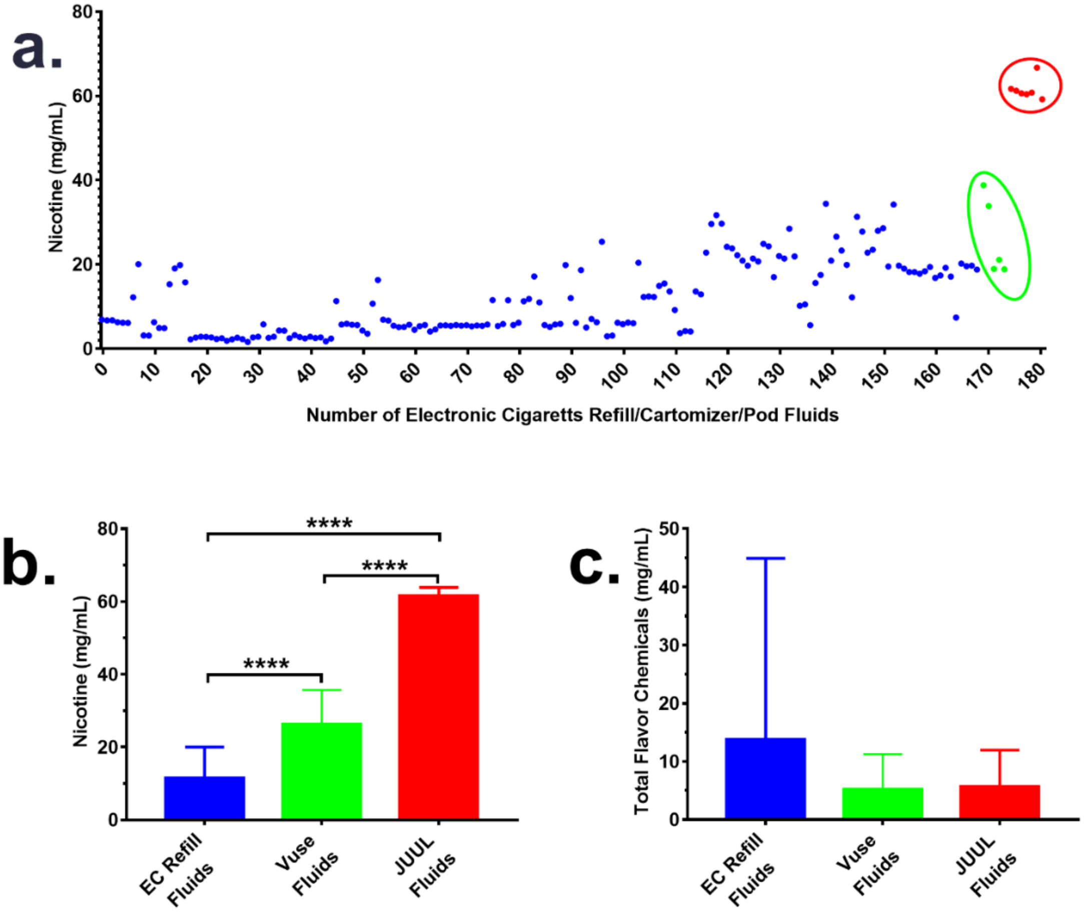

Figure 3.3: Nicotine and total flavor chemical concentrations in EC products. (a) Nicotine concentrations in 182 EC products. Red dots represent eight JUUL products; green dots represent 5 Vuse cartomizer fluids, and blue dots represent 169 refill fluids from 34 brands. The *y*-axis shows nicotine concentrations in each EC product listed on the *x*-axis. (b) The mean concentrations of nicotine in 169 EC refill fluids from 34 brands (blue bar), five Vuse cartomizers (green bar), and eight JUUL pods (red bar). The mean concentrations of nicotine were significantly different in each group. **** = $p < 0.0001$. (c) The mean concentrations of total flavor chemicals in 169 EC refill fluids from 34 brands (blue bar), five Vuse cartomizers (green bar), and eight pod JUUL pods (red bar).

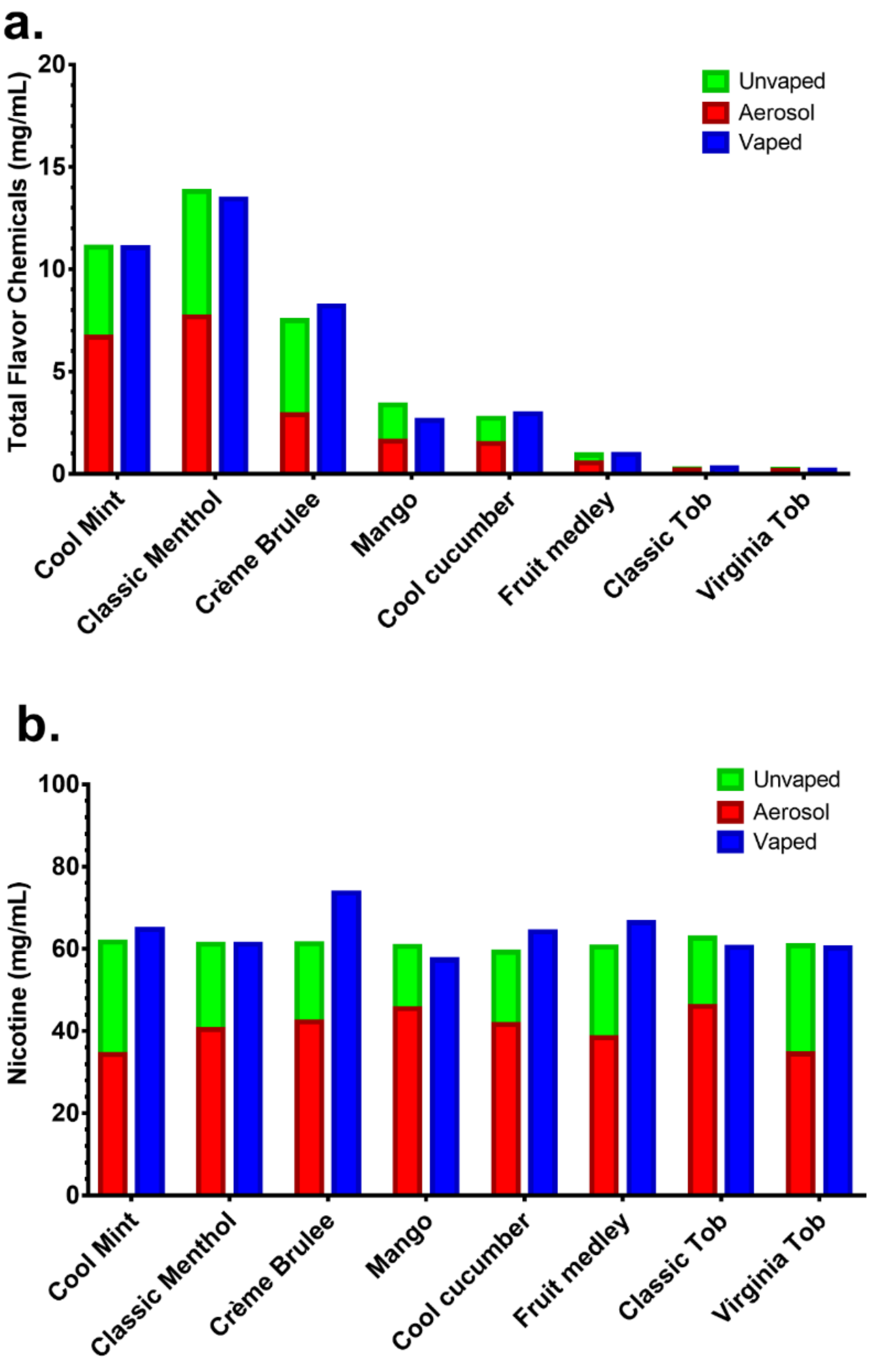

Figure 3.4: Total flavor chemical and nicotine concentrations in JUUL pod fluids and aerosols. (a) The total flavor chemical concentrations in unvaped pod fluids, vaped pod fluids, and aerosols. (b) Concentrations of nicotine in unvaped pod fluids, vaped pod fluids, and aerosols. The total flavor chemical concentrations and nicotine concentrations were very similar in the unvaped and vaped pod fluids. Each bar is mean concentration of two independent experiments.

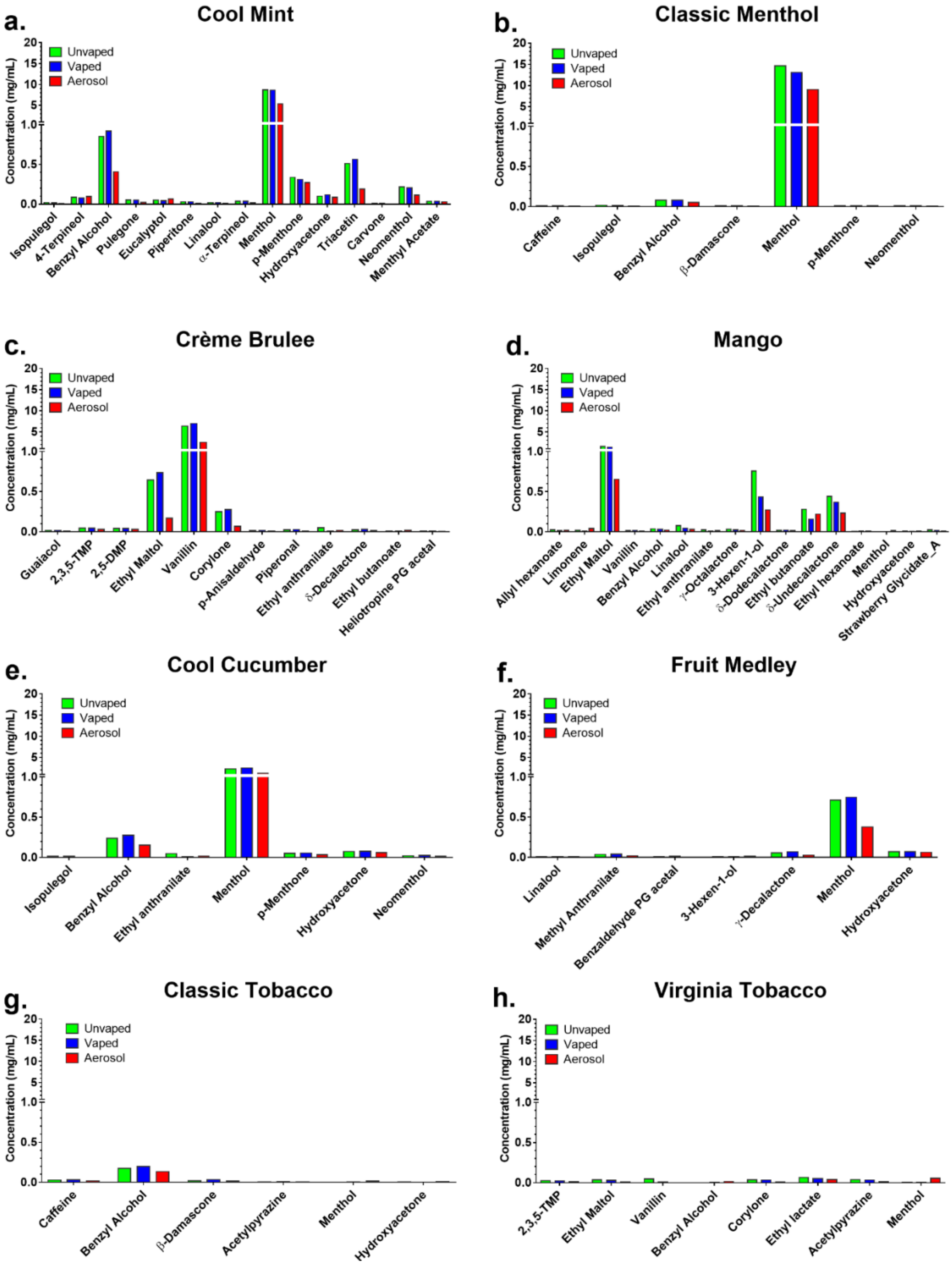
a.
Cool Mint
Concentration (mg/mL)
Unvaped
Vaped
Aerosol
Isopulegol
4-Terpineol
Benzyl Alcohol
Pulegone
Eucalyptol
Piperitone
Linalool
α-Terpineol
Menthol
p-Menthone
Hydroxyacetone
Triacetin
Carvone
Neomenthol
Menthyl Acetate
b.
Classic Menthol
Caffeine
Isopulegol
Benzyl Alcohol
β-Damascone
Menthol
p-Menthone
Neomenthol
c.
Crème Brulee
Guaiacol
2,3,5-TMP
2,5-DMP
Ethyl Maltol
Vanillin
Corylone
p-Anisaldehyde
Piperonal
Ethyl anthranilate
δ-Decalactone
Ethyl butanoate
Heliotropine PG acetal
d.
Mango
Allyl hexanoate
Limonene
Ethyl Maltol
Vanillin
Benzyl Alcohol
Linalool
Ethyl anthranilate
γ-Octalactone
3-Hexen-1-ol
δ-Dodecalactone
Ethyl butanoate
δ-Undecalactone
Ethyl hexanoate
Menthol
Hydroxyacetone
Strawberry Glycidate_A
e.
Cool Cucumber
Isopulegol
Benzyl Alcohol
Ethyl anthranilate
Menthol
p-Menthone
Hydroxyacetone
Neomenthol
f.
Fruit Medley
Linalool
Methyl Anthranilate
Benzaldehyde PG acetal
3-Hexen-1-ol
γ-Decalactone
Menthol
Hydroxyacetone
g.
Classic Tobacco
Caffeine
Benzyl Alcohol
β-Damascone
Acetylpyrazine
Menthol
Hydroxyacetone
h.
Virginia Tobacco
2,3,5-TMP
Ethyl Maltol
Vanillin
Benzyl Alcohol
Corylone
Ethyl lactate
Acetylpyrazine
Menthol
20
15
10
5
1.0
0.5
0.0

Figure 3.5. Concentrations of individual flavor chemicals in JUUL pod fluids and aerosols. (a) “Cool Mint”, (b) “Classic Menthol”, (c) “Crème Brulee”, (d) “Mango”, (e) “Cool Cucumber” (f) “Fruit Medley”, (g) “Classic Tobacco”, and (h) “Virginia Tobacco”. Most fluids contained 1-2 flavor chemicals >1 mg/mL, except the tobacco flavored products, which had very low concentrations of flavor chemicals. Flavor chemicals >1mg/mL transferred from unvaped pod fluids into the aerosols with 39 to 62% efficiency. Each bar is the mean concentration of two independent experiments.

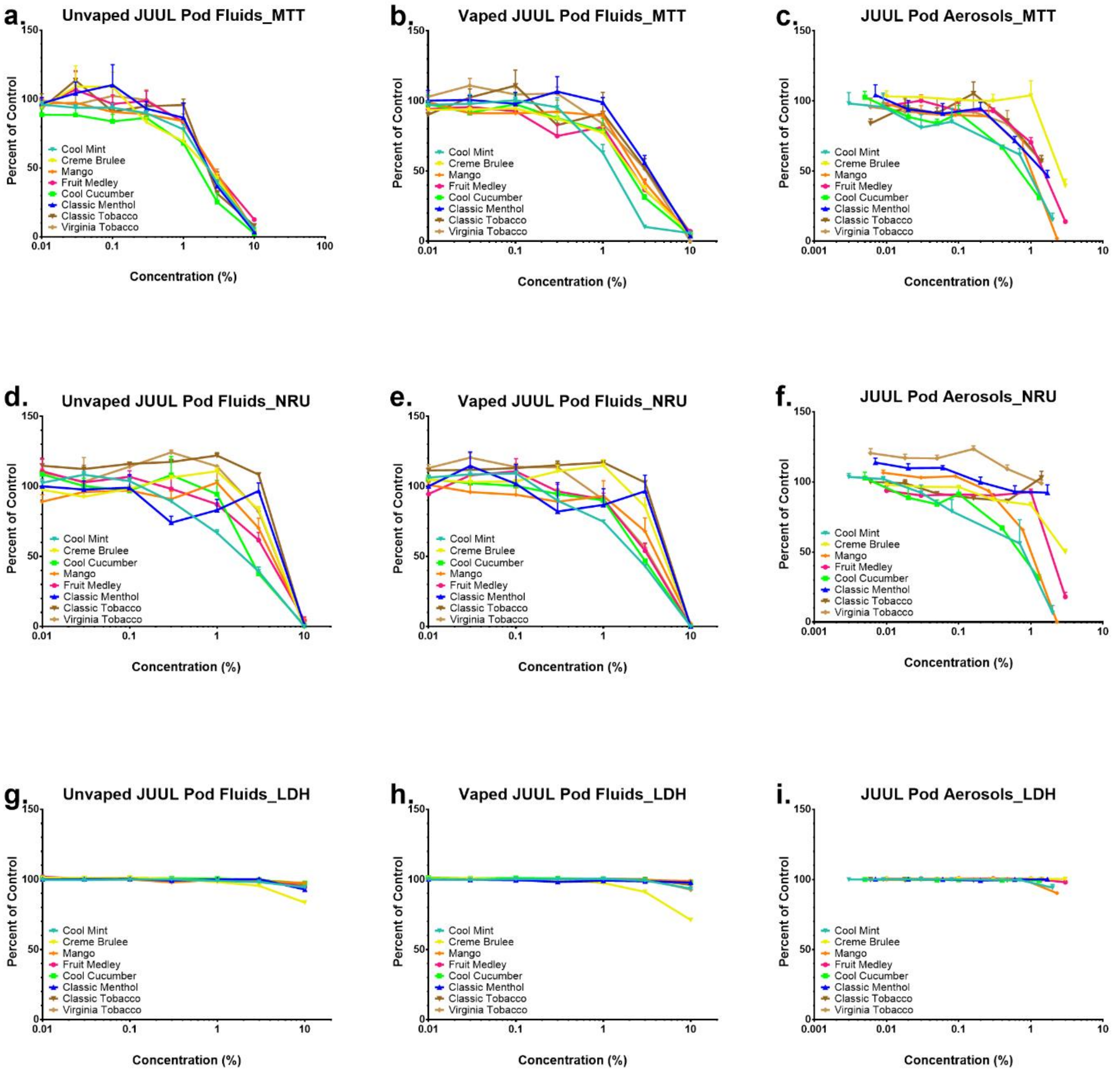

Figure 3.6. Concentration-response curves for BEAS-2B cells treated with JUUL pod fluids and aerosols. (a-c) MTT assay, (d-f) NRU assay, and (g-i) LDH assay for all eight pod variants. The *y*-axis shows the response of cells in each assay as a percentage of the untreated control. Each point is the mean ± standard error of the mean for three independent experiments.

Relationships between Chemicals and Cytotoxicity of Unvaped Pod Fluids

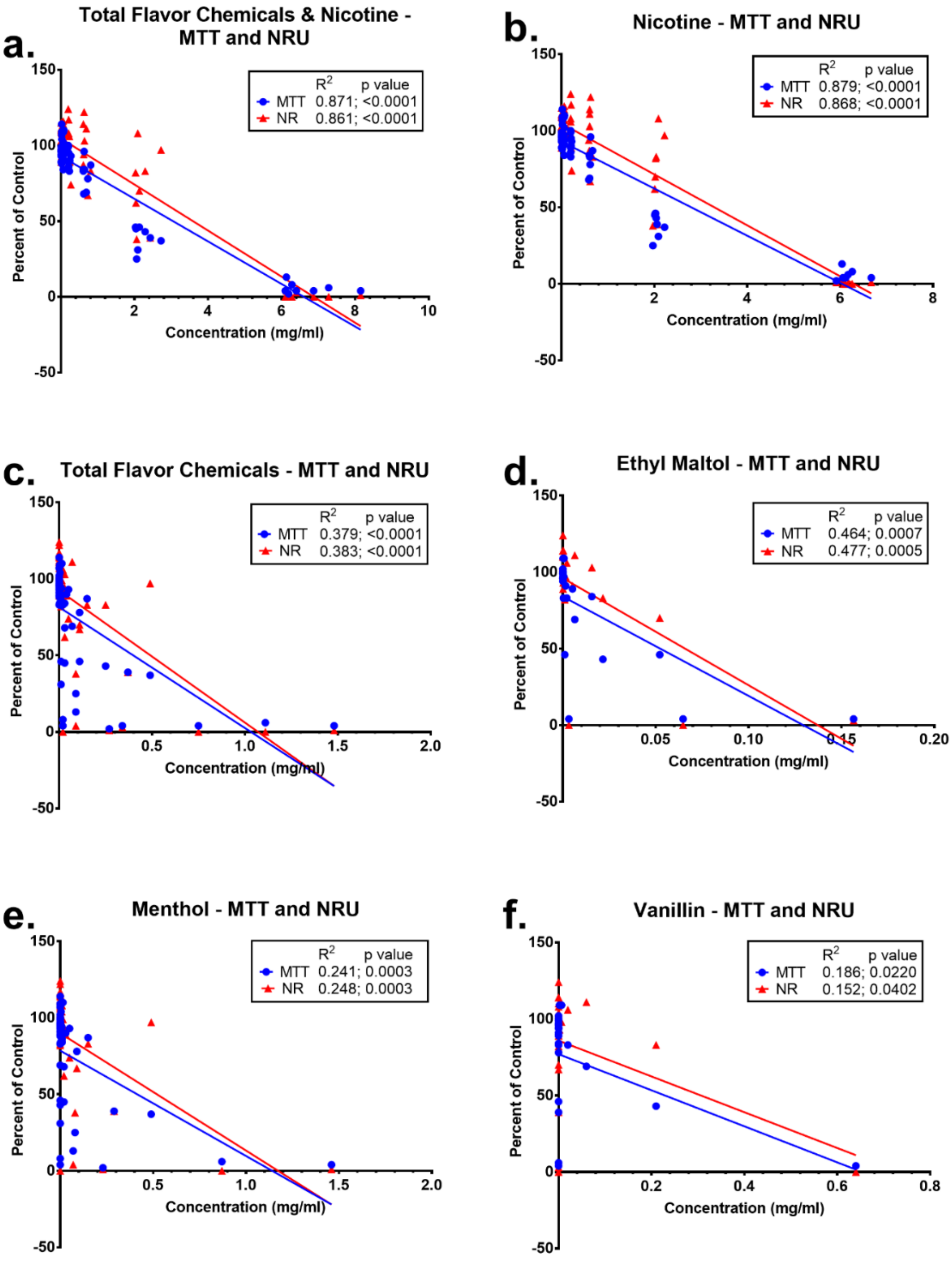

Figure 3.7. Relationship between cytotoxicity of unvaped pod fluids and concentrations of nicotine and the flavor chemicals. Linear regression analysis for cytotoxicity (*y*-axis, expressed as a percentage of the untreated control) in the MTT and NRU assays versus the concentrations of: (a) total flavor chemicals and nicotine, (b) nicotine only, (c) total flavor chemicals only, (d) ethyl maltol, (e) menthol, and (f) vanillin. Blue dots and red triangles represent concentrations tested in the MTT and NRU assay, respectively. Cytotoxicity was strongly correlated with total concentration of chemicals (flavor chemicals and nicotine) and with nicotine concentration only and weakly to moderately correlated with the concentrations of total flavor chemicals, ethyl maltol, menthol and vanillin. All correlations were significant ($p<0.05$).

Relationships between Chemicals and Cytotoxicity of Aerosols

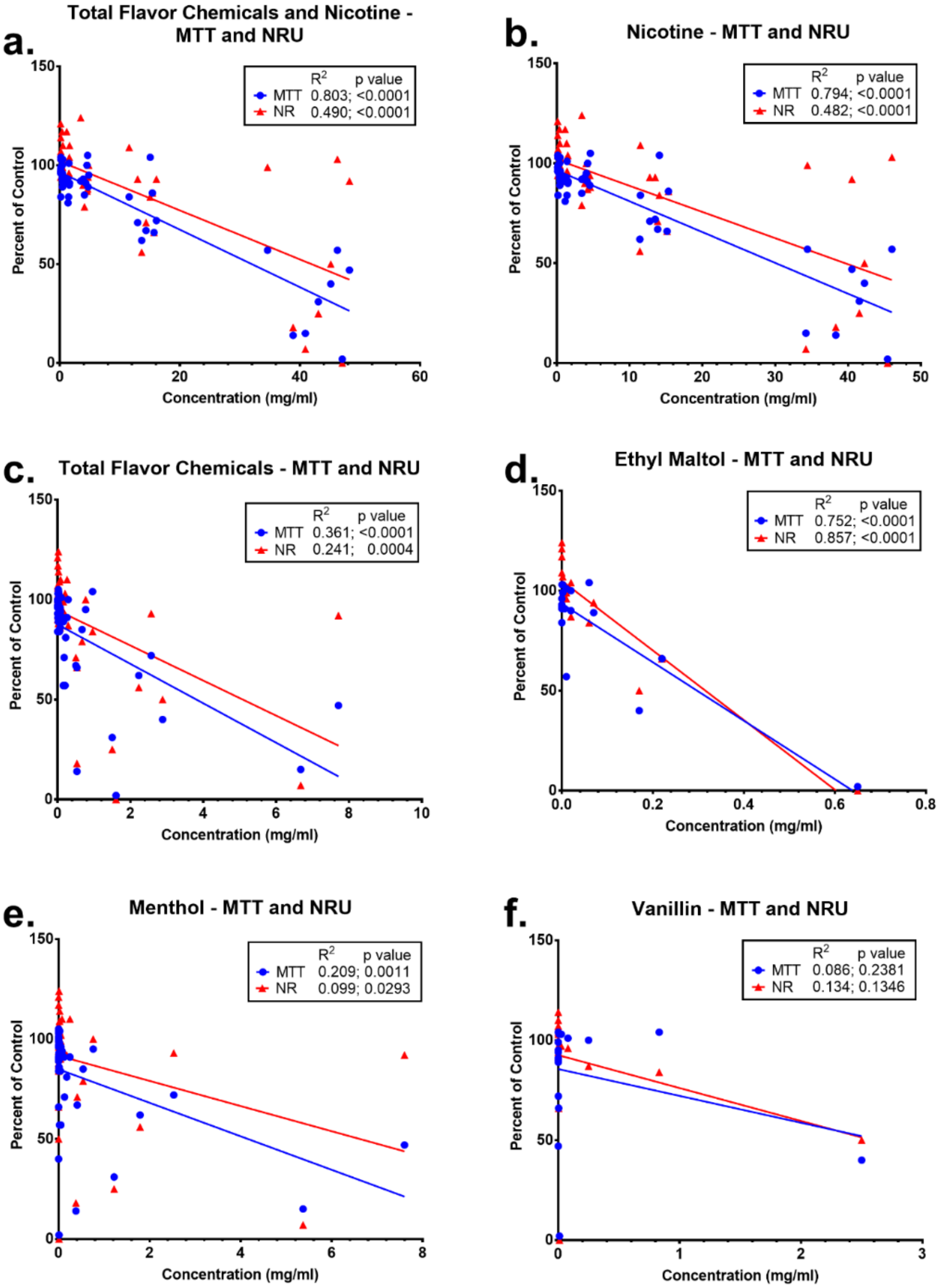

Figure 3.8. Relationship between cytotoxicity of pod aerosols and the concentrations of nicotine and the flavor chemicals. Linear regression analysis for cytotoxicity in the MTT and NRU assays versus the concentrations of: (a) total flavor chemicals and nicotine, (b) nicotine only, (c) total flavor chemicals only, (d) ethyl maltol, (e) menthol, and (f) vanillin. Blue dots and red triangles represent the concentrations tested in the MTT and NRU assay. Cytotoxicity (percent of control) was strongly correlated with the total concentration of chemicals (flavor chemicals and nicotine), nicotine concentration only, and ethyl maltol concentration. The correlations between cytotoxicity and the concentrations of total flavor chemicals and menthol were moderate and weak, respectively. The correlation between cytotoxicity and vanillin concentration was not significant.

Table 3.1. IC_{70} and IC_{50} (%) of JUUL pod fluids and aerosols in the MTT Assay

	Unvaped Fluids		Vaped Fluids		Aerosols		
JUUL Pod Flavors[a]	**IC_{70}**	**IC_{50}**	**IC_{70}**	**IC_{50}**	**IC_{70}**	**IC_{50}**	**Highest Conc. (%)**
"Cool Mint"	0.92	2.17	0.79	1.25	0.31	0.64	2
"Cool Cucumber"	1.10	1.43	1.23	1.93	0.33	0.68	1.3
"Mango"	1.52	2.57	1.61	2.58	0.65	0.93	2.3
"Classic Menthol"	1.48	2.33	2.14	3.24	0.67	1.51	1.7
"Virginia Tobacco"	1.54	2.61	1.66	2.88	0.85	2.17	1.4
"Classic Tobacco"	1.60	2.37	1.87	3.07	0.89	1.67	1.4
"Fruit Medley"	1.52	2.70	1.35	2.00	1.01	1.42	3
"Crème Brulee"	1.03	1.97	1.27	2.06	1.80	2.90	3

a = order of pod flavors ranked according to IC_{70} of aerosols

Table 3.2. IC_{70} and IC_{50} (%) of JUUL pod fluids and aerosols in the NRU Assay

JUUL Pod Flavors[a]	Unvaped Fluids		Vaped Fluids		Aerosols		
	IC_{70}	IC_{50}	IC_{70}	IC_{50}	IC_{70}	IC_{50}	Highest Conc. (%)
"Cool Mint"	1.32	1.81	1.21	2.18	0.20	0.54	2
"Cool Cucumber"	1.65	2.55	1.70	2.77	0.42	0.68	1.3
"Mango"	3.08	3.75	1.61	3.75	0.65	0.89	2.3
"Fruit Medley"	2.29	3.50	1.35	3.11	1.39	1.98	3
"Crème Brulee"	3.68	4.88	3.75	5.07	1.52	3.23	3
"Classic Menthol"	4.28	5.09	2.14	4.30	> 1.7	> 1.7	1.7
"Classic Tobacco"	4.82	7.94	1.87	7.84	> 1.4	n/a	1.4
"Virginia Tobacco"	3.69	4.91	1.66	3.21	n/a	n/a	1.4

a = order of pod flavors ranked according to IC_{70} of aerosols

Chapter 4

High concentrations of flavor chemicals are present in electronic cigarette refill fluids

Omaiye et al., 2019

Scientific Reports 2019; 21;9(1):2468.

ABSTRACT

We characterized the flavor chemicals in a broad sample of commercially available electronic cigarette (EC) refill fluids that were purchased in four different countries. Flavor chemicals in 277 refill fluids were identified and quantified by gas chromatography-mass spectrometry, and two commonly used flavor chemicals were tested for cytotoxicity with the MTT assay using human lung fibroblasts and epithelial cells. About 85% of the refill fluids had total flavor concentrations > 1 mg/ml, and 37% were > 10 mg/ml (1% by weight). Of the 155 flavor chemicals identified in the 277 refill fluids, 50 were present at ≥ 1 mg/mL in at least one sample and 11 were > 10 mg/ml in 54 of the refill fluids. Sixty-one% (170 out of 277) of the samples contained nicotine, and of these, 56% had a total flavor chemical/nicotine ratio >2. Four chemicals were present in 50% (menthol, triacetin, cinnamaldehyde) to 80% (ethyl maltol) of the samples. Some products had concentrations of menthol (“Menthol Arctic”) and ethyl maltol (“No. 64”) that were 30 times (menthol) and 100 times (ethyl maltol) their cytotoxic concentration. One refill fluid contained cinnamaldehyde at ~34% (343 mg/ml), more than 100,000 times its cytotoxic level. High concentrations of some flavor chemicals in EC refill fluids are potentially harmful to users, and continued absence of any regulations regarding flavor chemicals in EC fluids will likely be detrimental to human health.

INTRODUCTION

Electronic cigarette (EC) consumers inhale aerosols that usually contain nicotine, propylene glycol and/or glycerol, and blends of flavor chemicals that directly contact the lining of the mouth and respiratory system [1,2]. Thousands of refill fluids, which are used at full strength, are commercially available for refilling cartomizer and tank-style EC products[3]. Instances of adverse health effects, some of which involve the respiratory system, such as bronchiolitis obliterans and acute eosinophilic pneumonia, have been attributed to EC use [4,5]. Cultured cells and animal models exposed to EC fluids and aerosols show increased oxidative stress, inflammatory responses, and impaired pulmonary defenses that may contribute to adverse health effects [6-9].

The constituents of EC fluids and aerosols that cause adverse effects in cells and animals are beginning to be identified. Cytotoxicity of ECs has been linked to the presence of multiple flavor chemicals, including cinnamaldehyde [10-13]. As recently pointed out by the Flavor and Extracts Manufacturers' Association (FEMA), while many of the flavor chemicals used in EC refill fluids are on the FEMA GRAS (generally regarded as safe) list, the GRAS designation presumes ingestion and does not apply to inhalation [14, 15]. In addition, government agencies, such as the National Institute of Occupational Safety Health (NIOSH), have published inhalation exposure guidelines to protect workers who manufacture flavor chemicals from adverse health effects [16]. Clearly more data are needed to inform regulatory agencies and protect public health.

The purpose of this study was to identify and quantify the flavor chemicals in a broad spectrum (277) of EC refill fluids that were purchased in four countries to gain a better understanding of the range of chemicals and concentrations used in these products. Each flavor chemical was also classified based on organoleptic characteristics and their frequency of use in refill fluids. Two commonly used flavor chemicals were further evaluated for cytotoxicity using an *in vitro* model based on human respiratory cells.

MATERIALS AND METHODS

Sampling

A worldwide sample of 277 bottles of EC refill fluids was purchased from product lines offered by manufacturers in the USA, England, China, and Nigeria, and seven fluids were compounded for us by a vape shop in Riverside, CA to match popular flavor names not offered by the shop. The latter group of seven products was included to begin an examination of what may result from fluid "cloning" services offered by some EC vendors. Flavor chemicals were analyzed by gas chromatography-mass spectrometry (GC/MS), and two chemicals found at high concentrations were tested for cytotoxicity using the MTT assay with BEAS-2B bronchial epithelial cells and human pulmonary fibroblasts (hPF), as described previously.[10-12]

Identification and Quantification of Flavor Chemicals in EC Refill Fluids

For each refill fluid, 50 µl were dissolved in 0.95 ml of isopropyl alcohol (IPA) (Fisher Scientific, Fair Lawn, NJ). All diluted samples were shipped overnight on ice to Portland State University and analyzed using GC/MS on the day they were received. A 20 µl aliquot of internal standard solution (2000 ng/ µl of 1, 2, 3-trichlorobenzene dissolved in IPA) was added to each diluted sample before analysis. Using internal standard-based calibration procedures described elsewhere [52], analyses were performed with an Agilent 5975C GC/MS system (Santa Clara, CA). A Restek Rxi-624Sil MS column (Bellefonte, PA) was used (30 m long, 0.25 mm id, and 1.4 µm film thickness. A 1.0 µl aliquot of diluted sample was injected into the GC with a 10:1 split.

The injector temperature was 235 °C. The GC temperature program for analyses was: 40 °C hold for 2 min; 10 °C/min to 100 °C; then 12 °C/min to 280 °C and hold for 8 min at 280 °C, then 10 °C/min to 230 °C. The MS was operated in electron impact ionization mode at 70 eV in positive ion mode. The ion source temperature was 220 °C and the quadrapole temperature was 150 °C. The scan range was 34 to 400 amu. Each of the 178 target analytes was quantitated using authentic standard material and an internal standard (1, 2, 3-trichlorobenzene) normalized multipoint calibration.

Cell Culture

Human pulmonary fibroblasts (hPF) (ScienCell, Carlsbad, CA) were cultured in complete fibroblast medium supplemented with 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin prepared according to the manufacturer's protocol.[10,12] Prior to culturing, Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin CA) were coated with poly-L-lysine (PLL) prepared at a 20ul/10ml concentration and kept in the incubator to allow for even distribution and efficient coating of the culture flask. hPF cultures were maintained in 5% CO_2 at 37 °C and 95% relative humidity and the medium was replaced every 48 hours. At 80–90% confluency, cells were harvested using Dulbecco's Phosphate Buffered Saline (DPBS) for washing and incubated with 0.01% trypsin EDTA/DPBS (GIBCO, Invitrogen Carlsbad, CA) for 2 mins at 37^oC to allow detachment from the PLL coated surface of the culture flask. Detached cells were washed with culture medium and spun at 3,000g for 3 mins. The resulting supernatant was discarded, and cell pellets were resuspended in fresh culture medium for the MTT cytotoxicity experiments. Single cells were plated at a density of 3,000 cells/well (cells/$0.32cm^2$) based on a standard curve produced using a BioMate 3S

Spectrophotometer (Thermo Fisher Scientific, Chino, CA) and evenly dispersed in 96-well plates.

Human bronchial epithelial (BEAS-2B) cells were cultured in basal BEBM (Lonza, Walkersville, MD) supplemented with 2 ml bovine pituitary extract and 0.5 ml of insulin, hydrocortisone, retinoic acid, transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor (Lonza, Walkersville, MD). Nunc T-25 tissue culture flasks were coated overnight with BEBM, collagen, BSA and fibronectin prior to culturing and passaging cells. At 80% confluency, cells were harvested using DPBS for washing and incubated with 1.5 ml of 0.25% trypsin EDTA/DPBS and poly-vinyl-pyrrolidone for 3-4 mins at 37^{o}C to allow detachment. Cells were cultured in T-25 flasks at 75,000 cells/flask, and the medium was replaced the next day and then every other day. Plating for the MTT assay was done at 3,500 cells/well in pre-coated 96-well plates.

Cytotoxicity of Authentic Standards of Flavors Chemicals

Authentic standards of menthol and ethyl maltol (Sigma-Aldrich, St Louis, MO) were tested individually using the MTT assay with hPF and BEAS-2B cells. The MTT assay was performed over 3-fold dilutions with the highest concentration being 10% of the concentration found in the refill fluids. Concentrations above 10% were not used as they produced a vapor effect [9] that shifted the dose response curve to the left. Serial dilutions of authentic standard solutions in culture medium were arranged in 96-well plates with two negative controls next to the highest dose to check for a vapor effect [9]. Cells were allowed to attach for 24 hours, then treated for 48 hours after which 20 µl of MTT (Sigma-Aldrich, St Louis, MO) dissolved in 5 mg/ml of DPBS (Fisher Scientific, Chino, CA) were added to each well and incubated for 2 hrs at 37^{o}C. Solutions were removed, and 100 µl of dimethyl sulfoxide (DMSO) (Fisher, Chino, CA) were added to each well and gently mixed on a shaker. The assay was performed in triplicate, and the absorbance of control and treated wells was read against a DMSO blank at 570 nm using an Epoch micro-plate reader (Biotek, Winooski, VT). Each chemical was tested in three independent experiments.

Data Analysis

For the GC/MS results, the sample-mean values were analyzed using Prism software (GraphPad, San Diego). MTT data were normalized by setting treatment wells as percentages of the negative control (100%). Prism software (GraphPad, San Diego) was used to compute IC_{50}s using the log inhibitor vs. normalized response-variable slope with the top and bottom constraints set to < 100% and > 0%, respectively. Graphs were plotted using GraphPad Software. When significance was found using a one-way analysis of variance, each concentration was compared to the control using Dunnett's post hoc test.

RESULTS

Identification and quantification of flavor chemicals by gas-chromatography-mass spectrometry

Using authentic chemical materials purchased from chemical supply houses, analytical standards were prepared for 178 "target analytes", namely 177 known flavor chemicals (including triacetin) plus nicotine. One hundred and fifty-five flavor chemicals in over 22 organoleptic groups were identified in our sample of 277 refill fluids (Supplemental Table 1). The sum of the detected flavor chemical concentration values in the 277 products ranged from a low of 0.005 mg/ml to a high of 362 mg/ml (Supplemental Table 2). About 85% (236 of 277) of the samples had total flavor chemical concentrations in excess of 1 mg/ml (Figure 1A), in good agreement with a smaller sample set analyzed previously [17], and about 37% (102 of 277) were ≥ 10 mg/ml. The detected concentrations of individual flavor chemicals ranged from 0.00085 to 343 mg/ml. Forty-nine chemicals were found in some samples at concentrations between 1 – 9.9 mg/ml, and 11 were found in some samples at concentrations ≥10 mg/ml (Supplemental Table 1). About 2.5% (7 of 277) of the samples had total flavor chemical concentrations less than 0.1 mg/ml. The brand/manufacture and product names of all 277 EC refill fluids evaluated are presented in Supplemental Table 3.

The 177 flavor chemicals on the target analyte list could not include every flavor chemical in the 277 products that were analyzed. The propylene glycol and glycerol acetals of cinnamaldehyde, vanillin, and ethyl vanillin were frequently detected in the refill fluids containing substantial cinnamaldehyde, vanillin, and ethyl vanillin. For the seven products with concentrations of total target flavor chemical values of <0.1 mg/mL, only small amounts of 2-hexanal and a few other non-target flavor chemicals were detected, indicating they were truly low/non-flavored fluids.

Relationship of the total concentration of flavor chemicals to nicotine concentration

The total concentration of the flavor chemicals is plotted vs. nicotine concentration for the 170 refill fluids that contained nicotine in Figure 1B. Detected nicotine concentrations ranged from < 0.0006 mg/ml to 25.4 mg/ml. 116 out of the 170 products had nicotine concentrations ≥ 1 mg/ml (Figure 1B), while 54 had concentrations ≤ 1 mg/ml (Figure 1B). The nicotine and flavor chemicals that were < 1 mg/ml may have been incidental, caused by carryover during manufacturing, or picked up during storage. For those products that contained nicotine > 1 mg/ml, the ratio for total flavor chemicals/nicotine was greater than 2 for 56% of the samples, and for one product

("Cinnamon Bomb"), the ratio was 129. In Figure 1B, points lying above the diagonal line have a total flavor concentration/nicotine concentration ratio greater than 2. The data demonstrate that flavor chemicals are major ingredients of many EC refill fluids, and often present at total concentrations higher than that of nicotine.

Organoleptic properties and concentration ranges of 155 detected target analyte flavor chemicals

The 155 target analyte flavor chemicals detected in the samples were grouped into flavor categories using reported taste and odor descriptions (aka "organoleptic properties") (*http://www.thegoodscentscompany.com/*)[18], (Supplemental Table 1 and Figure 2A). The top five categories were "fruity" (21%), "floral" (12%), "spiced" (6%), "minty/menthol" (6%), and "herbal" (6%). "Popcorn", "musty", "phenolic", "campherous", "honey", "meaty", "smoky", "tropical", "earthy" and "odorless" flavor chemicals appeared only once and are grouped as "others". Organoleptic information was not available (N/A) for strawberry glycidate_A, strawberry glycidate_B, heliotropin PG acetal, 4-methylbenzyl alcohol, and aromadendrene.

We further evaluated the organoleptic distribution of those chemicals that were present at concentrations greater than 1 mg/ml (Figure 2B). The top categories in this analysis were "fruity", "minty/mentholic", "floral", "caramellic", and "spicy". In the "others" category, acetylpyrazine (popcorn), hemineurine (meaty) and syringol (smoky) were also present at concentrations greater than 1 mg/ml.

Frequency distribution, chemical class, and hazard classification of the 155 detected target analyte flavor chemicals

The frequency with which each of the 155 detected target analyte flavor chemicals appeared in refill fluids is shown in Supplemental Table 1, Figure 3A and

Supplemental Figure 1. The chemicals in Figure 3A appeared in at least 21 different products out of 277 total. The 13 most frequently used flavor chemicals that appeared over 100 times in descending order of frequency were: ethyl maltol, ethyl butanoate, vanillin, linalool, ethyl acetate, (3z)-3-hexen-1-ol, γ-decalactone, maltol, benzaldehyde PG acetal, corylone, benzyl alcohol, δ-decalactone, and ethyl vanillin (Fig 3A). The chemicals in Supplemental Figure 1 appeared in 20 or fewer products.

Flavor chemicals were grouped into chemical classes using their structural properties (Figure 3B). We used parent compound structures to classify those flavor chemicals that could be placed in more than one chemical group. About 39 % (60 of 155) were esters followed by terpenes and ketones, which were both 16 %. One flavor chemical each was classified as a pyrrole, acid, xanthine, thiazole and benzopyrone.

Using available safety information [18], all the flavor chemicals were grouped in terms of potential to cause harm (Figure 3C). This hazard classification is based on: (1) the Dangerous Substances Directive [19] for pure substances; and, (2) the Dangerous Preparations Directive [20] for mixtures. Some provisions of both directives related to classification, packaging and labeling of dangerous substances and preparations were amended and replaced by the Regulation on the Classification, Labeling and Packaging (CLP) of Substances and Mixtures [21], which was enacted in 2008 with enforcement beginning in 2009. According to these directives the categories applicable to the flavor chemicals in our study included; (1) “irritants”, (2) “harmful”, (3) “toxic/harmful and dangerous to the environment”, and (4) not determined (Figure 3C and 4). Most of the chemicals were “irritants” and “harmful”, and three (limonene, strawberry glycidate_A and strawberry glycidate_B) were both “irritants” and “dangerous to the environment”. One chemical, allyl hexanoate, was “toxic and dangerous to the environment”. Irritants are chemicals that can potentially destroy living tissues at significant doses. Whether or not any of these chemicals would adversely affect EC users would depend on their concentration, extent consumption, and sensitivity of the user.

Flavor chemicals > 10 mg/ml in EC refill fluids

A heat map was created to visualize the concentrations and frequency of use in refill fluids of the 11 chemicals that were present in at least one product at a concentration > 10 mg/ml (Figure 4A). The heat map shows: (1) 11 chemicals with individual concentrations >10 mg/ml in at least one refill fluid, (2) the relative frequency with which they were found, and (3) their concentrations in each product. Some chemicals appeared frequently at concentrations > 10 mg/ml (e.g., ethyl maltol and ethyl vanillin), while others appeared at > 10 mg/mL in only one product (e.g., ethyl acetate and p-menthone).

Data on the inhalation toxicity of flavor chemicals are scarce, therefore we ranked these chemicals on the *y*-axis (most to least toxic) based on previously published peer reviewed oral toxicity data in rats (Figure 4A) [18]. Nine were categorized as harmful or irritants. Four of these chemicals were present in 50% (menthol, triacetin, cinnamaldehyde) and 80% (ethyl maltol) of the samples. Two of these flavor chemicals had no available oral toxicity data (ND). One product, which was compounded in a local vape shop and sold as a refill fluid, had 343 mg/ml (~34%) of cinnamaldehyde, which is more than 100,000 times the cytotoxic level we reported previously [10,12].

Cytotoxicity of Menthol and Ethyl Maltol

Because ethyl maltol was in almost all products, often at concentrations > 1 mg/ml, and because menthol was highest in concentration (after cinnamaldehyde which was previously tested), authentic standards of each were evaluated for cytotoxicity using the MTT assay with human pulmonary fibroblasts (hPF) and human lung epithelial cells (BEAS-2B). The results are summarized in Figures 4B and 4C, for which the highest concentration on the *x*-axis is only 10% of the concentration found in at least one of the refill fluids. Both flavor chemicals were highly cytotoxic at concentrations 30 (menthol) and 100 times (ethyl maltol) lower than the highest concentrations in the refill fluids. BEAS-2B cells (IC_{50} = 0.15) were somewhat more sensitive to ethyl maltol than hPF (IC_{50} = 0.28).

DISCUSSION

EC manufacturers have about 16,000 flavor chemicals from which to choose [15]. Our data provide a simpler picture: (1) the number that were used in our sample of 277 refill fluids was 155, not thousands; (2) in any given product, the number of flavor chemicals typically ranged from 0 to 50; and (3) while some constituents were present at rather low concentrations, 11 were found at concentrations > 10 mg/ml. When evaluating just those chemicals that were over 1 mg/ml, the number/product ranged from 0 to 10. Moreover, the total concentrations of flavor chemicals exceeded the nicotine concentration in over half of the products. These data demonstrate that flavor chemicals are a major component of currently marketed EC refill fluids and their health effects on EC users should be addressed.

Of particular importance in our study is the finding that some products have individual flavor chemicals in concentrations > 10 mg/ml, and many of these chemicals were found in many of the samples (e.g., ethyl maltol was in 24.5% of the products at ≥ 10 mg/ml, and menthol was in 22.6% of the products at ≥ 10 mg/ml) (Figure 4). Based on the results of the MTT assay, menthol and ethyl maltol were present at concentrations that would be cytotoxic in 34% (26 of 76) and 40% (66 of 164) of the refill fluids that contained menthol and ethyl maltol, respectively. While the MTT data cannot be translated directly to *in vivo* human effects, they do raise concern about the potential for these chemicals to cause harm to users at the concentrations currently used in some refill fluids. Moreover, chronic exposure to high concentrations of flavor chemicals may be far more damaging than the effects seen in our acute experiments.

Further evidence that the concentrations of some flavor chemicals used in EC refill fluids may exceed safe levels can be found by comparing our data to the concentrations in other consumer products. Although cinnamaldehyde has been approved by the FDA for use as a flavoring agent [22] and given FEMA GRAS status, some in the flavor industry and the Research Institute for Fragrance Materials have recommended that cinnamaldehyde not exceed 1% when used in skin cosmetic products[23, 24]. Cinnamaldehyde is usually found in body care and household products, such as detergents, creams and lotions, soaps and perfumes, in the range 0.001% - 0.8% [25]. Moreover, cinnamaldehyde is used in food products at concentrations ranging from 7.7 ppm (0.00077%) in ice creams to a 700 ppm (0.07%) in candy and up to a 6,400 ppm (0.64%) in fruits and juices [23, 26, 27]. In our refill fluid samples, two products had cinnamaldehyde concentrations of 118 mg/ml (11.8% or 118,000 ppm) and 343 mg/ml (34.3% or 343,000 ppm). We have previously reported that the cinnamaldehyde concentrations in a different set of refill fluid samples often exceeded 1% (range = 0.00022 – 14%) for cinnamon flavored refill fluids [10, 11]. Our current study further shows, in agreement with our earlier work [11] that cinnamaldehyde is more widely used in EC refill fluids than would be expected based on the names of the EC products. For example, cinnamaldehyde was found previously in fruity flavors, such as a product named “Blueberry Hills”, and in the current study was found in 70 of 277 (25%) products, even though only two products indicated “cinnamon” in their name. Cinnamaldehyde at concentrations found in EC products has also been shown to impair the function of immune cells in the respiratory system [13].

Like cinnamaldehyde, ethyl maltol is added to edible products such as beverages, ice cream, candy, baked goods, gelatin desserts, meat, chewing gum and related products in concentrations up to 0.0142%) [28], and the maximum concentrations of ethyl maltol in final formulations of soap, detergents, and creams and lotions are 0.06%, 0.006%, and 0.01%, respectively [29]. These concentrations of ethyl maltol in consumer products are far below the concentrations that we found (0.008 - 3.13%) in 46% of the of the refill fluids that we tested. Ethyl maltol increases free radical formation in EC aerosols [30], which could further increase the toxicity of products with this flavor chemical.

Menthol is commonly used in consumer products including tobacco cigarettes. Mentholated cigarettes generally have menthol concentrations < 7 mg/cigarette and many are < 0.002 mg/cigarette[31]. Menthol was present in 76 of our refill fluids at concentrations ranging from 0.002 to 68 mg/ml. Twelve out of the 76 refill fluids had concentrations greater than 10 mg/ml, which would exceed the concentrations normally found in conventional tobacco cigarettes flavored with menthol. Menthol produced cytotoxicity in the MTT assay at concentrations 30 times lower than the highest concentration found in the refill fluids we analyzed.

2,3-butanedione (diacetyl), which can cause bronchiolitis obliterans, also called “popcorn lung disease” [32 -35], has previously been found in EC products [36, 37]. We found diacetyl, as well as two related chemicals, acetoin and 2,3-pentanedione, in 54% of the refill fluids. Of these chemicals, diacetyl, acetoin and 2,3-pentanedione were present in

36% (54 of 150), 42% (63 of 150) and 22% (33 of 150), respectively. Assuming a consumer vapes 3.4 ml of a refill fluid [38] containing diacetyl at 0.32 mg/ml (highest concentration found in our study) and the transfer rate of diacetyl to the aerosol is 100%, the consumer would be exposed to 1.088 mg of diacetyl/day (equivalent to 85.83 ppb/8 hour average) which is well above the exposure limit of 5 ppb for 8 hours recommended by NIOSH [39]. Concentrations in refill fluids also exceeded the Short-Term (15 minute) Exposure Limit of 25 ppb for diacetyl [39]. These data raise concern about the potential for harm of some of the flavor chemicals that are present in refill fluids at relatively low concentrations.

Coumarin (1,2-benzopyrone) is another chemical of concern. It was present in 21 products at concentrations ranging from 0.007 to 5 mg/ml. Coumarin is currently prohibited as an additive in human food by the Federal Drug Administration (21CFR189.130) due to its hepatotoxicity, and when present, the food is deemed adulterated [40]. It’s prohibition in food supports the idea that it should likewise not be used in tobacco products, including ECs. Coumarin is often co-extracted from cinnamon with cinnamaldehyde and may have been a co-constituent inadvertently introduced into the products containing high concentrations of cinnamaldehyde

Our data show that both menthol and ethyl maltol are frequently used in refill fluids at concentrations that were cytotoxic to cultured human lung cells when tested with the MTT assay. Menthol and ethyl maltol have been reported in other brands of EC products[41-43], although their concentrations were not given. While most prior work on the toxicity of EC flavors has been done on intact fluids [9,44-46], several studies have examined the cytotoxicity of authentic standards of flavor chemicals present in EC fluids and aerosols [41,47,48].

Our cytotoxicity data with menthol and ethyl maltol can be compared to results reported previously. Both ethyl maltol and menthol altered calcium homeostasis in CALU3 lung epithelial cells by depleting the endoplasmic reticulum of Ca^{2+} and elevating cytosolic Ca^{2+} [41]. The effective concentration (EC_{50}) of menthol in the Ca^{2+} assay (3.02 mM) [41] was similar to the inhibitory concentration (IC_{50}) of menthol in our MTT assay (1.38 mg/ml or 8.8 mM). In contrast, the concentration of ethyl maltol (0.15 mg/ml or 1.07 mM) that produced an effect in our MTT assay was much lower than the effective concentration (21.14 mM) in the Ca^{2+} influx assay [41]. These differences with ethyl maltol could be related to the different cell types (BEAS-2B versus CALU3) that were used in the two studies. These data show that mitochondrial reductase activity (MTT assay) is very sensitive to ethyl maltol and demonstrate the importance of evaluating multiple toxicity endpoints.

Cinnamaldehyde, which was very high in concentration in several products in the current study, was shown previously to be highly cytotoxic and immunosuppressive when tested in vitro with lung cells [10,11,13,48]. Based on our prior data with the MTT assay[10,11], cinnamaldehyde is the most potent flavor chemicals we have tested, and it was found in 25% of all refill fluids in the current study.

Aerosolization of the flavor chemicals can increase aldehyde concentrations in EC aerosols [49], although this was not confirmed in a second study [50]. A previous study which compared the toxicity of EC aerosol produced at 3 versus 5 volts (4.3 W versus 11.9 W) showed a clear increase in toxicity at the higher voltage [48]. This observation would be consistent with the production of toxic reaction products upon aerosolization at the higher voltage and deserves further evaluation given the high concentration of flavor chemicals that we report here in many refill fluids and the increased popularity of tank style EC with variable power controls.

Conclusions

This paper is the first to identify and quantify the flavor chemicals in a broad spectrum of EC refill fluids that are sold worldwide. These data should help focus future work on the flavor chemicals that are frequently used and/or used at high concentrations. Our findings draw attention to the fact that EC serve the dual purpose of delivering both nicotine and flavor chemicals and that some of the flavor chemicals are used at concentrations far in excess of the acceptable levels found in other consumer products. The human health effects of inhalation of flavor chemicals at high concentrations are not well understood and will require further evaluation with attention to those chemicals that are frequently used in high concentration and cytotoxic *in vitro*.

There are now sufficient data to heighten concern about the unregulated use of flavor chemicals in refill fluids, especially at high concentrations. Given the current data, regulation of flavor chemicals in EC products should be addressed, as we have recommended previously [17]. Regulatory agencies could consider limiting the concentrations of flavor chemicals in EC products, requiring a list of flavor ingredients on product labels, restricting use of flavor chemicals that are cytotoxic at low concentrations, such as cinnamaldehyde, or banning the use of flavor chemicals in tobacco products, as suggested by others [51].

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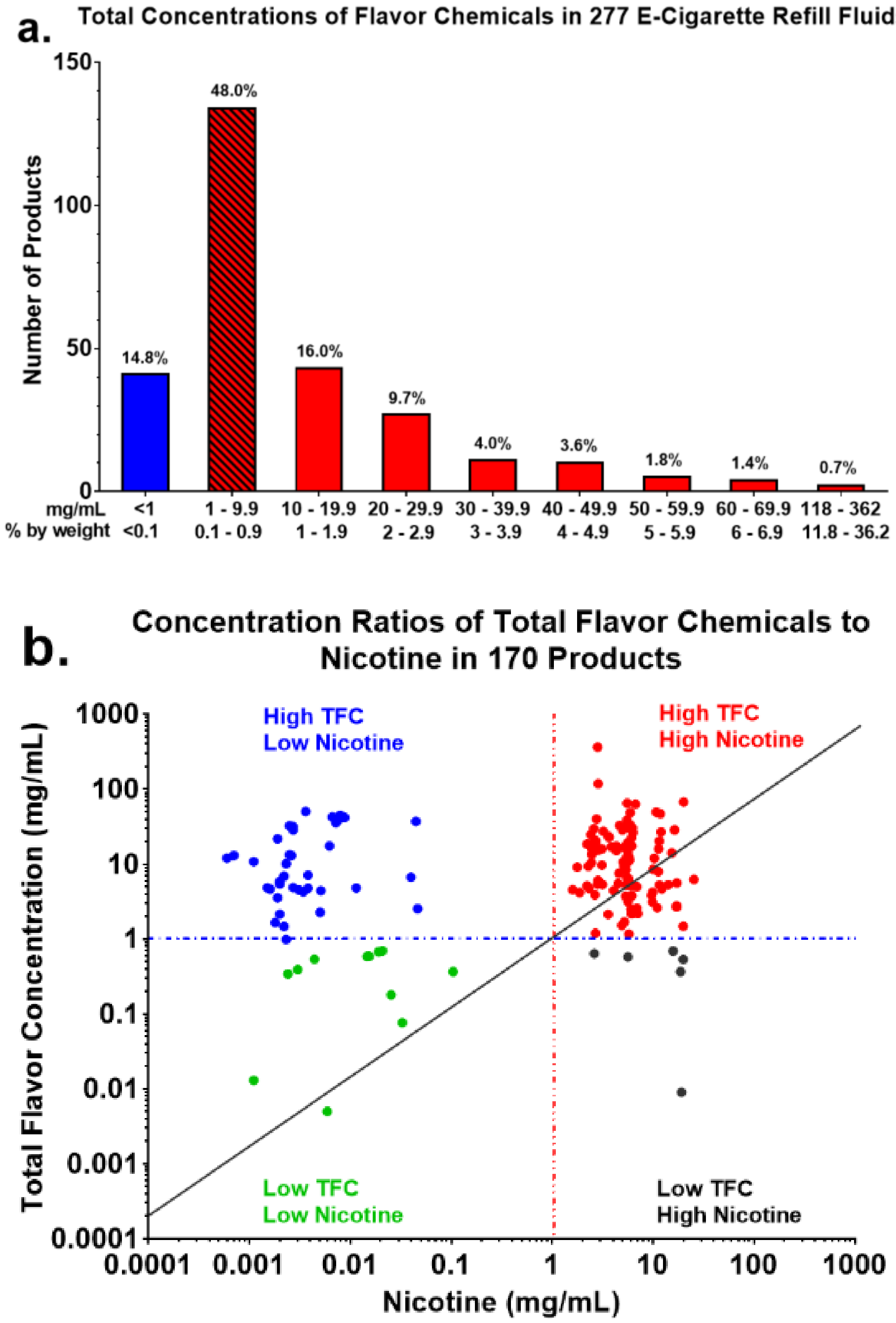
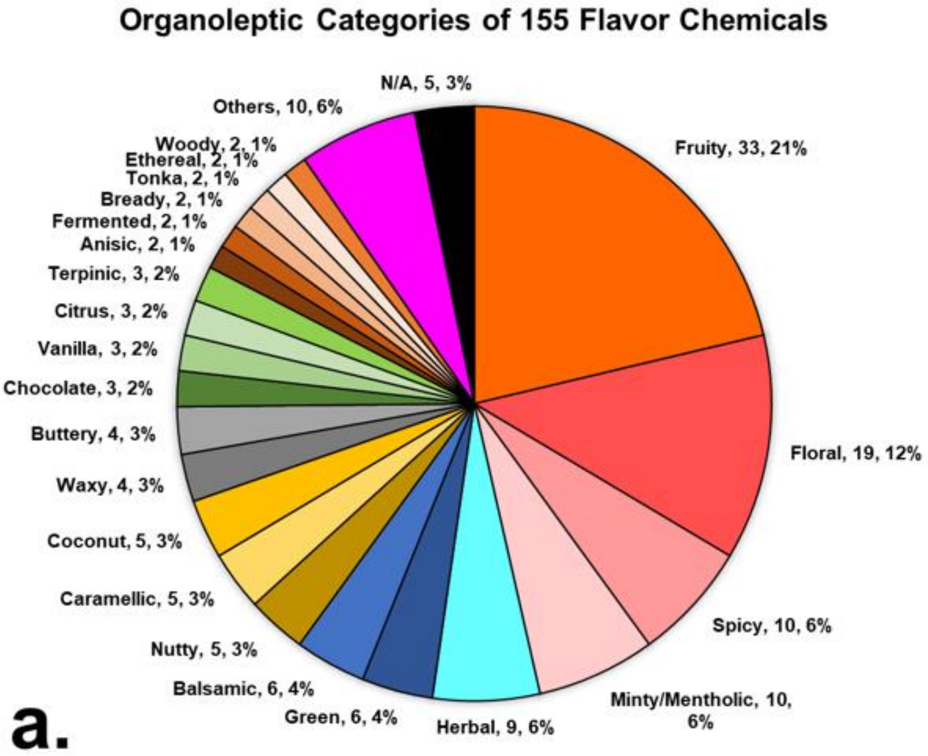


Figure 4.1: Total Concentrations of Flavor Chemicals and Nicotine in EC Refill Fluids. (A) The total concentration of flavor chemicals ranged from < 1 mg/ml to 362 mg/ml. Total weight concentration of the flavor chemicals (mg/ml) was determined for each product and plotted according to the ranges in the figure. The numbers above the frequency bars represent the percentage of products in each group. (B) The concentration of nicotine (*x*-axis) plotted against the total concentration of flavor chemicals (*y*-axis) for each product, which ranged from 0.005 – 362 mg/ml.


Organoleptic Categories of 155 Flavor Chemicals
N/A, 5, 3%
Fruity, 33, 21%
Floral, 19, 12%
Spicy, 10, 6%
Minty/Mentholic, 10, 6%
Herbal, 9, 6%
Green, 6, 4%
Balsamic, 6, 4%
Nutty, 5, 3%
Caramellic, 5, 3%
Coconut, 5, 3%
Waxy, 4, 3%
Buttery, 4, 3%
Chocolate, 3, 2%
Vanilla, 3, 2%
Citrus, 3, 2%
Terpinic, 3, 2%
Anisic, 2, 1%
Fermented, 2, 1%
Bready, 2, 1%
Tonka, 2, 1%
Ethereal, 2, 1%
Woody, 2, 1%
Others, 10, 6%
a.

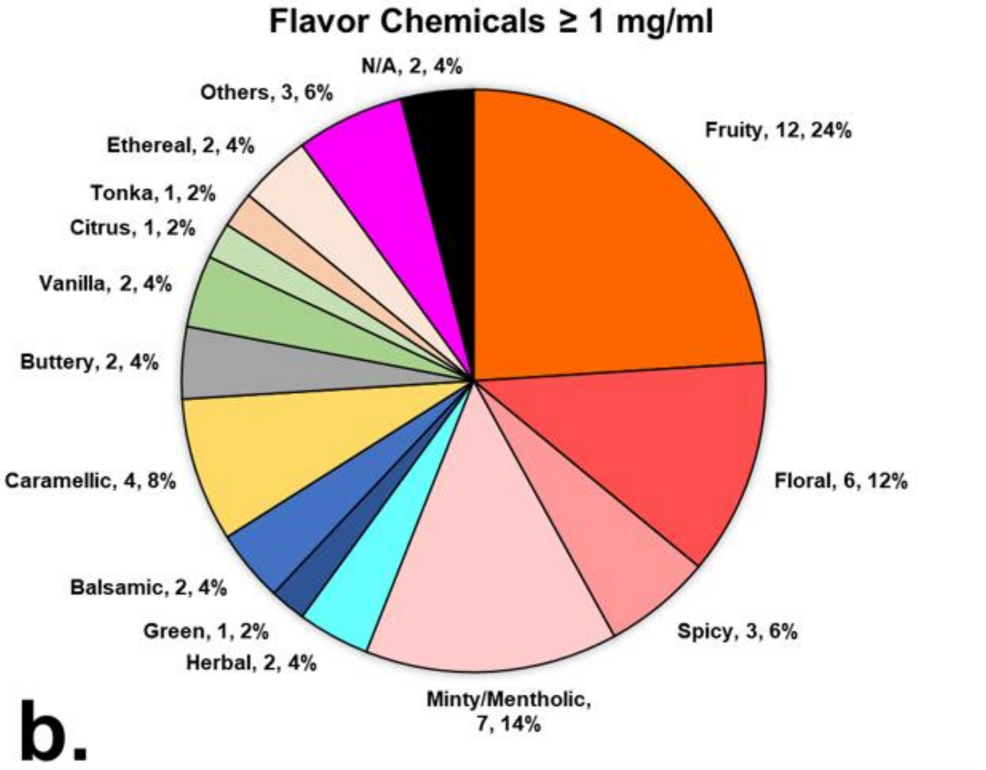

Flavor Chemicals ≥ 1 mg/ml
N/A, 2, 4%
Fruity, 12, 24%
Floral, 6, 12%
Spicy, 3, 6%
Minty/Mentholic, 7, 14%
Herbal, 2, 4%
Green, 1, 2%
Balsamic, 2, 4%
Caramellic, 4, 8%
Buttery, 2, 4%
Vanilla, 2, 4%
Citrus, 1, 2%
Tonka, 1, 2%
Ethereal, 2, 4%
Others, 3, 6%
b.

Figure 4.2: Organoleptic Properties of Flavor Chemicals Identified. (A) The taste and odor descriptions of flavor chemicals were obtained from an online database and charted to show the number and percentages of flavor chemicals in each category. The pie chart shows the top 4 tastes as fruity, floral, spicy and minty/mentholic. Nine chemicals grouped as “N/A” did not have any identified taste/description. (B) Flavor chemicals present in concentrations > 1mg/ml were then sorted to identify major and frequently used flavor categories.

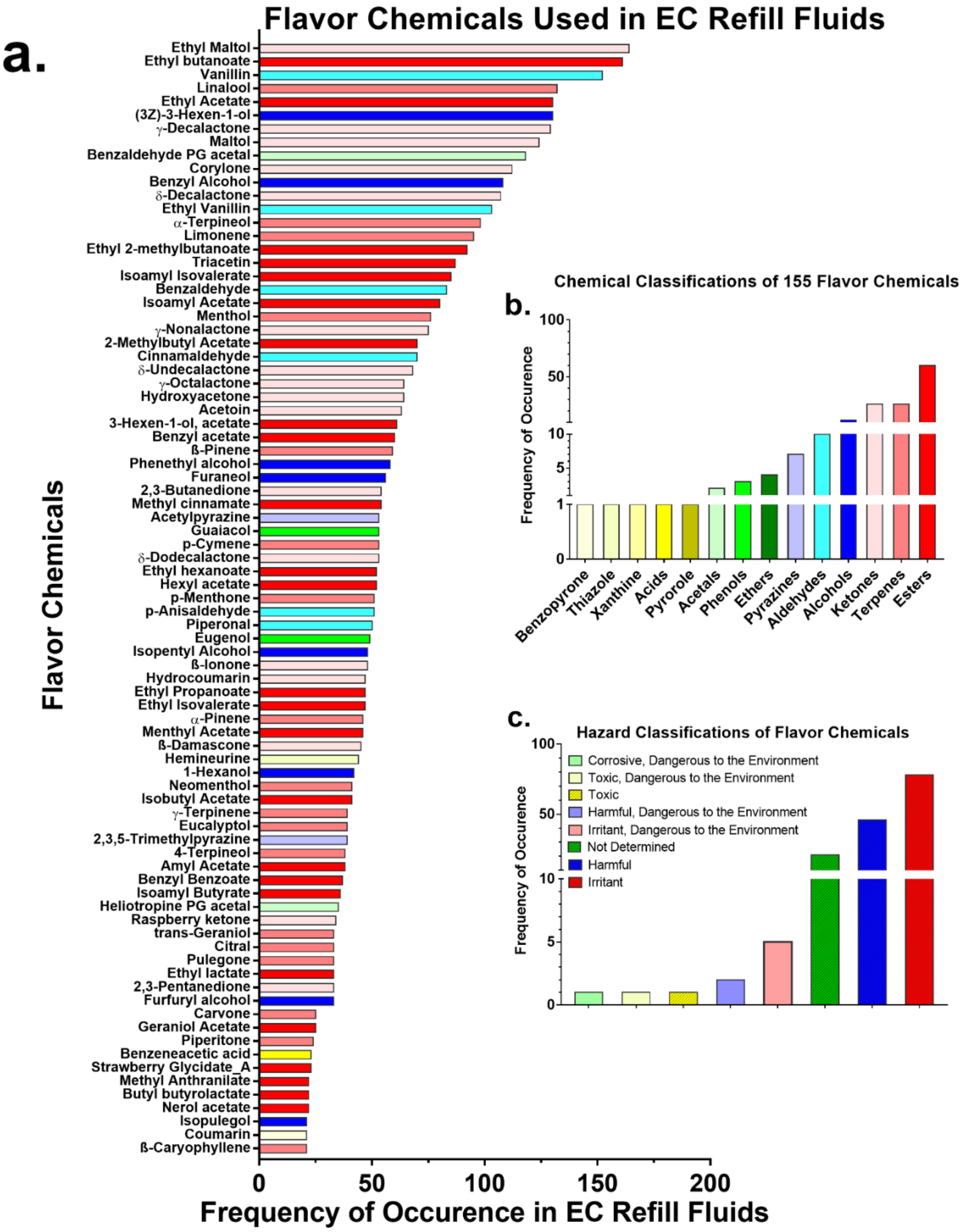

Flavor Chemicals Used in EC Refill Fluids
a.
Flavor Chemicals
Ethyl Maltol
Ethyl butanoate
Vanillin
Linalool
Ethyl Acetate
(3Z)-3-Hexen-1-ol
γ-Decalactone
Maltol
Benzaldehyde PG acetal
Corylone
Benzyl Alcohol
δ-Decalactone
Ethyl Vanillin
α-Terpineol
Limonene
Ethyl 2-methylbutanoate
Triacetin
Isoamyl Isovalerate
Benzaldehyde
Isoamyl Acetate
Menthol
γ-Nonalactone
2-Methylbutyl Acetate
Cinnamaldehyde
δ-Undecalactone
γ-Octalactone
Hydroxyacetone
Acetoin
3-Hexen-1-ol, acetate
Benzyl acetate
ß-Pinene
Phenethyl alcohol
Furaneol
2,3-Butanedione
Methyl cinnamate
Acetylpyrazine
Guaiacol
p-Cymene
δ-Dodecalactone
Ethyl hexanoate
Hexyl acetate
p-Menthone
p-Anisaldehyde
Piperonal
Eugenol
Isopentyl Alcohol
ß-Ionone
Hydrocoumarin
Ethyl Propanoate
Ethyl Isovalerate
α-Pinene
Menthyl Acetate
ß-Damascone
Hemineurine
1-Hexanol
Neomenthol
Isobutyl Acetate
γ-Terpinene
Eucalyptol
2,3,5-Trimethylpyrazine
4-Terpineol
Amyl Acetate
Benzyl Benzoate
Isoamyl Butyrate
Heliotropine PG acetal
Raspberry ketone
trans-Geraniol
Citral
Pulegone
Ethyl lactate
2,3-Pentanedione
Furfuryl alcohol
Carvone
Geraniol Acetate
Piperitone
Benzeneacetic acid
Strawberry Glycidate_A
Methyl Anthranilate
Butyl butyrolactate
Nerol acetate
Isopulegol
Coumarin
ß-Caryophyllene
0
50
100
150
200
Frequency of Occurence in EC Refill Fluids
Chemical Classifications of 155 Flavor Chemicals
b.
Frequency of Occurence
100
50
10
5
1
0
Benzopyrone
Thiazole
Xanthine
Acids
Pyrorole
Acetals
Phenols
Ethers
Pyrazines
Aldehydes
Alcohols
Ketones
Terpenes
Esters
c.
Hazard Classifications of Flavor Chemicals
Frequency of Occurence
100
50
10
5
0
Corrosive, Dangerous to the Environment
Toxic, Dangerous to the Environment
Toxic
Harmful, Dangerous to the Environment
Irritant, Dangerous to the Environment
Not Determined
Harmful
Irritant

Figure 4.3: Frequency Distribution, Chemical Classes, and Hazard Classification of the Flavor Chemicals. (A) The frequency with which individual flavor chemicals were found in at least 21 products. The *x*-axis is the number of refill fluids in which the chemicals were found, and the *y*-axis is sorted according to decreasing frequency of their occurrence. Frequency ranged from 21 – 164 with the highest being ethyl maltol. Chemicals appearing less frequently are shown in Supplemental Figure 1. (B) The chemical classes of the flavor chemicals (*x*-axis) are plotted versus the frequency of occurrence of each class of flavor chemicals (*y*-axis). (C) The classification of flavor chemicals into color coded hazard categories using the European safety data (x-axis) are plotted versus the frequency of occurrence of flavor chemicals in each hazard category (*y*-axis).

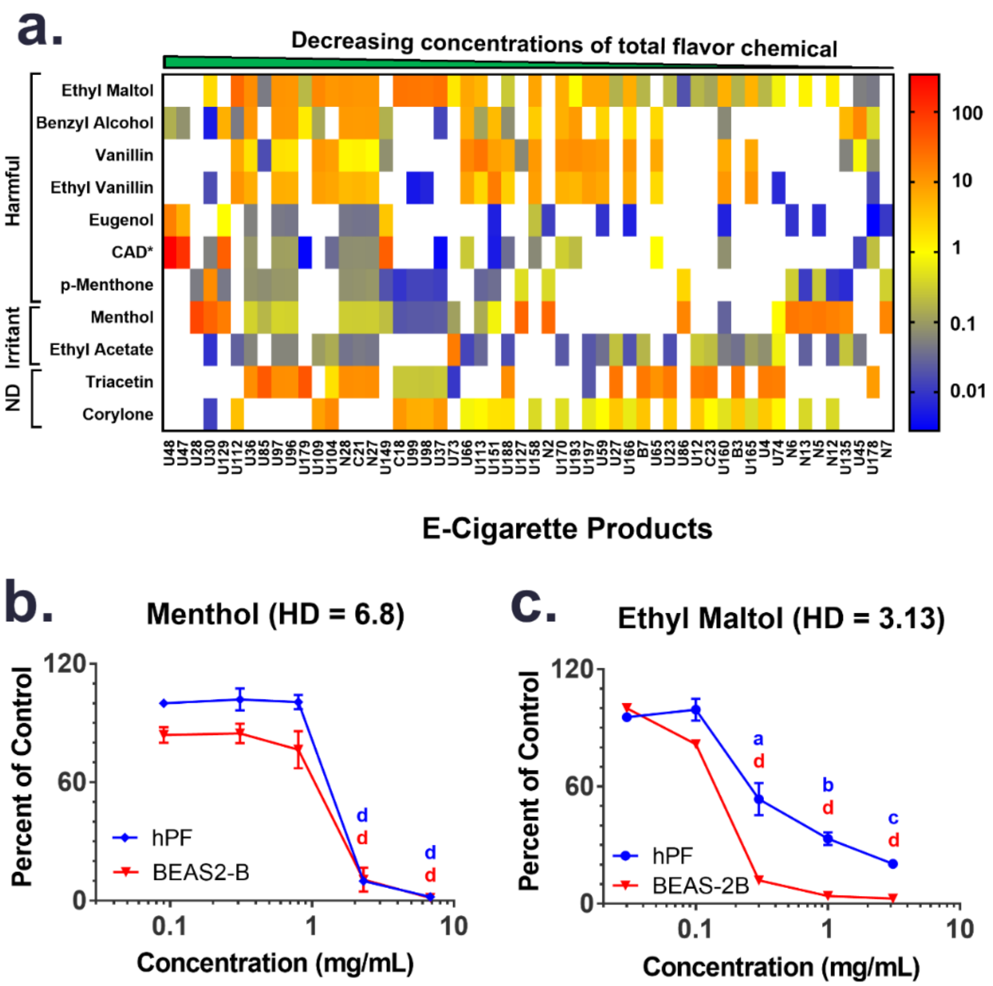


Figure 4.4: Heat Map and Cytotoxicity of Flavor Chemicals > 10 mg/ml. (A) The *x*-axis of the heat map shows individual refill fluid products with at least one flavor chemical >10 mg/ml. Total flavor concentration decreases from left to right. The *y*-axis is ordered from high to low toxicity for the individual flavor chemicals based on the LD_{50} oral dose for rats (from peer reviewed articles on the Good Scents database (***www.thegoodscentscompany.com***) and grouped according to the European CLP regulation criteria; harmful; irritant, and not determined (ND). Concentration of individual flavor chemicals: > 1mg/ml are shown as yellow cells, > 10 mg/ml are orange to red cells. The country of each product's origin is designated on the *x*-axis labels by U = USA, N = Nigeria, C = China, and B = Britain. Cinnamaldehyde is abbreviated CAD. (B, C) Dose-response curves for menthol (B) and ethyl maltol (C) tested with hPF and BEAS-2B cells in the MTT assay. The highest concentration tested is 10% of that found in the refill fluid that contained the highest concentration. Each point is the mean ± standard error of the mean of three independent experiments. Points with different letters are significantly different from points without letters and points with different letters are significantly different from each other. a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = p < 0001

Chapter 5

Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids

Hua and Omaiye et al., 2019

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ABSTRACT

We identified the most popular electronic cigarette (EC) refill fluids using an Internet survey and local and online sales information, quantified their flavor chemicals, and evaluated cytotoxicities of the fluids and flavor chemicals. “Berries/Fruits/Citrus” was the most popular EC refill fluid flavor category. Twenty popular EC refill fluids were purchased from local shops, and the ingredient flavor chemicals were identified and quantified by gas chromatography-mass spectrometry. Total flavor chemical concentrations ranged from 0.6 to 27.9 mg/ml, and in 95% of the fluids, total flavor concentration was greater than nicotine concentration. The 20 most popular refill fluids contained 99 quantifiable flavor chemicals; each refill fluid contained 22 to 47 flavor chemicals, most being esters. Some chemicals were found frequently, and several were present in most products. At a 1% concentration, 80% of the refill fluids were cytotoxic in the MTT assay. Six pure standards of the flavor chemicals found at the highest concentrations in the two most cytotoxic refill fluids were effective in the MTT assay, and ethyl maltol, which was in over 50% of the products, was the most cytotoxic. These data show that the cytotoxicity of some popular refill fluids can be attributed to their high concentrations of flavor chemicals.

INTRODUCTION

Electronic cigarettes (EC) and their refill fluids (also called e-liquids) are relatively new tobacco products. In 2014, consumers could choose from over 400 models of EC and ~8,000 different refill fluid flavor names [1.] While many flavor chemicals in EC are reported safe for use in food [2], the National Institute for Occupational Safety and Health has warned food-processing workers that some inhaled flavor chemicals may cause lung disease [3], and the Flavor and Extract Manufacturers Association has strongly cautioned that their "Generally Recognized as Safe" (GRAS) certification is intended for exposure by ingestion, not inhalation [4].

Information on adverse health effects of ECs comes from several sources. Adverse systemic effects, including inflammatory lung and digestive diseases, have been linked to EC use in case reports [5]. A systematic review on EC health effects collated data on EC flavor chemicals that have cytotoxic effects as well as information on particles, harmful metals, tobacco specific nitrosamines, and toxic carbonyl-containing degradation products [6]. EC users have reported numerous negative effects of vaping on their health [7]. In an *in vitro* study, EC refill fluids varied in their cytotoxicities when tested with embryonic and adult cells; products with high concentrations of flavor chemicals were often the most toxic [8]. Cinnamaldehyde was subsequently identified in the most cytotoxic refill fluids [9, 10] and was found at toxic (in vitro) concentrations in a broad spectrum of refill fluids not suggesting "cinnamon", such as variations of "fruit", "berry", "coffee", "tobacco", and "sweet" [10]. Cinnamaldehyde was also immunosuppressive when tested with human respiratory cells [11]. Other flavor chemicals in EC refill fluid are also a concern. For example, diacetyl, which can cause *bronchiolitis obliterans* [12, 13], was found in a high percentage of randomly sampled refill fluids with flavor terms related to “buttery”, “caramel”, “fruity”, “alcohol” and “candy” [14, 15]. In an air-liquid interface model, toxicity was linked to flavor chemical content of refill fluids with “strawberry”-flavored fluids being the most cytotoxic [16]

Existing data suggest that high concentrations of flavor chemicals in EC may harm users. It is important to identify and understand which EC flavor types are commonly purchased, what their chemical compositions are, and what their potential toxicities are. Here we: (1) evaluated EC users’ flavor preferences based on an Internet survey and data from local and Internet vape shops, (2) identified and quantified the flavor chemicals in 20 popular refill fluids, (3) established which of the popular fluids are cytotoxic, and (4) identified the flavor chemical ingredients that are individually cytotoxic at concentrations found in the popular refill fluids.

