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Identification, Quantification, and Evaluation of the Cytotoxicity of Electronic Cigarette Exhaled Aerosol Residue (ECEAR)

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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Dedication

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ABSTRACT OF THE DISSERTATION

Identification, Quantification, and Evaluation of the Cytotoxicity of Electronic Cigarette Exhaled Aerosol Residue (ECEAR)

by

Careen Khachatoorian

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
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Dr. Prue Talbot, Chairperson

Electronic cigarettes (ECs) produce EC exhaled aerosol residue (ECEAR), which is the residue left on surfaces when EC users have exhaled or stopped vaping. It contains nicotine, nicotine alkaloids, tobacco specific nitrosamines (TSNAs), as well as flavor chemicals. The purpose of this dissertation was to identify and quantify chemicals in ECEAR and to detect cytotoxicity of ECEAR using *in vitro* models. Firstly, refill fluids and their aerosols were analyzed to quantify flavor chemicals and nicotine and to determine their transfer efficiency to aerosols. Human participants then vaped the same refill fluids to create exhale which was used to identify and quantify what is retained and what contributes to ECEAR. Not only did nicotine and cinnamaldehyde transfer better than other flavor chemicals, they were better retained by users, especially cinnamaldehyde. Participants were divided into two topographies: mouth and lung inhalers. The mouth inhalers exhaled more chemicals, while lung inhalers retained almost all inhaled flavor chemicals and nicotine. Keratinocytes and EpiDerm tissues were exposed to refill fluids at various concentrations and times. Cytotoxicity was induced at a 1% concentration of Churrios refill fluid and reactive oxygen species (ROS) were increased in keratinocytes. EpiDerm skin tissues had increases in the secretion of IL-1α, IL-6, and MMP-9 upon exposure to Dewberry Cream and Churrios refill fluids, indicating an inflammatory

response. Churrios ECEAR extract also increased IL-1α secretion. These data show that refill fluids and ECEAR can induce an inflammatory response and the production of ROS in skin cells. The concentrations of nicotine and tobacco alkaloids were then measured in ECEAR collected from three field sites. A vape shop sample exposed for 1 month contained as much as 108 mg of nicotine/m^2, and the concentration increased with exposure time. The living room of an individual EC user had ECEAR, although concentrations were lower than in the vape shop. Lastly, ECEAR was detected and quantified in a business located near a vape shop in a multiunit tenant building, indicating that EC aerosols can move through ventilation systems and contaminate indoor surfaces away from their origin. In conclusion, ECEAR builds up on indoor surfaces and can affect the skin of those actively or passively exposed.

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Chapter 1: Introduction

What are Electronic Cigarettes?

Electronic cigarettes (ECs) are noncombustible nicotine delivery devices that were first marketed as a smoking cessation tool and a healthy alternative to conventional cigarettes (U.S. FDA, 2020). Since their emergence into the U.S. market in 2007, these novel devices have quickly gained worldwide popularity, becoming a multi-billion-dollar industry (U.S. FDA, 2020). Approximately 5% of U.S. adults reported being current e-cigarette users (Al Rifai M, 2020) but even more troublesome is that 3.05 million American high school students are currently using ECs.

ECs, which are battery-powered, can deliver varying concentrations of nicotine, flavor chemicals, and solvents as an aerosol. When the user takes a puff, a heating element is activated converting the liquid in the EC into an aerosol that the user holds in

1st generation 2nd generation 3rd generation 4th generation

Figure 1.1 Different generations of ECs

the mouth or inhales (Williams and Talbot, 2011) . ECs do not contain tobacco or require combustion, but some do mimic the design of a conventional cigarette (Trtchounian et al., 2010). This kind of EC, often called a “cig-a-like”, is classified as a first generation “closed” system that can be disposable or refillable (Fig. 1.1). Second generation ECs are usually called “clearomizers” and have a clear tank that allows the user to fill the tank with the fluid of their choice. Third generation ECs, also known as mods, are completely customizable with respect to voltage, wattage, amperage, air intake, and batteries. Some mods operate at very low resistance (subohms), and these ECs are often used in vaping competitions because they produce large clouds of aerosol. Fourth generation ECs which include the pods can be disposable or reusable (Fig. 1.1). Each type of EC generates different amounts of aerosol with variable chemical components. The pod-based ECs (e.g., JUUL) use benzoic acid with nicotine ranging from 0-60 mg/mL to create a nicotine salt, which is much easier to inhale and does not produce much irritation on the throat. They also have a sleek and compact design that many companies have come to replicate. Flavored JUUL pods, such as “Mango” and “Crème Brulee”, have recently been banned by the U.S. Federal Drug Administration (FDA) due to their unethical marketing to young children and teenagers, but menthol and tobacco flavored JUUL pods are still available for purchase (FDA, 2020). Recently, the EC marketplace has opened up to new competitors selling disposable flavored ECs, such as Puff Bars. These do not fall under the FDA ban on flavors as they are disposable and single use.

In the United States, ECs fall under the Tobacco Control Act. The FDA made this determination in a “deeming rule”. This meant ECs were deemed subject to the act that went into effect on August 8th, 2016 (Sharpless, 2019). Also on Aug. 8th, it became immediately illegal to sell ECs and other ENDS to people younger than 18. Retailers

became legally responsible for requiring age verification by photo ID for individuals under 27 to purchase a tobacco product.

Refill Fluids, E-liquids, and Juices

"Refill fluids" can be purchased separately and added to empty cartridges, cartomizers or tanks by the user, while most pods are sold prefilled with e-juice. All fluids contain solvents, such as propylene glycol (PG) or vegetable glycerin/glycerol (VG/G), that allow the flavors and nicotine to dissolve so that aerosolization of the fluid occurs upon heating (Uryupin et al., 2013). PG is an FDA approved food additive that is used in cosmetics and medicines. It can also be found in antifreeze and de-icing agents for cars, planes, and boats. However, short term exposure to PG causes eye, throat, and airway irritation (Wieslander et al., 2001;Vardavas et al., 2012) and long-term exposure can result in children developing asthma (Choi et al., 2010). VG/G is a solvent used instead of, or in combination with PG in EC fluids for aerosol production. The FDA considers VG relatively safe to ingest, and it is used in ECs to carry flavors such as tobacco, menthol, coffee, chocolate, cinnamon, and vanilla. These solvents also carry nicotine into the lungs of the user.

Nicotine

Nicotine is a weak base, and in its ionized state (acidic), doesn't rapidly cross membranes. When nicotine reaches the lungs, it is rapidly absorbed because of the dissolution of nicotine at a pH of 7.4. Nicotine binds to the nicotinic acetylcholine receptors (nACHRs) that trigger the release of dopamine. These receptors are expressed by endothelia, pulmonary epithelia, immune, and muscle cells (Benowitz and

Fraiman, 2017; Bhatnagar, 2016;Lam et al., 2016). In addition, nicotine has many effects: it raises blood pressure and heart rate, curbs appetite, increases basal metabolic rate, activates bowel movements, lowers urine production, promotes blood coagulation, stimulates breathing, increases pain sensitivity and may cause nausea and vomiting. In the brain, nicotine promotes release of several neurotransmitters causing various psychological effects, which may lead to dependence (Benowitz, 2010). Use of nicotine-containing e-cigarettes raises the heart rate and increases the pulse of the user in the first 5 minutes (Schaller et al., 2013).

Nicotine is the addictive component of conventional cigarettes and ECs; however, large amounts of nicotine are lethal (60 mg in adults; 6 mg in children), but recently these numbers have been revised to 500-1000 mg for adults and 10 mg for children (Mayer, 2013). Nicotine concentrations vary in cartridges/cartomizers. Usually, an EC contains between 0 and 24 mg/mL, while some refill fluids have up to 100 mg/ml of nicotine. For JUUL ECs, a nicotine base and a weak acid such as benzoic acid is used to form a nicotine salt (Voos et al., 2019) that delivers nicotine and flavor for efficient nicotine absorption. JUUL pods contain 61.6 mg/mL of nicotine in its 5% nicotine pod (Cao et al., 2020), although a previous study found concentrations between 59.2 and 66.7 mg/mL (Omaiye et al., 2019b).

Flavors

As of 2017, there were about 20,000 unique e-liquid flavors available for sale in the Netherlands (Havermans et al., 2019). Figure 1.2 shows various refill fluids. A recent analysis of U.S. data showed that the most common e- liquid flavor categories among adult EC users were menthol/mint (37.4%), fruit (31.2%), and candy/other sweets

(16.2%)(Schneller et al., 2018) while another study found the most popular refill fluid flavor to be “Berries/Fruits/Citrus” using internet survey and local and online sales information (Hua et al., 2019). Sweet flavors are particularly appealing to youth. Many EC products contain more than one flavoring chemical (average is approximately 6), and those with sweet flavors contain more chemicals compared with tobacco and menthol flavored liquids (Czoli et al., 2019).

There are high concentrations of flavor chemicals in some e-liquids (Behar et al., 2016) (Omaiye et al., 2019b) and adverse effects have been observed in these studies, such as depolymerization of microtubules in human pulmonary fibroblasts. “Dewberry

Figure 1.2 Various flavors of e-liquids. https://vaporferver.com/e-liquid-reviews/

Cream” is a refill fluid with 47 flavor chemicals including ethyl maltol, maltol, vanillin, ethyl vanillin, benzyl alcohol, and furaneol in high concentrations (>1 mg/ml), which were all cytotoxic in the MTT assay at 1 mg/mL (Hua et al., 2019). Cinnamaldehyde, a flavor chemical used in many e-juices was found in 51% of the products sampled (Behar et al.,

2016) and there is evidence that cinnamaldehyde induces cytotoxicity in *in vitro* exposure studies (Bahl et al., 2012; Behar et al., 2014; Behar et al., 2016; Rowell et al., 2017; Omaiye et al., 2019b). It also increases inflammatory responses, impairs neutrophil phagocytotic function, and impairs ciliary motility in bronchial epithelial cells (Clapp and Jaspers, 2017; Clapp et al., 2019; Lerner et al., 2015; Muthumalage et al., 2018). One study found cinnamaldehyde in 20 out of 39 e-liquids tested with concentrations ranging from $1.7x10^{-5}$ to 1.1M (Behar et al., 2016). Aldehyde flavor chemicals, such as cinnamaldehyde and benzaldehyde, react and degrade during heating of the e-liquid to produce toxic chemicals, such as formaldehyde, methylglyoxal, acrolein, and acetaldehyde (Gillman et al., 2016; Erythropel et al., 2018; Flora et al., 2016; Goniewicz et al., 2013; Jensen and Luo, 2015).

Chemicals in EC Aerosols

Chemicals present in EC aerosol include nicotine, solvents, particulate matter such as ultra-fine particles, volatile organic chemicals (VOCs) (Hutzler et al., 2014; Kosmider et al., 2014; Logue et al., 2017; Schripp et al., 2012; Sleiman et al., 2016) carcinogens, such as formaldehyde and acrolein (Goniewicz et al., 2013), heavy metals like nickel and lead (Williams et al., 2017; Williams et al., 2013; U.S. Department of Health and Human Services. Centers for Disease Control and Prevention, 2016) and flavoring agents such as diacetyl and cinnamaldehyde, similar to what is found in unheated refill fluids. The processes of oxidation and/or dehydration of PG and G creates several additional chemicals, such as formaldehyde, acetaldehyde and acrolein (Flora et al., 2016; Goniewicz et al., 2014; Jensen and Luo, 2015).

VOCs

The most common volatile organic chemicals (VOCs) in EC aerosols are benzene, toluene, ethylbenzene, and p,m, xylene (McAuley et al., 2012). Benzene is a known human carcinogen and can be produced in cigarette smoke. It has also been found in two refill tank systems formed from solvents (PG and G) and additives (benzoic acid and benzaldehyde). Levels of benzene ranged between 1.8 $\mu g/m^3$ and 5000 $\mu g/m^3$, in this study (Pankow et al., 2017). Although these levels are much lower than what is found in traditional cigarettes (200,000 $\mu g/m^3$), it is a cause for concern due to the repeated exposures.

Aldehydes

Formaldehyde, acetaldehyde, and acrolein are three toxic low-molecular weight aldehydes present in cigarette smoke (700–800 µg/cigarette) as well as e-cigarette aerosols (8.2 to 40.4 µg /10 puffs) (Ogunwale et al., 2017). Many chemicals in refill fluids increase due to the heating and aerosolization process leading to toxic aldehyde emissions. It is known that both VG and PG undergo pyrolysis at high temperatures to yield low molecular weight carbonyl compounds including formaldehyde and acetaldehyde. Additionally, small amounts of acrolein can also be produced from PG and VG at higher temperatures (Paine et al., 2007). According to Kosmider et al. 2014, levels of formaldehyde in vapors from high voltage ECs were in the range of levels reported in traditional cigarettes and an increase from 3.2 to 4.8 volts resulted in increased formaldehyde, acetaldehyde, and acetone levels (Kosmider et al., 2014).

These results were in line with other studies done on different ECs (Havel et al. 2017; Qu et al. 2018). Studies suggest that the level of aldehydes in EC aerosols can approach those from traditional cigarettes, up to 344.6 µg for formaldehyde and 206.3 µg for acetaldehyde, if the EC device is used at higher power settings, such as 5 V and more, or during dry puff conditions (Farsalinos et al., 2015b).

Metals

EC heating coils are usually made of nichrome, combination of nickel (Ni), and chromium (Cr), and stainless steel. Toxic metals such as cadmium (Cd) and lead (Pb) have been detected in EC aerosol from the leaching of heated coils (Prokopowicz et al., 2018). Using scanning electron microscopy with liquids from different brands or single-use, rechargeable, and pod devices from different years, copper and zinc were found to be elevated in liquids from devices containing brass. Cr, Ni, Cu, Zn, Sn, and Pb were reported up to 0.396, 4.04, 903, 454, 0.898, and 13.5 µg/g respectively. Elevated metal concentrations in the liquid were also elevated in aerosol from the corresponding device (Gray et al., 2019). Ni and Cr are toxic for humans and classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC); inhalation of these metals is associated with chronic bronchitis and reduced lung function (Agency for Toxic Substances & Disease Registry Public Health Statement for Chromium, 2020). In addition, cartomizer fluid with tin particles was also tested on human pulmonary fibroblasts and found to be cytotoxic (Williams et al., 2013).

TSNAs

Tobacco-specific nitrosamines (TSNAs) are chemicals that remain in the tobacco leaves after the curing process. Figure 1.3 shows the chemical structures of nicotine, its alkaloids, and TSNAs. N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), TSNAs that are known carcinogens, are included on the FDA's list of harmful and potentially harmful constituents (HPHCs) in tobacco products (FDA, 2020). NNK and NNN are present in both refill fluids (Kim and Shin, 2013) and EC aerosols (Goniewicz et al., 2014; McAuley et al., 2012), however, the concentrations are usually low compared to cigarette smoke. Interestingly, a study has shown that as the nicotine concentration increased from 0 to 16 mg in an EC cartridge, the number and concentration of TSNAs increased as well (Laugesen, 2008). One brand of EC contained small amounts of all four TSNAs, and only NNK and NNN were found in another brand (Westenberger 2009, Dept Health & Human Services FDA), while NNN and NNK were detected in 9 out of 12 EC brands in another study (Goniewicz et al., 2013). The maximum concentrations of total TSNAs in replacement liquids of E-cigarettes were measured at 86.92 µg /L in 105 replacement liquid brands of 11 companies (Kim and Shin, 2013). Finally, a study found that the TSNA levels present in

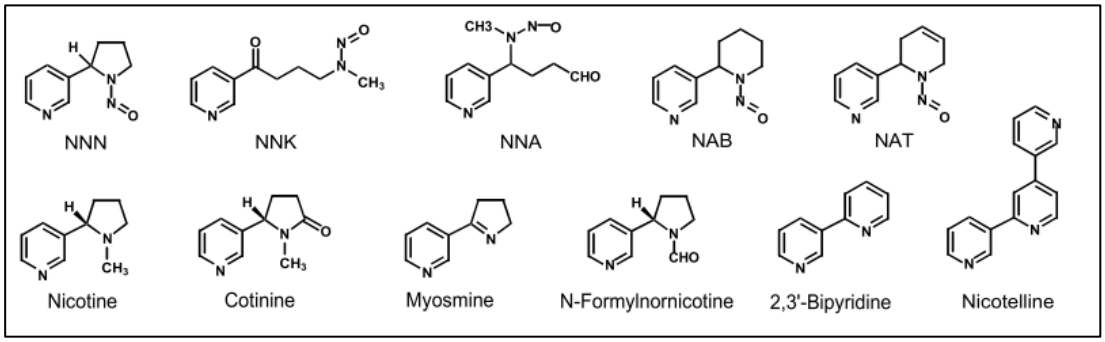

Figure 1.3 Nicotine, alkaloids, and tobacco specific nitrosamines

the aerosol are similar to those present in the liquid (Farsalinos et al., 2015a). TSNAs continue to be studied in ECs and EC aerosols, due to their harmful effects.