MATERIALS AND METHODS

Design, Recruitment, and Analysis for the Online Survey

Our study was approved by the Institutional Review Board at UC Riverside. Informed consent was not required for the survey which did not involve direct interaction with human subjects. An online survey was created with Survey Monkey using filter logic. The survey contained questions pertaining to EC user: (1) preference for refill fluid flavors and (2) conventional smoking and EC use history.

To obtain a broad cross section of EC users, survey participants were recruited from: (1) UC Riverside between May 2015 to August 2015 via email; and (2) various online health forums (WebMD, DailyStrength, eHealthForum, and Student Doctor Network), and (3) sites with special interest groups related to EC use or survey volunteering (Craigslist, Reddit). The resulting EC user data were analyzed to determine user demographics, EC usage history, and flavor preferences.

Identification of EC flavor preferences in Southern California shops and online stores

Each product was assigned an inventory number and stored at 4ºC. For each refill fluid, 50 µl was diluted with 0.95 ml of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ) for an overall dilution ratio of 20 to 1. All diluted samples were shipped overnight on ice to Portland State University and analyzed on the day they were received using gas chromatography/mass spectrometry (GC/MS) to identify and quantify the flavor chemicals. Twenty µl of internal standard (2000 ng/µl of 1,2,3-trichlorobenzene) were added into each sample before GC/MS analysis. Using internal standard-based calibration procedures described elsewhere [39], analyses were performed with an Agilent 5975C GC/MS system (Santa Clara, CA). A Restek Rxi-624Sil MS column (Bellefonte, PA) was used (30 m long, 0.25 mm id, and 1.4 µm film thickness). One µl of each sample was injected into the GC with a 10:1 split. The injector temperature was 235°C. The GC temperature program for all analyses was: 40°C hold for 2 min; 10°C/min to 100°C; then 12°C/min to 280°C; hold for 8 min at 280 °C; then 10ºC/min to 230ºC. The MS was operated in electron impact ionization mode at 70 eV in positive ion mode. The ion source temperature was 220°C. The scan range was 34 to 400 amu. Each target analyte (178 total) was quantitated using authentic standards (pure chemicals) and an internal standard (1,2,3-trichlorobenzene) normalized multipoint calibration. All reported concentration values were based on the 20:1 dilution sample except for overloaded peaks at 20:1 dilution, in which case quantitation was based on a 400:1 dilution sample.

Cell Culture

Dose-response cytotoxicity experiments were performed using mouse neural stem cells (mNSC) and human bronchial epithelial cells (BEAS-2B). The mNSC and BEAS-2B measure cytotoxicity in a neurological and respiratory model. In addition, mNSC provide information on a stem cell population as well as data that can be compared to our earlier studies [8], and it is robust in moderate throughput assays. Cytotoxicity was measured using the MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which measures the reduction of a yellow tetrazolium bromide to a purple formazan. This assay is widely used to screen for toxicity.

The mNSC were cultured in Dulbecco's Eagle's Medium (DMEM) (Lonza Walkersville, MD) supplemented with 10% fetal bovine serum, 5% horse serum, and 1% each penicillin-streptomycin (GIBCO, Invitrogen, Carlsbad, CA) and sodium pyruvate. Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin, CA) were used to culture cells, and medium was replaced every 48 hours. At 80% confluency, cells were harvested using Dulbecco's phosphate buffered saline (DPBS) for washing and incubated with 0.05% trypsin EDTA/DPBS (GIBCO, Invitrogen Carlsbad, CA) for 2 mins at 37^{o}C to allow detachment from the culture flask. For the MTT assay, plating was done at 1,500 cells/well in 96 well plates.

Human BEAS-2B cells were purchased from the American Type Culture Collection (ATCC), USA. The cell line was cultured in basal bronchial epithelial cell basal medium (BEBM) (Lonza, Walkersville, MD) supplemented with 2 ml bovine pituitary extract and 0.5 ml of: insulin, hydrocortisone, retinoic acid, transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor (Lonza, Walkersville, MD). Nunc T-25 tissue culture flasks were coated overnight with BEBM, collagen, bovine serum albumin (BSA) and fibronectin prior to culturing and passaging cells. At 80% flask confluency, cells were harvested using DPBS for washing and incubated with 1.5 ml of 0.25% trypsin EDTA/DPBS and poly-vinyl-pyrrolidone for 3-4 mins at 37^{o}C to allow detachment. Cells were cultured in T-25 flasks at 75,000 cells/flask, and medium was replaced the next day and then every other day. Plating for the MTT assay was done at 3,500 cells/well in pre-coated 96-well plates.

Cytotoxicity of EC refill fluids

The cytotoxicities of the 20 EC refill fluids were evaluated in 96-well plates using the MTT assay [37,40]. Serial dilutions (0.001 - 1%) of refill fluids were made in culture medium and arranged in 96-well plates with a negative control (culture medium only) adjacent to the high and low concentration to check for a vapor effect. The high dose of 1% was chosen as preliminary experiments showed that it did not produce a vapor effect in this study. mNSC were added to non-coated 96-well plates at 1,500 cells/well, allowed to attach for 24 hours, then treated for 48 hours with serial dilutions of refill fluids. After treatment, 20 µl of MTT (Sigma-Aldrich, St Louis, MO) dissolved in 5 mg/ml of DPBS (Fisher Scientific, Chino, CA) were added to each well and incubated for 2 hrs at 37ºC. Solutions were removed and 100 µl of dimethyl sulfoxide (DMSO) (Fisher, Chino, CA) were added to each well and gently mixed by pipetting until homogenous. Absorbance of control and treated wells was read against a DMSO blank at 570 nm using an Epoch micro-plate reader (BioTek, Winooski, VT).

Cytotoxicity of authentic standards of flavor chemicals

A heat map of the flavor chemicals found in the two most cytotoxic refill fluids and their clones was examined to identify potentially toxic flavor chemicals that were present in high concentrations. Authentic standards of each chemical (ethyl maltol, maltol, ethyl vanillin, vanillin, benzyl alcohol, and furaneol) (Sigma-Aldrich, St. Louis, MO) diluted in culture medium were tested individually using the MTT assay with mNSC and BEAS-2B cells. Toxicity assays were performed over a concentration range that included the concentration of each chemical found in the refill fluids.

Data analysis

The MTT assay was performed in three independent experiments for each refill fluid and authentic standard chemical. Data were normalized by setting treatment wells as percentages of the negative control (100%). Graphs were plotted using GraphPad Software (GraphPad, San Diego), and significance was obtained using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test in which treated groups were compared to the lowest concentration. The ANOVAs were used to determine which concentrations of refill fluid or authentic standard produced a significant effect in the MTT assay. GraphPad Prism software was also used to compute IC_{50s} with the log inhibitor vs. normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively.

RESULTS

Demographics and flavor preferences of EC users in the online survey

We conducted an online survey to identify the most popular flavors of EC refill fluids. Of 2,753 participants, 853 were current EC users (Supplemental Table 1). Most EC users were between ages 18-22 (49.5%), male (72.0%), and listed "some college" as their highest education (39.0%). The most represented ethnic groups were White/Caucasian (43.0%), followed by Asian (23.4%), and Hispanic/Latino (19.0%). 87.0% were ever cigarette users. 68.0% were ever cigarette users that no longer smoked. 53.1% listed ECs and as aids to quit smoking. 49.0% listed nicotine replacement products as aids to quit smoking. EC use was "influenced by friends" (54.0%) and some believed "vaping is safer than smoking" (55.0%) (Supplemental Table

1). Most users described their health as “very good/good” (73.0%) and vaped daily (82.0%) for at least 1 month to 2 years (74.0%). Most participants described their use of EC as “regularly, at least once a day” (57.0%) and for 31-59 minutes a day (22.0%) or 1-3 hours a day (22.0%). In users’ decisions to vape, refill fluids flavors were deemed “very important” or “important” (78.0%). The most popular models of EC were tanks/mods (47.0%). Over half of the EC users (54.0%) currently used EC products, while 19.0% were former users, and 27.0% were dual users of both EC and conventional cigarettes.

EC users (N = 789) indicated their flavor preferences from 18 possible flavor categories (Figure 1A). They were able to select more than one flavor. The top six flavor preferences were “Berries/Fruits/Citrus” (N = 559), “Sweet” (N = 406), “Bakery/Dessert” (N = 321), “Mint/Menthol” (N = 298), “Candy” (N = 293), and “Buttery/Cream/Caramel/Vanilla” (N = 274) (Figure 1A). The two least popular flavors were “Nuts” (N = 44) and “Savory/Dinner Food” (N = 21). Flavor preferences were similar irrespective of the users’ age (Figure 1B).

Popular flavors in local and online shops

To confirm the results of the online survey, 17 EC vape shops in southern California were contacted by telephone or visited to obtain information on their top-selling refill fluids. Each local shop reported 5-10 top-selling refill fluid categories, the majority of which were “Berries/Fruits/Citrus” (54 local; 57 online) (Figure 1C). The Internet was used to determine flavor profiles when shops could not provide these data. In addition, nine popular online shops were visited, and the flavor profiles for 5-10 top-selling fluids were identified as Berries/Fruits/Citrus (Figure. 1C). Flavor categories that were not among the most popular are not included in Figure 1C.

Based on the above data, 20 top-selling refill fluids were purchased from four shops in Riverside County, CA. One local shop specialized in “cloning” brand-name EC fluids, while the other shops sold fluids that were made by a refill fluid manufacturer. We distinguish these products as “cloned” and “authentic”, respectively. It is important to consider cloned products because they are often less expensive than their authentic branded counterparts, and some shops sell mainly cloned products. Supplemental Table 2 shows the flavor profile and general flavor category for each product purchased in local shops.

Identification and quantification of flavor chemicals in the 20 popular refill fluids

A total of 99 flavor chemicals were identified and quantified in the 20 EC refill fluids purchased in local shops (Figure 2; Supplemental Table 3). The general flavors associated with each chemical are given in Supplemental Table 3, and the target flavors not found in any of the products are given in Supplemental Table 4. The total concentration of the flavor chemicals in each product, which is given at the top of the columns in the heat map (Figure 2), ranged from 0.63 mg/ml (“Bird Brains”) to 27.9 mg/ml (authentic “Dewberry Cream”). The *x*-axis of the heat map is sorted based on the total flavor chemical concentration (highest on the left).

On the *y*-axis of the heat map, the 99 chemicals were ranked by their safety classification (Toxic, Harmful, Irritant, and No data) as posted on the Good Scents Flavor Company website [17], which provides peer reviewed information for the flavor, food, and fragrance industry. Within each safety classification, chemicals are listed from most to least toxic based on rat oral LD_{50}, also posted on the Good Scents website. For most flavor chemicals, one LD_{50} value was available, but if multiple were given, we chose the LD_{50} value reported in the journal *Food and Cosmetics Toxicology*. Rat oral data were used for ranking because they were available for most chemicals in the heat map, while inhalation LD_{50} data were seldom available. The *y*-axis ranking was useful for predicting which chemicals would be most toxic and therefore most interesting to pursue; however, it is not intended to imply that the chemicals in refill fluids produce the same effects as in the rat oral data. The chemicals with the highest concentrations and highest predicted toxicities are in the upper left quadrant of the heat map.

“Bird Brains” had the fewest flavor chemicals (N = 22), while authentic “Dewberry Cream” had the most (N = 47). In some cases, these chemicals were very low in concentration (e.g., maltol in “Bird Brains”), while in others the concentrations exceeded 1 mg/ml (e.g., ethyl maltol in “Dewberry Cream”). Thirteen percent of the flavor chemicals were present at concentrations higher than nicotine in some samples.

The frequency with which individual chemicals were found in the 20 popular products varied. Some were found in all or almost all refill fluids (e.g., maltol and ethyl acetate), while others were only in 2-3 products (e.g. ethyl lactate and citral) (Figure 3). Of the 99 chemicals identified in the popular products, 28 appeared in at least 10 of 20 products, indicating that a subset of flavor chemicals is used frequently. Those chemicals that appeared in only one product are shown in Supplemental Table 5.

Data were also analyzed according to their chemical class (Figure 3 insert). Most flavor chemicals were esters, and many were terpenes, phenols, alcohols, ketones, and aldehydes. The “other” category included benzopyrone, pyrazine, pyrone, and thiazole. While not shown in Figure 3, some chemicals belong to more than one class, such as vanillin, which is both an aldehyde and phenol.

In Figure 4, the flavor chemical data were filtered to include only those refill fluids (15 of 20) that had at least one chemical at a concentration ≥ 1 mg/ml. Filtering at this level reduced the number of flavor chemicals from 99 to 18, which we further considered in this study.

Identification of cytotoxic EC refill fluids

The cytotoxicities of the 20 popular refill fluids were evaluated using the MTT assay, which measures mitochondrial reductase activity. Decreases in the MTT assay relative to untreated controls are indicative of cytotoxicity due to decreases in mitochondrial metabolism and/or cell survival. The concentrations required for a 30% (IC_{70}) and 50% (IC_{50}) reduction in the MTT assay were determined for each refill fluid (Figure 5; Supplemental Figure 1). Some products (e.g., “Bird Brains”) showed no cytotoxicity (Figure 5A; Supplemental Figure 1A). Most refill fluids (e.g., “Ho! Ho! Watermelon”) reached at least an IC_{70} (caused 30% inhibition vs control), indicating they were cytotoxic by ISO standard 10993-5 [18] (Figure 5B). Four refill fluids (“Dewberry Cream”, “Dewberry Cream” clone, “Mega Melons”, and “Kiberry Yogurt”) (Figures 5C, D, E) reached at least IC_{50} values (caused 50% inhibition vs. control), again indicating cytotoxicity. Figure 5E summarizes the cytotoxicity data relative to the untreated control for cells treated with a 1% concentration of each refill fluid. Table 1 shows the IC_{70} and IC_{50} values for all 20 products. When tested independently, propylene glycol, glycerol, and nicotine were not cytotoxic at concentrations found in the 1% refill fluid solutions, (Supplemental Figure 2).

Relationship between cytotoxicity and the total number and total concentration of flavor chemicals.

Cytotoxicity was examined as a function of the total number of flavor chemicals (Figure 6A) and total concentration of flavor chemicals (Figure 6B) in each product. The correlations (R^2) between cytotoxicity and the total number of flavor chemicals in a refill fluid or the total concentration of flavor chemicals in each product were 0.42 and 0.54, respectively. The p values of the correlation coefficients were 0.002 (Figure 6A) and 0.0002 (Figure 6B), indicating they were statistically significant.

Identification of cytotoxic flavor chemicals

Figure 7A allows a direct comparison of the two most cytotoxic authentic refill fluids and their corresponding clones. Total flavor concentrations in Dewberry Cream (27.9 mg/ml) and its clone (20.17 mg/ml) were similar; however, “Mega Melons” (authentic) had a higher total flavor concentration (14.59 mg/ml) than its clone (“Melon Mania”) (3.96 mg/ml). In no case were the flavor chemicals in the clones an exact match in number or concentration to their authentic counterpart.

We hypothesized that chemicals that were high in concentration in the upper region of Figure 7A would contribute to the cytotoxicity observed in the MTT assay. Six chemicals (ethyl maltol, maltol, vanillin, ethyl vanillin, benzyl alcohol, and furaneol) were > 1 mg/ml in “Dewberry Cream” and/or “Mega Melon” and slightly lower in the less toxic clones. Authentic standards of these chemicals were tested in the MTT assay using mNSC and human BEAS-2B cells (Figures 7B – 7G). The highest concentration for each authentic standard was chosen to match the highest concentration found in authentic “Dewberry Cream” and “Mega Melons”. In support of our hypothesis, all six authentic standards were cytotoxic at the concentrations found in the refill fluids. mNSC were slightly more sensitive than BEAS-2B to ethyl maltol and benzyl alcohol. For all other chemicals, dose-response curves were similar for the two cell types. Based on the IC_{50} data, the chemicals that were the most toxic from high to low were: ethyl maltol, furaneol, maltol, ethyl vanillin, vanillin, and benzyl alcohol.

Although their predicted toxicities based on the rat-oral data were lower than the chemicals in the above assays, ethyl butanoate, triacetin, acetoin, and ethyl acetate, were evaluated in a secondary MTT screen (Figures 7H - 7K). Both ethyl butanoate and triacetin were cytotoxic at the highest concentrations found in the 20 products, while the other two chemicals were not cytotoxic.

Figure 8 shows the cytotoxicity (% survival) for each of the refill fluids at 1%, the concentration of each flavor chemical at 1%, and the cytotoxicity of each flavor chemical based on the authentic standard data. In general, when the parent refill fluid was cytotoxic at 1%, it contained flavor chemicals that could account for its cytotoxicity (e.g., "Dewberry Cream" had a cytotoxic level of ethyl maltol). Exceptions to this, such as "North Shore", which was cytotoxic (51% of control) but did not have a cytotoxic level of flavor chemicals, suggest that our target list of chemicals does not contain some of the flavors that are used in refill fluids, that chemicals act additively or synergistically to produce cytotoxicity, or that another factor, such as a metal [19], caused cytotoxicity in this product.

Since refill fluids are used in ECs without dilution (100%), Figure 9 is included to show the actual concentration of each flavor chemical in the undiluted parent refill fluid and the cytotoxicity that would be predicted for each chemical at the actual concentration used by EC vapers to produce aerosol. At actual flavor concentrations, all refill fluids would be predicted to be cytotoxic. Ethyl maltol, furaneol, and maltol were always present at concentrations that would be cytotoxic, and these three chemicals were used frequently (maltol for example was in 18 of 20 products tested).

Relationship between the cytotoxicity of each refill fluid at a 1% concentration and each authentic standard chemical

Each highly cytotoxic refill fluid contained one or more of the six toxic flavor chemicals at concentrations that were as cytotoxic as authentic standards in the MTT assay (Figure 8). In general, moderately cytotoxic refill fluids had lower concentrations (e.g., “Lava Flow” and “WTF” clone) or non-cytotoxic concentrations (e.g., “Blueberry Hills” and “Unicorn Puke”) of the six toxic chemicals. Non-cytotoxic products or those that did not differ from the control by more than 30% either had none of the six toxic flavor chemicals (e.g., “Bird Brains”) or had low levels (e.g., “Overnight”). These data demonstrate a positive relationship between the concentration of ethyl maltol and the cytotoxicity of the refill fluids in which it was used.

The cytotoxicity of refill fluids (1% concentration) was plotted as a function of the flavor chemical concentration in each fluid at 1% (Figures 10A - J). Dots are color-coded to toxicity of the refill fluids (red = highly cytotoxic, blue = moderately cytotoxic, green = non-cytotoxic), and the letter code with each dot correlates to a refill fluid in Figure 8. The cytotoxicity and ethyl maltol concentrations in each fluid were highly correlated (R^2 = 0.93; p value = <0.0001). This high correlation occurs because ethyl maltol was the most cytotoxic of the chemicals tested and it maintained its toxicity when tested in a refill fluid. The correlation coefficient was also significant for ethyl vanillin (R^2 = 0.68; p value = 0.0033), maltol (R^2 = 0.502; p value = 0.0010) and vanillin (R^2 = 0.49; p value = 0.0028) but decreased and was not significant for the remainder of the toxic chemicals (Figures 10E - J).

DISCUSSION

The online survey and identification of the top-selling refill fluids in local and Internet shops showed that EC users prefer “Berries/Fruit/Citrus” flavors with “Sweet”, “Candy”, “Bakery/Dessert”, and “Breakfast Cereal” also being popular. In the local and online shops, all top-selling products were in the “Berries/Fruit/Citrus” category. This is the first time that flavor popularity has been assessed using three independent methods, which proved to agree with each other and with other flavor surveys [20-22]. Identification of the popular flavor categories enables research to focus on those that are most relevant to EC users. Our study also identified popular EC products that are cytotoxic, examined the flavor chemicals in the most popular selling refill fluids, and identified specific flavor chemicals that contribute to cytotoxicity at concentrations found in the refill fluids. The dominant flavor chemicals (those > 1 mg/ml) that we identified in popular refill fluids are important because some, such as ethyl maltol and vanillin, were used frequently at cytotoxic concentrations. Ethyl butanoate, an inexpensive fruity flavoring widely used in the food industry, was in all 20 products, in some cases at cytotoxic concentrations.

The total flavor chemical concentrations exceeded 1 mg/ml (about 0.1%) in all top-selling refill fluids, except in “Bird Brains” (0.63 mg/ml). It is possible that “Bird Brains” had additional flavor chemicals that were not on our target list, and hence not quantified. Also noteworthy, total flavor chemical concentrations exceeded that of nicotine (1.2-3.3 mg/ml) in 19 of 20 products. These data identify flavor chemicals as major constituents of popular refill fluids and further show that the concentrations of flavor chemicals used by manufacturers vary significantly among products.

The total number of flavor chemicals identified (N = 99) in the 20 popular refill fluids is relatively small considering that there are thousands from which manufacturers could choose [2]. These data suggest that EC refill fluid manufacturers use a small subset of the available flavor chemicals in their products, which should help focus future research in this area. The majority of the flavor chemicals were esters, a class that imparts fruity flavors and aromas [23], consistent with fruity and berry flavors being the most popular. Those flavor chemicals that were < 1 mg/ml were likely introduced as minor constituents or impurities in the flavor chemical ingredients used to compound the refill fluids or as accents in a more complex flavoring base.

Our study examined the toxicity of refill fluids and authentic standards of the pure flavor chemicals at concentrations similar to those found in refill fluids. 12 of 20 (60%) popular refill fluids produced an IC_{70} and four produced an IC_{50} in the MTT assay (Table 1). These 16 products (80% of total tested) would be classified as cytotoxic by ISO protocol #10993-5 [18]. While some earlier studies reported little cytotoxicity for refill fluids and their aerosols [24, 25], in our study and other recent papers, refill fluids frequently produced cytotoxicity when tested in vitro [8-10, 16, 26]. Our study further showed that the cytotoxicity of the refill fluids was correlated with the total number and the total concentration of flavor chemicals.

The cytotoxicity observed in most refill fluids was isolated to individual flavor chemicals that were tested as authentic standards. The overall hierarchy of potency for the 10 tested chemicals was: ethyl maltol > furaneol > maltol > ethyl vanillin > vanillin > benzyl alcohol > ethyl butanoate > triacetin > acetoin > ethyl acetate. The toxicity of refill fluids was correlated with the concentration of ethyl maltol, ethyl vanillin, maltol and vanillin, further supporting the idea that toxicity, as measured in the MTT assay, was due to the flavor chemicals. The low correlation coefficient of the remaining six chemicals (e.g., benzyl alcohol) and refill fluid toxicity was due to the presence of the more cytotoxic flavor chemicals, such as ethyl maltol. As an example, the products that had benzyl alcohol also had cytotoxic concentrations of ethyl maltol and maltol that reduced R^2 (Figure 10F).

As a further example of the correlation between flavor chemicals and cytotoxicity, “#Crawlie Tuesday” had a high number and high concentration of flavor chemicals but had only one flavor chemical (furaneol) that would cause cytotoxicity at a 1% concentration (Figure 8). Interestingly, the predicted cytotoxicity of #Crawlie Tuesday at 1% test solution based on its furaneol concentration would be ~$IC_{70,}$ and the actual measured inhibitory concentration for this product was 72.5%. The predicted and observed values are remarkably close further supporting the idea that toxicity can be attributed to furaneol.

While heating refill fluids can increase toxicity by formation of carbonyls through decomposition of flavor chemicals or glycerol/propylene glycol [27, 28], the authentic flavor chemicals examined in our study showed toxicity independent of reaction products produced by heat. Maltol and ethyl maltol are especially important as they were detected in > 50% of our refill fluids, and they were among the most toxic of the authentic standards tested. Other studies have also reported that vanillin, ethyl vanillin, and ethyl maltol are often used in EC products[28, 29], further supporting the idea that refill fluid manufacturers use a relatively small subset of the flavor chemicals available. Potential harm due to flavor chemicals is further supported by an *in vitro* study in which maltol increased secretion of IL-8 from BEAS-2B cells and decreased barrier function in human bronchial epithelial cells [30] and by animal studies in which maltol produced long-term adverse health effects in rats and dogs [31] and elicited liver and kidney damage in mice [32].

While we have focused on flavor chemicals present in refill fluids at high concentrations, some flavor chemicals may be harmful at low doses. 2,3-Butanedione (diacetyl) was present in 6 of 20 products at concentrations of 0.0187– 0.0989 mg/ml, and the related flavorings, acetoin and 3,2-pentanedione, were in 8 of 20 and 6 of 20 refill fluids, respectively. Others have reported 2,3-butanedione in refill fluids [15] and in EC aerosols [14], also at relatively low concentrations. Although these chemicals were generally minor constituents (< 1 mg/ml), 2,3-butanedione is of concern because it has been linked to *bronchiolitis obliterans* (popcorn lung) [12, 13].

Our data do not address the toxicity of flavor chemicals in aerosols. However, we have found that flavor chemicals transfer very efficiently into EC aerosols [33], and that refill fluid toxicity accurately predicts aerosol toxicity in about 74% of the cases [34]. These studies further showed that the solvents, in particular glycerol, increased toxicity when aerosols were produced in a tank style EC (iClear 16D dual coil clearomizer with Innokin battery) at higher power and that flavor chemicals produce potentially toxic reaction products when heated to create aerosols [34]. Thus, in aerosols, dominant flavor chemicals may combine with pyrolysis products from both the flavor chemicals and solvents to increase cytotoxicity beyond what was shown in the current study.

All 20 products would be predicted to be cytotoxic at 100% strength based on the concentrations of flavor chemicals in these products (Figure 9), and this would be relevant to dermal exposure, in which fluids are not diluted. Even “Bird Brains”, which had low levels of flavor chemicals, had sufficient maltol (0.032 mg/ml) to be cytotoxic at full strength. In fact, maltol was used in 18 of 20 products at concentrations that would be predicted to be cytotoxic in the undiluted refill fluid. However, the concentrations of the flavor chemicals reaching the lungs and other organs have not yet been directly measured in humans and are probably quite variable given the large differences reported in EC user puffing topography [35]. Table 2 summarizes the concentrations of flavor chemicals in refill fluids (maximum observed concentration is given for each chemical), the amount of each chemical a user would be exposed to if they inhaled 3.4 ml/day (average consumption reported previously) for 2 days [36], and how the in vitro IC_{70} and IC_{50} compare to the estimated consumption. As can be seen in Table 2, the intake of flavor chemicals is high enough to be a concern based on the *in vitro* cytotoxicity data.

Little is known about the specific effects of inhaled flavor chemicals on cells of the respiratory system or disease progression of EC users. Most toxicological work with flavor chemicals has been done on ingestion, and those studies that have evaluated inhalation toxicity have generally used animal models, not humans. Of the eight chemicals we tested in the MTT assay, only two have been examined in inhalation studies with rats, in which fatality was the endpoint[17]. For vanillin, inhalation of 41 mg/kg/2 hours was fatal in rats, whereas a much higher dose (3300 mg/kg) produced fatality by ingestion[17], demonstrating that for this example, the FEMA GRAS designation would not be valid for inhalation. The best characterized of the flavor chemicals with respect to human effect is diacetyl, which as mentioned above, has been linked to bronchiolitis obliterans in humans[12,13]. Diacetyl was present in EC refill fluids at relatively low concentrations, which nevertheless are high enough to be a concern. Many of the flavor chemicals in EC products are aldehydes, which are highly reactive and usually cause irritation and inflammation of the respiratory epithelium[17]. Cinnamaldehyde is particularly noteworthy as it is highly toxic *in vitro* at low concentrations[10, 33]. EC users have apparently experienced adverse health effects with its use as some bloggers have recommended avoiding products with cinnamon flavors, which are also known to rapidly etch plastic tanks, indicative of its reactivity.

The MTT assay is frequently used to evaluate cytotoxicity [37] and to provide information on the health of mitochondria. Because many lung diseases are characterized by defects in mitochondrial function [38], the MTT also provides insight into possible diseases that could be linked to flavor chemicals. For example, oxidative phosphorylation is often impaired in COPD, asthma, and lung cancer [38]. While there have been relatively few case reports related to EC use, those that do exist often include lung disease and most of these involve inflammation [5.] Mitochondria play a key role in lung homeostasis and proper functioning of lung immune cells [38], and one study has linked impairment of innate immune cell response to cinnamaldehyde [11], a flavor often used in EC products [10]. The limited data currently available demonstrate that flavor chemicals do affect mitochondrial function in vitro and establish the need for a better understanding of this finding on disease progression in EC users.

Our study was done using submerged cultures which are particularly valuable for screening purposes and for identification of those flavor chemicals that would be most interesting to study further in air-liquid interface systems, which we are currently doing, and in human inhalation studies. It will also be important in future work to determine if reaction products form heated flavor chemicals that could affect the cytotoxicity of aerosols and if flavor chemicals produce adverse effects in vivo.

Conclusions

“Berries/Fruits/Citrus” flavored refill fluids were the most popular in three independent methods of analysis. The 20 popular refill fluids contained 22 to 47 different flavor chemicals with their total concentrations ranging from 0.63 to 27.9 mg/ml.

Eighteen flavor chemicals were present in at least one refill fluid at a concentration >1 mg/ml. 80% of the 20 popular flavors were cytotoxic in the MTT assay. The four most cytotoxic refill fluids contained various combinations of the six chemicals (ethyl maltol, furaneol, maltol, ethyl vanillin, benzyl alcohol, and vanillin) that were cytotoxic as authentic standards. Most of these chemicals were present in the cytotoxic refill fluids at concentrations > 1 mg/ml. Maltol and ethyl maltol, which were highly toxic, were present in 19 and 13 of the 20 refill fluids, respectively. The cytotoxicity of refill fluids was directly correlated with ethyl maltol concentrations in the fluids. These data raise concerns about the safety of popular EC refill fluids as those tested all contained concentrations of flavor chemicals that would be cytotoxic at the concentration in the undiluted fluids (Figure 9). Although flavor chemicals have been used for many years in foods, their introduction into products that are heated and inhaled presents new potential health concerns. Our data may facilitate establishing concentration limits of the dominant flavor chemicals used in EC refill fluids and requirements for labeling the flavor chemicals included in each product.

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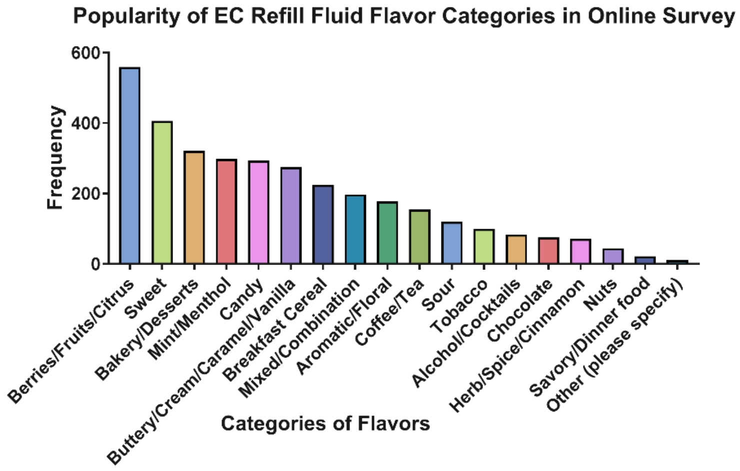
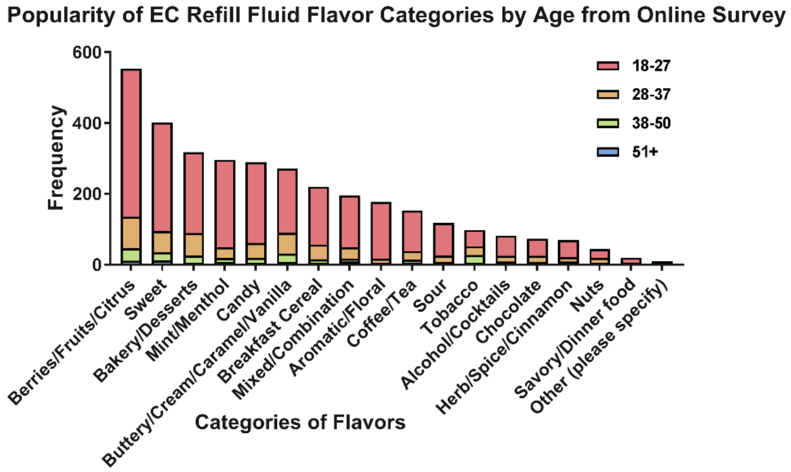
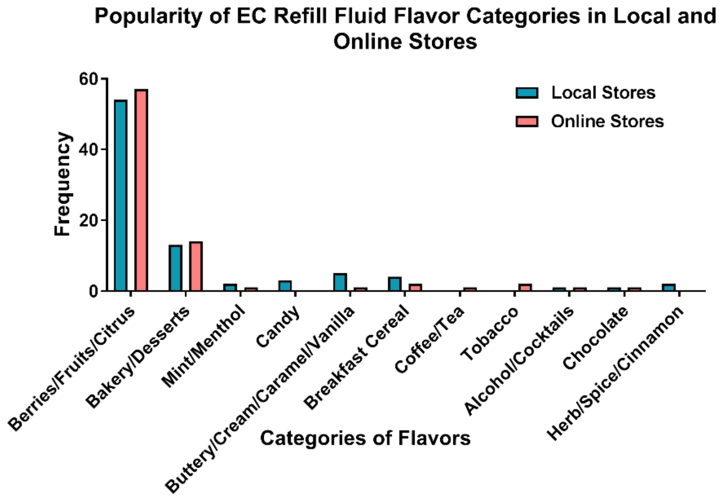

Popularity of EC Refill Fluid Flavor Categories in Online Survey
Frequency
600
400
200
0
Berries/Fruits/Citrus
Sweet
Bakery/Desserts
Mint/Menthol
Candy
Buttery/Cream/Caramel/Vanilla
Breakfast Cereal
Mixed/Combination
Aromatic/Floral
Coffee/Tea
Sour
Tobacco
Alcohol/Cocktails
Chocolate
Herb/Spice/Cinnamon
Nuts
Savory/Dinner food
Other (please specify)
Categories of Flavors

A.


Popularity of EC Refill Fluid Flavor Categories by Age from Online Survey
Frequency
600
400
200
0
18-27
28-37
38-50
51+
Berries/Fruits/Citrus
Sweet
Bakery/Desserts
Mint/Menthol
Candy
Buttery/Cream/Caramel/Vanilla
Breakfast Cereal
Mixed/Combination
Aromatic/Floral
Coffee/Tea
Sour
Tobacco
Alcohol/Cocktails
Chocolate
Herb/Spice/Cinnamon
Nuts
Savory/Dinner food
Other (please specify)
Categories of Flavors

B.


Popularity of EC Refill Fluid Flavor Categories in Local and Online Stores
Frequency
60
40
20
0
Local Stores
Online Stores
Berries/Fruits/Citrus
Bakery/Desserts
Mint/Menthol
Candy
Buttery/Cream/Caramel/Vanilla
Breakfast Cereal
Coffee/Tea
Tobacco
Alcohol/Cocktails
Chocolate
Herb/Spice/Cinnamon
Categories of Flavors

C.

Figure 5.1. Frequency distribution of popular flavors from survey, local shops, and online stores. (A) Results from the online survey. (B) Popularity of flavors among different age groups in the online survey. (C) Results from the local and online stores. Frequency on the *y*-axis refers to the number of times each flavor category (*x*-axis) appeared in the population.

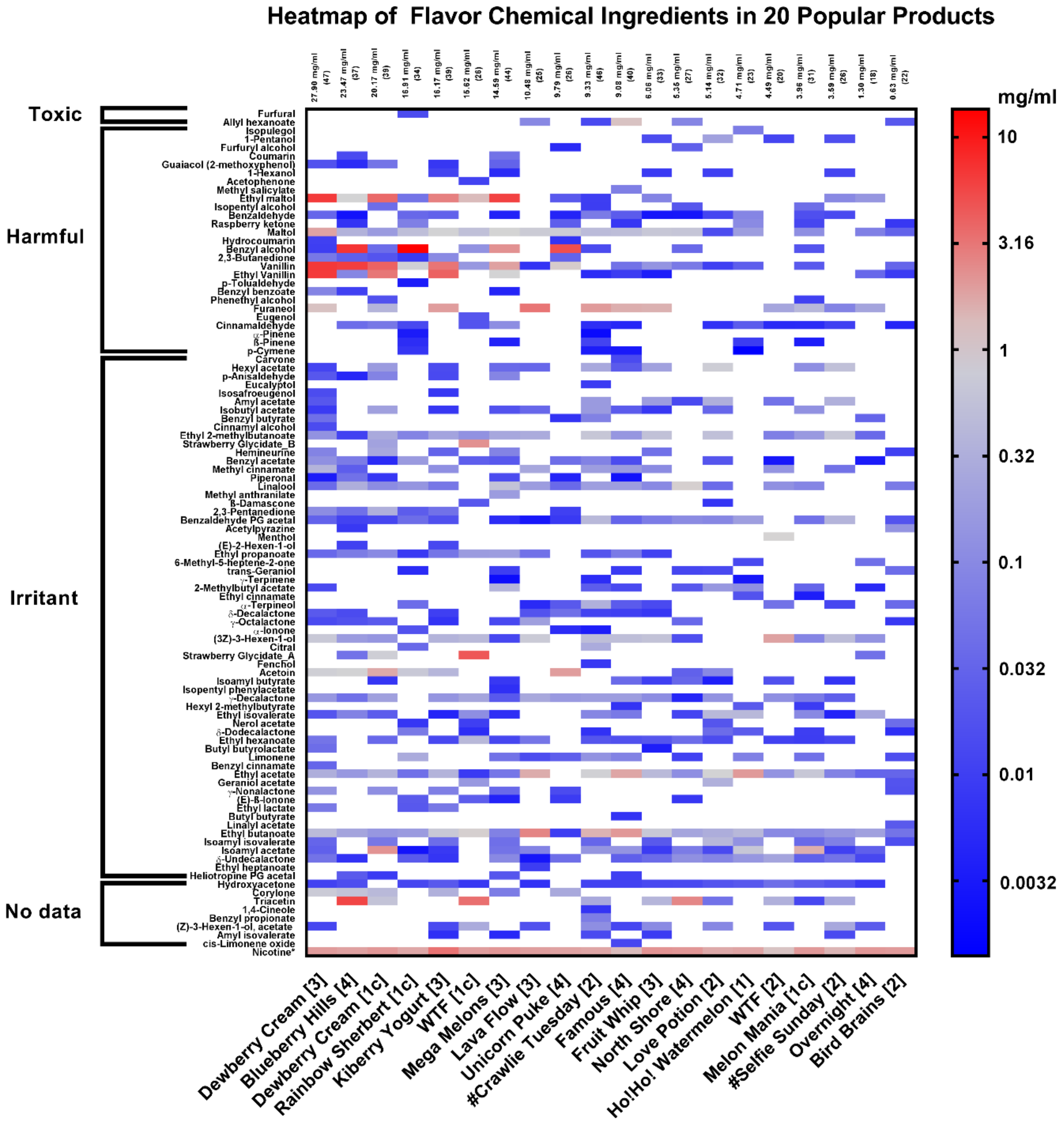
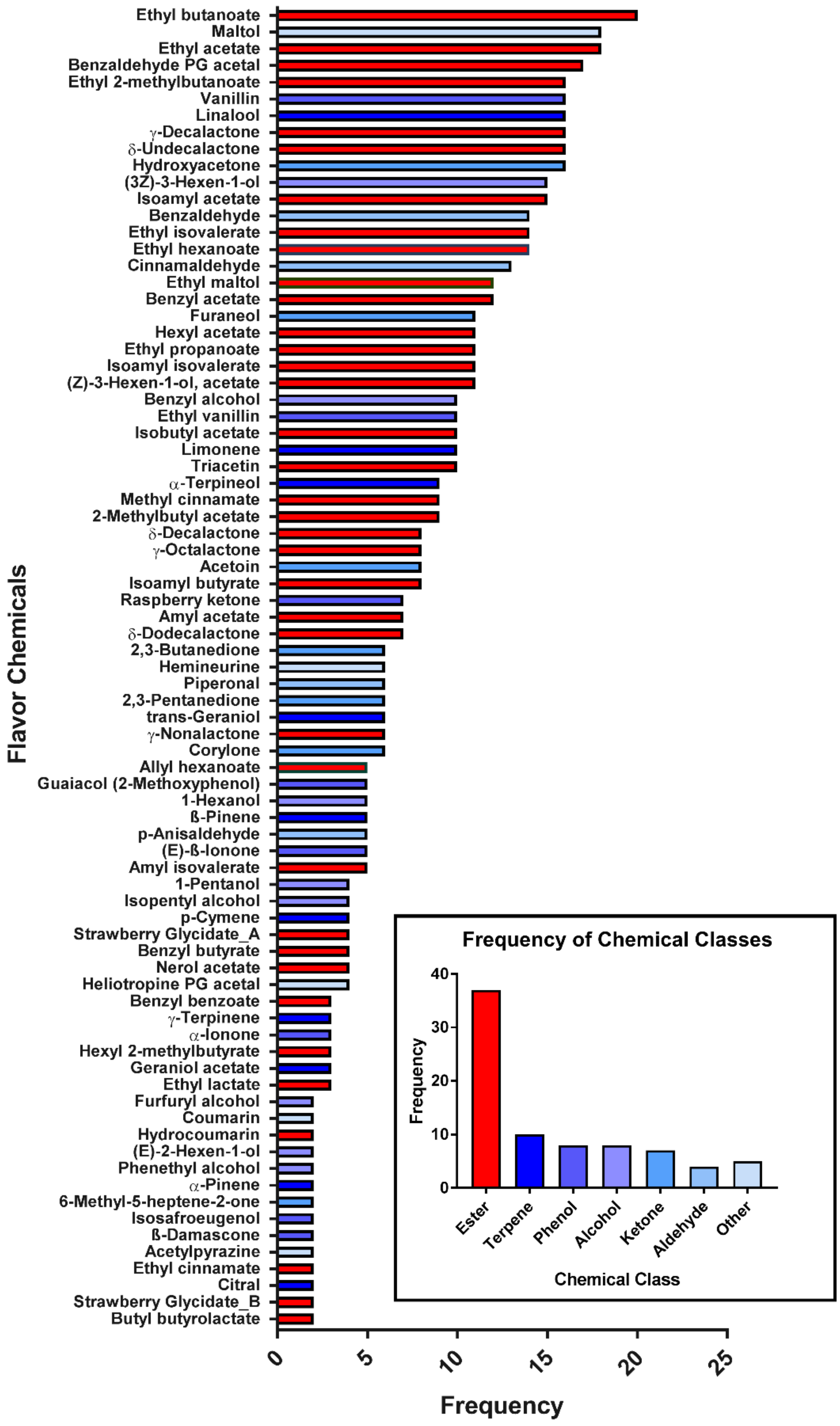


Figure 5.2. **Heat map showing flavor chemical concentrations in 20 popular refill fluids.** Chemicals are ordered on the *y*-axis according to their toxicity (based on LC_{50} data from oral exposure in rats) and within each class, they are ranked from most to least toxic. Products (*x*-axis) are ordered according to the weight (mg/ml) of all the flavor chemicals in each product with the highest concentration at the left. Numbers 1-4 with product names denote stores where refill fluids were purchased, and “C” indicates a cloned product. Rainbow Sherbet is a clone of Unicorn Puke and Melon Mania is a clone of Mega Melons. The total chemical concentration (mg/ml) and the number of individual chemicals is indicated at the top of each column. Nicotine, which is not a flavoring, is in the bottom row for comparison.



Frequency Distribution of Flavor Chemicals in 20 Popular Products

Figure 5.3. Frequency distribution of flavor chemicals within popular products and their chemical class. Chemicals are ranked according to their frequency in popular products for all data. The inset shows the class to which each chemical belongs.

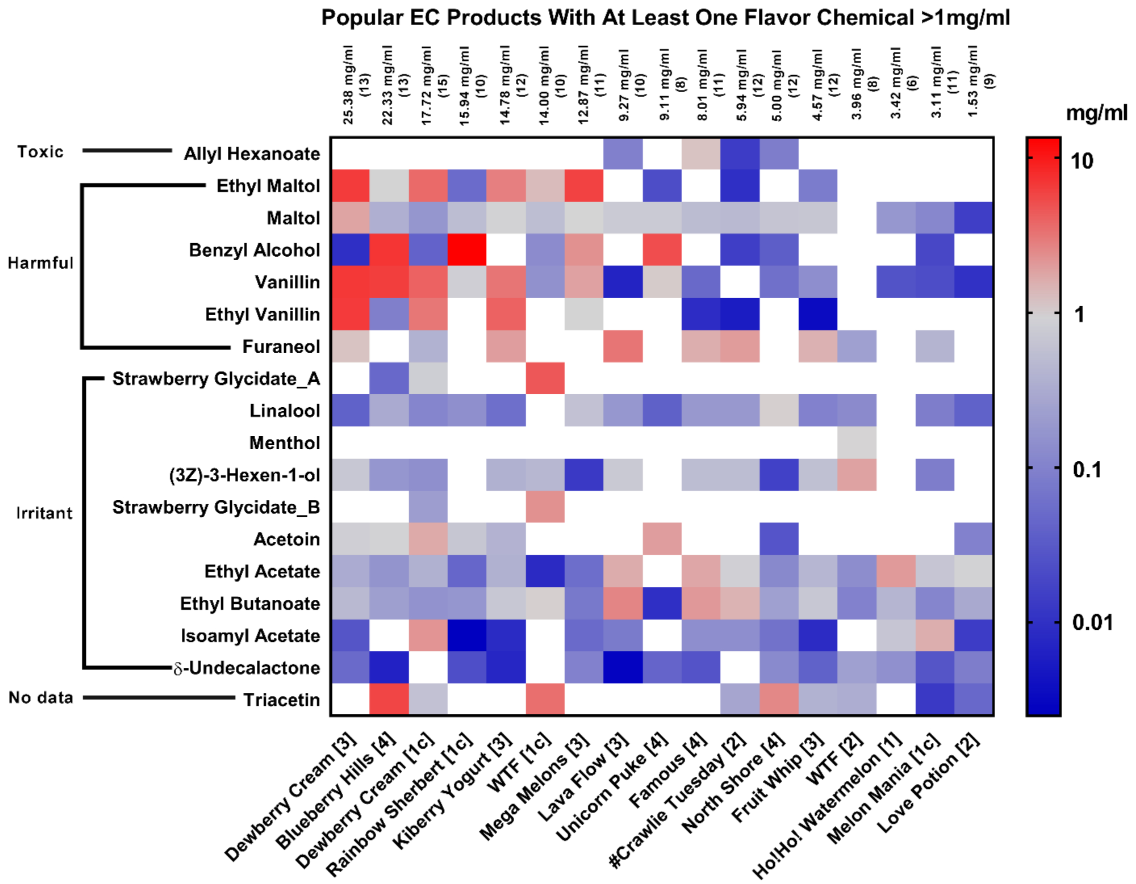


Figure 5.4. Heat map of popular EC refill fluids with at least one flavor chemical > 1 mg/ml. These products were considered the dominant flavor chemicals in the 20 popular refill fluids that were analyzed. They are ranked on the *y*-axis according to rat oral toxicity and on the *x*-axis according to total concentration (mg/ml) of the flavor chemicals.

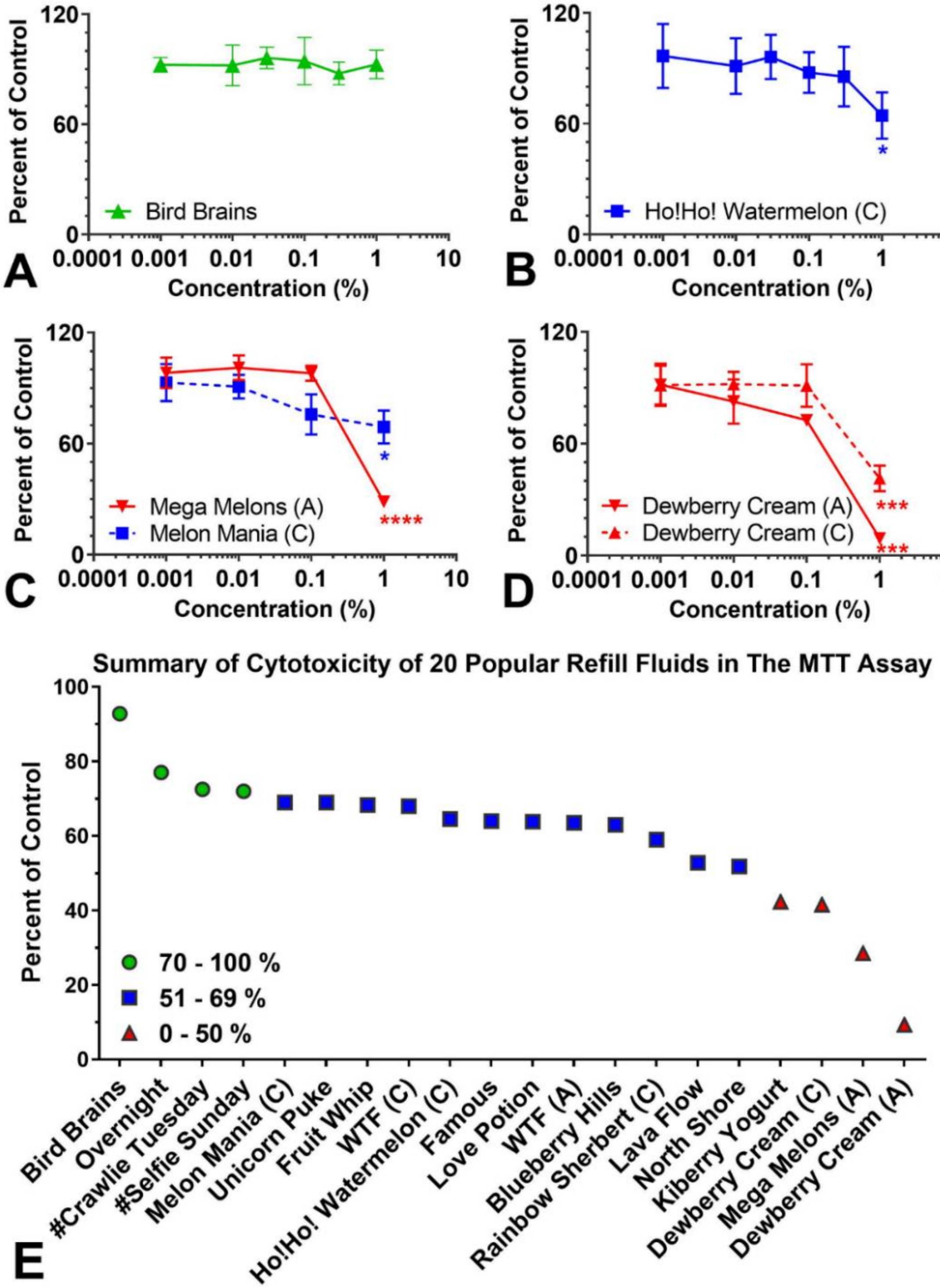

A
B
C
D
E
Percent of Control
Concentration (%)
0.0001 0.001 0.01 0.1 1 10
0
60
120
Bird Brains
Ho!Ho! Watermelon (C)
*
Mega Melons (A)
Melon Mania (C)
****
Dewberry Cream (A)
Dewberry Cream (C)
***
***
Summary of Cytotoxicity of 20 Popular Refill Fluids in The MTT Assay
0
20
40
60
80
100
70 - 100 %
51 - 69 %
0 - 50 %
Bird Brains
Overnight
#Crawlie Tuesday
#Selfie Sunday
Melon Mania (C)
Unicorn Puke
Fruit Whip
WTF (C)
Ho!Ho! Watermelon (C)
Famous
Love Potion
WTF (A)
Blueberry Hills
Rainbow Sherbert (C)
Lava Flow
North Shore
Kiberry Yogurt
Dewberry Cream (C)
Mega Melons (A)
Dewberry Cream (A)

Figure 5.5. Cytotoxic refill fluids identified using mNSC. (A-D) Representative MTT dose response curves for products that were not cytotoxic (A), cytotoxic reaching IC_{70} (B), and highly cytotoxic reaching IC_{50} (C, D). (E) Summary of cytotoxicity screening results showing products that had little effect (green dots), reached an IC_{70} (blue squares), or reached an IC_{50} (red triangles). The most cytotoxic products were Dewberry Cream, Dewberry Cream clone, Mega Melons, and Kiberry Yogurt. Rainbow Sherbet is a clone of Unicorn Puke and Melon Mania is a clone of Mega Melons. Each graph is the mean ± the std error of the mean for three independent experiments. * = $p<0.05$, *** = $p< 0.001$, **** = $p< 0.0001$

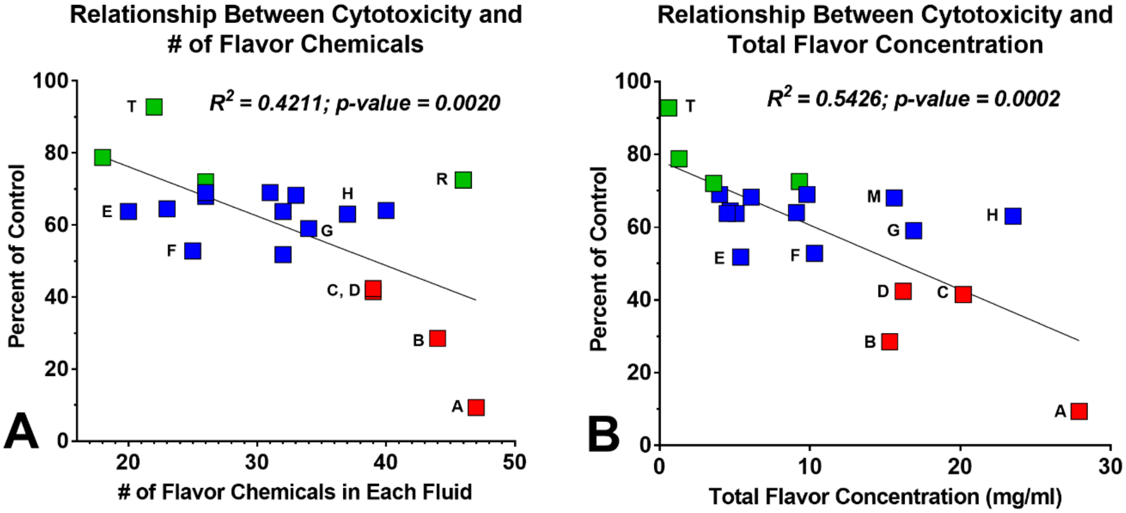


Figure 5.6. The relationship between cytotoxicity and the total number of favor chemicals and the total concentration of flavor chemicals. Cytotoxicity is plotted as a function of the total number of flavor chemicals (A) and the total concentration of flavor chemicals (B) in each of the popular refill fluids. Green dots indicate fluids that were not significantly cytotoxic, blue dots are fluids that reach an IC_{70}, and red dots are fluids that reached an IC_{50}. Letters with each point correspond to the products listed in Figure 8 and Figure 9.

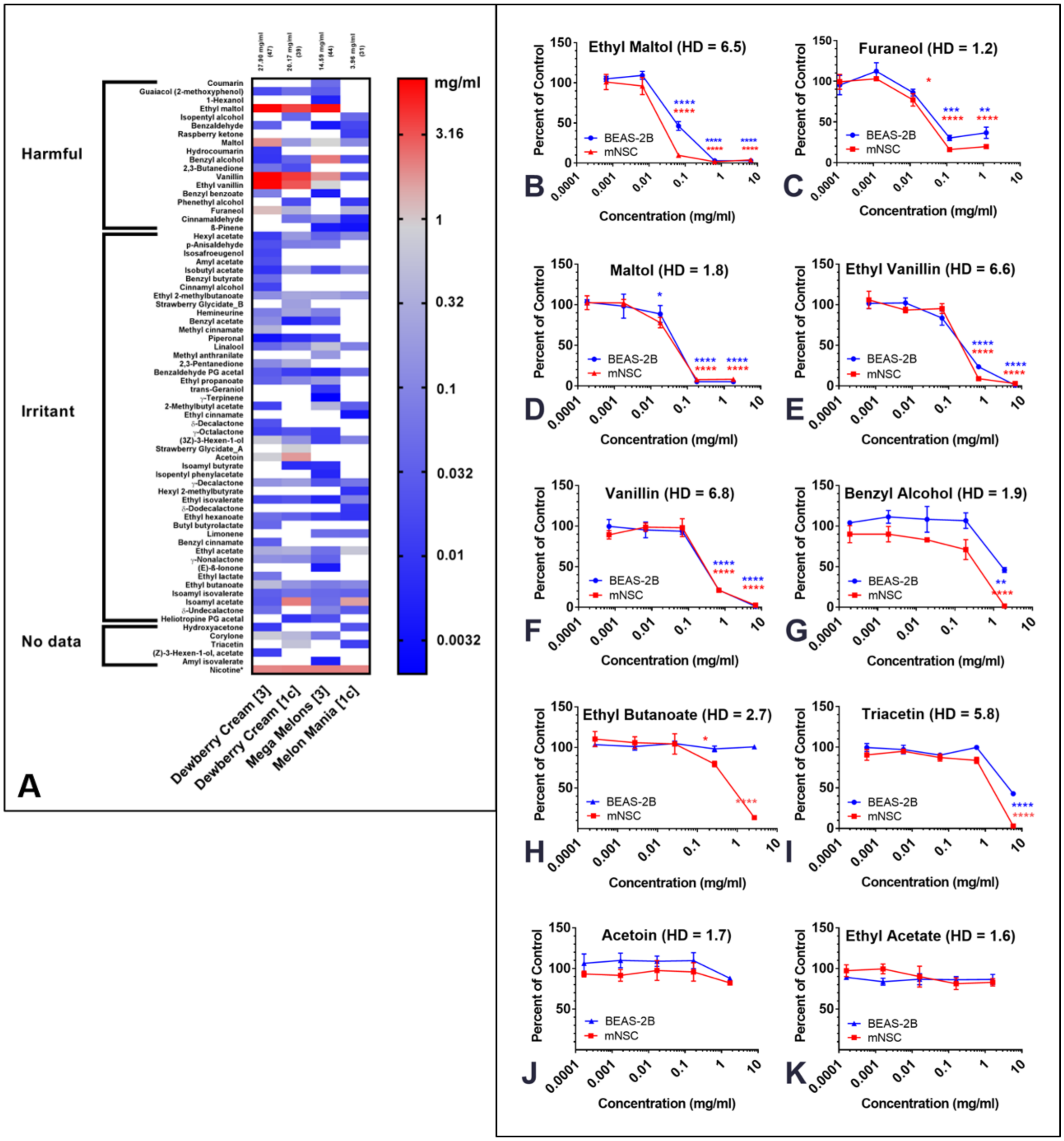
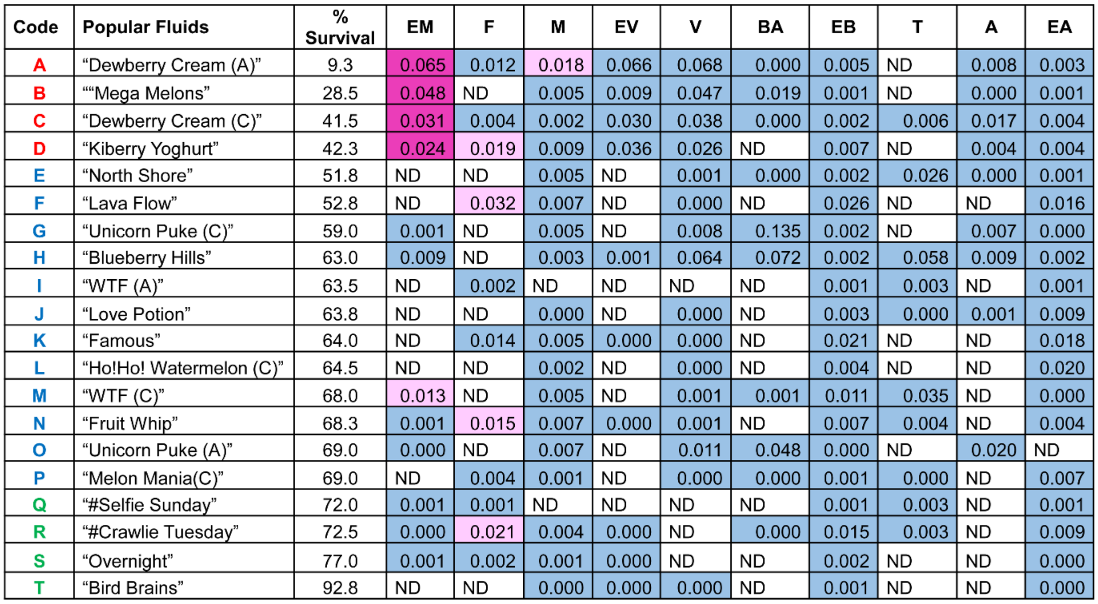
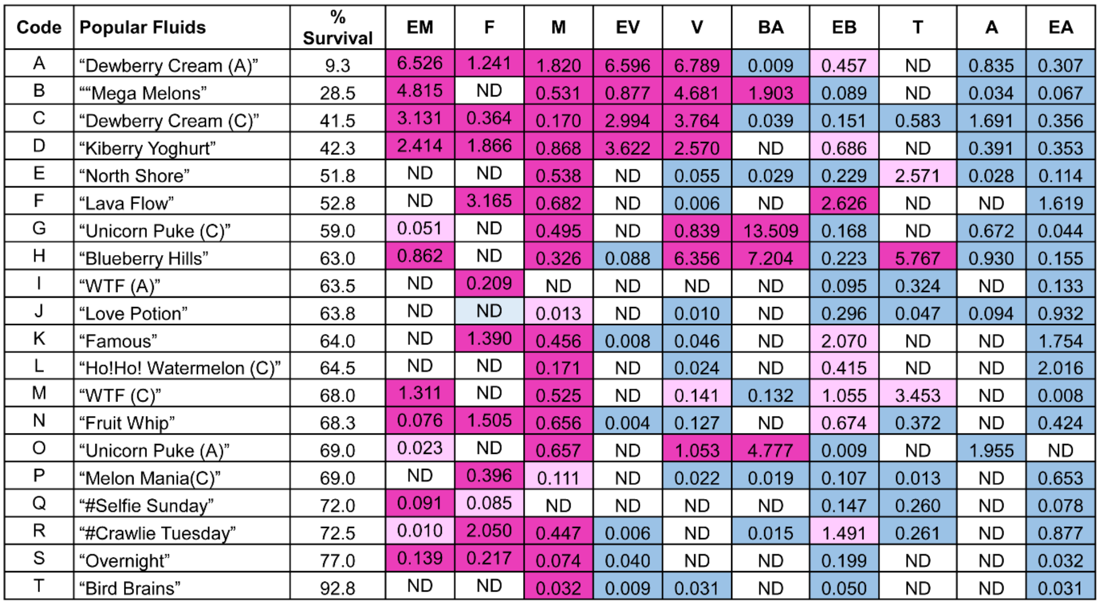

27.90 mg/ml (47)
20.17 mg/ml (39)
14.59 mg/ml (44)
3.96 mg/ml (31)
Harmful
Irritant
No data
Coumarin
Guaiacol (2-methoxyphenol)
1-Hexanol
Ethyl maltol
Isopentyl alcohol
Benzaldehyde
Raspberry ketone
Maltol
Hydrocoumarin
Benzyl alcohol
2,3-Butanedione
Vanillin
Ethyl vanillin
Benzyl benzoate
Phenethyl alcohol
Furaneol
Cinnamaldehyde
ß-Pinene
Hexyl acetate
p-Anisaldehyde
Isosafroeugenol
Amyl acetate
Isobutyl acetate
Benzyl butyrate
Cinnamyl alcohol
Ethyl 2-methylbutanoate
Strawberry Glycidate_B
Hemineurine
Benzyl acetate
Methyl cinnamate
Piperonal
Linalool
Methyl anthranilate
2,3-Pentanedione
Benzaldehyde PG acetal
Ethyl propanoate
trans-Geraniol
γ-Terpinene
2-Methylbutyl acetate
Ethyl cinnamate
δ-Decalactone
γ-Octalactone
(3Z)-3-Hexen-1-ol
Strawberry Glycidate_A
Acetoin
Isoamyl butyrate
Isopentyl phenylacetate
γ-Decalactone
Hexyl 2-methylbutyrate
Ethyl isovalerate
δ-Dodecalactone
Ethyl hexanoate
Butyl butyrolactate
Limonene
Benzyl cinnamate
Ethyl acetate
γ-Nonalactone
(E)-ß-Ionone
Ethyl lactate
Ethyl butanoate
Isoamyl isovalerate
Isoamyl acetate
δ-Undecalactone
Heliotropine PG acetal
Hydroxyacetone
Corylone
Triacetin
(Z)-3-Hexen-1-ol, acetate
Amyl isovalerate
Nicotine*
Dewberry Cream [3]
Dewberry Cream [1c]
Mega Melons [3]
Melon Mania [1c]
mg/ml
3.16
1
0.32
0.1
0.032
0.01
0.0032
A
Ethyl Maltol (HD = 6.5)
Furaneol (HD = 1.2)
Maltol (HD = 1.8)
Ethyl Vanillin (HD = 6.6)
Vanillin (HD = 6.8)
Benzyl Alcohol (HD = 1.9)
Ethyl Butanoate (HD = 2.7)
Triacetin (HD = 5.8)
Acetoin (HD = 1.7)
Ethyl Acetate (HD = 1.6)
Percent of Control
Concentration (mg/ml)
BEAS-2B
mNSC
0.0001
0.001
0.01
0.1
1
10
0
50
100
150
B
C
D
E
F
G
H
I
J
K

Figure 5.7. Chemicals in “Dewberry Cream” and “Mega Melons” and their cytotoxicity. (A) Heat map showing the flavor chemicals and their concentrations in the two most cytotoxic refill fluids and their clones. Chemicals are ordered on the *y*-axis according to their toxicity and within each class, they are ranked from most to least toxic. Products (*x*-axis) are ranked according to the total flavor chemicals concentration with the highest on the left. The total flavor chemical concentration and number of individual flavor chemicals are indicated at the top of the heat map. Nicotine is in the bottom row for comparison. (B-G) Dose response curves of authentic standard chemicals present in the highest concentrations in the two most toxic refill fluids and their clones. (H-K) Dose response curves for four chemicals frequently used or present at over 1 mg/ml in refill fluids. Each graph is the mean ± the standard error of the mean for three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p< 0.001$, **** = $p< 0.0001$. HD = high dose tested.



Code	Popular Fluids	% Survival	EM	F	M	EV	V	BA	EB	T	A	EA
A	“Dewberry Cream (A)”	9.3	0.065	0.012	0.018	0.066	0.068	0.000	0.005	ND	0.008	0.003
B	““Mega Melons”	28.5	0.048	ND	0.005	0.009	0.047	0.019	0.001	ND	0.000	0.001
C	“Dewberry Cream (C)”	41.5	0.031	0.004	0.002	0.030	0.038	0.000	0.002	0.006	0.017	0.004
D	“Kiberry Yoghurt”	42.3	0.024	0.019	0.009	0.036	0.026	ND	0.007	ND	0.004	0.004
E	“North Shore”	51.8	ND	ND	0.005	ND	0.001	0.000	0.002	0.026	0.000	0.001
F	“Lava Flow”	52.8	ND	0.032	0.007	ND	0.000	ND	0.026	ND	ND	0.016
G	“Unicorn Puke (C)”	59.0	0.001	ND	0.005	ND	0.008	0.135	0.002	ND	0.007	0.000
H	“Blueberry Hills”	63.0	0.009	ND	0.003	0.001	0.064	0.072	0.002	0.058	0.009	0.002
I	“WTF (A)”	63.5	ND	0.002	ND	ND	ND	ND	0.001	0.003	ND	0.001
J	“Love Potion”	63.8	ND	ND	0.000	ND	0.000	ND	0.003	0.000	0.001	0.009
K	“Famous”	64.0	ND	0.014	0.005	0.000	0.000	ND	0.021	ND	ND	0.018
L	“Ho!Ho! Watermelon (C)”	64.5	ND	ND	0.002	ND	0.000	ND	0.004	ND	ND	0.020
M	“WTF (C)”	68.0	0.013	ND	0.005	ND	0.001	0.001	0.011	0.035	ND	0.000
N	“Fruit Whip”	68.3	0.001	0.015	0.007	0.000	0.001	ND	0.007	0.004	ND	0.004
O	“Unicorn Puke (A)”	69.0	0.000	ND	0.007	ND	0.011	0.048	0.000	ND	0.020	ND
P	“Melon Mania(C)”	69.0	ND	0.004	0.001	ND	0.000	0.000	0.001	0.000	ND	0.007
Q	“#Selfie Sunday”	72.0	0.001	0.001	ND	ND	ND	ND	0.001	0.003	ND	0.001
R	“#Crawlie Tuesday”	72.5	0.000	0.021	0.004	0.000	ND	0.000	0.015	0.003	ND	0.009
S	“Overnight”	77.0	0.001	0.002	0.001	0.000	ND	ND	0.002	ND	ND	0.000
T	“Bird Brains”	92.8	ND	ND	0.000	0.000	0.000	ND	0.001	ND	ND	0.000

Figure 5.8. Concentrations (mg/ml) of flavor chemicals in 1% refill fluids and their cytotoxicity. Color code indicates the cytotoxicity of flavor chemicals at the concentrations found in 1% refill fluids. Magenta = concentrations that would reach an IC_{50}; Light pink = concentrations that would reach an IC_{70}, Blue = no cytotoxic effect. ND = indicates chemical was not detected in the GC-MS analysis. Code alphabet colors match summary of cytotoxicity of popular refill fluids in the MTT assay (Figure 5E). Red = 0 - 50%, Blue = 51 - 69%, and Green = 70 - 100%. Flavor names: EM = ethyl maltol; F = furaneol; M = maltol; EV = ethyl vanillin; V = vanillin; BA = benzyl alcohol; EB = ethyl butanoate; T = triacetin; A = acetoin; EA = ethyl acetate



Code	Popular Fluids	% Survival	EM	F	M	EV	V	BA	EB	T	A	EA
A	“Dewberry Cream (A)”	9.3	6.526	1.241	1.820	6.596	6.789	0.009	0.457	ND	0.835	0.307
B	““Mega Melons”	28.5	4.815	ND	0.531	0.877	4.681	1.903	0.089	ND	0.034	0.067
C	“Dewberry Cream (C)”	41.5	3.131	0.364	0.170	2.994	3.764	0.039	0.151	0.583	1.691	0.356
D	“Kiberry Yoghurt”	42.3	2.414	1.866	0.868	3.622	2.570	ND	0.686	ND	0.391	0.353
E	“North Shore”	51.8	ND	ND	0.538	ND	0.055	0.029	0.229	2.571	0.028	0.114
F	“Lava Flow”	52.8	ND	3.165	0.682	ND	0.006	ND	2.626	ND	ND	1.619
G	“Unicorn Puke (C)”	59.0	0.051	ND	0.495	ND	0.839	13.509	0.168	ND	0.672	0.044
H	“Blueberry Hills”	63.0	0.862	ND	0.326	0.088	6.356	7.204	0.223	5.767	0.930	0.155
I	“WTF (A)”	63.5	ND	0.209	ND	ND	ND	ND	0.095	0.324	ND	0.133
J	“Love Potion”	63.8	ND	ND	0.013	ND	0.010	ND	0.296	0.047	0.094	0.932
K	“Famous”	64.0	ND	1.390	0.456	0.008	0.046	ND	2.070	ND	ND	1.754
L	“Ho!Ho! Watermelon (C)”	64.5	ND	ND	0.171	ND	0.024	ND	0.415	ND	ND	2.016
M	“WTF (C)”	68.0	1.311	ND	0.525	ND	0.141	0.132	1.055	3.453	ND	0.008
N	“Fruit Whip”	68.3	0.076	1.505	0.656	0.004	0.127	ND	0.674	0.372	ND	0.424
O	“Unicorn Puke (A)”	69.0	0.023	ND	0.657	ND	1.053	4.777	0.009	ND	1.955	ND
P	“Melon Mania(C)”	69.0	ND	0.396	0.111	ND	0.022	0.019	0.107	0.013	ND	0.653
Q	“#Selfie Sunday”	72.0	0.091	0.085	ND	ND	ND	ND	0.147	0.260	ND	0.078
R	“#Crawlie Tuesday”	72.5	0.010	2.050	0.447	0.006	ND	0.015	1.491	0.261	ND	0.877
S	“Overnight”	77.0	0.139	0.217	0.074	0.040	ND	ND	0.199	ND	ND	0.032
T	“Bird Brains”	92.8	ND	ND	0.032	0.009	0.031	ND	0.050	ND	ND	0.031

Figure 5.9. Projected cytotoxicity of flavor chemicals at concentrations (mg/ml) found in refill fluids. Color code indicates the projected cytotoxicity of flavor chemicals at the concentrations found in refill fluids. Magenta = concentrations that would reach an IC_{50}; Light pink = concentrations that would reach an IC_{70}, Blue = no cytotoxic effect. ND = indicates chemical was not detected in the GC-MS analysis. Flavor names: EM = ethyl maltol; F = furaneol; M = maltol; EV = ethyl vanillin; V = vanillin; BA = benzyl alcohol; EB = ethyl butanoate; T = triacetin; A = acetoin; EA = ethyl acetate.

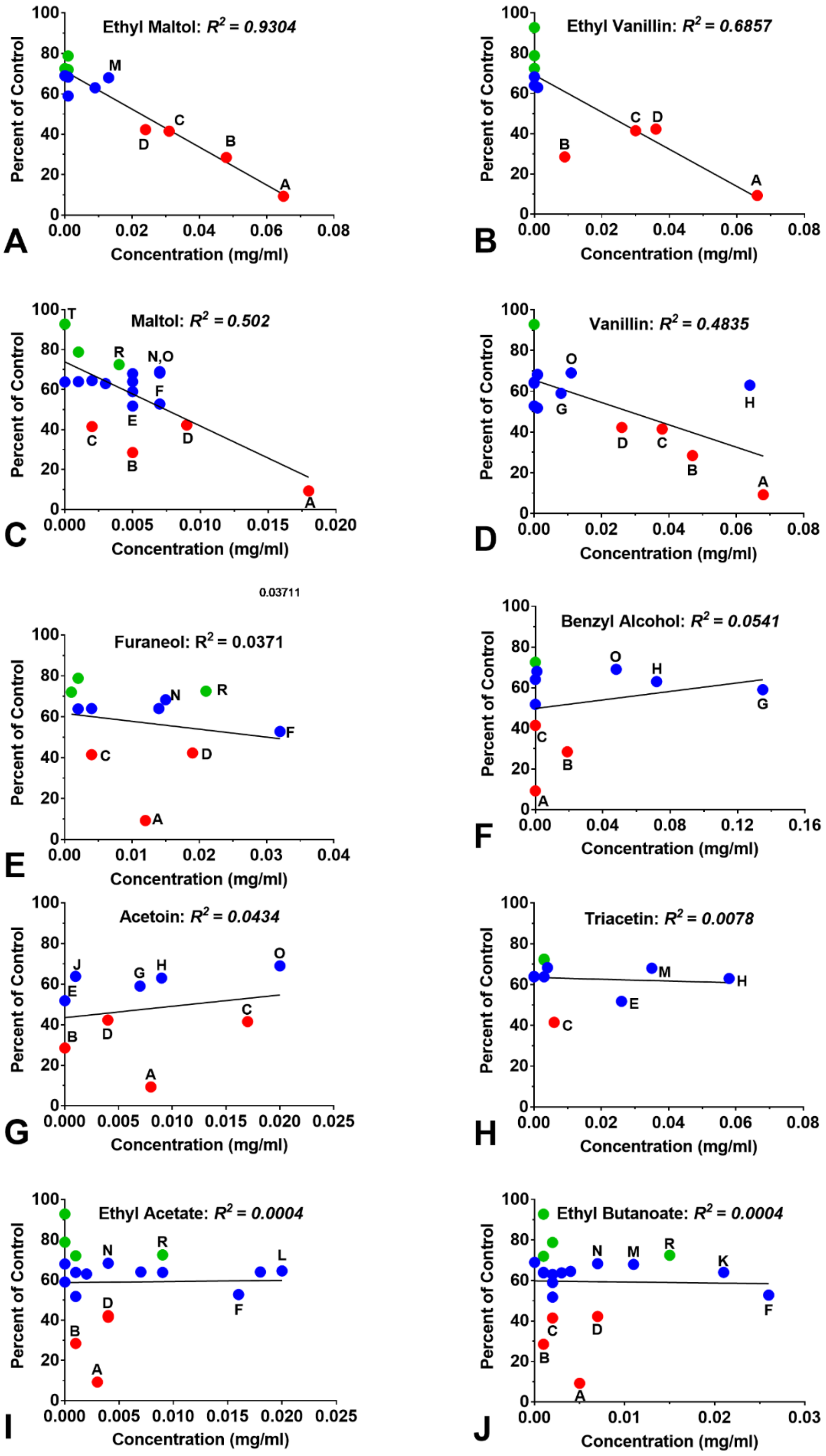

A
Ethyl Maltol: $R^2 = 0.9304$
Percent of Control
Concentration (mg/ml)
M
C
D
B
A
B
Ethyl Vanillin: $R^2 = 0.6857$
Percent of Control
Concentration (mg/ml)
C
D
B
A
C
Maltol: $R^2 = 0.502$
Percent of Control
Concentration (mg/ml)
T
R
N,O
F
E
C
D
B
A
D
Vanillin: $R^2 = 0.4835$
Percent of Control
Concentration (mg/ml)
O
G
H
D
C
B
A
0.03711
E
Furaneol: $R^2 = 0.0371$
Percent of Control
Concentration (mg/ml)
N
R
F
C
D
A
F
Benzyl Alcohol: $R^2 = 0.0541$
Percent of Control
Concentration (mg/ml)
O
H
G
C
B
A
G
Acetoin: $R^2 = 0.0434$
Percent of Control
Concentration (mg/ml)
J
E
G
H
O
B
D
C
A
H
Triacetin: $R^2 = 0.0078$
Percent of Control
Concentration (mg/ml)
M
H
E
C
I
Ethyl Acetate: $R^2 = 0.0004$
Percent of Control
Concentration (mg/ml)
N
R
L
D
F
B
A
J
Ethyl Butanoate: $R^2 = 0.0004$
Percent of Control
Concentration (mg/ml)
N
M
R
K
F
C
D
B
A

Figure 5.10. Relationship between the cytotoxicity of each refill fluid at 1% concentration and the concentrations of each authentic standard chemical. Green dots indicate fluids that were not significantly cytotoxic, blue dots are fluids that reach an $IC_{70,}$ and red dots are fluids that reached an IC_{50}. Letters associated with dots correspond to products in Figure 8. Because refill fluids are mixtures of cytotoxic chemicals, only ethyl maltol (the most toxic of the authentic standards) had a high correlation coefficient. The p values for ethyl maltol, maltol, ethyl vanillin and vanillin indicate that the correlations are statistically significant. Correlation coefficients for the other chemicals were affected by the presence of ethyl maltol.

Table 5.1: Inhibitory concentrations (IC$_{70}$ and IC$_{50}$) of EC Refill Fluids

Code	***Popular Fluids***	**mNSC**	
		IC$_{70}$[1](mg/ml)	**IC$_{50}$[2](mg/ml)**
A	“Dewberry Cream (A)”	0.13	0.18
B	“Mega Melons”	0.42	0.59
C	“Dewberry Cream (C)”	0.46	0.72
D	“Kiberry Yoghurt”	0.43	0.71
E	“North Shore”	0.36	>1
F	“Lava Flow”	0.55	>1
G	“Unicorn Puke (C)”	0.37	>1
H	“Blueberry Hills”	0.61	>1
I	“WTF (A)”	0.64	>1
J	“Love Potion”	0.64	>1
K	“Famous”	0.57	>1
L	“Ho!Ho! Watermelon (C)”	0.68	>1
M	“WTF (C)”	0.96	>1
N	“Fruit Whip”	0.90	>1
O	“Unicorn Puke (A)”	0.97	>1
P	“Melon Mania (C)”	0.84	>1
Q	“#Selfie Sunday”	>1	>1
R	“#Crawlie Tuesday”	>1	>1
S	“Overnight”	>1	>1
T	“Bird Brains”	>1	>1

[1] Code alphabet colors matches summary of cytotoxicity of popular refill fluids in the MTT assay (Figure 5E). Red = 0 – 50%, Blue = 51-69%, and Green = 70 -100%

[2] IC$_{70}$ values read directly off the dose response curves.

[3] IC$_{50}$ values obtained after non-linear fit using log (inhibitor) vs. normalized response - variable slope model

Table 5.2: Extrapolated Daily Consumption of Flavor Chemicals by EC Users

Flavor Chemicals	Concentration in Refill Fluids (mg/ml)[1]	Projected Human Consumption (mg/ 2 day)[2]	mNSC		BEAS-2B	
			IC_{70}[3] (mg/ml)	IC_{50}[4] (mg/ml)	IC_{70} (mg/ml)	IC_{50} (mg/ml)
Ethyl Maltol	6.5	44.2	0.01	0.03	0.03	0.06
Furaneol	1.2	8.2	0.02	0.03	0.03	0.11
Maltol	1.8	12.2	0.02	0.04	0.03	0.04
Ethyl Vanillin	6.6	44.8	0.13	0.24	0.11	0.25
Vanillin	6.8	46.2	0.15	0.38	0.15	0.32
Benzyl Alcohol	1.9	13	0.20	0.31	0.72	1.87
Ethyl Butanoate	2.7	18.4	0.37	0.72	> 2.7	> 2.7
Triacetin	5.8	39.4	0.84	1.24	4.30	5.20
Acetoin	1.7	11.6	> 1.7	> 1.7	> 1.7	> 1.7
Ethyl Acetate	1.6	10.8	> 1.6	>1.6	> 1.6	> 1.6

[1] mg/ml = highest concentration of flavor chemicals in the two most cytotoxic refill fluids.

[2] mg/2 day = exposures based on consumption of 3.4 mL of EC refill fluid over a 2-day period.

[4] IC_{70} = values read directly off the dose response curves.

[3] IC_{50} = values obtained after non-linear fit using log (inhibitor) vs. normalized response - variable slope model

Chapter 6

Electronic Cigarette Refill Fluids Sold Worldwide: Flavor Chemical Composition, Toxicity, and Hazard Analysis

Omaiye et al., 2020

Chem Res Toxicol. 2020 21;33(12):2972-2987

ABSTRACT

Flavor chemicals in electronic cigarette fluids (ECs), which may negatively impact human health, have been studied in a limited number of countries/locations. To gain an understanding of how the composition and concentrations of flavor chemicals in ECs are influenced by product sale location, we evaluated refill fluids manufactured by one company (Ritchy LTD) and purchased worldwide. Flavor chemicals were identified and quantified using gas chromatography-mass spectrometry (GC-MS). We then screened the fluids for their effects on cytotoxicity (MTT assay) and proliferation (live-cell imaging) and tested authentic standards of specific flavor chemicals to identify those that were cytotoxic at concentrations found in refill fluids. One hundred twenty-six flavor chemicals were detected in 103 bottles of refill fluid, and their number per/bottle ranged from 1 - 50 based on our target list. Two products had none of the flavor chemicals on our target list, nor did they have any non-targeted flavor chemicals. Twenty-eight flavor chemicals were present at concentrations ≥ 1 mg/mL in at least one product, and 6 of these were present at concentrations ≥ 10 mg/mL. The total flavor chemical concentration was ≥ 1 mg/mL in 70% of the refill fluids and ≥ 10 mg/mL in 26%. For sub-brand duplicate bottles purchased in different countries, flavor chemical concentrations were similar and induced similar responses in the in vitro assays (cytotoxicity and cell growth inhibition). The levels of furaneol, benzyl alcohol, ethyl maltol, ethyl vanillin, corylone, and vanillin were significantly correlated with cytotoxicity. The margin of exposure calculations showed that pulegone and estragole levels were high enough in some products to present a non-trivial calculated risk for cancer. Flavor chemical concentrations in refill fluids often exceeded concentrations permitted in other consumer products. These data

support the regulation of flavor chemicals in EC products to reduce their potential for producing both cancer and non-cancer toxicological effects.

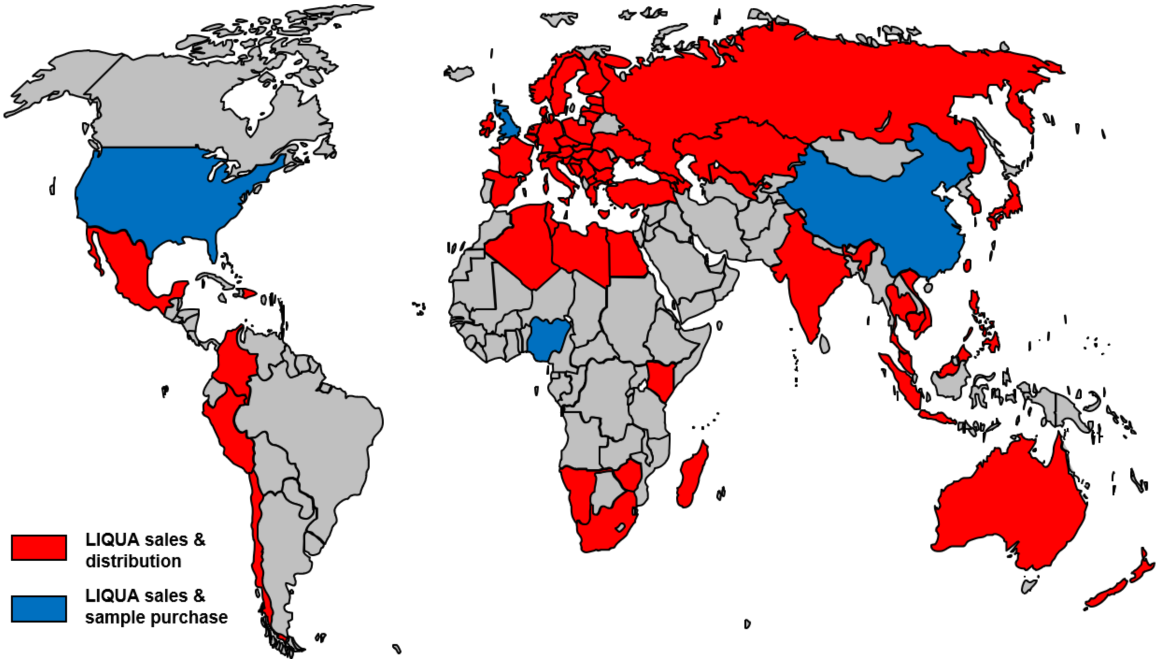


Figure 6.1. Graphical abstract

INTRODUCTION

Adverse health effects have been linked to electronic cigarette (EC) use in prior experimental studies on cells, animals, and humans,[1,2] case reports,[3] and Internet posts.[4,5] The recent epidemic of "electronic cigarette or vaping product use associated lung injury" (EVALI) has further heightened concerns about the safety of ECs.[6-9] The Centers for Disease Control and Prevention (CDC) suggested that poor quality counterfeit and black-market products are linked to some EVALI cases[10] and further recommended that vaping products not be used until the causes of EVALI are determined.[11] We have previously shown that some EC refill fluids contain very high concentrations of some flavor chemicals[12,13] and that the presence of some flavor chemicals at high levels is significantly correlated with cytotoxicity.[14] Although flavor chemicals have not been directly linked to EVALI, we did previously conclude that the high concentrations of flavor chemicals used in some EC refill fluids may cause adverse health effects.[13,15] While many flavor chemicals in EC products are GRAS (generally regarded as safe) for ingestion; their safety has not been evaluated for inhalation.[16] Some EC products have flavor chemical concentrations that far exceed those acceptable for ingestion, for example, we have found cinnamaldehyde in one product at 343 mg/mL.[13]

Most prior studies on EC flavor chemicals have been done using products purchased in one country, often the USA, and have generally focused on identification only. In this study, all products were manufactured by one company, and purchases were made in four different countries. We compared the flavor chemicals in each product to determine: (1) if there were variations in content and concentration with country, (2) if products were cytotoxic, (3) if specific flavor chemicals contributed to cytotoxicity, (4) if any flavor chemicals or co-constituents were present in high enough concentrations to be a risk factor for cancer and (5) how flavor chemicals in the current study compared to those we have examined previously.

MATERIALS AND METHODS

Product Selection and Collection

105 LIQUA brand EC refill fluids manufactured by Ritchy LTD (www.ritchy.com)[17] were evaluated. Products were purchased in four countries (NG = Nigeria, US = the United States, UK = the United Kingdom, and CN = China) chosen to represent different geographical regions and to allow comparison between varying levels of quality control and regulation of consumer products. Within countries, states/provinces are designated as follows: KS = Kansas, USA; CA = California, USA; LG = Lagos, Nigeria; GB = Great Britain, UK; GD = Guangdong, China; and XE = Xiamen, China. Within states/provinces, duplicate bottles are indicated numerically, e.g., 1, 2. EC refill fluids were stored at 4 °C in the dark until analyzed.

Evaluation and Quantification of Flavor Chemicals using GC/MS

For each refill fluid, 50 µl was dissolved in 0.95 ml of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ). Chemical analysis was performed with an Agilent 5975C GC/MS system (Santa Clara, CA) using internal standard-based calibration procedures and methods previously described in detail.[18,19] The method analyzes 177 flavor chemicals plus nicotine.

Culturing of mNSC and BEAS-2B Cells

Mouse neural stem cells (mNSC) are sensitive to EC refill fluids,[20] amenable to high-throughput screening, and are an excellent model for neurological development. mNSC were cultured in Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin CA) containing growth medium prepared using methods previously described.[19] For the MTT experiments, cell concentrations were determined using a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Chino, California, USA)-based standard curve, and single cells were plated at 1500 cells/well in 96-well plates. For live-cell imaging in a BioStation CT (Nikon Instruments, Melville NY), mNSC were seeded at 5000 cells/well in 24-well uncoated culture plates and allowed to attach overnight before imaging. Seeding densities were adjusted to achieve ~80-85% confluency at the end of the experiments.

Human bronchial epithelial cells (BEAS-2B, ATCC, USA), which are often used in inhalation toxicology studies, were cultured in bronchial epithelial cell growth medium using protocols previously described.[19] At 80% confluency, cells were harvested and plated at 3500 cells/well in pre-coated 96-well plates for the MTT assay.

MTT Cytotoxicity Assay

Direct effects of EC refill fluids or authentic standards of flavor chemicals on mitochondrial reductases were evaluated in concentration-response experiments that included untreated wells to control for vapor effects.[21] After seeding and overnight attachment, cells were either treated with 0%, 0.001%, 0.1%, 0.03%, 0.1%, 0.3%, and 1% refill fluids solutions or 10-fold dilutions of the actual concentration of authentic standard solution made up in culture medium. All treatments were incubated for 48 hours at 37 ºC. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, St Louis, MO) was added to wells and incubated for 2 hours at 37ºC. Solutions were removed from wells, and 100 µl of dimethyl sulfoxide (DMSO) were added to each well to solubilize formazan crystals. Absorbance readings were taken against a DMSO blank at 570 nm using an Epoch microplate reader (Biotek, Winooski, VT). The MTT assay quantifies the conversion of a yellow tetrazolium salt (MTT) to purple formazan. For each variable tested, three independent experiments were performed.

Live Cell Imaging of mNSC

For non-invasive analysis of cell morphology, motility, and survival, live-cell imaging was performed using a 10x phase contrast objective in a BioStation CT using automatic Z-focus. After attachment, mNSC were treated with refill fluid solutions at 0.1%, 0.3% and 1% made up in culture medium. Images were taken at 5 - 8 regions in each well once every 2 hours for 48 hours to collect time-lapse data for analysis. Evaluation of mNSC confluency, morphology, and survival was compared in control and treated groups using CL Quant software (DR Vision, Seattle, WA).

Data Analysis

For GC/MS analysis, each sample was analyzed twice, and the means were plotted using Prism software (GraphPad, San Diego). For the MTT assay, data were normalized to the negative control (100%), and treatment groups were expressed as percentages of the negative control. IC_{50}s were computed using the log inhibitor vs. normalized response-variable slope in GraphPad Prism, and IC_{70}s were evaluated visually. Statistical significance in the MTT assay was determined using a one-way analysis of variance (ANOVA), and when there was significance, treated groups were compared to the untreated control. In the live-cell imaging assay, significance was evaluated using a two-way ANOVA in which the variables were time and treatment.

RESULTS

Total Flavor Chemical Concentrations and Total Number of Flavor Chemicals

The number and concentrations of flavor chemicals in 105 refill fluids were evaluated (Figure 1). Each refill fluid was grouped into a product flavor category and compared for variability based on country of purchase. Refill fluid categorization was done according to flavors and types on the manufacturer's website (Table 1). Products are sorted from left to right in Figure 1 in order of decreasing total concentrations of flavor chemicals. Based on our target analyte list, the total number (1-50) and concentration (0.0047 – 54.5 mg/mL) of flavor chemicals varied among products. Two "Q American Blend Tobacco" products did not have any chemicals on our target analyte list.

Total flavor chemical concentration and number in original LIQUA flavors were high in "Two Apple" and "Peach" (Figure 1a), "Mints," and Two Mints" (Figure 1b), and "RY4 Tobacco" (Figure 1c), and "Sweet Accelerator" (Figure 1d), and Cheesecake (Figure 1e). Within the mint/menthol groups, the total concentration of flavor chemicals varied with "Mints" (54 mg/mL), having over twice the total concentration of the other products (range = 11 – 27 mg/mL). In all these products, total flavor chemical concentration was > 10 mg/mL, and the total number of flavor chemicals was > 10. In contrast, low total concentrations of flavor chemicals were found in various categories (e.g., Fruity Freshness, Indulgent Dessert, Energy Enjoyment, Juicy Berries, and Classic Tobacco) (Figure 1a, c, e-f). Based on the duplicate samples we processed, the total number of flavor chemicals and their concentrations were similar in most products with the same flavor name irrespective of country of origin (e.g., "Two Apples," "Peach," and "Ry4 Tobacco"). However, there were some exceptions, such as "Apple" (US-KS2 and

US-CA), which was purchased in different cities within the USA and had different flavor chemical concentrations.

Individual Flavor Chemical Concentrations in LIQUA Refill Fluids

The concentrations of flavor chemicals across all products ranged from 0.001 – 44.3 mg/mL (Supplementary Figure 1, Supplementary Table 1 and 2). All products with ≥ 10 mg/mL in total flavor chemicals contained 3-9 dominant flavor chemicals (i.e., chemicals present at > 1 mg/mL), and the most frequently occurring were ethyl maltol, triacetin, corylone, ethyl vanillin, vanillin, and menthol (Figure 2). When comparing flavor chemical concentrations across duplicate products purchased in different countries, concentrations of specific chemicals were generally similar (e.g., triacetin, ethyl maltol, ethyl lactate, and menthol). However, we did find some differences. For example, the concentration of corylone was about five times lower in the "Peach" product purchased in the UK than in those from the two US sites and China. Moreover, for "Ry4 Tobacco", the concentrations of corylone and furaneol varied with the location of purchase.

Frequency of Occurrence, Hazard Classification, and Chemical Class of Flavor Chemicals

The frequency of occurrence of the 126 flavor chemicals is shown in Figure 3a and Supplemental Figure 2. In descending order of frequency, the most frequently used flavor chemicals, which appeared in at least 30 products, were triacetin (52%), ethyl butanoate (46%), ethyl maltol (43%), γ-decalactone and δ-decalactone (39%), hydroxyacetone (36%), vanillin and ethyl acetate (34%), 3-Hexen-1-ol (Z) and linalool (32%), corylone (30%), and phenethyl alcohol (29%) (Figure 3a). Less frequently used flavor chemicals that appeared in fewer than 6 products are shown in Supplementary Figure 2. Using publicly available safety information, (www.goodscents.com) [22] flavor chemicals were grouped according to their potential to cause harm (Figure 3a). Most of the flavor chemicals identified were either “irritants” (red bars) or “harmful” (blue bars). At the same time, two were “irritant and dangerous to the environment” (pink bars), 2 were “harmful and dangerous to the environment” (cyan bars), and one (furfural) was “toxic” (yellow) (Figure 3a). Additional information on flavor chemicals less frequently used is included in Supplementary Figure 2. Esters, terpenes, and ketones were the most abundant chemical classes (Figure 3b).

Cytotoxicity using mNSC and BEAS-2B in the MTT assay

The cytotoxicity of 16 refill fluids that contained at least one flavor chemical ≥ 1 mg/mL and total flavor chemical concentrations ≥ 10 mg/mL is shown in Figure 4. The MTT assay, which evaluates the metabolic activity of mitochondria, was performed using mNSC and BEAS-2B cells after 48 hours of exposure to dilutions of refill fluids in submerged culture. Absorbances that are lower than the untreated controls indicate that the treatment decreased mitochondrial reductase activity. Cytotoxic refill fluids and their inhibitory concentrations at 70 % (IC_{70}) and 50 % (IC_{50}) are shown in Figure 4 and Table 2. “Two Apples” (Figure 4a-c) and “Ry4” (Figure 4d-f) were the most cytotoxic refill fluids and duplicates from multiple countries produced similar results in the MTT assay. When cytotoxicity was observed, the mNSCs were generally more sensitive to the effects of the refill fluids than the BEAS-2B cells. Even though “Cheesecake,” “Peach,” “Mints” and “Honeydew” contained relatively high concentrations of flavor chemicals, they produced little to no response in either cell type in the MTT assay.

Effect of Refill Fluids on Cell Growth Using Live-Cell Imaging

Non-invasive analysis of mNSC growth was performed using time-lapse images of cells taken over 48 hours. “Two Apples” from Nigeria and China, and “Ry4” from the USA and

China inhibited cell growth in a concentration-dependent manner irrespective of country of origin (Figure 5a-d). In the treatment group with "Two Apples," 2-way ANOVA revealed statistical significance as early as 12 hours and 20 hours for cells treated with EC refill fluid solutions at 1% (red lines) and 0.3% (blue lines). The effect observed when cells were treated with 0.1% solutions was statistically different from the control starting at 34 hours (Figure 5a). Micrographs show images taken at 0, 24, and 48 hours. Compared to the untreated group, 0.3 and 1 % concentrations inhibited cell growth early in the experiment (Figure 5b). The effects of "Ry4 Tobacco" on mNSC growth at 1% and 0.3% were similar with p values < 0.0001 starting at 10 hours (Figure 5c). 0.1% differed significantly from the control beginning at 20 hours (Figure 5c and 5d). Peach did not significantly alter growth in any treatment (Figure 5e and 5f).

Mixtures of Flavor Chemicals Sometimes Reduced Toxicity

To evaluate the effects of authentic standards of flavor chemicals individually and as mixtures, BEAS-2B cells were treated with concentrations of specific flavor chemicals that were dominant in "Peach" (Figure 6a) and "Mint" (Figure 6b). Inhibitory concentrations at 70 % (IC_{70}) and 50 % (IC_{50}) which are indicators of cytotoxicity[23] are shown in Table 3. Individually, triacetin (22 mg/mL), corylone (3.7 mg/mL), and γ-decalactone (1 mg/mL) at the concentrations found in "Peach" would be cytotoxic to BEAS-2B cells. However, when combined, there was no effect in the MTT assay (Figure 6a). Similarly, the concentrations of triacetin (44 mg/mL) and carvone (8.7 mg/mL) in "Mint" are high enough to induce significant cytotoxic effects individually, but when combined, the mixture was non-cytotoxic (Figure 6b).

Relationship between Cytotoxicity of LIQUA Products and Flavor Chemical Concentration

Regression analysis was performed to determine if cytotoxicity correlated with total flavor chemical concentrations (Figure 7a), the total number of flavor chemicals (Figure 7b), and the concentration of individual flavor chemicals (Figure 7c-j). The correlations were grouped into 3 categories: (1) high ($R^2 \geq 0.5$), (2) moderate (R^2 0.11 - 0.5), and (3) low ($R^2 \leq 0.1$). Cytotoxicity was strongly correlated with total flavor chemical concentration ($R^2 = 0.56$) for mNSC and moderately correlated for BEAS-2B cells ($R^2 = 0.39$) (Figure 7a). The relationship between the total number of flavor chemicals and cytotoxicity was moderate for BEAS-2B ($R^2 = 0.19$) and not correlated for mNSC ($R^2 = 0.04$) (Figure 7b). The concentrations of six flavor chemicals (furaneol, benzyl alcohol, ethyl maltol, ethyl vanillin, corylone, and vanillin) were high to moderately correlated with cytotoxicity for both cell types (p values <0.0001) (Figure 7c-h). Although carvone was not very cytotoxic in the MTT assay, its concentration did correlate with cytotoxicity for mNSC cells, but not for BEAS-2B cells (Figure 7i). Triacetin concentrations, which were high in “Peach” flavored products, were not correlated with cytotoxicity for BEAS-2B cells ($R^2 = 0.001$) or mNSC ($R^2 = 0.052$) (Figure 7j).

The Margin of Exposure Assessment of Potential Carcinogens in Refill Fluids

Some refill fluid chemicals are known or probable carcinogens. The Margin of Exposure (MOE) approach aids risk managers in prioritization and is used by the FDA and other expert groups to access the cancer risk of food additives.[24-27] The MOE is the ratio of a reference point for an adverse effect to the estimated daily intake or exposure of a chemical in humans. Reference points obtained from experimental or epidemiological data based on dose-response curves include the BenchMark Dose (BMD), the No

Observed Adverse Effect Level (NOAEL), or the Low Observed Adverse Effect Level (LOAEL). For MOEs below 10,000, cancer risk needs to be considered. We calculated MOEs for β-myrcene, hydrocoumarin, estragole, and pulegone based on an available BMD that caused a 10% increase in tumor incidence in animal models ($BMDL_{10}$) and NOAELs and a user consumption of 3.4 or 5 mL of fluid/day for a body weight of 60 kg [15,24,28-30] (Table 4). The MOEs for β-myrcene and hydrocoumarin were >10,000 in all samples (Figure 8a and 8b), indicating a low cancer risk. In contrast, some products had pulegone and estragole concentrations that were well below 10,000, meaning there is a cancer risk associated with these products (Figure 8c and 8d). Q Menthol (pulegone) and Two Apple (estragole) had extremely low MOEs.

Comparison of the Dominant Flavor Chemicals in Three Refill Fluid Studies

In the current study, concentrations of the flavor chemicals were averaged and plotted as a function of their frequency (Figure 9a). The dominant flavor chemicals separated into three groups. Ethyl maltol and triacetin were most frequently, followed by vanillin, corylone, menthol, ethyl vanillin, benzyl alcohol, and ethyl lactate, while carvone, furaneol, and isobutyl alcohol were infrequently used.

The individual dominant flavor chemicals (not averaged) were compared across our current and two previous studies (Supplemental Figure 4). Twenty-seven dominant flavor chemicals were identified in the present study bringing the total number across our three studies on refill fluids to 37 (Figure 9b).[12,14] Of these, five flavor chemicals (benzyl alcohol, ethyl maltol, menthol, triacetin, and vanillin) were used in at least one product at > 1 mg/mL in all three studies (Figure 9b). Ten dominant chemicals (eugenol, p-menthone, maltol, (3Z)-3-Hexen-1-ol, corylone, ethyl acetate, ethyl butanoate, ethyl vanillin, furaneol, and isoamyl acetate) were in two of the three studies, and 13 (1-

hexanol, 4-terpineol, acetylpyrazine, benzaldehyde PG acetal, butyl acetate, carvone, ethyl lactate, ethyl propanoate, hexyl acetate, isobutyl acetate, limonene, methyl anthranilate, γ-decalactone) were found only in the current study. Other chemicals present in only one study of our prior studies at > 1 mg/mL included acetoin, allyl hexanoate, linalool, strawberry glycidate_A and _B,[14] and benzaldehyde, cinnamaldehyde, ethyl cinnamate, and p-anisaldehyde.[12]

DISCUSSION

This study is the first to identify and quantify the flavor chemicals in refill fluids manufactured under one brand and purchased worldwide. In general, the flavor chemicals and their concentrations were similar in duplicate bottles of refill fluids from each country. One bottle of "Apple" from Kansas, USA (US-KS2) was an exception in that it had twice the total concentration of flavor chemicals than "Apple" bottles purchased at other locations (Supplemental Table 3), These differences may be due to instability or reactivity of the flavor chemicals in these products, mislabeling, human error in compounding, or the use of different batches of ingredients during production at plants in Italy and China. While some of the "Ritchy" refill fluids that we previously purchased in Nigeria were counterfeits,[17] all the products in the current study were manufactured by Ritchy LTD. Generally, the flavor chemicals and their concentrations were similar irrespective of the country of purchase.

One of our objectives was to determine which flavor chemicals are used frequently in refill fluids and to establish their concentration ranges by amalgamating data from our prior and current studies. We categorize flavor chemicals as "dominant" when they are 1 mg/ml or higher. Dominant chemicals are likely added intentionally to create the desired flavor profile. Chemicals at low concentrations (< 1 mg/ml) may be added intentionally or may be co-constituents of the dominant flavors. For example, pulegone, a potential carcinogen, [31] is often found at low concentrations in menthol-flavored products, but it is not likely added intentionally during manufacture. One hundred thirty-seven flavor chemicals were quantified in our prior[12,14] and current studies (164 refill fluids total) (Supplementary Table 4). These refill fluids represent a convenience sample,[12] the most popular flavors in southern California vape shops,[14] and products manufactured by one company and sold worldwide (current study). Of the 137 flavor chemicals identified in the three studies, 37 were present at concentrations > 1 mg/ml and were distributed among the studies (Figure 9b). This number of flavor chemicals reinforces our earlier conclusions that a relatively small number of flavor chemicals are used in the manufacture of a broad range of EC refill fluid products.[13,15] In contrast to our prior studies, triacetin was the most frequently used flavor chemical in the current LIQUA study, where it exceeded 44 mg/mL in one product. In all studies, esters were the most used chemical class with terpenes, ketones, alcohols, and aldehydes also identified. The five dominant flavor chemicals in our three studies (menthol, ethyl maltol, benzyl alcohol, triacetin, and vanillin) have also appeared in products analyzed in other labs,[32-37] supporting the conclusion they are commonly used.

Most products have at least one flavor chemical that is > 1 mg/ml. Tobacco-flavored products are sometimes an exception, having few flavor chemicals at low concentrations.[13,19] The LIQUA “Ry4 Tobacco” product was unusual in having four dominant flavor chemicals. Products that are a single flavor, such as menthol, peach, or cinnamon, often use one dominant flavor chemical to create the desired profile (e.g., LIQUA Peach has mainly triacetin). An exception would be LIQUA “Two Apple” which had four dominant flavor chemicals. Products with names that obscure the flavor profile, such as Dewberry Cream[14] or Cheesecake (current study), often use multiple dominant flavor chemicals to create a more complex profile. Interestingly, LIQUA “Peach” and “Q Pina Colada” have very similar flavor chemicals with triacetin (~20 mg/ml) being the dominant flavor chemical in both. Presumably, some of the flavor chemicals with lower concentrations contribute to the taste and enable the users to distinguish between the two flavors. In general, the total concentration and the total number of flavor chemicals in LIQUA “Q” and “HP” products were lower than in the regular LIQUA products.

The concentrations of flavor chemicals in some LIQUA products were higher than those typically used or permitted in other consumer goods, such as fragrances and food.[13] Triacetin, ethyl maltol, and corylone were used at concentrations averaging 6 mg/mL, 4 mg/mL, and 2 mg/mL, respectively (Figure 9a). While triacetin should not exceed 2% in cosmetics for external use,[38] its concentration in LIQUA “Mint” was 4.4% (44 mg/mL). Ethyl maltol concentrations in edible products and cosmetics should not exceed 0.015%.[39,40] However, LIQUA concentrations were 0.015% or higher in 60% (26 of 44) of the products containing ethyl maltol, with one product containing 2.6%. These concentrations exceed the MTT NOAEL (0.007 mg/mL) for ethyl maltol.[14] Ethyl maltol has been linked to free radical formation,[41] which could increase the cytotoxicity of these products. Likewise, the maximum average concentration of corylone in chewing gum for example, is 0.015 mg/mL,[22] while in some LIQUA refill fluids, concentrations ranged between 0.03 to 10.2 mg/mL.

Flavor chemicals that were not dominant (i.e., < 1 mg/ml) may also have significant health effects, including the potential to cause cancer with chronic use. Hydrocoumarin (dihydrocoumarin or 3,4 -dihydrocoumarin), a derivative of coumarin which is prohibited in human food[42] increased kidney and liver neoplasms in male rats and female mice, respectively.[26] β-myrcene is a naturally occurring acyclic monoterpene which increased kidney and liver neoplasms in male rats and mice,[43] resulting in its prohibition in food.[24] Because the MOEs for hydrocoumarin and β-myrcene in LIQUA products were >10,000, they do not appear to present a cancer risk to EC users. In contrast, the MOEs for both pulegone and estragole were far below 10,000 in some LIQUA products, consistent with cancer risk. The "Q" version of refill fluids, which are Ritchy's higher quality products, had the lowest MOEs, indicating that more expensive products are not necessarily safer. Pulegone levels in other EC products have likewise produced MOEs below the safe threshold.[44] Pulegone, a naturally occurring oxygenated monoterpene, is a major constituent of pennyroyal plant oil extracts and several other mint plants[45] and has been classified as a type 2B carcinogen by the International Agency for Research on Cancer. [45] Estragole a naturally occurring chemical found in spices, plants, and essential oils, [46-48] is a rodent hepatocarcinogen at high doses.[47,49,50] While the Joint FAO/WHO Expert Committee on Food Additives concluded further research is needed to assess the risk of estragole to humans,[51] the European Medicines Agency recommended keeping exposures to the lowest levels possible.[48]

Other flavor chemicals that are not carcinogens may cause health effects, even at low concentrations. Diacetyl (2,3, butanedione) and cinnamaldehyde were less frequently found in LIQUA products than in our other studies and ranged in concentration between 0.005 – 0.057 mg/mL and 0.003 – 0.112 mg/mL, respectively. While probably not added intentionally, diacetyl causes bronchiolitis obliterans in humans,[52,53] and cinnamaldehyde is highly cytotoxic in vitro, having IC_{50s} within the LIQUA range when tested in the MTT assay with human embryonic stem cells (0.0529 mg/mL) and human pulmonary fibroblasts (0.0489 mg/mL).[54] Cinnamaldehyde also inhibits ciliary beating in bronchial epithelial cells and impairs innate immune function.[55,56] Triacetin, the most frequently used flavor chemical in the LIQUA products, ranged in concentration from 0.005 to 44.333 mg/mL, a concentration significantly higher than triacetin in our other EC studies.[12,14] Triacetin is a clear, colorless, oily GRAS human food and cosmetic additive that produces eye and skin irritation in humans but is non-toxic in animals when administered orally or dermally.[57-59] While triacetin has relatively low cytotoxicity in vitro,[14] upon heating, it produces acetic acid, which catalyzes the formation of acrolein, formaldehyde hemiacetals, and acetaldehyde from propylene glycol and glycerol.[60] We are currently determining if triacetin increases the concentrations of reaction products in LIQUA aerosols. While our cytotoxicity data is based on refill fluids, other factors may affect results when heated aerosol are used. [61,62] For example, additional chemicals that can be toxic, such as 2, 3 butanedione, acetaldehyde, formaldehyde, and acrolein [63-68], may form upon heating and could alter cellular responses. In addition, 100% of the flavor chemicals may not transfer to aerosol so that users are exposed to lower concentration than those in the fluids. [19.] These factors notwithstanding, in one study that compared refill fluids and aerosols, the cytotoxicity of the fluids accurately predicted that of the aerosols in 74% of the samples when one EC devise was tested. [69]

The cytotoxicity of refill fluids generally correlates with the concentration of cytotoxic flavor chemicals,[13,14,19] and this was observed in the current study for “Two Apples” and “Ry4 Tobacco” in the both the MTT and cell growth assays. These products contained high concentrations of ethyl maltol, benzyl alcohol, ethyl vanillin, and corylone, which were themselves directly correlated with cytotoxicity in the MTT assay. In contrast, “Peach,” with high levels of triacetin (~20 mg/ml), was not cytotoxic in the MTT or proliferation assays, even though a concentration of triacetin lower than 20 mg/ml was cytotoxic when tested individually as an authentic standard. This observation may be explained by the fact that three of the “Peach” chemicals that were cytotoxic individually (corylone, triacetin, decalactone) produced no effect when tested in a mixture (Figure 6). A similar neutralizing effect was observed when carvone and triacetin were combined (Figure 6). Both mixtures in Figure 6 contained high concentrations of triacetin, which may decrease cytotoxicity in mixtures or the presence of solvents. Previously, a similar unexpected decrease in cytotoxicity was observed when benzyl alcohol, which was cytotoxic by itself, was used in a refill fluid.[14] This type of antagonism usually occurs when the chemicals in a mixture interact with each other to inhibit uptake or interaction with a target.[70] Antagonism appears to be rare in EC refill fluid mixtures; however, it should be studied further as “Peach” aerosols may be cytotoxic due to reaction products formed during heating.

The MTT assay measures mitochondrial reductase activity and is widely used to evaluate mitochondrial function and cell health.[71] The inhibition of cell growth by “Two Apples” and “RY4 Tobacco” may have occurred due to the reduction in ATP levels by poorly functioning mitochondria. Although not measured in this study, disruption of mitochondrial function can lead to increases in reactive oxygen species, inflammation, altered expression of genes in the electron transport chain, abnormal Ca^{2+} elevation, and glutathione depletion.[72] These changes underlie diseases of the respiratory system including chronic obstructive pulmonary disease, asthma, and lung cancer.[72,73]

This study examined products sold worldwide from one manufacturer (Ritchy). The use of flavor chemicals and their concentrations may differ for refill fluids made by other companies. In addition, it is possible that LIQUA products had additional flavor chemicals that were not on our target list.

In summary, flavor chemicals in LIQUA products were generally similar in all countries of purchase. The flavor chemicals on our target list varied in total flavor chemical concentration (range = 0.0047 – 54.5 mg/mL) and the number of flavor chemicals per product (range 1 – 50) in 103 of the refill fluids we analyzed. No target and non-target flavor compound was detected in two tobacco flavored refill fluids (American Blend and Q American Blend from US-KS). Twenty-seven flavor chemicals were dominant (used in at least one product at ≥ 1 mg/mL), and triacetin was the most frequently used, often at high concentrations. Thirty-seven chemicals not identified in our prior work were present in LIQUA products. Toxicities of refill fluids correlated with total flavor chemical concentrations and with specific individual flavor chemicals (e.g., furaneol and ethyl maltol) and resulted in inhibition of mitochondrial reductases and cell proliferation. In two refill fluids, antagonism appeared to reduce the potency of individually cytotoxic flavor chemicals. In some products, flavor chemical concentrations exceeded those used in other consumer products. Pulegone and estragole, which were likely co-constituents of dominant flavor chemicals, had MOEs consistent with a risk for cancer. The regulation of flavor chemicals could improve the safety of these EC refill fluids.

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Total Number and Total Concentration of Flavor Chemicals

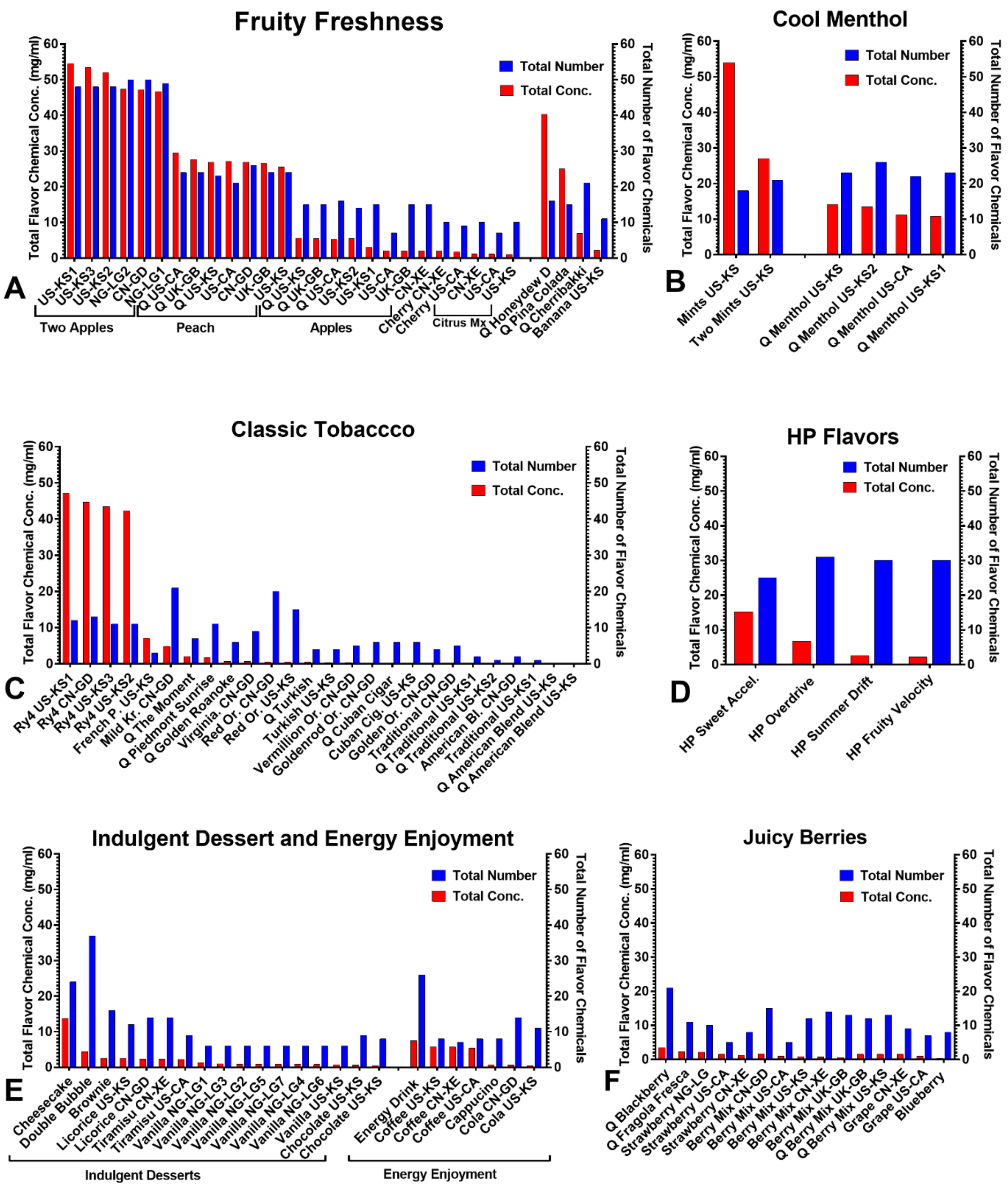


Figure 6.2. Total number and total concentrations of flavor chemicals in 103 LIQUA refill fluids. Total flavor chemical concentrations ranged from 0.0047 – 54.5 mg/mL mg/ml, and the total number of flavor chemicals ranged from 1 - 50. (a) Fruity Freshness, (b) Cool Menthol, (c) Classic Tobacco, (d) LIQUA HP, (e) Indulgent Desserts, Energy Enjoyment, and (f) Juice berries. The x-axis of each graph shows the flavor name and purchase location of each refill fluid (also see Supplemental Table 2). The left y-axis shows the concentration of total flavor chemicals ordered according to decreasing concentration from left to right within each flavor category. In contrast, the right y-axis shows the total number of flavor chemicals in each product. Each bar is the mean of two independent measurements.

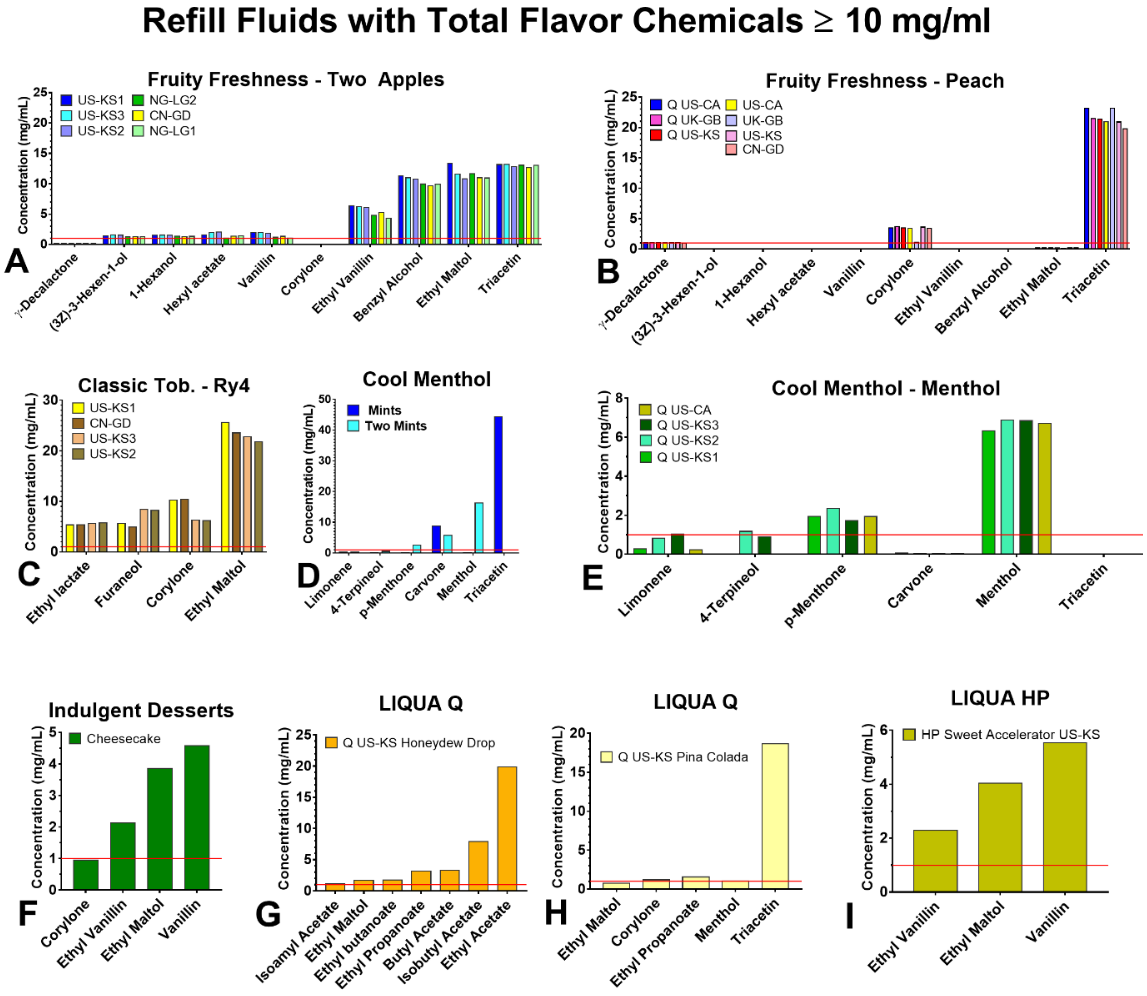


Figure 6.3. Individual flavor chemicals in refill fluids with a total concentration of flavor chemicals ≥ 10 mg/mL (a) Two Apples, (b) Peach, (c) Ry4 Tobacco, (d) Mints and Two Mints, (e) Q Menthol (Authentic), (f) Cheesecake, (g) Q Honeydew Drop, (h) Q Pina Colada, (i) HP Sweet Accelerator. The x-axis shows flavor chemicals that were > 1 mg/mL, and the y-axis shows the concentration of individual flavor chemicals.

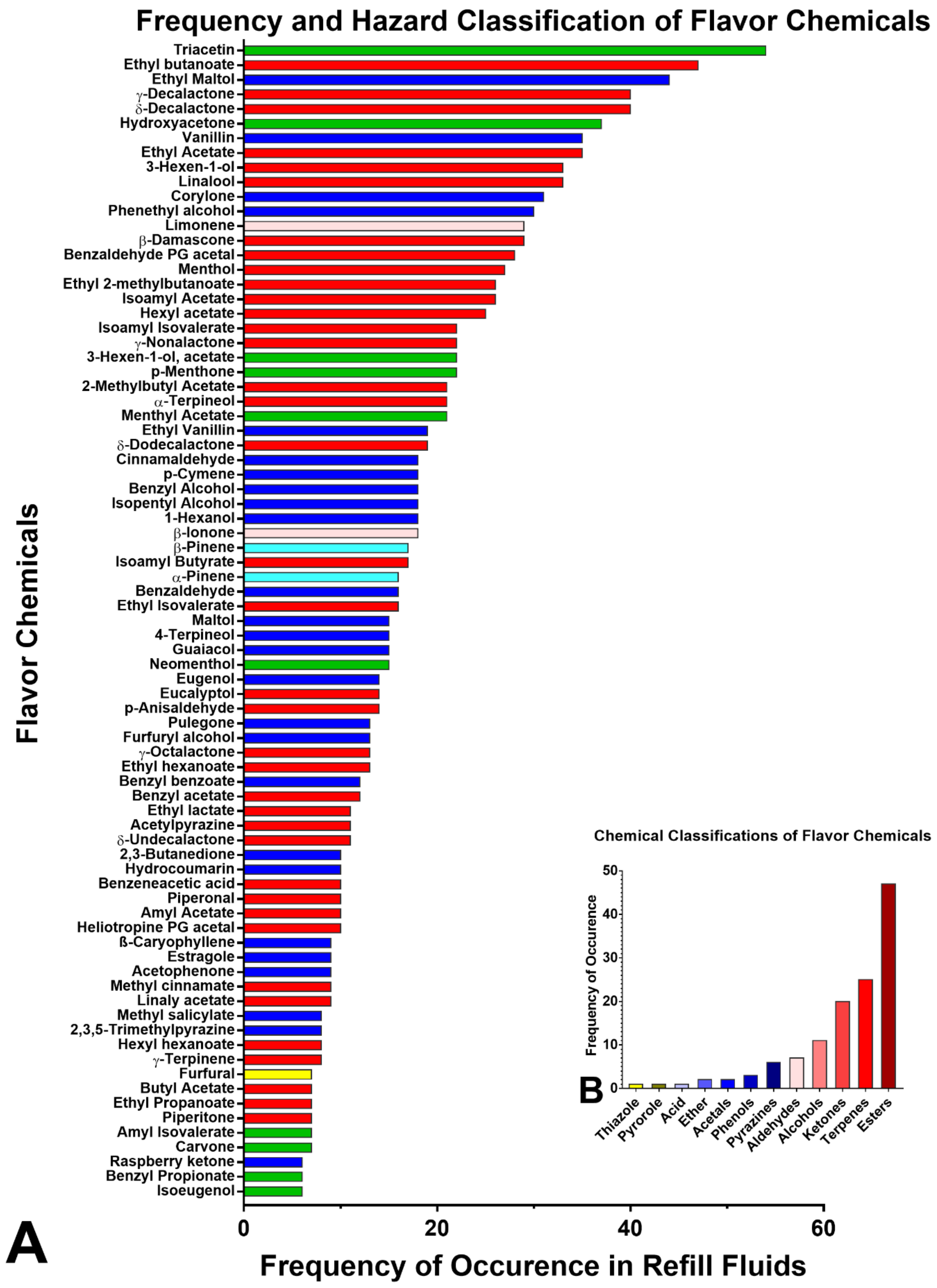

Frequency and Hazard Classification of Flavor Chemicals
Flavor Chemicals
Triacetin
Ethyl butanoate
Ethyl Maltol
γ-Decalactone
δ-Decalactone
Hydroxyacetone
Vanillin
Ethyl Acetate
3-Hexen-1-ol
Linalool
Corylone
Phenethyl alcohol
Limonene
β-Damascone
Benzaldehyde PG acetal
Menthol
Ethyl 2-methylbutanoate
Isoamyl Acetate
Hexyl acetate
Isoamyl Isovalerate
γ-Nonalactone
3-Hexen-1-ol, acetate
p-Menthone
2-Methylbutyl Acetate
α-Terpineol
Menthyl Acetate
Ethyl Vanillin
δ-Dodecalactone
Cinnamaldehyde
p-Cymene
Benzyl Alcohol
Isopentyl Alcohol
1-Hexanol
β-Ionone
β-Pinene
Isoamyl Butyrate
α-Pinene
Benzaldehyde
Ethyl Isovalerate
Maltol
4-Terpineol
Guaiacol
Neomenthol
Eugenol
Eucalyptol
p-Anisaldehyde
Pulegone
Furfuryl alcohol
γ-Octalactone
Ethyl hexanoate
Benzyl benzoate
Benzyl acetate
Ethyl lactate
Acetylpyrazine
δ-Undecalactone
2,3-Butanedione
Hydrocoumarin
Benzeneacetic acid
Piperonal
Amyl Acetate
Heliotropine PG acetal
ß-Caryophyllene
Estragole
Acetophenone
Methyl cinnamate
Linaly acetate
Methyl salicylate
2,3,5-Trimethylpyrazine
Hexyl hexanoate
γ-Terpinene
Furfural
Butyl Acetate
Ethyl Propanoate
Piperitone
Amyl Isovalerate
Carvone
Raspberry ketone
Benzyl Propionate
Isoeugenol
A
0
20
40
60
Frequency of Occurence in Refill Fluids
Chemical Classifications of Flavor Chemicals
B
Frequency of Occurence
50
40
30
20
10
0
Thiazole
Pyrorole
Acid
Ether
Acetals
Phenols
Pyrazines
Aldehydes
Alcohols
Ketones
Terpenes
Esters

Figure 6.4. Frequency of occurrence, hazard, and chemical classification of flavor chemicals. (a) The frequency with which individual flavor chemicals were found in at least 6 products. The x-axis is the number of refill fluids in which the chemicals were found, and the y-axis is sorted according to decreasing frequency of their occurrence, which ranged from 6 - 54 with the highest being triacetin. Chemicals appearing less frequently (≤ 5 times) are shown in Supplemental Figure 2. Colored bars represent hazard categories using the European Union safety guidelines; red = irritant, blue = harmful, yellow = toxic, green = not determined, pink = irritant and dangerous to the environment, cyan = harmful and dangerous to the environment. light yellow = toxic and dangerous to the environment. (b) The chemical classes of the flavor chemicals (x-axis) are plotted versus the frequency of occurrence of each class of flavor chemicals (y-axis).

Cytotoxicity of EC Fluids with High Flavor Chemical Concentrations

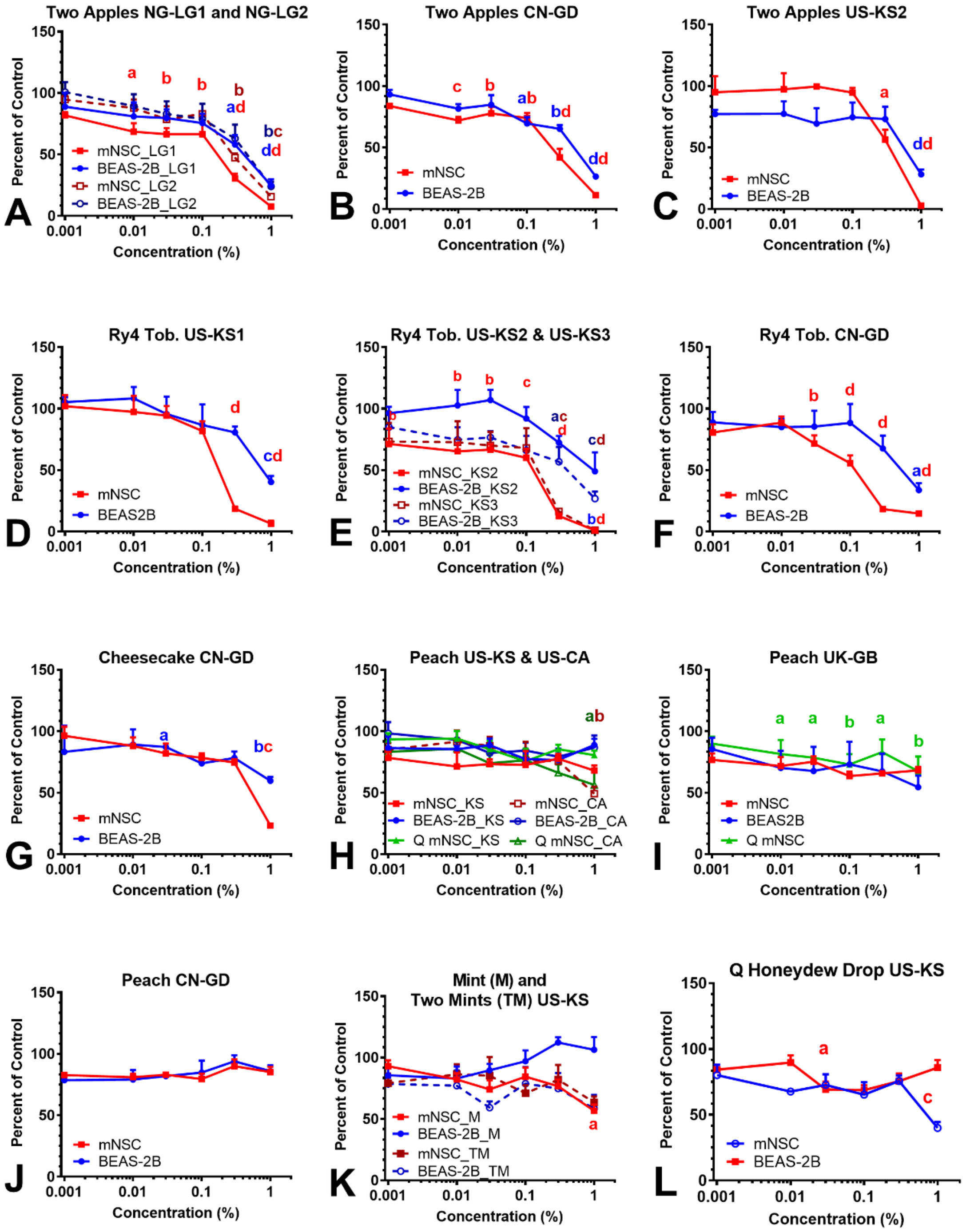
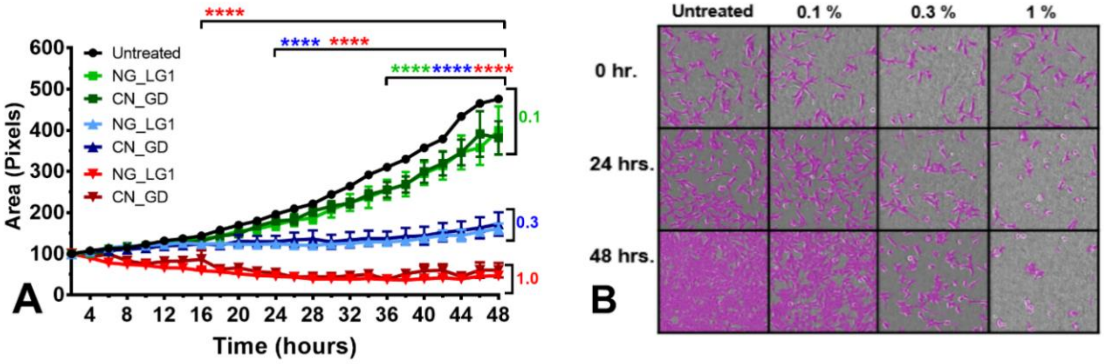


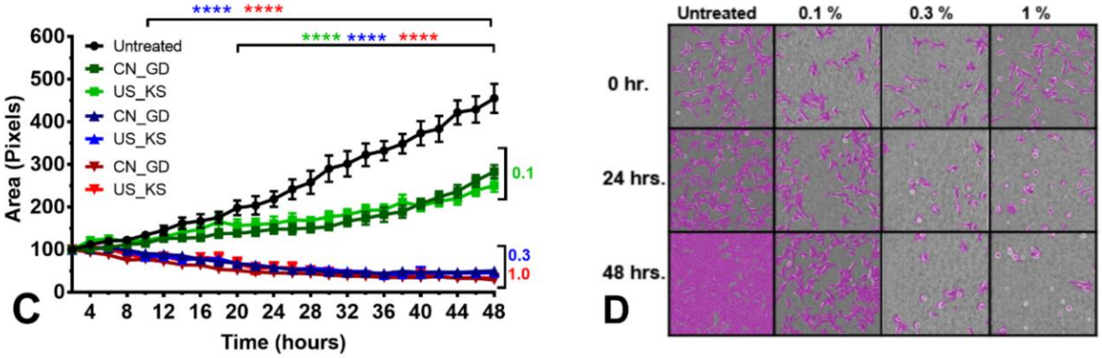
Figure 6.5. Refill fluid cytotoxicity using mNSC and BEAS-2B in the MTT assay. Concentration-response curves for (a-c) Two Apples, (d-f) Ry4 Tobacco, (g) Cheesecake, (h-j) Peach, Mints (k), and Q Honeydew Drop (l) tested with mNSC and BEAS-2B cells. The numbers after each cell type (e.g., 1 and 2 or 2 and 3) in Figures 4a and 4e indicate duplicate bottles from the same country. In Figure 4k, M = “Mint” and TM = “Two Mint”. Each point is the mean ± standard error of the mean of three independent experiments. Points with letters are significantly different from the untreated control, and points with different letters show degrees of statistical significance. ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$, ^{d} $p < 0001$.

mNSC Treated with Refill Fluids in a Live Cell Imaging Assay

Two Apples from Nigeria and China



Ry4 Tobacco USA and China



Peach (1%) from USA and UK

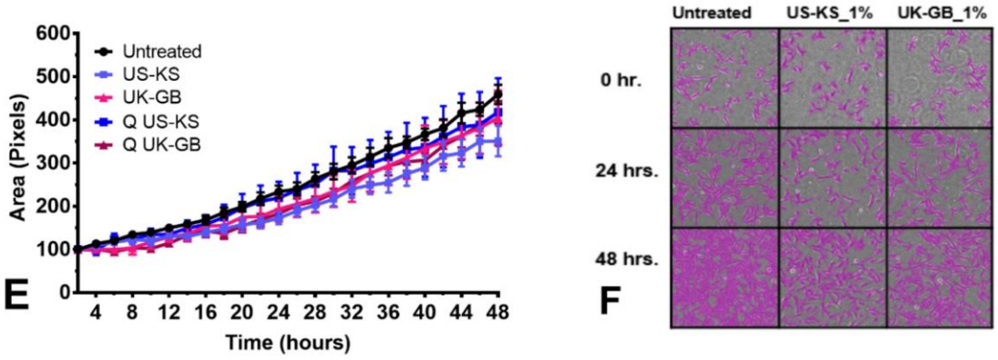


Figure 6.6. Effect of refill fluids on cellular growth using mNSC in the live-cell imaging assay. Time-lapse imaging was performed for mNSC cells treated with (a-b) Two apples, (c-d) Ry4 Tobacco, (e-f) Peach. The x-axis shows the duration of the experiment, and the y-axis shows the mean of the percent increase in cell area (growth) over 48 hours as determined using CL-Quant software.

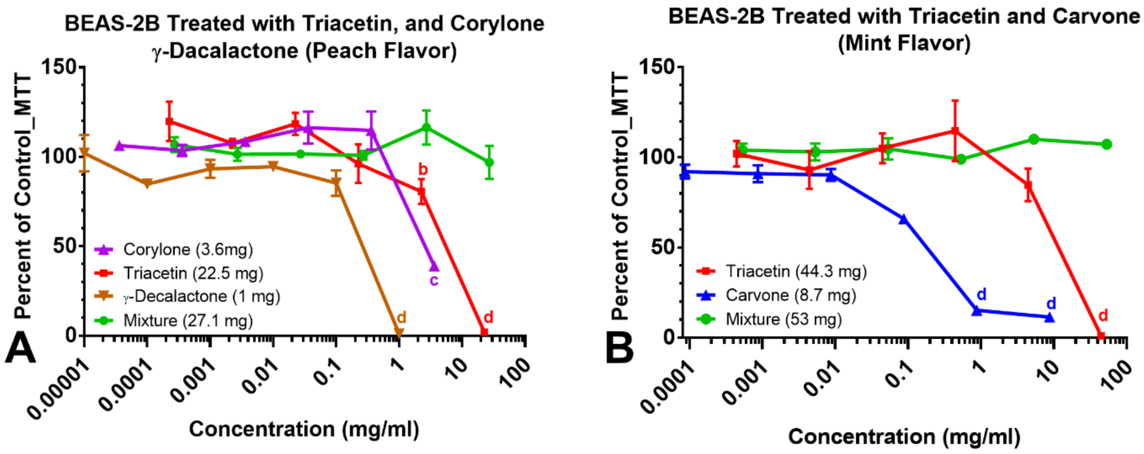


Figure 6.7. Concentration-response curves of dominant (> 1 mg/mL) flavor chemicals and mixtures in "Peach" and "Mint" and their cytotoxicity. Concentration-response curves of authentic standards of chemicals present in the highest concentrations in (a) Peach flavors and (b) Mint flavor. The curves show the dynamic response of BEAS-2B cells to authentic standards as individual flavor chemicals; corylone, triacetin and γ-decalactone (a), triacetin and carvone (b) and their mixtures (a and b). Each curve on the graph is the mean ± the standard error of the mean for at least three independent experiments. ^{a} p < 0.05, ^{b} p < 0.01, ^{c} p < 0.001, ^{d} p < 0001.

Flavor Chemicals and Cytotoxicty Relationships

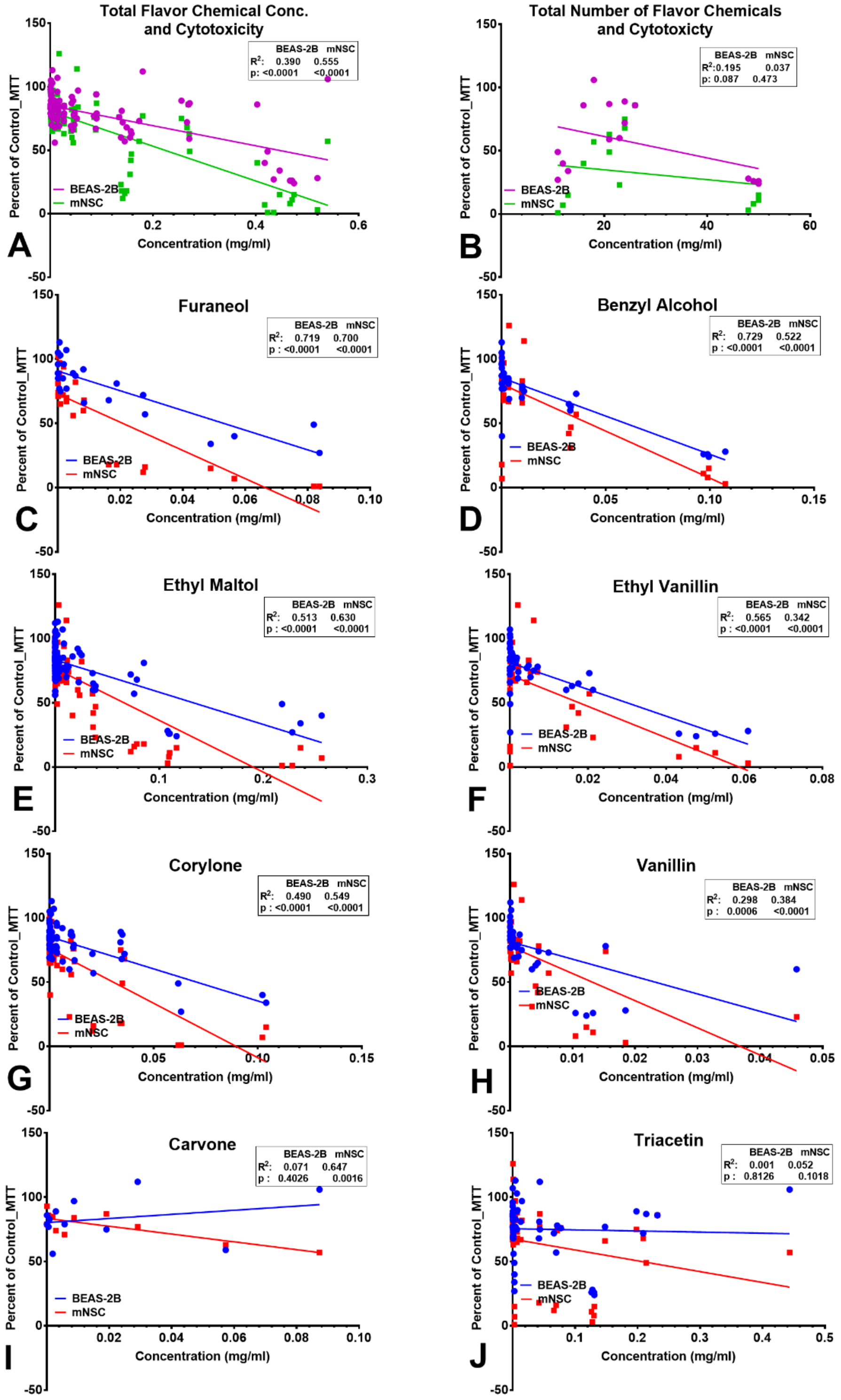


Figure 6.8. Linear regression analysis of refill fluid cytotoxicity and flavor chemical composition. Cytotoxicity at 1% refill fluid concentration is plotted as a function of (a) the total number of favor chemicals, (b) the total concentration of favor chemicals, (c) furaneol, (d) benzyl alcohol, (e) ethyl maltol, (f) ethyl vanillin, (g) corylone, (h) vanillin, (i) carvone, (j) triacetin. Correlation coefficients were high and statistically significant for furaneol, benzyl alcohol, ethyl maltol, and corylone with both cell lines. (c-f and h). The regression analysis revealed a statistically high and moderate correlation for ethyl vanillin with BEAS-2B and mNSC, respectively. While the correlation for vanillin was moderate and significant with both cell lines, for corylone, it was high for BEAS-2B and low for mNSC. (h-i). There was no relationship between triacetin concentration and cytotoxicity with the 1% refill fluid solution.

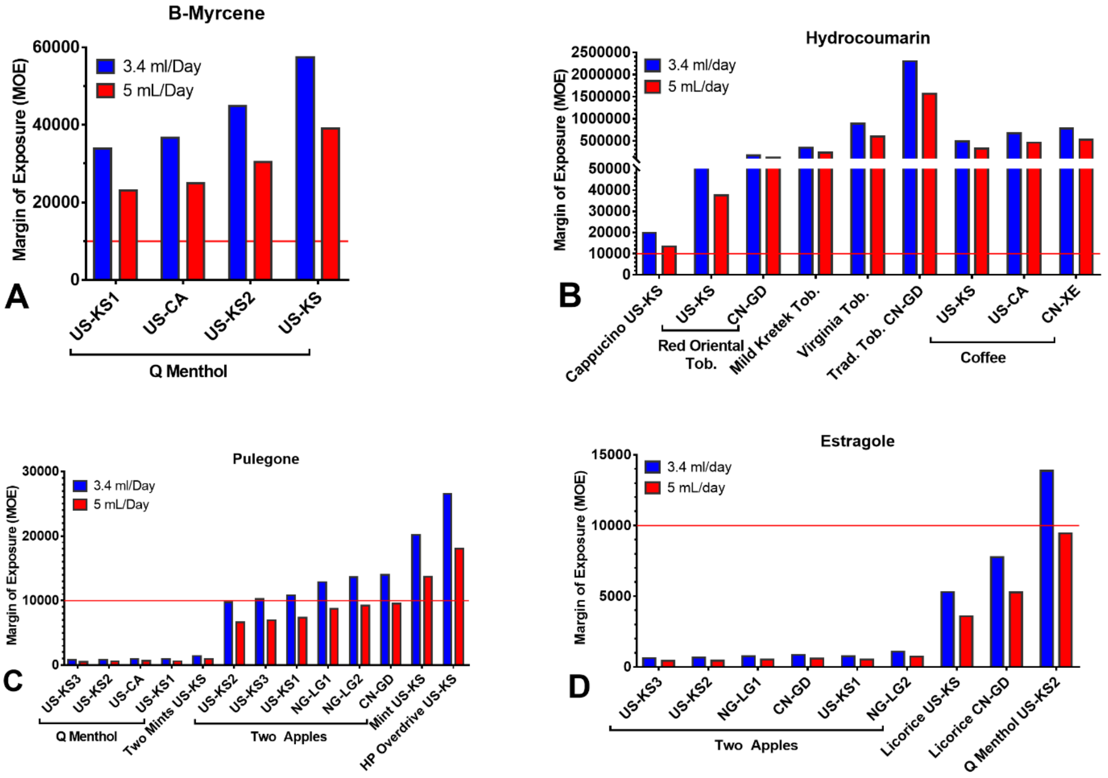


Figure 6.9. The margin of exposure (MOE) for four potential carcinogens or food additives in LIQUA products. (a) β-Myrcene, (b) Hydrocoumarin, (c) Estragole, and (d) Pulegone. MOEs below the threshold of 10,000 indicates a high carcinogenic potential and concern for human health.

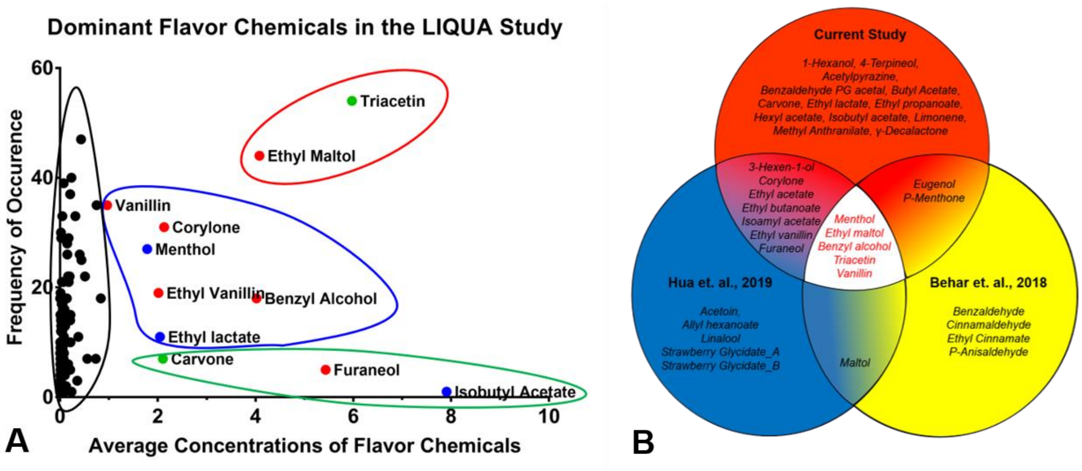


Figure 6.10. Dominant flavor chemicals in three refill fluid studies. (a) average concentrations and the number of dominant flavor chemicals in the LIQUA study. The x-axis represents the average concentration of dominant flavor chemicals (> 1 mg/ml) found in the LIQUA EC library, and the y-axis is the frequency of occurrence of each dominant flavor chemicals. Outlines represent different groupings of average concentration and frequency of dominant flavor chemicals; red = high concentration and high frequency, blue = high concentration and mid-frequency, green = high concentration and low frequency, black = low concentration and varying frequency. Colored dots represent hazard classification according to European CLP safety criteria; red = harmful, blue = irritants, green = not determined. (b) Dominant flavor chemicals in three refill fluid libraries. Each chemical in the Venn diagram was present in at least one product in the library at > 1 mg/mL.

Table 6.1. LIQUA EC Refill Fluids and Their Respective Flavor Categories

Company	EC Fluid Categories	Flavors
LIQUA Original	Fruity Freshness	Two Apples, Peach, Apple, Banana, Cherry, Citrus Mix
	Cool Menthol	Mints, Two Mints,
	Classic Tobacco	Ry4 Tob., French Pipe Tob., Mild Kretek Tob., Virginia Tob., Red Oriental Tob., Turkish Tob., Vermillion Tob., Cuban Cigar Tob., Goldenrod Tob., Golden Oriental Tob., Traditional Tob., American Blend Tob.
	Indulgent Desserts	Cheesecake, Licorice, Tiramisu, Brownie, Vanilla, Chocolate
	Energy Enjoyment	Energy drink, Coffee, Cappuccino, Cola
	Juice Berries	Strawberry, Berry Mix, Grape, Blueberry
LIQUA Q		Peach, Apple, Menthol, Golden Roanoke Tob., Turkish Tob., Havana Libre, Traditional Tob., American Blend Tob., Berry Mix, Honeydew Drop, Pina Colada, Cherribakki, Double Bubble, Blueberry Jack, Fragola Fresca, The Moment.
LIQUA HP		Sweet Accelerator, Overdrive, Summer Drift, Fruity Velocity

Table 6.2. IC_{70}s and IC_{50}s for Cytotoxic Refill Fluids

Refill Fluids	**Country Code**[1]	**BEAS-2B (%)**		**mNSC (%)**		**Q mNSC (%)**[2]	
		IC_{70}	**IC_{50}**	**IC_{70}**	**IC_{50}**	**IC_{70}**	**IC_{50}**
"Two Apples"	NG-LG1	0.17	0.34	0.02	0.10		
	CN-GD	0.08	0.30	0.12	0.17		
	NG-LG2	0.20	0.39	0.15	0.26		
	US-KS2	0.33	0.68	0.23	0.33		
"Ry4 Tobacco"	US-KS2	0.36	0.89	0.00	0.05		
	US-KS3	0.07	0.23	0.07	0.08		
	CN-GD	0.29	0.58	0.04	0.09		
	US-KS1	0.44	0.77	0.12	0.17		
"Cheesecake"	CN-GD	0.548	>1	0.35	0.47		
"Peach"	US-CA	>1	>1	0.39	>1	0.22	>1
	US-KS	>1	>1	0.43	>1	-	>1
	UK-GB	0.20	>1	0.05	>1	0.88	>1
	CN-GD	>1	>1	>1	>1		
"Mint	US-KS	-	-	0.77	>1		
"Two Mints"	US-KS	0.99	>1	0.02	>1		
"Q Honeydew Drop"	US-KS	-	-	0.01	>1		

[1] Country Code: NG-LG = Lagos, Nigeria; CN-GD = Guangdong, China; US-KS = Kansas, USA; US-CA = California, USA; UK-GB = Great Britain, United Kingdom.

[2] The highest concentration tested was 1% of the EC refill fluids.

Table 6.3. IC_{70}s and IC_{50}s for Authentic Standards (mg/mL)

Flavor Chemical	In house fluid formulation[1]	Concentration (mg/mL)	BEAS-2B	
			IC_{70}	IC_{50}
Triacetin	"Mint"	44.3	6.18	11.49
Carvone		8.7	0.064	0.163
Triacetin + Carvone		53	N/A	N/A
Triacetin	"Peach"	22.5	2.95	5.09
Corylone		3.6	1.37	3.36
g-decalactone		1	0.15	0.24
Triacetin + Corylone + g-decalactone		27.1	N/A	N/A

[1] In house fluid formulation is a combination of the dominant flavor chemicals in LIQUA "Mint" and "Peach" EC products.

Table 6.4: Summary of MOE for Potential Carcinogens/Food Additives in LIQUA Products[1]

	Carcinogen/ Food Additive	Daily Consumption		Reference Point	Study Ref.
		3.4 mL	5 mL		
Q Menthol US-KS1	B myrcene	33916	23063	64 mg/kg bw/day (BMDL$_{10}$)[2]	FDA, 2018[24]
Q Menthol US-CA		36669	24935		
Q Menthol US-KS2		44818	30476		
Q Menthol US-KS		57476	39084		
Cappuccino US-KS	Hydrocoumarin	19732	13418	150 mg/kg bw/day (NOAEL)[3]	NTP, 1993[29]
Coffee US-KS		483481	328767		
Coffee US-CA		674410	458599		
Coffee CN-XE		778547	529412		
Red Oriental CN-GD		55118	37480		
Red Oriental CN-GD		169412	115200		
Mild Kretek CN-GD		344894	234528		
Virginia CN-GD		882353	600000		
Traditional CN-GD		2301790	1565217		
Q Menthol US-KS	Pulegone	788	536	13.39 mg/kg bw/day (NOAEL)[3]	FDA, 2018[24]
Q Menthol US-KS2		837	569		
Q Menthol US-KS1		918	624		
Q Menthol US-CA		938	638		
Two Mints US-KS		1413	961		
Mints US-KS		20196	13733		
Two Apple US-KS2		9724	6612		
Two Apple US-KS3		10251	6971		
Two Apples US-KS1		10839	7371		
Two Apples NG-LG1		12877	8756		
Two Apples NG-LG2		13619	9261		
Two Apples CN-GD		13982	9508		
HP Overdrive US-KS		26550	18054		
Two Apple US-KS3	Estragole	637	433	3.3 mg/kg bw/day (BMDL$_{10}$)[2]	van den Berg, 2014[28]
Two Apple US-KS2		668	454		
Two Apples NG-LG1		764	520		
Two Apples CN-GD		863	587		
Two Apples US-KS1		769	523		
Two Apples NG-LG2		1086	739		
Licorice US-KS		5294	3600		
Licorice CN-GD		7765	5280		
Q Menthol US-KS2		13866	9429		

[1] *MOEs below the threshold of 10,000 indicates a high carcinogenic potential and concern for human health.*

[2] *BMDL$_{10}$ = Benchmark Dose Level with a lower confidence limit of 10%.*

[3] NOAEL = No Observed adverse Effect Level.

Chapter 7

Flavor chemicals, synthetic coolants, and pulegone in popular mint and menthol flavored e-cigarettes

Omaiye et al., 2021

Tobacco Control 2021

ABSTRACT

Background. The FDA recently banned flavors from pod-style electronic cigarettes (e-cigarettes), except for menthol and tobacco. JUUL™ customers quickly discovered that flavored disposable e-cigarettes from other manufacturers, such as Puff, were readily available. Our goal was to compare flavor chemicals, synthetic coolants, and pulegone in mint/menthol-flavored e-cigarettes from JUUL™ and Puff, evaluate the cytotoxicity of the coolants and perform a cancer risk assessment for pulegone, which was present in both JUUL™ pods and disposable Puff product.

Methods. Identification and quantification of chemicals were performed using gas chromatography/mass spectrometry. Cytotoxicity of the coolants was evaluated with BEAS-2B cells using the MTT assay. The cancer risk of pulegone was calculated using the Margin of Exposure (MOE).

Results. Menthol was the dominant flavor chemical (> 1 mg/mL) in all products from both manufacturers. Minor flavor chemicals (< 1 mg/mL) differed in the JUUL™ and Puff fluids and may produce flavor accents. The concentrations of WS-3 and WS-23 were higher in Puff than in JUUL™. WS-23 was cytotoxic in the MTT assay at concentrations 90 times lower than concentrations in Puff fluids. The risk for cancer (MOE < 10,000) was greater for mint than menthol products and greater for Puff than JUUL.

Conclusions. Switching from JUUL™ to Puff e-cigarettes may expose users to increased harm due to the higher levels of WS-23 and pulegone in Puff products. Cancer risk may be reduced in e-cigarettes by using pure menthol rather than mint oils to produce minty flavored e-cigarette products.

INTRODUCTION

JUUL™ was the first popular pod-style e-cigarette with a large share of its sales going to middle and high school students.[1-5] JUUL™ initially marketed eight flavors of pods, including “Cool Mint” and Classic Menthol”, which were later replaced by “Mint” and “Menthol”, respectively.[6] The rapid spike in JUUL™ popularity concerned parents, public health officials, and regulatory agencies, leading JUUL™ in 2019 to remove all flavors from their product line in the US, except for “Classic Tobacco,” “Virginia Tobacco,” and “Menthol.” Puff products, which appear similar to JUUL™, did not fall under the Food and Drug Administration’s limitations on flavors, and many JUUL™ users switched to Puff, which rapidly became a dominant e-cigarette brand.[7-9] In spite of their popularity, we know little about the relative safety of Puff and JUUL™ products.

This study compares three classes of chemicals in Puff and JUUL™ e-fluids. These include flavor chemicals, in particular menthol, two synthetic coolants, and pulegone, a potential carcinogen that has been reported in mint-flavored e-cigarettes.[10,11] Because the use of menthol is permitted by the Family Smoking Prevention and Tobacco Control Act of 2009,[12] it is one of the most widely used flavor chemicals in tobacco products,[13] sometimes appearing in e-cigarettes that are not explicitly labeled “mint” or “menthol”.[14] The cooling properties and pleasant minty flavor of menthol may make smoking initiation easier among novice users.[15,16] Although generally regarded as safe (GRAS) for ingestion by the Flavor and Extract Manufacturers Association (FEMA).[17] menthol is often used in e-cigarette products at high concentrations,[14] which are cytotoxic in vitro.[14,18,19]

The synthetic coolants WS-3 (N-ethyl-p-menthane-3-carboxamide; CAS # 39711-79-0) and WS-23 (2-isopropyl-N,2,3-trimethylbutyramide; CAS # 51115-67-4) are popular cooling agents, were initially developed by Wilkinson Sword Ltd. in the 1970s.[20] These coolants are considered safe for ingestion by FEMA and are used extensively in consumer products, including breath fresheners, confectionaries, and cosmetics.[21-23] WS-3 and WS-23 activate the TRPM8 and TRPA1 receptors, creating a cool relaxing sensation,[24] while imparting little or no flavor to products that are ingested. WS-23 has been reported in JUUL™ pods purchased in the European Union,[25] but was not found in JUUL™ pods purchased in the US.[6] Bloggers have discussed the addition of coolants to e-cigarette fluids, suggesting they are more widely used than generally recognized.[26-28] However, apart from one report on JUUL™,[25] very little is known about the identities and concentrations of coolants used in e-fluids, and the range of concentrations of these coolants in JUUL™ and Puff e-cigarette has not previously been compared.

Mint oil, which is often used in e-cigarettes to create “mint” flavor, can contain pulegone, [29,30] a known carcinogen.[31,32] In several recent studies, a Margin of Exposure (MOE) analysis found pulegone to be sufficiently high in some e-cigarettes to present a cancer risk,[10,11] which motivated us to examine pulegone in JUUL™ and Puff products.