Figure 1.4 EC user produces "clouds" of EC aerosols, usually made when mixing various amounts of PG and VG. Image: Shutterstock.com PublicDomain

Particulate Matter and Ultra Fine Particles (UFP)

Increases in device power settings, such as voltage and wattage, and user topography, such as increased puff duration and flow rate, lead to increased aerosol emissions and particle concentrations (Y. Chen et al., 2018; Gillman et al., 2016; H. V. Nguyen and Sheikh, 2018; Zhao et al., 2016). Particle concentrations also increase with higher puff flow rates, longer puff durations, and greater interpuff intervals (Chen et al., 2018; Gillman et al., 2016; Zhao et al., 2016). High VG, produces ultrafine particles, contributes to the "cloud chasing" phenomenon usually done with high powered customizable ECs (50-250 watts) with low nicotine concentrations (2-8 mg/mL) (Figure 1.4). Users inhale and exhale large amounts of aerosol to determine who can blow the largest clouds. However, pod-based or disposable systems do not produce large volumes of aerosol. They are non-customizable, low powered devices (4-7 watts) with

high nicotine concentrations (24-56 mg/mL), and low VG/high PG concentrations (Floyd and Marcham, 2018).

The heating of the EC causes volatile droplets to form due to PG's high vapor pressure. Therefore, not only are these volatiles inhaled but coagulation causes the high number of particles to be reduced, while particle size increases (Floyd et al., 2018; Mikheev et al., 2016). The size and level of particulates in EC aerosols have a bimodal particle size distribution of ultrafine particles (UFPs) and submicron particles (96–175 nm) (Mikheev et al., 2016), with power and coil resistance greatly affecting EC aerosol count and mass distribution of particles. This can be of concern as UFP can deposit deep in the lung and long-term exposure to UFP is associated with chronic inflammatory diseases such as COPD (Traboulsi et al., 2017). A dosimetry study estimated that an average of 6.25×10^{10} particles are deposited in the bronchioles of the lungs after one puff from an EC (Manigrasso et al., 2015). These EC particles can settle and lead to surface deposition creating additional exposure of EC users and passive exposure of non-users (Goniewicz and Lee, 2015) but, increasing ventilation or air exchange rate can allow for the evaporation of particles and reduce particle concentration and size (Floyd et al., 2018; Ingebrethsen et al., 2012; Nguyen et al., 2019). Similarly, increasing temperature or decreasing relative humidity can also enhance evaporation and reduce particle size (Schripp et al., 2013; Wright et al., 2016).

In vitro toxicity to EC

Toxicity caused by refill fluids and the aerosols that are generated may be the result of many factors including the composition of the fluid, the temperature, puff duration and volume, and the cell type. Many studies have looked into the cytotoxicity of refill fluids and aerosols from ECs using *in vitro* studies. The toxicity of aerosolized flavored liquids and individual flavor chemicals can alter biological functions of respiratory cells, leading to the loss of transepithelial resistance, increase in reactive oxygen species (ROS), and an increase in inflammatory markers (Behar et al., 2018; Lerner et al., 2015; Gerloff et al., 2017; Rowell et al., 2017; Sassano et al., 2018; Sherwood and Boitano, 2016; Nair et al., 2020).

A screening study of 35 products had variations in cytotoxicity of refill fluids to human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) using the MTT assay (Bahl et al., 2012). Stem cells, more than other cell types, seemed to be highly affected upon exposure in other studies as well (Behar et al., 2016). A more recent study found that out of the 126 flavor chemicals detected in 103 bottles of refill fluid, the levels of furaneol, benzyl alcohol, ethyl maltol, ethyl vanillin, corylone, and vanillin were significantly correlated with cytotoxicity with mNSCs and human bronchial epithelial cells (BEAS-2B) (Omaiye et al., 2020). Menthol and tobacco aerosol-treated mNSCs showed an increase in superoxide production, induction of calcium influx, and hyperfusion of mitochondria (Zahedi et al., 2018).

Not only are the refill fluids cytotoxic at various concentrations, but the aerosols produced from the heating of the fluids have been shown to induce toxicity. An *in vitro* study demonstrated that isolated human alveolar macrophages exposed to EC vapour

induces inflammation and reduces phagocytosis which could mean that an EC user is more susceptible to pulmonary infections (Scott et al., 2018). In a gene expression profiling study done on primary human bronchial epithelial cells (HBECs) grown at Air Liquid Interface, EC aerosol exposure decreased expression of genes involved in cilia assembly and movement. The aerosol also induced gene-expression changes in bronchial airway epithelium. Interestingly, changes were generally less pronounced than the effects of traditional cigarette exposure and were more pronounced in EC products containing nicotine than those without nicotine (Moses et al., 2017).

In vivo toxicity to EC

Mouse models are an important next step to understand in vivo exposures and toxicities of ECs. Studies have demonstrated that chronic inhalation of EC aerosol may lead to cardio-renal and hepatic disease, increased inflammation, and organ damage (Crotty Alexander et al., 2018), as well as decreased cardiac fractional shortening and ejection fraction (Espinoza-Derout et al., 2019). Chronic inflammation is thought to drive the development and progression of lung cancer and COPD and exposure to cigarette smoke causes airway inflammation. A 3-day exposure to EC aerosols showed higher levels of Muc5ac, a predominant gel-forming mucin that is induced during allergy, in mice (Glynos et al., 2018). Interestingly, Muc5ac also increased in the airways of smokers and EC users (Reidel et al., 2018). In a similar study, Lerner et al. (2016) exposed mice to EC aerosol containing nicotine for 5 hours/day for 3 consecutive days. This brief exposure regime resulted in reduced lung glutathione levels and increases in

certain pro-inflammatory cytokines (IL-6, MCP-1, IL-1α and IL-13) in BAL fluid (Lerner et al., 2016).

A lipidomics study on mice demonstrated that EC aerosols composed solely of solvents (PG and VG), without nicotine, responded with a decrease in innate immunity and a disruption in lipid homeostasis (Madison et al., 2019). Moreover, aerosols decrease the production of the antiviral protein SPLUNC1 by epithelial cells and thus increase susceptibility to infection by rhinovirus, a respiratory virus that is the primary cause of the common cold (Madison et al., 2019). A follow up study focused on a 70/30% mix of VG/PG evaluated genes related to immunotoxicity of mice and showed an increase in markers of lung inflammation, such as IL-6, and lipid-based immune mediators. In addition, when vanilla was added to the solvents, there were increases in the percentage of NK, DC, CD4 and CD19 immune lung cells (Szafran et al., 2020). Similar to the in vitro studies, the in vivo studies also showed that EC users could be more susceptible to pathogens and bacterial infections.

Health effects of EC use

Several case reports, studies, reviews, and data mined from Internet forums have researched the health effects related to EC use (Hua et al., 2013; Hua and Talbot, 2016; Pisinger and Døssing, 2014; Li et al., 2016). These reports found respiratory, cardiac, and digestive system effects, but also highlighted the (un)intentional poisonings and injuries due to explosion. A thorough review by the Electronic Nicotine Delivery Systems Committee for the National Academies of Sciences, Engineering, and Medicine was done on ECs (National Academies of Sciences, Engineering, and Medicine, 2018).

This review was to inform the public of the health risks and benefits of ECs. It addressed many questions related to health, specifically looking at cardiovascular disease, cancers, respiratory and oral diseases, and developmental and reproductive effects.

Many pulmonary problems such as acute eosinophilic pneumonia, bronchiolitis obliterans, and primary spontaneous pneumothorax have been reported in EC users (Landman et al., 2019). Acute eosinophilic pneumonia is characterized by filling of the alveolar airspaces with eosinophil-rich infiltrate and has been reported in a study of 22 patients (Philit et al., 2002; Bonilla et al., 2019). Bronchiolitis obliterans is caused by inflammation and obliteration of small airways. chronic inhalation of diacetyl, a buttery flavor “generally recognized as safe” for ingestion, can lead to the development of bronchiolitis obliterans or “popcorn lung,”. Diacetyl as well as 2,3-butadione have been found in e-liquids and refill fluids (Allen et al., 2016).

There is growing evidence that EC exposure decreases host defense mechanisms similar to cigarette smoking. Vaping decreases the expression of immune genes in nasal scrape biopsies including chemokine (C-X-C Motif) ligand 2 (CXCL2), chemokine (C-X3-C Motif) receptor 1 (CX3CR1), as well as cluster of differentiation 28 (CD28) (Martin et al., 2016). More recently, researchers have been using biomarkers to study the effects of ECs. Biomarkers of harm, 8-isoprostane and 8-OHdG, and urinary biomarkers of exposure such as cotinine and urinary metals were detected in higher levels in EC users compared to non-smokers. Overall, these studies suggest that ECs can disturb the respiratory system, immune response, and production of biomarkers.

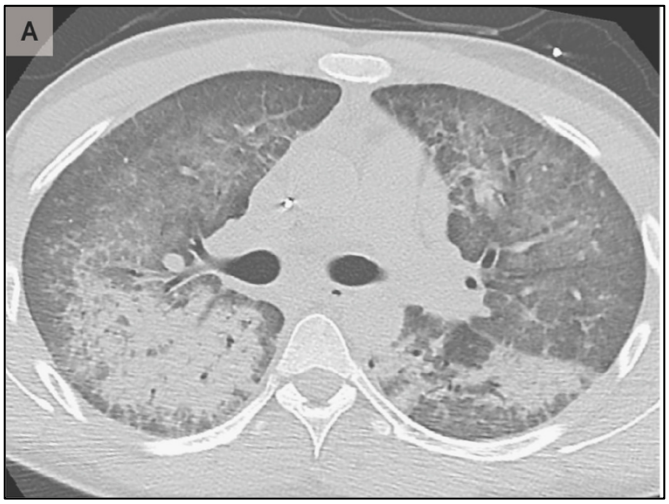

Figure 1.5 Computed tomographic scan of chest obtained from patients with vaping-associated lung injury. Image: Reproduced with permission from Imaging of Vaping-Associated Lung Disease. N Engl J Med 2019; 381:1486-1487. Doi:10.1056/NEJMc1911995, Copyright Massachusetts Medical Society.

EVALI

Starting with the first case in August 2019, young patients were hospitalized with shortness of breath, cough, chest pain, fever and chills amongst other symptoms (Yale Medicine, 2020). After chest imaging, patients showed acute eosinophilic pneumonia, diffuse alveolar damage, organizing pneumonia, and lipoid pneumonia. Many patients had bronchial dilation typical of diffuse alveolar damage and diffuse ground-glass opacity determined through computed tomographic scans of the chest (Figure 1.5). All pathological findings from the clinical study done on cases of these patients were attributable to vaping (Henry et al., 2019).

This national outbreak due to the use of ECs was called EVALI (e-cigarette, or vaping, product use-associated lung injury). Nationwide, 82% of patients hospitalized with EVALI reported using a product with THC and vitamin E acetate, an additive to THC-containing EC or vaping products (Krishnasamy et al., 2020). Some hospitalized patients reported only the use of nicotine containing ECs, therefore it is possible that

there was underreporting of THC containing products due to personal or legal reasons. The long-term effects of EVALI on lung health are unknown as of now; however, there is evidence that chronic use of ECs can lead to serious adverse health effects. In some cases, patients have required lung transplants. In 2019, there were 2,807 confirmed hospital admission cases in the United States and 68 deaths due to EVALI. The median age at hospital admission was 24 years, but the median age of death was 49.5 years (CDC, 2020). Additionally, EC use and dual use of EC and cigarettes are significant underlying risk factors for COVID-19 (Gaiha et al., 2020). These findings demonstrate the potential consequences that chronic EC use can have on human health, as well as the need to evaluate the long-term effects of ECs and EVALI.

Exhaled EC Aerosols

While less is known about the composition of exhaled EC aerosol or secondhand aerosol, several studies have found PG, VG, VOCs, metals and nicotine, as well as $PM_{2.5}$ particles and ultrafine particles in exhaled aerosol (Saffari et al., 2014; Schripp et al., 2012). Most studies evaluating exhaled breath of EC users have used machines to model exhaled aerosols (Palmisani et al., 2019; Son et al., 2020b), while only a few studies quantified selected constituents in the exhaled breath of human participants (Papaefstathiou et al., 2019; Long, 2014; Marco and Grimalt, 2015; St Helen et al., 2016b; Gallart-Mateu et al., 2016). In a modeled vape bar study, formaldehyde and acrolein exceeded the permissible acute 1-hour exposure levels of the California OEHHA levels when a high voltage EC device was used. The 8-hour levels were also

calculated for lower voltage ECs and showed high levels of the same chemicals (Logue et al., 2017). When investigating vape shops and vaping conventions, $PM_{2.5}$ particles reached concentrations of 600 to 800 $\mu g/m^3$ (Li et al., 2020; Nguyen et al., 2019; Soule et al., 2017). An *in vitro* study concluded that $PM_{2.5}$ may be the primary constituent that mediates e-cigarette-induced platelet activation and aggregation (Szołtysek-Bołdys et al., 2014). As mentioned before, there is also high variability in the size distributions of exhaled particles and levels of VOCs from different EC users due to devices, EC settings, and liquids (Papaefstathiou et al., 2019). These exhaled chemicals and particles can be re-inhaled by EC users or inhaled by non-users leading to passive exposure and have the potential to negatively impact health.

The individual and population harms of ECs are important to study to determine what the user retains and subsequently exhales into the environment. In one study, the retention of nicotine, PG, and VG in EC users was 93.8%, 91.7%, and 84.4%, respectively (St Helen et al., 2016a). Machine puffing was used to generate EC aerosols and estimate the potential exposure but did not consider the fraction of the aerosol retained in the respiratory tract (McAuley et al., 2012; Pellegrino et al., 2012; Zhang et al., 2012; Czogala et al., 2014; Bekki et al., 2014; Geiss et al., 2015). Other studies incorporated respiratory tract deposition by reporting levels of selected constituents from environmental samples collected in a room where EC had been used (Czogala et al., 2014; Ballbè et al., 2014; Schober et al., 2014; Maloney et al., 2016). However, the amount and rate of nicotine delivered may depend on user topography, such as puff duration, or the nicotine concentration, or the flavor (Dawkins and Corcoran, 2014; Hiler et al., 2017; Hajek et al., 2017; St Helen et al., 2016b; Voos et al., 2019). The retention of nicotine is influenced by several factors, such as the pH of the refill fluids or

protonation by benzoic acid (Helen et al., 2018; Duell et al., 2018; Pankow et al., 2017). The pH of EC aerosols varies from 4.85–9.6, and aerosols that are more alkaline (pH 6.5 or higher) result in nicotine existing primarily in the free-base form (unprotonated) which crosses the cell membrane for more rapid absorption (Stepanov and Fujioka, 2015). Newer ECs such as JUUL and PuffBar contain a nicotine base and a weak organic acid (benzoic acid) that forms a nicotine salt (Pankow et al., 2017). Nicotine salts are less irritating to the lungs when inhaled, making them more palatable to naïve users and enabling delivery of higher concentrations of nicotine (Omaiye et al., 2019a; Rao et al., 2020). It is possible that users of acid containing pods would get even more nicotine than they would from a traditional cigarette. This is an area of concern since younger generations are using pod based and disposable ECs that contain benzoic acid and higher nicotine concentrations, which may lead readily to addiction.

Passive Exposure to EC Aerosols and ECEAR

Not only are active users exposed to EC aerosols, but bystanders may also be passively exposed. Figure 1.6 shows how ECs are used in outdoor spaces, such as restaurants or cafes, passively exposing others surrounding the EC user to exhaled aerosol. Secondhand exposures to EC exhaled aerosols are common in homes, cars, bars, and workplaces (Giovenco et al., 2014), where it is possible to passively expose vulnerable populations such as children or pregnant mothers (Drehmer et al., 2017) (Wang et al., 2017). Ballbé and colleagues measured salivary cotinine in nonusers who lived with exclusive EC users and found significantly higher levels of cotinine in these nonusers, 0.19 ng/ml, compared to nonusers, 0.07 ng/ml, living in homes where no

smoker or EC user was living (Ballbè et al., 2014). Other studies have found evidence of secondhand exposure to harmful chemicals, such as metals and TSNAs (Chen et al., 2018; Saffari et al., 2014). A bystander exposure experiment modeled a daily car trip of one hour in a car with two EC users and concluded that bystanders may experience irritation to the upper respiratory tract and eyes, and systemic effects of nicotine, including an increased heart rate and higher systolic blood pressure when exposure took place in confined spaces (Visser et al., 2019).

Figure 1.6 Passive exposure to EC Aerosols and ECEAR. Image: ©iStockphoto/mediaphotos.

When EC users exhale, the chemicals that are in the aerosol settle on indoor surfaces where they accumulate, and form EC exhaled aerosol residue (ECEAR). Passive exposure to ECEAR can occur through inhalation, dermal absorption, or ingestion even after EC use has stopped. Very little data exist on ECEAR and most studies measure $PM_{2.5}$ and chemicals in surface wipes after the use of EC (Melstrom et al., 2017; Marcham et al., 2019; Son et al., 2020a). *Ad libitum* EC use by three participants in an 1858 ft^3 room for 2 hours lead to deposition of nicotine in the amounts of 2.1 ng/100cm^2/hour using disposable and 4 ng/100cm^2/h using tanks ECs. In the same study, residue on cloth samples had 44.4 ng/100 cm^2/h using disposables and 69.6 ng/100 cm^2/h using tanks (Melstrom et al., 2017). Nicotine can also react with ambient nitrous acid (HONO) to form TSNAs in the environment (Bahl et al., 2014) (Sleiman et al., 2010). Surface wipes from the inside of 5 vape shops measured nicotine, NNA, and NNK at levels of 223.6 ± 313.2 μg/m^2, 4.78 ± 11.8 ng/m^2, and 44.8 ± 102.3 ng/m^2, respectively (Son et al. 2020). When materials left inside a vape shop were measured, substantial amounts of nicotine (0-2073 μg/m^2) had deposited (Son et al., 2020a). However, another study done by the CDC in a vape shop with exceptional ventilation and cleaning procedures did not detect any nicotine with surface wipes (Zwack et al., 2017). This is likely due to the thorough cleaning procedures and dedicated exhaust systems inside the vape shop and is not representative of most vape shops. Therefore, it is crucial for vape shops or any indoor spaces where ECs are used

to have dedicated exhaust systems in place so that aerosols do not settle and re-emit into the air.

Indoor settings where ECs are used could cause involuntary inhalation, ingestion, or dermal uptake of EC aerosols and ECEAR chemicals. This is especially true for employees and customers inside vape shops and family members inside a home where ECs are used. Direct dermal uptake of nicotine from air can exceed that of inhaled nicotine (Bekö et al., 2017a). Wearing nicotine contaminated clothes could result in an over 17-fold higher nicotine exposure than wearing uncontaminated clothes (Bekö et al., 2017a). Most worrisome is the exposure that toddlers and small children receive in a home with ECEAR due to their frequent mouthing of fabrics and touching surfaces and floors (Bekö et al., 2017b). In addition, children's dermal exposure to nicotine is more likely to be associated with poisoning symptoms than accidental nicotine ingestion (Woolf et al., 1997).

EC Exposure to The Skin

With more EC hobbyists modifying and creating their own devices and liquids, skin is constantly contacted by refill fluids through spillage, leakage, or just handling the fluids (Figure 1.7). Interestingly, some of the top symptoms and disorders in a study that mined health data reported inflammation of the skin (e.g., itching and eczema) possibly due to direct exposure and allergic reactions to EC products (Hua et al., 2020; Hua et

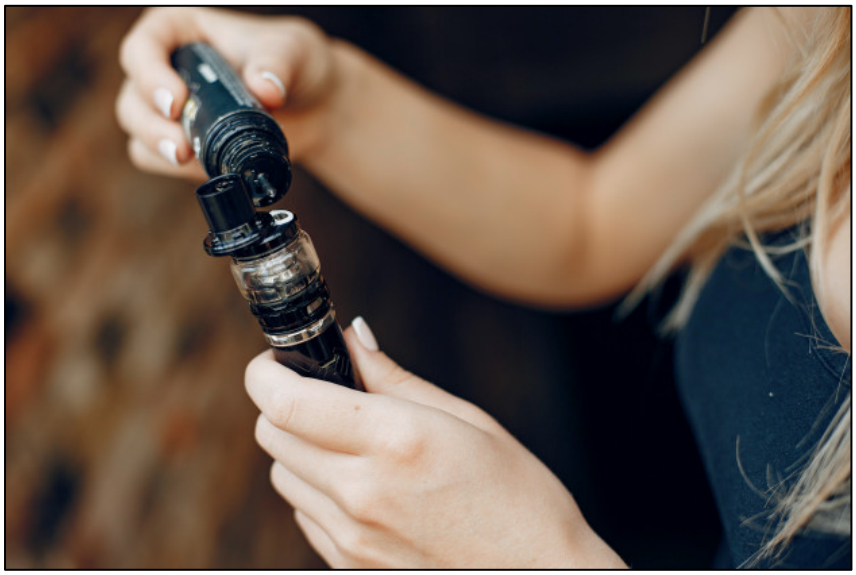

Figure 1.7 EC user refills an EC with e-juice Image: shutterstock.com

al., 2013). Dermal exposure to e-liquids have been reported in epidemiologic studies, indicating local skin irritation and even painful burns with skin blistering after EC device explosions (Hughes and Hendrickson, 2019). Leaky cartridge reservoirs could also be a cause of the skin irritation due to the high amounts of nicotine, solvents, and flavor chemicals (Trtchounian et al., 2010; EU Health Programme, 2016).

The skin is the most likely route of exposure to refill fluids and the residue that settles on surfaces from exhaled aerosols. The skin, which is made up of many layers of cells, including the corneum, granulosum, spinosum, and basal (Figure 1.8) protects against environmental pollutants. The stratum basale, the innermost layer, consists of basal cells that continually divide to push older cells up toward the surface of the skin. This layer contains melanocytes which increase melanin production upon sun exposure. The squamous cell layer or stratum spinosum, the thickest layer of the epidermis, is made up of keratinocytes that contain keratins which provide protection. The stratum granulosum and lucidum contain granulocytes that are pushed towards the surface of the skin. Finally, the stratum corneum, the outermost layer of the epidermis, is made up

of 10 to 30 thin layers of continually shedding, dead keratinocytes. Although the skin is the body's best protection from the environment, it can be permeated and irritated. Direct exposure to chemicals can induce inflammatory responses and irritation.

Nicotine is water and lipid soluble and can be easily absorbed through the epidermis (Zorin et al., 1999; Kuswahyuning and Roberts, 2014) . Maina et al. showed that refill fluid contamination occurs to the skin even with a low dose of nicotine (8%) (Maina et al., 2016) . Another study reported nicotine absorption of 2 – 4 mg/ml into skin exposed to lemon lime e-liquid (24 mg/ml of nicotine) for 4 hrs. (Frasch and Barbero, 2016). Even a short contact time of 10 minutes can produce transdermal absorption of nicotine (Maina et al., 2017). Although there are some preliminary studies on skin

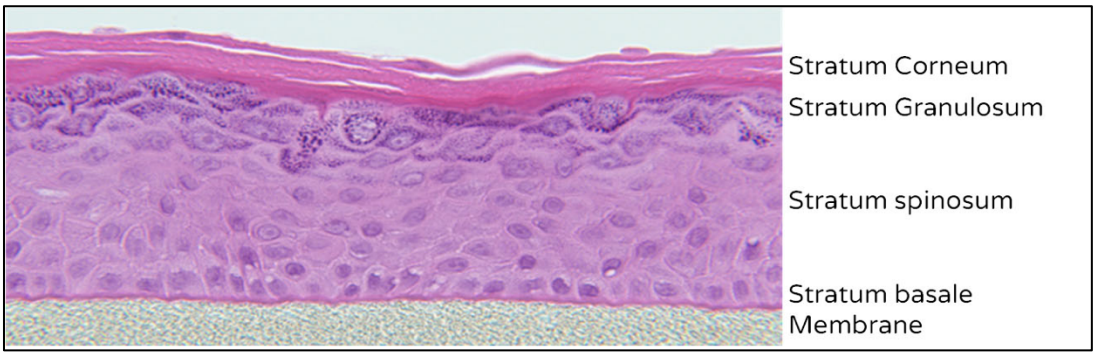

Figure 1.8 MatTek EpiDerm™ skin tissue histological section. Image: Mattek.com

exposures to EC components, the irritation to skin needs to be studied to know the extent of toxicity to solvents and/or flavor chemicals.

Reconstructed human epidermis (RhE) models have recently emerged as a way to replace rabbit skin testing, giving researchers an insight into skin response without the use of animal models. RHE is defined as an in vitro three-dimensional reconstruction of human epidermis composed of primary keratinocytes extracted from human skin. Cells

are differentiated into a stratified human epidermis at the air-liquid interface (Huet et al., 2018). These models are histologically and functionally similar to in vivo human epidermis. MatTek EpiDerm™ is made of normal human epidermal keratinocytes (NHEK) derived from neonatal-foreskin tissue. EpiDerm™ is grown using Dulbecco's modified Eagle's medium (DMEM) and cultured at the air-liquid interface (ALI). In 2013, Casas et al. published a proof-of concept study that evaluated a modification of the EpiDerm™ skin irritation protocol and confirmed ability of the RhE *in vitro* assay to identify skin irritants at concentrations similar to those of substances extracted from medical device polymers (Casas et al., 2013). Then Kandarova et al. optimized and pre-validated the EpiDerm™ Skin irritation test (EPI-200-SIT-MD) for medical devices to detect the presence of skin irritants at low levels in medical device extracts (Kandárová et al., 2018). Models using RHE allow for the study of toxicants, informing researchers of inflammation via interleukins (ILs) or by producing a defect in the epidermal barrier. Therefore, 3D skin tissues are a great in vitro tool to study the toxic effects of EC refill fluids and residues on the skin.

PURPOSE OF THE DISSERTATION

The research presented here aims to identify and quantify ECEAR chemicals by tracking flavor chemicals and nicotine from their origin in a refill fluid to the residue created by an EC user's exhale. I then examined the toxicology of EC fluids and ECEAR in relation to skin and identified and quantified the chemicals in ECEAR in field sites. Chapter 2 assesses the flavor chemicals and nicotine concentrations in refill fluids, aerosols, exhale produced by participants, the retention of those chemicals and each

user's contribution to ECEAR. Chapter 3 provides a toxicological assessment of refill fluids and ECEAR on 2D cells culture and EpiDerm™ 3D tissues. Chapter 4 identifies and quantifies nicotine, nicotine alkaloids, and TSNAs in a vape shop and a living room of an EC user. Chapter 5 identifies and quantifies ECEAR in a multi-unit tenant building next to a vape shop, proving that EC aerosols move from their point of origin to adjacent spaces.

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Chapter 2

Tracing the Movement of Electronic Cigarette Flavor Chemicals and Nicotine from Refill Fluids to Aerosol, Lungs and Exhale

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Abstract:

Background: Electronic cigarettes (ECs) have been linked to lung diseases, including COVID-19, with little understanding of exposure, retention, and exhalation of EC aerosol chemicals. EC users exhale flavor chemicals and nicotine, which accumulate on indoor surfaces as ECEAR (electronic cigarette exhaled aerosol residue). The retention of chemicals and composition of ECEAR have not been well characterized.

Objectives: To determine retention of flavor chemicals and nicotine by identifying and comparing the chemical composition of refill fluids, inhaled aerosols, and exhaled aerosols, and then determine the contribution to ECEAR.

Methods: Flavor chemicals and nicotine were identified and quantified by GC-MS in two different refill fluids, smoking machine-generated aerosols, and aerosols exhaled by 10 human participants (average age 21; 7 males). Machine generated aerosols were made with varying puff durations and two wattages (40 and 80). Participants generated exhale *ad libitum*; their exhale was measured, and chemical retention was modeled.

Results:

"Dewberry Cream" had five dominant (≥ 1 mg/ml) flavor chemicals (maltol, ethyl maltol, vanillin, ethyl vanillin, furaneol), while "Cinnamon Roll" had one (cinnamaldehyde). Nicotine transferred well to aerosols irrespective of topography; however, transfer efficiencies of flavor chemicals depended on the chemical, puff volume, puff duration, pump head, and EC power. Participants could be classified as "mouth inhalers" or "lung inhalers" based on their retention and exhale of flavor chemicals and nicotine. Only mouth inhalers exhaled sufficient concentrations of flavor chemicals/nicotine to contribute to chemical deposition on environmental surfaces. Lung inhalers had high retention and exhaled low concentrations of chemicals.

Discussion: These data help distinguish two types of EC users. Lung inhalers retain more harmful chemicals than mouth inhalers, who make a significant contribution to ECEAR. This study adds to our knowledge of chemical exposure during vaping.

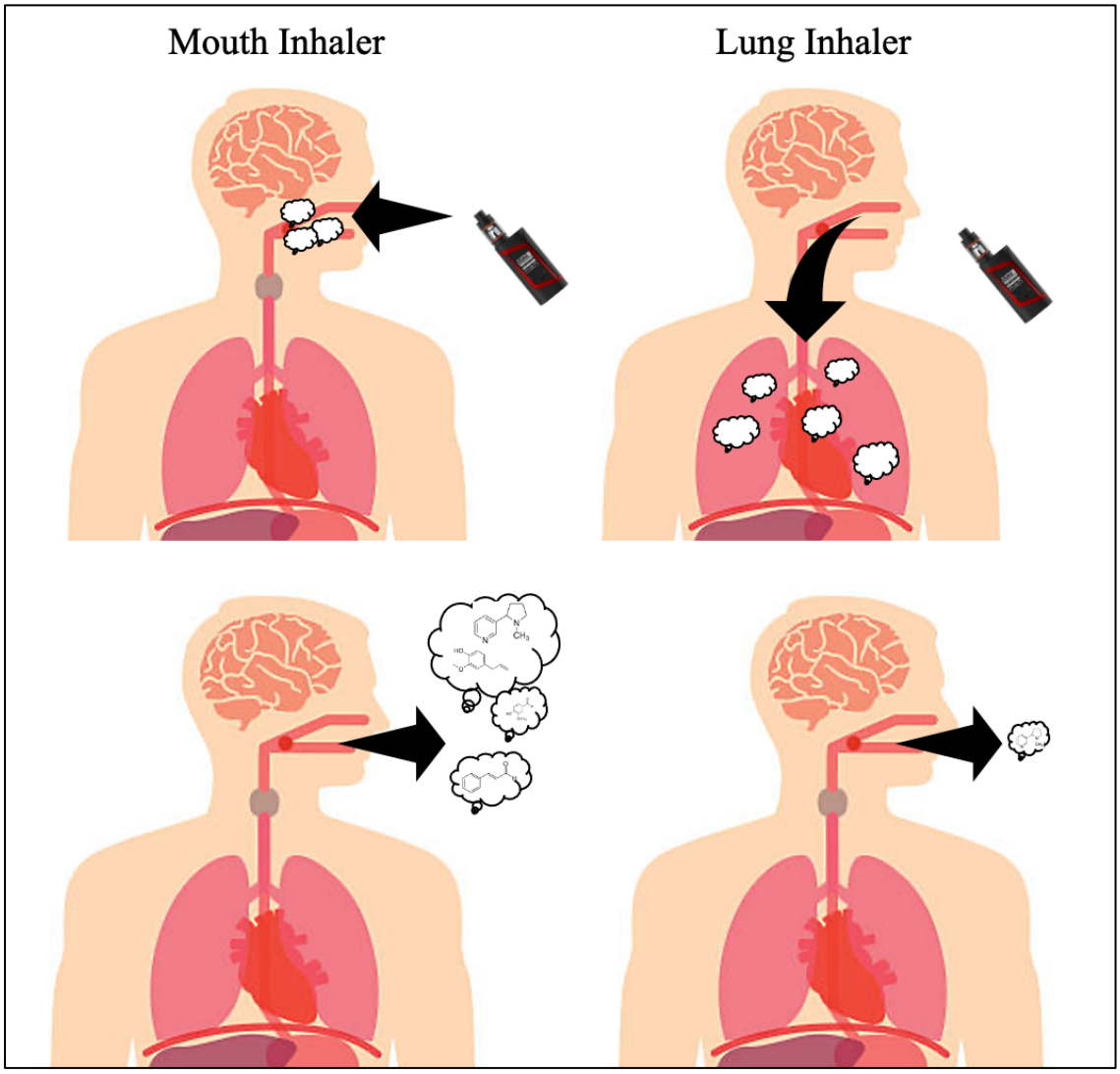


Figure 2.1 Graphical Abstract

INTRODUCTION

Electronic cigarettes (ECs) are battery powered nicotine delivery devices that produce an inhalable aerosol. The battery heats a metal coil(s) surrounded by a cotton wick saturated with fluid. The user then inhales aerosol usually containing nicotine, propylene glycol (PG), glycerol (G), flavor chemicals, metals, particulate matter, and volatile organic chemicals (VOCs) (Goniewicz et al., 2013; Goniewicz et al., 2014; Trehy et al., 2011; Vansickel et al., 2018; Lerner et al., 2015; Pellegrino et al., 2012; Williams et al., 2013). The VOCs include toxic aldehydes, such as formaldehyde and acrolein, that are produced by thermal dehydration of glycerin and/or glycols (McAuley et al., 2012; Uchiyama et al., 2013). Many EC devices are customizable and allow the user to vary the voltage, wattage, and amperage (Bitzer et al., 2017), which can alter the transfer of fluid chemicals to the aerosol (Zhao et al., 2016) and may also increase the production of toxic reaction products (Logue et al., 2017).