This study compares menthol, WS-3, and WS-23, and pulegone in menthol and minty-flavored products made by JUUL™ and Puff to gain insight into their relative safety. Specifically, we have compared the following: (1) concentrations of the flavor chemicals, (2) the concentrations and cytotoxicity of WS-3 and WS-23, and (3) the MOEs, which predict cancer risk.

METHODS

Sample Acquisition

In 2018 and 2019, JUUL™ “Cool Mint”, “Classic Menthol”, and their replacements “Mint”, and “Menthol” were purchased online (www.juul.com) and from local stores in

Riverside, CA, and Portland, OR. Of the four minty/menthol-flavored pods produced by JUUL™, only "Menthol" is currently available. JUUL™ "Cool Mint", "Classic Menthol", "Mint", and "Menthol" pods were analyzed to compare chemical composition in all minty/menthol JUUL™ pods. All pods were stored in the dark and analyzed close to the time of purchase.

Two types of disposable Puff devices were purchased; the 1.3 mL Puff Bar "Menthol" labeled to deliver 300 puffs/device and the 3.2 mL Puff Plus "Cool Mint" labeled to deliver 800 puffs/device. Puff devices were purchased at vape shops in Los Angeles, CA, and Riverside, CA, in 2020. All devices were stored in the dark and analyzed close to the time of purchase.

Identification and Quantification of Chemicals Using (GC/MS)

E-cigarette fluids were extracted from the pods and devices, and 50 µL was dissolved in 0.95 mL of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ). Chemical analysis was performed with an Agilent 5975C GC/MS system (Santa Clara, CA) using internal standard-based calibration procedures and methods previously described in detail.[6,33] The method analyzes 180 flavor chemicals plus nicotine.

Culturing of BEAS-2B Cells

Human bronchial epithelial cells (BEAS-2B) from American Type Culture Collection (ATCC), Manassas, VA were cultured in a growth medium made with 500 mL of Airway Epithelial Cell Basal Medium supplemented with 1.25 mL HLL supplement containing human serum albumin (500 µg/mL), linoleic acid (0.6 µM), and lecithin (0.6 µg/mL), 15 mL of L-glutamine (6 mM), 2 mL of extract P (0.4%), and 5.0 mL Airway Epithelial Cell Supplement containing epinephrine (1.0 µM), transferrin (5 µg/ml), T3 (10 nM), hydrocortisone (0.1 µg/ml), rh EGF (5 ng/mL), and rh Insulin (5 µg/mL) from ATCC, Manassas, VA. Nunc T-25 tissue culture flasks (Thermo Scientific, Waltham, MA) were coated overnight with a coating medium made with basal medium (69.3%) (ATCC, Manassas, VA), collagen (29.7%) (Sigma-Aldrich, St Louis, MO), bovine serum albumin (0.99%) (Sigma-Aldrich, St Louis, MO), and fibronectin (0.01%) (Sigma-Aldrich, St Louis, MO) before culturing and passaging cells. At 85 - 90% confluency, cells were harvested using Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (Lonza, Walkersville, MD) for washing and incubated with a trypsin solution containing Trypsin-EDTA (0.25% trypsin/0.53 mM EDTA) from ATCC, Manassas, VA, and 0.5% poly-vinyl-pyrrolidone (Sigma-Aldrich, St Louis, MO), for 3 mins at 37^{o}C to allow detachment. Cells were cultured in T-25 flasks at 75,000 cells/flask, and the medium was replaced every other day. Cells were then plated at 10,000 cells/well in pre-coated 96-well tissue culture plates (Thermo Scientific, Waltham, MA.) and allowed to attach overnight before a 24-hour treatment.

MTT Cytotoxicity of WS-3 and WS-23

The effects of WS-3 and WS-23 on mitochondrial reductases were evaluated in concentration-response experiments. BEAS-2B cells were seeded, allowed to attach overnight, and treated with 0.5 – 5 mg of each coolant/mL of culture medium for 24 hours at 37 ºC. After treatment, 20 µL of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St Louis, MO) dissolved in 5 mg/mL of DPBS (Fisher Scientific, Chino, CA) were added to wells and incubated for 2 hours at 37 ºC. Solutions were removed from wells, and 100 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific, Chino, CA) were added to each well and gently mixed on a shaker to solubilize formazan crystals. Absorbance readings of control and treated wells were taken against a DMSO blank at 570 nm using an Epoch microplate reader (Biotek, Winooski, VT). The MTT assay quantifies the conversion of a yellow tetrazolium salt (MTT) to purple formazan. For each coolant tested, three independent experiments on different passages of the same culture were performed.

The Margin of Exposure (MOE) Calculations for Pulegone

To assess the cancer risk associated with pulegone in pod/device fluids, the MOE was calculated using the no-observed adverse effect level (NOAEL) of pulegone and the estimated exposure dose (EED) from pods/devices. Regulatory agencies, including the FDA use the MOE to assess the cancer risk of food additives.[31] Chemicals with MOE values below 10,000 require strategies to limit exposure. The risk associated with pulegone content in JUUL™ and Puff e-cigarettes was evaluated using a daily EED of 1 – 3 mL,[34-37] a NOAEL of 13.39 mg/kg and an adult body weight of 60 kg.[31,32]

Data Analysis and Statistics

For GC/MS data, and the means and standard deviations for at least three pods/devices were plotted using Prism software (GraphPad, San Diego, CA). For the MTT assay, treatment groups were expressed as percentages of the negative control. IC_{50}s were computed using the log inhibitor vs. normalized response-variable slope in GraphPad Prism, and IC_{70}s were evaluated visually. Statistical significance in the MTT assay was determined in GraphPad using a one-way analysis of variance (ANOVA) on the raw data. When means were significant ($p < 0.05$), treated groups were compared to the untreated control using Dunnett’s post hoc test.

RESULTS

Concentrations of Flavor Chemicals in JUUL™ and Puff E-cigarettes

Menthol was the dominant flavor chemical in the JUUL™ and Puff samples (concentration range 5 - 14 mg/mL) (figure 1A). Menthol concentrations were similar in all products, except Puff Bar “Menthol” in which the concentration was lower. Other flavor chemicals were generally < 1 mg/mL (figures 1B and C), except for triacetin and p-menthone, which were >1 mg/mL in Puff Plus “Cool Mint” and Puff Bar “Menthol”, respectively (figure 1B). In JUUL™ fluids, minor flavor chemicals (< 1 mg/mL) were generally present in the two “mint” flavors from JUUL™ but absent or lower in concentration in the “menthol” flavors. Puff products had more minor flavor chemicals than JUUL™ (figures 1B and C). In Puff, minor flavor chemicals were generally higher in the “Menthol” devices (figure 1B and 1C). Estimated concentrations of flavor chemicals identified at levels below the LOQ (20 µg/mL for 50 µl samples) are shown in supplementary table S1.

WS-3 and WS-23 Concentrations in JUUL™ and Puff

While WS-3 was absent in all JUUL™ pods, WS-23 was present in JUUL™ “Menthol” pods at an average concentration of 0.1 mg/mL (figure 2A). Both coolants were in Puff fluids at much higher concentrations. WS-23 in Puff Plus “Cool Mint” averaged 36 mg/m/L with one device having 45 mg/mL of WS-23. In the other Puff groups, the average concentrations of WS-3 and WS-23 were similar and ranged between 4.3 - 7.2 mg/m/L.

Cytotoxicity of WS-3 and WS-23

The cytotoxicity of WS-3 and WS-23 was evaluated using the MTT assay in conjunction with ISO protocol #10993-5, which measures mitochondrial reductase activity (figure 2B).[38] BEAS-2B cells were tested using concentrations of coolant that were lower than those found in the e-cigarettes. While concentrations of WS-3 below 5 mg/mL produced little to no response in the MTT assay, BEAS-2B cells were adversely affected by all concentrations of WS-23 that were tested (IC_{70} = 0.59).

Hazard Analysis of Pulegone in JUUL™ and Puff E-Cigarettes

The concentrations of pulegone in JUUL™ pods and disposable Puff fluids ranged from 0.002 – 0.2 mg/mL and were higher in the "mint" labeled products (figure 1). For "menthol" products from both manufacturers, only the 3 mL/day exposure scenario for Puff Bar "Menthol" generated a MOE < 10,000, which is below the safety threshold (figure 3A). In contrast, for all "mint" flavored samples, most scenarios produced a MOE < 10,000 (figure 3B). For all scenarios for both mint and menthol-flavored products, the MOEs for Puff were consistently lower than those for JUUL™, suggesting a greater risk with Puff.

Concentrations of Flavor Chemicals in Edible Consumer Products

Synthetic coolants and menthol in edible consumer goods were compared to concentrations in JUUL™ and Puff e-cigarette fluids (figure 4). Concentrations of menthol in JUUL™ and Puff were similar but between 14 to 543 times higher than in other consumer products (figure 4A). WS-23 in Puff was 450 times higher than concentrations in JUUL™ pods, and 23 to 4500 times higher than the concentration in edible consumer products (figure 4B). WS-3, which was absent in JUUL™ pods, was 2 to 688 times higher in Puff when compared to edible products (figure 4C).

DISCUSSION

Four main observations come from our comparison of three classes of chemicals in JUUL™ and Puff e-cigarettes. First, in both brands, menthol was the dominant flavor chemical in mint and menthol-flavored fluids, which likely have similar, although not identical, minty flavors. Secondly, while low concentrations of WS-23 were present in JUUL™ "Classic Menthol", both WS-3 and WS-23 were present at much higher concentrations in Puff products with the concentration of WS-23 exceeding that of menthol in Puff Plus "Cool Mint". Third, WS-23 was cytotoxic in the MTT assay at concentrations well below those found in Puff devices. Fourth, pulegone concentrations in mint products from JUUL™ and Puff were high enough to present a cancer risk based on MOE evaluations. While the FDA flavor ban has reduced sales of JUUL™ to minors, young users appear to have rapidly adopted other brands, such as Puff,[22,] which has high concentrations of WS-23 and concerning levels of pulegone. Ironically, the flavor ban may have caused youth to migrate to a potentially more harmful e-cigarette.

Since the dominant flavor chemical in mint and menthol-flavored JUUL™ and Puff products was menthol, banning the sale and distribution of mint-flavored pods may not adequately address the widespread use of this popular flavor. While current federal regulations limit the distribution and sale of flavored cartridge-based pod products, such as JUUL™, they do not solve the problem that "menthol" flavored e-cigarettes are apparently similar, although not identical to "mint." Consequently, a minty flavor is still sold by JUUL™ as "Menthol" and is also available as "mint" in disposable devices from other manufacturers, such as Puff. Although our study deals only with JUUL™ and Puff, any e-cigarette manufacturer can produce menthol-flavored pods or cartridges that may be an acceptable substitute for "mint."

FEMA has designated menthol and synthetic coolants (WS-3 and WS-23) as GRAS (generally regarded as safe) for ingestion, and they are widely used in food and cosmetic products.[17] As pointed out previously, the concentrations of flavor chemicals in e-cigarettes are often very high.[14,39] Menthol and WS-23 concentrations in both brands exceeded those used in most edible consumer products (figure 4).[22,23] While acceptable exposure to GRAS chemicals is based on ingestion data, the acceptable exposures when inhaled are generally unknown and are likely to be much lower, [40,41] raising concerns about the delivery of coolants in e-cigarettes. Unlike the US, several countries (Canada and Germany) have avoided potential problems with coolants by banning their use in tobacco products. [42,43]

The concentrations of menthol in JUUL™ and Puff are high enough to affect cell health. In numerous studies with various cell types, menthol inhibited proliferation and/or caused cell death.[44,45] Menthol concentrations in JUUL™ and Puff would be cytotoxic in the MTT assay based on prior reports with BEAS-2B cells (IC_{70} = 1.38 mg/mL) and A549 cells (IC_{50} = 0.98 mg/mL – aerosol data).[14,18] Even at concentrations below the MTT NOAEL, menthol, when delivered in a PG aerosol using an e-cigarette, binds to TRPM8 receptors on BEAS-2B cells allowing calcium influx and downstream activation of oxidative stress and inflammatory responses.[46] The reported adverse effects of menthol in humans have generally been derived from studies comparing mentholated vs. non-mentholated tobacco cigarettes and have ranged from it being an irritant to causing cancer, although the data supporting the latter claim have been ambiguous.[44] In 2011, it was concluded by the FDA's Tobacco Products Scientific Advisory Committee (TPSAC) that menthol is not a carcinogen.[47] Nevertheless, the inhalation of menthol does have an effect on humans. For example, inhalation of a high dose of menthol by a 13-year-old boy resulted in adverse central nervous system effects.[48] Workers in a throat lozenge manufacturing plant reported that menthol was an irritant that affected their eyes, nasal passages, throats and larynxes.[49] Ingestion of menthol at high doses has resulted in abdominal discomfort, convulsions, nausea, vertigo, ataxia, drowsiness and coma. [49,50] In future studies, it will be important to determine if the high concentrations of menthol inhaled in the context of EC aerosols produce health effects that have not yet been recognized.

High concentrations of WS-23 and WS-3 appeared in our EC fluid data for the first time in Puff and are likewise concerning, as they produce cytotoxic effects in the MTT assay at concentrations below those in Puff e-cigarettes. In contrast, the concentration of WS-23 in JUUL™ "Classic Menthol" was not high enough to produce an IC_{70} in the MTT assay. The cytotoxicity that could be ascribed to menthol in the six products we tested would be roughly equivalent. However, the toxicity ascribable to WS-23 would be many times greater in the Puff products than in JUUL™, suggesting that the removal of most JUUL™ flavors inadvertently motivated users to try other products, such as Puff, that may be more harmful.

Pulegone in EC fluids is a concern because of its known carcinogenicity.[31,32] Our data are based on acute exposures and do not directly assess the long-term effects of e-cigarette chemicals on human health. Calculation of the MOE enables a prediction to be made about the possibility of cancer developing with long-term exposure to individual chemicals and is useful to regulatory agencies in prioritizing their cancer risk.[31,51-53] As MOE values fall below 10,000, the possibility of cancer developing increases. Products labeled "menthol" had concentrations of pulegone that produced MOEs above 10,000, indicating they are not likely to cause cancer in users. However, Puff Bar "Menthol" was much closer to the 10,000 cut off than the JUUL™ products, which ranged from 100,000 to >300,000. In contrast, products labeled "mint" generally had MOEs below 10,000, and in all cases, MOEs for Puff were lower than those for JUUL™. These data are consistent with the interpretation that the mint products were flavored with mint oil, which usually contains pulegone,[29,30] while menthol-flavored products were likely made from crystalline menthol, which would have higher purity and lower concentrations of pulegone. These data support the idea that using pure menthol rather than mint oil in e-fluids would reduce the risk of developing cancer, which could provide a basis for the regulation of additives to mint/menthol-flavored products. Since our MOE calculations are based on pulegone ingestion, our values probably underestimate inhalation exposure, which generally produces a stronger effect to toxicants, including carcinogens.[40,41]

Our data are based on concentrations of chemicals in e-liquids, which we have previously shown generally correctly predicts the cytotoxicity of aerosols.[18] The concentrations of flavor chemicals and coolants received by a user will depend on the transfer efficiency of each chemical to the aerosol and its retention by the user. Therefore, the actual doses inhaled during vaping may be lower than the concentrations we report in the e-liquid. The frequency of vaping will also affect the overall exposure a user receives. These factors will eventually need to be determined to understand the concentrations of flavor chemicals, coolants fully, and pulegone users of JUUL™ and Puff products receive.

In summary, flavor chemicals in JUUL™ "Cool Mint," "Mint," "Classic Menthol," and "Menthol," and in Puff Plus "Cool Mint" and Puff Bar "Menthol" were similar, but not identical, with menthol being the dominant flavor chemical in all products tested. Synthetic coolants are being added to e-cigarettes, sometimes at high concentrations that exceed those used in other consumer products and produced in vitro cytotoxicity. Regulation of mentholated e-cigarettes is now complicated by the sale of "mint-like" flavors under the name "menthol," the lack of regulation of flavor chemicals in disposable e-cigarettes, the presence of cytotoxic concentrations of synthetic coolants in menthol and mint e-cigarettes, and the presence of pulegone in mint-flavored products at concentrations that may be a cancer risk.

What This Paper Adds

- We compared the flavor chemicals, coolants (WS-3 and WS-23), and pulegone in mint and menthol-flavored Puff (disposable) and JUUL™ (pod) e-cigarettes.
- Menthol was the dominant flavor chemical in all products suggesting users may interchange mint and menthol products to achieve a "minty" flavor.
- Unlike JUUL™, Puff products contained cytotoxic concentrations of the synthetic coolant WS-23 and concentrations of pulegone that present a greater cancer risk based on MOE analysis.
- Restriction of JUUL™ flavors may have inadvertently caused a migration of users to a potentially more harmful product.
- The use of pure menthol instead of mint oil in e-cigarette fluids may reduce cancer risk.

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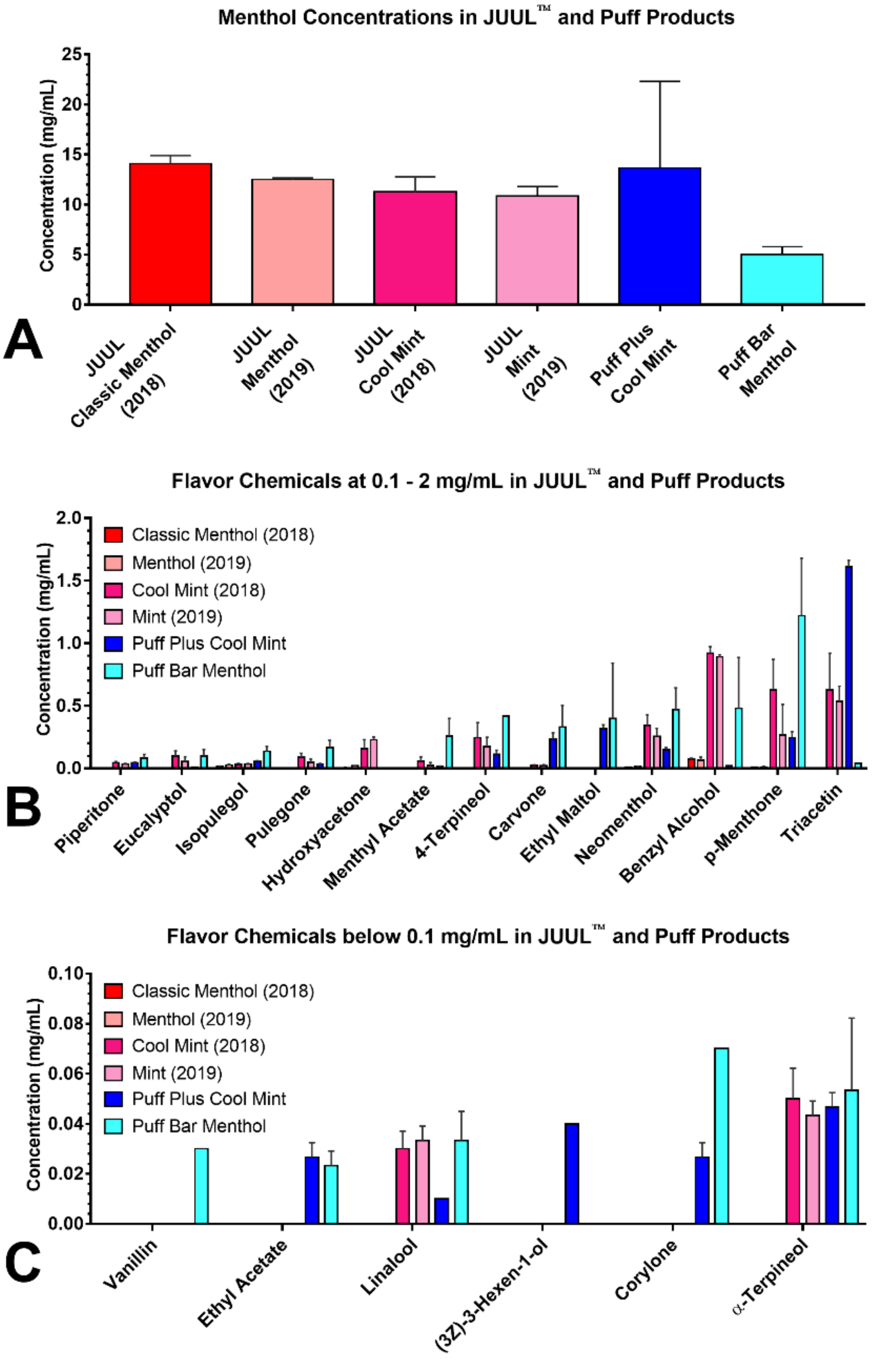

Menthol Concentrations in JUUL™ and Puff Products
Concentration (mg/mL)
25
20
15
10
5
0
A
JUUL Classic Menthol (2018)
JUUL Menthol (2019)
JUUL Cool Mint (2018)
JUUL Mint (2019)
Puff Plus Cool Mint
Puff Bar Menthol
Flavor Chemicals at 0.1 - 2 mg/mL in JUUL™ and Puff Products
Concentration (mg/mL)
2.0
1.5
1.0
0.5
0.0
Classic Menthol (2018)
Menthol (2019)
Cool Mint (2018)
Mint (2019)
Puff Plus Cool Mint
Puff Bar Menthol
B
Piperitone
Eucalyptol
Isopulegol
Pulegone
Hydroxyacetone
Menthyl Acetate
4-Terpineol
Carvone
Ethyl Maltol
Neomenthol
Benzyl Alcohol
p-Menthone
Triacetin
Flavor Chemicals below 0.1 mg/mL in JUUL™ and Puff Products
Concentration (mg/mL)
0.10
0.08
0.06
0.04
0.02
0.00
Classic Menthol (2018)
Menthol (2019)
Cool Mint (2018)
Mint (2019)
Puff Plus Cool Mint
Puff Bar Menthol
C
Vanillin
Ethyl Acetate
Linalool
(3Z)-3-Hexen-1-ol
Corylone
α-Terpineol

Figure 7.1. Flavor chemicals in JUUL™ and Puff "mint" and "menthol" e-cigarette fluids. (A) Menthol was the dominant flavor chemical in all six products. (B) Chemicals present at concentrations ranging 0.1 - 2 mg/mL. (B) Chemicals present at concentrations lower than 0.1 mg/mL. Data are means ± the standard deviations of at least three samples for each group.

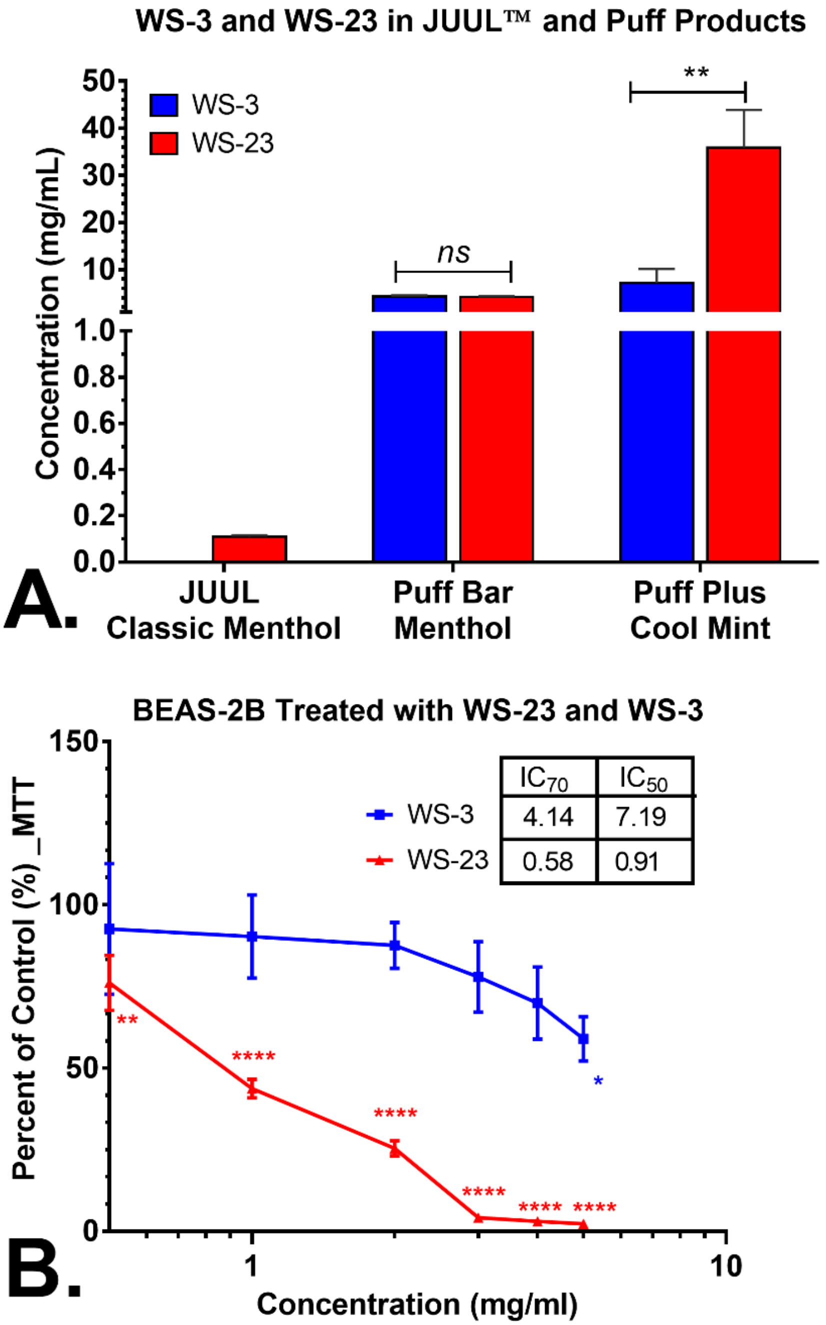


Figure 7.2. Synthetic coolant concentrations in e-cigarette fluids and their toxicities. (A) WS-23 and WS-3 were higher in Puff fluids than in JUUL™ pods. (B) Cytotoxicity of WS-3 and WS23 in the MTT assay. Data are the means ± the standard deviations of at least three independent biological experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

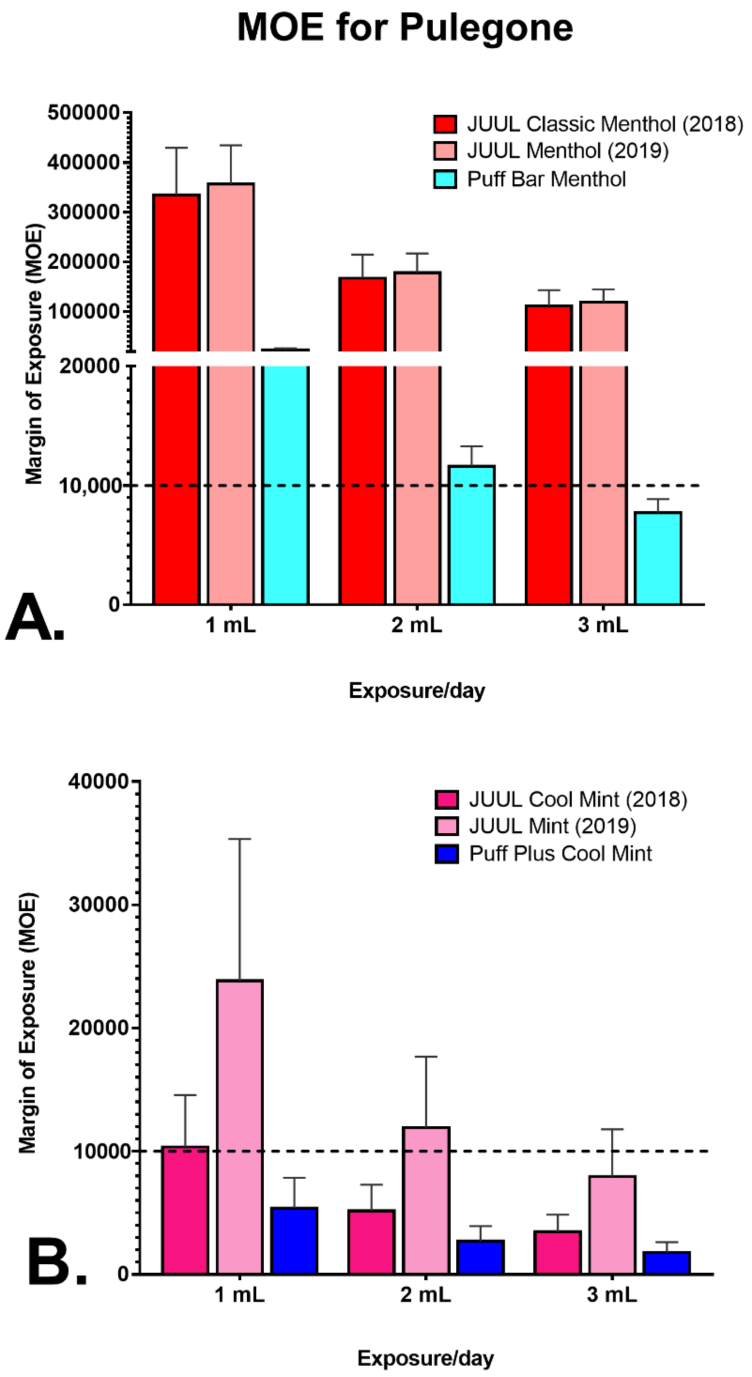


Figure 7.3. The Margin of Exposure (MOE) for pulegone in JUUL™ and Puff products. (A) MOE for "menthol" labeled JUUL™ and Puff e-cigarette fluids. (B) MOE for "mint" labeled JUUL™ and Puff e-cigarette fluids. MOEs below the threshold of 10,000 indicate a high carcinogenic potential and concern for human health.

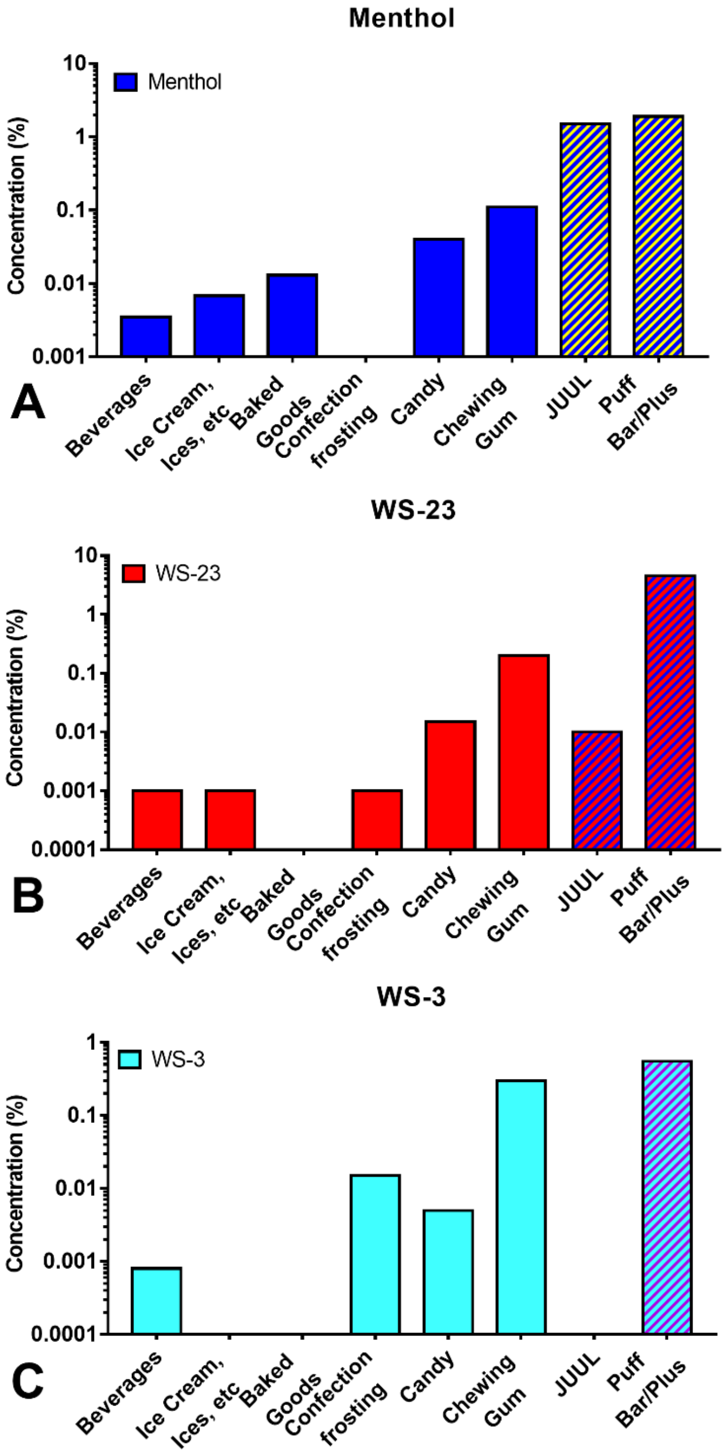


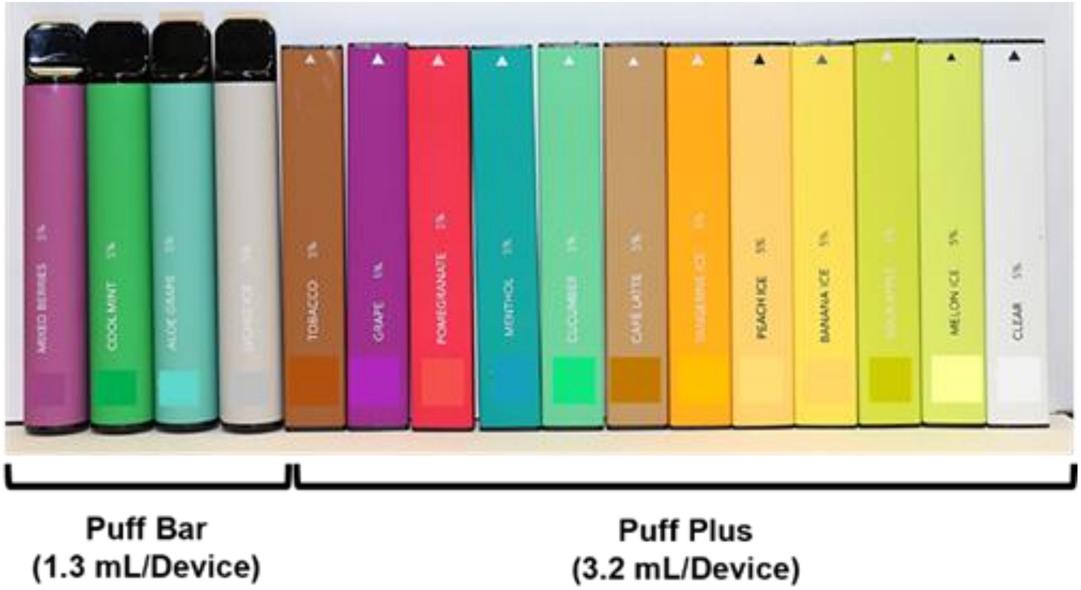
Figure 7.4. Concentrations of flavor chemicals in JUUL™ and Puff e-cigarette fluids and edible consumer products. (A) Menthol. (B) WS-23 (C) WS-3.

Chapter 8

Disposable Puff Bar Electronic Cigarettes: Chemical Composition and Toxicity of E-liquids and a Synthetic Coolant (WS-23)

ABSTRACT

The popularity of disposable fourth-generation electronic cigarettes (ECs) among young adults and adolescents has been rising since the ban on flavored cartridge EC products such as JUUL™. While the constituents and toxicity of some cartridge-based fourth-generation ECs like JUUL™ have been studied, limited data exist for disposable ECs such as Puff. The purpose of this study was to determine flavor chemicals, synthetic coolants, and nicotine concentrations in 16 disposable Puff devices, evaluate the cytotoxicity of the different flavors from the Puff brand using in vitro assays and investigate the health risks of synthetic coolants in EC products. Gas chromatography/mass spectrometry was used to identify and quantify chemicals in Puff EC fluids. A hundred and twenty-six flavor chemicals were identified in Puff fluids, and 16 were >1 mg/mL. WS-23 was present in all products, and concentrations ranged from 0.8 to 45.1 mg/mL. WS-3 concentrations ranged from 1.5 – 16.4 mg/mL in 6/16 products. Nicotine concentrations ranged from 40.6 – 52.4 (average 44.8 mg/mL). All unvaped fluids were cytotoxic at dilutions between 0.1 - 10% in the MTT and NRU assays when tested with BEAS-2B lung epithelial cells. The cytotoxicity of Puff fluids was highly correlated with total chemical concentrations, nicotine, WS-23, both synthetic coolants, and synthetic coolants, plus ethyl maltol. Lower concentrations of WS-23 than were in the fluids adversely affected cell growth and morphology. Concentrations of synthetic coolants exceeded levels used in consumer products. The margin of exposure data showed that WS-3 and WS-23 concentrations were high enough in Puff products to present a health hazard. Our study demonstrates that disposable Puff ECs have high levels of cytotoxic chemicals. The data support the regulation of flavor chemicals and synthetic coolants in ECs to limit potentially harmful health effects.


MIXED BERRIES 5%
COOL MINT 5%
ALOE GRAPE 5%
TOBACCO 5%
GRAPE 5%
POMEGRANATE 5%
MENTHOL 5%
CUCUMBER 5%
CAFE LATTE 5%
PEACH ICE 5%
BANANA ICE 5%
MELON ICE 5%
CLEAR 5%
Puff Bar
(1.3 mL/Device)
Puff Plus
(3.2 mL/Device)

INTRODUCTION

Electronic cigarettes (ECs), which contain nicotine, solvents, and flavor chemicals, continue to evolve and grow in popularity, especially among young adults.1-6 The popularity of 4th generation EC products and their disposable spinoffs, especially among young users, has been attributed to flavored and “icy” fluids, useability, and device features that facilitate stealth use.7-12 EC fluids and aerosols generated from multiple devices contain higher concentrations of chemicals than used in other consumer products, such as foods, cosmetics, and medicines.13-15 ECs and their constituents are cytotoxic to cells, induce inflammatory responses, increase oxidative stress, cause cellular senescence, and negatively affect cell membrane channel potentials.16-23 Despite concern over the use of flavor chemicals in ECs, the chemicals used in EC fluids continue to change and are largely unregulated. Even though JUUL™ dominates the EC market with 63% of current sales,24,25 projections show that disposables, such as Puff Bar, are likely to continue to increase their sales through 2028.26

The technology used by manufacturers of fourth-generation ECs, such as JUUL™ and Puff Bar, is innovative. Nicotine is combined with an acid(s) to reduce the amount of free-base nicotine, making the resulting aerosol less harsh. The use of acids allows manufacturers to increase nicotine concentrations (e.g., 61 mg/mL in JUUL™)27,28 while making it less harsh to users,29-31 thereby increasing the likelihood of addiction. To reduce sales of JUUL™ to young users, the Food and Drug Administration (FDA) enacted a ban on cartridge-based flavored EC pods in 2020.32 Consumers and suppliers quickly discovered a loophole in the ban, which did not cover “disposable” flavored EC products, such as Puff ECs.33,34 The market for disposable pods continues to grow, with dozens of products offered by multiple purveyors.35,36

Although Puff ECs are the most widely used of the 4th generation disposable products, very little is known about their fluids' chemical composition and toxicity. The purpose of our study was to: (1) identify and quantify nicotine, flavor chemicals, and synthetic coolants in Puff fluids, (2) determine the toxicity of the Puff fluids in multiple assays, (3) evaluate the transfer efficiency of synthetic coolants to aerosols, and (4) perform MOE risk assessment analysis on synthetic coolants in Puff products.

MATERIALS AND METHODS

Materials

Isopropyl alcohol (IPA), Dulbecco's Phosphate Buffered Saline (DPBS), Dimethyl sulfoxide (DMSO), EtOH, and acetic acid were purchased from Fisher Scientific (Chino, CA). Analytical grade N-Ethyl -p-menthane-3-carboxamide (WS-3) (CAS # 39711-79-0; catalog # E0796; Lot: SYXVH-SP) and 2-Isopropyl-N,2,3-trimethylbutyramide (WS-23) (CAS # 51115-67-4; catalog # I0729; Lot: LTNPJ-DP) both > 98% pure were purchased from Tokyo Chemical Industry Co. LTD. (Portland, OR). BEAS-2B cells were obtained from American Type Cell Culture (ATCC, USA). Bronchial epithelial basal medium (BEBM) and supplements were purchased from Lonza (Walkersville, MD). Collagen (30 mg/mL), bovine serum albumin (BSA, 10 mg/mL) and fibronectin (10 mg/mL), poly-vinyl-pyrrolidone (PVP), MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Neutral red dye, Tris-HCl, Tris-base, lithium lactate, Tetrazolium salt (INT), phenazine methosulfate (PMS), and β-nicotinamide adenine dinucleotide (NAD) sodium salt were purchased from Sigma-Aldrich (St Louis, MO).

Sample Acquisition

Sixteen disposable Puff EC devices were purchased from vape shops in Los Angeles, CA, and Riverside, CA, in 2020. Twelve Puff Bar flavors ("Tobacco," "Grape," “Pomegranate,” “Cucumber,” “Café Latte,” “Tangerine Ice,” “Peach Ice,” “Banana Ice,” “Sour Apple,” “Melon Ice,” “Menthol,” and “No Flavor” (“Clear”) were labeled to contain 1.3 mL of fluids and advertised to deliver 300 puffs/device. Four Puff Plus flavors (“Mixed Berries,” “Aloe Grape,” “Cool Mint,” and “Lychee Ice”) were labeled to contain 3.2 mL of fluids and advertised to deliver 800 puffs/device. All devices were inventoried, stored in the dark at room temperature, and analyzed within 2 – 3 weeks of purchase.

Authentic standards of both WS-3 and WS-23 were dissolved in propylene glycol (80%) and distilled water (<20%) to simulate lab-made refill fluids. A propylene glycol control blank was prepared with 80% propylene glycol and 20% distilled water.

Aerosol Production and Capture Using an Impinger Method

The transfer efficiency of synthetic coolants from lab-made fluids into the aerosols was evaluated using a fourth generation Baton V2 open pod system equipped with a 350 mAh rechargeable battery, a 1.5 mL refillable pod, and a 1.6-ohm coil that produces an aerosol at 3.7V(volts)/8.6W (watts). Refillable pods were filled with lab-made fluids and pre-conditioned by taking three puffs before making aerosol solutions. The generated aerosol was bubbled through and captured in isopropyl alcohol (IPA) for chemical analysis. The WS-3 and WS-23 aerosol materials captured in IPA (referred to as “aerosol”) were collected at room temperature in two tandem 125 mL impingers, each containing 25 mL of IPA. The Baton V2 pod system was connected to a Cole-Parmer Masterflex L/S peristaltic pump and was puffed using a 4.3 s puff duration,21 inter-puff intervals of 60 s, and an airflow rate of 10 –13 mL/s. To reduce the likelihood of “dry puffing,” fluid level was monitored, and the device was not vaped beyond ¾ of the pod. The pods were weighed before and after aerosol production to collect at least 10 mg for

GC/MS analysis. Aerosol solutions were stored at −20 °C until shipped to Portland State University for analysis.

Gas chromatography/mass spectrometry (GC/MS)

Puff ECs containing fluid-saturated wicks were dissected to expose the atomizers. The fluid-saturated wicks were centrifuged in MinElute spin columns (Qiagen, Valencia, CA) at 3,000 revolutions/minute for 3 minute to separate the fluid from the wick. The extracted fluid was analyzed using previously described gas chromatography/mass spectrometry (GC/MS) methods.28,37 50 µL of each sample were dissolved in 0.95 mL of IPA and shipped overnight on ice to Portland State University, where they were analyzed on the day they were received. A 20 µL aliquot of internal standard solution (2000 ng/µL of 1,2,3-trichlorobenzene dissolved in IPA) was added to each diluted sample before analysis. Using internal-standard-based calibration procedures described elsewhere,37 analyses for 178 flavor-related target analytes, two synthetic coolants, and nicotine were performed with an Agilent 5975C GC/MS system (Santa Clara, CA). A Restek Rxi-624Sil MS column (Bellefonte, PA) was used (30m long, 0.25 mm id, and 1.4 µm film thickness). A 1.0 µL aliquot of diluted sample was injected into the GC with a 10:1 split. The injector temperature was 235 °C. The GC temperature program for analyses was: 40 °C hold for 2 min, 10 °C/min to 100 °C, then 12 °C/min to 280 °C and hold for 8 min at 280 °C, then 10 °C/min to 230 °C. The MS was operated in electron impact ionization mode at 70 eV in positive ion mode. The ion source temperature was 220 °C, and the quadrupole temperature was 150 °C. The scan range was 34 to 400 amu. Each of the 181 (178 flavor chemicals, two synthetic coolants, and nicotine) target analytes was quantitated using authentic standard material.

In October 2019, two synthetic coolants (WS-3 and WS-23) and triethyl citrate were added to our GC/MS target list, which is used to identify and quantify flavor chemicals. GC/MS data collected for multiple EC libraries from 2016 to September 2019 were re-evaluated to estimate the concentrations of synthetic coolants (WS-3 and WS-23) and triethyl citrate using the average response factors generated for them between October 2019 and December 2019.

Human Bronchial Epithelial Cells (BEAS-2B)

Experiments were performed using BEAS-2B cells (passages 20 - 34), often used for toxicological testing. BEAS-2B cells exposed to menthol in submerged culture gave similar results to 3D EpiAirway exposed at the air-liquid interface38 and therefore represent a good cell type for initiating work on the synthetic coolants. BEAS-2B cells were cultured in BEGM supplemented with 2 ml of bovine pituitary extract and 0.5 ml each of insulin, hydrocortisone, retinoic acid, transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor. Nunc T-25 tissue culture flasks were coated overnight with BEBM fortified with collagen (30 mg/mL), bovine serum albumin (BSA, 10 mg/mL), and fibronectin (10 mg/mL) before culturing. Cells were maintained at 30 - 90% confluence at 37o C in a humidified incubator with 5% carbon dioxide. For sub-culturing, cells were harvested using DPBS for washing and incubated with 1.5 ml of 0.25% trypsin EDTA/DPBS and PVP for 3–4 mins at 37°C to allow detachment. Cells were counted using a hemocytometer and cultured in T-25 flasks at 75,000 cells/flask. The medium was replaced the next day and then every other day.

For in-vitro assays, cells were cultured and harvested at 80-90% confluency, using protocols previously described.15 For the MTT, NRU, and LDH assays, cells were plated at 10,000 cells/well in pre-coated 96-well plates and allowed to attach overnight before a

24-hour treatment. BEAS-2B cells were plated at 42,000 cells/well in pre-coated 24-well plates for the live-cell imaging experiments.

Cytotoxicity and Cell Viability Assays

The effects of Puff fluids on the activity of mitochondrial reductase, neutral red uptake, and lactate dehydrogenase release were evaluated. In the culture medium, serial dilutions of EC fluids (10%, 3%, 1%, 0.3%, 0.1%, 0.03%) were arranged in 96-well plates with negative controls (0%) placed next to the highest and lowest concentrations to check for vapor effect.39 BEAS-2B cells were seeded and allowed to attach for 24 hrs. Cells were exposed to treatments for 24 h before the MTT, NRU, and LDH assays were performed.

The MTT assay measures the activity of mitochondrial reductases, which converts water-soluble MTT salt to a formazan that accumulates in viable cells. After treatment, 20 µL of MTT reagent dissolved in 5 mg/mL of DPBS were added to wells and incubated for 2 hours at 37ºC. Solutions were removed from wells, and 100 µl of DMSO were added to each well and gently mixed on a shaker to solubilize formazan crystals. Absorbance readings of control and treated wells were taken against a DMSO blank at 570 nm using an Epoch microplate reader (Biotek, Winooski, VT). For each synthetic coolant tested, three independent experiments were performed.

The NRU assay measures the uptake of neutral red dye, which accumulates within the lysosomes of viable living cells. Following the exposure of cells to treatments, all medium was removed. A working solution of 40 µg of neutral red stock/mL of cell culture medium was prepared and incubated at 37 °C overnight to dissolve the neutral red. Cells were incubated with 150 µL of neutral red solution for 2 hours. Cells were washed with PBS, and 150 µL of lysis buffer (50% EtOH/49% deionized H2O/1% acetic acid) were added to each well and gently mixed to achieve complete dissolution. Absorbance readings of wells were recorded at 540 nm using an Epoch microplate reader (Biotek). The LDH assay measures lactate dehydrogenase released into the culture medium due to plasma membrane damage. Reagents and solutions were prepared using an in-house recipe developed by OPS Diagnostics (Sigma-Aldrich). 200 mM TRIS (22.2 g Tris-HCl, 10.6 g Tris-base, and 50 mM lithium lactate) at a pH of 8 were prepared in water. Tetrazolium salt (INT) was dissolved in DMSO (33 mg/mL), phenazine methosulfate (PMS) was dissolved in water (9 mg/mL), and β-nicotinamide adenine dinucleotide (NAD) sodium salt was dissolved in water (3.7 mg/mL). The three reagents (INT, PMS, and NAD) were combined to make the INT/PMS/NAD solution. 50 µL of all reagents were added to empty wells, followed by 50 µL of medium from treated and control wells. Absorbance readings were recorded at 540 nm and 620nm using an Epoch microplate reader (Biotek).

Growth and Morphology Assays

Non-invasive cell growth and morphology analyses of live cells were performed using 10x and 20x phase contrast objectives in a BioStation CT using automatic Z-focus.40 After attachment, BEAS-2B cells were treated with Puff EC fluids (0.1 - 10%), or with WS-23 (0.045 - 4.5 mg/mL) solutions dissolved in cell culture medium. Images were taken every 2 hours for 48 hours to collect time-lapse data for analysis. Evaluation of BEAS-2B growth and morphology was compared in control and treated groups using CL Quant software (DR Vision, Seattle, WA).40-42 Data from the treated groups were normalized to untreated controls.

Solubility of WS-23 and WS-3 in Water and Culture Medium

WS-23 was dissolved in molecular grade water or culture medium at concentrations of 0.45, 4.5, 7 ,or 9 mg/mL, and 500 ul of each solution was added to 48-well plates with a glass bead in each well to aid in focusing the liquid with a stereoscopic microscope. For WS-3, 0.02 mg/mL was dissolved in water and cell culture medium to confirm its reported solubility. Images were taken with a stereoscopic microscope, and the presence of residues was compared for both solvents.

Statistical Analyses

For GC/MS data, data points are averages of measurements from fluids obtained from three devices. All values below the limit of quantification (LOQ) were excluded from the data. Cytotoxicity analyses were performed using three different cell passages, and each experiment was done at least three times. Data were statistically analyzed with a one-way analysis of variance (ANOVA). When significance was found ($p < 0.05$), each concentration was compared to the untreated control with Dunnett's post-hoc test using Prism software (GraphPad, San Diego).

RESULTS

Total Concentrations of Nicotine and Flavor Chemicals

Based on flavor names, Puff ECs were grouped into five categories: tobacco, fruity, berries, menthol, and unflavored. The concentrations of nicotine, total flavor chemicals, and synthetic coolants were analyzed (Figure 1a). The average nicotine concentration in disposable Puff devices (44.8 mg/mL ± 2.5 SD) was lower than in previously evaluated JUUL™ pods (61 mg/mL) but higher than in cartomizer and refill fluids we have examined28 (Figure 1a). The total concentration of flavor chemicals in Puff fluids was highly variable and ranged from 0.7 (Cucumber) to 34.3 (Tobacco) mg/mL (Figure 1a).

Fruit-flavored products were highly variable in total concentrations and dominant chemicals (> 1 mg/mL). Seven flavor chemicals, including ethyl maltol and ethyl acetate in Aloe Grape, accounted for 80% of the sum of flavor chemicals. Minty flavored Puff ECs contained two dominant flavor chemicals: menthol, and the other was p-Menthone in “Cool Mint” and triacetin in “Menthol.” While “Lychee Ice” and “Melon Ice” contained only ethyl maltol as the dominant flavor chemical, “Peach Ice” and “Clear” contained γ-undecalactone and menthol, respectively.

Synthetic Coolants: WS-3 and WS-23

WS-3 and WS-23 were identified and quantified in both “ice,” and “non-ice” flavored Puff EC fluids (Figure 1b). WS-23 was present in all 16 products at concentrations ranging from 0.8 mg/mL in Tobacco to 45.1 mg/mL in Cool Mint. The levels of both synthetic coolants in “Cucumber” and “Menthol” were similar (5.1 mg/mL and 4.3 mg/mL, respectively) and are shown using yellow bars in Figure 1b. WS-3 concentrations in 6/16 products were generally lower than WS-23 ranging from 1.5 mg/mL in “Tangerine Ice” to 16.4 mg/mL in “Clear” (Figure 1b). The concentrations of WS-3 in Banana Ice, Mixed Berries, and Caffe Latte were below the LOQ (0.02 mg/mL). The combined concentrations of WS-3 and WS-23 in products that contained both synthetic coolants ranged from 0.9 – 55.8 mg/mL.

EC products purchased and analyzed between 2016 – 2019 were re-evaluated to identify and estimate the concentrations of WS-3 and WS-23 in cartomizers, pods, and refill fluids (Table S3). Out of over 600 EC samples analyzed in our lab, both synthetic coolants were found in 13 products: WS-3 (n = 5) and WS-23 (n = 8) (Table S3). The concentrations of the synthetic coolants ranged from 0.2 to 1.7 mg/mL for WS-3 and 0.1

to 3.9 mg/mL for WS-23. Triethyl citrate was more frequently found in refill fluids at elevated levels and ranged from 0.05 – 11.5 mg/mL (Table S3).

Propylene Glycol and Glycerol Concentrations

All Puff EC fluids contained propylene glycol (PG) and glycerol (G). The concentrations ranged from 158 – 371 and 310 – 437 mg of solvent per mL of undiluted Puff EC fluid for PG and G, respectively (Figure 1c). The sum of both solvents in Puff ECs ranged from 544 – 740 mg/mL. The percentage ratio of PG: G was approximately 30:70 in one product, 40:60 in three products, and 50:50 in 12 products (Figure 1c).

Contributions of Chemicals to the Total Sum of Chemicals in Each Product

Chemicals in Puff ECs were grouped into four categories: nicotine, synthetic coolants (WS-3 and WS-23), flavor chemicals, and solvents (PG and G) (Figure 1d), and the percentage contribution of each group to the total sum of chemicals was calculated. Nicotine accounted for 5% of the total content in Aloe Grape to 7% in Tangerine Ice, Sour Apple, and Caffe latte. The remaining 12 products contained 6% nicotine (Figure 1d). Synthetic coolant contribution to the total chemicals ranged from 0.1% in Tobacco to 6% in Cool Mint and Clear (unflavored product). In 75% of the product, synthetic coolant concentration to the total content was greater than 1% (Figure 1d). Flavor chemicals contributed between 0.09 - 4.2%, with more than half of the products higher than 1%. Solvents accounted for the most chemicals ranging from 87% in Cool Mint to 93% in Cucumber.

Individual Flavor Chemicals in Puff Bar Fluids

Seventy-one percent (129/181) of the chemicals on our target analyte list were identified in Puff EC fluids. Forty-two flavor chemicals detected below the LOQ are listed in Table S1. Further analysis was performed on 87 flavor chemicals above the LOQ (Figure 1e and Figure S1). Except for “Sour Apple,” “Tangerine Ice,” and “Cucumber,” all Puff ECs had at least one dominant flavor chemical (> 1 mg/mL) (Figure 1e). Ethyl maltol, menthol, vanillin, ethyl propionate, ethyl butanoate, triacetin, methyl anthranilate, (3Z)-3-hexen-1-ol were present in at least two products at > 1 mg/mL. p-Menthone, ethyl lactate, corylone, isoamyl acetate, benzyl alcohol, ethyl acetate, ethyl vanillin, and γ-undecalactone were present in one product at > 1 mg/mL. The concentrations of dominant flavor chemicals varied between Puff EC flavors and ranged from 1 – 15 mg/mL. Ethyl maltol was > 1 mg/mL in 50% of the products evaluated. Less dominant flavor chemicals (0.02 – 0.99 mg/mL) are shown in Figure S1. While the frequency of all chemicals detected ranged from 1 to 16, the total number of chemicals per product ranged from 4 to 40 (Figure 1e, Figure S1, and Table S1).

Major and minor non-target chemicals were investigated for all Puff EC flavors. Benzoic acid, acetic acid, 2-hydroxypropyl acetate, 1,2-propanediol-2-acetate, 2-Hydroxypropane-1,3-diyl diacetate, and glycerol 1,2-diacetate were identified as major non-target compounds (Table S2). Vanillin and ethyl vanillin PG and G acetals were present as minor non-target compounds in products that contained ≥ 5 mg of each chemical/ml of fluid (Figure 1e and Table S2).

Cytotoxicity of Puff EC Fluids

Cytotoxicity of Puff EC fluids was evaluated with BEAS-2B cells using the MTT, NRU, and LDH assays (Figure 2 and Table 1). Products were considered cytotoxic if they had an effect of 30% less than the untreated control (the IC70).43 Puff EC fluids were cytotoxic in the MTT and NRU assays, and IC_{70} and IC_{50} values were reached at fluid concentrations between 0.09 – 1.35 % and 0.14 – 1.24%, respectively (Figure 2 and

Table 1). Cell viability was evaluated using the LDH assay, and no significant effects were observed (Figure 2a-p).

Relationship between Chemical Concentrations and Cytotoxicity

Linear regressions were performed to determine the contributions of nicotine, flavor chemicals, and synthetic coolants to the cytotoxicity observed with Puff EC fluids (Figure 3). The chemical concentrations and cytotoxicity data for the 0.03 - 1% range were used to perform the regression analysis. Regression coefficients (R2) for concentration versus cytotoxicity were considered high (≥ 0.5), moderate (0.1 − 0.4), or low (≤ 0.1). High and moderate correlations were observed between cytotoxicity and concentrations of the total chemicals and flavor chemicals (Figures 3a and b). The regression analysis for nicotine only, a combination of synthetic coolants and WS-23, showed high and moderate correlations with significant p-values (Figure 3c – e). WS-3 and ethyl maltol concentrations were moderately correlated to cytotoxicity with significant p-values (Figures 3f and g). For products with both synthetic coolants and ethyl maltol, the relationship between cytotoxicity and concentration was high and statistically significant (Figure 3h). Regression analyses were performed for all other dominant flavor chemicals (Figure S2). The correlation coefficients ranged from moderate (Figure S2 a – k) to no relationship (Figure S2 l – o) with almost no significant p-values.

Effect of WS-23 and Puff EC Fluids on Cell Growth and Morphology

Non-invasive analysis of BEAS-2B cell growth was performed using time-lapse images taken over 48 hours (Figure 4a – f). The typical epithelial monolayer was observed for untreated control cells (Figure 4b, d, f). WS-23 significantly inhibited cell growth in a concentration-dependent manner (Figures 4a and b). Significance was observed as early as 20 hours for cells treated with 10 % (red lines), 28 hours for 3% (blue lines), 40 hours for 1% and 0.3%, and 48 hours for 0.1% fluid solutions (Figure 4a and b). Cells appeared normal in all concentrations except in the 1.5mg (3%) and 4.5 mg (10%) treatments, where the cells appeared elongated and rounded, respectively. Micrographs showing segmented images taken at 0, 24, and 48 hours are presented in Figures S3 a.

When Puff EC fluids with high levels of WS-23 (“Cool Mint”) and equal levels of WS-23 and WS-3 (“Cucumber”) were tested, varying effects were observed. The effects of “Cool Mint” fluid (WS-23 = 45 mg/mL) and “Cucumber” fluid (equal concentrations of WS-3 and WS-23 = 5.1 mg/mL) were evaluated in a live-cell imaging assay. Cell growth was significantly affected starting at about 12 hours for both treatments at concentrations > 1% (Figure 4c - f). BEAS-2B cells exposed to various concentrations of Puff Plus Cool Mint (Figure 4d) revealed elongated morphologies at 1%, rounded at 3%, or appeared fixed at 10% starting at the first time point and extending throughout the experiment. The morphologies observed with Puff Bar Cucumber fluid (Figure 4f) were either stressed and elongated (1%), rounded (3%), or fragmented (10%). Micrographs showing segmented images taken at 0, 24, and 48 hours are presented in Figures S3 b and c.

Transfer Efficiency of Aerosolized Synthetic Coolants

Refill fluids made in-house using 80% PG, water, and authentic standards of each synthetic coolant were analyzed using GC/MS to identify and quantify chemicals in the fluids and corresponding aerosols (Figure 5). Generally, the transfer efficiency of aerosols produced with the Baton V2 pod device was high (Figure 5a). The mean of two experiments revealed that WS-23 transferred to an aerosol with 70% efficiency, while WS-3 transferred with 90% efficiency. (Figure 5b). Puff Bar is also a low powered EC and likely has similar transfer efficiencies. Transfer efficiency can vary with many factors, including power, and may be higher in second and third generation ECs.

Margin of Exposure (MOE) Evaluation for Synthetic Coolants

The MOE, which aids risk assessors in prioritizing the potential exposure risk to food additives,44,45 was used to evaluate the potential risk from daily exposure to WS-3 and WS-23. The MOE approach considers a reference point (e.g., the NOAEL) from experimental data, an estimated daily exposure dose to the chemical or additive, and an average adult body weight of 60 kg. Daily consumption range of 0.5 mL (less than half the fluid in a Puff Bar device) to 15 mL, a high daily consumption for free-base nicotine EC fluids was used. Using NOAEL values determined from orally administered WS-3 and WS-23 in rats, we calculated MOEs for WS-23 (NOAEL = 5 mg/kg/bw) and WS-3 (NOAEL = 8 mg/kg/bw)46 based on a 100% transfer from EC fluid mixture into the aerosol. A MOE below the 100 threshold for a food additive is considered high risk requiring prioritization and mitigation by regulatory agencies. MOEs for WS-23 were <100 for all flavors except Tobacco at 1mL consumption per day (Figure 6a). For other nicotine-salt-based disposable devices and free-base nicotine fluids, daily consumption of 3mL/day generated MOEs < 100. In contrast, MOEs calculated for WS-3 were < 100 in 5/6 products considering a 1mL consumption per day (Figure 6b). Daily consumption of 3mL/day generated WS-3 MOEs < 100 in only 25% of the samples for other free-base nicotine fluids.

DISCUSSION

Our study investigated the chemicals in fluids from fourth-generation disposable Puff ECs and their toxicological effects. Over 100 chemicals, including nicotine and two synthetic coolants, were identified in 16 “ice” and “non-ice” flavors. Nicotine concentrations in Puff fluids were generally lower than previously reported in fourth-generation cartridge-based fluids.[27,28,47] However, nicotine concentrations in Puff and JUUL™[28] were higher than in free-base nicotine EC refill fluids.[48–52] Two synthetic coolants (WS-3 and WS-23), often used in cosmetics, personal hygiene products, and edibles, were present in Puff EC fluids at concentrations higher than recommended for consumer products.[46] The concentrations of WS-23 that inhibited mitochondrial reductases and cell growth were well below the concentrations in the Puff EC fluids. Concentration-response curves for toxic effects were significantly correlated with nicotine, ethyl maltol, and WS-23 concentrations. For most Puff ECs, the MOEs for the synthetic coolants were below the acceptable threshold of 100 for food additives, indicating a potential health risk.

Flavor chemicals in EC fluids and aerosols are frequently found in high concentrations and often account for a significant fraction of the total chemicals in EC products.[14,18] We have previously categorized "dominant flavor chemicals" as those at concentrations ≥ 1 mg/mL.[17] JUUL™ products generally had 0-1 dominant flavor chemical/product.[28] In contrast, most (n=13) Puff ECs had more than one dominant flavor chemical, and nine Puff e-cigarettes had two or more/products. Three Puff Bars ("Sour Apple," "Tangerine Ice," and "Cucumber") did not have any dominant flavor chemicals. Puff Bar Tobacco contained the highest total flavor chemical concentration, with dominant chemicals being ethyl maltol, vanillin, corylone, and ethyl vanillin. In contrast, JUUL™ Classic and Virginia Tobacco did not have any dominant flavor chemicals,[28] similar to other previously examined tobacco-flavored refill fluids.[15] While menthol was the dominant flavor chemical in "minty" Puff e-cigarettes, its concentration was two times higher in Puff Plus "Cool Mint" than in Puff Bar "Menthol". p-Menthone, which may be added to enhance the minty flavor, was also dominant in Puff Plus "Cool Mint" and previously found at high levels in LIQUA

"Cool Menthol" refill fluids.[15] Triacetin, a dominant flavor chemical in Puff "Menthol," may have been added to produce a fruity accent, or in the case of "Sour Apple," it may have formed in part by a reaction between acetic and propylene glycol. In our prior studies, triacetin was not used frequently in American manufactured e-fluids.[14] However, it was the most commonly used flavor chemical in a Russian brand (Ritchy LTD), distributed worldwide with high concentrations in fruity-flavored fluids (13 to 22.5 mg/mL) and a mint-flavored product without menthol (44.3 mg/mL).[15] Ethyl maltol, a dominant and frequently used flavor chemical in multiple EC libraries,[15] was in almost all Puff products at > 1 mg/mL. In some previous studies, ethyl maltol was the most cytotoxic flavor chemical in the MTT assay, and its concentration was correlated with the cytotoxicity of JUUL™ and LIQUA EC fluids.[15,18,28]

Some of the dominant flavor chemicals in Puff and JUUL™ ECs frequently appeared in high concentrations in our prior studies (e.g., menthol, ethyl maltol, benzyl alcohol, vanillin, and triacetin).[13,15,17] Ethyl acetate and 3Z-3-Hexen-1-ol were found in most Puff products, generally at concentrations < 1 mg/mL. Ethyl acetate, which has low cytotoxicity in the MTT assay, was also present in most products in popular refill fluids.[18]

Both JUUL™ and Puff EC fluids contained benzoic acid, and two Puff flavors ("Sour Apple" and "Aloe Grape") also had acetic acid. In addition, both 2-hydroxypropyl acetate and 1,2-propanediol-2-acetate were major non-target chemicals in "Sour Apple," "Aloe Grape," "Tangerine Ice," and "Peach Ice." Both compounds are acetates of propylene glycol, which may be added as solvents or fruit flavorants, or form as reaction products between propylene glycol and acetic acid. Since acetic acid was a major non-target, they may be reaction products.

Synthetic coolants were rarely used in earlier generations of EC products. When present, their concentrations were about 0.2 mg/mL in cartomizer fluids and 0.1 – 3.9 mg/mL in refill fluids, with WS-23 generally being higher than WS-3 (Table S3). WS-3 and WS-23 concentrations in Puff ECs sold in the USA were greater than those in JUUL™ pods in Europe or the USA.[19,53] The synthetic coolants were present in all Puff ECs, while only 2 of 8 JUUL™ flavors had synthetic coolants, which were significantly lower in concentration. WS-3 and WS-23 do not add flavor but impart a cooling sensation and were found in "ice" and "non-ice" fruit, berries, and tobacco flavored Puff EC flavors. Concentrations of chemicals recently reported generally agreed with our data, except for menthol in "Cool Mint," which was 22 times higher in our samples.[54]This observation suggests batch-to-batch variations in Puff products. The constituents of EC fluids are rapidly evolving. In 2018, JUUL™ products contained very high nicotine concentrations combined with benzoic acid, which was not the case with refill fluids before the introduction of JUUL™. Some Puff ECs contain synthetic coolant concentrations that are ~450 times higher than the concentrations in JUUL™ (45.1 mg/ml in Puff Plus Cool Mint vs. 0.1 mg/ml in JUUL Classic Menthol).[19] The concentrations of nicotine, synthetic coolants, and flavor chemicals in Puff ECs are concerning and demonstrate the need for more attention to evolving EC constituents.

Fourth-generation JUUL™ pods are characterized by high concentrations of nicotine (~61 mg/mL).[28] Likewise, nicotine was relatively high in concentration in Puff products (40.6 – 52.4 mg/mL). In a related study, nicotine in Puff ECs ranged from 29.4 – 40.7 mg/mL,[52] while another study found 83.4 mg/mL.[55] Differences in reported concentrations for Puff ECs may be due to the methods used to quantify nicotine or variations in manufacturing different batches. In both studies, the reported nicotine concentrations are high relative to earlier generation products. PG/G ratios are similar to those reported previously for Puff products. [55]

Chemicals in EC products impair cell processes and induce inflammatory responses in multiple cell types.[16-23] The concentrations of flavor chemicals and synthetic coolants in EC products are high enough to affect cell growth and morphology during acute exposure. In the current study, the cytotoxicity of Puff EC fluids in the MTT and NRU assays was significantly correlated with total chemical concentration and individual chemicals (nicotine, WS-3, WS-23, and ethyl maltol). The IC_{50}s of fluids were lower when compared to similar flavors from JUUL in the MTT and NRU assays.[28] We previously showed that the IC_{50} is reached for nicotine at 0.9 mg/mL in the MTT assay.[18] The nicotine concentrations in Puff ECs are high enough to contribute to the toxicity of the fluids at the medium to high concentrations tested in the current study. Ethyl maltol, a frequently used dominant chemical,[15] impairs the activity of mitochondrial reductases in BEAS-2B and mouse neural stem cells, with IC50s of 0.06 and 0.03 mg/mL in the MTT assay, respectively. The concentrations of ethyl maltol in Puff EC fluids are well above the IC_{50}s reported previously.[18] Both synthetic coolants in Puff ECs were evaluated for cytotoxicity, and WS-23 had a significant effect on mitochondrial metabolism at concentrations 90 times lower than those in Puff EC fluids (IC_{50} = 1 mg/mL). Our live-cell imaging analysis shows that WS-23 significantly affected cell growth and morphology shortly after the onset of treatment.

There are conflicting reports on websites regarding the solubility of WS-23. PubChem and the Food and Agriculture Organization (FAO) of the United Nations report it is insoluble in water.[56,57] The Good Scents Company and ChemHub websites report its solubility to be 0.45 mg/mL in water[58,59]. In contrast, European and Chinese websites[60-62] have reported the solubility of WS-23 to be ~ 7 mg/mL, which is higher than the highest concentration we tested (4.5 mg/mL). To verify that WS-23 was dissolved at 4.5 mg/mL in our experiments, we tested its solubility in water and BEAS-2B culture medium at various concentrations (Figure 7). At 4.5 mg/mL, WS-23 was completely dissolved in water and culture medium (Figure 7 c - f). At 7 mg/mL, WS-23 was soluble in water but not in culture medium (Figure 7 g and h). At 9 mg/mL, a concentration above all reported solubilities, the chemical was partially soluble in water and insoluble in culture medium (Figure 7 i and j). These data show that WS-23 was completely dissolved in our experiments at the highest concentration tested and further show that its reported solubility is incorrect on some websites.

Menthol and structurally related synthetic coolants such as WS-3 activate the TRPM8 channels located on cells, allowing ion influx and creating a cooling sensation, followed by activation of downstream inflammatory responses.[38] WS-23 differs structurally from menthol yet imparts a cooling sensation. However, the lower potency of WS-23 to activate TRPM8 channels compared to menthol[63-65;] may indicate that other targets, including promiscuous TRP channels outside the M8 subfamily, may be involved in its effects on cells. Since these synthetic coolants, like flavor chemicals, were not originally intended for use in inhalatable products, minimal data exist on their adverse effect in humans after inhalation. A recent rat inhalation study found no significant effects of WS-23 on body weight, food consumption, and relative organ weights after a 4-hr acute exposure and a 14-day observation period.[66] In the same study, a 28-day subacute exposure followed by 28-days of recovery found no significant differences in body weight, food consumption, blood parameters, serum biochemistry, urine, pulmonary function, organ weight, and bronchoalveolar lavage fluid (BALF).[66] However, the high dose used in the rat study

(342.85 mg/m^3) was one eighth the concentration ($2{,}813$ mg/m^3) calculated for air exposure based on the highest concentration of WS-23 (45.1 mg/mL) in our study (assuming a 40 mL puff, 2.5 mg/puff, an aerosol density of 1g/mL and WS-23 concentration). The concentration in the rat study may not have been sufficient to produce an effect and/or the chosen endpoints may not have been affected. Similar animal exposure experiments using higher doses would be helpful.

Flavor chemicals are used in EC products at levels that exceed concentrations in other consumer products.[15,19] While these flavor chemicals are designated "Generally Regarded As Safe" (GRAS) for ingestion, the Flavor Extract Manufacturers Association (FEMA) does not endorse their use for inhalation.[67] The concentrations of dominant flavor chemicals in Puff fluids were generally higher than those in edible products, except for ethyl vanillin in imitation vanilla extracts, which are diluted before use (Table S4 and S5).[68-72] Ethyl maltol, which imparts a sweet flavor, is frequently used at high concentrations in EC products.[13,14,17,19] In edibles (e.g., beverages, candy, chewing gum, ice cream, baked goods) and cosmetics (e.g., soaps, detergent, lotions, and perfume products), it is recommended that ethyl maltol concentrations not exceed 0.4%.[68-73] However, ethyl maltol in Puff fluids ranged from 0.007– 0.99% and exceeded ingestible concentrations in 77% of the products when present. Ethyl maltol and some other flavor chemicals (e.g., ethyl vanillin and γ-decalactone) increase free radical formation in EC aerosols[74] and contributes to the toxicity of EC fluids.[15,17,18]

Like flavor chemicals, synthetic coolants are designated GRAS and used in edible and skincare products.[71,72] Even though their safety designation does not apply to inhalation, they have been used in tobacco products at 263 – 2300 ng/stick[75] concentrations. The evolution of EC products has seen increased levels of synthetic coolants, especially with fourth-generation disposable products. WS-23 is used at 0.0008 – 0.3% in beverages, hard candy, confectionaries, and chewing gums.[71] However, in Puff ECs, concentrations ranged from 0.08 – 4.51%. WS-3, another popular synthetic coolant, was found in fewer Puff ECs (38%) at 0.14 – 1.64% concentrations, exceeding maximum levels regarded as safe in beverages, ice creams, confectioneries, candy, and chewing gum (range = 0.001 – 0.12%).[72] In the current study, the concentrations of synthetic coolants were up to thousands of times higher than in edible products and toxic in in-vitro assays at concentrations lower than those found in Puff fluids.[18] Consumers may be unwittingly exposed to high levels of synthetic coolants in "non-ice" Puff ECs. Long-term studies with humans will be needed to fully understand the health effects of chronic inhalation of high concentrations of synthetic coolants.

Risk assessors use the margin of exposure (or safety) to evaluate carcinogenic risk or chemical safety based on predicted or estimated exposure levels. Since minimal data exist for inhalation exposures and toxicity, parameters based on oral administration of a chemical in experimental animals are often used.[76] Non-genotoxic and non-carcinogenic chemical substances with MOEs less than 100 are generally considered a health risk. The concentrations of synthetic coolants in inhaled tobacco products exceed those in edible products. Calculated MOEs for WS-3 and WS-23 are well below 100 for almost all Puff products at 1 mL of fluid/day, thereby presenting a safety risk to consumers. Mint and "ice" flavored Puff had the lowest MOEs, consistent with higher concentrations of synthetic coolants. Puff products that contained both synthetic coolants at levels that generated MOEs below the 100 thresholds would increase the exposure risks to users. Because the oral and inhalation toxicities are not always equivalent, route-to-route extrapolations routinely used by regulatory agencies[77,78] may be required for a more realistic exposure model in humans. Considering the increased sensitivity of the respiratory tract to toxicants, the MOE values calculated for Puff ECs underestimate exposure.[77,78] The Joint FAO/WHO Expert Committee on Food Additives concluded that further research is needed to assess the risk of synthetic coolants to humans. [76]

Future work should evaluate the use and concentrations of synthetic coolants in new EC products as they evolve. It would also be informative to examine exposure at the air-liquid interface using aerosolized synthetic coolants.

In summary, our data show that the fluid composition of ECs is evolving, with the most recent major change being the inclusion of high concentrations of synthetic coolants, which were toxic in our in vitro assays. The ban on flavored cartridge-based EC products caused a migration of adolescents and young adults from cartridge-based products like JUUL™ to disposable ECs like Puff, which is exempt from the flavor ban. These new disposable ECs, exemplified by the Puff brand studied here, have much higher concentrations of synthetic coolants than those found in JUUL™. The high levels of nicotine, flavor chemicals, and synthetic coolants, which exceeded those used in other consumer products, raise a concern about the safety of Puff products. Product manufacturers are increasing the youth-attracting synthetic coolant content of ECs, while the inhalation risks remain unknown. This practice, in effect, represents a large, uncontrolled experiment in the lungs of youth and other consumers and highlights the need for regulation to protect public health.

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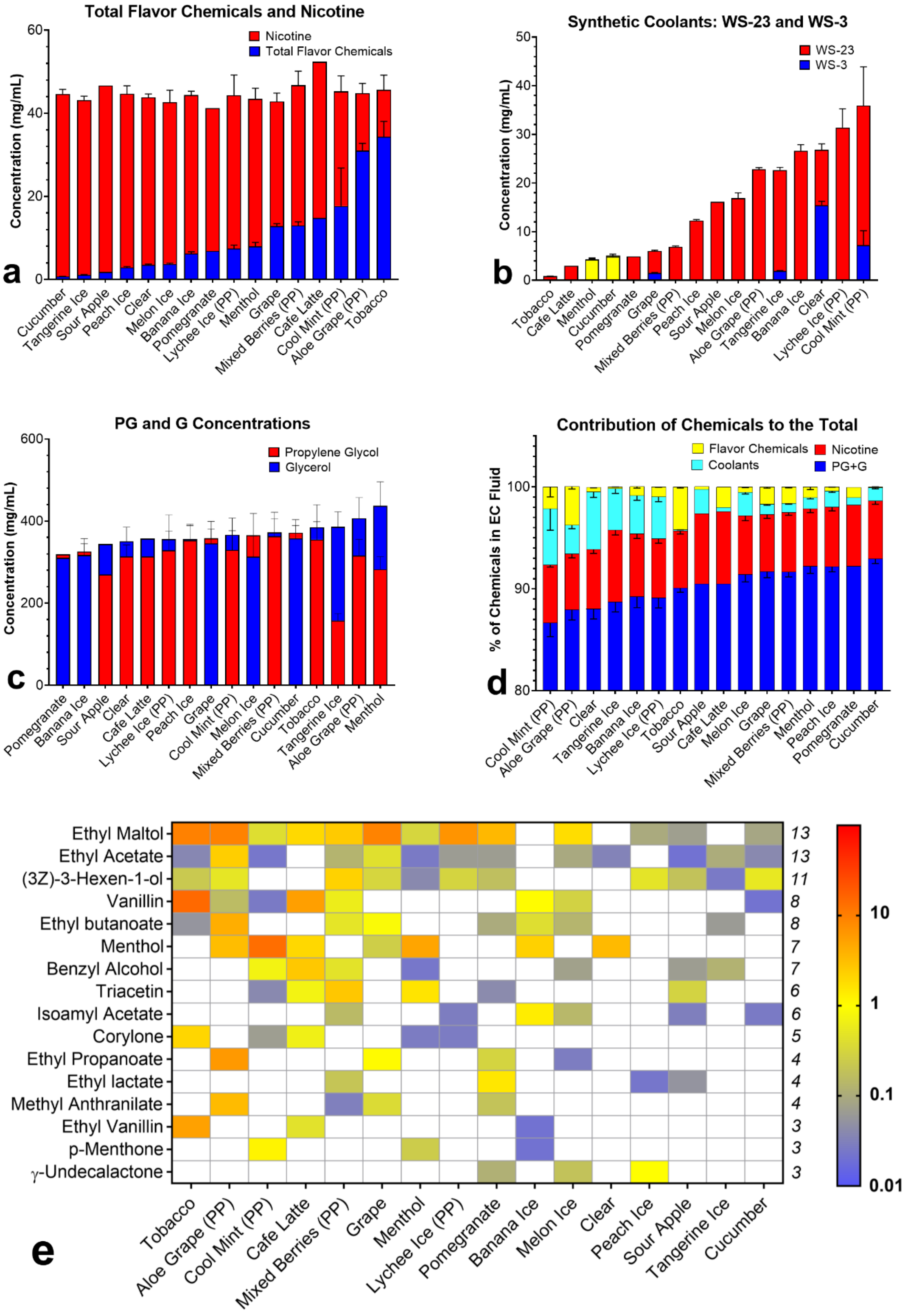

Total Flavor Chemicals and Nicotine
Nicotine
Total Flavor Chemicals
Concentration (mg/mL)
60
40
20
0
a
Cucumber
Tangerine Ice
Sour Apple
Peach Ice
Clear
Melon Ice
Banana Ice
Pomegranate
Lychee Ice (PP)
Menthol
Grape
Mixed Berries (PP)
Cafe Latte
Cool Mint (PP)
Aloe Grape (PP)
Tobacco
Synthetic Coolants: WS-23 and WS-3
WS-23
WS-3
Concentration (mg/mL)
50
40
30
20
10
0
b
Tobacco
Cafe Latte
Menthol
Cucumber
Pomegranate
Grape
Mixed Berries (PP)
Peach Ice
Sour Apple
Melon Ice
Aloe Grape (PP)
Tangerine Ice
Banana Ice
Clear
Lychee Ice (PP)
Cool Mint (PP)
PG and G Concentrations
Propylene Glycol
Glycerol
Concentration (mg/mL)
600
400
200
0
c
Pomegranate
Banana Ice
Sour Apple
Clear
Cafe Latte
Lychee Ice (PP)
Peach Ice
Grape
Cool Mint (PP)
Melon Ice
Mixed Berries (PP)
Cucumber
Tobacco
Tangerine Ice
Aloe Grape (PP)
Menthol
Contribution of Chemicals to the Total
Flavor Chemicals
Nicotine
Coolants
PG+G
% of Chemicals in EC Fluid
100
90
80
d
Cool Mint (PP)
Aloe Grape (PP)
Clear
Tangerine Ice
Banana Ice
Lychee Ice (PP)
Tobacco
Sour Apple
Cafe Latte
Melon Ice
Grape
Mixed Berries (PP)
Menthol
Peach Ice
Pomegranate
Cucumber
Ethyl Maltol
Ethyl Acetate
(3Z)-3-Hexen-1-ol
Vanillin
Ethyl butanoate
Menthol
Benzyl Alcohol
Triacetin
Isoamyl Acetate
Corylone
Ethyl Propanoate
Ethyl lactate
Methyl Anthranilate
Ethyl Vanillin
p-Menthone
γ-Undecalactone
13
13
11
8
8
7
7
6
6
5
4
4
4
3
3
3
10
1
0.1
0.01
e
Tobacco
Aloe Grape (PP)
Cool Mint (PP)
Cafe Latte
Mixed Berries (PP)
Grape
Menthol
Lychee Ice (PP)
Pomegranate
Banana Ice
Melon Ice
Clear
Peach Ice
Sour Apple
Tangerine Ice
Cucumber

Figure 8.2. Chemical concentrations in Puff EC fluids. (a) Total flavor chemicals ranged from 0.7 – 34.3 mg/mL, and nicotine concentrations ranged from 41.2 – 52.3 mg/mL. (b) WS-3 and WS-23 concentrations ranged from 1.5 – 15.5 mg/mL and 0.9 – 35.9 mg/mL, respectively. The x-axis is sorted by increasing total flavor chemical concentration and WS-23 in Figures 1a and 1b. Yellow bars in Figure 1b indicate equal levels of synthetic coolants. (c) PG and G concentrations ranged from 158 – 371 mg/mL and 310 – 437 mg/mL, respectively. (d) The percentage of each chemical class of chemicals in Puff products: flavor chemicals = 0.1 – 4.2%, synthetic coolants = 0.1 = 5.6%, nicotine = 5.5 – 7.1%, and solvents = 86.7 – 92.9%. (e) Heat map of individual flavor chemicals ordered on the y-axis according to the frequency of occurrence of dominant flavor chemicals. Products are ranked according to decreasing total weight (mg/mL) of the flavor chemicals on the *x*-axis from left to right. “PP” on the flavor name on the x-axis indicates “Puff Plus” products. Graphs show the means ± the standard deviation of three independent measurements (n = 3), except for Sour Apple, Pomegranate, and Café Latte, each based on one measurement.