The possible effects of EC use on human health have been reviewed (Pisinger and Døssing, 2014; Gotts et al., 2019), and recent infodemiological data show the occurrence of health issues in EC users over the last 7 years (Hua et al., 2020). The relationship between reported health and flavor chemicals/nicotine is of interest due to their frequent use at high concentrations (Behar et al., 2018; Omaiye et al., 2019; Davis et al., 2015; Hua et al., 2019) and reported toxicity. For example, vanillin, ethyl vanillin, and ethyl maltol are often used in EC products (Khlystov and Samburova, 2018; Tierney et al., 2016) and are cytotoxic to human pulmonary fibroblasts in the MTT assay (Behar et al., 2018). Ortho-vanillin and maltol increased secretion of IL-8 from BEAS-2B cells and decreased barrier function in human bronchial epithelial cells exposed *in vitro* (Gerloff et al., 2017). Flavor chemicals in aerosolized refill fluids (cinnamaldehyde,

vanillin, and ethyl vanillin) were toxic to CALU3 cells after five puffs and caused dose-dependent decreases in cell viability (Rowell et al., 2017). Pure menthol, when aerosolized in a cloud chamber, increased mitochondrial protein oxidation, expression of the antioxidant enzyme SOD2, and activation of NF-κB, in air-liquid interface cultures of BEAS-2B (Nair et al., 2020). Some EC flavor chemicals are known to damage human lung tissue. For example, inhalation of diacetyl leads to bronchiolitis obliterans, a serious and irreversible lung disease (Allen et al., 2016). Although not directly linked to flavor chemicals/nicotine, vaping does cause e-cigarette or vaping product use-associated lung injury (EVALI) (Balmes, 2019) and has been associated with COVID-19, which has a higher probability of occurring in those who have used ECs (Wang et al., 2020).

While most research focus has been on inhalable aerosols, EC users also exhale aerosol that settles on indoor surfaces where it accumulates as EC exhaled aerosol residue (ECEAR). ECEAR contains nicotine, tobacco specific nitrosamines (TSNAs), solvents, and particles (Son et al., 2020; Khachatoorian et al., 2018; Bush and Goniewicz, 2015; Khachatoorian et al., 2019; Goniewicz and Lee, 2015; Sempio et al., 2019). ECEAR chemicals increased in concentration in a vape shop over a month-long period of monitoring, and concentrations were highest in heavily used areas (Khachatoorian et al., 2019). An EC user's living room also had residue containing nicotine and tobacco alkaloids, albeit at a lower concentration than the vape shop. ECEAR can also accumulate away from its site of origin. Nicotine, other alkaloids, and TSNAs transferred from a vape shop in a mini mall to an adjacent business where they deposited on paper and cotton towels (Khachatoorian et al., 2018). As far as we know, no studies have looked at flavor chemicals in ECEAR, even though their concentrations are high in many in refill fluids (Hua et al., 2019). The effects of ECEAR on human health

are unknown, but its accumulation in indoor environments presents the opportunity for active and passive exposure.

Given the high concentrations of nicotine and flavor chemicals in EC fluids and their demonstrated toxicity, it is important to determine how efficiently they transfer to aerosols, how well they are retained by users (exposure), and if they are exhaled into the environment where they settle on surfaces, forming ECEAR. The goal of our study was to obtain a complete overview of the movement of flavor chemicals and nicotine from refill fluids into aerosols, then into users' respiratory systems, and finally into their exhale where it could contribute to ECEAR. To do this, we quantified flavor chemicals and nicotine in two refill fluids, then determined the effects of topography on their transfer into machine-generated aerosols. Human exposures were determined by measuring the concentrations of these chemicals in exhale and modeling retention using information on transfer efficiency and exhale.

MATERIALS & METHODS:

i. *Refill Fluids*

"Dewberry Cream" was purchased at a local vape shop that sold products made by refill fluid manufacturers, while "Cinnamon Roll with Cinnamon Bomb" was purchased at a local vape shop that custom mixes its refill fluids. Both shops were located in Riverside County, CA. "Dewberry Cream" by Kilo was chosen because it has many flavor chemicals, including vanillin, ethyl vanillin, maltol and ethyl maltol, and a high total flavor chemical concentration (Hua et al., 2019. "Cinnamon Roll with Cinnamon Bomb", which we refer to as "Cinnamon Roll", was chosen because it has only one dominant flavor chemical (cinnamaldehyde) and cinnamon-flavored refill fluids can adversely affect cultured cells (Behar et al., 2014) (Behar et al., 2016) (Wavreil and Heggland, 2019) (Clapp et al., 2019) (Fetterman et al., 2018). Each refill fluid was labeled to have 6 mg of nicotine/mL and 70/30 G/PG ratio.

ii. EC Aerosol Production and Capture

For aerosol production, we used a SMOK Alien 220W Mod (variable voltage (0.35-8V) with two high amperage flat top 18650 batteries. The mod was used with a SMOK V8 Baby-Q2 (0.4) single coil tank atomizer inside a SMOK Baby Beast tank. The smoking machine was a Cole-Parmer Masterflex L/S peristaltic pump used with a standard or high-performance pump head. When set to 40 watts, aerosols were generated at 4.3 volts, 0.4 ohms, and 9.9 amps. When set to 80 watts, aerosols were generated at 6.1 volts, 0.4 ohms, and 14.1 amps. The tank was loaded with 3 mL of refill fluid each time aerosol was produced, and the EC was primed with three puffs. The tank was washed with water and ethanol, and the V8 Baby Beast coil was replaced between

each refill fluid. Puff durations were 1, 2 and 4.3 seconds; the latter is a reported average for EC consumers (Hua et al., 2013).

The standard pump head (low volume pump head) generated a flow rate of 13 mL/sec to produce puff volumes of 13 mL (1 sec), 26 mL (2 sec), and 56 mL (4.3 sec). The high-performance pump head (high volume pump head) generated a flow rate of 40 mL/sec to produce puff volumes of 40 mL (1 sec) and 80 mL (2 sec).

For flavor analysis, aerosols were collected at room temperature in two 125 mL impingers, each containing 25 mL of isopropanol (IPA). The tank was weighed before and after aerosol production to collect a mass concentration of at least 15 mg/mL for GC/MS analysis. Aerosol solutions were collected, aliquoted, and stored at −20 °C until analyzed.

iii. Identification and Quantification of Flavor Chemicals Using GC/MS.

Refill fluids, aerosols, and exhale were analyzed by GC/MS. Internal standard-based calibration procedures similar to those described elsewhere were used (Tierney et al., 2016) (Omaiye et al., 2019), and analyses for 176 flavor-related target analytes and nicotine were performed with an Agilent (Santa Clara, CA) 5975C GCMS system. The capillary column used was a Restek (Bellefonte, PA) RXI-624Sil MS (30 m long, 0.25 mm id, and 1.4 μm film thickness). For each refill fluid sample, 50 μL was dissolved in 950 μL of isopropanol (Fisher Scientific, Fair Lawn, New Jersey, USA). Prior to analysis, 20 μL of internal standard solution (2 μg/μL of 1,2,3-trichlorobenzene in isopropyl alcohol) was added into the 1 mL diluted refill samples, the aerosol and exhaled extract aliquots. 1 μL of the sample was injected into the GC/MS with a 10:1 split. The injector temperature was 235°C. The GC temperature program for all analyses was as follows: 40°C hold for 2 min; 10°C/min to 100°C; 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 at electron ionization mode. The ion source temperature was 226°C. The scan range was from 34 to 400 amu. Each target analyte was quantitated using authentic standard material, and an internal standard (1,2,3-trichlorobenzene) normalized multipoint calibration.

iv. Participants

Ten of eleven recruited participants (3 women and 7 men) completed the exhale portion of the study. The average age was 21 years (SD = 2.8; median = 20; range = 18-28). The ethnicity of the participants was: eight Asian, one African American, and one Caucasian. All participants self-reported no use of combustible cigarettes for the duration of the study and were told to abstain from using ECs 1 hour before the experiment. Six of the participants had used combustible cigarettes in the past. One of the six used a cigarette once a month during the study and the other five reported no current use. Two of the participants had used cigars in the past. The inclusion criteria were: (1) experienced EC users (at least 3 months), and (2) must use at least 3 mg of nicotine in their current EC. Participants were excluded if they were: (1) pregnant or breastfeeding, (2) under the age of 18 or over 75 years, (3) never users of EC nicotine, or (4) experiencing any medical conditions. All participants signed informed consent before admission into the study. The project was approved by the UCR Internal review Board (IRB # HS-12-023). Participants were coded to identify puffing topography and were compensated after four sessions of vaping.

v. EC Exhaled Aerosol Production and Capture

Participants were asked not to use any ECs or cigarettes an hour before coming to the lab. Upon arrival, participants vigorously washed their mouths and gargled for 30 sec with water. A 2 feet piece of plastic tubing with a mouthpiece was attached to two

impingers connected to each other by a short piece of tubing. Each impinger contained 25 mL of IPA. The first session (control) involved collection of 30 puffs of exhale in the impingers at 1 puff/minute without any EC use. After the last puff, the sample was collected from each impinger and stored in glass vials for chemical analysis. The next four sessions involved using the SMOK Alien with the Baby Beast tank at 40 or 80 watts for each refill fluid ("Dewberry Cream" and "Cinnamon Roll"). The tank was primed with three puffs before each use. Volunteers were asked to use the EC at 1 puff/minute at 40 or 80 watts during different sessions. The puff duration was sampled two to three times during each session. At the end of a session, IPA was collected from each impinger and used to wash residual aerosols from inside the tubing and impingers. 1mL from each impinger was then aliquoted into GC sample vials for chemical analysis. The impingers, tubing, and V8 Baby Beast tank were washed with water and 75% ethanol and left to dry for the next session. Each volunteer was given a new tube and V8 Baby coil for each refill fluid. The coil in the tank was changed between each participant and each refill fluid. The SMOK Alien box mod and tank were changed once during the study.

vi. Calculating Transfer Efficiency, Percent Retention, and ECEAR

To determine the transfer efficiency of flavor chemicals and nicotine, aerosol fluid flavor or nicotine concentrations were divided by the refill fluid flavor or nicotine concentrations. The transfer rate was multiplied by 100 to get percent transfer efficiency.

Tank weights, which were recorded before and after each session, were subtracted to find the total weight of EC fluid consumed. Potential mass delivered was calculated by multiplying the fluid consumed by the refill fluid flavor chemical or nicotine concentration. Actual mass delivered was calculated by multiplying the transfer rate by the potential mass delivered. Total mass in the exhaled aerosol was calculated by

multiplying the fluid consumed by the concentration in the exhaled sample. The percent retention was calculated by the following equation:

$$\left[\frac{(actual\ mass\ delivered - total\ mass\ in\ exhaled\ aerosol)}{actual\ mass\ delivered}\right] x\ 100$$

ECEAR was calculated by subtracting the percent retention from 100.

RESULTS

1. Refill Fluid Characterization

"Dewberry Cream" and "Cinnamon Roll" had 47 and 36 flavor chemicals, respectively. Heatmaps show all flavor chemicals (y-axis) detected and quantified in the refill fluid (x-axis) (Fig. 2.2). The total quantity of flavor chemicals and nicotine are listed at the top of each column in mg/mL.

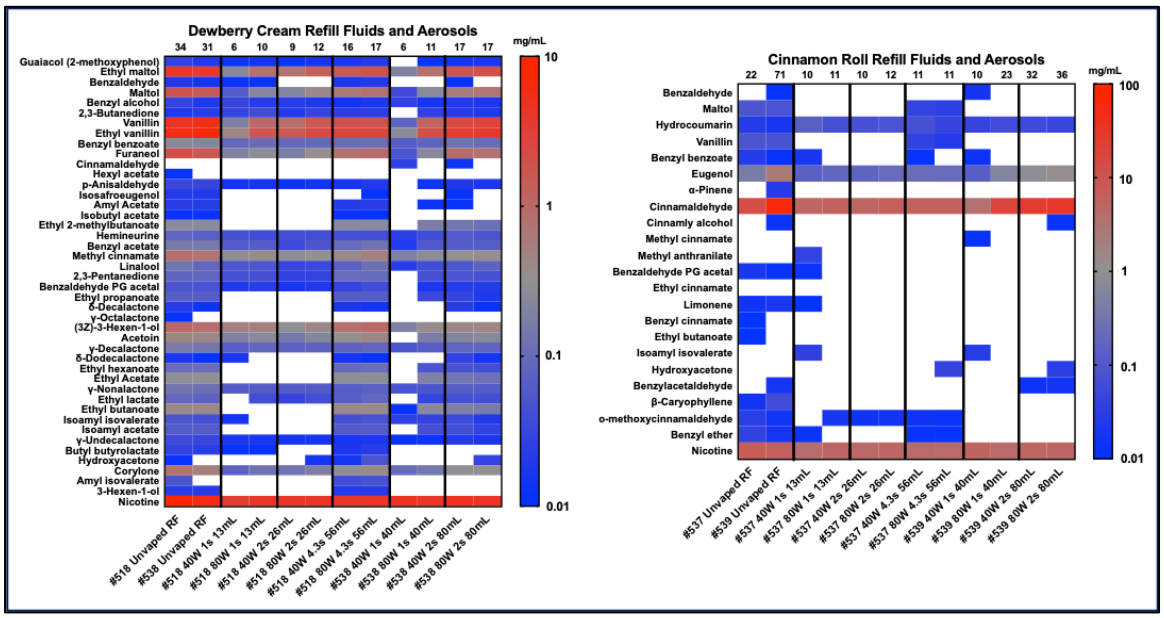


Figure 2.2: Heatmaps showing concentrations of flavor chemicals and nicotine in "Dewberry Cream" (A) and "Cinnamon Roll" (B) refill fluids and aerosols made at 40 or 80-watts. Puff durations were either 1, 2, or 4.3 seconds, while puff volume was either 13, 26, 56, 40, or 80 mL. Flavor chemicals are listed on the left y-axis and concentrations are in mg/mL. The top x-axis shows the total mg of flavor chemicals including nicotine in each column.

i. Dewberry Cream

"Dewberry Cream" is distributed in vape shops nationally and can be purchased online. Its flavor profile is described as mixed berries, honeydew, and cream. Bottles #518 and #538 were purchased at different times. Although their total flavor chemical

concentrations varied by 3 mg, the concentrations of the dominant flavor chemicals were similar in each bottle (Fig. 2.2). Dewberry Cream (#518) contained > 1 mg/mL of ethyl vanillin (6.1 mg/mL), vanillin (4.7 mg/mL), ethyl maltol (4.4 mg/mL), maltol (1.9 mg/mL), furaneol (2.1 mg/mL), and (3Z)-3-hexen-1-ol (1 mg/mL). Although labeled as 6 mg/mL of nicotine, the actual concentration was 8.7 mg/mL. "Dewberry Cream" (#518) was the refill fluid that participants used to create exhaled aerosols. Both "Dewberry Cream" #518 and #538 were used to create aerosols to determine transfer efficiency.

ii. Cinnamon Roll with Cinnamon Bomb

"Cinnamon Roll with Cinnamon Bomb" was custom mixed for us on two occasions at a local vape shop in Riverside, CA. The mixture's flavor profile was described as mostly cinnamon with some sweet flavors. Bottles #537 and #539 were not identical and had different concentrations of cinnamaldehyde and eugenol, the two dominant flavor chemicals (Fig. 2.2). "Cinnamon Roll" (#537) contained 13.5 mg/mL of cinnamaldehyde, and although labeled as 6 mg/mL nicotine, the actual concentration was 7.5 mg/mL. Other flavor chemicals in "Cinnamon Roll" were eugenol (0.4 mg/mL), maltol (0.09 mg/mL), vanillin (0.08 mg/mL), and hydrocoumarin (0.02 mg/mL). "Cinnamon Roll" (#537) was the refill fluid that participants used to create exhaled aerosols. Both "Cinnamon Roll" #537 and #539 were used to create aerosols to determine transfer efficiency.

2. Aerosol Characterization

iii. E-Liquid Aerosolized and EC Setting

The amount (mg) of refill fluid aerosolized with the Smok Alien V8 baby beast tank from 30 puffs with the low and high-volume pump heads is shown in Table 2.1.

Pump Head		Dewberry Cream (mg)	Cinnamon Roll (mg)
low volume pump head	1s 40 watt	80	60
	1s 80 watt	330	300
	2s 40 watt	280	380
	2s 80 watt	620	680
	4.3s 40 watt	1170	930
	4.3s 80 watt	1700	1040
high volume pump head	1s 40 watt	90	70
	1s 80 watt	420	440
	2s 40 watt	320	330
	2s 80 watt	920	840

Table 2.1: Amount of refill fluid aerosolized at different EC settings

3. Transfer Efficiency

Figure 2.3 shows that the transfer efficiency of the major flavor chemicals and nicotine from the refill fluids to the aerosol was affected by topography. Two different pump heads were used to create aerosol to get a range of flow rates. The low flow rates are shown in Figure 2.3A, C, E, G, I, K, M, O and Q, while the high flow rates are shown in Figure 2.3B, D, F, H, J, L, N, P and R. The 40-watt setting is almost always lower in transfer efficiency than the 80-watt setting.

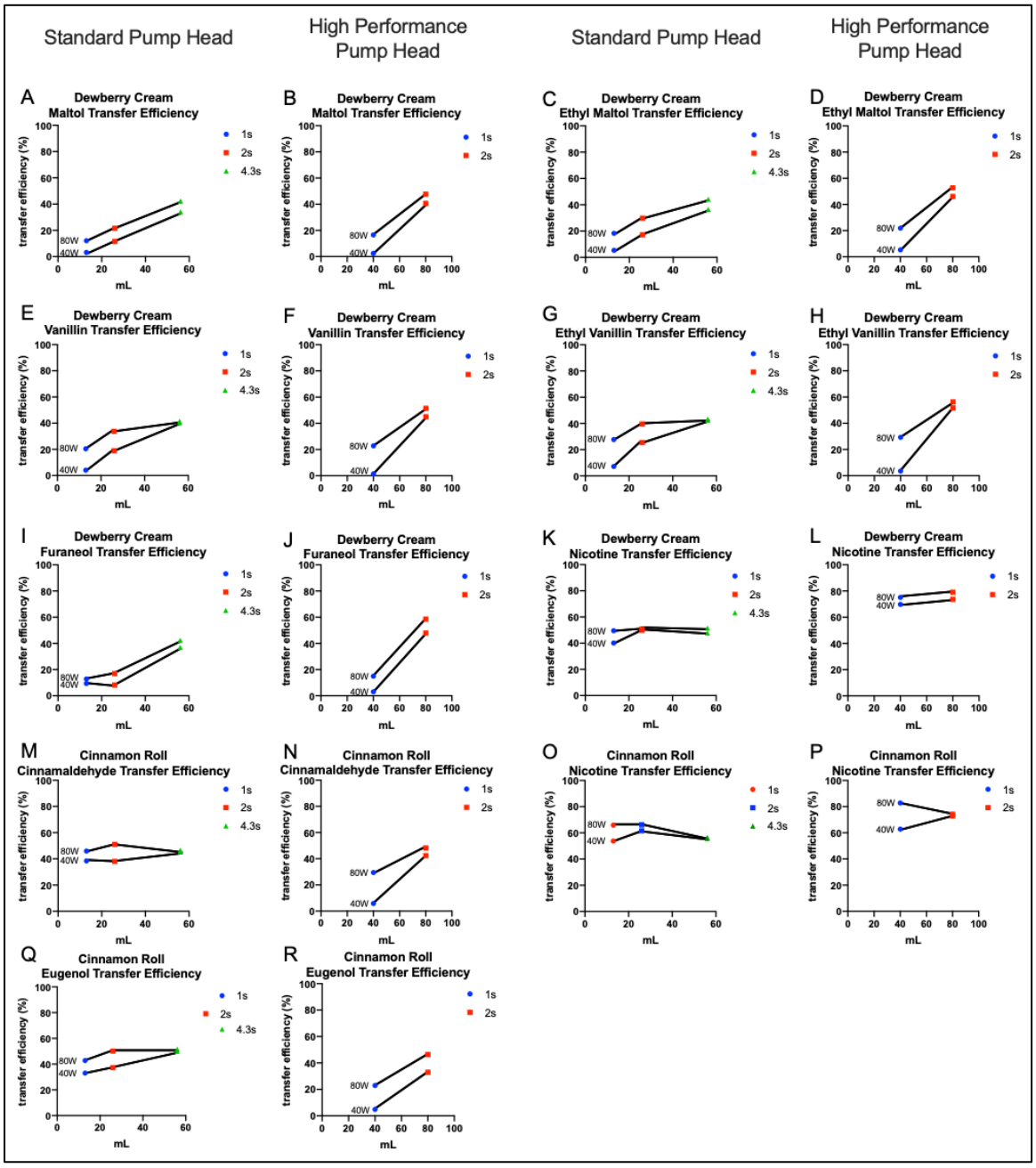


Figure 2.3: Transfer Efficiency of major flavor chemicals in "Dewberry Cream" and "Cinnamon Roll". Aerosols made with the low volume pump head are shown in A, C, E, G, I, K, M, O and Q, while aerosols made with the high-volume pump head are shown in B, D, F, H, J, L, N, P and R. Volume is shown on the x axis and transfer efficiency (in percentage) is shown on the y axis.

4. Low Flow Rate

Maltol, ethyl maltol, vanillin, ethyl vanillin, and furaneol have similar patterns for each puff duration, EC setting, and puff volume (Fig. 2.3A, C, E, G, I). Lower puff durations had a lower transfer efficiency than higher puff durations with the low volume pump head. Cinnamaldehyde was consistently between 38 -51% transfer efficiency for each puff volume (Fig. 2.3M). Similarly, eugenol was between 33-51%. Finally, nicotine was consistently between 40-51% and 54-66% for “Dewberry Cream” (Fig. 2.3K) and “Cinnamon Roll” (Fig. 2.3O), respectively.

5. High Flow Rate

Maltol (Fig. 2.3B), ethyl maltol (Fig. 2.3D), vanillin (Fig. 2.3F), ethyl vanillin (Fig. 2.3H), and furaneol (Fig. 2.3J) have similar patterns for each puff duration, EC setting, and puff volume. The lower puff duration (1 second) had a lower transfer efficiency than the higher puff duration (2 seconds). When using the high flow pump head, 1 second puff durations were lower or equal to the transfer efficiency of 1 second puff durations with the low flow pump head. Cinnamaldehyde (Fig. 2.3N) had a 5% transfer efficiency for the 1 second 40-watt setting, but when the wattage increased to 80, the transfer efficiency increased to 30%. In a similar pattern, eugenol had a 5% transfer efficiency for the 1 second 40-watt setting, but when the wattage increased to 80, the transfer efficiency increased to 23%. Nicotine had a consistent transfer efficiency between 70-79% for “Dewberry Cream” (Fig. 2.3L) and between 63-82% for “Cinnamon Roll” (Fig. 2.3P).

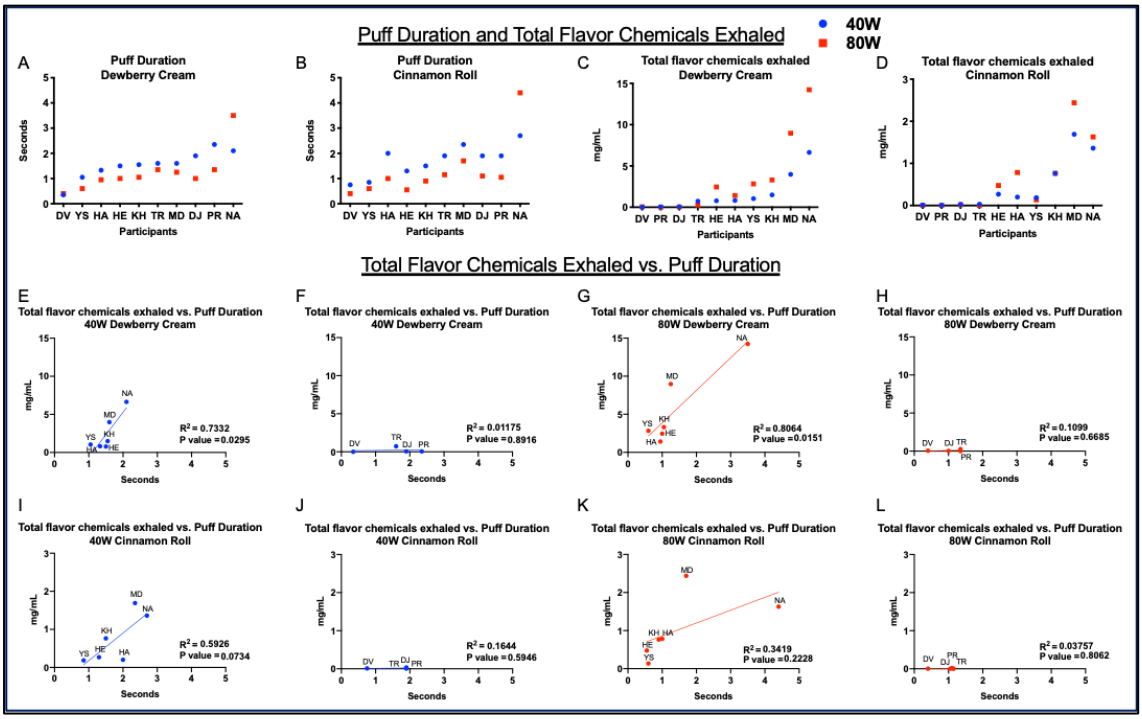


Figure 2.4: Participant topography: puff duration and total flavor chemicals exhaled. A and B show each participant's puff duration for Dewberry Cream and Cinnamon Roll. C and D show the concentration of the total flavor chemicals exhaled (mg/ml). E through L show the relationship between the total flavor chemicals exhaled and puff duration. Mouth inhalers are shown in E, G, I, and K, while lung inhalers are shown in F, H, J, and L.

6. *Exhaled Aerosol*

iv. Puff Duration

Participants used the SMOK Alien at a low and high wattage with "Dewberry Cream" and "Cinnamon Roll" refill fluids. Control analysis of exhaled aerosols showed minimal to no detectable levels of nicotine or flavor chemicals (Appendix A Table S1). Each participant took 30 puffs at 1 puff/minute of either "Dewberry Cream" or "Cinnamon

Roll" refill fluid at 40-watts or 80-watts, and puff duration was sampled for each participant (Fig. 2.4A and 2.4B). A total of 5 puffing sessions/participant, including one control session, was documented and analyzed. Most participants had puff durations between 0.5-2 s. For each participant, puff durations for both wattages were similar with deviations generally no more than 0.5 seconds. Puff duration was generally longer for the 40-watt setting for both refill fluid flavors. One participant, NA, a higher puff duration (2.1s for 40W "Dewberry Cream", 3.5s for 80-watt "Dewberry Cream", 2.7s for 40-watt "Cinnamon Roll", and 4.4s for 80-watt "Cinnamon Roll") than the others at both settings and for both refill fluid flavors. The average puff duration for all participants was 1.4 ± 0.27 s. The average puff duration for "Dewberry Cream" (DC) at 40-watts = 1.5 ± 0.56 s, DC at 80-watts = 1.2 ± 0.85 s, CR at 40-watts = 1.7 ± 0.62 s, and CR at 80-watts =1.3 ± 1.15 s.

v. Total Flavor Chemicals

Total flavor chemical concentration in the exhale was generally higher for the 80-watt setting (Fig. 2.4 C, D). Four participants (DV, PR, DJ, and TR) exhaled almost no flavor chemicals, and we categorized these as "lung inhalers" (i.e., all of the aerosol likely reached the alveoli of the lungs). Six of the participants (HE, HA, YS, KH, MD, and NA) exhaled a fraction of the flavor chemicals that they inhaled, and these were categorized as "mouth inhalers" (i.e., intake went mainly into the mouth but did not fully penetrate into the lungs). The mouth inhalers exhaled 1 to 15 mg of the total flavor chemicals. The average concentration of flavor chemicals exhaled for Dewberry Cream and Cinnamon Roll at 40-watts by mouth inhalers increased as wattage increased (Dewberry Cream = 2.5 ± 2.4 to 5.5 ± 5 mg and Cinnamon Roll = 0.7 ± 0.6 mg to 1 ± 0.8 mg). The average concentration of flavor chemicals exhaled by lung inhalers was low

and similar between the two wattages (Dewberry Cream 40 watts = 0.2 ± 0.3 mg, 80-watts = 0.09 ± 0.1 mg; Cinnamon Roll = 40-watts = 0.02 ± 0.01 mg, 80-watts = 0.007 ± 0.007 mg). The average total flavor chemicals exhaled for all participants was 1.5 ± 1.87 mg and averages increased with increasing wattage (Dewberry Cream = 40-watts = 1.6 ± 2.13 mg, 80-watts = 3.4 ± 4.68 mg, and Cinnamon Roll = 40-watts = 0.5 ± 0.61 mg, 80-watts = 0.6 ± 0.83 mg).

vi. Total Flavor Chemicals Exhaled Vs. Puff Duration

The total flavor chemicals exhaled vs. puff duration for each refill fluid and EC setting are shown in Figure 2.4E-L. Participants were separated based on whether they were “mouth inhalers” (Fig. 2.4E, G, I, K) or “lung inhalers” (Fig. 2.4F, H, J, L). Dewberry Cream refill fluid puffed at 40 (Fig. 2.4E) and 80 watts (Fig. 2.4G) had significant correlation for the amount of flavor chemicals exhaled and puff duration for mouth inhalers. Cinnamon Roll puffed at the 40-watt (Fig. 2.4I) was not correlated flavor chemical concentration but had a p value close to 0.05. Cinnamon Roll at 80 watts (Fig. 2.4K) was not significant, but when reanalyzed without the outlier (MD), the p value decreased from 0.22 to 0.03 indicating a correlation. There was no correlation of exhaled chemicals and puff duration for the lung inhalers (Fig. 2.4F, H, J, L).

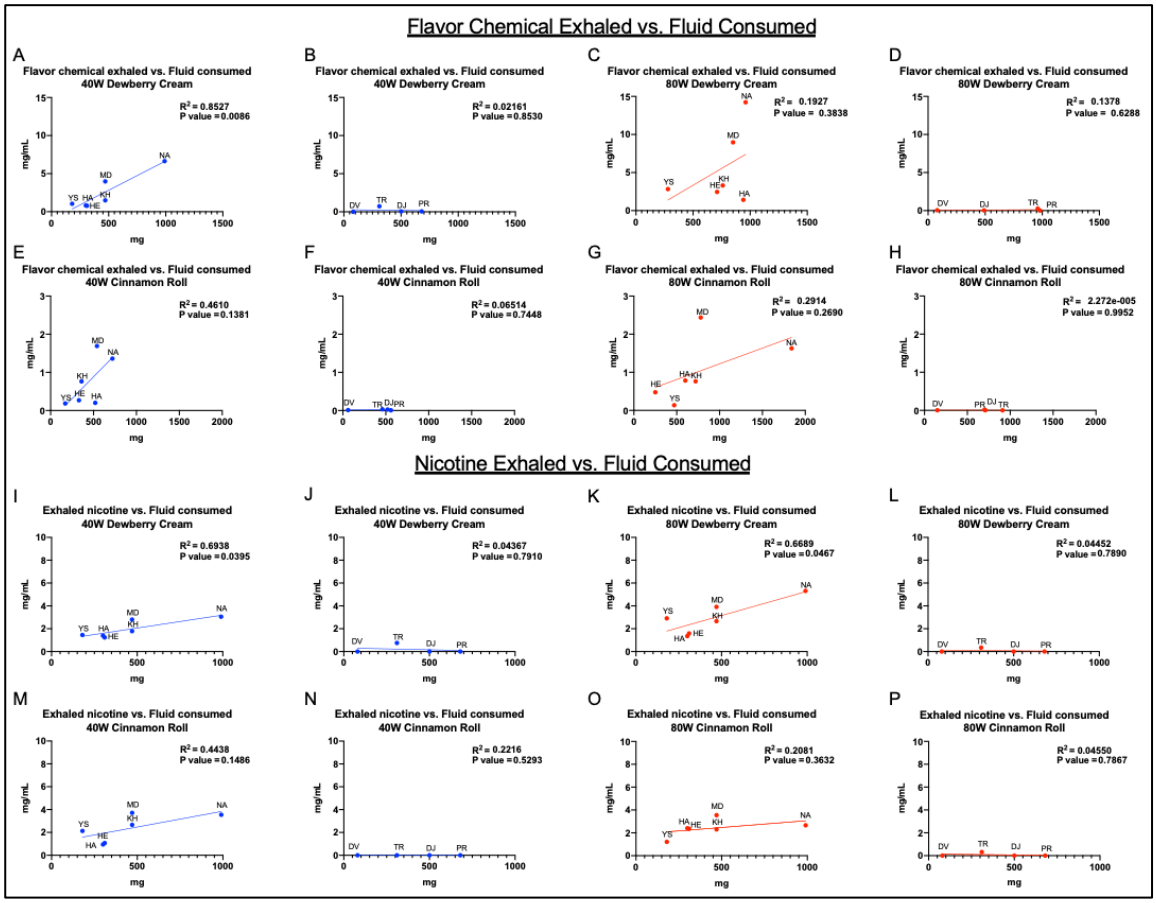


Figure 2.5: Participant Topography: fluid consumed and chemical exhaled. Relationship between the refill fluid consumed and the flavor chemicals exhaled for both EC settings and refill fluids (A-H). Relationship between fluid consumed and nicotine exhaled for both EC settings and refill fluids (I-P). Mouth inhalers are shown in A, C, E, G, I, K, M, O and lung inhalers are shown in B, D, F, H, J, L, N, and P.

vii. Fluid Consumed Vs. Flavor Chemicals Exhaled

The average fluid consumed for all participants was 567 ± 112 mg. The average fluid consumed was lower at the 40-watt setting and higher at the 80-watt setting (DC 40-watts = 429 ± 261 mg, DC 80-watts = 705 ± 308 mg, CR 40-watts = 424 ± 197 mg, and CR 80-watts = 713 ± 462 mg). Figure 2.5A-H shows the relationship between the

amount of refill fluid consumed and the concentration of flavor chemicals exhaled. For lung inhalers there was no correlations between how much fluid was consumed and the amount of flavor chemicals exhaled (Fig. 2.5B, D, F, H). For mouth inhalers, “Dewberry Cream” at the 40-watt setting showed significant correlation ($R^2 = 0.85$, $p = 0.008$) (Fig 2.5A). Mouth inhaler data appeared to be linearly related but were not significantly correlated. However, when Figure 2.5G was re-analyzed without the outlier (MD), the $p = 0.03$, indicating significance.

viii. Fluid Consumed Vs. Nicotine Exhaled

Exhaled nicotine was quantified and compared to the amount of refill fluid consumed (Fig. 2.5I-P). For the lung inhalers, there was no correlation between nicotine exhaled and the amount of fluid consumed (Fig. 2.5J, L, N, P). For the mouth inhalers, there was a significant correlation for the Dewberry Cream refill fluid and nicotine exhaled at both 40 (Fig. 2.5I) and 80 (Fig. 2.5K) watts, while there was no correlation for Cinnamon Roll at either wattage (Fig. 2.5M and 2.5O).

i. Percent Retention and Contribution of Exhale to ECEAR modeling

We computed retention and ECEAR for each of the topographies. The percent retention was calculated and averaged for all participants (Fig. 2.6). Lung inhalers had ~100% retention for flavor chemicals and nicotine for each setting/topography. Mouth inhalers retained variable percentages of specific flavor chemicals and nicotine. For mouth inhalers, cinnamaldehyde was retained better than nicotine and other flavor chemicals (Fig. 2.6G), and nicotine (Fig. 2.6E and 2.6F) was retained better than maltol, ethyl maltol, vanillin and ethyl vanillin (Fig. 2.6A-D).