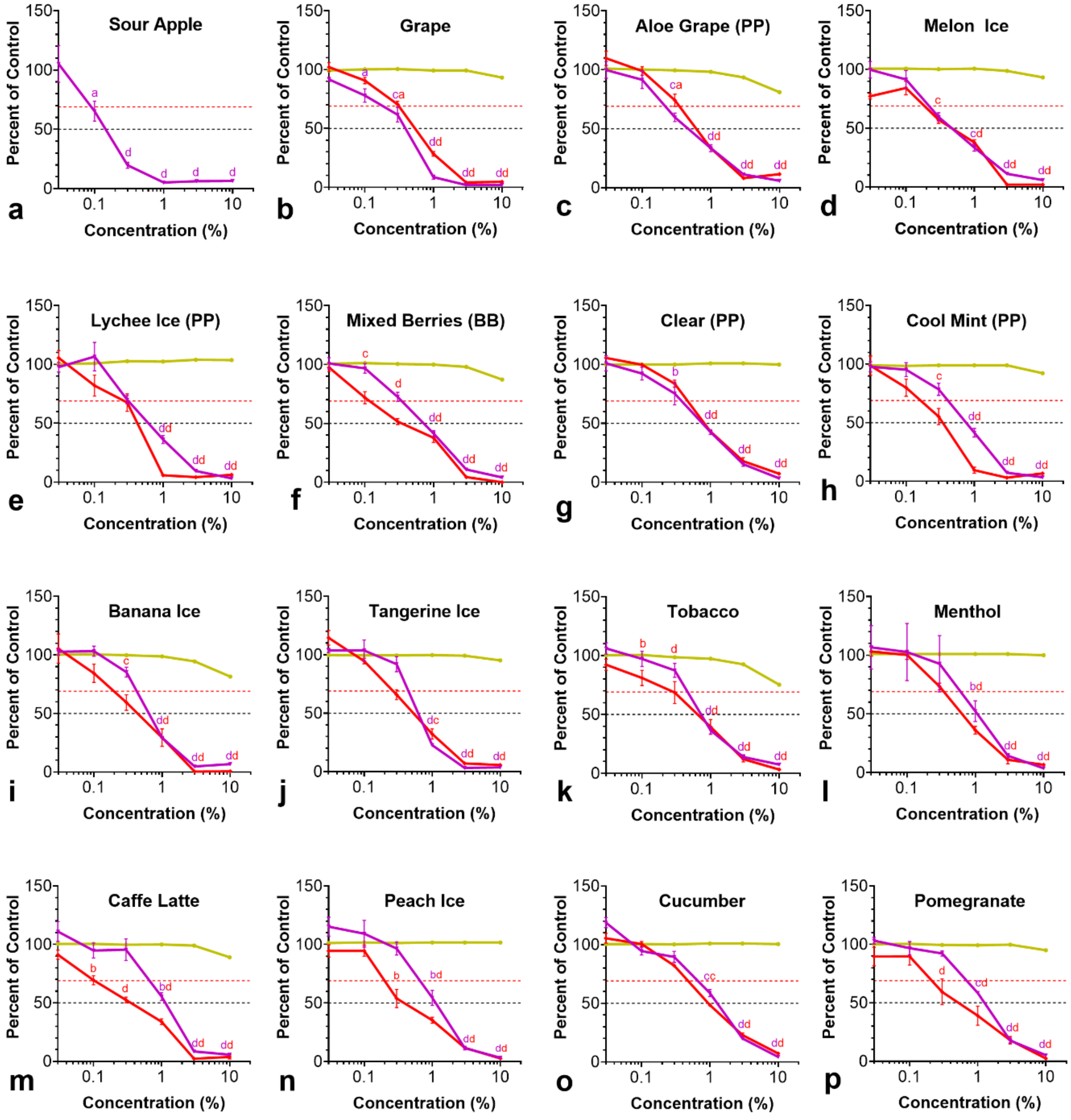


Figure 8.3. MTT, neutral red, and LDH assay concentration-response curves for BEAS-2B cells treated with Puff EC fluids. Purple line = the MTT assay. Red line = the neutral red assay. Yellow line = the LDH assay. The y-axis shows the response of cells in each assay as a percentage of the untreated control. The concentrations tested were 0.03%, 0.1%, 0.3%, 1%, 3%, and 10%. Each point is the mean ± standard error of the mean of at least three independent experiments. Red and black dotted lines on each graph represent IC_{70}s and IC_{50}s, respectively. For statistical significance, a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$.

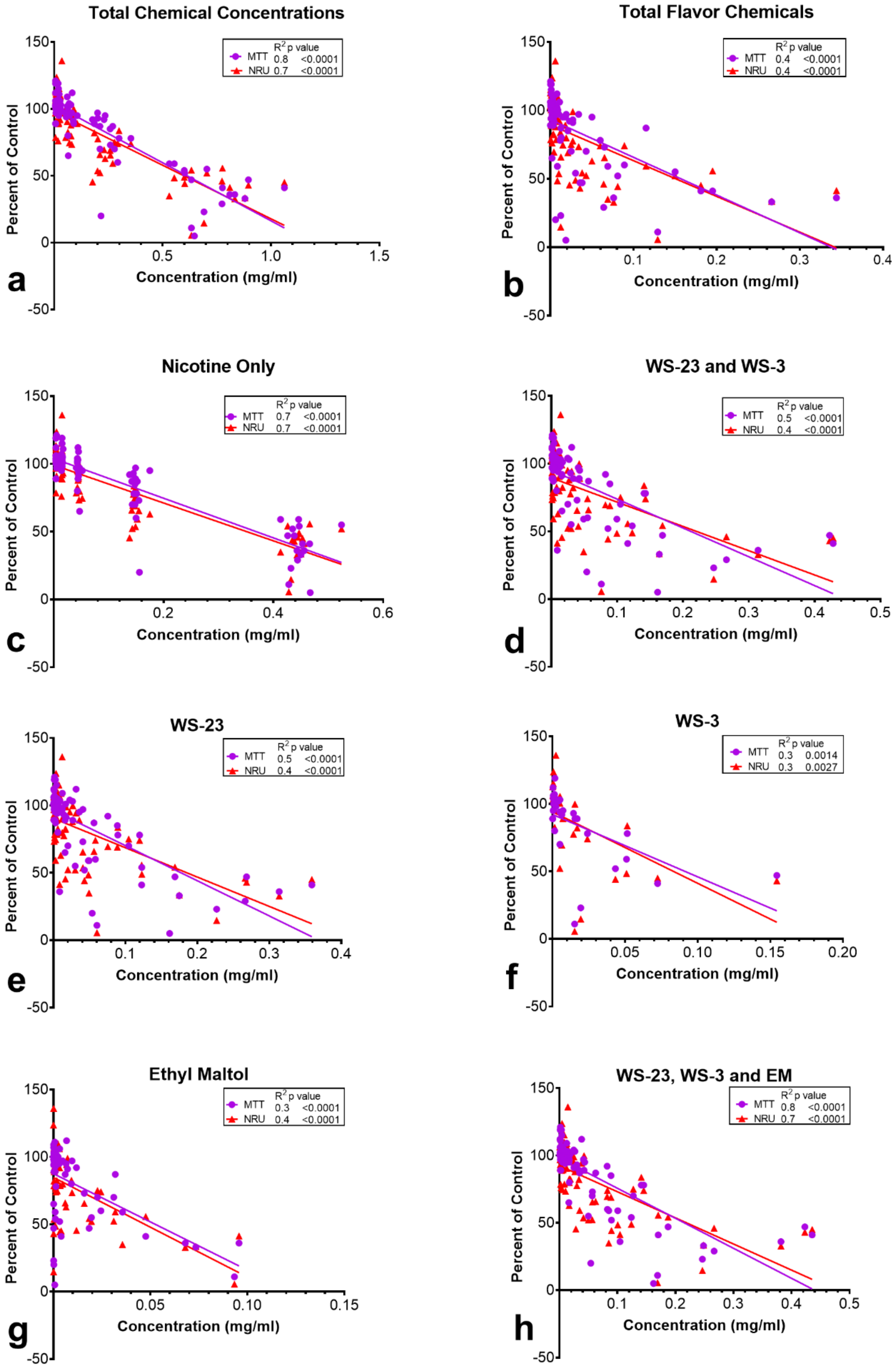

Total Chemical Concentrations
R^2 p value
MTT 0.8 <0.0001
NRU 0.7 <0.0001
Percent of Control
150
100
50
0
-50
0.5
1.0
1.5
Concentration (mg/ml)
a
Total Flavor Chemicals
R^2 p value
MTT 0.4 <0.0001
NRU 0.4 <0.0001
Percent of Control
0.1
0.2
0.3
0.4
Concentration (mg/ml)
b
Nicotine Only
R^2 p value
MTT 0.7 <0.0001
NRU 0.7 <0.0001
Percent of Control
0.2
0.4
0.6
Concentration (mg/ml)
c
WS-23 and WS-3
R^2 p value
MTT 0.5 <0.0001
NRU 0.4 <0.0001
Percent of Control
0.1
0.2
0.3
0.4
0.5
Concentration (mg/ml)
d
WS-23
R^2 p value
MTT 0.5 <0.0001
NRU 0.4 <0.0001
Percent of Control
0.1
0.2
0.3
0.4
Concentration (mg/ml)
e
WS-3
R^2 p value
MTT 0.3 0.0014
NRU 0.3 0.0027
Percent of Control
0.05
0.10
0.15
0.20
Concentration (mg/ml)
f
Ethyl Maltol
R^2 p value
MTT 0.3 <0.0001
NRU 0.4 <0.0001
Percent of Control
0.05
0.10
0.15
Concentration (mg/ml)
g
WS-23, WS-3 and EM
R^2 p value
MTT 0.8 <0.0001
NRU 0.7 <0.0001
Percent of Control
0.1
0.2
0.3
0.4
0.5
Concentration (mg/ml)
h

Figure 8.4. Relationship between the toxicity of Puff EC fluids in the MTT assay and the chemical concentrations of nicotine, WS-3, WS-23, and ethyl maltol in the Puff fluids. Linear regression analysis for cytotoxicity in the MTT assays (y-axis, expressed as a percentage of the untreated control) versus the concentrations of (a) total chemicals, (b) total flavor chemicals, (c) nicotine only, (d) WS-3 and WS-23, (e) WS-23 only, (f) WS-3 only, (g) ethyl maltol, and (h) synthetic coolants and ethyl maltol. Toxicity was strongly correlated ($R^2 \geq 0.5$) with the total chemicals, nicotine only, synthetic coolants, WS-23, and synthetic coolants plus ethyl maltol. Total flavor chemicals, WS-3, and ethyl maltol were moderately correlated with toxicity ($R^2 < 0.5$). All correlations were significant ($p < 0.05$). Linear regression analyses for toxicity versus other dominant flavor chemicals are shown in Figure S2.

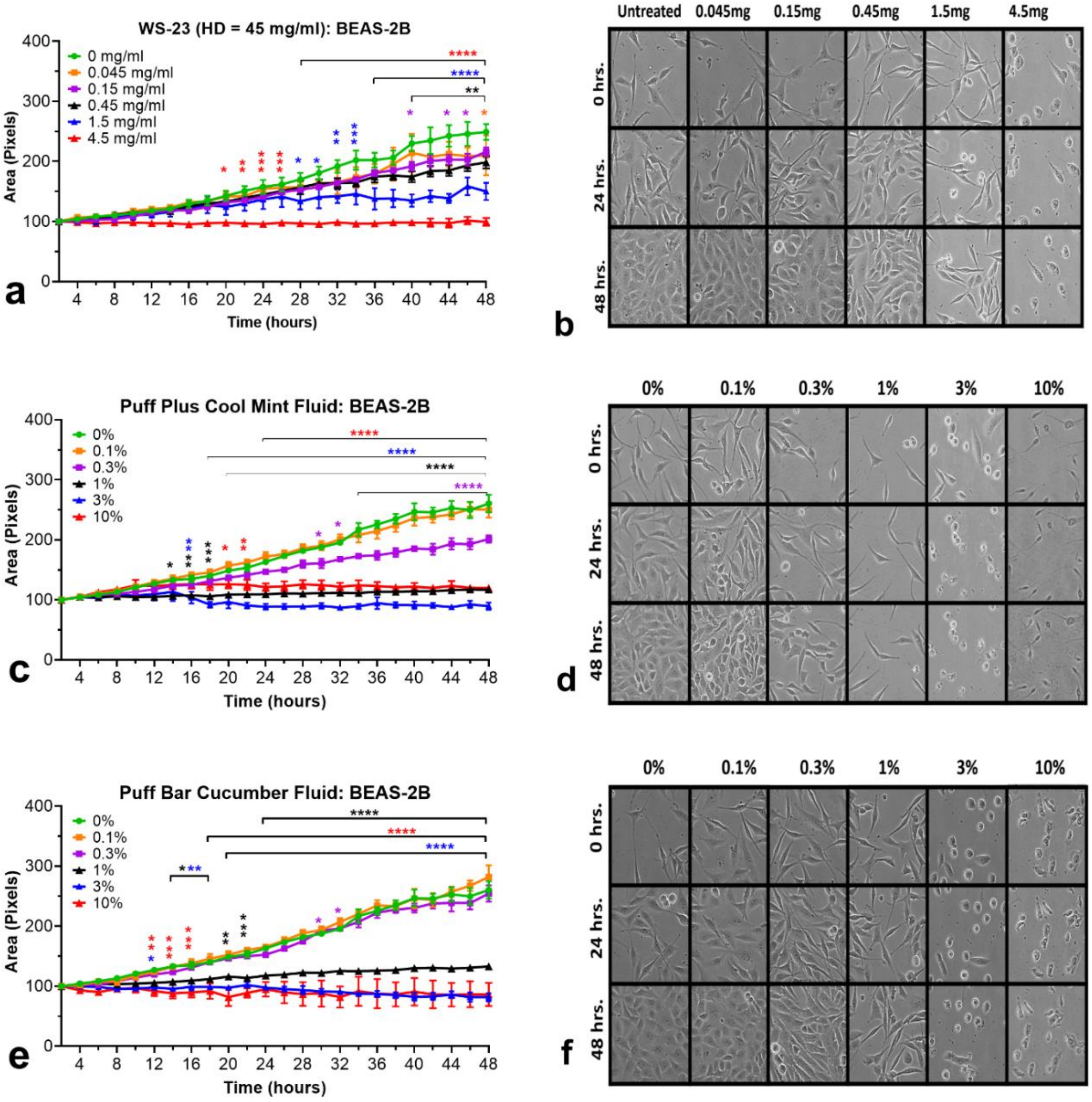


Figure 8.5. Effects of synthetic coolants and Puff EC fluids on cell growth and morphology in the live-cell imaging assay. Time-lapse imaging was performed with WS-23 (a and b), Puff Plus Cool Mint (c and d), and Puff Bar Cucumber (e and f). The cell growth experiments (a, c, e) x-axis shows the duration of the experiment. The y-axis shows the mean of the percent increase in cell area (growth) over 48 hours as determined using CL-Quant software. For cell morphology data (b, d, f), the x-axis shows the treatment concentration and the y-axis 24 h time intervals. Each point is the mean of at least three experiments ± the SEM. * = $p < 0.05$; ** = $p<0.01$; *** = $p < 0.001$; **** = $p<0.0001$.

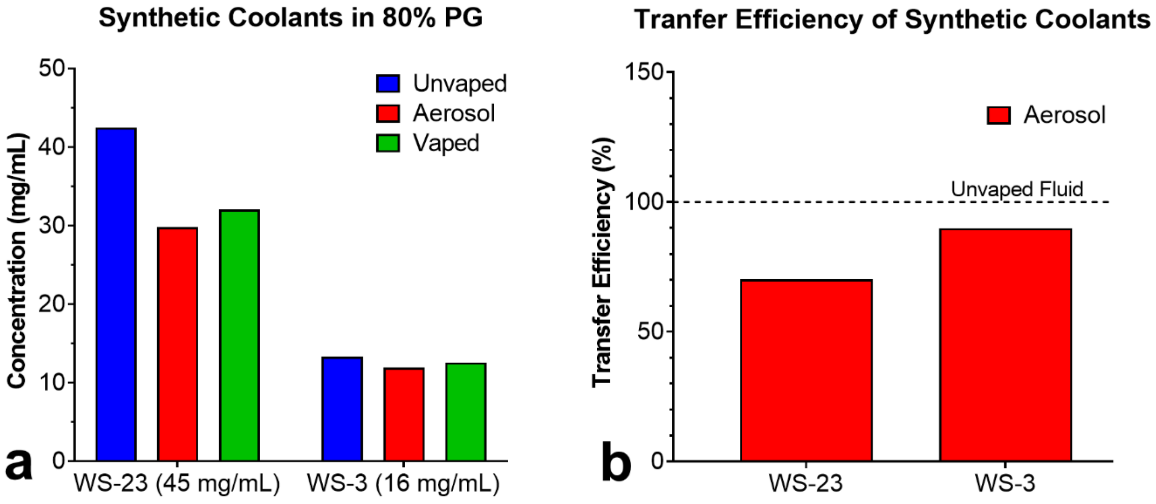
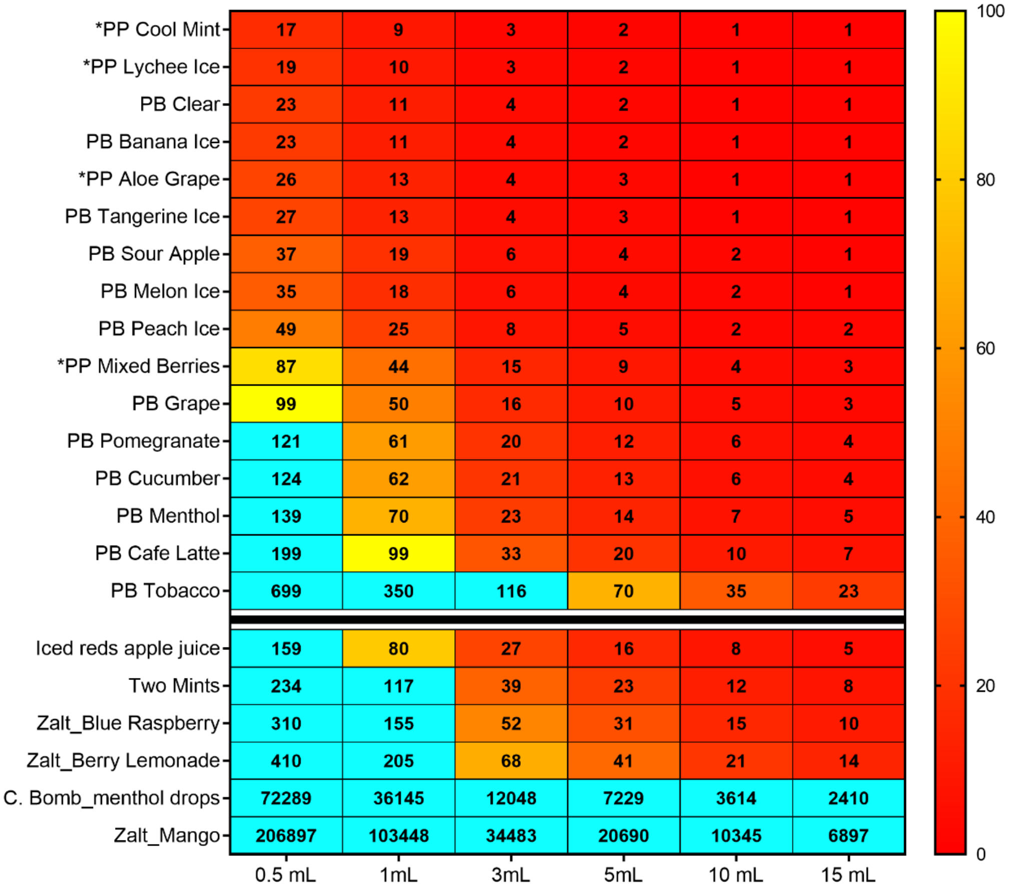


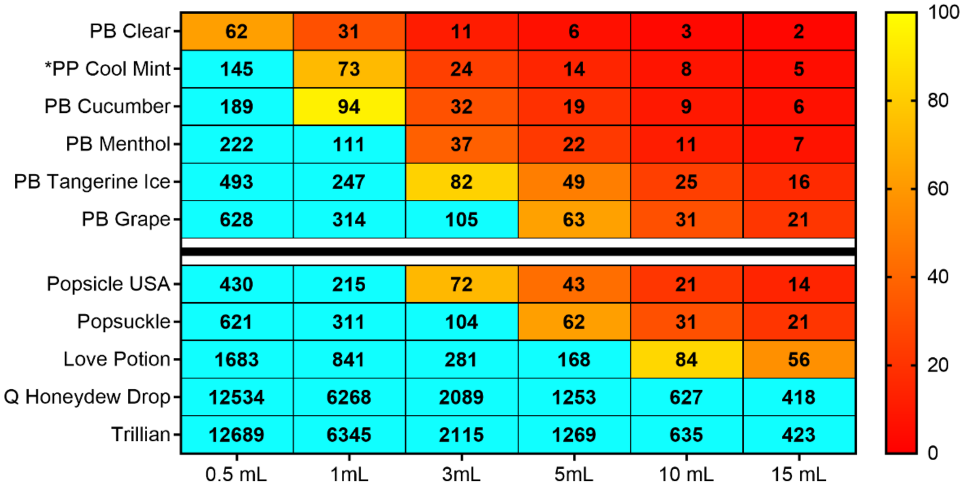
Figure 8.6. Synthetic coolants in lab-made refill fluids and their corresponding aerosols. (a) Concentrations of WS-23 and WS-3 in unvaped fluids, vaped fluids, and aerosols. (b) Transfer efficiency of WS-23 and WS-3 to aerosols. Aerosols were made using a fourth generation Baton V2 open pod EC operating at 3.7V(volts)/8.6 watts. Each bar is a mean of two measurements.

MOE Calculations for WS-23



	0.5 mL	1mL	3mL	5mL	10 mL	15 mL
*PP Cool Mint	17	9	3	2	1	1
*PP Lychee Ice	19	10	3	2	1	1
PB Clear	23	11	4	2	1	1
PB Banana Ice	23	11	4	2	1	1
*PP Aloe Grape	26	13	4	3	1	1
PB Tangerine Ice	27	13	4	3	1	1
PB Sour Apple	37	19	6	4	2	1
PB Melon Ice	35	18	6	4	2	1
PB Peach Ice	49	25	8	5	2	2
*PP Mixed Berries	87	44	15	9	4	3
PB Grape	99	50	16	10	5	3
PB Pomegranate	121	61	20	12	6	4
PB Cucumber	124	62	21	13	6	4
PB Menthol	139	70	23	14	7	5
PB Cafe Latte	199	99	33	20	10	7
PB Tobacco	699	350	116	70	35	23
Iced reds apple juice	159	80	27	16	8	5
Two Mints	234	117	39	23	12	8
Zalt_Blue Raspberry	310	155	52	31	15	10
Zalt_Berry Lemonade	410	205	68	41	21	14
C. Bomb_menthol drops	72289	36145	12048	7229	3614	2410
Zalt_Mango	206897	103448	34483	20690	10345	6897

MOE Calculations for WS-3



	0.5 mL	1mL	3mL	5mL	10 mL	15 mL
PB Clear	62	31	11	6	3	2
*PP Cool Mint	145	73	24	14	8	5
PB Cucumber	189	94	32	19	9	6
PB Menthol	222	111	37	22	11	7
PB Tangerine Ice	493	247	82	49	25	16
PB Grape	628	314	105	63	31	21
Popsicle USA	430	215	72	43	21	14
Popsuckle	621	311	104	62	31	21
Love Potion	1683	841	281	168	84	56
Q Honeydew Drop	12534	6268	2089	1253	627	418
Trillian	12689	6345	2115	1269	635	423

Figure 8.7. The margin of exposure (MOE) for WS-3 and WS-23 in EC products. (a) WS-23, (b) WS-3. MOEs below the threshold of 100 indicate a potential human health risk. The blue boxes are MOEs that were above the threshold of 100. EC products listed below the black horizontal bar indicate refill fluids and the Zalt brand of disposable ECs. “C” in “C. Bomb” in Figure 1a = Cinnamon, “PP” = Puff plus, and “PB” = Puff Bar.

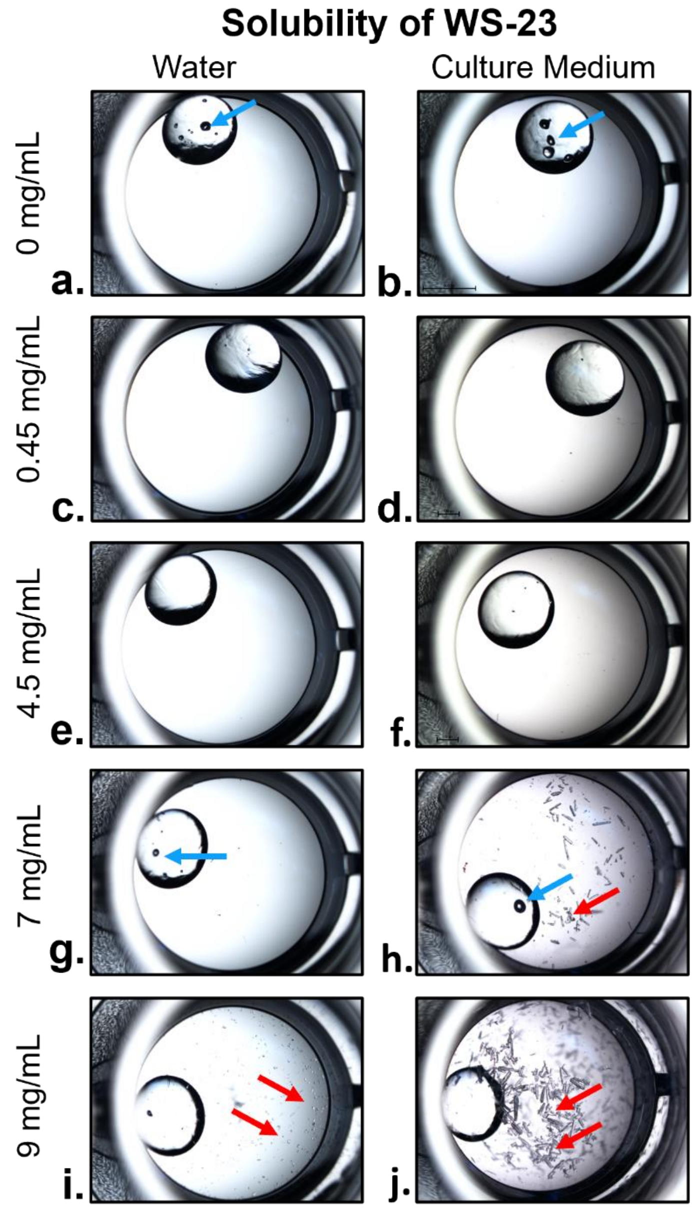


Figure 8.8. Stereoscopic microscope images of droplets of water or culture medium containing various concentrations of WS-23 to show solubility (a-j). Both 0.45 mg/mL and 4.5 mg/mL of WS-23 were soluble in water and culture medium (c-f). 7 mg/mL of WS-23 was soluble in 1 mL of water but not in BEAS-2B medium (g, h). Precipitates were present in both water (red arrows) and the culture medium containing 9 mg/mL (i, j). Blue arrows show air bubbles within the glass beads. The highest concentration used in our study was 4.4 mg/mL.

Table 8.1. IC$_{50s}$ and IC$_{70s}$ for BEAS-2B Cells Treated with Puff EC Fluids

Refill Fluids	MTT (%)		NRU (%)		LDH (%)	
	IC$_{50}$	IC$_{70}$	IC$_{50}$	IC$_{70}$	IC$_{50}$	IC$_{70}$
Sour Apple	0.15	0.09	0.53	0.32	>10	>10
Grape	0.33	0.18	0.64	0.35	>10	>10
Aloe Grape	0.51	0.22	0.41	0.19	>10	>10
Melon Ice	0.51	0.21	0.38	0.36	>10	>10
Lychee Ice	0.64	0.31	0.35	0.12	>10	>10
Mixed Berry	0.72	0.35	0.90	0.47	>10	>10
Clear (PP)	0.77	0.38	0.31	0.17	>10	>10
Cool Mint	0.75	0.41	0.42	0.20	>10	>10
Banana Ice	0.68	0.42	0.55	0.26	>10	>10
Tangerine Ice	0.67	0.44	0.57	0.30	>10	>10
Tobacco	0.80	0.46	0.67	0.34	>10	>10
Menthol	1.08	0.61	0.32	0.10	>10	>10
Café latte	1.10	0.66	0.48	0.20	>10	>10
Peach Ice	1.11	0.66	1.04	0.49	>10	>10
Cucumber	1.24	0.67	0.55	0.21	>10	>10
Pomegranate	1.23	0.68	0.53	0.32	>10	>10

[1] The highest concentration tested was 1% of the EC refill fluids.

Chapter 9:

Evolution of e-cigarette fluid (e-liquid) constituents – clever uses of flavor chemicals?

ABSTRACT

Background. The increased popularity of electronic cigarettes (e-cigarettes) has been linked to the abundance of flavored products that are attractive to adolescents and young adults. In the last decade, e-cigarette designs have evolved through four generations that include modifications in battery power, e-liquid reservoirs, and atomizer units. E-cigarette liquids (e-liquids) have likewise evolved in terms of solvent use/ratios, concentration and number of flavor chemicals, use of nicotine salts and acids, the recent increased use of synthetic cooling agents, and the introduction of synthetic nicotine. Our current objective was to evaluate and compare the evolving composition of tobacco-flavored e-liquids over the last 10 years.

Methods. Our extensive database of flavor chemicals in e-liquids was used to identify trends and changes in flavor chemical composition and concentrations.

Results. Tobacco-flavored products purchased in 2010 and 2011 generally had very few flavor chemicals, and their concentrations were generally very low. In tobacco-flavored refill fluids purchased in 2019 and Puff Bar Tobacco e-cigarettes, the total number and concentration of flavor chemicals were higher than expected. Products with total flavor chemicals > 10 mg/mL contained 1 – 5 dominant flavor chemicals (>1 mg/mL). The most frequently used flavor chemicals in tobacco e-liquids were fruity and caramellic.

Conclusions. There is a need for continuous surveillance of e-liquids, which are evolving in often subtle and harmful ways. Chemical constituents of tobacco flavors should be monitored as they clearly can be doctored by manufacturers to have a taste that would appeal to young users.

INTRODUCTION

Understanding the compositions and toxicities of electronic cigarette (e-cigarette) liquids (e-liquids) is important in developing effective regulatory policies regarding vaping. However, e-liquid formulations continue to evolve rapidly, including the use of new ingredients expressly designed to circumvent regulatory law, such as synthetic nicotine[1,2] or the repurposing of synthetic coolants that Wilkinson Sword developed for topical use in shaving cream.[3-6] Flavor chemicals are particularly important since product flavors, such as fruit, candy, and sweet, attract students and young adults who might otherwise not use e-cigarettes.[7-9] The rapid rise in JUUL's popularity[10] has prompted the enactment of flavor bans both locally and nationally,[11] with the FDA issuing an enforcement policy to remove cartridge-based flavored e-cigarettes (except for menthol and tobacco flavors) from the market.[12] JUUL withdrew its popular fruity and sweet flavors before the FDA enforcement policy, leaving only their "Menthol" and "Virginia Tobacco" flavors on the market. However, fruity and sweet flavors continue to be sold by companies, such as Puff, that market disposable products not covered by the FDA's enforcement policy on characterizing flavors in cartridge-style e-cigarettes.[12] Some e-cigarettes (menthol and tobacco manufactured by Vuse and Logic have been given FDA market authorization based on data suggesting they are less harmful than tobacco cigarettes. These flavors were probably authorized because they do not appeal to youth[9], and they may help e-cigarette users with smoking cessation.[11,13]

Given the recent limitations on flavored e-cigarettes sales, our goal was to determine if an FDA authorized flavor, specifically tobacco, was evolving in a way that would appeal to youth by incorporating sweet and fruity flavor chemicals. To accomplish this, we examined the flavor chemicals in tobacco-flavored refill fluids over the last decade and in two popular pod-style e-cigarettes and determined if flavor chemical use has evolved in a manner that could increase the popularity of tobacco-flavored products, especially among young consumers.

METHODS

During the past 10 years, we have identified, quantified, and toxicologically evaluated >200 chemicals in e-liquids in many hundreds of products purchased in the United States and worldwide.[2,14-23] This work has been consolidated in the UCR/PSU Electronic Cigarette Data Collection, a unique and extensive knowledge base on flavor chemicals, acids, consequent reaction products (RxPs), and metals found in e-liquids and aerosols. We have previously used this knowledge base to publish on the unusually high concentrations of flavor chemicals used in many e-liquids,[20] and the sudden market presence of the “Wilkinson Sword” coolants WS-3 and WS-23 in Puff brand e-cigarettes.[3] The current study compared the number and concentrations of flavor chemicals in 63 tobacco-flavored e-cigarette refill fluids purchased between 2011 and 2019 and two popular disposable/pod-style e-cigarettes (JUUL and Puff). Specifically, the flavor chemical concentrations in each tobacco-flavored product were extracted from the Electronic Cigarette Data Collection and compared across products and time of purchase.

The refill fluids were selected from two libraries; a convenience library purchased online [16,18] and a worldwide library of one brand of refill fluids.[22] The JUUL and Puff tobacco products were included due to their popularity among young adults and adolescents.[24-28] All products were shipped and stored at room temperature and analyzed within a month of receipt.

RESULTS

Total concentrations of flavor chemicals in refill fluids

Flavor chemicals were identified and quantified in 63 tobacco-flavored refill fluids purchased between 2011 and 2019 (supplementary table S1). Figure 1 shows the total concentrations of the flavor chemicals in each product. Most (63%) of the refill fluids purchased before 2019 had low total concentrations of flavor chemicals (< 2 mg/mL) and 84% were <5 mg/ml. There were six notable exceptions: (1) duplicate bottles of "Marcado" purchased in 2011 and 2012 with ~ 20.3 mg/mL); (2) "Artic Menthol" purchased in 2011 with 19.1 mg/mL); and four LiQua "RY4 Tobacco" products purchased in 2016 with 42.3 – 47.2 mg/mL). In contrast, of 13 products purchased in 2019, 54% had total flavor chemical concentrations > 10 mg/mL.

Concentrations of individual flavor chemicals in refill fluids

The individual flavor chemicals used in tobacco-flavored refill fluids purchased between 2011 and 2019 are shown in figures 2 and 3, in which blank cells indicate the chemical was not detected.[16,18,19,22] In the 2011-2012 group, duplicate bottles of "Macardo" had elevated cinnamaldehyde and eugenol, while one "Arctic Menthol" product with benzaldehyde had a high concentration of benzaldehyde PG acetal. While acetals may form at room temperature during storage, the concentration found in "Arctic Menthol" was likely added as a flavor compound. All other products had low total concentrations of flavor chemicals (figure 2).

Figure 3 shows products purchased in 2015, 2016, and 2019. "American Blend" flavors purchased in multiple countries in 2015 and 2016 had neither flavor chemicals nor nicotine, suggesting those products were unflavored. "Traditional Tobacco" refill fluids contained 1 – 4 flavor chemicals below the limit of quantification. The absence of flavor chemicals is unusual and was not observed in the other flavor categories studied previously.[18,19,21] Most flavor chemicals were present at very low concentrations (< 1 mg/mL). Nine flavor chemicals that were used mainly in products purchased in 2016 and 2019 had concentrations > 2 mg/mL, and these included: ethyl maltol (sweet or caramel), cinnamaldehyde (cinnamon), benzaldehyde PG acetal (fruity), corylone (caramellic, maple), triacetin (creamy), furaneol (sweet, caramellic), ethyl lactate (sweet, fruity), and eugenol (spicey, clove).

Frequency of occurrence and odor description of flavor chemicals

The frequency with which 55 flavor chemicals were used in tobacco-flavored refill fluids is shown in figure 4. The dominant flavor chemicals (> 1 mg/mL in at least one product) are indicated by an asterisk. The five most frequently used flavor chemicals were ethyl maltol (60%), corylone (44%), menthol (33%), vanillin (25%), maltol, and triacetin (24%). Based on odor type, flavor chemicals with a fruity or caramellic flavor were used most frequently. The “Other” category in the insert includes flavor chemicals that appeared only once (popcorn, anisic, ethereal, woody, musty, herbal, meaty, phenolic, and citrus). Based on odor/taste description information,[29] flavor chemicals used in tobacco-flavored e-cigarette refill fluids are sweet (figure 4).

Fourth-generation pod-style e-cigarettes

Flavor chemicals were compared in JUUL and Puff e-cigarettes, two popular disposable/pod-style 4th generation e-cigarettes (figure 5, supplementary table S1). JUUL has marketed two tobacco flavors, “Classic” and “Virginia,” containing negligible flavor chemicals. Total flavor chemical concentrations for both JUUL products were under 0.35 mg/mL, and the concentrations of the individual chemicals were, in most cases, ≤ 0.05 mg/mL (figure 5A, B). Different flavor chemicals were used in the “Classic” vs. “Virginia Tobacco” products, suggesting these chemicals were added intentionally to create distinct tastes for each product.

In contrast, Puff “Tobacco” had 27 different flavor chemicals with a total concentration of 34.3 mg/mL (figure 5A, B), which is higher than the other Puff products we evaluated.[3] Individual chemicals ranged in concentration from 0.03 to 15 mg/mL. Four flavor chemicals (vanillin, ethyl maltol, ethyl vanillin, and corylone) which were the highest in concentrations (range = 2.07 - 15 mg/mL), are typically used in sweet-flavored e-cigarette products, such as Dewberry Cream (figure 5B).[21] For the dominant flavor chemicals found in both brands, the fold increase in Puff vs. JUUL was 300 for vanillin, 239 for ethyl maltol, and 41 for corylone. The total number of flavor chemicals used in Puff Bar “Tobacco” was greater than 94% percent of the refill fluids. The vanillin and ethyl vanillin concentrations in Puff Bar Tobacco were higher than in refill fluids containing these chemicals. A comparison of dominant flavor chemicals in Puff Bar “Tobacco” to previously evaluated Kilo “Dewberry Cream” [21] revealed an identical flavor profile (figure 5C).

DISCUSSION

Our goal was to determine if flavor chemical use in tobacco-flavored e-cigarette products has changed during the past 10 years as flavor restrictions have come into play. Our main finding is the recent inclusion of decidedly non-tobacco flavor chemicals in products labeled “tobacco.” This change coincides with the national public health concern regarding the rapid adoption of JUUL products by students and young adults attracted to these pod-style e-cigarettes with appealing flavors.[30] Surveys found that many young adults and students started JUULing because they found the flavors attractive.[31] In contrast, tobacco-flavored pods are not generally attractive to young users,[32,] which may be why recent FDA authorizations were granted for tobacco-flavored e-cigarettes manufactured by Vuse and Logic.[33] The chemicals in high concentrations in tobacco-flavored e-cigarettes were ethyl maltol, corylone, vanillin, and ethyl vanillin. These chemicals were often found in our samples at concentrations much higher than in other consumer products, such as cosmetics and ingestibles.[20, 34-36] As we have shown previously, these chemicals are totally absent in U.S. commercial tobacco cigarettes;[37] therefore, their use is not to replicate tobacco cigarette flavor but appears to be to create a sweet flavor, attractive to a broad base of customers.

The flavor chemicals in Puff "Tobacco" are remarkably similar to those in "Dewberry Cream," a flavor popular with young e-cigarette users.[21] The Puff "Tobacco" flavored e-liquid has a higher total concentration of flavor chemicals (~35 mg/mL) than Dewberry Cream (27 mg/mL), which had the highest total flavor chemical concentration in popular products purchased in southern California.[21] Concern has been raised previously about the safety of flavor chemicals when inhaled at these high concentrations.[20] Although these particular flavors are Generally Regarded As Safe (GRAS) by the Flavor Extract Manufacturers Association (FEMA) for ingestion, FEMA has not evaluated them for inhalation toxicity.[38] The concentrations at which these flavor chemicals are used in tobacco products exceed levels usually used in other consumer products.[20,34-36] We have shown that ethyl maltol produces cytotoxicity in the MTT assay at concentrations lower than those in many of the products purchased in 2019, LiQua Ry4, and Puff e-cigarettes.[21]

The inclusion of high levels of distinctly non-tobacco flavor chemicals in e-cigarette products labeled as "tobacco" flavored is not limited to Puff; the practice was also observed in a small number of refill fluids. The LiQua "Ry4 Tobacco" refill fluids had a total flavor chemical concentration of ~45 mg/mL, mainly due to ethyl maltol (> 22 mg/mL). The LiQua Ry4 products were among the most cytotoxic of any fluids we have tested in that line or other brands.[22] Other tobacco-flavored refill fluids in the LiQua companies' product line did not have a high concentration of flavor chemicals. Ry4 refill fluids are generally blended to have vanilla and caramel accents, but in the case of LiQua Ry4, the concentrations of accent flavors were usually high.

It is clear from this and other recent studies that e-liquids are evolving in a manner that appears to broaden their appeal to young users and avoid regulatory obstacles. More specifically, the changes in e-liquids that have occurred in the last 10 years appear designed to (1) intensify the user experience (e.g., using novel coolants);[3,5] (2) facilitate nicotine delivery (e.g., using acids to allow inhalation of high nicotine levels[39,40] and/or, (3) appeal to a broader market that includes young vapers (e.g., using fruity/sweet flavor chemicals in "tobacco" flavored products (this study). In an effort to comply with the FDA regulation of fruity and sweet flavored products that appeal to youth, JUUL reduced its product line and now sells only two flavors, "Menthol" and "Virginia Tobacco." However, the FDA regulation on flavors did not include disposable pod-style e-cigarettes like Puff, which quickly filled the vacuum created by a reduction in JUUL flavors. Ironically, the limited availability of fruity/sweet JUUL products drove young users to an arguably more dangerous product with high nicotine concentrations, synthetic coolants, and pulegone, a carcinogen.[3] Additionally, the Puff Bar tobacco-flavored product with high concentrations of vanillin, ethyl maltol, ethyl vanillin, and corylone is likely appealing to young people and may become a staple should other Puff flavors be removed from the market in the future.

In spite of the FDA's enforcement policy on flavored e-cigarette products, which was issued in January 2020, many e-cigarettes, including Puff, are readily available today. Our data show that Puff sells tobacco-flavored e-cigarettes that appear to be designed to appeal to youth. As long as products without FDA authorization remain on the market, there is a possibility that flavors, such as tobacco, will be manipulated to make them fruity or sweet and thereby broaden their appeal.

Only Vuse and Logic products have received FDA authorization for their Premarket Tobacco Applications (PMTA),[33] We do not know if the FDA identifies and quantifies flavor chemicals before authorizing PMTAs, but this probably should be done for two reasons. First flavor chemicals are often used in e-liquids, without safety data, at concentrations much higher than those found in other consumer products.[3,19,23] Once an ENDS product receives FDA authorization, its formulation cannot be changed; otherwise, a new application for authorization is required. However, following authorization of a product, periodic surveillance independent of manufacturers would be needed to be certain that e-liquids are not modified in a way that would broaden their appeal.

What This Paper Adds

- Little is known about the evolution of the use of flavor chemicals in e-liquids, information which is critical to their regulation.
- There has been a recent stealth use of high concentrations of sweet and fruity flavor chemicals in "tobacco-flavored" products.
- There is a need for continued surveillance of e-liquids, particularly tobacco-flavored liquids, which may be manipulated to circumvent policies on flavor use.

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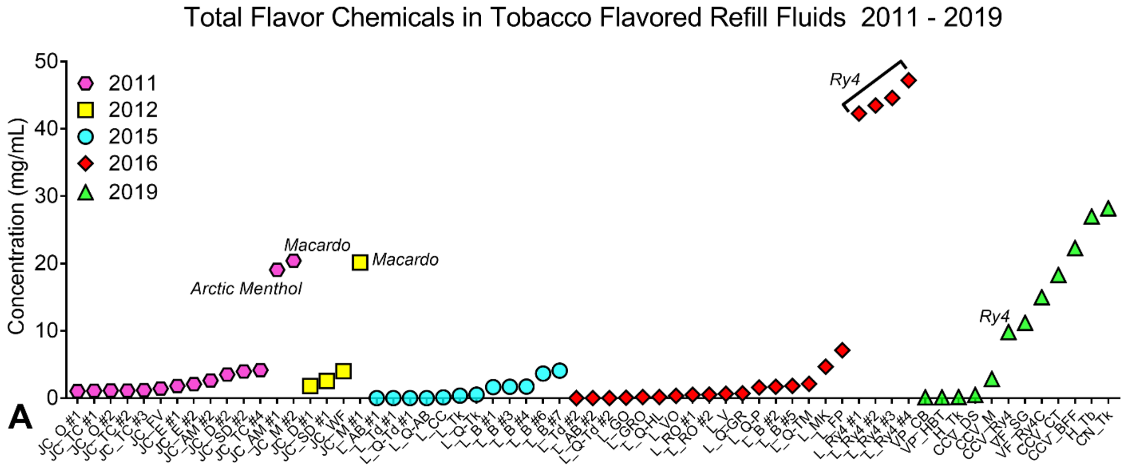
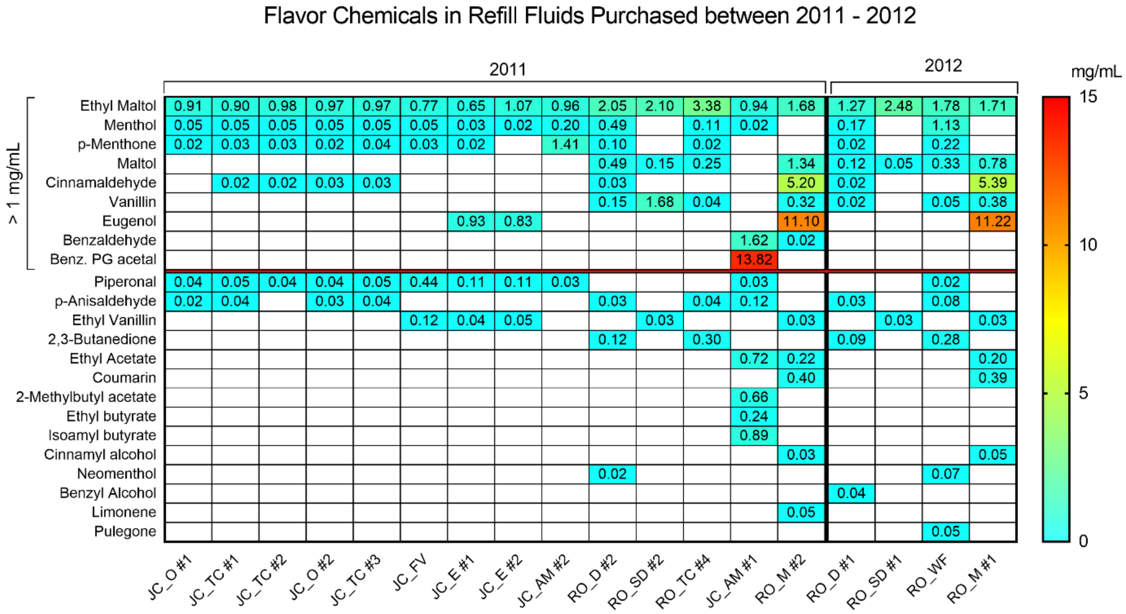


Figure 9.1. **The total concentration of flavor chemicals in tobacco-flavored refill fluids purchased between 2011 and 2019.** The *y*-axis shows concentrations in mg/mL, and the *x*-axis is ordered by increasing concentrations from left to right within each year. Codes represent products as described in Table S1. While total concentrations ranged from 0 – 47 mg/mL, most tobacco flavored refill fluids had low total concentrations of flavor chemicals until 2019, when over 54% of the products analyzed had concentrations > 10 mg/mL.



Flavor Chemicals in Refill Fluids Purchased between 2011 - 2012

	2011														2012			
	JC_O #1	JC_TC #1	JC_TC #2	JC_O #2	JC_TC #3	JC_FV	JC_E #1	JC_E #2	JC_AM #2	RO_D #2	RO_SD #2	RO_TC #4	JC_AM #1	RO_M #2	RO_D #1	RO_SD #1	RO_WF	RO_M #1
Ethyl Maltol	0.91	0.90	0.98	0.97	0.97	0.77	0.65	1.07	0.96	2.05	2.10	3.38	0.94	1.68	1.27	2.48	1.78	1.71
Menthol	0.05	0.05	0.05	0.05	0.05	0.05	0.03	0.02	0.20	0.49		0.11	0.02		0.17		1.13	
p-Menthone	0.02	0.03	0.03	0.02	0.04	0.03	0.02		1.41	0.10		0.02			0.02		0.22	
Maltol										0.49	0.15	0.25		1.34	0.12	0.05	0.33	0.78
Cinnamaldehyde		0.02	0.02	0.03	0.03					0.03				5.20	0.02			5.39
Vanillin										0.15	1.68	0.04		0.32	0.02		0.05	0.38
Eugenol							0.93	0.83						11.10				11.22
Benzaldehyde													1.62	0.02				
Benz. PG acetal													13.82					
Piperonal	0.04	0.05	0.04	0.04	0.05	0.44	0.11	0.11	0.03				0.03				0.02	
p-Anisaldehyde	0.02	0.04		0.03	0.04					0.03		0.04	0.12		0.03		0.08	
Ethyl Vanillin						0.12	0.04	0.05			0.03			0.03		0.03		0.03
2,3-Butanedione										0.12		0.30			0.09		0.28	
Ethyl Acetate													0.72	0.22				0.20
Coumarin														0.40				0.39
2-Methylbutyl acetate													0.66					
Ethyl butyrate													0.24					
Isoamyl butyrate													0.89					
Cinnamyl alcohol														0.03				0.05
Neomenthol										0.02							0.07	
Benzyl Alcohol															0.04			
Limonene														0.05				
Pulegone																	0.05	

Figure 9.2. Heat map showing individual flavor chemicals in refill fluids purchased in 2011 and 2012. The *y*-axis shows flavor chemicals ordered by high vs. low concentrations, and the *x*-axis represents product codes as described in Table S1. Most flavor chemicals were present in low concentrations.

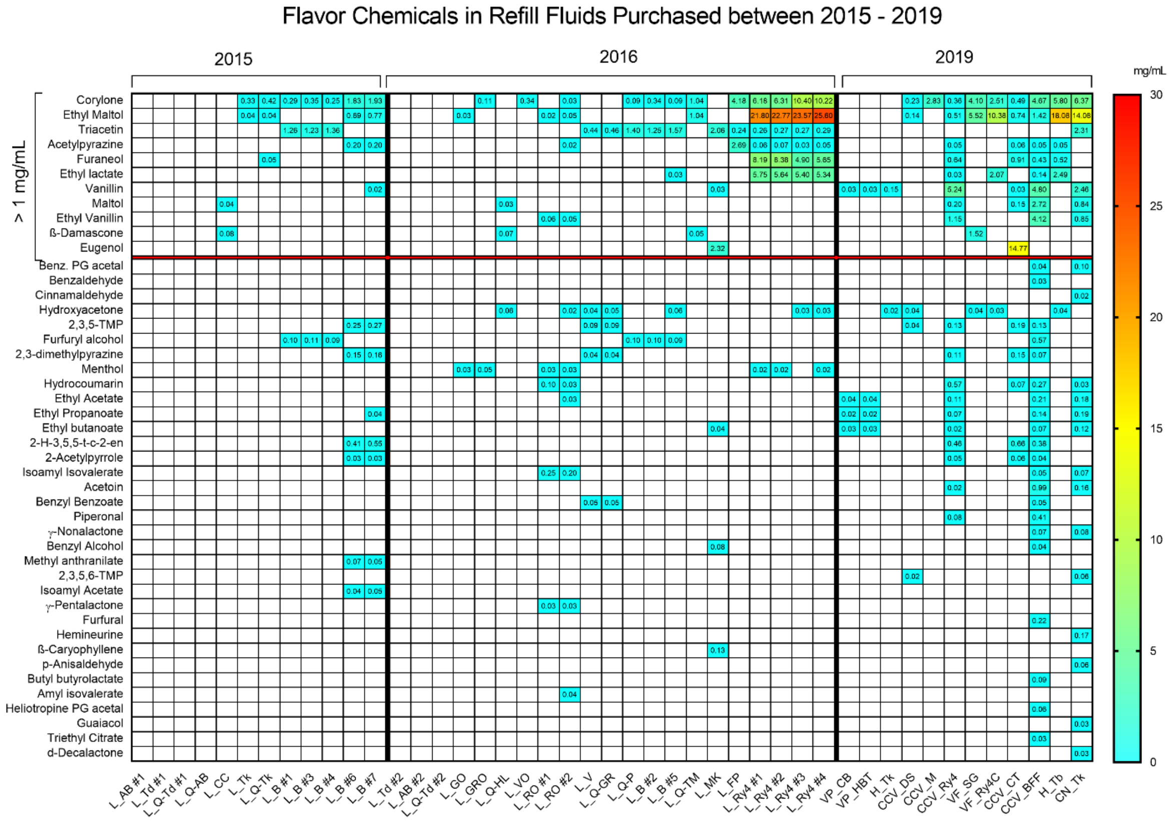


Figure 9.3. Heat map showing individual flavor chemicals in refill fluids purchased in 2015, 2016, and 2019. The *y*-axis shows flavor chemicals ordered by high vs. low concentrations, and the *x*-axis represents product codes as described in Table S1. Most flavor chemicals were present in low concentrations. However, increases in the concentrations of several commonly used flavor chemicals are seen in products purchased in 2016 and 2019. 2-H-3,5,5-t-c-2-en = 2-Hydroxy-3,5,5-trimethyl-cyclohex-2-en; TMP = Trimethylpyrazine

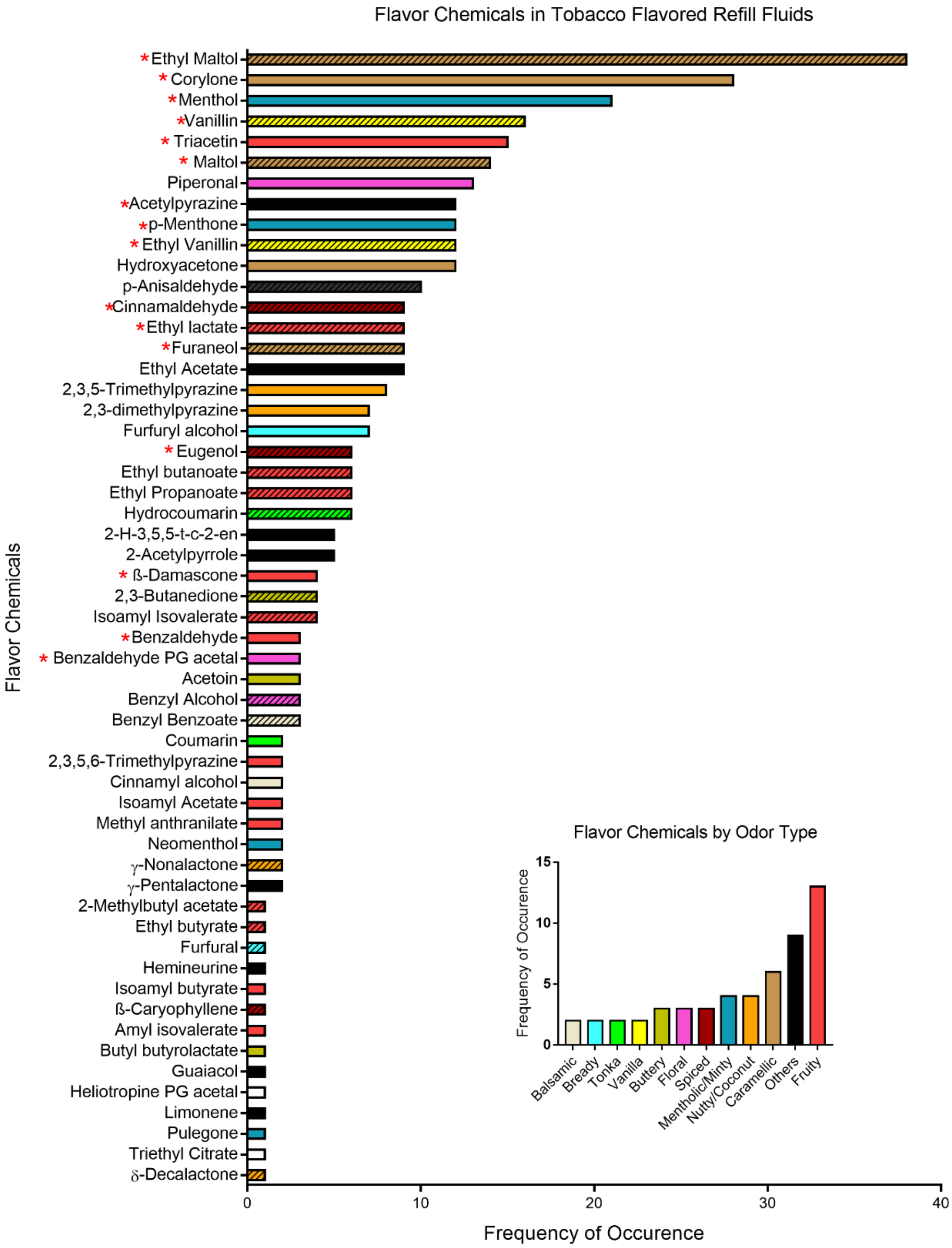
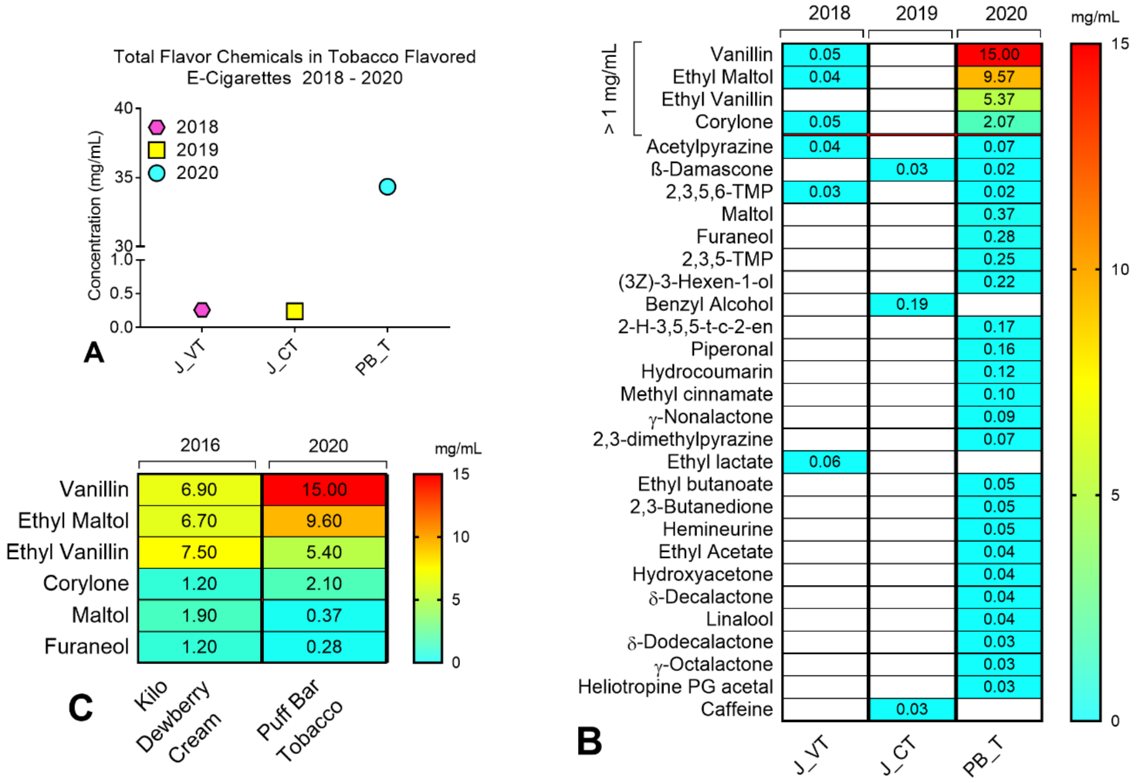

Flavor Chemicals in Tobacco Flavored Refill Fluids
Flavor Chemicals
*Ethyl Maltol
*Corylone
*Menthol
*Vanillin
*Triacetin
*Maltol
Piperonal
*Acetylpyrazine
*p-Menthone
*Ethyl Vanillin
Hydroxyacetone
p-Anisaldehyde
*Cinnamaldehyde
*Ethyl lactate
*Furaneol
Ethyl Acetate
2,3,5-Trimethylpyrazine
2,3-dimethylpyrazine
Furfuryl alcohol
*Eugenol
Ethyl butanoate
Ethyl Propanoate
Hydrocoumarin
2-H-3,5,5-t-c-2-en
2-Acetylpyrrole
*ß-Damascone
2,3-Butanedione
Isoamyl Isovalerate
*Benzaldehyde
*Benzaldehyde PG acetal
Acetoin
Benzyl Alcohol
Benzyl Benzoate
Coumarin
2,3,5,6-Trimethylpyrazine
Cinnamyl alcohol
Isoamyl Acetate
Methyl anthranilate
Neomenthol
γ-Nonalactone
γ-Pentalactone
2-Methylbutyl acetate
Ethyl butyrate
Furfural
Hemineurine
Isoamyl butyrate
ß-Caryophyllene
Amyl isovalerate
Butyl butyrolactate
Guaiacol
Heliotropine PG acetal
Limonene
Pulegone
Triethyl Citrate
δ-Decalactone
0
10
20
30
40
Frequency of Occurence
Flavor Chemicals by Odor Type
Frequency of Occurence
15
10
5
0
Balsamic
Bready
Tonka
Vanilla
Buttery
Floral
Spiced
Mentholic/Minty
Nutty/Coconut
Caramellic
Others
Fruity

Figure 9.4. Frequency distribution for 55 flavor chemicals found in 63 tobacco-flavored refill fluids. The *x*-axis is the number of products, and the *y*-axis is sorted according to decreasing frequency of their occurrence. Representative color codes based on odor type are shown in the insert. Frequency ranged from 1 to 38, with the highest being ethyl maltol. The asterisks indicated chemicals found at > 1 mg/mL in at least one product, and hatched bars indicate flavor chemicals that produce a sweet taste.
2-H-3,5,5-t-c-2-en = 2-Hydroxy-3,5,5-trimethyl-cyclohex-2-en; TMP = Trimethylpyrazine.



B

	2018 J_VT	2019 J_CT	2020 PB_T
Vanillin	0.05		15.00
Ethyl Maltol	0.04		9.57
Ethyl Vanillin			5.37
Corylone	0.05		2.07
Acetylpyrazine	0.04		0.07
ß-Damascone		0.03	0.02
2,3,5,6-TMP	0.03		0.02
Maltol			0.37
Furaneol			0.28
2,3,5-TMP			0.25
(3Z)-3-Hexen-1-ol			0.22
Benzyl Alcohol		0.19	
2-H-3,5,5-t-c-2-en			0.17
Piperonal			0.16
Hydrocoumarin			0.12
Methyl cinnamate			0.10
γ-Nonalactone			0.09
2,3-dimethylpyrazine			0.07
Ethyl lactate	0.06		
Ethyl butanoate			0.05
2,3-Butanedione			0.05
Hemineurine			0.05
Ethyl Acetate			0.04
Hydroxyacetone			0.04
δ-Decalactone			0.04
Linalool			0.04
δ-Dodecalactone			0.03
γ-Octalactone			0.03
Heliotropine PG acetal			0.03
Caffeine		0.03	

C

	2016 Kilo Dewberry Cream	2020 Puff Bar Tobacco
Vanillin	6.90	15.00
Ethyl Maltol	6.70	9.60
Ethyl Vanillin	7.50	5.40
Corylone	1.20	2.10
Maltol	1.90	0.37
Furaneol	1.20	0.28

Figure 9.5. The total flavor chemical concentrations and individual chemicals in JUUL and Puff products. (A) Total flavor chemical concentrations in JUUL and Puff e-cigarettes. (B) Concentrations of individual flavor chemicals in JUUL and Puff e-cigarettes. (C) Dominant flavor chemicals in Kilo Dewberry Cream and Puff Bar Tobacco. The *y*-axis shows concentrations in mg/mL, and codes represent the products as described in Table S1.

Chapter 10

Design features and elemental/metal analysis of the atomizers in pod-style electronic cigarettes

Omaiye et al., 2021

PLoS One. 2021 Mar 9;16(3):e0248127

doi: 10.1371/journal.pone.0248127

ABSTRACT

Background: The atomizers of electronic cigarettes (ECs) contain metals that transfer to the aerosol upon heating and may present health hazards. This study analyzed 4th-generation EC pod atomizer design features and characterized their elemental/metal composition. **Methods:** Eleven EC pods from six brands/manufacturers were purchased at local shops and online. Pods were dissected and imaged using a Canon EOS Rebel SL2 camera. Elemental analysis and mapping of atomizer components was done using a scanning electron microscope coupled with an energy dispersive x-ray spectrometer. **Results**: EC pods varied in size and design. The internal atomizer components were similar across brands except for variations occurring mainly in the wicks and filaments of some products. The filaments were either Elinvar (nickel, iron, and chromium) (36.4%), nichrome (36.4%), iron-chromium (18.2%), or nickel (9%). Thick wires present in 55% of the atomizers were mainly nickel and were joined to filaments by brazing. Wire-connector joints were Elinvar. Metal air tubes were made of Elinvar (50%), nickel, zinc, copper, and tin (37.5%), and nickel and copper (12.5%). Most of the wick components were silica, except for two pods (PHIX and Mico), which were mainly ceramic. Connectors contained gold-plated nickel, iron-chromium multiple alloys of nickel, zinc, gold, iron, and copper. Wick chambers were made of Elinvar. Outer casings were either nickel, copper-tin, or nickel-copper alloys. Magnets were nickel with minor iron, copper, and sulfur. Some frequently occurring elements were high in relative abundance in atomizer components.**Conclusions:** The atomizers of pods are similar to previous generations, with the introduction of ceramic wicks and magnets in the newer generations. The elements in EC atomizers may transfer into aerosols and adversely affect health and accumulate in the environment.

INTRODUCTION

The external appearance, design, battery power, atomizers, and nicotine delivery of electronic cigarettes (ECs) have evolved over the last decade, with four generations recognized [1-3]. Most first-generation or cig-a-like/cartomizer products (e.g., NJOY, V2 Cigs, BluCig, Mark Ten, and Vuse) are similar in size and resemblance to tobacco cigarettes. They contain an atomizer designed to produce an aerosol by heating e-liquids rather than burning tobacco [4-6]. Second-generation ECs or clearomizers (e.g., Ego C Twist) have larger atomizers/tanks with some models (e.g., Vuse) lacking solder joints, polyfil fibers, and microprocessors. Larger fluid reservoirs and batteries are used in the third-generation products (mods), such as iTaste MVP, Smok Alien, and iPV6X [1]. Third generation ECs generally lack thick wires, fibers, and sheaths and are user-friendly with variable power settings.

Fourth-generation ECs or pods, which now comprise a significant share of the EC market [7-9], have relatively low-powered batteries, an e-liquid reservoir, and an atomizer/mouthpiece. Pods can be prefilled (closed-system), refillable (open-system), or disposable. Pod fluids differ from prior generation fluids in that they contain high concentrations of nicotine (~ 50 – 60 mg/mL) [2] and acid, which protonates the nicotine and makes the aerosol less harsh [10]. The combination of high nicotine delivered in an acidic aerosol may increase the possibility of addiction of novice users [10,11].

Potentially harmful elements/metals, including nickel, lead, cadmium, arsenic, and chromium, are in atomizers of the first three EC generations [6,12,13] and vape fluids [14-19]. Upon heating, elements/metals in vape fluids can transfer into aerosols and increase in concentrations in the fluid after vaping [18,19]. The concentration of these elements in aerosols varies with EC products, and it is usually higher in third-generation products, which operate at higher power [12, 13, 18-21].

Despite the ability of harmful metals to transfer into EC fluids and aerosols, extensive reviews of current literature on the health effects of ECs have presented little information on the impact of inhaled elements on users [22-24]. Fourth-generation ECs remain very popular with high school students and adolescents and dominate the current EC market [25, 26]. However, we have very little knowledge about the elemental composition of their atomizers. Our goals for this study were to: (1) characterize the atomizer components and design features of popular pod ECs, and (2) identify and map the elements/metals in their atomizers.

MATERIALS AND METHODS

Selection and purchase of EC pod devices

In 2019, popular prefilled and refillable pod EC products were identified in multiple reviews on vape forums, blogs, company websites, and then purchased online from the manufacturer's website or vape shops from reputable third-party vendors. The products selected for evaluation were PHIX (ECS Global LLC., Los Angeles, CA), Kilo 1K (Kilo E-liquid Inc., La Mirada, CA), KWIT Stick (Aspire., USA), Suorin Air (Shenzhen Blumark Technology Co., Ltd, China), Suorin Drop (Shenzhen Blumark Technology Co., Ltd, China), Suorin Edge (Shenzhen Blumark Technology Co., Ltd, China), SMOK Mico (Shenzhen IVPS Technology CO., Ltd, China), SMOK Infinix (Shenzhen Blumark Technology Co., Ltd, China), and SMOK NORD (Shenzhen Blumark technology Co., Ltd, China). Two replacement coils were evaluated within the SMOK NORD brand, a 1.4-ohm coil filament designed for mouth-to-lung (MTL) vaping and a 0.6-ohm mesh filament for sub-ohm vaping. Upon receipt, all products were inventoried and stored at room temperature until analyzed. Multiple EC devices and pods from the same brand were purchased simultaneously to ensure that the analysis was performed on products from the same purchase batch.

Dissection, scanning electron microscopy, and elemental analysis of EC pod atomizers

Prefilled (closed system) EC pods were emptied of the liquid. The atomizers of all pods were then carefully dissected to expose the internal components of interest. Each component was then photographed using a Canon EOS Rebel SL2.

The dissected components were mounted on aluminum pin stubs covered with conductive carbon tape to prevent charging during the analysis [5]. The edges of any plastic components were covered with carbon conductive paint to minimize charging under the electron beam during SEM imaging. Morphological and elemental analyses were performed using a Thermo Fisher Scientific Co. NovaNano-SEM 450 Scanning Electron Microscope (SEM) equipped with an Oxford Instruments Inc. energy dispersive X-ray spectrometer (EDS) fitted with an X-Max50 50 mm^2 SDD detector with an energy resolution of 126 eV at MnK-α located at the Central Facility for Advanced Microscopy and Microanalysis at the University of California at Riverside. SEM images were obtained using a secondary electron mode with a dedicated detector at 15 kV. EDS spectra and elemental maps were acquired and processed with the Oxford Instruments Inc. Aztec Synergy v.4 software package to qualitatively reveal the distribution of chemical elements within the sample area. Elemental identification is based on the system's ability to identify and differentiate specific element peaks with an atomic number of 5 or higher on the spectra. The detection limit for the EDS method is about 0.1 wt. %. For ease and clarity of data analysis, we have set an arbitrary threshold value of 5 wt. %. Elements present above the > 5% threshold are denoted as “major.” Those below the threshold are “minor.” Quantification of the elements was performed by processing the acquired EDS spectra using the standard-less routine and Oxford Instruments Inc. factory-supplied table of elemental standards incorporated in the Aztec software.

RESULTS

Design and components of prefilled and refillable pod ECs

The design and pod components of 11 products purchased in 2019 were compared (S1 Fig and Fig 1). Pod design shapes included rectangle, diamond, square, and teardrop shapes. Discreet airholes were located at different sites on each device. (S1 Fig). The rectangular or duck-billed shape mouthpieces were located close to the reservoir, which held 0.7 – 3 ml of fluid. Generally, low volume reservoirs were present in closed-pod systems, while higher volume reservoirs were in the refillable and open-system pods.

All products had filaments, which are required for generating aerosols. Most products had metal air tubes; however, the air tube was plastic in three products. The wicks were either cotton, silica, or ceramic. Some products had two wicks: cotton and ceramic (yellow and magenta for PHIX) or cotton and silica (yellow and light pink for Kilo 1K) (Fig 1). A connector pin was present in all pods except JUUL™, which had a connector plate. The thick wire was either brazed to the filament or joined to the connector component. (Fig 1). Four products contained a wick chamber, which held the wick in place. A magnet, which secured the pod to the battery, was present in three products (Fig 1).

Elemental analysis of pod EC atomizers

The design and layout of the atomizer components varied among products (Fig 2). An air tube (metal or plastic) (black arrows), filament (red arrows), and a wick (cotton or silicon) (blue arrows) were present in all brands. SMOK Mico and PHIX had additional ceramic wicks (orange arrows) (Fig 2D and 2L). The connector plate/pins (pink arrows in Fig 2B, 2Q, and 2S) located at the base of all pods provided a path for the current to flow from the atomizer's battery. All pods with a thick wire had a filament-wire joint, a wire-connector joint, or both. All Suorin products had plastic air tubes and large cotton wicks (Fig 2Q-U). PHIX, SMOK (Infinix, and NORD) had a chamber that housed the wick and filament (Fig 2C, 2M, and 2O). In SMOK NORD, this chamber served the same purpose as the air tube. A magnet, which secures the pod to the battery when the device is in use, was present in the Kilo 1K, Suorin, and SMOK brands. An outer casing (green arrow), which held the atomizer components together, was present in PHIX and SMOK (Infinix and Mico) pods (Fig 2C, 2I, and 2K).

The elemental composition of 11 pod ECs was analyzed using SEM and EDS (Figs 3-6, Table 1, and S2-S5 Figs). Examples of the major (silicon, oxygen, gold, and nickel) and minor (aluminum, chromium, and iron) peaks are shown in EDS spectra for the wick and connector plate components of JUUL™ atomizers (S2 Fig). The relative abundance of elements based on the EDS spectra for each atomizer component is summarized in Fig 3. The blue and light blue squares are elements with major and minor abundance, respectively. Pink squares are components made of plastic. Dark gray squares indicate components absent in atomizers, and light gray squares represent components that were present but not analyzed. Due to similar organic materials being used in components such as the wick, only selected pods were evaluated in the SEM.

SEM images of all components and their corresponding elemental maps arranged from left to right based on high relative abundance are shown in Figs 4-6, and S3-S5 Figs.

Nickel, iron, and chromium alloys in the form of Elinvar (nickel, iron, chromium alloy) (36.4%), nichrome (nickel and chromium) (36.4%), stainless steel (iron and chromium) (18.2%), or nickel (9.1%) were the most abundant elements in the filaments (Figs 3A and 4A-FF). Some filaments also contained minor amounts of silicon, aluminum, titanium, and molybdenum. Within the SMOK (NORD, Mico, and Infinix) brand, filament composition and structure varied between products. While the 0.6-ohm NORD mesh filament was mainly iron and chromium (Figs 3A and 4E-H), the Mico was mainly nickel with minor amounts of silicon, aluminum, and iron (Fig 4I-M). The Infinix and the 1.4-ohm NORD coil filaments were mainly nichrome (Fig 4N-P, 4KK-NN). The filaments in the Suorin (Air, Edge, and Drop) pods contained iron, chromium, nickel, and silicon, with molybdenum present only in the Suorin Drop and Suorin Air filament (Fig 4S-X and S3 Fig).

When present, the thick wire brazed to the filament was predominantly nickel with minor amounts of silicon, aluminum, titanium, and molybdenum in some products (Fig 3A, Table 1, Fig 4CC-ZZ, and S3 Fig). Connector-wire joints contained major metals found in the thick wires and minor elements such as molybdenum, aluminum, and silicon (Fig 4OO-ZZ and S3 Fig).

Except for the plastic air tubes in the Suorin pods (Fig 3B), air tubes were either Elinvar, nickel, or nickel alloy containing tin, zinc, or copper as major elements (Figs 3B and 5A-X, S4 Fig). Minor amounts of zinc, copper, cobalt, oxygen, silicon, aluminum, sulfur, and vanadium were also present in some air tubes (Fig 3B). A nickel-coated brass (copper, zinc) air tube with little sulfur was found in KWIT Stick (Figs 3B and 5E-I).

Within the SMOK brand, air tube composition varied in the NORD, Mico, and Infinix. While the Infinix air tube was mainly Elinvar, the Mico was majorly nickel and tin (Figs 3B and 5J – X). The NORD variants were almost identical except for a higher abundance of copper in the pod with the 1.4-ohm NORD coil filament.

The wicks in pod atomizers were mainly oxygen and silicon. However, PHIX and KWIT Stick wicks also had significant amounts of sodium and carbon, respectively (Figs 3B and 5AA-SS, S4 Fig). Wicks in some products contained minor amounts of iron, chromium, potassium, zinc, aluminum, titanium, phosphorus, calcium, barium, chlorine, or magnesium. Most of the minor elements were present in the ceramic wicks, which had the heating coils embedded in them (Figs 3B and 5GG-SS, S4 Fig). Since all samples were prepared in the same manner, it is unlikely the carbon or the minor elements were from residual fluid, which would have been present on all samples and other components.

Most connector components (plate/pins) were mainly gold-plated nickel (Figs 3C, 6A-R). Additional elements, including iron, chromium, zinc, and copper, were also in high abundance in several brands (Fig 3C). Lower abundance elements in connector components included chromium, copper, zinc, tin, cobalt, silicon, aluminum, and molybdenum. A connector plate, which was present only in JUUL™, was comprised of nickel, iron, and gold with minor chromium. (Figs 1, 2B and 3C, S5 Fig)

Miscellaneous components found in some products included a wick chamber, outer casing, and magnet. The wick chambers in PHIX and SMOK Infinix were mainly Elinvar with minor aluminum (Fig 6S-AA). The outer casing in PHIX and SMOK (Infinix and Mico) was mainly nickel, copper, and tin (Fig 3C and 6BB-6PP). Minor levels of iron, chromium, aluminum, and calcium were also present. Magnets were mainly nickel with minor iron, copper, and sulfur (Fig 3C and 6QQ-UU).

Frequency of occurrence of elements in atomizers of fourth generation pod ECs

Information based on the relative abundances of elements in our study (Fig 3) was used to evaluate the frequency of 23 metals/elements in pod atomizer components (Fig 7). The nine most frequently found metals that appeared in 5 or more components in descending order of frequency were: nickel, chromium, iron, aluminum, gold, copper, zinc, molybdenum, titanium. All except aluminum, molybdenum, and titanium were in relatively high abundance (blue bars in Fig 7).

Comparison of atomizer components from fourth generation pod ECs

The components and method of joining components in atomizers of previous generations of ECs [1,6] were compared to the fourth generation ECs (Fig 8). Filament, thick wire, air tube, wick, and wire-wire joint were present and preserved across all EC generations (Fig 8A). Wire-air tube joint, sheath, and fiber were components found only in first-generation ECs. Consequently, connectors, connector-wire joint, wick chamber, magnet, and outer casing are evolving components that were present only in the fourth generation ECs (Fig 8A).

Within atomizers, brazing was used in all four generations for wire-wire joining. Soldering, coiling, welding, and clamping were used only in the earliest EC products (Fig 8B). Wire-air tube joints were only in the first generation, where all joining types except brazing were used. Both inner and outer fibers were present only in the first generation, and ceramic wicks were found only in the fourth generation (Fig 8B).

Elements in atomizer components across multiple EC generations

Fourteen elements (aluminum, calcium, cobalt, chromium, copper, iron, sodium, nickel, silicon, tin, titanium, zinc, carbon, and oxygen) have been identified in atomizer components from all generations (Fig 9). While silver, lead, and tungsten were present only in first-generation products, manganese was found in the first, second, and third generations. Magnesium was present in components of the first, second, and fourth generations. Barium, chlorine, vanadium, phosphorus, and sulfur were only in the fourth generation. However, gold, molybdenum, and potassium were identified in components of first and fourth generation ECs. There were no elements identified in either second or third generations, which were not found in other generations (Fig 9).

DISCUSSION

Our study compares the design features and elemental composition of atomizers in pod ECs from multiple popular manufacturers. The pod fluid reservoirs were either prefilled (JUUL™, PHIX, and Kilo 1K), prefilled and refillable (KWIT Stick), or refillable SMOK (Infinix, Mico, and NORD) and Suorin (Air, Edge, and Drop). Even though multiple components in the pods were similar (e.g., filament, air tube, wicks, and connector plate/pin), there were variations in fluid reservoirs, battery capacity, and elemental composition across our sample of prefilled and refillable pod components. A total of 23 elements were identified in the EC pod atomizers, of which 11 were considered dominant elements based on their relative abundance in EDS spectra.

Like earlier EC generations, the pod ECs varied in shape and size. The most striking design difference in the pods was the modern and futuristically shaped batteries (S1 Fig) [1, 4]. The pod batteries are smaller than those in clearomizers and mods, but larger than cig-a-likes. EC pod batteries operate at a relatively low fixed voltage, unlike clearomizers/mods that have higher variable voltages with higher potential to release atomizer elements into the aerosol [1].

Some pod atomizer components, such as the filament, thick wire, air tube, wick, and wire-wire joint, were preserved across generations (Fig 8A). Brazing, a wire joining method, and organic wicks were also found in all EC generations. Components such as the gold-plated connectors, connector-wire joint, wick chamber, magnets, and outer casing were observed for the first time in pods (Fig 8A). The insulating sheaths in previous models were absent in the fourth generation. Differences, such as the inclusion of ceramic wicks, connectors, and variations in filaments (coil vs. mesh), were seen in some pod atomizers.

Of 23 elements identified in pod atomizers from different manufacturers, 11 (48%) were present in relatively high abundance (nickel, chromium, iron, gold, copper, zinc, tin, oxygen, silicon, carbon, and sodium). Twelve elements (52%) were present in lesser amounts (aluminum, molybdenum, titanium, sulfur, cobalt, calcium, potassium, phosphorus, vanadium, barium chlorine, and magnesium). Except for gold, which was only present in the first and fourth generations, all the high abundance elements have been identified in previous EC generations. (Fig 9), [6]. While filaments in older EC atomizers were mostly nichrome [6], pod filaments were mainly Elinvar. The elemental composition of the JUUL™ filament and connector plate was in agreement with a previous report [27]. Thick wires, mostly copper and silver [6], have evolved into predominantly nickel. Wire-wire joints, which were previously mainly chromium, copper, nickel, tin, and zinc [6], are often Elinvar or iron-containing alloys. Previously used wire-air-tube joints consisting of mainly tin solder in most first-generation ECs have become obsolete. A new wire-connector joint made mainly of Elinvar is present in the fourth generation. In general, the dominant elements in the air tubes and wicks were similar in all generations.

There are several possible sources for the elements in EC aerosols. Atomizer fluids contain elements/metals prior to heating [13,15-19, 28]. Some of these elements are likely contaminants of the solvents, flavor chemicals, and nicotine that comprise the fluid. Others may leach into the fluid from the atomizer components. Some pod fluids have a low pH, which may facilitate the transfer of elements into fluids before vaping [10], although, in a recent comparison of fluids with pHs as low as 4.02 and as high as 6.79, high metal concentrations did not correlate with low pH [29]. Elements in EC atomizers can also be released into fluids during heating [18, 19] and then inhaled by users. The concentration of metals in EC aerosols can be manipulated by changing the power at which aerosols are generated and/or altering the metals used in the ECs. For example, there has been a gradual reduction in tin solder joints close to the filament and a corresponding decrease in tin in the aerosols [21]. It was recently suggested, based on single particle inductively coupled plasma mass spectrometry, that steel components in the atomizers, not the nichrome filament, are the source of chromium, iron, and nickel in fourth generation aerosols [30]. The plastic components in pod atomizers (e.g., air tubes and fluid reservoirs) may also leach metals and non-metals, such as plasticizers, into pod fluids, which could contribute to aerosol toxicity.

We evaluated a subset of currently marketed EC pods. Products not evaluated in our study may contain additional elements/metals. Likewise, counterfeit products, which were not included in our study, may differ from those produced by major manufacturers [31].

Metals in e-liquids do transfer into the aerosols of first, second, and third generation products [5, 12, 17 - 21] and are therefore inhaled by EC users. Metal transfer to aerosols has also recently been shown for fourth generation products, such as myblu™ and Vuse Alto® pods, which had elevated levels of chromium, nickel, copper, zinc, tin, and lead in their aerosols [29,30]. However, not all pods showed this transfer, e.g., the concentrations of these metals in JUUL™ aerosols were at or below the limit of detection and/or limit of calibration standard [29]. This variability between brands has also been shown for prior generations of ECs [32]. Variations in the liquid-to-aerosol transfer can also occur within pod brands. For example, nickel transferred more efficiently to aerosols made using myblu™ Intense Mint-sation than those made with myblu™ Intense Tobacco Chill [29].

Elements such as arsenic, lead, cadmium chromium, cobalt, nickel, and silica have been linked to human illnesses, including cardiovascular diseases, immune system suppression, lung injury, cancer, renal damage, neurotoxicity, and silicosis [33-42]. The metals in EC products have not yet been directly linked to these illnesses, and such linkage may be challenging to demonstrate given the high variability in metal transfer to EC aerosols from different products and the variations in user topography [43], which also affects metal concentrations in aerosols [44]. A recent risk assessment study based on published concentrations of metals in EC products concluded that nickel and chromium are high enough in EC liquids and aerosol to present a cancer risk and that nickel, chromium, and manganese may also present non-cancer health risks [45]. In addition, human urine samples from EC users had higher concentrations of zinc than those from nonsmokers, and zinc concentration was positively correlated with increased DNA oxidation, suggesting a potential increased risk for disease in the EC user group

[46]. Data clearly show that EC liquids and aerosols contain elements/metals known to cause disease with chronic exposure. However, because of the variability between and within EC products, it is difficult for users to identify products that may be safer to use.

In summary, we characterized the design features of pod EC, then mapped 23 elements/metals in the atomizers of pods from six manufacturers. The elements/metals in atomizers are important for two reasons. First, chronic exposure could adversely affect human health. Some of the elements/metals are known to produce disease, although this has not yet been demonstrated for the toxic elements in ECs. Secondly, EC pod products are eventually discarded into the environment, contributing to chemical pollution in water and soil. Understanding the health impact of the elements/metals in EC pods and their fate when discarded will be important when establishing regulations on their use and disposal.

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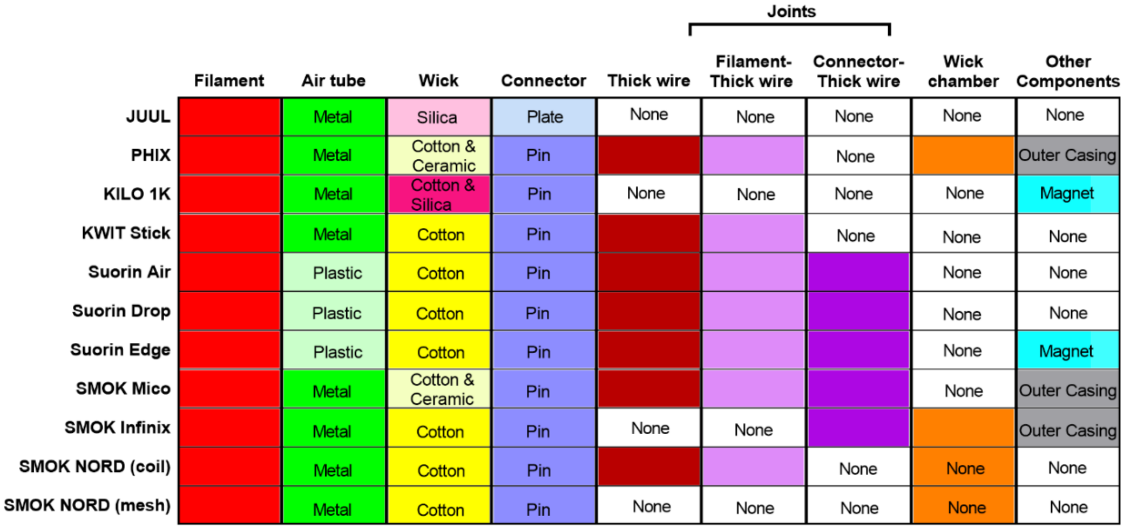


						Joints			
	Filament	Air tube	Wick	Connector	Thick wire	Filament-Thick wire	Connector-Thick wire	Wick chamber	Other Components
JUUL	🟥	Metal	Silica	Plate	None	None	None	None	None
PHIX	🟥	Metal	Cotton & Ceramic	Pin	🟥	🟪	None	🟧	Outer Casing
KILO 1K	🟥	Metal	Cotton & Silica	Pin	None	None	None	None	Magnet
KWIT Stick	🟥	Metal	Cotton	Pin	🟥	🟪	None	None	None
Suorin Air	🟥	Plastic	Cotton	Pin	🟥	🟪	🟪	None	None
Suorin Drop	🟥	Plastic	Cotton	Pin	🟥	🟪	🟪	None	None
Suorin Edge	🟥	Plastic	Cotton	Pin	🟥	🟪	🟪	None	Magnet
SMOK Mico	🟥	Metal	Cotton & Ceramic	Pin	🟥	🟪	🟪	None	Outer Casing
SMOK Infinix	🟥	Metal	Cotton	Pin	None	None	🟪	🟧	Outer Casing
SMOK NORD (coil)	🟥	Metal	Cotton	Pin	🟥	🟪	None	None	None
SMOK NORD (mesh)	🟥	Metal	Cotton	Pin	None	None	None	None	None

Figure 10.1. Atomizer components that were present in multiple brands of pod ECs. ECs are listed on the left axis, and pod components are listed above each column.

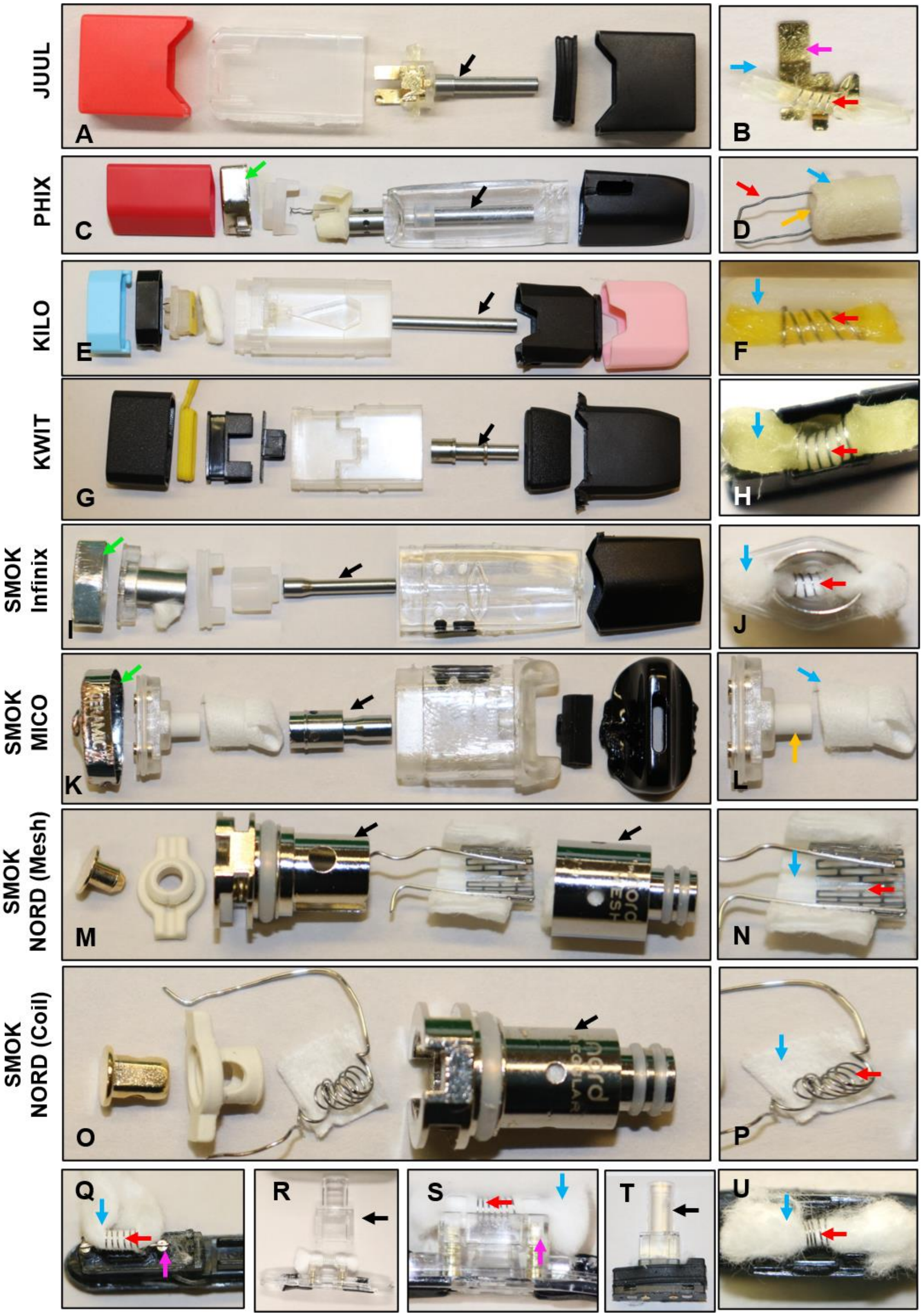

JUUL
A
B
PHIX
C
D
KILO
E
F
KWIT
G
H
SMOK Infinix
I
J
SMOK MICO
K
L
SMOK NORD (Mesh)
M
N
SMOK NORD (Coil)
O
P
Q
R
S
T
U

Figure 10.2. Anatomy and internal design of atomizers from pod ECs. JUUL™ (A-B), PHIX (C-D), Kilo 1K (E-F), KWIT Stick (G-H), SMOK Infinix (I-J) SMOK Mico (K-L), SMOK NORD 0.6-ohm (mesh) filament (M-N), SMOK NORD 1.4-ohm (coil) filament (O-P), Suorin Air (Q), Suorin Drop (R-S) Suorin Edge (T-U). JUUL™, PHIX, and Kilo 1K are prefilled. KWIT Stick is prefilled and refillable. SMOK and Suorin are refillable. Specific key components are indicated by colored arrows: air tube = black; cotton/silica wick = blue; ceramic wick = orange; filament and thick wires = red; connector plate/pin= pink; outer casing = green.

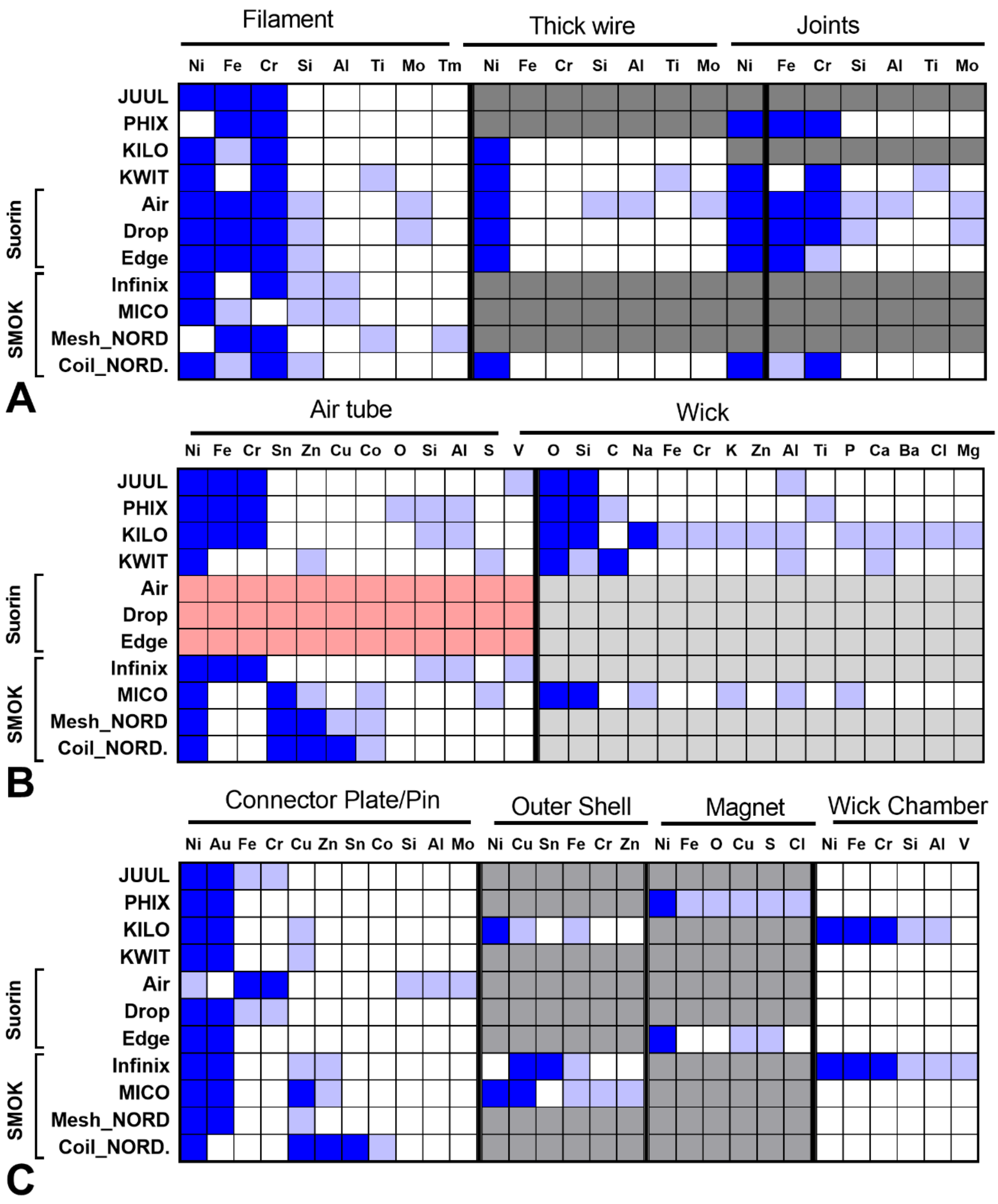


Figure 10.3. Heat maps showing the elemental composition of atomizer components in pod ECs. Blue squares = elements that were major peaks in the EDS spectra. Light blue squares = elements that were minor peaks in spectra. Dark gray squares = components that were not present. Light gray squares = components that were present but not analyzed because they were identical to other components. Pink squares = components that were made of plastic and not analyzed.

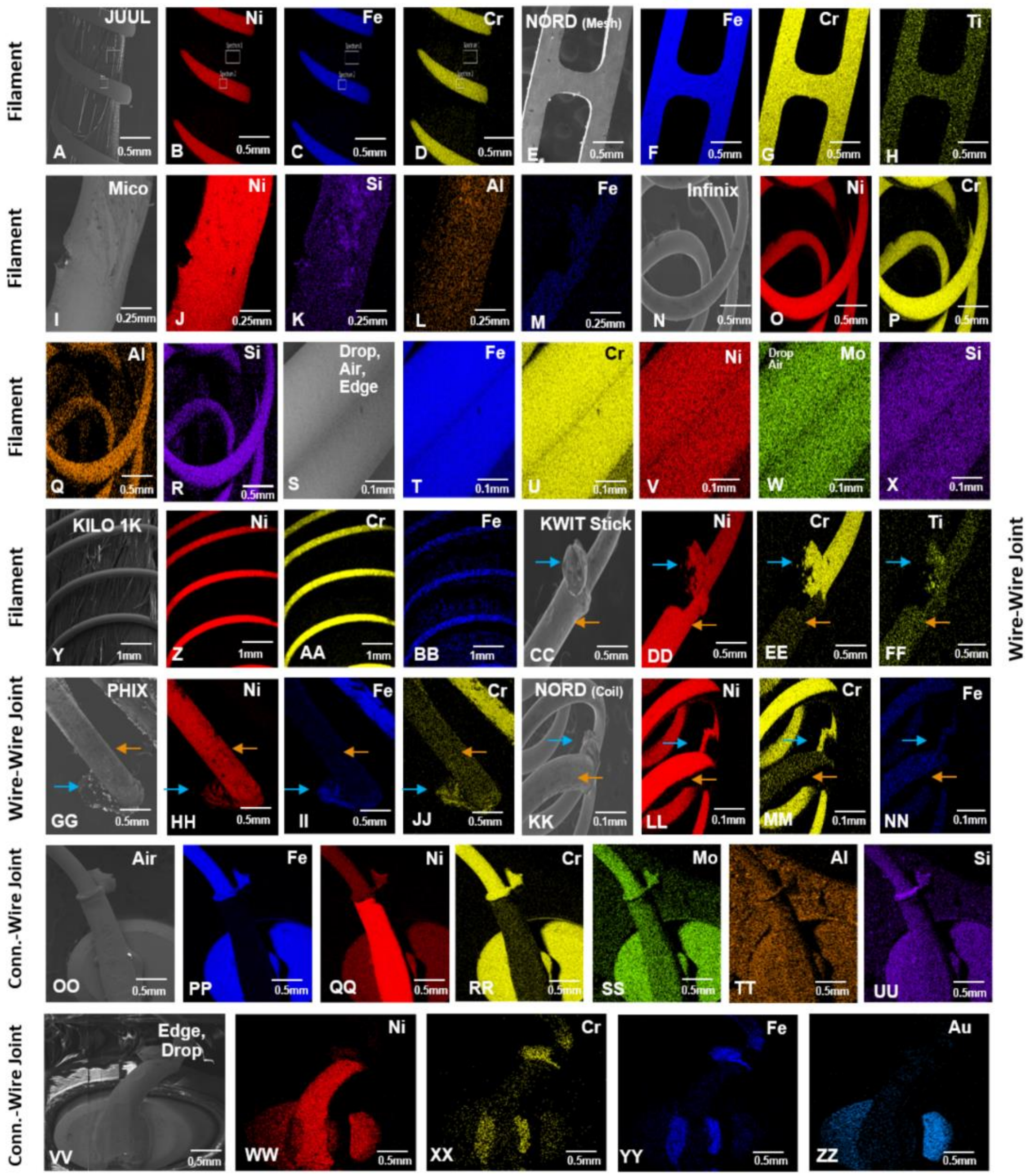

Filament
JUUL
A 0.5mm
Ni
B 0.5mm
Fe
C 0.5mm
Cr
D 0.5mm
NORD (Mesh)
E 0.5mm
Fe
F 0.5mm
Cr
G 0.5mm
Ti
H 0.5mm
Filament
Mico
I 0.25mm
Ni
J 0.25mm
Si
K 0.25mm
Al
L 0.25mm
Fe
M 0.25mm
Infinix
N 0.5mm
Ni
O 0.5mm
Cr
P 0.5mm
Filament
Al
Q 0.5mm
Si
R 0.5mm
Drop, Air, Edge
S 0.1mm
Fe
T 0.1mm
Cr
U 0.1mm
Ni
V 0.1mm
Drop Air
Mo
W 0.1mm
Si
X 0.1mm
Filament
KILO 1K
Y 1mm
Ni
Z 1mm
Cr
AA 1mm
Fe
BB 1mm
KWIT Stick
CC 0.5mm
Ni
DD 0.5mm
Cr
EE 0.5mm
Ti
FF 0.5mm
Wire-Wire Joint
Wire-Wire Joint
PHIX
GG 0.5mm
Ni
HH 0.5mm
Fe
II 0.5mm
Cr
JJ 0.5mm
NORD (Coil)
KK 0.1mm
Ni
LL 0.1mm
Cr
MM 0.1mm
Fe
NN 0.1mm
Conn.-Wire Joint
Air
OO 0.5mm
Fe
PP 0.5mm
Ni
QQ 0.5mm
Cr
RR 0.5mm
Mo
SS 0.5mm
Al
TT 0.5mm
Si
UU 0.5mm
Conn.-Wire Joint
Edge, Drop
VV 0.5mm
Ni
WW 0.5mm
Cr
XX 0.5mm
Fe
YY 0.5mm
Au
ZZ 0.5mm

Figure 10.4. SEM images and EDS elemental maps of the filaments and joints from pod EC atomizers. For filaments, JUUL™ (A) was made of nickel (B), iron (C), and chromium (D). SMOK NORD (mesh) (E) was made of iron (F), chromium (G), and titanium (H). SMOK Mico (I) was made of nickel (J), silicon (K), aluminum (L), and iron (M). SMOK Infinix (N) was made of nickel (O), chromium (P), aluminum (Q), and silicon (R). Suorin Drop, Air, and Edge (S) were made of iron (T), chromium (U), nickel (V), molybdenum (W), and silicon (X). Kilo 1K (Y) was made of nickel (Z), chromium (AA), and iron (BB). KWIT Stick (CC) was made of nickel (DD), chromium (EE), and titanium (FF). For filament-wire joints, KWIT Stick (CC) was made of nickel (DD) and chromium (EE). PHIX (GG) was made of nickel (HH), iron (II), and chromium (JJ). SMOK NORD (coil) (KK) was made of nickel (LL), chromium (MM), and iron (NN). Suorin Air (OO) was made of iron (PP), chromium (RR), molybdenum (SS), aluminum (TT), and silicon (UU). Suorin Edge and Drop (VV) were made of chromium (XX) and iron (YY). For connector-wire joints, Suorin Air (OO) was made of a droplet of iron (PP), nickel (QQ), chromium (RR), molybdenum (SS), aluminum (TT), and silicon (UU). Suorin Edge and Drop (VV) were made of nickel (WW), chromium (XX), and iron (YY). Orange arrows in CC – NN show thick wires, while blue arrows show joints between the thick wire and filament.

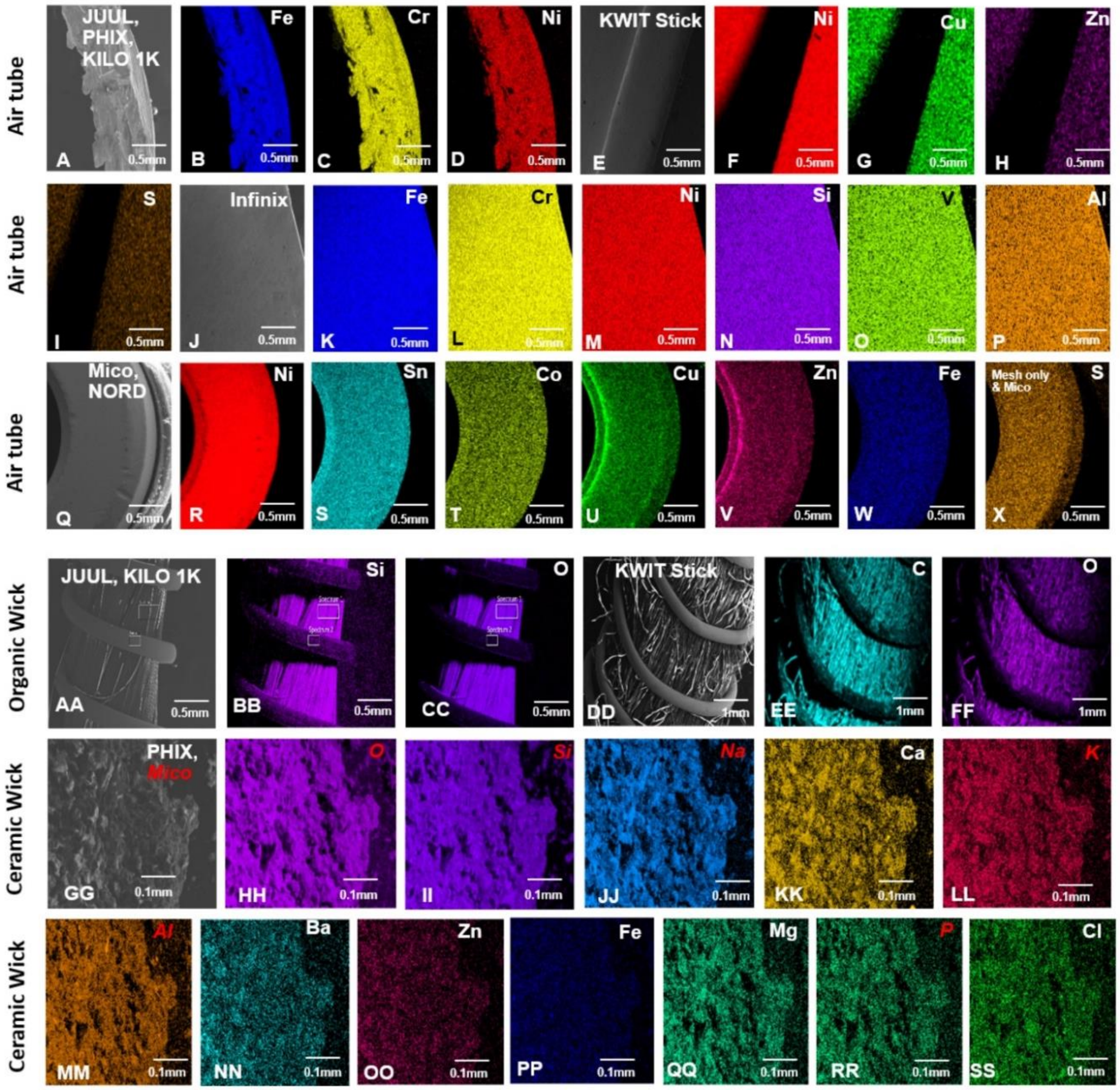


Figure 10.5. SEM images and EDS elemental maps of the air tubes and wicks from pod EC atomizers. JUUL™, PHIX, and Kilo 1K (A) were made of iron (B), chromium (C), and nickel (D). KWIT Stick (E) was made of nickel (F), copper (G), zinc (H), and sulfur (I). SMOK Infinix (J) was made of iron (K), chromium (L), nickel (M), silicon (N), vanadium (O), and aluminum (P). SMOK Mico and NORD (Q) were made of nickel (R), tin (S), cobalt (T), copper (U), zinc (V), iron (W), and sulfur (X). Organic wicks in JUUL™ and Kilo 1K (AA) were made of silicon (BB) and oxygen (CC), and KWIT (DD) was made of carbon (EE) and oxygen (FF). PHIX and SMOK Mico (GG) had ceramic wicks consisting of iron (HH), silicon (II), sodium (JJ), calcium (KK), potassium (LL), aluminum (MM), barium (NN), zinc (OO), iron (PP), magnesium (QQ), phosphorus (RR), and chlorine (SS). Elements highlighted in red for ceramic wicks were present only in SMOK Mico.

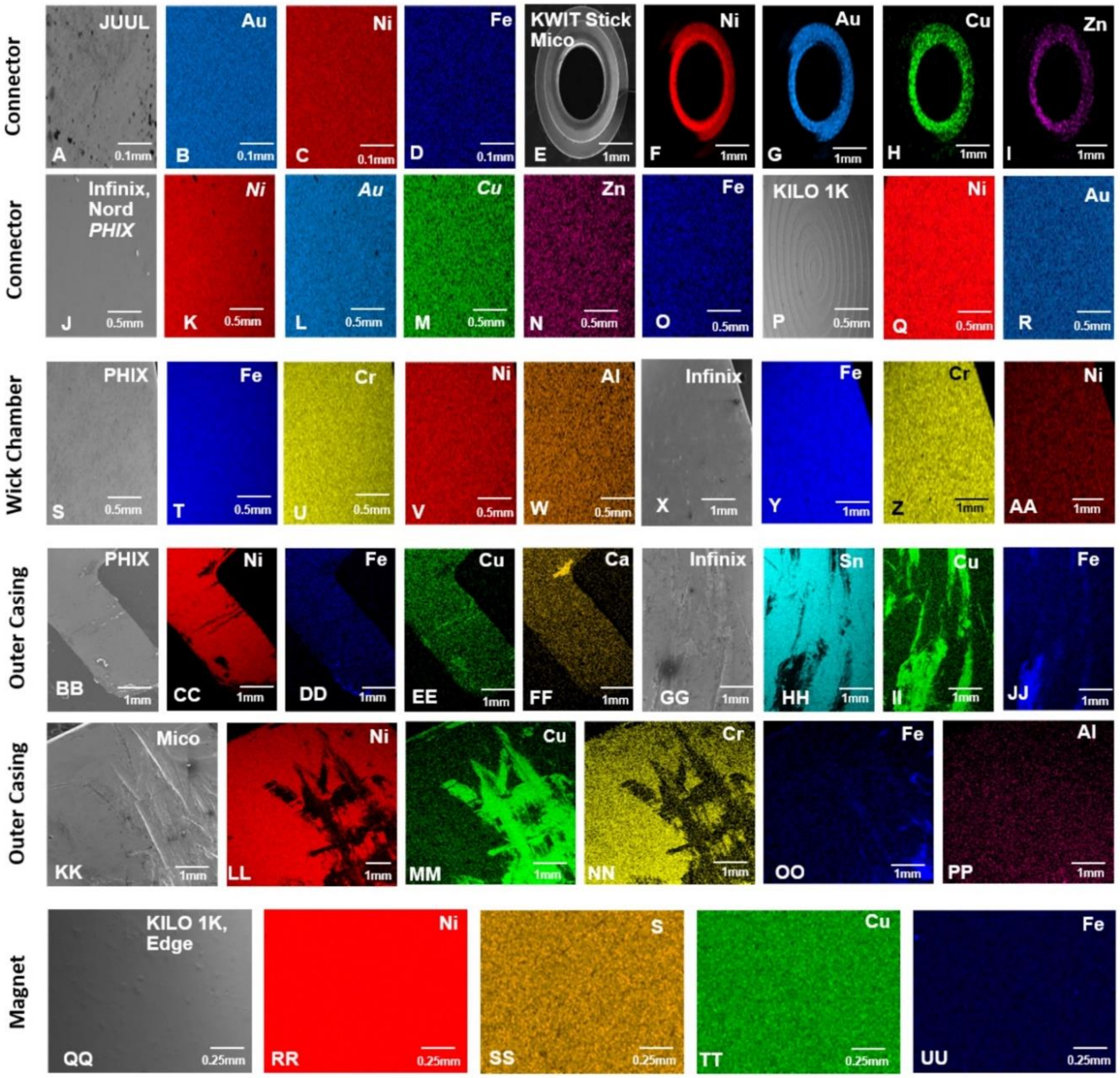


Figure 10.6. SEM images and EDS elemental maps of miscellaneous components from pod EC atomizers. Connectors present in JUUL™ (A) were made of gold (B), nickel (C), and iron (D). KWIT Stick and SMOK Mico (E) were made of nickel (F), gold (G), copper (H), and zinc (I). SMOK Infinix, SMOK NORD, and PHIX (J) were made of nickel (K), gold (L), copper (M), zinc (N), and iron (O). Kilo 1K (P) was made of nickel (Q) and gold (R). Wick chambers present in PHIX (S) was made of iron (T), chromium (U), nickel (V), and aluminum (W). SMOK Infinix (X) was made of iron (Y), chromium (Z), and nickel (AA). The outer casing present in PHIX (BB) was made of nickel (CC), iron (DD), copper (EE), and calcium (FF). SMOK Infinix (GG) was made of tin (HH), copper (II), and iron (JJ). SMOK Mico (KK) was made of nickel (LL), copper (MM), chromium (NN), iron (OO), and aluminum (PP). Magnet components present in Kilo and Suorin Edge (QQ) were made of nickel (RR), sulfur (SS), copper (TT), and iron (UU).

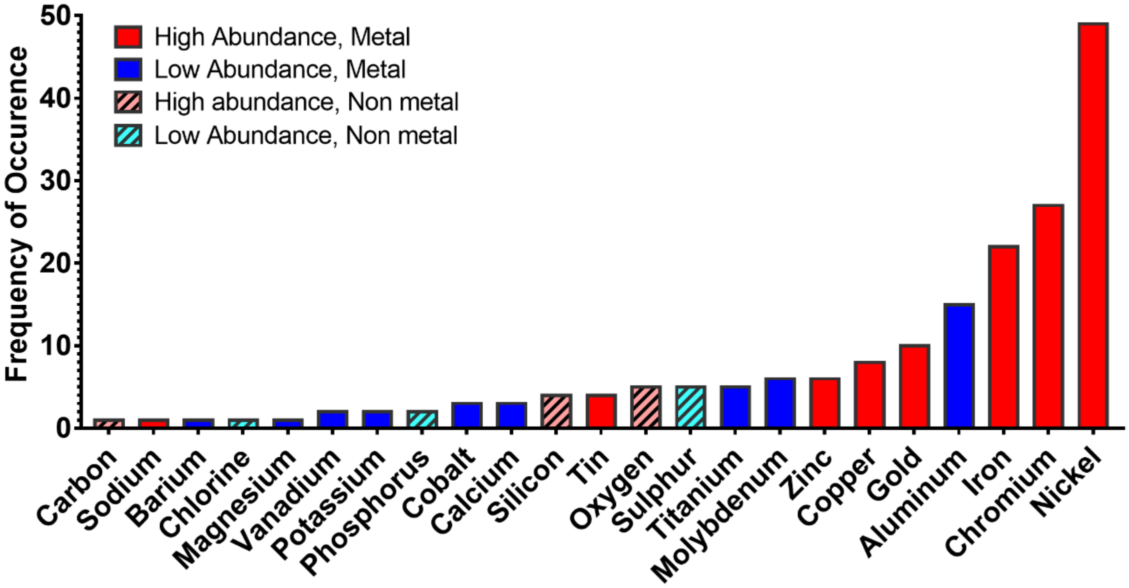
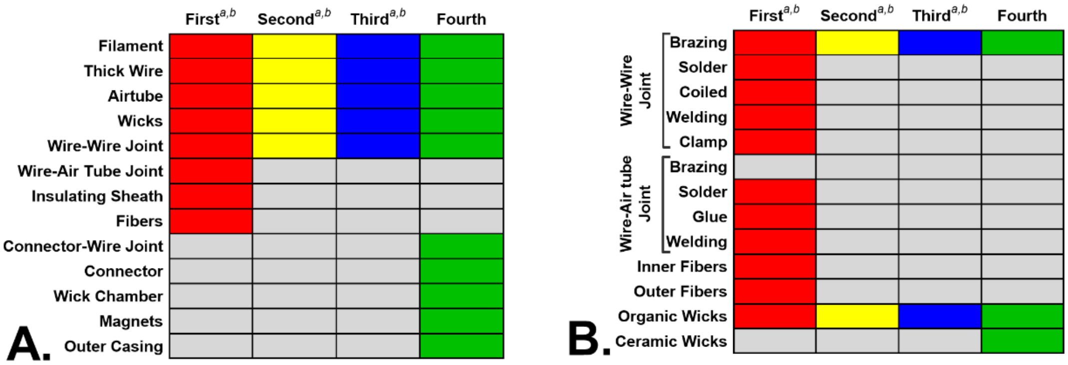


Figure 10.7. Frequency of occurrence of elements in pod EC atomizers. The x-axis shows metal/elements sorted according to increasing frequency of their occurrence (1 – 49), with nickel being the highest. Bars are coded based on each element's relative abundance. Solid bars are metals, while hatched bars are non-metals. Red bars = metals in major abundance, blue bars = metals in minor abundance, red hatched bars = non-metals in major abundance, hatched blue bars = non-metals in minor abundance.



A.

	First^{a,b}	Second^{a,b}	Third^{a,b}	Fourth
Filament	🟥	🟨	🟦	🟩
Thick Wire	🟥	🟨	🟦	🟩
Airtube	🟥	🟨	🟦	🟩
Wicks	🟥	🟨	🟦	🟩
Wire-Wire Joint	🟥	🟨	🟦	🟩
Wire-Air Tube Joint	🟥			
Insulating Sheath	🟥			
Fibers	🟥			
Connector-Wire Joint				🟩
Connector				🟩
Wick Chamber				🟩
Magnets				🟩
Outer Casing				🟩

B.

		First^{a,b}	Second^{a,b}	Third^{a,b}	Fourth
Wire-Wire Joint	Brazing	🟥	🟨	🟦	🟩
	Solder	🟥			
	Coiled	🟥			
	Welding	🟥			
	Clamp	🟥			
Wire-Air tube Joint	Brazing				
	Solder	🟥			
	Glue	🟥			
	Welding	🟥			
	Inner Fibers	🟥			
	Outer Fibers	🟥			
	Organic Wicks	🟥	🟨	🟦	🟩
	Ceramic Wicks				🟩

Figure 10.8. Atomizer components, methods of joining, and types of components in four EC generations. (A) Atomizer components in four EC generations. (B) Types of joining methods, fibers, and wicks in four EC generations. The columns indicate the four EC generations (first = cartomizers/disposables, second = clearomizers/tanks, third = mods, fourth = pods). The y-axis lists major atomizer components. [a] = Williams et al. 2019a; [b] = Williams et al. 2019b

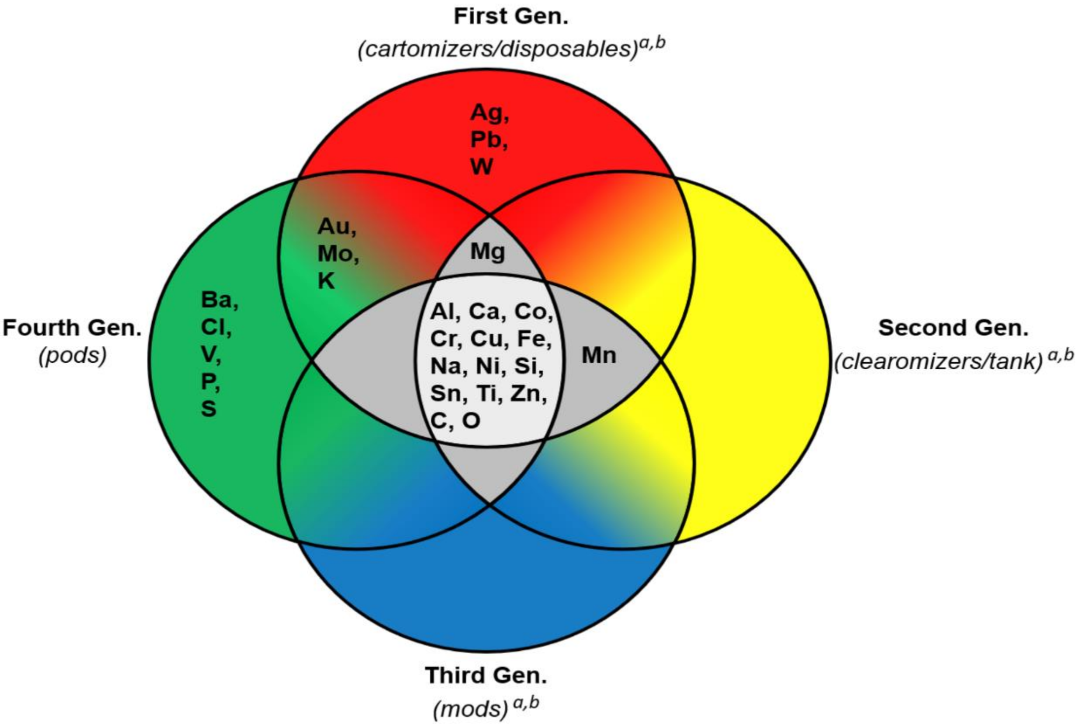


Figure 10.9. Venn diagram showing elements identified in atomizer components of four EC generations. Al = aluminum, Co = cobalt, Cr = chromium, Cu = copper, Fe = iron, Ni = nickel, Sn = tin, Ti = titanium, Zn = zinc, Ag = silver, Pb = lead, W = tungsten, V = vanadium, Au = gold, Mo = molybdenum, Mn = manganese, Mg = magnesium, C = carbon, Ca = calcium, Na = sodium, O = oxygen, Si = silicon, Ba = barium, Cl = chlorine, P = phosphorus, S = sulfur, K = potassium. [a] = Williams et al. 2019a; [b] = Williams et al. 2019b

Table 10.1. Summary of element/metal analysis of pod EC atomizer components.

Brand	Product	Filament	Thick Wire	Joints	Air tube	Wick	Connector Plate/Pin	Miscellaneous
Pax Labs	JUUL™	Ni, Fe, Cr	None	None	Ni, Fe, Cr (*V*)	O, Si	Ni, Fe, Au (*Cr*)	
Kilo E-liquid Inc.	KILO 1K	Ni, Cr (*Fe*)	None	None	Ni, Fe, Cr	O, Si (*C, Ti*)	Ni, Au	
Aspire	KWIT Stick	Ni, Cr (*Ti*)	Ni (*Al, Ti*)	Ni, Cr (*Ti*)	Ni, Cu (*Zn, S*)	O, C (Si, Al, Ca)	Ni, Zn, Au (*Cu*)	**Outer shell:** Ni (*Fe, Cu, Ca*)
ECS Global LLC	PHIX	Fe, Cr	Ni	Ni, Fe, Cr	Ni, Fe, Cr	O, Si, Na, (*Fe, K, Zn, Al, P, Ca, Ba, Cl Mg*)	Ni, Au (*Cu*)	**Wick Chamber:** Ni, Fe, Cr (*Al*). **Outer shell:** Ni (*Fe, Cu, S*)
SMOK	Infinix	Ni, Cr (*Si, Al*)	None	None	Ni, Fe, Cr (*Si, Al, V*)		Ni, Au (*Si, Zn, Al, Cu*)	**Wick Chamber:** Ni, Fe, Cr (*Al*) **Outer shell:** Cu, Sn (*Fe*)
SMOK	MICO	Ni (*Fe, Si, Al*)			Ni, Zn, Cu, Sn (*Fe, Co, S*)	O, Si (*K, Al, Na, P*)	Ni, Au, Cu (*Zn*)	**Outer shell:** Ni, Cu (minor: Fe, Cr, Al).
SMOK.	NORD (coil).	Ni, Cr (*Fe, Si*)	Ni	Ni, Cr (*Fe*)	Ni, Zn, Cu, Sn (*Fe, Co*)		Ni, Zn, Au, Cu (*Fe*)	
SMOK	NORD (mesh)	Fe, Cr (*Ti*)	None	None	Ni, Zn, Cu, Sn (*Fe, Co, S*)		Ni, Zn, Au (*Fe, Cu*)	
Suorin	Air	Ni, Fe, Cr (*Si, Mo*)	Ni (*Si, Al, Mo*)	Ni, Fe, Cr (*Si, Al, Mo*)	None		Fe, Cr (*Ni, Si, Al, Mo*)	
Suorin	Edge	Ni, Fe, Cr (*Si*)	Ni	Ni, Fe, Cr	None		Ni, Au	**Magnet:** Ni (*Cu, S*)
Suorin	Drop	Ni, Fe, Cr (*Si, Mo*)	Ni	Ni, Fe, Cr	None		Ni, Au (*Fe, Cr*)	

Full names of elements. Ni = Nickel, Fe = iron, Cr = chromium, Ti = Titanium, Si = silicon, Al = aluminum, Mo = molybdenum, V = Vanadium, Cu = copper, Zn = zinc, S = sulfur, Sn = tin, Co = cobalt, O = oxygen, Si = silicon, C = carbon, Ca = calcium, Na = sodium, K = potassium, P = phosphorus, Ca = calcium, Ba = barium, Cl = chlorine, Mg = magnesium, Au = gold. Elements *italicized* indicate minor relative abundance.

Chapter 11

Elemental Analysis of E-Liquids and Aerosols from Multiple Brands of Fourth Generation E-Cigarettes

ABSTRACT

Electronic cigarette (EC) liquids and aerosols contain chemical constituents, including metals, some of which are detrimental to human health. The e-liquids used in fourth-generation EC "pods" have evolved to include salt-based nicotine coupled with acids which makes the residual aerosols easier for consumers to inhale. These newer models of e-cigarettes which also contain flavor chemicals and synthetic coolants remain popular among adolescents and young adults. The objectives of our study were to identify and quantify chemical elements in pod ECs from multiple manufacturers/brands and in different flavor variants of JUUL. Inductively coupled plasma optical emission spectroscopy was used to identify and quantify 20 elements/metals in EC liquids and aerosols. All 20 elements were identified in at least one e-liquid or aerosol sample. Within JUUL, the total concentration of elements was determined in unvaped liquids (2,000 – 48,000 ug/L), vaped liquids (1,000 – 30,000 ug/L), and aerosols (500 – 24,000 ug/L). When comparing across multiple fourth generation brands, the total concentration of elements was 20 – 76,000 ug/L (unvaped liquids), 400 – 73,000 ug/L (vaped liquids), and 300 – 9,000 ug/L (aerosols). Unvaped KWIT Stick e-liquid contained the highest concentrations of both total elements and nickel (69,000 ug/L). In closed system pods, the transfer efficiency of total elements from the e-liquids to aerosols ranged from 6 – 89%, while the range within JUUL products was 7 – 98%. For open system pods, the concentrations of elements in aerosols were 0.2 – 35 times higher than in liquids samples. While silicon and potassium were the highest in concentration in JUUL liquids, nickel, and potassium were the highest in the liquids from other brands evaluated. Fourth-generation EC products vary in their elemental composition and concentration of hazardous metals. The increased concentration of elements in aerosols of open pod

system shows the release of metals into aerosols during vaping. Our study provides further evidence on the elemental composition of e-cigarettes metals, which users may inhale during a vaping of products that are currently popular.

INTRODUCTION

Since the introduction of electronic cigarettes (ECs) over a decade ago, several studies have shown that EC components contain hazardous metals and chemical elements that are also detected in e-liquids and aerosols (Williams et al., 2013, 2015, 2017; Lerner et al., 2015; Mikheev et al., 2016; Gray et al., 2019, 2022; Williams, Bozhilov & Talbot, 2019; Williams, Li & Talbot, 2019; Zhao et al., 2019, 2020; Halstead et al., 2020; Pearce et al., 2020; Gonzalez-Jimenez et al., 2021; Pappas et al., 2021). Earlier research on metals in e-cigarettes focused on first, second, and third generation devices as well as refill fluids, and revealed high levels of toxic chemical elements such as lead, nickel, zinc, and chromium that have the potential to negatively impact human health. ECs have continued to evolve in battery capabilities, e-liquid reservoirs, and chemical composition, and fourth-generation de-vices are designed to generate lower pH aerosols from salt-based nicotine solutions efficiently. While e-liquids generally contain nicotine, flavor chemicals, and solvents, fourth-generation ECs, such as JUUL, contain higher nicotine concentrations when compared to earlier devices and free-base nicotine e-liquids (Duell, Pankow & Peyton, 2018; Goniewicz et al., 2019) The high nicotine concentrations in fourth generation e-cigarettes results from a combination of the nicotine salts with acids such as benzoic acid which creates a low pH aerosol that is easier to inhale. The acids in e-liquids have been shown to affect metal concentrations and aid the transfer of metals from e-liquids to aerosols. Acid may also facilitate the leaching of metals from components thereby increasing concentrations in e-liquids and eventual transfer to resulting aerosols. Fourth-generation ECs from JUUL and Puff dominate the EC markets in market share and remain popular among young adults and adolescents (Cullen, Gentzke, et al., 2019; Cullen, Liu, et al., 2019; Wang et al., 2020, 2021; Park-Lee et al., 2021). The objectives of our study were to evaluate popular fourth generation e-liquids and their aerosols, identify and quantify elements/metals in closed system (prefilled) pods and refill fluids used in open system (refillable) pods, and compare element/metal concentrations in fourth-generation ECs to previous generations of ECs.

MATERIALS AND METHODS

Sample acquisition and storage

Fourth-generation ECs were purchased online from the manufacturer or locally from reputable third-party vendors. The six brands of prefilled pods that were evaluated included: KWIT Stick (Aspire., USA), Suorin Air (Shenzhen Blumark technology Co., Ltd, China), SMOK Infinix (Shenzhen Blumark Technology Co., Ltd, China), JUUL (Pax Lab, San Francisco, CA), KILO 1K (Kilo E-liquid Inc., La Mirada, CA), and PHIX (ECS Global LLC., Los Angeles, CA). All JUUL flavor variants marketed before the Food and Drug Administration enforcement policy on flavored cartridge-based ECs in 2020 were evaluated.

Two salt-based nicotine refill fluids (Mynto Ice Mango from Drip Fire and Mango Bomb from VGOD Salt Nic Labs) were purchased and used in SMOK Infinix and Suorin Edge refillable pods.

Three samples of each brand were purchased simultaneously to limit variations in purchase batch. All products were stored at room temperature and analyzed within a month of purchase.

Sample preparation, aerosol production, and capture using an impinger method

All liquid and aerosol samples for elemental analysis were made using nitric acid solutions prepared with Milli-Q System water (Millipore, USA) at 18.2 mOhm of resistance. Nitric Acid AR Select (ACS) for Trace Element Analysis was purchased from Macron Chemicals (Avanter Performance Materials, Inc, Center Valley, PA).

Elemental analysis was performed for liquids and aerosols from each pod product. For liquid analysis, 200 µL of e-liquid were removed from three unused pods and pooled (unvaped). Another 200 µL of e-liquid was collected from the same pods and pooled after vaping (vaped). Dilutions (0.5 mL: 9.5 mL) of the e-liquid were prepared with freshly made 2% nitric acid in deionized water and stored at room temperature for 1 – 2 days before analysis.

Before aerosol production, impingers were washed and soaked in 2% nitric acid for at least 3-5 days to seal the glass and prevent the leaching of elements into the sample (Williams et al., 2017; Williams, Bozhilov & Talbot, 2019). Since the devices performed differently, aerosols were generated at 10 - 13 mL/sec airflow rate. To reduce the chance of “dry puffs,” no more than ¾ of the e-liquid was vaped/pod. Each pod was primed by taking three puffs before weighing and aerosol generation. The aerosols generated from the pods were bubbled through 30 mL of 2% nitric acid solution in two impingers connected in tandem (Behar et al. 2018). The system was connected to a Cole-Parmer Master-flex L/S peristaltic pump set to take a 4.3-second puff at 1 puff / minute. A total of 180 puffs (60 each from triplicate pods) was collected for each product evaluated. The aerosol collection was performed at room temperature in a biosafety cabinet. Room air control samples were collected similarly. Aerosol solutions and room air samples were produced in triplicates and stored at room temperature in 15 mL conical tubes pre-sealed with 2% nitric acid.

The following elements were investigated: calcium, magnesium, potassium, arsenic, aluminum, boron, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, selenium, silicon, zinc, silver, tin, and titanium.

Quantification of elements/metals in e-liquids and aerosols using inductively coupled plasma optical emission spectrometry (ICP-OES)

The concentrations of elements/metals in the e-liquids and aerosols were analyzed using a Perkin-Elmer Optima 7300 DV inductively coupled plasma optical emission spectrometer (Perkin Elmer, USA). The ICP-OES was equipped with an autosampler, a Perkin-Elmer Nebulizer (N0777036 REV A, Cyclonic spray chamber Optima 5300DV, Quartz 7mm baffle drain line), and a segmented array charge-coupled device (SCD) detector as previously described (Williams et al., 2017, 2019). Elemental analysis was performed within 1-2 days of sample collection. Daily calibration of the ICP-OES was done using Perkin-Elmer Multi-element calibration standards Plus #2, #3, #4, and #5. Quality control checks on calibration were then run using NIST standard reference materials by Ultra Scientific (trace metal sample, QCI-700A, North Kingstown, RI). Running conditions were plasma flow = 15 L/min, auxiliary flow 0.2 L/min, nebulizer flow of 0.75 L/min, radiofrequency power 1450 W, sample flow rate = 0.80 mL/min, and a read delay time of 12 sec. For an internal standard, yttrium at 2.5 ppm was run in line with sample introduction into the nebulizer. Distilled deionized water with 1% nitric acid was run as the blank. Each sample was run in triplicate. When interference was observed for any element, additional peaks were monitored to identify the best wavelength for quantification. The concentrations of each element in the blank were subtracted from the measured concentrations in each sample. Samples of room air were made the same way as the EC aerosol samples and were run with each batch of samples, and room air values were subtracted from measured concentrations of each element in the aerosols.

In addition to instrument calibration standards, solutions (0.25 and 1.0 ppm) of 9 analytes (aluminum, chromium, copper, lead, nickel, selenium, silicon, tin, zinc) (Inorganic Ventures, Christiansburg, VA) were prepared in 2% nitric acid made with de-ionized water to assess the accuracy of measurements in the samples.

Data and statistical analysis

Data pre-processing was performed to subtract concentrations in control samples (nitric acid solution and room air) from experimental samples. When the element was present below the limit of quantification, it was treated as zero in further analysis. Graphs were plotted using GraphPad Prism software (San Diego, CA). The mean concentrations of elements in the unvaped, vaped, and aerosols samples were compared.

RESULTS

Characteristics of the fourth generation EC devices

The pods compatible with the ECs used in the current study were either prefilled with e-liquid (closed system) or empty and filled with salt-based nicotine refill fluids (open system) before analysis (Table 1). Information on the characteristics and specifications of the devices were obtained from the manufacturers websites and online vape blogs and forums. The original eight flavors of JUUL were still marketed during sample analysis.

Total concentration of elements/metals in JUUL e-liquids and aerosols

The total concentration of all elements and the total without silicon and potassium are reported in Figure 1. The total elements/metals concentration in e-liquids and aerosols from eight JUUL flavors varied among flavors and between pods of the same flavor (note the large standard deviations for Virginia Tobacco and Crème Brulee) (Figure 1A). The total concentration of elements ranged between 1,700 – 48,000 (unvaped), 1,300 – 30,000 (vaped), and 500 – 24,100 ug/L (aerosols) (Figure 1A). The transfer efficiency of elements from the e-liquids into aerosols varied among flavors and was generally less than 100%, with Mango flavor being the highest at 98% (Figure 1A).

Silicon was a major constituent of JUUL e-liquids and aerosols, and except for Classic Menthol and Classic Tobacco, silicon accounted for 90 – 95% of the total element concentration in all JUUL flavors. Potassium concentrations were highest in Classic Menthol and Classic Tobacco e-liquids and along with silicon accounted for 90 – 95% of the total elements. When both elements were removed from the analysis, the concentrations of the total elements were reduced to ranges of 0.1 – 0.5 (unvaped), 0.1 – 1.1 (vaped), and 0.02 – 1.3 (aerosols) (Figure 1B). Calcium in Crème Brulee aerosol contributed to the high total elements when silicon and potassium were removed. (Figure 1B).

Total Concentration of Elements/Metals in E-liquids and Aerosols from Other Brands

The total concentrations of all elements with and without nickel and potassium are reported in Figure 2. The total concentrations varied with brand and e-liquid flavor, with ranges of 40 – 69,000 ug/L (unvaped), 700 – 62,400 ug/L (vaped), and 700 – 8,700 ug/L (aerosols) (Figure 2A). The transfer efficiency of elements from the e-liquids into aerosols varied between brands and types of pods (closed vs. open systems) and was generally less than 100%. For open system pods, transfer efficiency varied with the device and e-liquid flavor and was higher for aerosol samples made with SMOK Infinix (93 – 2,067%) and Suorin Edge (38%). (Figure 2A).

Nickel and potassium were major contributors to the total concentration of elements from multiple brands. (Figure 2A). When both elements were removed from the analysis, the total element concentrations were reduced to 40 – 9,700 (unvaped), 700 – 9,000 (vaped), and 700 – 8,700 ug/L (aerosols) (Figure 2B). In general, the concentration of elements in vaped liquids increased in 5 of 7 products compared to the unvaped liquids. Although nickel accounted for over 70 - 85 % of the total element concentration in JUUL-Jones and KWIT Stick e-liquids, the transfer efficiency to the aerosol was low 10% - 23%.

The concentrations of elements in refill fluids for open system pods were lower than in e-liquids from closed system pods. While the concentration of elements generally increased in vaped e-liquids, concentrations were unchanged in Suorin Edge and decreased in KILO 1K and KWIT Stick.

Individual elements in all JUUL flavor variants

The elemental composition of unvaped JUUL e-liquids were similar; however, the number of elements varied between and within flavor categories (fruit, dessert, tobacco, and menthol) from 7 in Fruit Medley to 16 in Cool Mint (Figure 3).

While the elements in the unvaped and vaped were similar, the number of elements increased after vaping. While titanium, tin, silicon, and calcium were in all unvaped and vaped JUUL fluids, only tin and silicon were in all aerosols. Silicon was the highest in concentration in all JUUL pods, and its transfer efficiency to the aerosol varied between flavors ranging from 21% in Classic Tobacco to 73% in Mango. Some elements (cadmium, copper, aluminum, boron) appeared only in the aerosols of some pods. In general, the transfer efficiencies of most elements were less than 100%.

Individual Elements in Multiple Fourth Generation Products

Element composition and concentration varied in refill fluids, e-liquids, and aerosols. All but one (arsenic) of the 21 elements were present in at least one fluid or aerosol.In refill fluids used with SMOK Infinix and Suorin Edge open system pods, 11 elements were in unvaped liquids, 16 were in vaped liquids, and 15 were in aerosols. Mango Bomb refill fluids contained fewer elements than Mynto Ice Mango (Figure 4A). Tin, magnesium, and potassium were in all refill fluids and transferred to the aerosols, while silicon was in only vaped liquids and aerosols.

The variations in the performance of SMOK Infinix and Suorin Edge devices were evaluated using Mynto Ice Mango refill fluid. Seven elements (boron, calcium, magnesium, potassium, selenium, tin, and zinc) were in the refill fluid and transferred to aerosols generated from both devices (Figure 4A). Some elements in the unvaped liquid (aluminum and nickel) were undetected in the vaped fluids and aerosols. Other elements (chromium, copper, lead) were in vaped and aerosol samples from the SMOK Infinix. Cobalt and copper were present in aerosol samples only from the Suorin Edge. Potassium concentrations was higher in the Mynto Ice Mango, and transfer efficiency varied (19 – 449%) for SMOK Infinix

The number of elements in closed system pods varied slightly between fruity (15) and berry (17) flavor liquids. Zinc, silicon, calcium, magnesium, and sodium were in all e-liquids and transferred to the aerosols, while chromium and copper were in all samples from 3 of 4 devices. Nickel, aluminum, boron, and selenium were in all samples in 2 of 4 devices. (Figure 4B).

Copper, chromium, and nickel were in fluids and aerosols from mango-flavored pods. While aluminum and potassium were in fluids and aerosols from the JONES Clear

Mango pod vaped with a JUUL device, these elements did not transfer to the aerosol of the Mango Tango pod vaped with KWIT Stick. Boron and lead were in Mango tango fluids and aerosols, but only lead was present in the Clear Mango vaped fluid. Iron and tin in the fluids transferred only to the Mango Tango aerosol. Cobalt and manganese were only in the Clear Mango vaped fluid but in unvaped fluids in the Mango Tango. Two berry-flavored pods vaped with different devices (PHIX and Kilo 1K) differed in the elements present in all sample conditions. While calcium, magnesium, selenium, silicon, and zinc were in all conditions, boron and manganese were present in the fluid and aerosol samples from PHIX. While aluminum, chromium, copper, and potassium were in PHIX conditions, titanium was in Kilo 1K.

The elements in unvaped and vaped fluids from prefilled pods were similar; however, in a few cases, the number of elements increased after vaping and in the aerosol. Calcium, silicon, magnesium, and zinc were in all fluids and aerosols. Except for nickel in the KWIT Stick Mango Tango, silicon had the highest element concentration in all the sample conditions investigated. Its transfer efficiency to the aerosol ranged from 60 – 126 % and was higher in berry flavors than mango.

Transfer of elements in the fluids to the aerosols varied between flavors and devices. In KWIT Stick Mango Tango, Nickel was the highest element concentration in unvaped and vaped samples. Its transfer efficiency from unvaped mango-flavored prefilled pods to the aerosol ranged from 2 – 3 %. For refillable pods, nickel was detected in one aerosol sample made with SMOK Infinix, where it transferred at a 280% efficiency to the aerosol.

Comparison between Fluids and Aerosols from Multiple EC Generations.

Chemical elements in fluids and aerosols from all generations of ECs analyzed in our laboratory were compared. Eleven elements (aluminum, arsenic, boron, calcium, copper, magnesium, nickel, potassium, selenium, silicon, and tin) were present in prefilled fluids and aerosols (Figure 4A, B, and C). Chromium, iron, titanium, and zinc were present in all unvaped and vaped fluids but did not transfer into the cartomizer aerosols. Arsenic and mercury were present in only cartomizer fluids and transferred to the aerosol. Cadmium, cobalt, lead, manganese, silver, and sodium were in fourth-generation fluids and aerosols.

Fewer elements were shared between second, third, and fourth-generation products. Aluminum, calcium, selenium, and tin were present in all refill fluids used with clearomizers, mods and pods, and corresponding aerosols. (Figure 4D, E, and F). Sodium and magnesium were present in all conditions except for clearomizer aerosols. While silicon, copper, lead, and zinc were in all conditions for clearomizers and mods, silicon and copper were absent in unvaped pod refill fluids, and zinc was absent in unvaped mod refill fluids. Nickel was in all aerosols but absent in vaped pod fluids and unvaped clearomizer and mod fluids. All vaped fluids contained chromium and iron, which transferred only to mod and pod aerosols. Boron, potassium, manganese, titanium, and cobalt were in at least one condition for pod products, and arsenic was only in aerosols made with mod devices.

Comparison between Elements in Free-base and Salt-based Nicotine Refill Fluids

Like previous EC devices, fourth-generation ECs have evolved to contain reservoirs that can be refilled with salt-based nicotine refill fluids. Eight elements (aluminum, calcium, magnesium, nickel, potassium, selenium, tin, and zinc) have been quantified in free-base and salt-base nicotine refill fluids. While boron, manganese, and silver were present only in salt-based nicotine refill fluids, chromium, copper, lead, and silicon were present only in free-base nicotine fluids. Iron was pre-sent in only free-base nicotine fluids from black market brands. (Figure 4)

DISCUSSION

This study examines the fluids and aerosols from fourth-generation ECs. Twenty-one elements were investigated, and 17 were quantified in at least one fluid or aerosol sample. While some elements appeared in unvaped fluids, vaped fluids, and aerosols, others were present in one or two of all three conditions. The concentrations of elements in fluids varied between the type of pod (closed vs. open system) and the flavor of the fluids. The devices used in generating the aerosols also influenced the concentration and number of elements identified and quantified in the aerosols. Those found in high concentrations (>100 ug/L) in the unvaped fluids included nickel, silicon, tin, zinc, copper, magnesium, selenium, calcium, potassium, and lead. Additionally, the concentrations of iron, aluminum, chromium, and boron were higher in fluids after vaping. The transfer efficiency of elements to the aerosol was variable across brands and within the same brand. Although pod ECs operate at relatively low power, the number of elements in their aerosols was generally higher than in earlier generations. The concentrations of some elements, e.g., silicon and nickel had a major impact on the total concentration of elements. In JUUL products, a major fraction of the total concentration of elements was silicon which varied between flavor groups. When silicon concentrations are removed from the total concentrations, the range drops to 100 – 3,000 ug/L from 600 – 30,000 ug/L, with most products below 500 ug/L. This exclusion changes the distribution of total elements, going from Virginia Tobacco and Crème Brulee being the highest to Classic Menthol and Classic tobacco.

Silicon in JUUL fluids likely came from the wick, which is made of silicon and oxygen (Williams, Bozhilov & Talbot, 2019; Williams & Talbot, 2019). Variations in silicon concentrations in JUUL fluids may have been due to different batches of raw materials used to make the different JUUL pods. Variations may also be attributed to differences in the ages of the products before purchase and analysis. Previous research has shown the impact of fluid aging on elemental concentrations (ref). While our JUUL products were purchased and analyzed simultaneously, some products may have been sitting in the stores longer, causing the silicon wicks to break down and leach into the fluids. While the pods were handled carefully to prevent mechanical damage, accidental damage may have occurred to some pods before purchase, which could damage the wick and increase silicon in the liquid. Chemical constituents in the unvaped fluids may also affect the concentration of elements in fluids and aerosols. Nicotine increases the concentration of certain metal elements over a short period (Zhao et al., 2022).

E-liquids and aerosols from multiple generations of ECs contain metals (Williams et al., 2013, 2015, 2017; Goniewicz et al., 2014; Mikheev et al., 2016; Hess et al., 2017; Palazzolo et al., 2017; Talio et al., 2017; Dunbar et al., 2018; Kamilari et al., 2018; Kim et al., 2018; Olmedo et al., 2018; Gray et al., 2019, 2022; Na et al., 2019; Williams, Bozhilov & Talbot, 2019; Zhao et al., 2019, 2020; Halstead et al., 2020; Pearce et al., 2020; Zervas et al., 2020; Gonzalez-Jimenez et al., 2021) These studies show variability in metal composition of EC products within and between generation, brands, and flavors. Studies on fourth generation products (Dunbar et al., 2018; Gray et al., 2019, 2022; Zhao et al., 2019; Halstead et al., 2020; Pappas et al., 2021; Kapiamba et al., in press) are most relevant to the current study. In two studies using different analytical methods to evaluate metals in JUUL fluids (Dunbar et al., 2018; Gray et al., 2019) the levels of metals ranged from below the lowest calibration standards (LSTD) to lowest reportable limits (LRL) or limits of quantification to the highest levels reported in the studies. In agreement with these studies (Dunbar et al., 2018; Gray et al., 2019) our lead (Pb) levels were also below the detection and quantification limits. Cadmium, copper, and tin were below the LRL, LSTD, and LODD in both studies by Gray et al. However, other elements (cadmium; 0.001 – 0.003 mg/L), one (copper = 0.002 mg/L), and eight (tin; range = 0.029 – 0.071) were quantified in six JUUL flavors investigated in our study. The variations in these studies could result from different EC batches.

The current study included JUUL Cucumber flavor in which lead was quantified in the aerosol (4 ug/L). The presence of lead in only the aerosol samples may be due to contamination during the manufacturing process of the atomizers or thermal decomposition of the heating elements during aerosol production, leading to release into the aerosol. Among prefilled pods from other brands, only Mango Tango (KWIT Stick) fluid contained lead (610 ug/L), which increased in the vaped fluid (910 ug/L) and transferred to the aerosol (7 ug/L). PHIX, Kilo 1K, and JONES pods contained lead in the vaped fluids (range = 0.2 – 160 ug/L) which transferred into the aerosols (0.1 – 14 ug/L) except for Dewberry Fruit Ice vaped with the Kilo 1 K device. The differences can be attributed to the atomizer components and the effect of the heating coil during aerosolization.

Some metals are toxic, e.g., nickel, varied among brands and was highest in concentration (69000 ug/L) in KWIT. The concentrations in pods sometimes varied within a brand. Nickel was found five of eight JUUL (range = 0.02 to 8 ug/L) and four of six unvaped fluids from other brands (range = 2 – 61000 ug/L). The KWIT Mango Tango flavor had unusually high nickel concentrations in the unvaped fluid, which did not transfer efficiently to the aerosol. Nickel has been previously reported in fluids from cartomizers, clearomizers, and refill fluids (Williams et al., 2017; Olmedo et al., 2018; Gray et al., 2019). However, the concentration in the current study is the highest. Excluding nickel from the total element, concentrations revealed a wider distribution of total elements in fluids and efficient transfer from the unvaped fluids to the aerosol in three of eight products from the multiple brands evaluated. The air tube of the KWIT atomizer is predominantly nickel which may have degraded over time and contributed to the high levels in the fluids

Selenium, a constituent of EC solvents (propylene glycol and glycol), was absent in some JUUL Fruit Medley and Mango products, with the lowest total metal concentrations. In contrast, Mango and Crème Brulee had the highest concentrations of total elements in the aerosols. In addition to silicon, the highest element in all JUUL products, Classic Tobacco and Classic Menthol contained potassium, tin, magnesium, and calcium, which transferred efficiently to the aerosol. The variability observed with JUUL flavors may be due to differences in the metal composition of atomizer components and the chemical ingredients used in the fluids, which usually vary based on flavor.

Transfer efficiency was dependent on the EC device and the fluid's flavor. Some devices enhanced increased concentrations as observed with SMOK Infinix used with Mango Bomb fluid and JUUL used with the Jones Clear Mango pod. Increased concentrations in vaped fluids were observed in three products, with one product (PHIX) being up to twice the concentrations in the unvaped fluids. Increased concentrations of elements in fluids after vaping have been associated with heating the coil/filament and residual fluids, which contributes to leaching elements from the atomizer components.

Conclusions

This study investigated the concentrations of metals and chemical elements in prefilled fourth-generation EC pod and salt-based nicotine fluids used in refillable devices. Eight flavor variants from JUUL were evaluated along with fruit, tobacco and menthol flavors from other brands. Sample evaluation was performed under three conditions that showed variations in total element concentrations in the JUUL brand’s unvaped, vaped, and aerosols. The variation was less between brands except for KWIT Stick, which had--times as much nickel as the lowest concentration in other brands. While silicon, selenium, and tin transferred efficiently into the aerosols, other elements such as – and a—increased in the fluids after vaping. Large differences in the composition and concentration of elements in refill fluids that were not in contact with the atomizer compared to prefilled were observed. The difference in metal components of atomizing units, such as the coil and air tube, may be major contributors to the elements present in prefilled fluids. Data from our study adds to existing evidence that metals and elements can leach into unvaped fluids before heating, increase in concentration upon heating and transfer into the aerosol.

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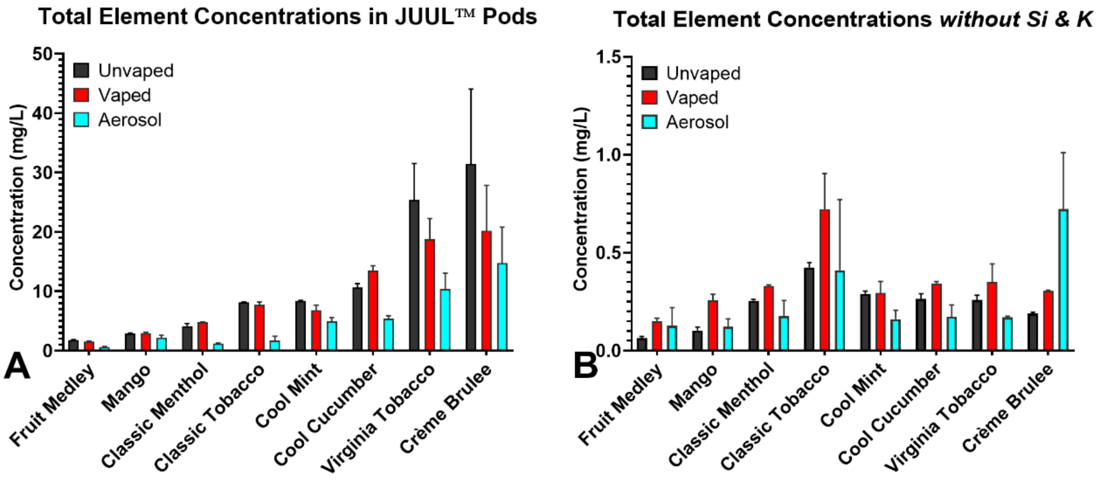

Figure 11.1. Total Concentration of Elements in JUUL E-liquids and Aerosols. (A) Concentrations of all elements/metals quantified. (B) Concentrations of elements/metals without silicon and potassium. The x-axis indicates the eight original JUUL flavors, and the y-axis shows concentrations of elements. Each bar is the mean +/- standard deviation of 3 independent pods

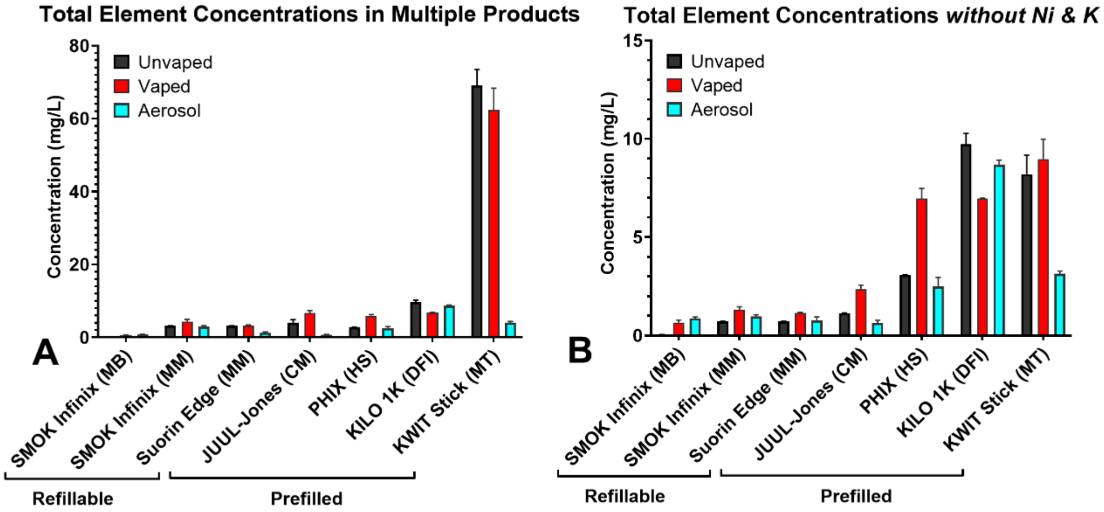

Figure 11.2. Total Concentration of Elements in Refill Fluids, E-liquids and Aerosols. (A) Concentrations of all elements/metals quantified. (B) Concentrations of elements/metals without nickel. The x-axis indicates six refillable and prefilled pod brands, and the y-axis represents concentrations of elements. Each bar is the mean +/- standard deviation of 3 independent runs/pods.

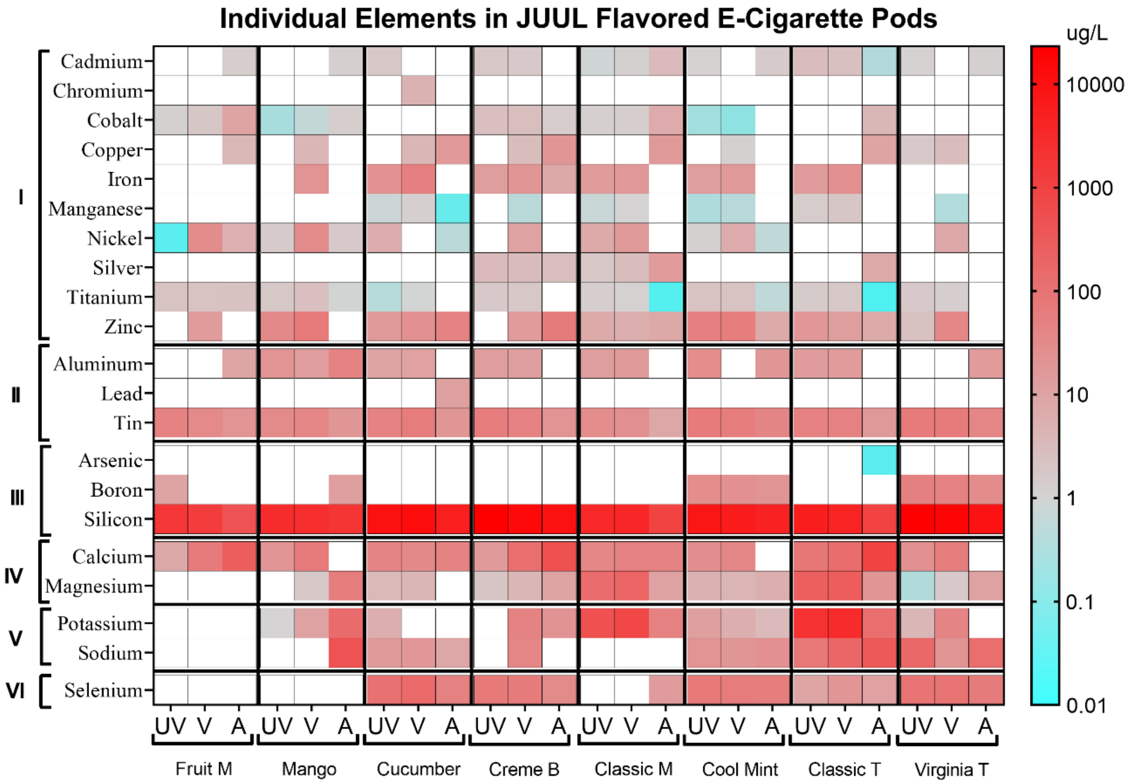

Figure 11.3. Chemical elements in JUUL pods. The x-axis on the heatmap shows JUUL flavors ordered according to flavor categories. U = unvaped, V = vaped and A = aerosol. Y-axis indicates metal groups based in the periodic table. I = transition, II = post-transition (basic), III = metalloids, IV = alkaline earth; V = alkali, and VI = non-metal. Each bar is the mean +/- standard deviation of 3 independent runs/pods.