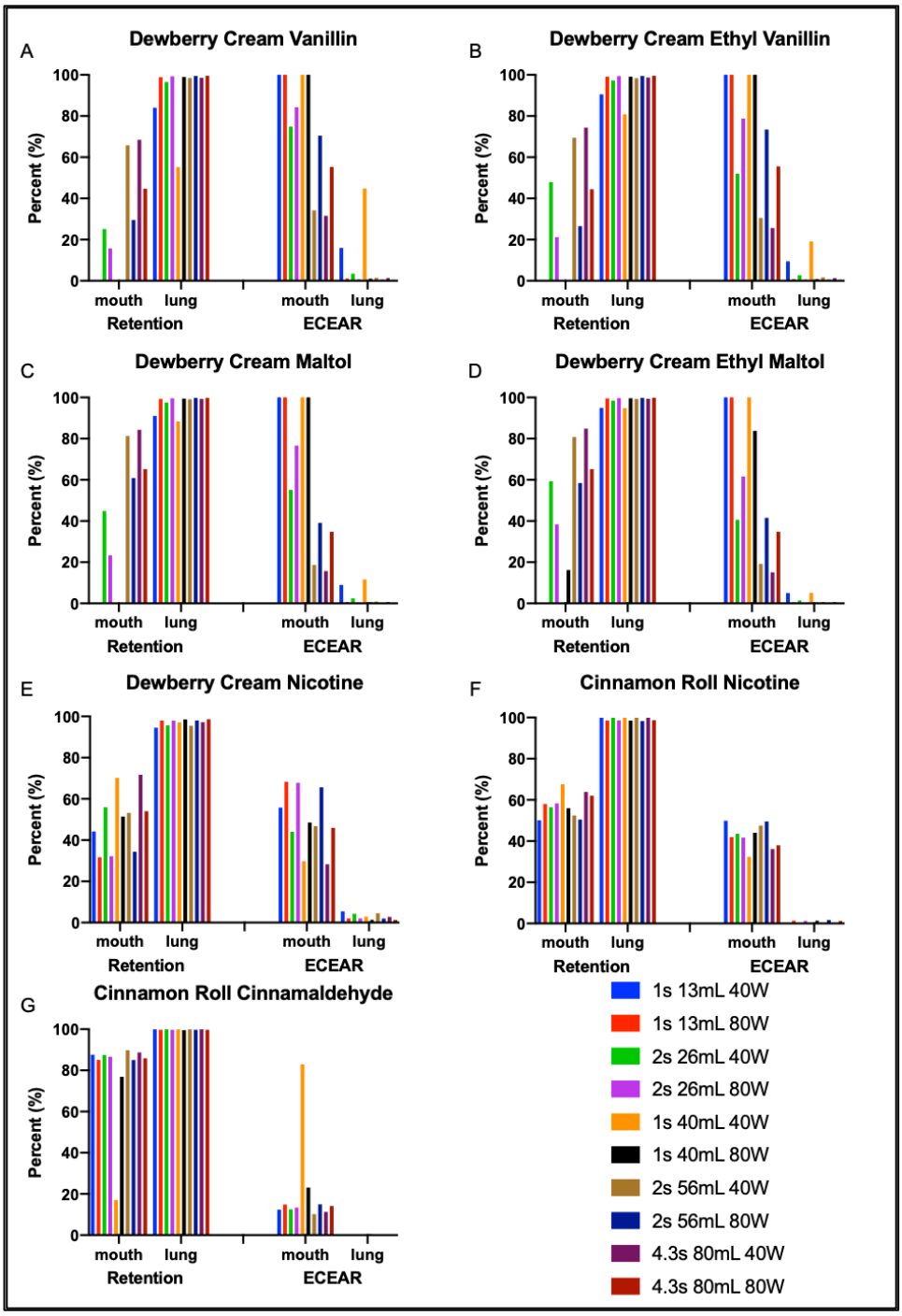


Figure 2.6: Retention and contribution to ECEAR modeling of major flavor chemicals by participants under several EC settings and conditions. Each participant's exhaled results were averaged for each topography to determine possible retention. Contribution to ECEAR was then calculated and averaged based on the amount retained. The y axis shows the percent retention or percent ECEAR while the x axis shows the participants averaged and separated by method of inhalation. EC settings, puff duration and puff volume are color coated.

The percent of inhaled aerosol that was exhaled and could contribute to ECEAR is also shown in Figure 2.6A-G. Lung inhalers did not contribute to ECEAR (Fig. 2.6A-G). However, mouth inhalers did contribute to ECEAR, and their contribution depended on flavor chemicals. Vanillin, ethyl vanillin, maltol, and ethyl maltol (Fig. 2.6A-D) contributed more to ECEAR than nicotine or cinnamaldehyde by mouth inhalers (Fig. 2.6E-G). There was very little contribution of cinnamaldehyde to ECEAR by mouth inhalers (Fig. 2.6G). The average nicotine contribution to ECEAR by mouth inhalers was 50% for “Dewberry Cream” and 42.5% for “Cinnamon Roll” (Fig. 2.6E, F).

7. Concentrations of Flavor Chemical and Nicotine in Exhale

The concentrations of specific flavor chemicals and nicotine in the exhale of the mouth and lung inhalers is shown in Figure 2.7. In most cases, exhaled concentrations were higher when vaping was done at 40 W. Mouth inhalers exhaled nicotine and flavor chemicals at concentrations > 1 mg/mL (Fig. 2.7A-L), while concentrations for lung inhalers (Fig. 2.7M-T) were < 1mg/mL and were often not detectable.

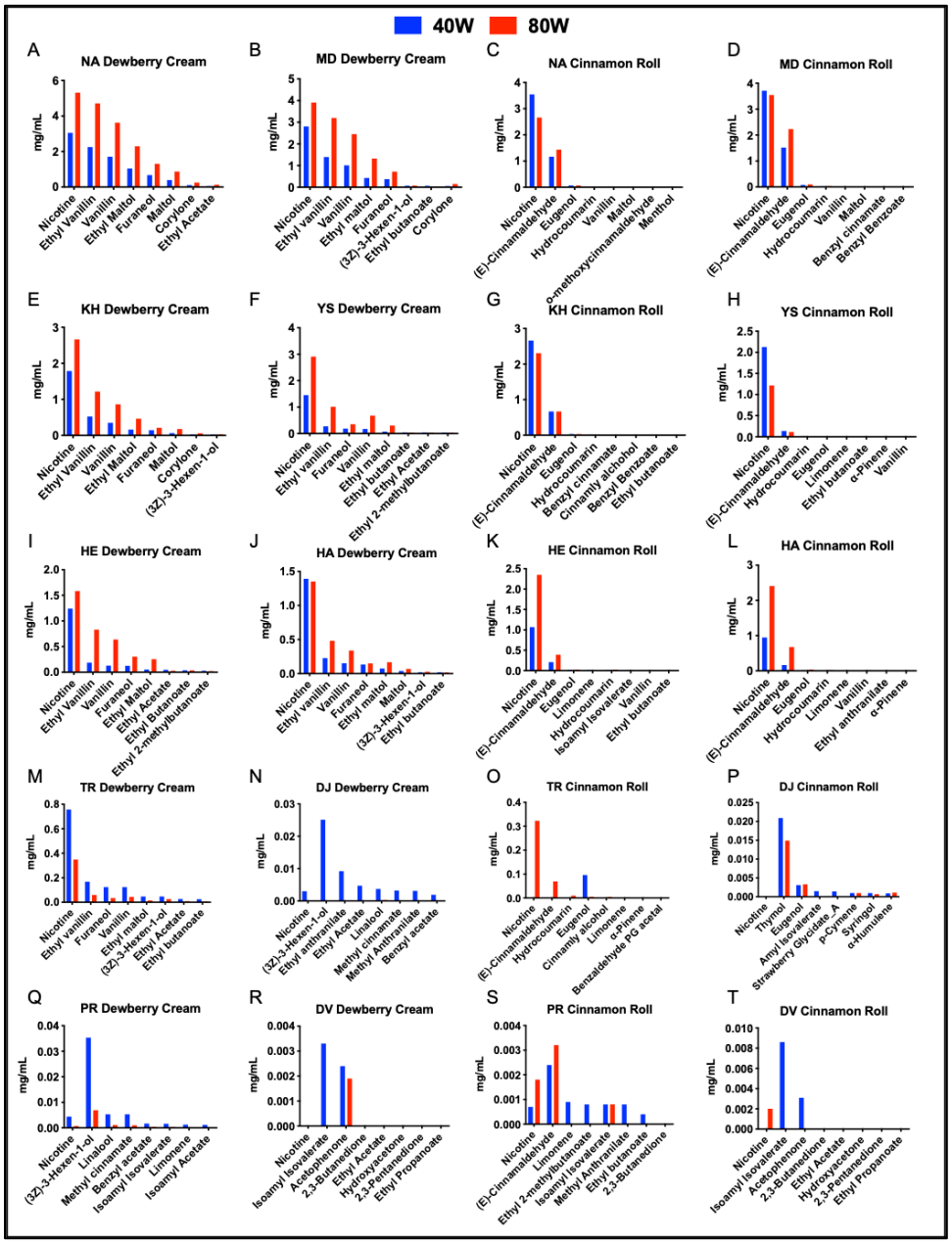


Figure 2.7: The concentration of major flavor chemicals emitted by each participant. 40-watt setting is shown in blue and 80-watt setting is shown in red.

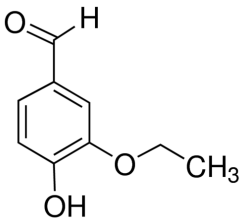
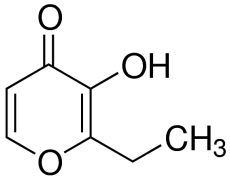
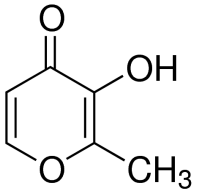
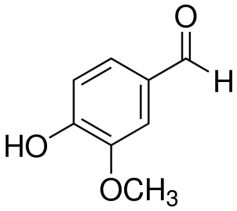
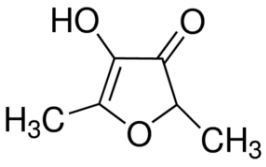
DISCUSSION

To the best of our knowledge, this is the first study to trace the movement of flavor chemicals/nicotine from refill fluids to exhaled aerosol. The EC settings and flavor chemicals in each refill fluid affected transfer efficiency and chemical retention. Participants either exhaled little or no nicotine/flavor chemicals or they exhaled up to half of what was found in the refill fluid. We interpret this to mean that the former group inhaled aerosol into their lungs where chemicals were efficiently absorbed (lung inhalers), while the latter group kept much of the aerosol in their mouths, then exhaled aerosol only partially depleted of chemicals (mouth inhalers). This distinction is important since chemical exposure varied considerably between the two types of inhalers and only the mouth inhalers contributed nicotine and flavor chemicals to ECEAR.

The flavor chemicals in “Dewberry Cream” were similar to those reported previously (Hua et al., 2019), with some bottle-to-bottle variation in total flavor chemical concentration (24, 25 and 28 mg/mL). In contrast, there was about a 5-fold difference in cinnamaldehyde concentration in bottles of “Cinnamon Roll” (#537 = 13.4 mg/ml and #539 = 61.4 mg/ml) purchased at different times in a local vape shop, where the compounding was not precisely controlled. Maltol, ethyl maltol, vanillin, and ethyl vanillin were detected in high concentrations in “Dewberry Cream” and are among the most potent flavor chemicals when tested *in vitro* with mouse neural stem cells and BEAS-2B cells in the MTT assay (Hua et al., 2019). Cinnamaldehyde, while present in Cinnamon Roll, was low in concentration compared to other cinnamon flavored products we have examined (Behar et al. 2016).

Transfer efficiency of flavor chemicals and nicotine from machine-vaped refill fluid to aerosols depended on the properties of the chemicals, EC wattage, the pump head, puff

duration and puff volume. Maltol, ethyl maltol, vanillin, and ethyl vanillin had similar patterns of transfer efficiency, which increased as puff volume, duration, and wattage increased. Nicotine transferred well and was not affected by these factors. Cinnamaldehyde and eugenol were similar to nicotine when the standard pump head was used. Of the chemicals tested, nicotine had the highest vapor pressure and hence lowest intermolecular forces (Table 2.2), which likely contributed to its high transfer efficiency. Eugenol and cinnamaldehyde had slightly lower vapor pressures, which may explain their efficient transfer with the standard pump head. However, like other flavor chemicals, eugenol and cinnamaldehyde did not transfer well with the high-performance pump head, probably due to the mechanics of the pump. For those chemicals with low vapor pressures (maltol, ethyl maltol, vanillin, and ethyl vanillin), transfer efficiency was also likely affected by the heat generated in the atomizers. Efficiency increased when puff duration and wattage increased, both factors which increase heat. These results fall in line with a study showing an increase in voltage from 3 to 6V increased aerosol generation across refill fluids and cartomizers (Havel et al., 2017). Although we tested only one brand of EC, transfer efficiencies would likely also be affected by EC brand.

Chemical	Structure	Vapor Pressure (mm HG at 25°C)
Ethyl Vanillin		0.00001
Ethyl Maltol		0.00022
Maltol		0.0005
Vanillin		0.002
Furaneol		0.008

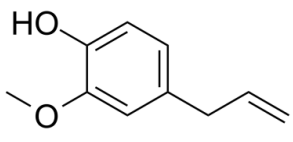
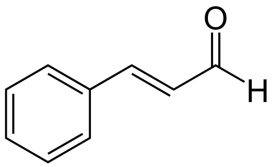
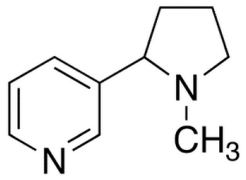
Eugenol		0.022
Cinnamaldehyde		0.0289
Nicotine		0.038

Table 2.2: Vapor pressures and structures of flavor chemicals and nicotine

EC puffing topography varied among participants but was usually similar between trials for each individual, in agreement with Behar et al. 2015 who showed that each participant had their own “fingerprint” that defined their puffing topography (Behar et al., 2015). Our participants had similar patterns of puff volume and exhale irrespective of the wattage or refill fluid they were using. In a preliminary *ad libitum* study, users had an average of 3.5 ± 1.4 seconds puff duration (St Helen et al., 2016), while another study evaluated YouTube videos for an average of 4.3 seconds puff duration (Hua et al., 2013). In our study, the average puff duration (1.4 ± 0.27 seconds) could be related to the younger age of our participants and/or their lack of cigarette smoking experience.