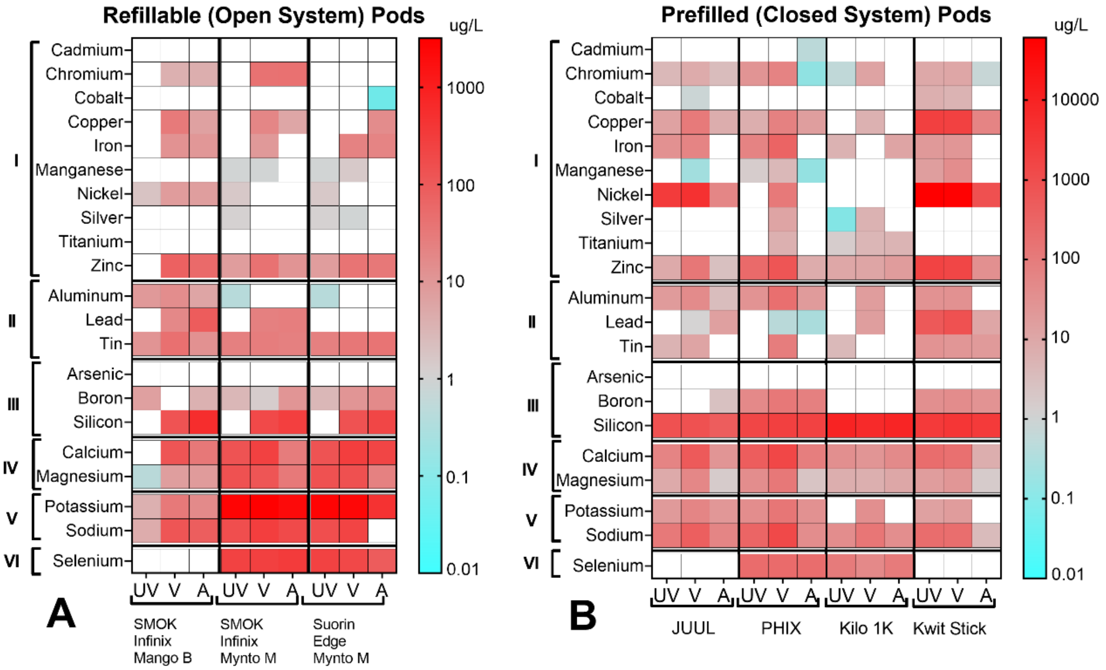

Figure 11.4. Chemical elements in refill fluids, e-liquids and aerosols from multiple brands. (A) open system pods (SMOK Infinix and Suorin Edge). (B) Closed system pods (JONES, PHIX, Kilo 1K, and KWIT Stick. The x-axis on each heatmap shows the element ordered according to concentration in unvaped liquid; U = unvaped, V = vaped and A = aerosol. The y-axis indicates metal groups based in the periodic table. I = transition, II = post-transition (basic), III = metalloids, IV = alkaline earth; V = alkali, and VI = non-metal. Each bar is the mean +/- standard deviation of 3 independent runs/pods

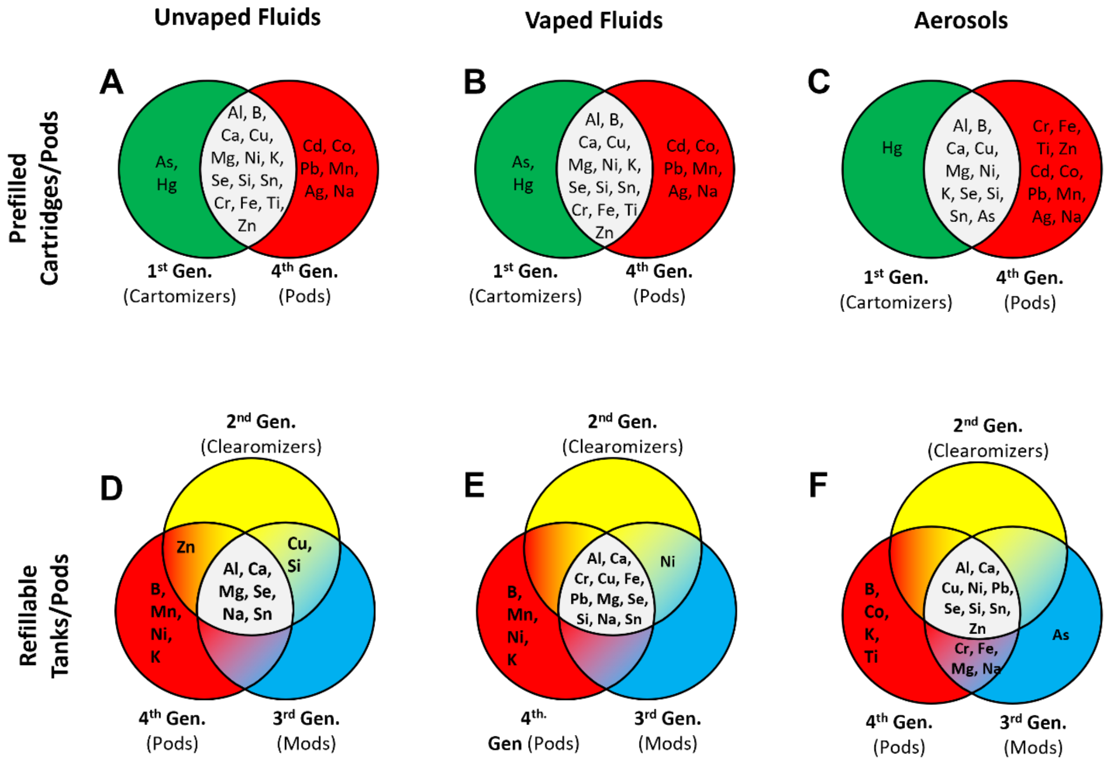

Figure 11.5. Chemical Elements in EC Fluids and Aerosols from Multiple Generations of ECs. (a – b) unvaped fluids, (c – d) vaped fluids, and (e – f) aerosols. Venn diagram showing elements identified in first and fourth generation prefilled devices (a, c, e) and second, third and fourth generation refillable devices (b, d, f). Al = aluminum, As = Arsenic, B = Boron, Cd = Cadmium, Co = cobalt, Cr = chromium, Cu = copper, Fe = iron, Hg = Mercury, Ni = nickel, Sn = tin, Ti = titanium, Zn = zinc, Ag = silver, Pb = lead, Mn = manganese, Mg = magnesium, Ca = calcium, Si = silicon, Se = selenium, K = potassium. 1st – 3rd generation data from Williams et al. 2020

Table 11.1. Characteristics of Fourth Generation EC Devices[1]

Brand/Manufacturer	**JUUL[2]**	**PHIX[2]**	**Kilo 1K[2]**	**Suorin Edge**	**SMOK Infinix**	**KWIT Stick[2]**
Pod System	Closed	Closed	Closed	Open	Open	Closed/Open
Liquid Capacity (*mL*)	0.7	1.5	1.5	1.5	2.0	1.0
Number of puffs per cartridge	200	400-450	n/a	n/a	n/a	n/a
Resistance (*ohm*)	n/a	1.4 – 1.5	1.8	1.4	1.4	>10
Puff Cut Off Time (seconds)	n/a	n/a	n/a	n/a	n/a	8
Activation method	Draw	Draw	Draw	Draw	Button	Draw
Battery type	Li-ion	n/a	n/a	n/a	n/a	n/a
Battery Capacity (*mAh*)	200	280	350	230	250	230
Watt-Hours (*Wh*)	0.7	n/a	n/a	n/a	n/a	n/a
Voltage (*Volts*)	3.7	3.7	n/a	3.3 – 4.2	3.3 – 4.2	3.5 – 4.2
Watts on a full charge	8.5	n/a	n/a	10	10 – 16	n/a
e-liquid Flavor[4]	Multiple[5]	HS[4]	DFI[4]	MM[4]	MB, MM[4]	MT[4]

[1] Information obtained from the manufacturer's website or other online sources.

[2] Products with prefilled pods.

[3] Liquid Flavors: HS = Hard Strawberry; DFI = Dewberry Fruit Ice; MM = Mynto Ice Mango; MB = Mango Bomb; MT = Mango Tango

[4] Multiple = Fruit Medley, Cucumber, Mango, Crème Brulee, Virginia Tobacco, Classic Tobacco, Classic Menthol, and Cool Mint.

Chapter 12: Conclusion

Chapter 2: Counterfeit Electronic Cigarette Products with Mislabeled Nicotine Concentrations

This is the first scientific report on the existence of counterfeit EC products with inaccurate nicotine labeling and invalid quality control certifications. Two novel issues in the e-cigarette industry, were identified; the production of counterfeit refill fluids under a brandjacked label and the inclusion of nicotine in 81.3% of the counterfeit products labeled 0 mg/mL. Using counterfeit products could unwittingly expose users to nicotine leading to unexpected and unwanted nicotine toxicity and poisoning in the cases of accidental ingestion by children. The inconsistencies in product color and labelling is useful for researchers, general public education, and for the establishment of regulatory measures for better control and monitoring of nicotine containing products and sales outlets.

Chapter 3: High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations

We demonstrated that the very popular JUUL e-cigarettes had high concentrations of nicotine and attractive flavor chemicals that are appealing to youth and adolescents. Irrespective of flavor type, nicotine concentrations were higher in eight JUUL e-liquids than in 174 previously evaluated refill and cartomizer fluids. Ethyl maltol and nicotine contributed significantly to the toxicity of JUUL fluids and aerosol in acute exposures. The data from this study emphasized the need for regulatory agencies to focus on high nicotine products like JUUL as chronic use could lead to adverse health effects such as nicotine addiction.

Chapter 4: High Concentrations of Flavor Chemicals are Present in Electronic Cigarette Refill Fluids

This study is the first to identify and quantify the flavor chemicals in a broad spectrum of EC refill fluids that are sold worldwide. With over 16,000 flavor chemicals that manufacturers can use in e-liquid formulations, our library of 277 refill fluids shows that 155 flavor chemicals are present in e-liquids at concentrations ranging from 0 to 343 mg/mL with the number of flavor chemicals in each product ranging between 0 – 50. The total flavor chemical concentrations exceeded the nicotine concentration in over half of the products, demonstrating that flavor chemicals are a major component of EC refill fluids. Flavor chemical concentrations in refill fluids often exceeded the maximum levels in other consumer products. The health effects of exposure to flavor chemicals in ECs is a concern, especially for those occurring at dominant concentrations of >1 mg/mL. Sufficient data is now available to tighten regulations on the use of flavor chemicals in inhalation products.

Chapter 5: Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids

Based on an online survey and local store visits, this study identified popular and top selling refill fluids to include “Berries/Fruit/Citrus,” “Sweet”, “Candy”, “Bakery/Dessert”, and “Breakfast Cereal”. Using acute exposures with high through put assays, we identified cytotoxic products and flavor chemicals contributing to cytotoxicity. Using linear regression models, we observed that cytotoxic chemicals correlated strongly with e-liquid cytotoxicity. The four most cytotoxic refill fluids contained various combinations of six flavor chemicals (ethyl maltol, furaneol, maltol, ethyl vanillin, benzyl alcohol, and vanillin) that were cytotoxic when tested as authentic standards. Two of the most cytotoxic chemicals were present in 65- 95% of the products. These data yet again emphasized the unintended use of high levels of flavor chemicals in popular e-liquids and the need to establish limits for the use of flavor chemicals in e-cigarette products. These data show that the cytotoxicity of some popular refill fluids can be attributed to their high concentrations of flavor chemicals.

Chapter 6: Electronic Cigarette Refill Fluids Sold Worldwide: Flavor Chemical Composition, Toxicity, and Hazard Analysis

This study is the first to identify and quantify the flavor chemicals in refill fluids manufactured under one brand (Ritchy) and purchased worldwide in four countries. With one exception, flavor chemicals and their concentrations were similar in duplicate bottles of refill fluids from each country. Previously certified counterfeit products contained similar levels of flavor chemicals when compared to their authentic counterpart. The total concentrations and number of flavor chemicals (range 0 – 50) varied significantly between products with two tobacco flavored refill fluids (American Blend and Q American Blend from US-KS) having no flavor chemicals (target or non-target). Thirty-seven chemicals not identified in our prior work were present in LIQUA products with triacetin being the most frequently used, often at high concentrations. While toxicities correlated with total flavor chemical concentrations and some flavor chemicals (e.g., furaneol and ethyl maltol), antagonism was observed to reduce the potency of individually cytotoxic flavor chemicals. Flavor chemical concentrations in refill fluids often exceeded concentrations permitted in other consumer products. Pulegone and estragole, which are carcinogens and likely co-constituents of dominant flavor chemicals, had MOEs < 10,000, consistent with high cancer risk. The regulation of flavor chemicals could improve the safety of EC refill fluids and reduce their potential to cause toxicological effects.

Chapter 7: Flavor chemicals, synthetic coolants, and pulegone in popular mint and menthol-flavored e-cigarettes

After the FDA enforcement policy on prefilled cartridge-based products like JUUL, consumers quickly switched the disposable products, such as Puff Bar, which were not included in the policy. Ironically, this migration of users to Puff Bar may have introduced them to a potentially more harmful product. Both menthol and non-menthol synthetic cooling agents (WS-3 and WS-23) were quantified in disposable and cartridge-based mint and menthol flavored JUUL and Puff products. Synthetic cooling agents have a focus for research and this study showed that WS-23 was cytotoxic in the MTT assay at concentrations 90 times lower than in e-liquids found in Puff devices. MOEs revealed that pulegone concentrations in mint products from JUUL™ and Puff were high enough to present a cancer risk and the use of pure menthol instead of mint oil in e-cigarette fluids may reduce this health risk.

Chapter 8: Disposable Puff Bar Electronic Cigarettes: Chemical Composition and Toxicity of E-liquids and WS-23.

This study investigated the chemicals in fourth-generation disposable Puff brand e-liquids and their toxicological effects. Nicotine concentrations were generally lower than previously reported in fourth-generation cartridge-based fluids. WS-3 and WS-23, concentrations were higher than recommended for consumer products. Inhibition of cell growth and mitochondrial reductase activity was attributable to WS-23 concentrations. Concentration-response curves for toxic effects were significantly correlated with nicotine, ethyl maltol, and WS-23 concentrations. For most Puff ECs, the MOEs for the synthetic coolants were below the acceptable threshold of 100 for food additives, indicating a potential health risk. EC formulations are quickly evolving, and it is important that future work focus on constituents that may accompany their evolution. Product manufacturers will continue to device new means to addict young users by altering fluid formulation to become more appealing. The data support the regulation of flavor chemicals and synthetic coolants in ECs to limit potentially harmful health effects.

Chapter 9: Evolution of e-cigarette fluid (e-liquid) constituents – clever uses of flavor chemicals?

The last few years have witnessed sweeping regulations to restrict the use of flavor chemicals and access to e-cigarettes by minors. As the FDA has moved to permit only menthol and tobacco products through the premarket authorization pathway, we sought to investigate how tobacco flavor constituents have changed in the last decade. We found that non-tobacco flavor chemicals (ethyl maltol, corylone, vanillin, and ethyl vanillin) have recently been added into products labeled “tobacco.” This change coincides with the national public health concern regarding the rapid adoption of JUUL products by students and young adults attracted to these pod-style ECs with appealing flavors. Tobacco-labelled pods are not generally attractive to young users, which may be the basis for the FDA granting authorizations for tobacco-flavored EC manufactured by Vuse and Logic. Since flavor chemicals are not used in U.S. commercial tobacco cigarettes, their use in ECs is not to replicate tobacco cigarette flavor, but rather to make “tobacco“ flavored ECs appealing to young EC users. The data clearly show one way that EC companies have manipulated e-liquid constituents to broaden product appeal and emphasize the need for continual surveillance to prevent doctoring by manufacturers after premarket authorizations are granted.

Chapter 10: Design features and elemental/metal analysis of the atomizers in pod-style electronic cigarettes

Our study compares the design features and elemental composition of atomizers in pod ECs from multiple popular manufacturers. The evolution of ECs has come with change to the outer and inner components with noticeable variations between generations. Newer generations now have ceramic wicks and magnetic component to help connect and hold the fluid reservoirs in place. A total of 23 elements were identified, mapped and characterized in the pod atomizers, of which 11 were considered dominant elements based on their relative abundance in EDS spectra. The elements in EC atomizers may have adverse health effects when inhaled in aerosols or accumulate in the environment and contribute to the pollution of land, water and air. Understanding the health impact of the elements/metals in EC pods and their fate when discarded will be important when establishing regulations on their use and disposal.

Chapter 11: Elemental Analysis of E-Liquids and Aerosols from Multiple Brands of Fourth Generation E-Cigarettes

This study provides further evidence on the metal/elemental composition of in e-liquid and aerosol which users may inhale during a vaping session. The concentrations of metals and chemical elements in prefilled fourth-generation devices including all previously marketed JUUL flavors, and mango flavored salt-based nicotine refill fluids were investigated. Sample evaluation was performed under three conditions that showed variations in total element concentrations in the JUUL brand’s unvaped, vaped, and aerosols. The variation was less between prefilled brands but high between vaped and unvaped fluid when compared to refillable pods. KWIT Stick had the highest concentration of nickel in all examined the e-liquids (~ 60 mg/mL) and concentrations were over 600 times higher than the lowest concentration in other brands. While silicon, selenium, and tin transferred efficiently into the aerosols, other elements increased in the fluids after vaping. The difference in metal components of atomizing units, such as the coil and air tube, may be major contributors to the elements present in prefilled fluids. Data from our study adds to existing evidence that metals and elements can leach into unvaped fluids before heating, increase in concentration upon heating, and transfer into the aerosol.

Appendix A: Supporting Information for Chapter 2

Table S2.1. Authentic and Suspected Authentic E-cigarette Refill Fluids[a]

#	[b]Co	Flavor	[c][Q] (mg/mL)	[d]Coloration	[e]QR	[f]EAN	[g]Mfr. Name	Product Name on Database
1	NG-L	Two Apples	0.4 ± 0.1	Yellow	C	C	RGHK	No record found
2	NG-L	Two Apples	0.6 ± 0.1	Light yellow	C	C	RGHK	No record found
3	NG-L	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
4	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
5	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
6	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
7	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
8	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
9	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
10	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
11	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
12	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
13	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
14	US-K	Two Apples	0.5 ± 0.0	Yellow	C	C	RGHK	No record found
15	US-K	Two Mints	ND	Clear	C	C	RGHK	No record found
16	US-K	Peach	ND	Clear	C	C	RGHK	No record found
17	US-K	Ry4 Tob.	ND	Pale yellow	C	C	RGHK	No record found
18	US-K	Red Oriental Tob.	ND	Light yellow	C	C	RGHK	No record found
19	US-K	Licorice	ND	Clear w/ BT	C	C	RGHK	No record found
20	US-K	Menthol	ND	Clear w/ YT	C	C	RGHK	Menthol (0mg)
21	US-K	Mints	ND	Clear	C	C	RGHK	Mints (0mg)
22	US-K	Apple	ND	Clear	C	C	RGHK	Apple (0mg)
23	US-K	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix (0mg)
24	US-K	Watermelon	ND	Clear	C	C	RGHK	Watermelon (0mg)
25	US-K	Watermelon	ND	Clear	C	C	RGHK	Watermelon (0mg)
25	US-K	Banana	ND	Clear	C	C	RGHK	Banana(0mg)
27	US-K	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
28	US-K	Blueberry	ND	Clear	C	C	RGHK	Blueberry(0mg)
29	US-K	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
30	US-K	Cola	ND	Clear	C	C	RGHK	Cola(0mg)
31	US-K	Energy Drink	ND	Clear w/ YT	C	C	RGHK	Energy Drink(0mg)
32	US-K	Bright Tobacco	ND	Clear	C	C	RGHK	Bright Tob(0mg)
33	US-K	Traditional Tob.	ND	Champagne	C	C	RGHK	Traditional Tob (0mg)
34	US-K	French Pipe Tob.	ND	Clear w/ YT	C	C	RGHK	French Pipe Tob. (0mg)
35	US-K	American Blend Tob.	ND	Clear w/ YT	C	C	RGHK	Amer Blend(0mg)
36	US-K	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
37	US-K	Chocolate	ND	Yellow	C	C	RGHK	Chocolate(0mg)
38	US-K	HP Overdrive	ND	Dark Brown	C	C	RGHK	No record found

39	US-K	HP Fruity Velocity	ND	Clear	C	C	RGHK	No record found
40	US-K	HP summer Drift	ND	Clear w/ YT	C	C	RGHK	No record found
41	US-K	HP Sweet Accelerator	ND	Clear w/ YT	C	C	RGHK	No record found
42	US-K	Q Apple	ND	Clear	C	C	RGHK	No record found
43	US-K	Q Peach	ND	Clear	C	C	RGHK	No record found
44	US-K	Q Menthol	ND	Clear	C	C	RGHK	No record found
45	US-K	Q Berry Mix	ND	Clear	C	C	RGHK	No record found
46	US-K	Q Pina Colada	ND	Clear	C	C	RGHK	No record found
47	US-K	Q Cherribakki	ND	Clear w/ YT	C	C	RGHK	No record found
48	US-K	Q The Moment	ND	Clear	C	C	RGHK	No record found
49	US-K	Q Havana Libre	ND	Clear	C	C	RGHK	No record found
50	US-K	Q Blackberry Jack	ND	Clear	C	C	RGHK	No record found
51	US-K	Q Double Bubble	ND	Clear	C	C	RGHK	No record found
52	US-K	Q Fragola Fresca	ND	Clear	C	C	RGHK	No record found
53	US-K	Q American Blend	ND	Clear w/ YT	C	C	RGHK	No record found
54	US-K	Q Honeydew Drop	ND	Clear w/ YT	C	C	RGHK	No record found
55	US-K	Q Golden Roanoke	ND	Clear w/ YT	C	C	RGHK	No record found
56	US-K	Q Piedmont Sunrise	ND	Clear	C	C	RGHK	No record found
57	US-K	Q Turkish Tob.	ND	Clear	C	C	RGHK	No record found
58	US-K	Q Traditional Tob.	ND	Champagne	C	C	RGHK	No record found
59	US-C	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix(0mg)
60	US-C	Cherry	ND	Clear	C	C	RGHK	Cherry(0mg)
61	US-C	Menthol	ND	Clear w/ YT	C	C	RGHK	Menthol(0mg)
62	US-C	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
63	US-C	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
64	US-C	Grape	ND	Clear	C	C	RGHK	Grape(0mg)
65	US-C	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
66	US-C	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
67	US-C	Tiramisu	ND	Clear w/ YT	C	C	RGHK	Tiramisu(0mg)
68	US-C	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob(0mg)
69	US-C	Peach	ND	Clear w/ YT	C	C	RGHK	No record found
70	US-C	Cuban Cigar Tob.	ND	Clear	C	C	RGHK	No record found
71	US-C	Q Apple	ND	Clear	C	C	RGHK	No record found
72	US-C	Q Peach	ND	Clear	C	C	RGHK	No record found
73	US-C	Q Menthol	ND	Clear	C	C	RGHK	No record found
74	GB-N	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob(0mg)
75	GB-N	Menthol	ND	Clear w/ BT	C	C	RGHK	Menthol(0mg)
76	GB-N	Apple	ND	Clear	C	C	RGHK	Apple(0mg)

77	GB-N	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
78	GB-N	Peach	ND	Clear	C	C	RGHK	No record found
79	GB-N	Q Apple	ND	Clear	C	C	RGHK	No record found
80	GB-N	Q Peach	ND	Clear	C	C	RGHK	No record found
81	GB-N	Q Berry Mix	ND	Clear	C	C	RGHK	No record found
82	CN-X	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob (0mg))
83	CN-X	Menthol	ND	Clear	C	C	RGHK	Menthol(0mg)
84	CN-X	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
85	CN-X	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix(0mg)
86	CN-X	Cherry	ND	Clear	C	C	RGHK	Cherry(0mg)
87	CN-X	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
88	CN-X	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
89	CN-X	Grape	ND	Clear	C	C	RGHK	Grape(0mg)
90	CN-X	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
91	CN-X	Tiramisu	ND	Clear w/ YT	C	C	RGHK	Tiramisu(0mg)
92	US-K	Turkish Tob.	ND	Clear	NC	C	RGHK	Turkish Tob. (0mg)

Note:

[a]#1 – 91 were verified to be authentic using all criteria. #92 could not be verified using the QR code

[b] Co =Country and location of product purchase (NG-L = Nigeria, Lagos; US-K = USA, Kansas; US-C = US, California; CN-X = China, Xiamen)

[c][Q] = Quantified nicotine concentration (± standard deviation) using HPLC (ND = Not Detected)

[d] Coloration = Color of the refill fluids (YT = yellow tint; GT = green tint; BT = brown tint)

[e] QR = Availability and verification of manufacturer's Quick Response Code (C = Correct code = Verified; NC/NB = No Code/No Box = Unverified)

[f] EAN = Availability and verification of company and product information using the European Article Number barcode (IC = Incorrect; NB = None)

[g] Mfr. Name = Name of manufacturer to which product EAN barcode is linked; RGHK = Ritchy Group Ltd HK; SLHK = Spoilt Ltd HK; RC:13 = Illegal/None; N/A = Not Available

Appendix B: Supporting Information for Chapter 3

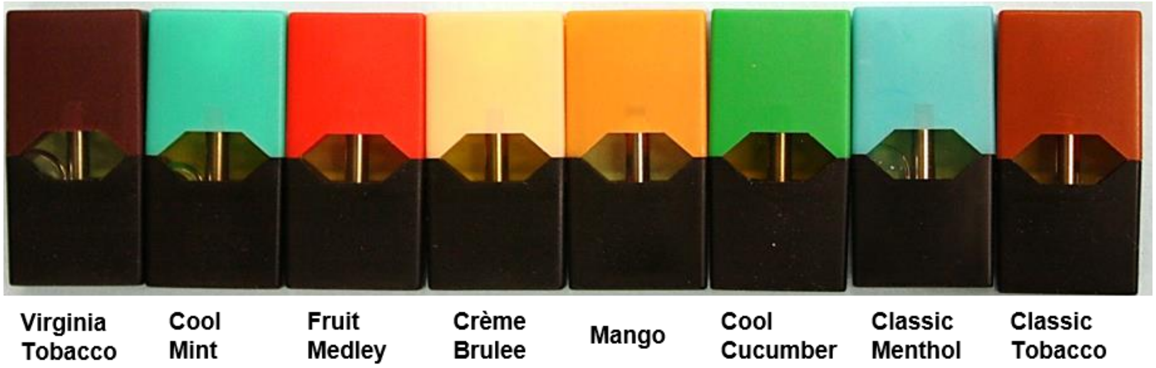

Figure S3.1. Images of eight JUUL pod flavors evaluated in this study.

Relationships between Chemicals and Cytotoxicity of Vaped Pod Fluids

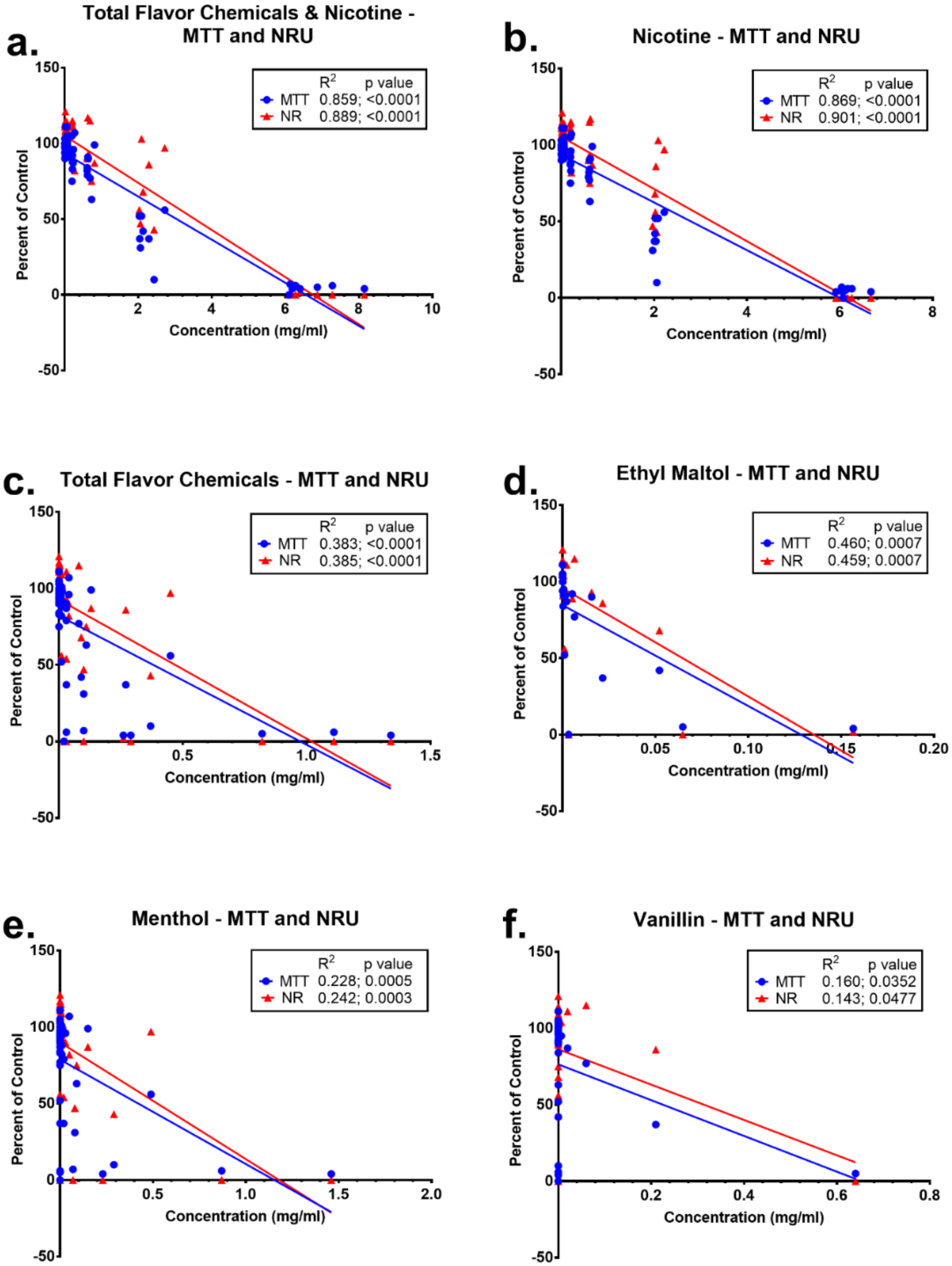

Figure S3.2. Relationship between cytotoxicity of vaped pod fluids and concentrations of nicotine and the flavor chemicals. Linear regression analysis for cytotoxicity (percentage of the untreated control) in the MTT and NRU assays versus the concentrations of: (a) total flavor chemical and nicotine, (b) nicotine only, (c) total flavor chemicals only, (d) ethyl maltol, (e) menthol, (f) vanillin. Blue dots and red triangles represent the concentrations tested in the MTT and NRU assays, respectively. Cytotoxicity was strongly correlated with the total concentration of chemicals (flavor chemicals and nicotine) and with nicotine concentration only and weakly to moderately correlated with the concentrations of total flavor chemicals, ethyl maltol, menthol, and vanillin. All correlations were significant ($p<0.05$).

Table S3.1: Brand/Manufacturer and Product Names of 83 EC Refill Fluid Products

Brand/Manufacturer	Product Name
Thrive	Ice Lemon Cole
Thrive	Ice Lemon Cole
Thrive	Ice Lemon Cole
Thrive	Banana
Thrive	Banana
Thrive	Banana
Johnson Creek	Arctic Menthol
Johnson Creek	Tundra
Lost Art Liquids	Gummy Glu
Lost Art Liquids	Unicorn Puke
Opulence Elixir	Popsicle
Kilo	Dewberry Cream
Ripe Vapes	HSAC
LiQua	Q Menthol
LiQua	Q Traditional Tob.
LiQua	Chocolate
LiQua	Cappucino
Canyon Crest Vape Shoppe	Unicorn Puke
Canyon Crest Vape Shoppe	Melon Mania
Canyon Crest Vape Shoppe	Cinnamon Bomb
Canyon Crest Vape Shoppe	Cinnamon Bomb Fiery
Canyon Crest Vape Shoppe	Dewberry Cream
Canyon Crest Vape Shoppe	Ho!Ho! Watermelon
Canyon Crest Vape Shoppe	WTF
Daze	#Selfie Sunday
Lion	Love Potion
Cuttwood	Bird Brains
Daze	#Crawlie Tuesday
WTF	WTF
Liquid State	Apple Butter
Cuttwood	Mega Melons
Cuttwood	Boss Reserve
Kilo	Dewberry Cream
Kilo	Fruit Whip
Kilo	Kiberry Yoghurt
Pop Drops	S'mores vape juice
Naked 100	Lava Flow
The Milkman	Churios
Cuttwood	Sugar Drizzle
Lost Art Liquids	Unicorn Puke
Q Vapor Labs	North Shore
Majestic	King Kong
Vape Mail	Overnight
Blue Label Elixir	Famous

Beetle Vapour Juice	Blueberry Hills
NJOY Artist Collection	Hedon's Bite
NJOY Artist Collection	Samba Sun
NJOY Artist Collection	Paramour
NJOY Artist Collection	Dragonscape
NJOY Artist Collection	Sacre Coeur
ANML	Looper
ANML	Carnage
Beard Vape Co	No. 51
Beard Vape Co	No. 05
Beard Vape Co	No. 71
Beard Vape Co	No. 64
Beard Vape Co	No. 88
Bombies	Bacco B
Bombies	Product X
Bombies	White Gummy B
Bombies	Black Out City
Bombies	Seven Seas
Bombies	Agent P
Bombies	Tiger Style
Bombies	Nana Cream
Bombies	Kiss The Ring
NicQuid	Banana Nut Bread
NicQuid	Blueberry
NicQuid	Soho
NicQuid	Strawberry Fuzz
NicQuid	Smoothol
NicQuid	Daybreak
NicQuid	Sinthol
NicQuid	Southern Freeze
NicQuid	Strawnanna Smoothie
NicQuid	Peach Lemonade
NicQuid	Maui
NicQuid	Midnight Express
NicQuid	Sublime
The Milkman	The Milkman
The Milkman	Churrios
The Mad Alchemist	Snow White's Demise
The Mad Alchemist	Dragons Breath
The Mad Alchemist	Zen
The Mad Alchemist	Twice in a Blue Moon
The Mad Alchemist	Eye of Newt
The Mad Alchemist	Custard Matter
Seduce Juice	Exodus 7:20
Seduce Juice	Snake Eyes

Seduce Juice	Blackjack
Seduce Juice	Snake Oil
Seduce Juice	Snake Venom
Seduce Juice	Caesar
Seduce Juice	White Walker
Seduce Juice	Jezebel
Seduce Juice	Snake Bite
Space Jam	Starship 1
Space Jam	Galactica
Twelve Vapor	Taurus
Twelve Vapor	Aquarius
Glas	Milk
Glas	Pound Cake
Glas	Pebbles

Appendix C: Supporting Information for Chapter 4

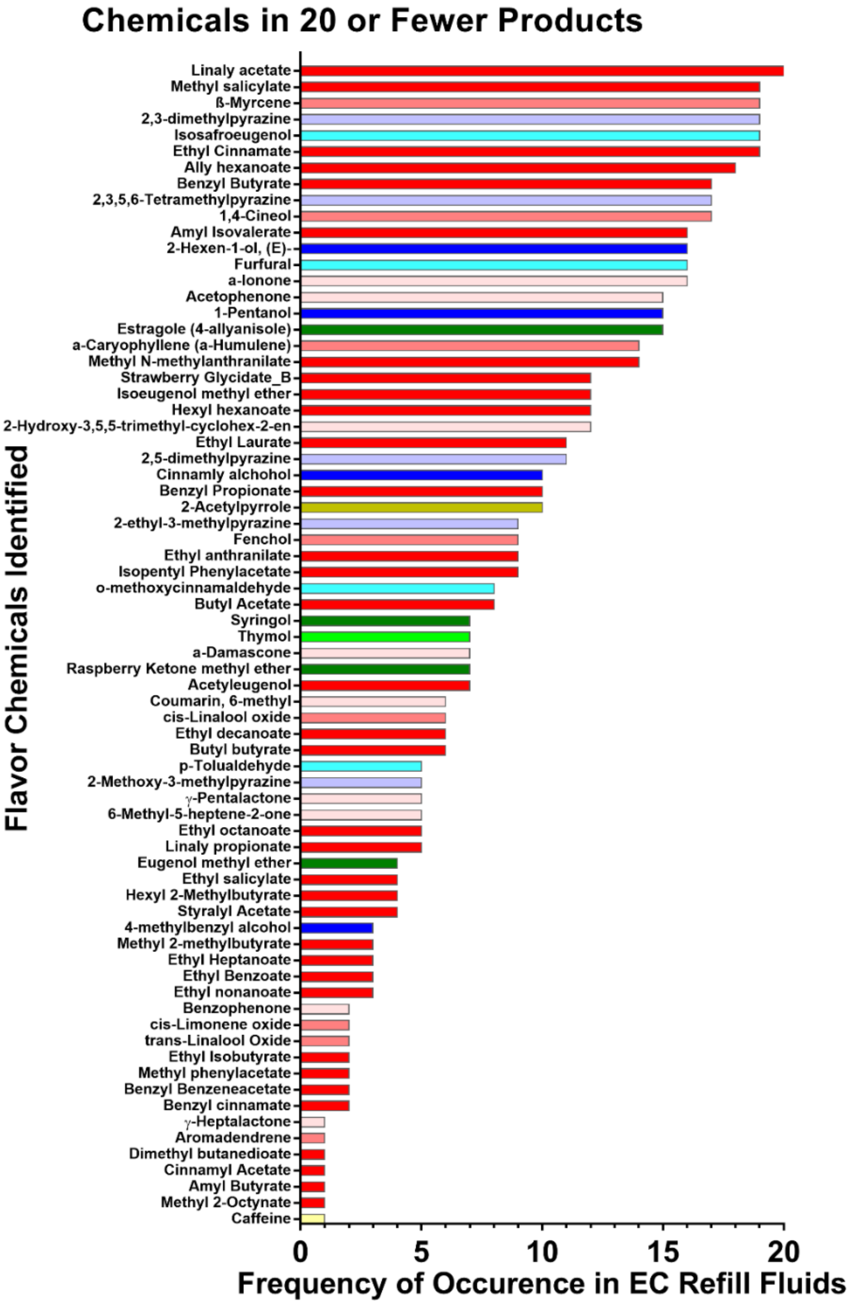

Figure S4.1: Frequency Distribution of 72 Flavor Chemicals. (A) shows 72 out of 155 flavor chemicals that occurred 20 or fewer times in at least one product. The *x*-axis is the number of refill fluids in which the chemicals were found, and the *y*-axis is sorted according to decreasing frequency of their occurrence. Frequency ranged from 1 – 20 with the highest being linalyl acetate to the lowest which occurred only once (δ-heptalactone, aromadendrene, dimethyl butanedioate, cinnamyl acetate, amyl butyrate, methyl 2-octynate and caffeine).

Table S4.1: Properties, Frequency and Concentration Ranges of 155 Flavor Chemicals

Organoleptic Properties	**Chemical Name**	**Frequency**	**≥ 10 mg/ml**		**1 - 9.9 mg/ml**		**≤ 1 mg/ml**	
			[a] **#**	[b] **%**	**#**	**%**	**#**	**%**
Fruity	*Triacetin*	*87*	*19*	*21.8*	*21*	*24.1*	*47*	*54.0*
	Ethyl butanoate	*161*			*14*	*8.7*	*147*	*91.3*
	Isoamyl acetate	*79*			*12*	*15.2*	*67*	*84.8*
	γ-Decalactone	*129*			*6*	*4.7*	*123*	*95.3*
	Hexyl acetate	*52*			*6*	*11.5*	*46*	*88.5*
	Ethyl lactate	*33*			*4*	*12.1*	*29*	*87.9*
	Ethyl propanoate	*47*			*2*	*4.3*	*45*	*95.7*
	Methyl anthranilate	*22*			*2*	*9.1*	*20*	*90.9*
	Allyl hexanoate	*18*			*2*	*11.1*	*16*	*88.9*
	Benzaldehyde	*83*			*1*	*1.2*	*82*	*98.8*
	2-Methylbutyl acetate	*70*			*1*	*1.4*	*69*	*98.6*
	Isobutyl acetate	*41*			*1*	*2.4*	*40*	*97.6*
	Amyl acetate	*38*					*38*	*100*
	Ethyl 2-methylbutanoate	*92*					*92*	*100*
	Isoamyl isovalerate	*85*					*85*	*100*
	δ-Undecalactone	*68*					*68*	*100*
	Ethyl hexanoate	*52*					*52*	*100*
	Ethyl isovalerate	*47*					*47*	*100*
	β-Damascone	*43*					*43*	*100*
	Isoamyl butyrate	*35*					*35*	*100*
	Raspberry ketone	*34*					*34*	*100*
	Benzyl butyrate	*17*					*17*	*100*
	2-Hexen-1-ol	*16*					*16*	*100*
	Amyl isovalerate	*16*					*16*	*100*
	Benzyl propionate	*10*					*10*	*100*
	Raspberry Ketone methyl ether	*7*					*7*	*100*
	Butyl butyrate	*6*					*6*	*100*
	p-Tolualdehyde	*5*					*5*	*100*
	Methyl 2-methylbutyrate	*3*					*3*	*100*
	Ethyl heptanoate	*3*					*3*	*100*
	Ethyl isobutyrate	*2*					*2*	*100*
	cis-Limonene oxide	*2*					*2*	*100*
	Amyl butyrate	*1*					*1*	*100*
Floral	*Benzyl alcohol*	*108*	*8*	*7.4*	*35*	*32.4*	*65*	*60.2*
	Benzaldehyde PG acetal	*118*			*7*	*5.9*	*111*	*94.1*
	Linalool	*132*			*1*	*0.8*	*131*	*99.2*
	Piperonal	*50*			*1*	*2.0*	*49*	*98.0*
	Geraniol acetate	*25*			*1*	*4.0*	*24*	*96.0*
	trans-Geraniol	*33*			*1*	*3.0*	*32*	*97.0*
	Benzyl acetate	*60*					*60*	*100*
	Phenethyl alcohol	*58*					*58*	*100*
	(E)-β-Ionone	*48*					*48*	*100*
	Benzeneacetic acid	*23*					*23*	*100*
	Nerol acetate	*33*					*33*	*100*
	α-Ionone	*16*					*16*	*100*
	Acetophenone	*15*					*15*	*100*

	Methyl N-methylanthranilate	*14*					*14*	*100*
	Ethyl anthranilate	*9*					*9*	*100*
	α-Damascone	*7*					*7*	*100*
	Linaly propionate	*5*					*5*	*100*
	Benzyl benzeneacetate	*4*					*4*	*100*
	trans-Linalool Oxide	*2*					*2*	*100*
Menthol/Minty	*Menthol*	*76*	*12*	*15.8*	*18*	*23.7*	*46*	*60.5*
	p-Menthone	*51*	*1*	*2.0*	*13*	*25.5*	*37*	*72.5*
	Carvone	*25*			*2*	*8.0*	*23*	*92.0*
	Neomenthol	*41*			*1*	*2.4*	*40*	*97.6*
	Menthyl acetate	*46*			*1*	*2.2*	*45*	*97.8*
	Pulegone	*33*			*1*	*3.0*	*32*	*97.0*
	Isopulegol	*21*			*1*	*4.8*	*20*	*95.2*
	Methyl salicylate	*19*					*19*	*100*
	Ethyl salicylate	*4*					*4*	*100*
	Ethyl benzoate	*3*					*3*	*100*
Spicy	*Cinnamaldehyde*	*70*	*4*	*5.7*	*2*	*2.9*	*64*	*91.4*
	Eugenol	*49*	*1*	*2.0*	*4*	*8.2*	*44*	*89.8*
	4-Terpineol	*38*			*1*	*2.6*	*37*	*97.4*
	β-Caryophyllene	*21*					*21*	*100*
	β-Myrcene	*19*					*19*	*100*
	Isoeugenol methyl ether	*12*					*12*	*100*
	o-methoxycinnamaldehyde	*8*					*8*	*100*
	Acetyleugenol	*7*					*7*	*100*
	Eugenol methyl ether	*4*					*4*	*100*
	Cinnamyl acetate	*1*					*1*	*100*
Caramellic	*Ethyl maltol*	*164*	*13*	*7.9*	*65*	*39.6*	*86*	*52.4*
	Corylone	*111*	*3*	*2.7*	*51*	*45.9*	*57*	*51.4*
	Maltol	*124*			*21*	*16.9*	*103*	*83.1*
	Furaneol	*56*			*20*	*35.7*	*36*	*64.3*
	Hydroxyacetone	*60*					*60*	*100*
Vanilla	*Vanillin*	*152*	*9*	*5.9*	*54*	*35.5*	*89*	*58.6*
	Ethyl vanillin	*103*	*4*	*3.9*	*45*	*43.7*	*54*	*52.4*
	Isosafroeugenol	*19*					*19*	*100*
Ethereal	*Ethyl acetate*	*130*	*1*	*0.8*	*5*	*3.8*	*124*	*95.4*
	Butyl acetate	*8*			*1*	*12.5*	*7*	*87.5*
Buttery	*Acetoin*	*63*			*13*	*20.6*	*50*	*79.4*
	2,3-Pentanedione	*33*			*1*	*3.0*	*32*	*97.0*
	2,3-Butanedione	*54*					*54*	*100*
	Butyl butyrolactate	*21*					*21*	*100*
Herbal	*1-Hexanol*	*42*			*7*	*16.7*	*35*	*83.3*
	Piperitone	*24*			*1*	*4.2*	*23*	*95.8*
	β-Pinene	*59*					*59*	*100*
	α-Pinene	*46*					*46*	*100*
	Eucalyptol	*39*					*39*	*100*
	Linalyl acetate	*20*					*20*	*100*
	1,4-Cineol	*17*					*17*	*100*
	Thymol$^{\$}$	*7*					*7*	*100*
	γ-Pentalactone	*5*					*5*	*100*

Balsamic	Methyl cinnamate	54	3	5.6	51	94.4
	Ethyl cinnamate	19	5	26.3	14	73.7
	Benzyl benzoate	37			37	100
	Cinnamyl alcohol	10			10	100
	Benzophenone[#]	2			2	100
	Benzyl cinnamate[#]	2			2	100
Tonka	Coumarin	21	4	19.0	17	81.0
	Hydrocoumarin	47			47	100
Green	(3Z)-3-Hexen-1-ol	130	8	6.2	122	93.8
	3-Hexen-1-ol, acetate	61			61	100
	Hexyl hexanoate	12			12	100
	Hexyl 2-methylbutyrate[#]	4			4	100
	Styralyl acetate	4			4	100
	Methyl 2-octynate	1			1	100
Citrus	Limonene	95	3	3.2	92	96.8
	Citral	33			33	100
	6-Methyl-5-heptene-2-one	5			5	100
Nutty	2,3,5-trimethylpyrazine	39			39	100
	2,3-dimethylpyrazine	19			19	100
	2,3,5,6-tetramethylpyrazine	17			17	100
	2-ethyl-3-methylpyrazine	9			9	100
	Dimethyl butanedioate	1			1	100
Coconut	δ-Decalactone	107			107	100
	γ-Nonalactone	75			75	100
	γ-Octalactone	64			64	100
	Coumarin, 6-methyl	6			6	100
	γ-Heptalactone	1			1	100
Waxy	Ethyl laurate	11			11	100
	Ethyl decanoate	6			6	100
	Ethyl octanoate	5			5	100
	Ethyl nonanoate	3			3	100
Terpinic	α-Terpineol	98			98	100
	p-Cymene	53			53	100
	γ-Terpinene	39			39	100
Chocolate	2,5-dimethylpyrazine	11			11	100
	Isopentyl phenylacetate	9			9	100
	2-Methoxy-3-methylpyrazine	5			5	100
Fermented	Isopentyl Alcohol	47			47	100
	1-Pentanol	15			15	100
Bready	Furfuryl alcohol	33			33	100
	Furfural*	16			16	100
Woody	α-Caryophyllene	14			14	100
	[c]2-Hy-3,5,5-t-cyclohex-2-en	12			12	100
Anisic	p-Anisaldehyde	50			50	100
	Estragole (4-allyanisole)	15			15	100
Popcorn	Acetylpyrazine	53	1	1.9	52	98.1
Meaty	Hemineurine	44	1	2.3	43	97.7
Smoky	Syringol	7	1	14.3	6	85.7

Phenolic	*Guaiacol (2-methoxyphenol)*	*53*			*53*	*100*
Tropical	*δ-Dodecalactone*	*53*			*53*	*100*
Musty	*2-Acetylpyrrole*	*10*			*10*	*100*
Camphoreous	*Fenchol*	*9*			*9*	*100*
Earthy	*cis-Linalool oxide*	*6*			*6*	*100*
Honey	*Methyl phenylacetate*	*2*			*2*	*100*
Odorless	*Caffeine*	*1*			*1*	*100*
	Strawberry glycidate_A	*23*	*1*	*4.3*	*22*	*95.7*
	Strawberry glycidate_B	*12*	*1*	*8.3*	*11*	*91.7*
	Heliotropine PG acetal	*35*			*35*	*100*
	4-methylbenzyl alcohol	*3*			*3*	*100*
	Aromadendrene	*3*			*3*	*100*

Note:

[a] # = frequency of occurrence of flavor chemicals in each concentration category

[b] % = Percentage of occurrence of flavor chemicals in each concentration category

[c] 2-Hydroxy-3,5,5-trimethyl-cyclohex-2-en is shortened as 2-Hy-3,5,5-t-cyclohex-2-en

Table S4.2: Total Concentration of Flavor Chemicals in 277 EC Refill Fluids

	Product Code	TFC (mg/ml)
1	U48	362.3
2	U47	118.2
3	U28	68.4
4	U30	67.5
5	U129	64.7
6	U112	63.1
7	U36	54.6
8	U85	54.0
9	U97	53.4
10	U96	52.0
11	U179	50.2
12	U109	49.5
13	U104	48.6
14	N28	47.4
15	U37	47.2
16	C21	47.1
17	U149	46.7
18	N27	46.7
19	C18	44.7
20	U99	43.5
21	U98	42.3
22	U73	40.3
23	U66	39.9
24	U113	38.5
25	U151	37.0
26	U188	35.6
27	U143	33.8
28	U127	33.8
29	U158	33.2
30	N2	32.6
31	U170	32.4
32	U193	31.5
33	U197	30.4
34	U59	30.1
35	U60	29.6
36	U27	29.5
37	U34	28.6
38	U166	28.6
39	U110	28.5
40	B7	27.7
41	U65	27.3
42	U23	27.1
43	U86	27.1
44	U12	26.9
45	C23	26.9
46	U160	26.9
47	B3	26.6
48	U165	26.3
49	U4	25.5
50	U74	25.1
51	U72	24.8
52	N6	23.4
53	N13	23.2
54	N5	22.7
55	U161	22.4
56	N12	21.8
57	U139	21.3
58	U49	20.7
59	U135	20.4
60	U141	19.5
61	U136	19.4
62	U69	19.3
63	U168	18.9
64	U57	18.8
65	U45	18.5
66	U138	18.3
67	U105	18.2
68	U122	18.1
69	U137	17.5
70	U178	17.5
71	U63	17.3
72	U62	17.0
73	U114	16.7
74	N7	17.0
75	U128	16.7
76	U102	15.9
77	U192	15.9
78	U51	15.7
79	U58	15.3
80	U93	15.2
81	U107	15.1
82	U119	14.3
83	U77	14.1
84	C26	13.8
85	U95	13.5
86	N23	13.3
87	U184	13.0
88	N24	13.0
89	U131	12.4
90	U130	12.1
91	N25	12.0
92	U183	12.0
93	U196	11.3
94	U25	11.2
95	U11	10.8
96	B1	10.7
97	U155	10.6
98	U163	10.6
99	U64	10.5
100	U67	10.4
101	U169	10.1
102	U186	10.1
103	U32	9.7
104	U2	9.7
105	U55	9.3
106	C2	9.3
107	U156	9.3
108	U14	9.1
109	U71	9.1
110	U150	8.5
111	U133	8.4
112	U194	8.3
113	U148	7.9
114	U121	7.5
115	U84	7.5
116	U146	7.4
117	U79	7.1
118	U39	6.9
119	U91	6.7
120	U126	6.7
121	U142	6.5
122	U116	6.4
123	U132	6.2
124	U61	6.1
125	U176	5.9
126	U7	5.8
127	C5	5.7
128	U103	5.7
129	U13	5.6
130	N8	5.6
131	U123	5.6
132	U187	5.6
133	U117	5.5
134	U100	5.5
135	U17	5.4
136	B8	5.4
137	U68	5.4
138	U144	5.4
139	N1	5.3
140	N11	5.3

141	U31	5.3
142	U124	5.3
143	N9	5.2
144	U26	5.2
145	N10	5.2
146	U53	5.1
147	U134	5.1
148	U111	5.0
149	C16	4.8
150	U152	4.8
151	U172	4.8
152	U173	4.8
153	U50	4.7
154	U29	4.7
155	U185	4.6
156	U56	4.6
157	U180	4.5
158	U120	4.4
159	U75	4.4
160	U171	4.3
161	U140	4.3
162	U175	4.3
163	N3	4.2
164	U52	4.2
165	U174	4.1
166	U46	4.0
167	U189	3.9
168	N22	3.8
169	U195	3.8
170	U118	3.7
171	U44	3.5
172	U177	3.5
173	U181	3.2
174	U125	3.2
175	U33	3.2
176	U145	3.0
177	U3	2.9
178	U182	2.9
179	N33	2.8
180	U147	2.7
181	N32	2.7
182	N34	2.7
183	U191	2.7
184	U88	2.7
185	U154	2.6
186	N4	2.6
187	U94	2.6
188	C25	2.6
189	N20	2.5
190	U10	2.5
191	U198	2.5
192	N15	2.4
193	N21	2.4
194	N14	2.4
195	C27	2.4
196	U87	2.3
197	C8	2.3
198	U76	2.3
199	U190	2.3
200	N19	2.2
201	N17	2.2
202	U20	2.2
203	N16	2.2
204	N18	2.2
205	U81	2.2
206	U92	2.2
207	U164	2.2
208	U43	2.2
209	U16	2.1
210	U108	2.1
211	N26	2.1
212	B5	2.0
213	C4	2.0
214	C9	1.9
215	C11	1.9
216	U1	1.7
217	U13	1.7
218	B2	1.7
219	C1	1.7
220	U21	1.7
221	C22	1.7
222	U42	1.7
223	U115	1.7
224	U35	1.5
225	B6	1.5
226	C7	1.5
227	U22	1.5
228	U159	1.5
229	U14	1.5
230	N29	1.4
231	U70	1.3
232	C10	1.2
233	U157	1.2
234	U18	1.1
235	C6	1.1
236	U38	1.0
237	U167	1.0
238	N31	1.0
239	U19	1.0
240	U15	1.0
241	N30	0.9
242	N36	0.9
243	N38	0.9
244	N35	0.8
245	N37	0.8
246	U9	0.8
247	U82	0.8
248	C3	0.7
249	C15	0.7
250	U41	0.7
251	U101	0.7
252	C24	0.7
253	B4	0.6
254	U54	0.6
255	C14	0.6
256	U89	0.6
257	U83	0.6
258	U106	0.6
259	U12	0.5
260	U90	0.5
261	U8	0.5
262	U6	0.4
263	C19	0.4
264	U80	0.4
265	U162	0.4
266	U153	0.3
267	U40	0.2
268	C13	0.2
269	U24	0.1
270	C12	0.1
271	C17	0.03
272	U15	0.01
273	U78	0.01
274	C20	0.01
275	U11	0.005
276	U5	0.000
277	U16	0.000

Table S4.3: Brand and Product Names of All 277 EC Refill Fluid Products

Brand/Manufacturer Name	Product Name
Canyon Crest Vape S	Cinnamon Bomb Fiery
Canyon Crest Vape S	Cinnamon Bomb
Flavor Art	Menthol Arctic
Johnson Creek	Tundra
NicQuid	Sinthol
Beard Vape Co	No. 64
LiQua	Two Apples
LiQua	Mints
LiQua	Two Apple
LiQua	Two Apple
Cosmic Fog	The Shocker
Beard Vape Co	No. 51
NJOY Artist Collection	Paramour
LiQua	Two Apples
LiQua	Two Apples
LiQua	Two Apples
The Mad Alchemist	Dragons Breath
LiQua	Ry4 Tob.
LiQua	Ry4 Tob.
LiQua	Ry4 Tob.
LiQua	Ry4 Tobacco
LiQua	Q Honeydew Drop
Cuttwood	Sugar Drizzle
Beard Vape Co	No. 88
The Mad Alchemist	Winters Bite
Twelve Vapor	Libra
NicQuid	Smoothol
Mystique Vapor	Prometheus
Seduce Juice	Snake Eyes
LiQua	Menthol
Cuttwood	Sugar Drizzle
Twelve Vapor	Aries
Glas	Pound Cake
Cuttwood	Boss Reserve
Kilo	Dewberry Cream
LiQua	Q Peach
Kilo	Dewberry Cream
Cuttwood	Boss Reserve
Beard Vape Co	No. 05
LiQua	Q Peach
The Milkman	Churios
LiQua	Peach
LiQua	Two Mints
LiQua	Q Peach
LiQua	Peach
LiQua	Peach
Seduce Juice	Snake Oil
Seduce Juice	Snake Bite
LiQua	Peach
LiQua	Q Pina Colada
Beetle Vapour Juice	Blueberry Hills
LiQua	Menthol
SMKING	Strawberry
LiQua	Menthol
Seduce Juice	Snake Venom
SMKING	Strawberry
Vapor Jerry's	Death Before Dishonor
Canyon Crest Vape S	Dewberry Cream
NicQuid	Sublime
Vapor Jerry's	Oh R'Lyeh
The Milkman	The Milkman
Majestic	King Kong
Cuttwood	Unicorn Milk
Liquid State	Apple Butter
Canyon Crest Vape S	Rainbow Sherbert
Vapor Jerry's	Berry Untraditional
NJOY Artist Collection	Dragonscape
Bombies	Kiss The Ring
The Milkman	Churrios
Cosmic Fog	Cola Gummy
Pop Drops	S'mores vape juice
LiQua	Menthol
Kilo	Kiberry Yoghurt

Bombies	Bacco B
NicQuid	Daybreak
NJOY Artist Collection	Hedon's Bite
Twelve Vapor	Taurus
Canyon Crest Vape S	WTF
Cuttwood	Mega Melons
LiQua	HP Sweet Accelerator
ANML	Looper
Bombies	Agent P
LiQua	Q Menthol
LiQua	Cheesecake
Naked 100	Naked 100
LiQua	Q Menthol
LiQua	Menthol
LiQua	Menthol
Space Jam	Andromeda
NicQuid	Strawnanna Smoothie
NicQuid	Southern Freeze
LiQua	Menthol
Space Jam	Starship 1
Glas	Milk
LiQua	Q Menthol
LiQua	Q Menthol
LiQua	Menthol
The Mad Alchemist	Eye of Newt
Seduce Juice	White Walker
Lost Art LiQuids	Unicorn Puke
Cuttwood	Mega Melons
Space Jam	Eclipse
LiQua	Menthol
Lost Art LiQuids	Unicorn Puke
LiQua	Menthol
Daze	#Crawlie Tuesday
The Mad Alchemist	Custard Matter
LiQua	Menthol
Blue Label Elixir	Famous
The Mad Alchemist	Zen
NicQuid	Maui

Twelve Vapor	Pisces
The Mad Alchemist	Snow White's Demise
LiQua	Energy Drink
Bombies	Nana Cream
Mystique Vapor	Cronos
LiQua	French Pipe Tob
LiQua	Q Cherribakki
LiQua	HP Overdrive
NicQuid	Strawberry Fuzz
Vapor Jerry's	Cut 'N Run
Bombies	White Gummy B
NicQuid	Peach Lemonade
Kilo	Fruit Whip
Cosmic Fog	Church
LiQua	Coffee
LiQua	Coffee
NJOY Artist Collection	Samba Sun
LiQua	Q Apple
LiQua	MB
NicQuid	Banana Nut Bread
Space Jam	Galactica
LiQua	Apple
Bombies	Black Out City
LiQua	Q Apple
LiQua	Coffee
Q Vapor Labs	North Shore
Mystique Vapor	Oceanus
LiQua	MB
Lost Art LiQuids	Gummy Glu
NicQuid	Blueberry
LiQua	MB
LiQua	MB
LiQua	Q Apple
Lion	Love Potion
NicQuid	Midnight Express
Beard Vape Co	No. 71
SMKING	Strawberry
LiQua	Mild Kretek Tob.
The Mad Alchemist	Chem Trail

Cosmic Fog	Lost Fog Streak
Cosmic Fog	Lost Fog Baie Creme
Johnson Creek	Arctic Menthol
Canyon Crest Vape S	Ho!Ho! Watermelon
Space Jam	Pluto
O.M.G E LiQuid	WTF
Cosmic Fog	Nutz
LiQua	Q Double Bubble
Bombies	Tiger Style
Vapor Jerry's	Jugo De Las Muerta
Cosmic Fog	Euphoria
Cosmic Fog	Kryptonite
LiQua	Bright Tobacco
Daze	#Selfie Sunday
Cosmic Fog	Lost Fog Neon Cream
Canyon Crest Vape S	Melon Mania
Twelve Vapor	Scorpio
LiQua	Bright Tobacco
Twelve Vapor	Aquarius
Bombies	Seven Seas
LiQua	Q Blackberry Jack
Cosmic Fog	Milk and Honey
NicQuid	Soho
Space Jam	Pulsar
Opulence Elixir	Popsuckle
Mystique Vapor	Hyperion
LiQua	Apple
Space Jam	Astro
LiQua	MB
LiQua	MB
LiQua	MB
LiQua	Watermelon
Mystique Vapor	Asteria
Twelve Vapor	Gemini
Thrive	Ice Lemon Cole
LiQua	Brownie
LiQua	HP Summer Drift

The Mad Alchemist	Twice in a Blue Moon
LiQua	Licorice
LiQua	Watermelon
Glas	Pebbles
Thrive	Ice Lemon Cole
Thrive	Ice Lemon Cole
LiQua	Watermelon
LiQua	Licorice
LiQua	Tiramisu
LiQua	Q Fragola Fresca
LiQua	Watermelon
Twelve Vapor	Cancer
Thrive	Banana
Thrive	Banana
Thrive	Banana
LiQua	Tiramisu
LiQua	Watermelon
LiQua	Banana
LiQua	HP Fruity Velocity
Seduce Juice	Jezebel
LiQua	Apple
LiQua	Strawberry
LiQua	Q The Moment
ANML	Carnage
LiQua	Apple
LiQua	Apple
LiQua	Cherry
LiQua	Bright Tob.
LiQua	Bright Tob.
LiQua	Bright Tob.
LiQua	Bright Tob.
LiQua	Bright Tob.
LiQua	Cherry
LiQua	Berry Mix
LiQua	Q Piedmont Sunrise
Bombies	Product X
LiQua	Q Berry Mix
LiQua	Q Berry Mix
LiQua	Grape

LiQua	Strawberry
Ripe Vapes	RF- HSAC
Seduce Juice	Blackjack
LiQua	Vanilla
Seduce Juice	Exodus 7:20
LiQua	Strawberry
Vape Mail	Overnight
LiQua	Berry Mix
LiQua	Grape
LiQua	Vanilla
LiQua	Citrus Mix
LiQua	Citrus Mix
LiQua	Citrus Mix
Cuttwood	Bird Brains
LiQua	Vanilla
LiQua	Vanilla
LiQua	Vanilla
LiQua	Berry Mix
LiQua	Vanilla
LiQua	Vanilla
LiQua	Vanilla
LiQua	Berry Mix
LiQua	Virginia Tobacco
LiQua	Q Golden Roanoke Tob.
LiQua	Cappucino
LiQua	Berry Mix
LiQua	Red Oriental Tobacco
LiQua	Cola
Cuttwood	Bird Brains
LiQua	Red Oriental Tobacco
LiQua	Chocolate
NJOY Artist Collection	Sacre Coeur
LiQua	Cola
LiQua	Q Turkish Tobacco
LiQua	Chocolate
LiQua	Turkish Tobacco
LiQua	Vermillion Oriental Tob.

LiQua	Blueberry
Seduce Juice	Caesar
The Mad Alchemist	Number 9
LiQua	Goldenrod Oriental Tob.
LiQua	Q Havana Libre Tob.
LiQua	Cuban Cigar Tobacco
LiQua	Golden Oriental Tob.
LiQua	Traditional Tobacco
LiQua	Q Traditional Tobacco
LiQua	Q Traditional Tobacco
LiQua	Traditional Tobacco
LiQua	American Blend Tob.
LiQua	Q American Blend Tob.
LiQua	American Blend Tob.

Appendix D: Supporting Information for Chapter 5

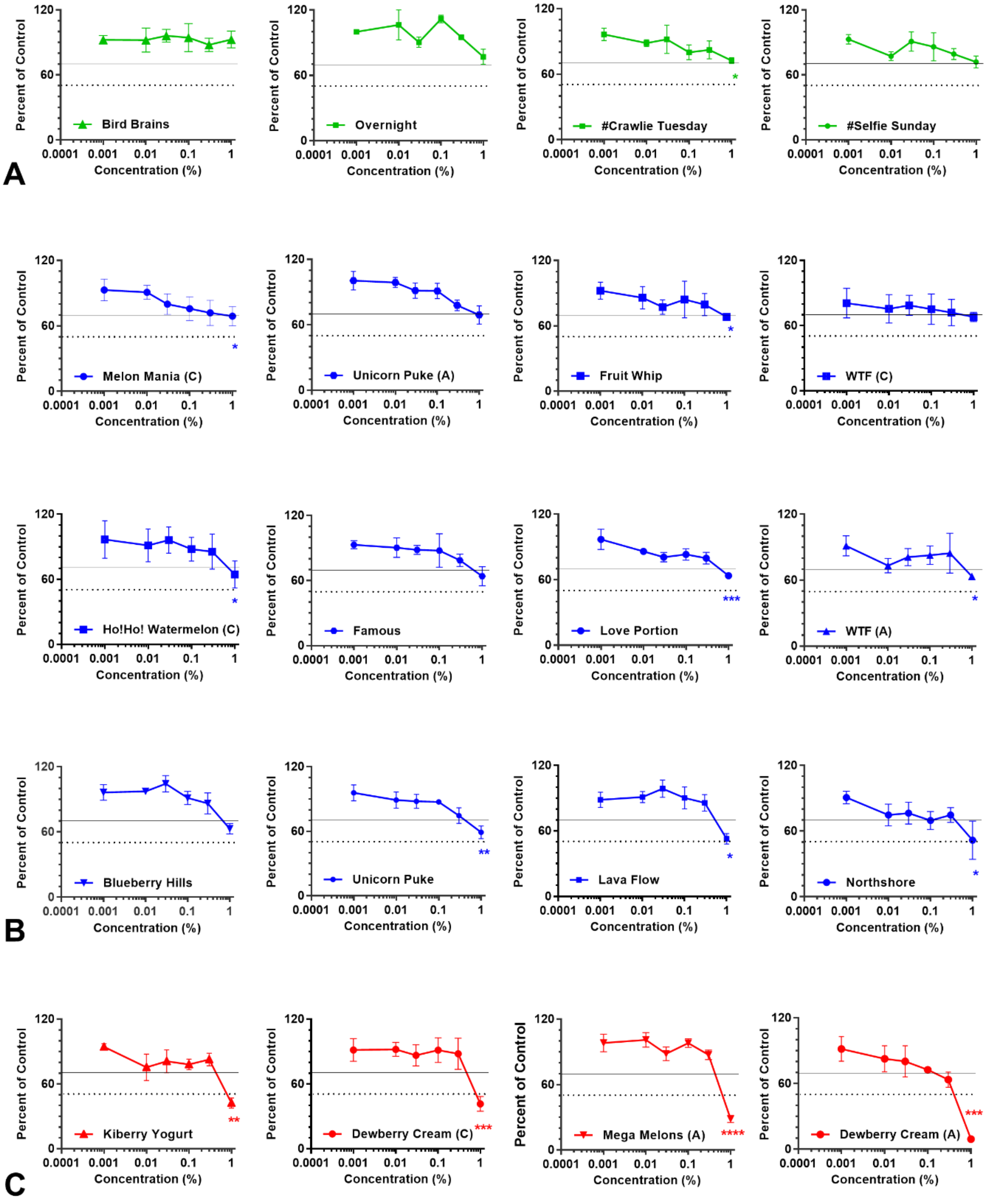

Figure S5.1. Dose-response curves for 20 popular refill fluids. (A) Non-cytotoxic fluids. (B) Fluids reaching an IC_{70}. (C) Fluids reaching an $IC_{50.}$ Horizontal bars are at the IC_{70} and IC_{50}. Each graph is the mean ± the std error of the mean for three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p< 0.001$, **** = $p< 0.0001$.

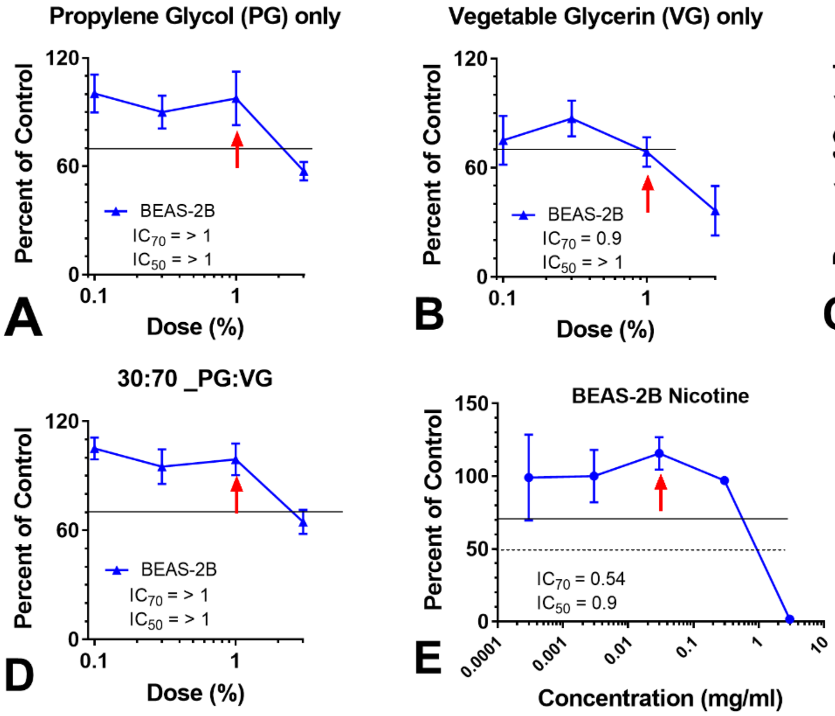

Figure S5.2. Dose-response curves for propylene glycol, glycerol, and nicotine. (A) Propylene glycol (PG). (B) Glycerol (C) 50:50 PG: VG (D) 30:70 PG: VG (E) Nicotine. The red arrows indicate the highest concentrations of solvents and nicotine that were used in the current study. Horizontal bars are at the IC_{70} and IC_{50}. Each graph is the mean ± the std error of the mean for three independent experiments.

Table S5.1. EC User Demographics

General Demographics (N=835)		**All EC Users**
Age at survey	<14	0 (0%)
	14-17	9 (1.08%)
	18-22	413 (49.46%)
	23-27	211 (25.27%)
	28-32	84 (10.06%)
	33-42	75 (8.98%)
	43-50+	43 (5.16%)
Gender	Male	599 (71.74%)
	Female	231 (27.66%)
	Other	5 (0.60%)
Race/Ethnicity	Asian	237 (28.38%)
	Black or African American	26 (3.11%)
	Hispanic/Latino	158 (18.92%)
	Indian	16 (1.92%)
	Middle Eastern	39 (4.67%)
	Native American or Alaska Native	80 (9.58%)
	Native Hawaiian or Pacific Islander	16 (1.92%)
	White/Caucasian	361 (43.23%)
	Other	13 (1.56%)
Highest Education	No high school	2(0.24%)
	Some high school	12 (1.44%)
	High school graduate or GED equivalent	122 (14.61%)
	Certification work	78 (9.34%)
	Some college	325 (38.92%)
	2-year college degree	74 (8.86%)
	4-year college degree	151 (18.08%)
	Some post-graduate education	22 (2.63%)
	Graduate or professional school	49 (5.87%)
EC Use History		
Age First Vaped	<14	3 (0.36%)
	14-17	218 (26.11%)
	18-22	413 (49.46%)
	23-27	211 (25.27%)
	28-32	84 (10.06%)
	33-42	75 (8.98%)
	43-50+	43 (5.16%)
Contributed to Vaping*	People in my family vape	74 (8.86%)
	Friends of mine vape	454 (54.37%)
	I saw people in the media vaping	128 (15.33%)
	I wanted to stop smoking conventional cigarettes	365 (43.71%)

	Vaping is a stress reliever	262 (31.38%)
	Vaping lowers my appetite	75 (8.98%)
	Vaping is safer than conventional cigarette smoking	462 (55.33%)
	I live or work near a vape shop	118 (14.13%)
	Other	55 (6.59%)
Length of EC Usage	<1 month	37 (4.46%)
	1-6 months	200 (24.10%)
	7-11 months	89 (10.72%)
	1 year	226 (27.23%)
	2 year	185 (22.29%)
	3-4 year	74 (8.92%)
	5+ year	19 (2.28%)
EC Frequency Use	Regularly, at least once a day	470 (56.63%)
	Occasionally	153 (18.43%)
	Rarely	139 (16.75%)
	Socially	68 (8.19%)
Hao often have you vaped in the past 30 days?		
	None	39 (4.70%)
	Less than once a week	128 (15.42%)
	A few times a week (less than five times a week)	212 (25.54%)
	Daily or almost daily	451 (54.34%)
Daily Vaping	Yes	677 (81.57%)
	No	153 (18.43%)
EC Use Daily	<10 minute intervals per day	131 (19.47%)
	10-30 minute sessions a day	128 (19.02%)
	31-59 minutes a day	163 (24.22%)
	1-3 hours a day	146 (21.69%)
	4-6 hours a day	56 (8.02%)
	7+ hours a day	51 (7.58%)

Table S5.2. Flavor Profiles for Popular Brands

Brand Names [StoreID]	Generic Flavor Profile and General Category
Dewberry Cream [3]	Honeydew; mixed berries; cream (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
Blueberry Hills [4]	Blueberry pop tart (Berries/Fruits/Citrus; Bakery/Dessert)
Dewberry Cream [1c]	Honeydew; mixed berries; cream (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
Rainbow Sherbet [1c]	Rainbow sherbet ice cream (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
Kiberry Yogurt [3]	Kiwi; yogurt (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
WTF [1c]	Strawberry; sourbelt candy (Berries/Fruits/Citrus; Candy)
Mega Melons [3]	Melon; cantaloupe; papaya; mango (Berries/Fruits/Citrus)
Lava Flow [3]	Strawberry; pineapple; coconut (Berries/Fruits/Citrus)
Unicorn Puke [4]	Rainbow sherbert ice cream (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
#Crawlie Tuesday [2]	Gummy worm candy (Berries/Fruits/Citrus; Candy)
Famous [4]	Strawberry; watermelon; pineapple; citrus (Berries/Fruits/Citrus)
Fruit Whip [3]	Apples; pear; berries; cream (Berries/Fruits/Citrus; Buttery/Creamy/Caramel/Vanilla)
North Shore [4]	Dragon fruit; guava; papaya (Berries/Fruits/Citrus)
Love Potion [2]	Apple; menthol (Berries/Fruits/Citrus; Menthol)
Ho!Ho! Watermelon [1]	Watermelon; assorted melons (Berries/Fruits/Citrus)
WTF [2]	Strawberry; sourbelt candy (Berries/Fruits/Citrus; Candy)
Melon Mania [1c]	Assorted melons (Berries/Fruits/Citrus)
#Selfie Sunday [2]	Apple (Berries/Fruits/Citrus)
Overnight [4]	Strawberry; watermelon; Jolly rancher candy (Berries/Fruits/Citrus; Candy)
Bird Brains [2]	Fruit loop cereal (Berries/Fruits/Citrus; Breakfast Cereal)

Table S5.3. Chemical Flavor Compounds and their Descriptive Taste Profiles

Flavor Chemical	**Descriptive Taste Profile(s)**
Furfural	*Bready, brown, burnt nuance, caramellic nuance, nutty, woody*
Allyl hexanoate	*Fresh, fruity, juicy nuance, pineapple, sweet*
Isopulegol	*Cool, mint*
1-Pentanol	*Bready, cereal, fruity undernote, fermented, intense fusel*
Furfuryl alcohol	*Burnt, creamy, caramellic, milky, powdery*
Coumarin	*aromatic nuance, bitter, burning*
Guaiacol (2-methoxyphenol)	*Bacon, phenolic, medicinal, savory, smoky, woody*
1-Hexanol	*Apple skin, fruity, green nuance, oily*
Acetophenone	*Almond, cherry pit-like, coumarinic nuances, fruity nuances, powdery*
Methyl salicylate	*Aromatic nuance, balsamic nuance, root beer, salicylate, sweet*
Ethyl maltol	*Burnt, cotton sugar, candy-like, jammy notes, strawberry notes, sweet*
Isopentyl alcohol	*Banana, cognac, etherial, fermented, fruity, fusel*
Benzaldehyde	*Sweet, oily, almond, cherry, nutty and woody*
Raspberry ketone	*Berry, blueberry, cotton candy nuances, fruity, jammy notes, raspberry, seedy nuances*
Maltol	*Berry notes, caramelic, cotton candy, fruity notes, jammy notes, sweet*
Hydrocoumarin	*Coconut, creamy, milky, sweet, vanilla*
Benzyl alcohol	*balsamic nuance, chemical, fruity nuances*
2,3-Butanedione	*Buttery, creamy, milky, sweet*
Vanillin	*Creamy, milky, phenolic, spicy, sweet, vanilla*
Ethyl vanillin	*Caramellic, creamy, smooth, sweet, vanilla*
p-Tolualdehyde	*Cherry, deep phenolic, fruity*
Benzyl benzoate	*Balsamic, berry nuances, fruity, powdery nuance*
Phenethyl alcohol	*Bready, floral, rosy, sweet*
Furaneol	*Caramelic, cotton candy, sweet*
Eugenol	*Phenolic nuances, clove, spicy, sweet, woody nuances*
α-Pinene	*Cedarwood, pine, sharp*
β-Pinene	*Camphoraceous, fresh, minty, piney, spicy, terpy, woody*
p-Cymene	*Citrus notes, green pepper, oregano, rancid, spice nuance, terpy, woody*
Carvone	*Caraway, minty, spicy*
Cinnamaldehyde	*Cinnamon, spicy*
Hexyl acetate	*Apple, banana peel, fresh, fruity, green, pear*
p-Anisaldehyde	*Almond, anise, mint*
Eucalyptol	*Eucalyptus*
Isosafroeugenol	*Creamy, anisic, sweet, vanilla-like*

Amyl acetate	*Apple, banana, ethereal, fruity, pear*
Isobutyl acetate	*Cereal, bready, fermented, fruity undertones, fusel*
Benzyl butyrate	*Apricot, fruity, pear, pineapple, sweet, tropical*
Cinnamyl alcohol	*Fermented, yeasty nuance, floral, green, honey, spicy*
Ethyl 2-methylbutanoate	*Berry, cherry notes, fruity, fresh, grape, pineapple, mango*
Strawberry Glycidate_B	*Berry, floral, fruity, strawberry, sweet, tutti frutti*
Hemineurine	*Brothy,meaty, metallic, nutty, roasted*
Benzyl acetate	*Balsamic undernotes, fruity, jasmine floral undernotes, sweet*
Methyl cinnamate	*Balsamic, cinnamyl, fruity, strawberry*
Piperonal	*Benzaldehyde, cherry, spicy, vanilla*
Linalool	*Aldehydic, citrus, floral, lemon, orange, waxy, woody*
Methyl anthranilate	*Berry nuances, concord grape, fruity, musty nuances, sweet*
β-Damascone	*Floral, green, herbal, minty, woody*
2,3-Pentanedione	*Buttery, caramellic, marshmellow nuance, molasses nuance, toasted*
Benzaldehyde PG acetal	*Floral*
Acetylpyrazine	*Bready, corn chip nuance, nutty, popcorn nuance, roasted, yeasty nuance*
Menthol	*Cool, mint*
(E)-2-Hexen-1-ol	*Fatty, fresh, fruity nuance, green nuance, juicy nuance*
Ethyl propanoate	*Apple nuance, bubble gum, etherial, fruity, grape nuance, sweet, winey*
6-Methyl-5-heptene-2-one	*Apple, banana, green bean, green, mistry, vegetative*
trans-Geraniol	*Floral, fruity, peach-like nuance, rosy, waxy*
γ-Terpinene	*Citrus, green, lime, oily, terpy, tropical fruity nuance*
2-Methylbutyl acetate	*Banana, ripe, fruity, juicy nuance, sweet*
Ethyl cinnamate	*Balsamic, berry, fruity, green,powdery, punch, spice, sweet*
α-Terpineol	*Anise, fresh, mint, oil*
δ-Decalactone	*Buttery, coconut, creamy, fatty, fruity nuance, milky, nutty*
γ-Octalactone	*Apricot, coconut, courmarin, creamy, fruity, lactonic, peach, toasted*
α-Ionone	*Berry, floral, fruity, raspberry, strawberry, violet*
(3Z)-3-Hexen-1-ol	*Fresh, fruity nuance, green nuance, raw nuance*
Citral	*Citrus, green, herbal, juicy, lemon peel, lime, woody*
Strawberry Glycidate_A	*Berry, floral, fruity, strawberry, sweet, tutti frutti*
Fenchol	*Fresh, pine*
Acetoin	*Buttery, creamy, dairy, milky, oily, sweet*

Isoamyl butyrate	*Berry notes, estry nuance, fruity, green apple, melon notes, sweet, waxy*
Isopentyl phenylacetate	*Chocolate nuances, dried fruit notes, honey*
γ-Decalactone	*Apricot, creamy, fatty nuance, fruity, peachy syrupy nuance*
Hexyl 2-methylbutyrate	*Apple, banana, fresh, fleshy nuance, green, unripe, fruity, waxy*
Ethyl isovalerate	*Apple, fruity, green, metallic, pineapple, spice, sweet*
Nerol acetate	*Floral, fruity, pear, rosy, soapy, tropical*
δ-Dodecalactone	*Buttery, creamy, dairy, fatty, nutty, peach,*
Ethyl hexanoate	*Banana, estry nuance, fruity, green, pineapple, sweet, waxy*
Butyl butyrolactate	*Cheesy nuances, creamy, dairy, fatty, milky, waxy*
Limonene	*Camphoraceous, citrus, herbal, terpene*
Benzyl cinnamate	*Balsamic, floral, fruity, spicy*
Ethyl Acetate	*Cherry, nuance, etherial, fruity, grape, nuance, sweet*
Geraniol Acetate	*Citrus nuances, floral, green, oily, rum nuances, soapy, waxy, wine nuances*
γ-Nonalactone	*Coconut, creamy, milky notes, waxy*
(E)-β-Ionone	*Berry, floral, fruity, powdery nuances, woody*
Ethyl lactate	*Creamy, caramellic nuance, fruity, sweet, pineapple*
Butyl butyrate	*Fatty, fresh, fruity, sweet*
Linalyl acetate	*Citrus, floral, green, herbal nuances, spicy nuances, terpy, waxy*
Ethyl butanoate	*Apple, etherial, fresh, fruity, sweet, tutti frutti*
Isoamyl isovalerate	*Apple, fruity nuance, green*
Isoamyl acetate	*Banana, estry nuance, fruity, green nuance, ripe nuance, sweet*
δ-Undecalactone	*Coconut, creamy, fatty, macadamia, nutty, peach, vanilla*
Ethyl heptanoate	*Banana, fruity, oily nuance, pineapple, spicy nuance, strawberry*
Heliotropine PG acetal	*Floral, fruity*
Hydroxyacetone	*Burnt, sweet, slightly green nuance*
Corylone	*Bready, caramelic, maple, nutty nuances, sweet*
Triacetin	*Creamy, oily nuance*
1,4-Cineole	*Camphoraceous, cooling, green, herbal, menthol, terpy*
Benzyl propionate	*Apple, banana, floral nuance, fruity, sweet, tutti frutti*
(Z)-3-Hexen-1-ol, acetate	*Apple, green, pear, tropical fruity nuance*
Amyl isovalerate	*Apple, fresh, fruity*
cis-Limonene oxide	*Cool, mint*

Table S5.4. List of Chemicals Not Included in Figure 2

(3Z)-3-Hexenyl formate
1,2-Dihydrolinalool
2,3,5,6-Tetramethylpyrazine
2,3,5-Trimethylpyrazine
2,3-Dimethylpyrazine
2,3-Hexanedione
2,5-Dimethylpyrazine
2-Acetylpyrrole
2-Ethyl-3-methylpyrazine
2-Hydroxy-3,5,5-trimethyl-cyclohex-2-one
2-Methoxy-3-methylpyrazine
2-Methylbenzofuran
2-Nonanone
3'-Methylacetophenone
4-Methylbenzyl alcohol
4-Terpineol
α-Caryophyllene (α-Humulene)
α-Damascone
β-Caryophyllene
β-Citronellal
β-Myrcene
γ-Heptalactone
γ-Pentalactone
Acetyleugenol
Amyl butyrate
Aromadendrene
Benzeneacetaldehyde
Benzeneacetic acid, ethyl ester
Benzoin ethyl ether
Benzophenone
Benzyl benzeneacetate
Benzyl dimethylcarbinyl butyrate
Benzyl ether
Benzylacetaldehyde
Butyl acetate
Caffeine
Cinnamyl acetate
cis-Linalool oxide
Citronellyl propionate
Coumarin, 6-methyl
D-Neomenthol
Dimethyl butanedioate
Estragole (4-allylanisole)
Ethyl 3-hydroxybutyrate
Ethyl anthranilate
Ethyl benzoate
Ethyl benzoylformate
Ethyl decanoate
Ethyl isobutyrate
Ethyl laurate
Ethyl nonanoate
Ethyl octanoate
Ethyl salicylate
Eugenol methyl ether
Gingerone
Hexanal
Hexyl hexanoate
Hexyl octanoate
Isoamyl propionate
Isoeugenol methyl ether
Linalyl propionate
Menthyl acetate
Methyl 2-methylbutyrate
Methyl 2-octynoate
Methyl N-methylanthranilate
Methyl phenylacetate
Myristicin
o-Methoxycinnamaldehyde
p-Menthanone
Pentyl propanoate
Piperitone
Pulegone
Raspberry ketone methyl ether
Styralyl acetate
Syringol
Thymol
trans-D-Limonene oxide
trans-Linalool oxide

Table S5.5. Flavor Chemicals Found in Only One Product

Chemical Category/Classification	Chemical Flavor Ingredient
Toxic	*Furfural*
Harmful	*Isopulegol* *Coumarin* *Acetophenone* *Methyl salicylate* *p-Tolualdehyde* *Eugenol*
Irritant	*Carvone* *Eucalyptol* *Cinnamyl alcohol* *Menthol* *Fenchol* *Isopentyl phenylacetate* *Benzyl cinnamate* *Butyl butyrate* *Linalyl acetate* *Ethyl heptanoate*
No data	*1,4-Cineole* *Benzyl propionate* *cis-Limonene oxide*

Appendix E: Supporting Information for Chapter 6

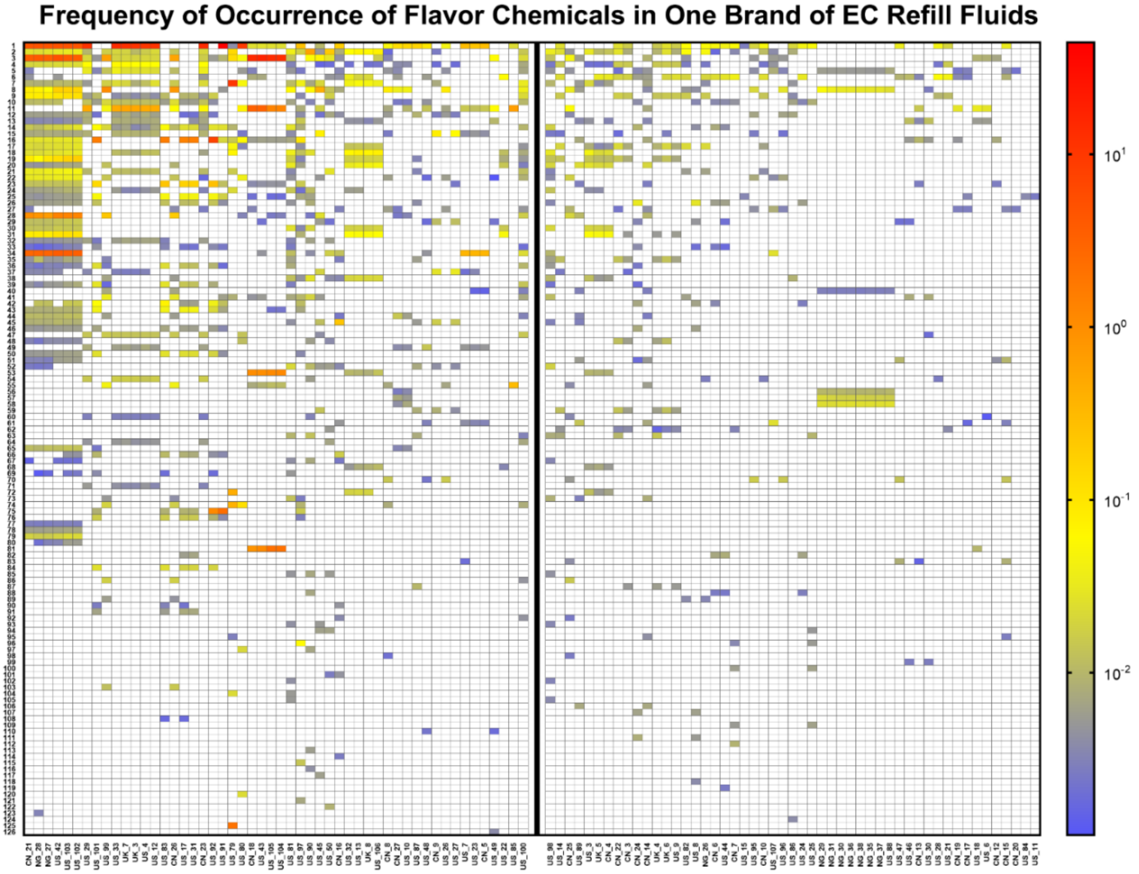

Figure S6.1. Heat map of flavor chemicals in 103 LIQUA refill fluids. Chemicals are ordered on the *y*-axis according to frequency of occurrence of 126 flavor chemicals from top to bottom. Products are ordered on the *x*-axis according to the total weight (mg/mL) of the flavor chemicals in each product with the highest concentration at the left. The vertical divider in the middle indicates products greater than 1 mg/mL to the left and < 1 mg/mL to the right. The color gradient on the right shows the concentrations of the flavor chemicals in the heat map. Values for the y-axis are given in Supplemental Table 1 and for the x-axis in Supplemental Table 2.

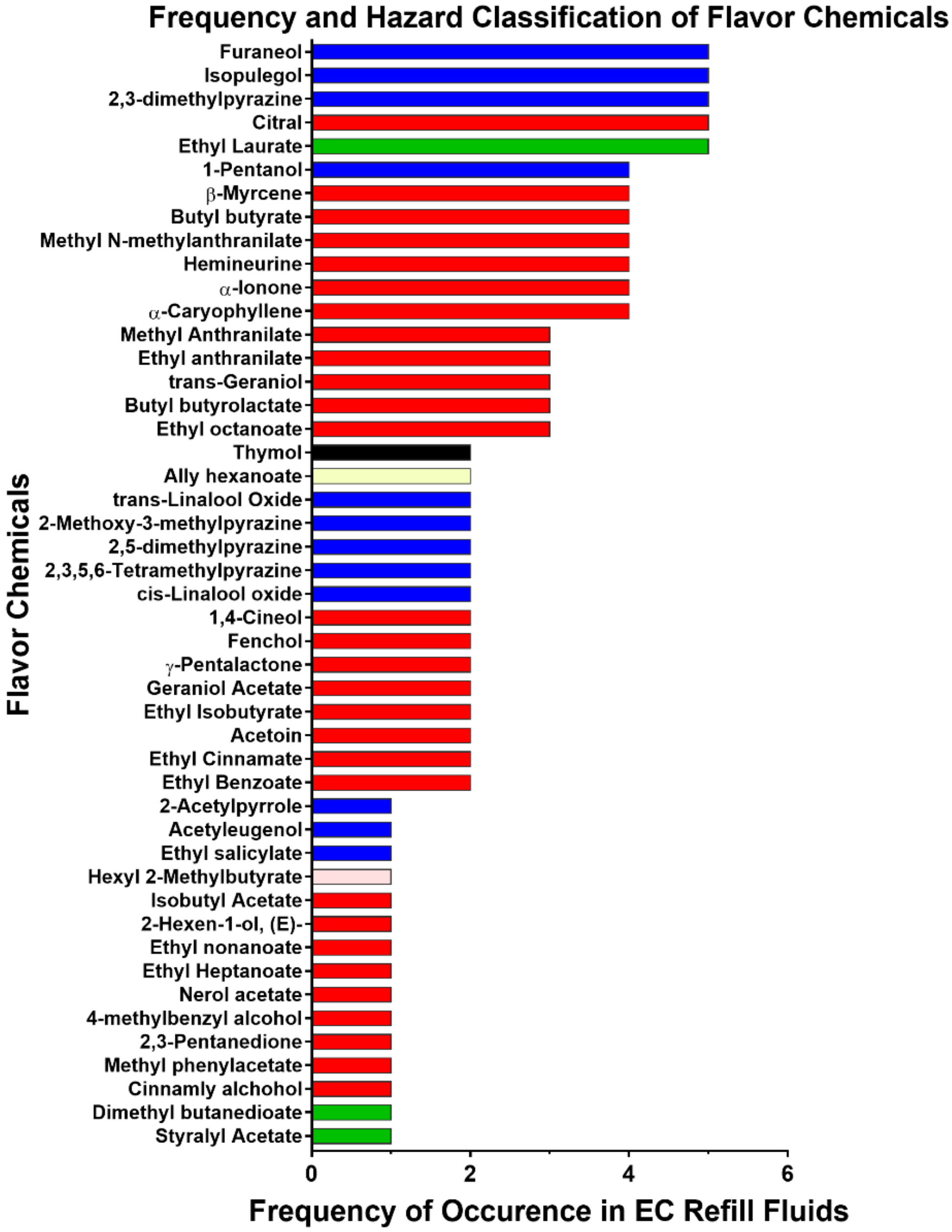

Figure S6.2. Frequency of occurrence of flavor chemicals in 5 or fewer products (continuation of Figure 3). The x-axis shows the number of refill fluids in which the chemicals were found. The y-axis is sorted according to decreasing frequency of occurrence, which ranged from 1 - 5, with the highest being furaneol. Colored bars represent hazard categories using the European Union safety guidelines; red = irritant, blue = harmful, yellow = toxic, green = not determined, pink = irritant and dangerous to the environment, cyan = harmful and dangerous to the environment, light yellow = toxic and dangerous to the environment. The chemical classes of the flavor chemicals are included in Figure 3b.

Table 6.1: Flavor Chemicals Identified in the Current LIQUA EC Study

	Flavor Chemical	CAS Number
1	Triacetin	102-76-1
2	Ethyl butanoate	105-54-4
3	Ethyl Maltol	11/8/4940
4	γ-Decalactone	706-14-9
5	δ-Decalactone	705-86-2
6	Hydroxyacetone	116-09-6
7	Ethyl Acetate	141-78-6
8	Vanillin	121-33-5
9	(3Z)-3-Hexen-1-ol	928-96-1
10	Linalool	78-70-6
11	Corylone	765-70-8
12	Phenethyl alcohol	60-12-8
13	β-Damascone	85949-43-5 (23726-91-2)
14	Limonene	138-86-3
15	Benzaldehyde PG acetal	2568-25-4
16	Menthol	15356-70-4
17	Ethyl 2-methylbutanoate	7452-79-1
18	Isoamyl Acetate	123-92-2
19	Hexyl acetate	142-92-7
20	Isoamyl Isovalerate	659-70-1
21	3-Hexen-1-ol, acetate, (Z)-	3681-71-8
22	γ-Nonalactone	104-61-0
23	p-Menthone	10458-14-7
24	2-Methylbutyl Acetate	624-41-9
25	Menthyl Acetate	16409-45-3
26	α-Terpineol	10482-56-1
27	delta-Dodecalactone	713-95-1
28	Ethyl Vanillin	121-32-4
29	Cinnamaldehyde, (E)-	14371-10-9
30	Isopentyl Alcohol	123-51-3
31	1-Hexanol	111-27-3
32	(E)-β-Ionone	79-77-6
33	p-Cymene	99-87-6
34	Benzyl Alcohol	100-51-6
35	Isoamyl Butyrate	106-27-4
36	β-Pinene	127-91-3
37	Benzaldehyde	100-52-7
38	Ethyl Isovalerate	108-64-5
39	α-Pinene	80-56-8 (7785-70-8)
40	Guaiacol	90-05-1

41	Maltol	118-71-8
42	4-Terpineol	20126-76-5
43	Neomenthol	2216-52-6
44	p-Anisaldehyde	123-11-5
45	Eugenol	97-53-0
46	Eucalyptol	470-82-6
47	γ-Octalactone	104-50-7
48	Ethyl hexanoate	123-66-0
49	Furfuryl alcohol	98-00-0
50	Pulegone	89-82-7
51	Benzyl Benzoate	120-51-4
52	Benzyl acetate	140-11-4
53	Ethyl lactate	97-64-3
54	δ-Undecalactone	104-67-6
55	Acetylpyrazine	22047-25-2
56	2,3-Butanedione	431-03-8
57	Piperonal	120-57-0
58	Heliotropine PG acetal	61683-99-6
59	Amyl Acetate	628-63-7
60	Benzeneacetic acid,	101-97-3
61	Hydrocoumarin	119-84-6
62	Acetophenone	98-86-2
63	Methyl cinnamate	103-26-4
64	Linalyl acetate	115-95-7
65	Estragole (4-allyanisole)	140-67-0
66	β-Caryophyllene	87-44-5
67	Methyl salicylate	119-36-8
68	Hexyl hexanoate	6378-65-0
69	γ-Terpinene	99-85-4
70	2,3,5-Trimethylpyrazine	14667-55-1
71	Furfural	98-01-1
72	Butyl Acetate	123-86-4
73	Amyl Isovalerate	25415-62-7
74	Ethyl Propanoate	105-37-3
75	Carvone	2244-16-8 (6485-40-1)
76	Piperitone	89-81-6
77	Isoeugenol methyl ether	93-16-3
78	Benzyl Propionate	122-63-4
79	Raspberry ketone	5471-51-2

80	Ethyl Laurate	106-33-2
81	Furaneol	3658-77-3
82	Citral	5392-40-5
83	2,3-dimethylpyrazine	5910-89-4
84	Isopulegol	89-79-2
85	α-Ionone	127-41-3
86	Hemineurine	137-00-8
87	1-Pentanol	71-41-0
88	Methyl N-methylanthranilate	85-91-6
89	Butyl butyrate	109-21-7
90	α-Caryophyllene	6753-98-6
91	β-Myrcene	123-35-3
92	Ethyl octanoate	106-32-1
93	Butyl butyrolactate	7492-70-8
94	trans-Geraniol	106-24-1
95	Ethyl anthranilate	87-25-2
96	Methyl Anthranilate	134-20-3
97	Ally hexanoate	123-68-2
98	2,5-dimethylpyrazine	123-32-0
99	2-Methoxy-3-methylpyrazine	2847-30-5
100	trans-Linalool Oxide	23007-29-6
101	Ethyl Benzoate	93-89-0
102	Ethyl Cinnamate	103-36-6
103	Acetoin	513-86-0
104	Ethyl Isobutyrate	97-62-1
105	Geraniol Acetate	105-87-3
106	γ-Pentalactone	108-29-2
107	Fenchol	1632-73-1
108	Thymol	89-83-8
109	cis-Linalool oxide	5989-33-3
110	2,3,5,6-Tetramethylpyrazine	1124-11-4
111	1,4-Cineol	470-67-7
112	Hexyl 2-Methylbutyrate	10032-15-2
113	Ethyl salicylate	118-61-6
114	Acetyleugenol	93-28-7
115	Cinnamyl alchohol	104-54-1
116	Methyl phenylacetate	101-41-7
117	2,3-Pentanedione	600-14-6
118	4-methylbenzyl alcohol	589-18-4
119	Nerol acetate	141-12-8
120	Ethyl Heptanoate	106-30-9
121	Ethyl nonanoate	123-29-5
122	Styralyl Acetate	93-92-5
123	Dimethyl butanedioate	106-65-0
124	2-Hexen-1-ol, (E)-	928-95-0
125	Isobutyl Acetate	110-19-0
126	2-Acetylpyrrole	1072-83-9

Table S6.2: Product Information for 105 LIQUA EC Refill Fluids Studied

Codes[1]	Refill Fluid Name and Site[2]	Location [3]
CN_21	Two Apples GD	
NG_28	Two Apples LG2	
NG_27	Two Apples LG1	
US_42	Two Apples KS1	
US_103	Two Apple KS3	
US_102	Two Apple KS2	
US_29	Peach CA	
US_101	Q Menthol KS2	Italy
US_99	HP Sweet Accelerator KS	Italy
US_33	Q Peach CA	Italy
UK_7	Q Peach GB	Italy
UK_3	Peach UK	
US_4	Peach KS	
US_12	Q Peach KS	Italy
US_83	Q Menthol KS3	Italy
CN_26	Cheesecake GD	
US_17	Q Menthol KS1	Italy
US_31	Q Menthol CA	Italy
CN_23	Peach GD	
US_92	Two Mints KS	
US_91	Mints KS	
US_79	Q Honeydew Drop KS	Italy
US_80	Q Pina Colada KS	Italy
CN_18	Ry4 Tob. GD	
US_43	Ry4 Tob. KS1	
US_105	Ry4 Tob. KS3	China
US_104	Ry4 Tob. KS2	China
US_81	Q Double Bubble KS	Italy
US_97	HP Overdrive KS	Italy
US_90	Energy Drink KS	
US_45	Q Cherribakki KS	Italy
US_50	Q Blackberry Jack KS	Italy
CN_16	Mild Kretek Tobacco GD	
US_32	Q Apple CA	Italy
US_13	Q Apple KS	Italy
UK_8	Q Apple GB	Italy
US_106	Apple 2 KS2	
CN_8	Tiramisu XE	
CN_27	Licorice GD	
US_10	Licorice KS	
US_87	Banana KS	
US_48	Q Piedmont Sunrise Tobacco KS	Italy
CN_9	Cherry XE	
US_26	Tiramisu CA	
US_27	Cherry CA	
US_7	Coffee KS	
US_23	Coffee CA	
CN_5	Coffee XE	
US_49	Q The Moment KS	Italy
US_22	Apple CA	
US_85	French Pipe Tobacco KS	
US_100	HP Summer Drift KS	Italy
US_98	HP Fruity Velocity KS	Italy
US_14	Q Berry Mix KS	Italy
CN_25	Brownie GD	
US_89	Red Oriental Tobacco GD	
US_3	Apple KS1	
UK_5	Apple GB	
CN_4	Apple XE	
CN_22	Berry Mix GD	
CN_3	Berry Mix XE	
CN_24	Cola GD	
CN_14	Red Oriental Tobacco KS	
UK_4	Berry Mix GB	
UK_6	Q Berry Mix GB	Italy
US_9	Berry Mix KS	
US_82	Q Fragola Fresca KS	Italy
US_8	Cola KS	
NG_26	Strawberry LG	

CN_6	Citrus Mix XE	
US_44	Citrus Mix KS	
CN_7	Grape XE	
US_15	Q Traditional Tobacco KS	Italy
US_95	Chocolate KS	
CN_10	Strawberry XE	
US_107	Cappuccino KS	
US_96	Chocolate KS	
US_86	Blueberry KS	
US_24	Citrus Mix CA	
US_25	Grape CA	
NG_29	Vanilla LG1	
NG_31	Vanilla LG3	
NG_30	Vanilla LG2	
NG_36	Vanilla LG5	
NG_38	Vanilla LG7	
NG_35	Vanilla LG4	
NG_37	Vanilla LG6	
US_88	Vanilla KS	
US_47	Q Golden Roanoke Tobacco KS	Italy

US_46	Q Havana Libre KS	Italy
CN_13	Goldenrod Oriental Tobacco KS	
US_30	Cuban Cigar Tobacco KS	
US_28	Strawberry CA	
US_21	Berry Mix CA	
CN_19	Vermillion Oriental Tobacco GD	
CN_17	Traditional Tobacco GD	
US_18	Q Turkish Tobacco KS	Italy
US_6	Turkish Tobacco KS	
CN_12	Golden Oriental Tobacco GD	
CN_15	Virginia Tobacco GD	
CN_20	American Blend Tobacco GD	
US_84	Q Traditional Tobacco KS2	Italy
US_11	Traditional Tobacco KS	
US_5	American Blend Tobacco KS	
US_16	Q American Blend Tobacco KS	Italy

[1] Codes identifies the country of purchase: NG = Nigeria; US = United States; GB = Britain; CN = China.

[2] Refill fluid Name and Site identifies the refill fluid and the state/province where the product was purchased: GD = Guangdong, China; LG = Lagos, Nigeria; KS = Kansas, USA; CA = California, USA.
GB = Great Britain, United Kingdom, XE = Xiamen, China.
Numbers after the site of purchases identifies duplicates or triplicates purchased from one location.

[3] Location = Country where refill fluids were manufactured. This information is not available for blank boxes.

Table S6.3: Concentrations of Flavor Chemicals in “Apple” EC Refill Fluids

	Q Apple (mg/ml)[1]			Apple (mg/ml)[2]				
Flavor Chemical	US-KS	UK-GB	US-CA	US-KS2	US-KS1	US-CA	UK-GB	CN-XE
Ethyl Acetate	0.8	0.7	0.6	0.5	0.2	ND	0.0	0.0
Ethyl butanoate	1.2	1.1	1.0	1.2	0.4	ND	0.1	0.1
γ-Decalactone	ND	0.0	0.0	0.0	0.0	ND	0.0	0.0
Isoamyl Acetate	1.2	1.2	1.1	1.2	0.6	0.1	0.2	0.2
2-Methylbutyl Acetate	0.0	ND	0.0	ND	0.0	ND	0.0	ND
Ethyl Maltol	ND	ND	ND	0.1	ND	ND	ND	ND
β-Damascone	0.0	0.0	0.0	0.0	0.0	ND	ND	0.0
Ethyl 2-methylbutanoate	0.1	0.1	0.1	0.1	0.0	ND	0.0	0.0
Hexyl acetate	0.2	0.2	0.1	0.2	0.1	0.0	0.1	0.1
1-Hexanol	0.8	0.8	0.9	0.9	0.8	0.7	0.7	0.7
Isopentyl Alcohol	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.1
Isoamyl Isovalerate	0.5	0.6	0.4	0.7	0.3	0.1	0.2	0.2
Hydroxyacetone	0.2	0.2	0.5	ND	ND	1.0	0.4	0.4
Hexyl hexanoate	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Butyl Acetate	0.2	0.2	0.2	ND	0.1	ND	0.0	0.0
Ethyl lactate	0.0	0.0	0.1	0.1	0.1	ND	0.0	0.0
Ethyl Isovalerate	0.2	0.2	0.2	0.2	0.1	ND	0.0	0.0
Total Flavor Conc.	5.6	5.4	5.2	5.5	2.9	2.1	2.0	2.0

[1] *For “Q Apple,” US-KS = Kansas, USA; UK-GB = Great Britain, United Kingdom; US-CA = California, USA*

[2] *For “Apple,” US-KS2 and US-KS1 are duplicate samples from Kansas, USA; CN-XE = Xiamen, China*

Table S6.4: Flavor Chemicals Identified in Three Independent EC Studies

1. Benzyl Alcohol
2. Ethyl Maltol
3. Menthol
4. Triacetin
5. Vanillin
6. (3Z)-3-Hexen-1-ol
7. Corylone
8. Ethyl Acetate
9. Ethyl butanoate
10. Ethyl Vanillin
11. Eugenol
12. Furaneol
13. Isoamyl Acetate
14. Maltol
15. p-Menthone
16. 1-Hexanol
17. 4-Terpineol
18. Acetylpyrazine
19. Benzaldehyde PG acetal
20. Butyl Acetate
21. Carvone
22. Ethyl lactate
23. Ethyl Propanoate
24. Hexyl acetate
25. Isobutyl Acetate
26. Limonene
27. Methyl Anthranilate
28. γ-Decalactone
29. Acetoin
30. Ally hexanoate
31. Linalool
32. Strawberry Glycidate_A
33. Strawberry Glycidate_B
34. Benzaldehyde
35. Cinnamaldehyde, (E)-
36. Ethyl Cinnamate
37. p-Anisaldehyde
38. 1,4-Cineol
39. 2,3,5,6-Tetramethylpyrazine
40. 2,3,5-Trimethylpyrazine
41. 2,3-dimethylpyrazine
42. 2,5-dimethylpyrazine
43. 2-Acetylpyrrole
44. 2-Methoxy-3-methylpyrazine
45. 4-methylbenzyl alcohol
46. Acetophenone
47. Acetyleugenol
48. Benzeneacetic acid,
49. Benzyl Propionate
50. cis-Linalool oxide
51. Dimethyl butanedioate
52. Estragole (4-allyanisole)
53. Ethyl anthranilate
54. Ethyl Benzoate
55. Ethyl Isobutyrate
56. Ethyl Laurate
57. Ethyl nonanoate
58. Ethyl octanoate
59. Eucalyptol
60. Fenchol
61. Hexyl hexanoate
62. Isoeugenol methyl ether
63. Isopulegol
64. Linalyl acetate
65. Methyl N-methylanthranilate
66. Methyl phenylacetate
67. Piperitone
68. Styralyl Acetate
69. Thymol
70. trans-Linalool Oxide
71. α-Caryophyllene
72. β-Caryophyllene
73. β-Myrcene
74. γ-Pentalactone
75. *(3E)-3- Hexenyl acetate*
76. *(E)-β-Ionone*
77. *1-Pentanol*
78. *2,3-Butanedione*
79. *2,3-Pentanedione*
80. *2-Hexen-1-ol, (E)-*
81. *2-Methylbutyl Acetate*
82. *3-Hexen-1-ol, acetate, (Z)-*
83. *6-Methyl-5-heptene-2-one*
84. *Amyl Acetate*
85. *Amyl Isovalerate*
86. *Benzyl acetate*
87. *Benzyl Benzoate*
88. *Benzyl Butyrate*
89. *Benzyl cinnamate*
90. *Butyl butyrate*
91. *Butyl butyrolactate*

92. *Cinnamyl alcohol*
93. *Citral*
94. *Coumarin*
95. *Ethyl 2-methylbutanoate*
96. *Ethyl butyrate*
97. *Ethyl Heptanoate*
98. *Ethyl hexanoate*
99. *Ethyl Isovalerate*
100. *Ethyl salicylate*
101. *Furfural*
102. *Furfuryl alcohol*
103. *Geraniol Acetate*
104. *Guaiacol*
105. *Heliotropine PG acetal*
106. *Hemineurine*
107. *Hexyl 2-Linalyl*
108. *Hydrocoumarin*
109. *Hydroxyacetone*
110. *Isoamyl Butyrate*
111. *Isoamyl Isovalerate*
112. *Isopentyl Alcohol*
113. *Isosafroeugenol*
114. *Menthyl Acetate*
115. *Methyl cinnamate*
116. *Methyl salicylate*
117. *Neomenthol*
118. *Nerol acetate*
119. *p-Cymene*
120. *Pentyl acetate*
121. *Phenethyl alcohol*
122. *Piperonal*
123. *p-Tolualdehyde*
124. *Pulegone*
125. *Raspberry ketone*
126. *trans-Geraniol*
127. *α-Ionone*
128. *α-Pinene*
129. *α-Terpineol*
130. *β-Damascone*
131. *β-Pinene*
132. *γ-Nonalactone*
133. *γ-Octalactone*
134. *γ-Terpinene*
135. *δ-Decalactone*
136. *δ-Dodecalactone*
137. *δ-Undecalactone*

Red = 3 studies; Blue = 2 studies; Green = Current Study; Purple = Hua et. al., 2019; Orange = Behar et. al., 2018. Black Bold = < 1 mg/ml in current study; *Black Italics* = < 1 mg/ml in two other studies

Appendix F: Supporting Information for Chapter 7

Table S7.1. Flavor Chemicals (µg/mL) Below the Limit of Quantification

	JUUL™ Pods				**Puff**	
Compound Name	“Cool Mint” [a]	“Mint” [b]	“Classic Menthol” [c]	“Menthol” [d]	Bar “Menthol” [e]	Plus “Cool Mint” [f]
β-Caryophyllene	-	4.7	3.7	-	-	18.23
Limonene	2.4	2.8	-	-	2.83	16.93
Methyl Anthranilate	-	-	-	-	-	14.65
trans-Geraniol	-	-	-	-	-	13.25
Ethyl lactate	-	-	-	-	12.9	-
Caffeine	-	-	12.7	11.3	-	-
Hydroxyacetone	-	-	-	-	10.27	8.17
β-Damascone	-	-	-	10	-	-
(E)-β-Ionone	-	-	-	-	2	10.2
Ethyl Vanillin	-	-	-	-	9.73	-
Ethyl Maltol	-	-	8.3	7.7	-	-
cis-Linalool oxide	-	-	-	-	-	7
Methyl salicylate	-	0.6	-	-	6.5	-
Ethyl Propanoate	-	-	-	-	-	6.55
Ethyl butanoate	1	-	-	-	3.67	6.35
1-Hexanol	-	-	-	-	6.03	1.4
β-Pinene	0.8	1.6	-	-	1.67	5.73
Eugenol	-	-	-	-	-	5.25
(3Z)-3-Hexen-1-ol	5	5	-	-	-	-
Vanillin	4.9	-	2.5	-	-	-
Benzyl acetate	-	-	-	-	-	4.85
Estragole	-	-	-	-	-	4.8
Acetylpyrazine	-	-	-	4.8	1.57	4.6
α-Pinene	0.9	0.8	-	-	1	4.3
δ-Decalactone	0.7	-	1.3	4	-	-
Triethyl Citrate	-	-	-	-	3.5	-
Phenethyl alcohol	2.5	3	-	-	3.17	3.53
Methyl cinnamate	-	-	-	-	3.2	-
Thymol	4.1	4.5	1.6	-	3.13	3.03
β-Myrcene	4.4	-	-	-	-	3
δ-Dodecalactone	2.1	-	-	-	-	-
γ-Undecalactone	-	-	-	-	2.1	-
Ethyl Cinnamate	-	-	-	-	-	1.9
Ethyl 2-methylbutanoate	-	-	-	-	1	1.95
Isobutyl Acetate	-	-	-	-	1.93	-
2-Acetylpyrrole	-	-	-	-	1.7	-
γ-Decalactone	-	-	-	-	1.67	-
α-Caryophyllene	-	-	-	-	-	1.65
p-Cymene	1.5	-	-	-	-	1.63
Piperonal	-	-	-	1.5	-	-
Fenchol	-	-	-	-	-	1.4
Isopentyl Alcohol	-	3.5	-	-	1.13	-
Isoamyl Acetate	-	-	-	-	-	1.05
Ethyl Isovalerate	-	-	-	-	-	1
2-Hexen-1-ol, (E)-	-	-	-	-	0.97	-

γ-Terpinene	1.9	0.9	4.3	-	0.5	0.9
2,3,5,6-Tetramethylpyrazine	-	-	-	-	-	0.9
Benzaldehyde	1	1.3	-	-	-	0.6

Note: Concentrations of flavor chemicals are an average of 3 - 5 different pods

[a]= “Cool Mint” purchased in 2018

[b]= “Mint” purchased in 2019

[c]= “Classic Menthol” purchased in 2018

[d]= “Menthol” purchased in 2019

[e]= “Menthol” is a Puff Bar variant

[f]= “Cool Mint” is a Puff Plus variant

Appendix G: Supporting Information for Chapter 8

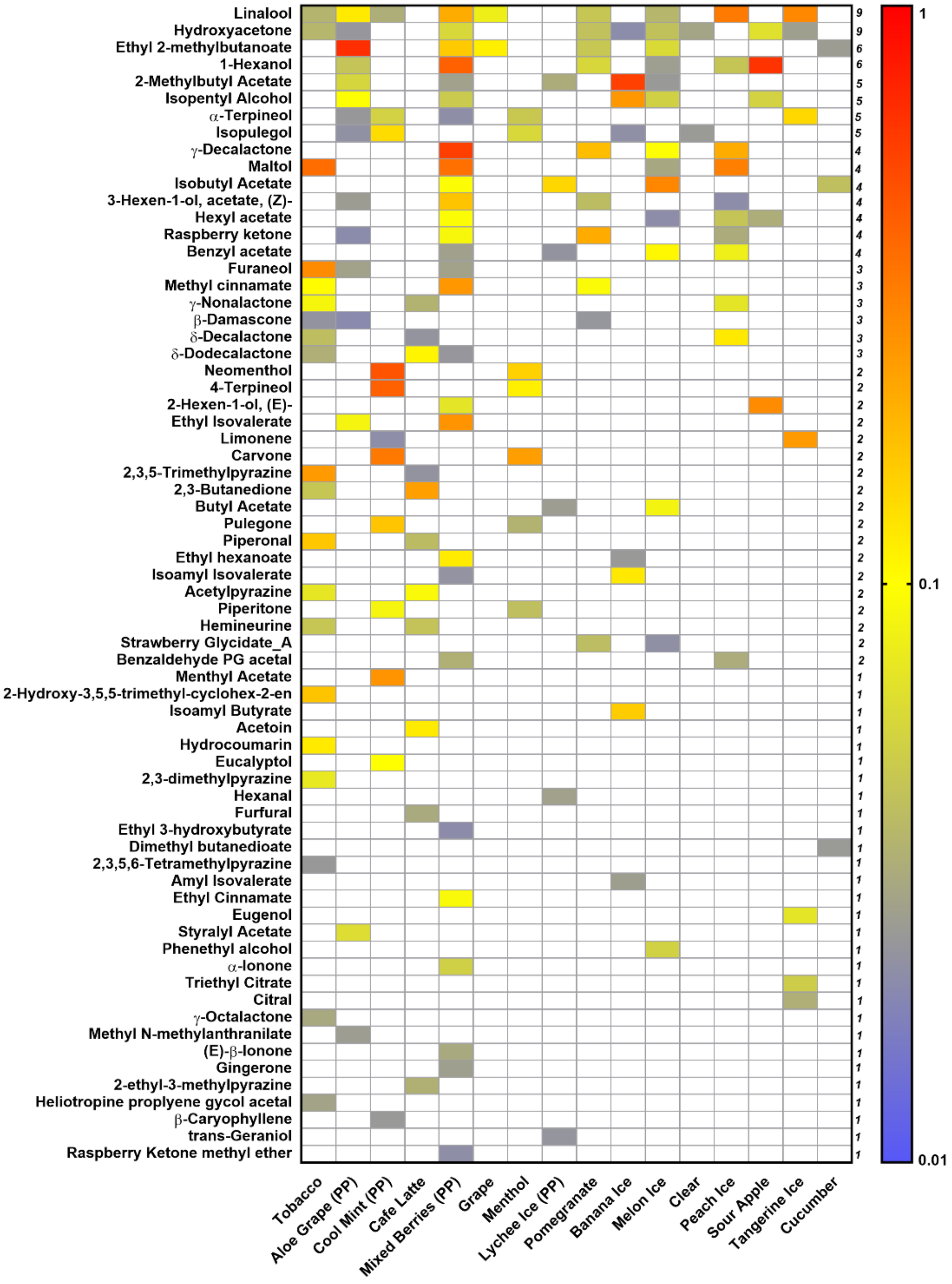

Figure 8.1. Heat map of 87 flavor chemicals above the LOQ in Puff EC fluids. Chemicals are ordered on the y-axis according to frequency of occurrence of flavor chemicals from top to bottom. Products are ordered on the x-axis according to the total weight (mg/mL) of the flavor chemicals in each product with the highest concentration at the left. The color gradient on the right shows the concentrations of the flavor chemicals in the heat map

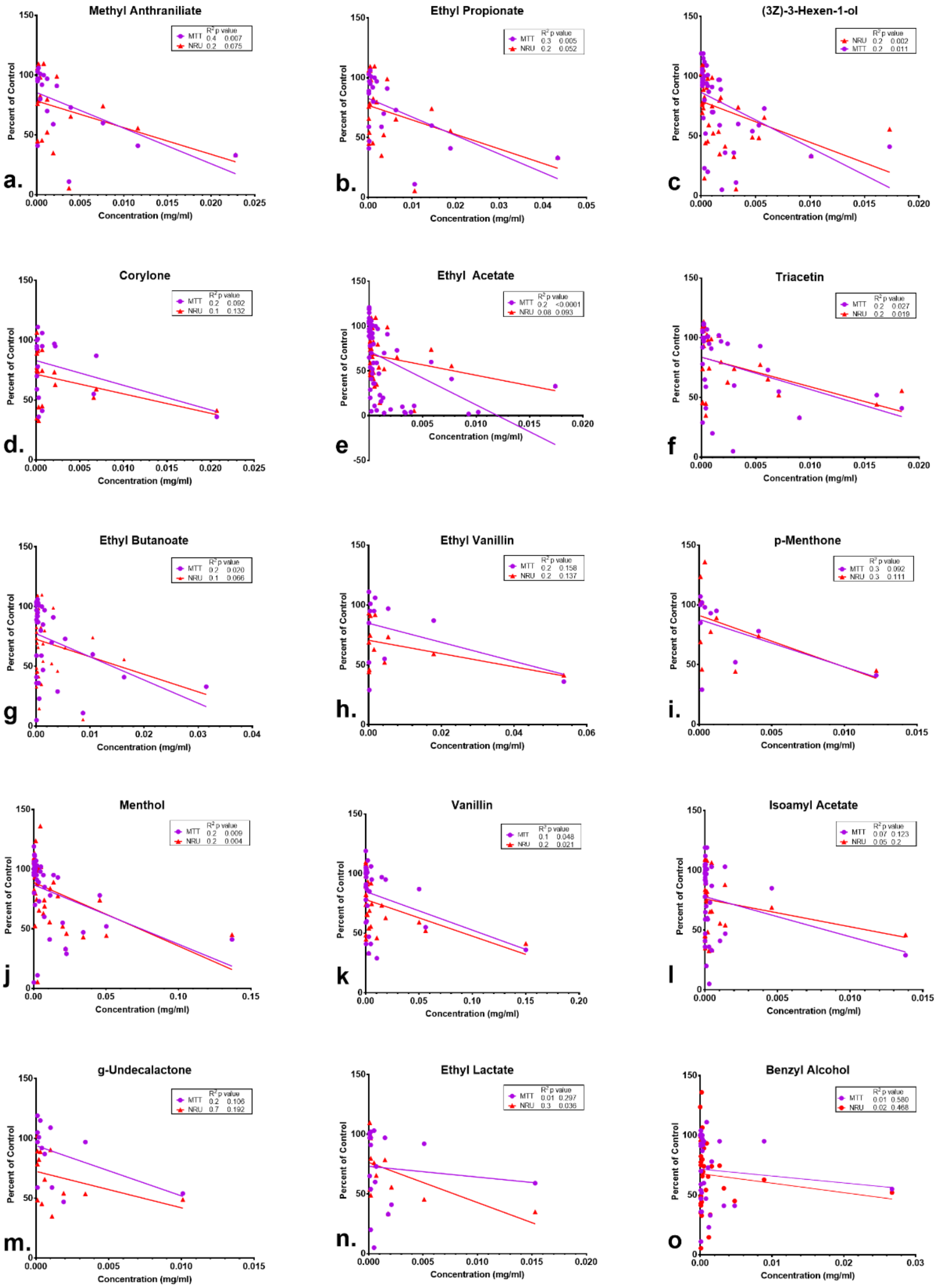

Figure 8.2. Regression analyses of other dominant flavor chemicals

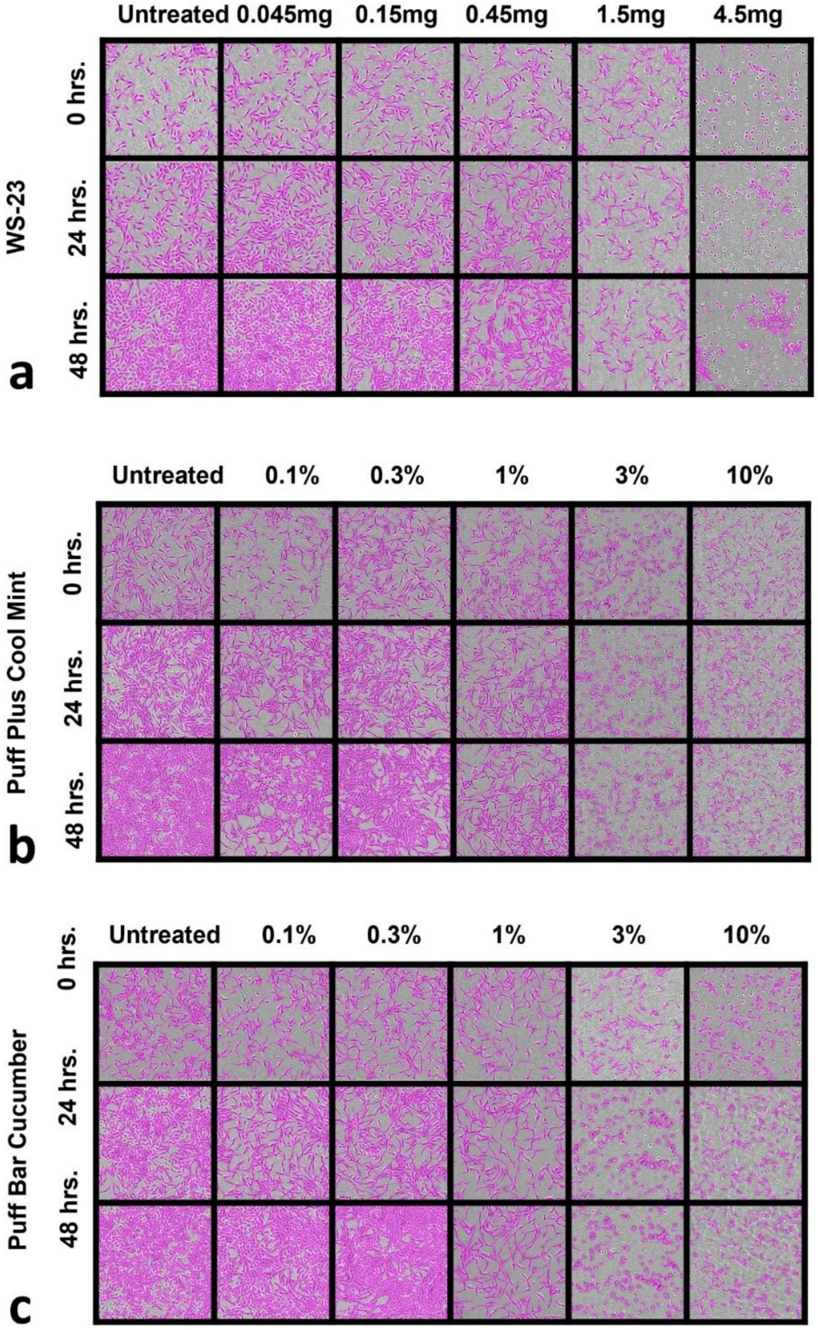

Figure 8.3. Micrographs showing segmented cells in the live cell imaging assay taken at 0, 24, and 48 hours

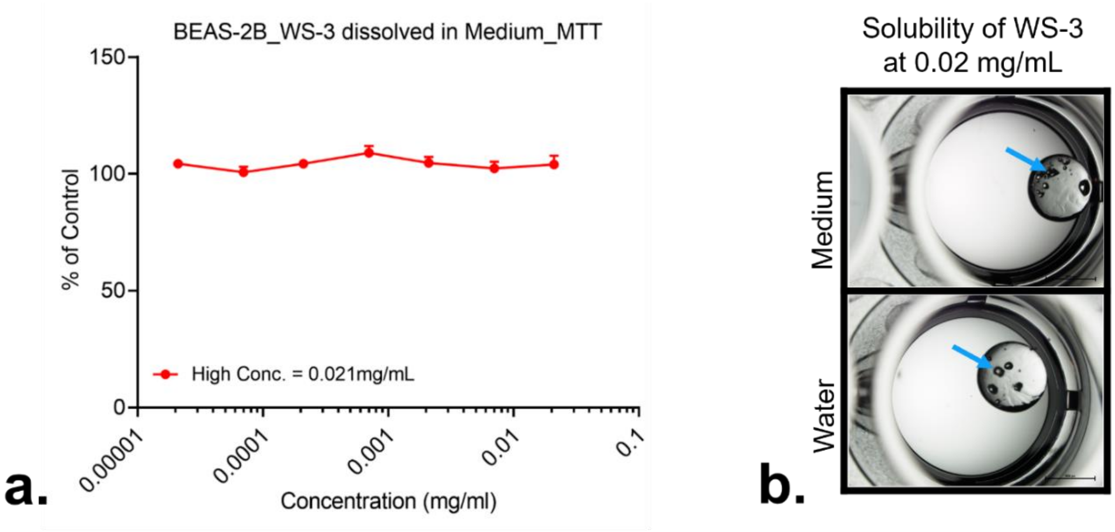

Figure S8.4. MTT assay concentration-response curve and solubility of WS-3. (a) MTT concentration-response curve for BEAS-2B cells treated with WS-3. The y-axis shows the response of cells as a percentage of the untreated control. Each point is the mean ± standard error of the mean of three independent experiments. (b) the solubility of WS-3 in culture medium and water at the highest concentration (0.02 mg/mL) used in the MTT assay. Each sample contains a glass bead to enable focusing on the liquid. Each bead has several black air bubbles (arrows). No precipitate is present in the medium or water solution containing WS-3 at 0.02 ug/mL

Table S8.1. Flavor Chemicals Detected Below the Limit of Quantification (0.02 mg/mL)

Flavor Chemical	CAS Number	Frequency
Benzaldehyde	100-52-7	10
2-Acetylpyrrole	1072-83-9	9
Furfuryl alcohol	98-00-0	6
1,2-Dihydrolinalool	18479-51-1	5
6-Methyl-5-heptene-2-one	110-93-0	4
Ethyl Benzoate	93-89-0	4
Strawberry Glycidate B	77-83-8	4
Benzeneacetic acid, ethyl ester	101-97-3	3
α-Pinene	80-56-8 (7785-70-8)	3
β-Pinene	127-91-3	3
γ-Terpinene	99-85-4	3
2,5-dimethylpyrazine	123-32-0	2
Benzyl Benzoate	120-51-4	2
Butyl butyrate	109-21-7	2
Ethyl anthranilate	87-25-2	2
Ethyl Heptanoate	106-30-9	2
Ethyl octanoate	106-32-1	2
Fenchol	1632-73-1	2
Guaiacol (2-methoxyphenol)	90-05-1	2
Isoeugenol methyl ether	93-16-3	2
Methyl 2-methylbutyrate	868-57-5	2
p-Anisaldehyde	123-11-5	2
p-Cymene	99-87-6	2
Thymol	89-83-8	2
β-Myrcene	123-35-3	2
1,4-Cineol	470-67-7	1
1-Pentanol	71-41-0	1
2-Nonanone	821-55-6	1
Acetophenone	98-86-2	1
Ally hexanoate	123-68-2	1
Amyl Acetate	628-63-7	1
Benzyl Butyrate	103-37-7	1
cis-Linalool oxide	5989-33-3	1
Coumarin, 6-methyl	92-48-8	1
Estragole (4-allyanisole)	140-67-0	1
Geraniol Acetate	105-87-3	1
Isosafroeugenol	94-86-0	1
Methyl phenylacetate	101-41-7	1

[1] Frequency = number of times the flavor chemical appeared in at least one Puff Bar EC pod fluid

Table S8.2. Major and Minor Non-target Chemicals in Puff EC Fluids

Sample	Major Non-targets	Minor Non-target
Sour Apple	Benzoic acid Acetic acid 2-Hydroxypropyl acetate 1,2-Propanediol-2-acetate	
Aloe Grape (PP)	Benzoic acid Acetic acid 2-Hydroxypropyl acetate 1,2-Propanediol-2-acetate	acetin (mixture of 2 isomers) 2-Hydroxypropane-1,3-diyl diacetate
Menthol	Benzoic acid 2-Hydroxypropane-1,3-diyl diacetate Glycerol 1,2-diacetate	Neoisomenthol, menthone isomer, 2-Hydroxypropyl acetate, 1,2-Propanediol-2-acetate
Pomegranate	Benzoic acid	Methyl (3-oxo-2-pentylcyclopentyl) acetate
Cafe Latte	Benzoic acid	vanillin PG and GL acetals
Tobacco	Benzoic acid	vanillin PG and GL acetals ethyl vanillin PG and GL acetals
Melon Ice	Benzoic acid	(6Z)-Nonen-1-ol 2-(hydroxymethyl)-5-oxidanyl-2,3-dihydropyran-4-one
Cool Mint (PP)	Benzoic acid	Neoisomenthol, menthone isomer
Tangerine Ice	Benzoic acid 2-Hydroxypropyl acetate 1,2-Propanediol-2-acetate	
Peach Ice	Benzoic acid 2-Hydroxypropyl acetate 1,2-Propanediol-2-acetate	
Clear	Benzoic acid	
Cucumber	Benzoic acid	
Grape	Benzoic acid	
Banana	Benzoic acid	
Lychee Ice (PP)	Benzoic acid	
Mixed Berries (PP)	Benzoic acid	

Table S8.3: Coolant Concentrations Other EC Products

	Refill Fluids	Pod Fluids	Cartomizer Fluids
WS-3			
Green Smoke Menthol			0.18
Green Smoke Menthol			0.20
Q Honeydew Drop	0.08 ± 0.014		
Love Potion	0.58 ± 0.101		
Popsuckle	1.65 ± 0.480		
WS-23			
Zalt Mango		<LOQ	
Cinnamon Bomb with menthol drops		<LOQ	
JUUL Cool Cucumber		0.03	
JUUL Classic Menthol		0.11 ± 0.02	
Zalt Berry Lemonade		1.46	
Zalt Blue Raspberry		1.94	
Two Mints	2.58 ± 0.24		
Iced reds apple juice	3.87 ± 0.86		

Table S8.4: Flavor Profiles of Dominant Chemicals in Puff EC Fluids

Chemical	CAS #	FEMA #	FEMA Flavor Profile
Ethyl Maltol	4940-11-8	3487	Fruit
Ethyl Acetate	141-78-6	2414	Aromatic, Brandy, Contact Glue, Grape
(3Z)-3-Hexen-1-ol	928-96-1	2563	Grass, Green Fruit, Green Leaf, Herb, Unripe Banana
Vanillin	121-33-5	3107	Vanilla
Ethyl butyrate	105-54-4	2427	Apple, Butter, Cheese, Pineapple, Strawberry
Menthol	15356-70-4	2665	Mint, cool
Benzyl Alcohol	100-51-6	2137	Boiled Cherries, Moss, Roasted Bread, Rose
Triacetin	102-76-1	2007	Fruity (www.thegoodscentscompany.com)
Isoamyl Acetate	123-92-2	2055	Apple, Banana, Glue, Pear
Corylone	765-70-8	2700	Caramellic (www.thegoodscentscompany.com)
Ethyl Propanoate	105-37-3	2456	Apple, Pineapple, Rum, Strawberry
Ethyl lactate	97-64-3	2440	Cheese, Floral, Fruit, Pungent, Rubber
Methyl Anthranilate	134-20-3	2682	Flower, Honey, Peach
Ethyl Vanillin	121-32-4	2464	Floral
p-Menthone	10458-14-7	2667	Green, Fresh, Mint
γ-Undecalactone	104-67-6	3091	Apricot, Fruit
WS-23	51115-67-4	3804	Cooling
WS-3	39711-79-0	3455	Cooling

Table 8.5. Chemicals in EC Fluids and Average Maximum Levels (ppm) Generally Regarded as Safe for their Intended Uses

	Puff EC Fluids							Beverages		
Chemical Name	**EC (Low)**	**EC (High)**	**Chewing Gum**	**Hard Candy**	**Frozen Dairy, Ices**	**Baked Goods**	**Gelatins, Puddings**	**Non-Alcoholic**	**Alcoholic**	**Others**
Ethyl Maltol	70.6	9898	83	27.9	144	152	119	12.4	18.6	140
Ethyl Acetate	20.4	2653.3	10000	7500	110	211	200	67	200	5000
(3Z)-3-Hexen-1-ol	23.4	2411.5		5	3.7	5		1		
Vanillin	21	16539.9	445	200	95	220	120	97	450	0
Ethyl butanoate	44.6	4506.4	1400	98	44	93	54	28		
Menthol	251.2	18739.9	1100	400	68	130		35		
Benzyl Alcohol	23	2671.5	1200	47	160	220	45	15		
Triacetin	39.1	2814.6	4100	560	2000	1000		190		
Isoamyl Acetate	24.9	1538.9	2700	190	56	120	100	28		
Corylone	24.4	2109.6	15	18	5.6	13	14	11		30
Ethyl Propanoate	27.8	6588.4	1100	78	29	110	15	7.7		
Ethyl lactate	22.1	1525.2	3100	28	17	71	8.3	5.4	1000	35
Methyl Anthranilate	26.6	3423.4	2200	56	21	20	23	16	0.2	
Ethyl Vanillin	20.8	5860	110	65	47	63	74	20	100	28000
p-Menthone	20.9	1488.1	8.7	71	33	52		7.7		
γ-Undecalactone	114	1059.8	90	11	3	7.1	7.5	4.4		
WS-23	832.9	45143.8	3000	50					8	150
WS-3	1442.5	16356.4	1200	100	10		10	10	10	10

Notes: Others include meat sauces, icings and toppings, soft candy, confectionery, frostings, syrups, jams and jellies, imitation vanilla, sweet sauces, fats and oils, meat products, poultry, milk products

References: Oser and Ford, 1977 (ethyl maltol); Cohen et. al., 2020 (ethyl acetate, vanillin); Hall and Oser 1968 ((3Z)-3-Hexen-1-ol, ethyl butanoate, menthol, benzyl alcohol, triacetin, isoamyl acetate, corylone, ethyl propanoate, ethyl lactate, methyl anthranilate, ethyl vanillin, p-menthone, γ-undecalactone); Smith et al., 1996 (WS-23); Newberne et. al., 1998 (WS-3)

Appendix H: Supporting Information for Chapter 9

Table S9.1: Product Information for Tobacco Flavored Refill Fluids and E-Liquids

	Company/Brand	Name of Refill Fluid	Purchase Year	Label Code
1	Johnson Creek	JC Original	2011	JC_O #1
2	Johnson Creek	Tennessee Cured	2011	JC_TC #1
3	Johnson Creek	Tennessee Cured	2011	JC_TC #2
4	Johnson Creek	JC Original	2011	JC_O #2
5	Johnson Creek	Tennessee Cured	2011	JC_TC #3
6	Johnson Creek	French Vanilla	2011	JC_FV
7	Johnson Creek	Espresso	2011	JC_E #1
8	Johnson Creek	Espresso	2011	JC_E #2
9	Johnson Creek	Arctic Menthol	2011	JC_AM #2
10	Red Oak	Domestic	2011	RO_D #2
11	Red Oak	Swiss Dark	2011	RO_SD #2
12	Red Oak	Tennessee Cured	2011	RO_TC #4
13	Johnson Creek	Arctic Menthol	2011	JC_AM #1
14	Red Oak	Marcado	2011	RO_M #2
15	Red Oak	Domestic	2012	RO_D #1
16	Red Oak	Swiss Dark	2012	RO_SD #1
17	Red Oak	Wisconsin Frost	2012	RO_WF
18	Red Oak	Marcado	2012	RO_M #1
19	LiQua	American Blend Tob.	2015	L_AB #1
20	LiQua	Traditional Tob.	2015	L_Td #1
21	LiQua	Q Traditional Tob.	2015	L_Q-Td #1
22	LiQua	Q American Blend Tob.	2015	L_Q-AB
23	LiQua	Cuban Cigar Tob.	2015	L_CC
24	LiQua	Turkish Tob.	2015	L_Tk
25	LiQua	Q Turkish Tob.	2015	L_Q-Tk
26	LiQua	Bright Tob.	2015	L_B #1
27	LiQua	Bright Tob.	2015	L_B #3
28	LiQua	Bright Tob.	2015	L_B #4
29	LiQua	Bright Tob.	2015	L_B #6
30	LiQua	Bright Tob.	2015	L_B #7
31	LiQua	Traditional Tob.	2016	L_Td #2
32	LiQua	American Blend Tob.	2016	L_AB #2
33	LiQua	Q Traditional Tob.	2016	L_Q-Td #2
34	LiQua	Golden Oriental Tob.	2016	L_GO
35	LiQua	Goldenrod Oriental Tob.	2016	L_GRO

36	LiQua	Q Havana Libre (Cuban Cigar)	2016	L_Q-HL
37	LiQua	Vermillion Oriental Tob.	2016	L_VO
38	LiQua	Red Oriental Tob.	2016	L_RO #1
39	LiQua	Red Oriental Tob.	2016	L_RO #2
40	LiQua	Virginia Tob.	2016	L_V
41	LiQua	Q Golden Roanoke (Virginia Tob.)	2016	L_Q-GR
42	LiQua	Q Piedmont Sunrise (Bright leaf Tob.)	2016	L_Q-P
43	LiQua	Bright Tob.	2016	L_B #2
44	LiQua	Bright Tob.	2016	L_B #5
45	LiQua	Q The Moment (Tob. purity)	2016	L_Q-TM
46	LiQua	Mild Kretek Tob.	2016	L_MK
47	LiQua	French Pipe Tob.	2016	L_FP
48	LiQua	Ry4 Tob.	2016	L_Ry4 #1
49	LiQua	Ry4 Tob.	2016	L_Ry4 #2
50	LiQua	Ry4 Tob.	2016	L_Ry4 #3
51	LiQua	Ry4 Tob.	2016	L_Ry4 #4
52	Vape Place	Captain Black Tob.	2019	VP_CB
53	Vape Place	House Blend Tob.	2019	VP_HBT
54	Halo	Turkish Tobacco (Robust Tob.)	2019	H_Tk
55	Canyon Crest VS	Desert Ship	2019	CCV_DS
56	Canyon Crest VS	Marvel	2019	CCV_M
57	Canyon Crest VS	Ry4	2019	CCV_Ry4
58	VaporFi	Sahara Gold Tob.	2019	VF_SG
59	VaporFi	Ry4 Caramel Tob.	2019	VF_Ry4C
60	Canyon Crest VS	Clove Tob.	2019	CCV_CT
61	Canyon Crest VS	Black Flag Fallen	2019	CCV_BFF
62	Halo	Tribeca (Smooth Tob.)	2019	H_Tb
63	Charlie Noble	Turkish Tob. Fig Almond	2019	CN_Tk
64	JUUL	Virginia Tob.	2018	J_VT
65	JUUL	Classic Tob.	2019	J_CT
66	Puff Bar	Tobacco	2020	PB_T

Appendix I: Supporting Information for Chapter 10

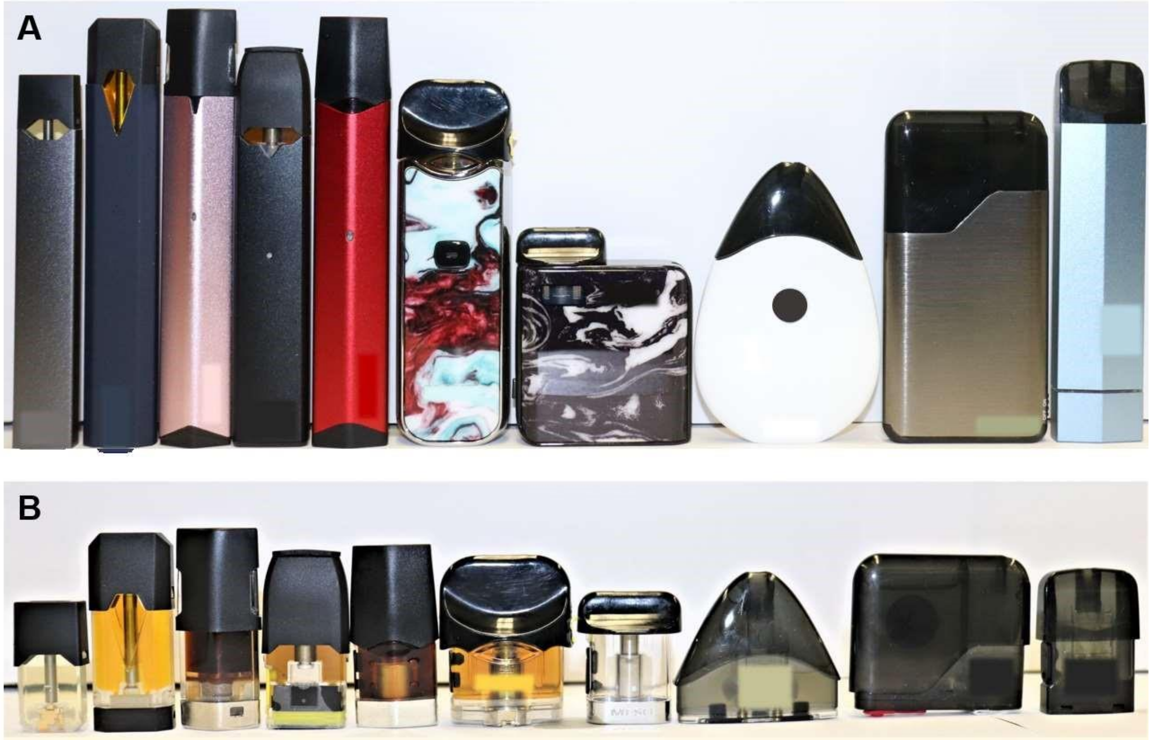

Figure S10.1. Fourth-generation pre-filled and refillable EC pod products. (A) ready to use pod devices with batteries, and (B) pods only arranged by brand from left to right as follows; JUUL, KILO 1K, PHIX, KWIT Stick, SMOK Infinix, SMOK NORD, SMOK Mico, Suorin Drop, Suorin Air, and Suorin Edge. (TIF)

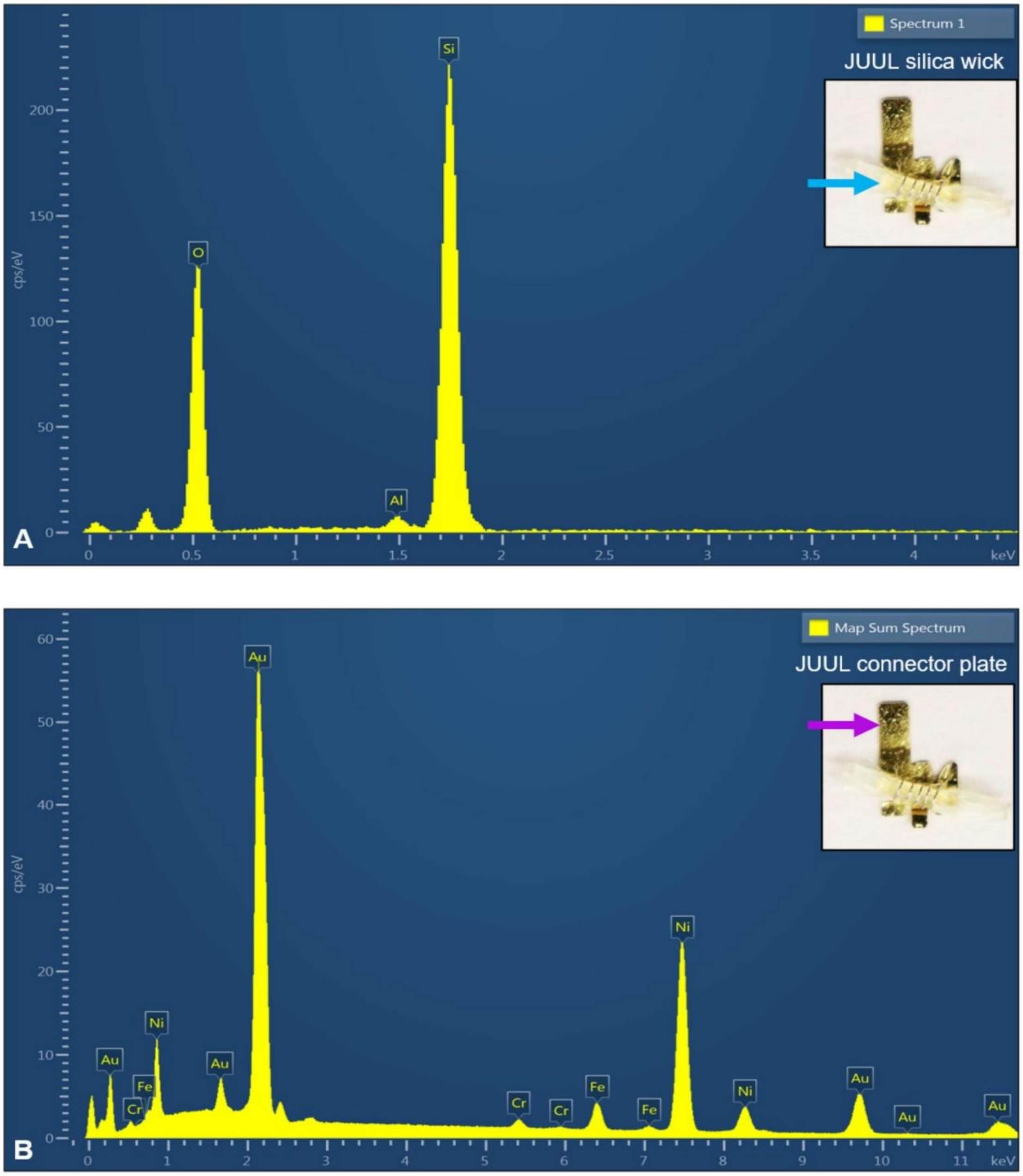

Figure S10.2. EDS spectra of JUUL atomizer components. (A) wick, which comprises silicon and oxygen, and (B) the connector plate, mainly nickel coated with gold. The Blue arrow indicates the wick, and the purple arrow indicates the connector plate

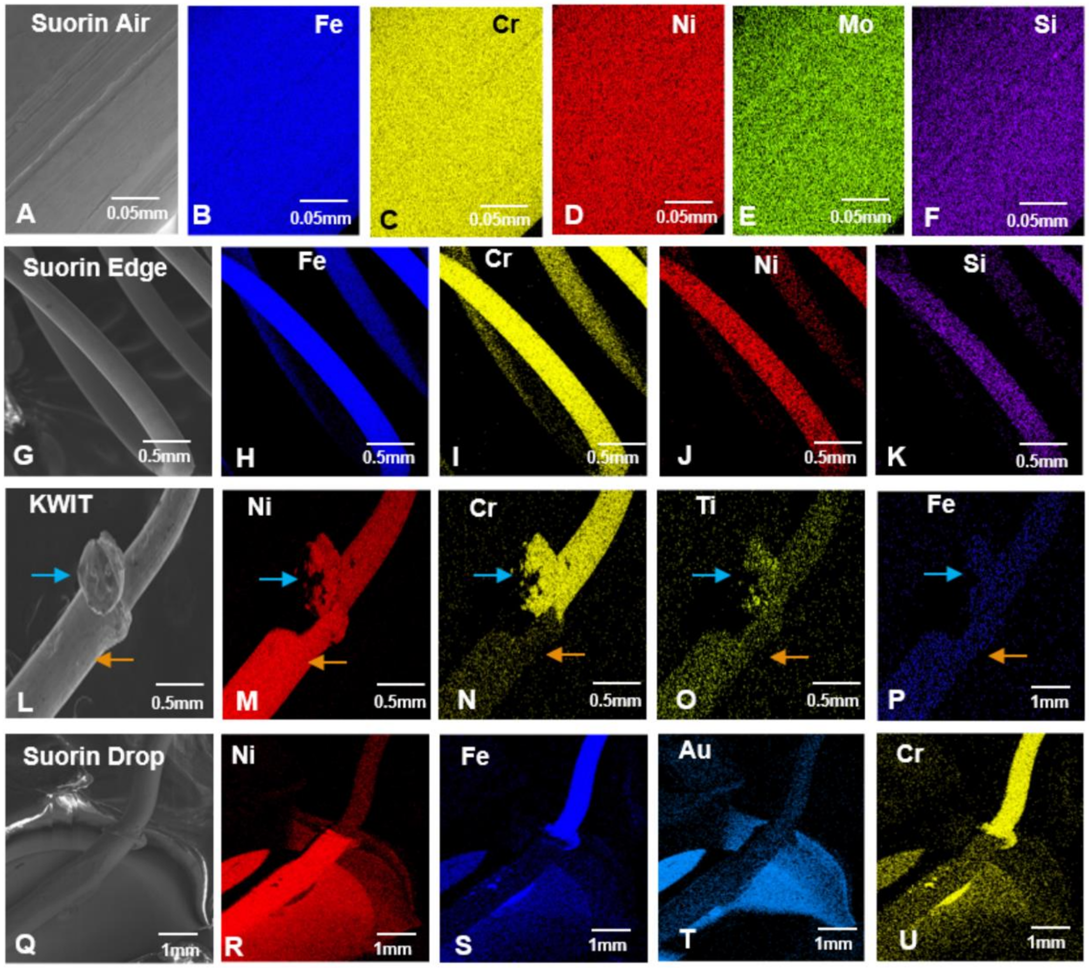

Figure S10.3. Scanning electron microscopy images and EDS elemental maps of the filaments and joints. For filaments, Suorin Air (A) was made of iron (B), chromium (C), nickel (D), molybdenum (E), and silicon (F). Suorin Edge (G) was made of iron (H), chromium (I), nickel (J), and silicon (K). For the wire-to-wire joints, KWIT Stick (L) was made of nickel (M), chromium (N), titanium (O), and iron (P). For connector-to-wire joints of the Suorin Drop (Q) was made of iron (S) and chromium (U) but not nickel (R) and gold (T).

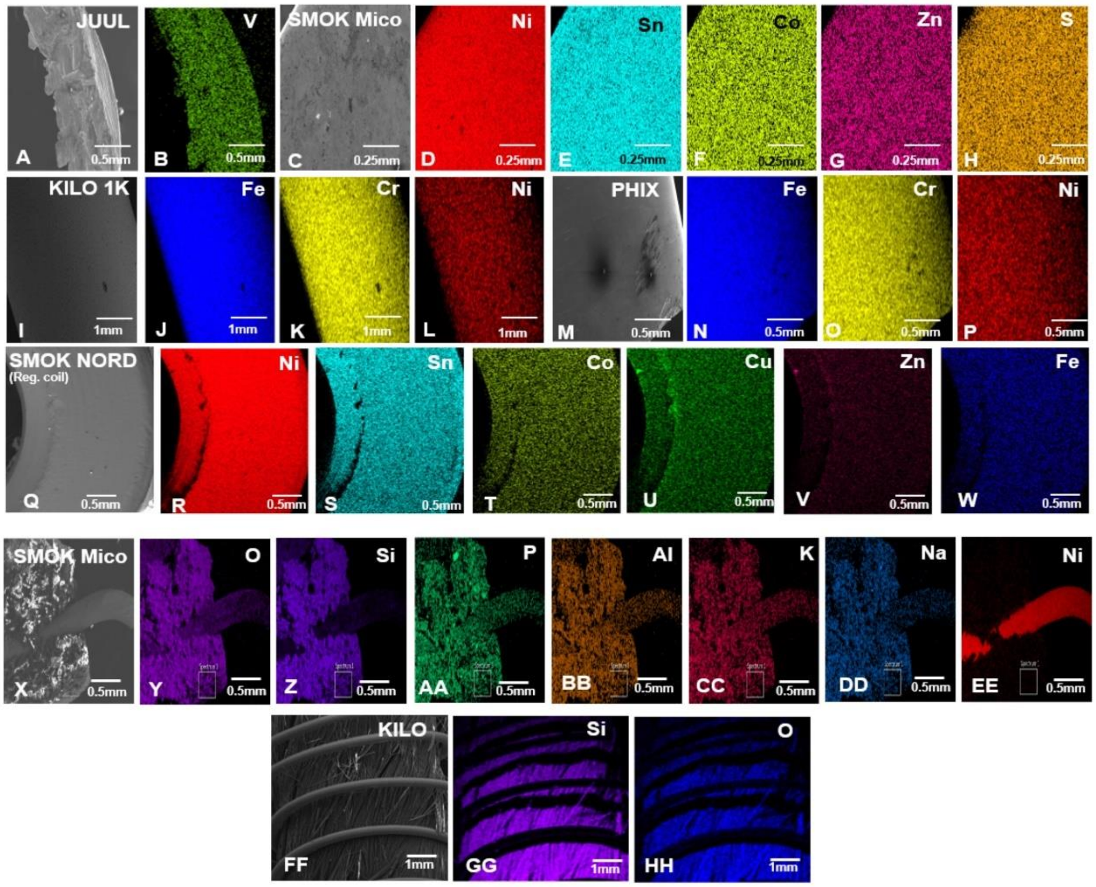

Figure S10.4. Scanning electron microscopy images and EDS elemental maps of the air-tubes and wicks. For the air tubes, JUUL (A) also contained vanadium (B). (C) SMOK Mico was made of nickel (D), tin (E), cobalt (F), zinc (G), and sulfur (H). (I) KILO 1K was made of iron (J), chromium (K), and nickel (L). (M) PHIX was made of iron (N), chromium (O), and nickel (P). (Q) SMOK NORD (regular coil) was made of nickel (R), tin (S), cobalt (T), copper (U), zinc (V), and iron (W). The SMOK Mico ceramic wick (X) contained oxygen (Z), silicon (AA), phosphorus (BB), aluminum (CC), potassium (DD), sodium (EE), but not nickel (Y). The KILO 1K organic wick (FF) was silicon and (GG), and oxygen (HH)

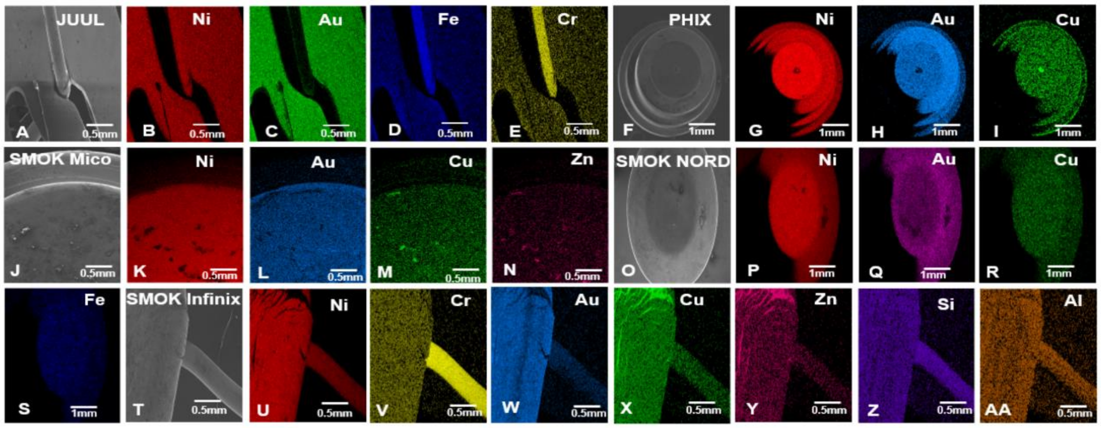

Figure S10.5. Scanning electron microscopy images and EDS elemental maps of connector components. Connectors present in JUUL (A) were made of nickel (B), gold (C), iron (D), and chromium (E). PHIX (F) was made of nickel (G), gold (H), and copper (I). SMOK Mico (J) was made of nickel (K), gold (L), copper (M), and zinc (N). SMOK NORD (O) was made of nickel (P), gold (Q), copper (R), zinc, and iron (S). SMOK Infinix (T) was made of nickel (U), gold (W), copper (X), zinc (Y), silicon (Z), and aluminum (AA) but not chromium (V).

Appendix J: Supporting Information for Chapter 11

Table S11.1: Properties of the elements investigated

Element	**Elemental Class**	**Limit of Detection (mg/L)**	**Melting Point** (°C)	**Boiling Point** (°C)
Aluminum	Post-Transition	0.0043	660	2519
Boron	Metalloids	0.0028	2075	4000
Cadmium	Transition	0.00069	321	767
Calcium	Alkaline Earth	0.0026	842	1484
Chromium	Transition	0.0020	1907	2671
Cobalt	Transition	0.0010	1495	2927
Copper	Transition	0.0024	1084	2562
Iron	Transition	0.00066	1538	2861
Lead	Post-Transition	0.0067	327	1749
Magnesium	Alkaline Earth	0.00047	650	1090
Nickel	Transition	0.00081	1455	2913
Potassium	Alkali	0.0017	64	759
Silicon	Metalloids	0.0027	1414	2900
Silver	Transition	0.0020	962	2162
Sodium	Alkali	0.0111	98	883
Tin	Post-Transition	0.0040	232	2602
Titanium	Transition	0.00035	1668	3287
Vanadium	Transition	0.0011	1910	3407
Zinc	Transition	0.00050	420	907