The concentration of exhaled flavor chemicals increased when the ECs were operated at a higher wattage. Nicotine exhale also varied with the wattage and refill fluid consumed. Based on the exhale data, there were two categories of vapers – those who exhaled some flavor chemicals and those who exhaled little or no flavor chemicals. It has been suggested that naïve vapers using first generation ‘cig-a-like’ ECs had buccal rather than pulmonary absorption (Bullen et al., 2010; Vansickel et al., 2012). By quantifying the exhale of the participants, we were able to distinguish mouth vs. lung inhalation. Our participants were young (average age 21), and only one participant reported the use of tobacco cigarettes once a month. Therefore, it is possible that the “mouth inhalers” have not yet learned how to inhale into their lungs for maximum nicotine retention or they intentionally chose not to do this as they engage in vaping as a social activity.

We modeled chemical retention for 10 topographies (Fig. 2.6) and found that retention varied among chemicals and between user topographies (i.e., lung vs mouth inhalers). Cinnamaldehyde was retained better than other flavor chemicals by the

“mouth inhalers”, suggesting that it is more soluble and/or reactive than the other aldehydes (e.g., vanillin or ethyl vanillin). This is concerning because cinnamaldehyde induces loss of ciliary motility and impairs mucociliary transport leading to respiratory infections (Clapp et al., 2019). Cinnamon-flavored refill fluids were also the most toxic of 36 refill fluids screened in vitro with three different cell types in the MTT assay (Bahl et al., 2012) and some cinnamon flavored products have very high concentrations of cinnamaldehyde, up to 343 mg/ml (Omaiye et al., 2019). It may be difficult for users to avoid exposure to cinnamaldehyde, as it has been reported in refill fluids that do not indicate a cinnamon flavor, such as Black Cherry or Caramel (Behar et al., 2016).

The retention of flavor chemicals and nicotine was about 100% for all “lung inhalers”, while retention for “mouth inhalers” was variable, but never 100%. In fact, nicotine was better retained than all flavor chemicals except cinnamaldehyde. These data add to the information needed to evaluate human exposure to EC aerosols. The amount and rate of nicotine delivered may depend on the user topography, such as puff duration, or the nicotine concentration or the flavor (Dawkins and Corcoran, 2014; Hiler et al., 2017; Hajek et al., 2017; St Helen et al., 2017; Voos et al., 2019). The retention of nicotine may be influenced by various factors, such as the pH of refill fluids or protonation by benzoic acid (Helen et al., 2018; Duell et al., 2018; Pankow et al., 2017), which is particularly relevant to pod-style products that contain acids and high nicotine concentrations.

Exhaled aerosol settles on surfaces forming ECEAR, which can remain for months (Khachatoorian et al., 2018). In previous studies, ECEAR had nicotine concentrations ranging from 0.03 to 0.949 μg/cm^2 depending on the surface (Marcham et al., 2019), while an EC user’s home had 7.7±17.2 μg/m^2 (Bush and Goniewicz, 2015). However, our previous study showed nicotine accumulated to a concentration of 108 mg/m^2 after 1

month inside a vape shop and up to 1,181 μg/m^2 inside a living room field site after 3 months (Khachatoorian et al., 2019). The exhaled flavor chemicals and nicotine in ECEAR are mostly likely contributed by mouth inhalers. Nevertheless, lung inhalers do exhale a visible puff of aerosol, which may contain mainly solvents. Other chemicals that were not measured in this study that could contribute to ECEAR include solvents, metals, and reaction products, such as formaldehyde, and acetaldehyde (Son et al., 2020; Li et al., 2020; Geiss et al., 2015).

While our focus was on ECEAR, the suspended exhale from EC users could also cause passive secondhand exposure to non-vapors. This idea is supported by studies in which non-vaping participants who were exposed to secondhand EC aerosols had alterations in respiratory mechanics and increases in salivary and urinary cotinine, urinary trans-3'-hydroxycotinine, and acrolein metabolites (Johnson et al., 2019; Tzortzi et al., 2018).

In conclusion, this is the first study to quantify flavor chemicals and nicotine in refill fluids, aerosols, and EC users' exhale and then deduce their retention and contribution to ECEAR. The transfer of flavor chemicals with low vapor pressures to aerosols was dependent on puff duration, puff volume, user topography, pump head, and EC wattage, while nicotine transfer was not significantly affected by these factors. Analysis of exhaled chemicals enabled identification of mouth and lung inhalers. Mouth inhalers exhaled chemicals and contributed to ECEAR, while lung inhalers retained almost all the inhaled flavor chemicals and nicotine. Since the retention of toxic chemicals is higher in lung inhalers, harm reduction could be achieved if lung inhalers switched to mouth inhalation; however, this would increase the concentration of chemicals in ECEAR, which may affect those who are passively exposed to EC chemicals. These data contribute to our

understanding of EC chemical transfer, retention, and contribution to ECEAR and are important to inform EC users, the public, and government agencies of potential exposures to chemical produced by ECs.

LIMITATIONS OF THE STUDY

Our study is based on a relatively small sample size comprised of predominantly young Asian males. Future studies could be expanded to include a more ethnically diverse population of EC users and more females. While our data are based on a single brand of EC, numerous brands spanning four generations are available, and should be evaluated in the future to determine how results are affected by brand. The introduction of pod-style ECs and loopholes in the flavor ban have led to the increased use of disposable pod-style ECs with many flavors and higher nicotine concentrations than were used in our study (US Dept. of Health & Human Services). Pod based products would be particularly interesting to examine in the future since these advanced devices can deliver higher nicotine concentrations to EC users (Yingst et al., 2019a;Yingst et al., 2019b).

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Chapter 3

Oxidative Stress and Inflammatory Response in Keratinocytes and a 3D Human Skin Model Upon Exposure to Electronic Cigarette Refill Fluids and Residues

Khachatoorian et al.

ABSTRACT

Background: The effects of electronic cigarette (EC) refill fluids, aerosols, and residue on human skin are poorly understood.

Objective: The goal of this study was to characterize the effects of EC refill fluids and EC exhaled aerosol residue (ECEAR) on cultured keratinocytes and a 3D human skin model (MatTek EpiDerm™).

Methods: Flavor chemical and nicotine quantification of Dewberry Cream and Churrios refill fluids was done by GC-MS. Keratinocytes were exposed to different concentrations of both fluids to determine cytotoxicity with the MTT assay and reactive oxygen species (ROS) production with CellROX® and ROS-Glo™ H2O2 assays. MatTek EpiDerm™ skin tissues were exposed to various concentrations of Dewberry Cream and Churrios refill fluids and lab made refill fluids for either 4 or 24 hours. Cytotoxicity was determined with the MTT and LDH assay and inflammatory markers were quantified with ELISAs. ECEAR was produced by a participant who used both Dewberry Cream and Churrios over 5 days in a lab-controlled setting. EpiDerm™ was exposed to ECEAR and ECEAR extract to determine cytotoxicity and inflammatory marker secretion.

Results: Major flavor chemicals (>1mg/mL) in Dewberry Cream were maltol, ethyl maltol, vanillin, ethyl vanillin, furaneol, while Churrios contained ethyl maltol, benzyl alcohol, vanillin, and ethyl vanillin. Churrios was cytotoxic to keratinocytes, and both fluids induced ROS production in the medium and cells. Dewberry Cream and Churrios did not alter the histology of EpiDerm™; however, both fluids caused secretion of inflammatory markers (IL-1α, IL-6, and MMP-9). Three exposure protocols were used with propylene glycol (PG)/ethyl maltol. None produced an effect in the MTT or LDH assay, however all increased secretion of IL-1α and MMP-9. Individual flavor chemicals

with PG were not cytotoxic in the MTT assay but did induced secretion of IL-1α, which was attributable to PG. ECEAR did not induce cytotoxicity in the MTT or LDH assay; however, ECEAR extract from Churrios both refill fluids induced secretion of IL-1α.

Conclusions: Refill fluids increased ROS and stimulated secretion of inflammatory cytokines in keratinocytes and EpiDerm™. IL-1α secretion was attributed to PG. ECEAR also activated an inflammatory response in the EpiDerm. These data are consistent with studies indicating chemicals in EC refill fluids can cause irritation and damage to the skin.

INTRODUCTION

The skin is made up of four layers of cells that include the corneum, granulosum, spinosum, and basal. Keratinocytes, which are located in the basal layer and move up to the corneum as they mature, are the main barrier protecting the organism against pathogens and chemical/physical damage. Epidermal keratinocytes proliferate and synthesize proteins and large amounts of lipids to establish the stratum corneum (Hornig-Do et al., 2007). Due to their location at the boundary between the human body and environment, keratinocytes are directly exposed to pro-oxidants and may be especially vulnerable to damage by reactive oxygen species (ROS) (Thiele et al., 1999). Although the skin is a protective barrier, it can be permeated and irritated.

Electronic cigarettes (ECs) have gained worldwide popularity since their introduction in 2004 (Trtchounian and Talbot, 2010; Grana et al., 2014; US Food and Drug Administration, 2020). While there are now four generations of ECs (Williams et al., 2019; National Academies of Sciences, Engineering, and Medicine, 2018), the main components of all ECs are similar and consist of a cartridge/tank/pod with liquid (refill fluid), a battery, and a heating element (atomizer). Refill fluids contain solvents (usually propylene glycol (PG) and/or glycerol (G)), flavor chemicals, and nicotine, which usually ranges in concentration from 0 to 60 mg (National Academies of Sciences, Engineering, and Medicine, 2018; Omaiye et al., 2019a). However, nicotine concentration can be much higher. A vial of refill fluid solution was reported to contain 1080 mg of nicotine (Cameron et al., 2014), while an unlabeled bottle of DIY nicotine contained over 100 mg/ml (Davis et al., 2015). The user can either buy new cartridges/pods/tanks or refill used products. Products from all generations have been reported to leak fluids, which come in direct contact with the skin of users (Trtchounian et al., 2010; Hughes and

Hendrickson, 2019). In addition, fluid can spill onto the skin during refilling (EU Health Programme, 2016).

One of the top disorders in a study that mined health data from an EC Internet website was inflammation of the skin (e.g, itching and eczema) (Hua et al., 2020), possibly due to direct exposure to e-liquids followed by an allergic reaction. Refill fluids can contain high concentrations of flavor chemicals, sometimes as high as 100 mg/mL (Behar et al., 2016). Flavor chemicals like diacetyl and acetoin can cause skin problems, such as rashes or the development of dermatitis, and are present in a many e-fluids and aerosols (US Department of Labor, Occupational Safety and Health Administration 2009; Farsalinos et al., 2014; Melvin et al., 2020). Nicotine is also a skin irritant that can be absorbed through the skin in water (Zorin et al., 1999; Kuswahyuning and Roberts, 2014) or from leaked refill fluid (Maina et al., 2016). Nicotine in refill fluids can remain on the skin even after vigorous hand washing (Maina et al., 2017). Nickel has also been identified as the source of skin rashes in EC users (Maridet et al., 2015; Shim and Kosztyuova, 2018).

Finally, EC users exhale puffs of aerosol that settle on indoor surfaces. We refer to this as EC exhaled aerosol residue (ECEAR) (Khachatoorian et al., 2018; Khachatoorian et al., 2019; Khachatoorian et al., 2020). The skin would usually be the first point of contact for ECEAR and is likely the main route of exposure. ECEAR contains nicotine, flavor chemicals, solvents, nicotine alkaloids, and tobacco specific nitrosamines (TSNAs) (Son et al., 2020; Khachatoorian et al., 2018; Khachatoorian et al., 2019; Khachatoorian et al., 2020; Bush and Goniewicz, 2015; Goniewicz and Lee, 2015; Sempio et al., 2019). ECEAR chemicals increased in concentration in a vape shop over a month-long period of monitoring, and concentrations reached up to 108 mg of

nicotine /m^2 in heavily used areas (Khachatoorian et al., 2019). ECEAR can also move through spaces and vents, collecting and accumulating on surfaces away from its point of origin (Khachatoorian et al., 2018). Therefore, ECEAR accumulation in indoor environments presents the opportunity for active and passive exposure, especially through the skin. In addition, it is well documented that tobacco smoke contributes, to photoaging and skin cancer (Pavlou et al., 2009; Yin et al., 2000). The health effects of accumulated ECEAR have not been studied in spite of its environmental accumulation over the past 15 years.

Our goal was to identify the flavor chemicals in two popular brands of refill fluid, test these products using both submerged cultures of human keratinocytes and air-liquid interface (ALI) exposure of EpiDerm™ 3D tissues, and evaluate how ECEAR extracts affect these skin models. We specifically examined endpoints that included cytotoxicity, oxidative stress, and inflammation.

METHODS

Refill fluids

Refill fluids were purchased at a local vape shop. Dewberry Cream by Kilo was chosen because it has a high total concentration of flavor chemicals (Hua et al., 2019) and high concentrations of widely used flavor chemicals, including vanillin, ethyl vanillin, maltol and ethyl maltol. Churrios by the Milk Man was chosen because cinnamon-flavored refill fluids adversely affect cultured cells and respiratory tissues in animal studies (Behar et al., 2016; Wavreil and Heggland, 2019; Clapp et al., 2019; Fetterman et al., 2018). Each refill fluid had a labeled nicotine concentration of 6 mg/mL. Dewberry Cream was labeled 70/30 glycerin/propylene glycol (VG/PG) ratio and Churrios was labeled MAX VG.

Lab made refill fluids

Custom refill fluids were made in our lab using 70% PG plus one of the following flavor chemicals: 1.7 mg/mL maltol (Sigma Aldrich, MO), 5.2 mg/mL ethyl maltol (Sigma Aldrich, MO), 7 mg/mL vanillin (Sigma Aldrich, MO), 7.5 mg/mL ethyl vanillin (Sigma Aldrich, MO), and 4.2 mg/mL benzyl alcohol (Sigma Aldrich, MO). These concentrations of flavor chemicals were chosen based on their concentrations in Dewberry Cream and Churrios refill fluid. PG and ethyl maltol at the above concentrations were used to test exposure protocols. The purity of all standards was first confirmed using GC/MS.

Identification and quantification of flavor chemicals in refill fluids using GC/MS.

Dewberry Cream and Churrios refill fluids were analyzed by GC/MS. Internal standard-based calibration procedures similar to those described elsewhere were used (Tierney et al., 2016; Omaiye et al., 2019b; Brown and Cheng, 2014), and analyses for 176 flavor-related target analytes and nicotine were performed with an Agilent (Santa Clara, CA)

5975C GCMS system. The capillary column used was a Restek (Bellefonte, PA) Rxi-624Sil MS (30 m long, 0.25 mm id, and 1.4 µm film thickness). For each refill fluid sample, 50 µL was dissolved in 950 µL of isopropanol (Fisher Scientific, Fair Lawn, New Jersey, USA). Prior to analysis, 20 µL of internal standard solution (2 µg/µL of 1,2,3-trichlorobenzene in isopropyl alcohol) was added into the 1 mL diluted refill samples, the aerosol and exhaled extract aliquots. 1 µL of the sample was injected into the GC/MS with a 10:1 split. The injector temperature was 235°C. The GC temperature program for all analyses was as follows: 40°C hold for 2 min; 10°C/min to 100°C; 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 at electron ionization mode. The ion source temperature was 226°C. The scan range was from 34 to 400 amu. Each target analyte was quantitated using authentic standard material, and an internal standard (1,2,3-trichlorobenzene) normalized multipoint calibration. The limit of quantification was 20 µg/ml.

Culturing Keratinocytes

CCD 1106 KERTr (ATCC® CRL-2309™) transformed human keratinocytes cells were cultured on poly-L-lysine coated flasks using the supplier's protocol in keratinocyte-Serum Free Medium (Gibco 17005-042) with added Keratinocytes Supplements (Gibco 37000-015) including Bovine Pituitary Extract (BPE; Gibco 13028-014) and human recombinant epidermal growth factor (EGF; Gibco 10450-013) further supplemented with a 35 ng/mL of human recombinant epidermal growth factor (EGF; BD cat# 354052). Cells were incubated in a 37°C/5% CO_2/95% relative humidity incubator for 24 to 48 hours before processing the cells for experiments. In experiments, keratinocytes were dispersed into single cells and seeded at a density of 1,000 cells/well in a 96 well plate

or 5,000 cells/well in an Ibidi µ-Slide 8 Well (Germany). Cells were counted using a hemocytometer.

Cytotoxicity of refill fluids using the MTT Assay

Refill fluids were tested for cytotoxicity using the MTT assay. The enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase. Cells were seeded and incubated for 24 hrs then exposed to refill fluids for 24 hrs. After exposures, 20 µl of 1 mg/mL of MTT reagent dissolved in phosphate buffered saline was added to each well in a 96 well plate for 2 hrs, then, formazan crystals were solubilized in dimethyl sulfoxide, and absorbance was read at 570 nm. For each refill fluid tested, three independent experiments were performed.

ROS-Glo™ H_2O_2 Assay

The Promega ROS-Glo™ H_2O_2 Assay (Madison, WI) was used to measure hydrogen peroxide in culture media, culture media plus refill fluid, and culture media plus refill fluid and cells. The non-lytic protocol provided by the manufacturer was followed. Controls reactions without cells were used to determine spontaneous hydrogen peroxide reaction. These were: medium only, medium with positive control, medium with 1% Dewberry Cream, and medium with 1% Churrios. Cells were seeded on a 96 well plate at 1000 cells/well and allowed to attach for 24 hrs., after which they were incubated in medium only, medium with positive control, medium with 1% Dewberry Cream, and medium with 1% Churrios for 24 hrs. 10 µM menadione was used as a positive control. 20µl of H_2O_2 Substrate solution was added to all samples at 18 hrs. (final 6 hrs. of treatment). When treatment was done, 50µl of media from each sample well was combined with 50µl of

ROS-Glo™ Detection Solution in a separate plate and incubated for 20 minutes at room temperature. Synergy™ HTX Multi-Mode Microplate Reader (BioTek, VT) was used to read relative luminescence units (RLU) (Gain: 135, bottom optics). Each sample was done in duplicate, average luminescence is reported.

Live cell imaging

InVitrogen CellROX™ Green Reagent (Carlsbad, CA) was used to assay oxidative stress in cells exposed to Dewberry Cream and Churrios refill fluids. Cell ROX^{TM} Green reagent is a dye which binds to DNA upon oxidation. For live imaging, cells were plated on µ-Slide Ibidi 8-well chambers (Ibidi) at approximately 5,000 cells/well. After 24 hrs. of attachment to the slide, cells were exposed to Dewberry Cream and Churrios refill fluids for 24 hrs. 50µM menadione was used as a positive control. Cells were exposed to 5 µM CellROX Green for 30 minutes, then rinsed three times with PBS (+). ThermoFisher NucBlue® (Waltham, MA) Live reagent (Hoechst® 33342 dye) was used to stain the nuclei of all cells. Cells were rinsed three times with PBS (+) and medium was added to each well. Fluorescent images were collected using a Nikon TI Eclipse inverted microscope equipped with a LiveCell temperature and CO_2-regulating, heated stage (Pathology Devices Inc).and fresh medium. Cells were imaged live using a Nikon Eclipse Ti-E microscope with a 37°C, 5% CO2, and 90% relative humidity-regulated stage top LiveCell Incubation Chamber (Pathology Devices, Inc., San Diego, CA). The images were collected using a Nikon 40x objective with 0.75 NA (model: CFI Plan Fluor). A high-resolution Andor Zyla VSC-04941 camera (Andor, Belfast, UK) was used to capture images. Excitation illumination was from a Nikon INTENSILIGHT C-HGFIE lamp. Nikon Elements was used to combine DAPI and TRITC images, and FIJI was used to sharpen.

EpiDerm™ Culture

EpiDerm™ is a skin tissue model used to replace in vivo rabbit skin. Experiments are done in air liquid interface with exposures applied to the apical side while medium feeds the basal side of tissues. Twenty-four-well EpiDerm^{TM} Skin cultures (Part No. EPI-100) in a 3-D epidermis model from MatTek Corporation (Ashland, Massachusetts) were transferred from agarose to EPI-100-NMM medium and incubated at 37 ± 1 °C, 5 ± 1% CO2, 95% relative humidity (RH) for 60 ± 5 min. Tissues were transferred to fresh medium and incubated at 37 ± 1 °C, 5 ± 1% CO2, 95% RH overnight (18 ± 3 h). The *In Vitro* EpiDerm^{TM} Skin Irritation Test (EPI-200-SIT) was followed to determine irritation (Kandárová et al., 2009).

EpiDerm™ Exposure to Flavor Chemicals and ECEAR

All samples were done in triplicate (three tissues) and treated for either 4 hrs. only or 4 and 24 hrs. at 37 ± 1 °C, 5 ± 1% CO2, 95% RH. All experiments had with a negative control (30 µl of DPBS) and a positive control (30 µl 5% SDS). For refill fluid experiments, EpiDerm™ tissues were exposed for 4 and 24 hrs. to three concentrations (10%, 30%, and 100%) of Dewberry Cream and Churrios. For the concentration experiments, EpiDerm™ tissues were exposed to PG ethyl maltol mixture (5.2 mg/mL of ethyl maltol in 70% PG) for 4 hrs. with three protocols (1) 30 µL for 4 hrs., (2) 30 µL for 2 hours followed by another 30 µL for 2 additional hours, or (3) 60 µL for 4 hrs. For the lab made refill fluid experiments EpiDerm™ tissues were exposed to authentic standards of the dominant flavor chemicals in Dewberry Cream and Churrios with protocol 1. Finally, for the ECEAR experiments, EpiDerm™ tissues were exposed to ECEAR or 30 µL ECEAR extract for 4 and 24 hrs. ECEAR paper towel was cut to 0.6 cm^2 and applied to

the apical side of tissues, then 30 µl of water was applied on top of the paper towel to ensure adhesion to tissues for 4 or 24 hrs.

Cytotoxicity of Refill fluids and Flavor Chemical using the MTT Assay with EpiDerm™

The enzymatic reduction of MTT was measured as described above with the following modifications. After each exposure, EpiDerm™ inserts were washed with PBS, loaded with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide according to the MatTek EPI-200-SIT (Skin Irritation Test) protocol, then placed in a 24-well plate containing 300 µL of MTT (1 mg/mL) and incubated for 3 hrs. at 37 °C, 5% CO2. After incubation, tissues were transferred into 24-well plates containing 2.0 mL of Isopropyl alcohol (IPA) (Kandárová et al., 2009) and samples were read in a spectrophotometer at an absorbance of 570 nm. The percent of control was determined using the equation: % of control = OD (treated tissue)/OD (untreated tissue) x 100.

Histology of EpiDerm™ Tissues

EpiDerm™ tissues were exposed to DPBS (negative control), 5% sodium dodecyl sulfate (SDS positive control), 100% Dewberry Cream, and 100% Churrios refill fluid for 24 hrs. Tissues were fixed in 10% paraformaldehyde overnight in 4 °C and shipped to MatTek for hematoxylin and eosin (H&E) staining (MatTek histology characterization sample preparation procedure). Histology slides were imaged using a DS-Fi1 color camera on a Nikon Eclipse TI inverted microscope using a 20x 0.45 NA (model: CFI Plan Apochromat VC 20X). Nikon Elements software was used to process images.

*IL-1*α, IL-6, *and MMP-9 Secretion from EpiDerm™*

After exposures to refill fluids, media were collected from EpiDerm™ cultures, aliquoted into Eppendorf tubes, and stored at -80 °C for later analysis of IL-1α, IL-6, and MMP-9. R&D Systems Quantikine ELISA Human IL-1α/IL-1F1 kit, Biolegend ELISA MAX™ Deluxe Set Human IL-6 kit, and R&D Systems Quantikine ELISA Human MMP-9 kit were used. Medium only was run to confirm no reaction with reagents. Standard curves were generated using a four-parameter logistic curve fit in GraphPad Prism software (GraphPad, San Diego, California, USA). Media from tissues were run in duplicate on each ELISA, and results were averaged. Each tissue was considered an independent experiment, and three independent experiments were done for each endpoint.

LDH Assay with EpiDerm™

Cell media were collected and immediately assayed using the OPS Diagnostics Lactate Dehydrogenase Protocol. Tris-HCl (Sigma-Aldrich, T-3253) and Tris-base (Sigma-Aldrich, T4661) were combined to make TRIS. Iodonitrotetrazolium chloride (INT, Sigma I-8377) dissolved in DMSO (Sigma D-8779), phenazine methosulfate (PMS, Sigma P-9625), nicotinamide adenine dinucleotide (NAD, Sigma N-0632), and lithium lactate (Sigma L-1500) were prepared before use and stored at -20°C. 50µl of 100mM TRIS were added to 50 µl of 50 mM lithium lactate and 50 µl of PMS, INT, NAD. 50 µl of sample were added to each well and after 5 minutes, the absorbance was read on a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, VT). The cytotoxicity was calculated:

$$\frac{Abs\ (x) - Abs\ (Negative\ CN)}{Abs\ (Positive\ CN) - Abs\ (Negative\ CN)}\ x\ 100$$

Participant Recruitment for ECEAR Generation

A 21-year-old Asian male was recruited for the exhale/ECEAR generation portion of the study. The participant self-reported no use of combustible cigarettes during the study and was told to abstain from using ECs for 1 hour before the reporting to our lab. The inclusion criteria were: (1) experienced EC users (at least 3 months) and (2) must use at least 3 mg of nicotine in his current EC. The participant would have been excluded if they were: (1) pregnant or breast feeding, (2) under the age of 18 or over 75 years, (3) a never-user of EC with nicotine, or (4) experiencing any medical conditions. The participant signed informed consent before admission into the study. The project was approved by the UCR Internal review Board (IRB # HS-12-023).

ECEAR Collection

In each session, the participant exhaled into tubing attached to an acrylic exhale chamber for each 2-hour session on 5 different days. The EC was an Innokin iTaste MVP 3.0 battery with variable voltage (3V–9V) and wattage (6–30 watts) and with fresh unused Innokin iClear16D dual coil clearomizers (or tanks). A new tank was filled with 2 ml of either Dewberry Cream or Churrios before the first session and used throughout the 5 days. It was not refilled. The EC was set on 6 volts, 1.9 ohms, and 18.9 watts. The exhale chamber was a rectangular acrylic box on wheels that had two ventilation holes. A 2 ft piece of Cole-Parmer Masterflex tubing L/S 18 tubing was attached to the hole on the side of the chamber to allow the participant to exhale into the chamber. A fresh piece of Bounty paper towel placed on the floor of the exhale chamber for 5 days of ECEAR collection.

ECEAR Extraction

The paper towel was collected 20 minutes after the last exhale on the last day and either extracted right away (ECEAR extract) or cut into smaller pieces (ECEAR) for EpiDerm™ exposure. To obtain ECEAR extract, paper towel was cut into smaller pieces for an extraction concentration of 0.1 g/mL of EpiDerm™ Assay Medium. 15 mL Falcon tubes with paper towel and medium were shaken for 1 hour then medium was transferred into 1.5 ml Eppendorf tubes and stored at -80°C for later EpiDerm™ exposures. The rest of the paper towel was stored immediately upon collection in a Ziploc bag inside a mylar bag in -80 °C freezer.

Data Analysis

All cytotoxicity assays were carried out using three independent experiments each with different passages of cells, and each experiment had triplicate points. Keratinocyte MTT and CellROX™ data were statistically analyzed with 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, CA). Data (EpiDerm™ MTT and ELISA) which did not satisfy the assumptions of ANOVA (normal distribution of data and homogeneity of variances) were transformed by Box-Cox transformation after which a one-way ANOVA was applied in MiniTab 17.0 (MiniTab Inc, PA). Dunnett's post hoc test was used to compare exposures to negative controls. Means were considered significantly different when p values were equal to or less than 0.05. All graphs were made using GraphPad Prism 8.0 software (GraphPad, San Diego, California, USA).

RESULTS

Flavor Chemicals in "Dewberry Cream" and "Churrios" Refill Fluids

Of the 177 flavor chemicals on our target list, Dewberry Cream and Churrios had 30 and 19, respectively. The heatmaps show the flavor chemicals (left y-axis) detected in the refill fluids, and the color gradient scale (right y-axis) shows their concentrations in mg/mL (Figure 3.1A, B).

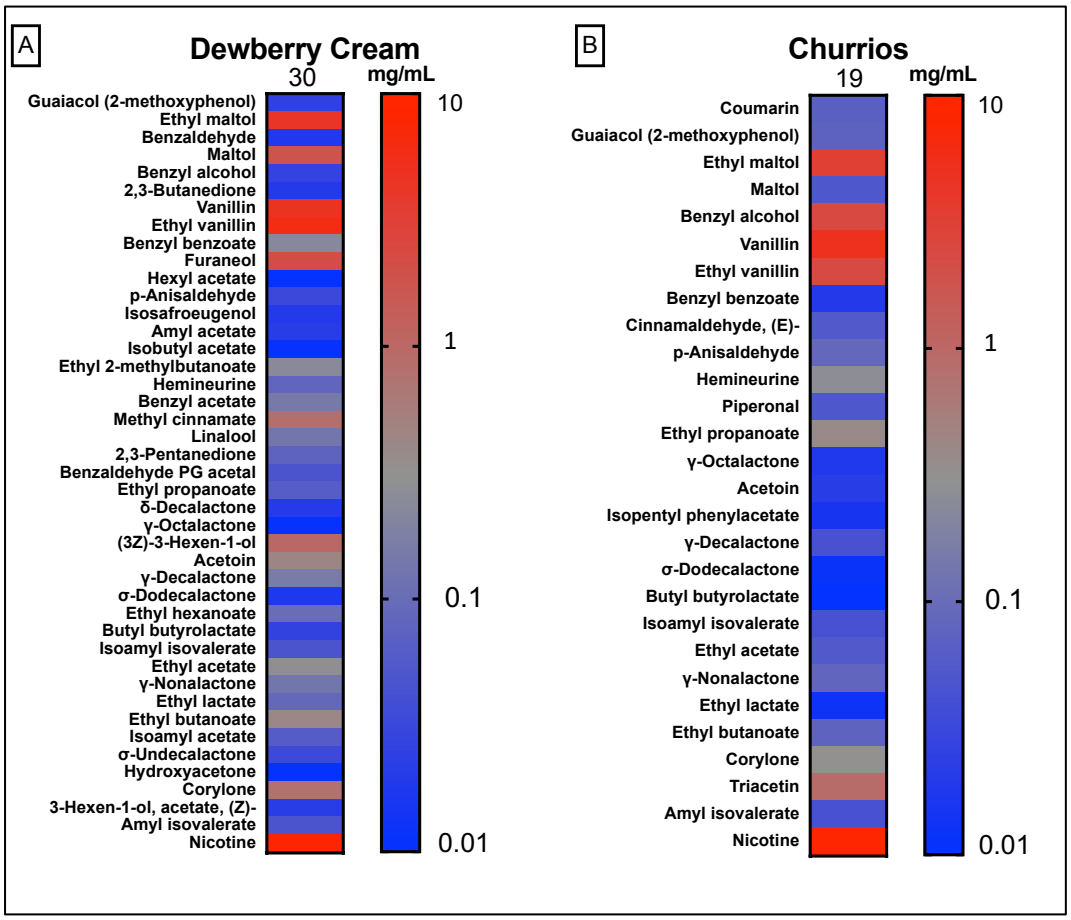

Figure 3.1: Flavor chemicals in Dewberry Cream and Churrios. Heatmaps showing concentrations of flavor chemicals and nicotine in "Dewberry Cream" (A) and "Churrios" (B) refill fluids. Flavor chemicals are listed on the left y-axis and concentrations are in mg/mL. Right y-axis shows a gradient color map from 10 mg/ml to 0.01 mg/mL.

The flavor profile of Dewberry Cream is described by the manufacturer as mixed berries, honeydew, and cream. The total concentration of flavor chemicals in Dewberry Cream was 30 mg/mL (Figure 3.1A). Five flavor chemicals had concentrations > 1 mg/mL (7.5 mg/ml ethyl vanillin, 7 mg/mL vanillin, 5.2 mg/mL ethyl maltol, 1.7 mg/mL maltol, 1.6 mg/mL furaneol). Although labeled as 6 mg of nicotine/mL, the measured concentration was 6.7 mg/mL.

The flavor profile of Churrios is described by the manufacturer as brown sugar, sweet cinnamon with fresh milk, and honey-infused cereal base. The total concentration of flavor chemicals in Churrios was 19 mg/mL (Figure 3.1B). Four flavor chemicals were present in Churrios at concentrations > 1 mg/mL (4.6 mg/mL ethyl maltol, 6.2 mg/mL vanillin, 2.2 mg/mL ethyl vanillin, and 4.2 mg/mL benzyl alcohol). Churrios contained 0.25 mg/mL of cinnamaldehyde, and although labeled as 6 mg of nicotine/mL, the measured concentration was 7.8 mg/mL.

Cytotoxicity of Refill Fluids When Exposed to Keratinocytes

The cytotoxicity of Dewberry Cream and Churrios refill fluids was measured using human keratinocytes in conjunction with the MTT assay. Keratinocytes were exposed to 0.001, 0.01, 0.1, and 1% of Dewberry Cream or Churrios (Figure 3.2A). While Dewberry Cream did not significantly affect the cells at any concentration tested, there was a significant increase in cytotoxicity in the 1% Churrios group. In both the 0.01 and 0.1% Dewberry Cream and Churrios, there was an increase in mitochondrial reductase activity, although this was not significantly different than the untreated control.

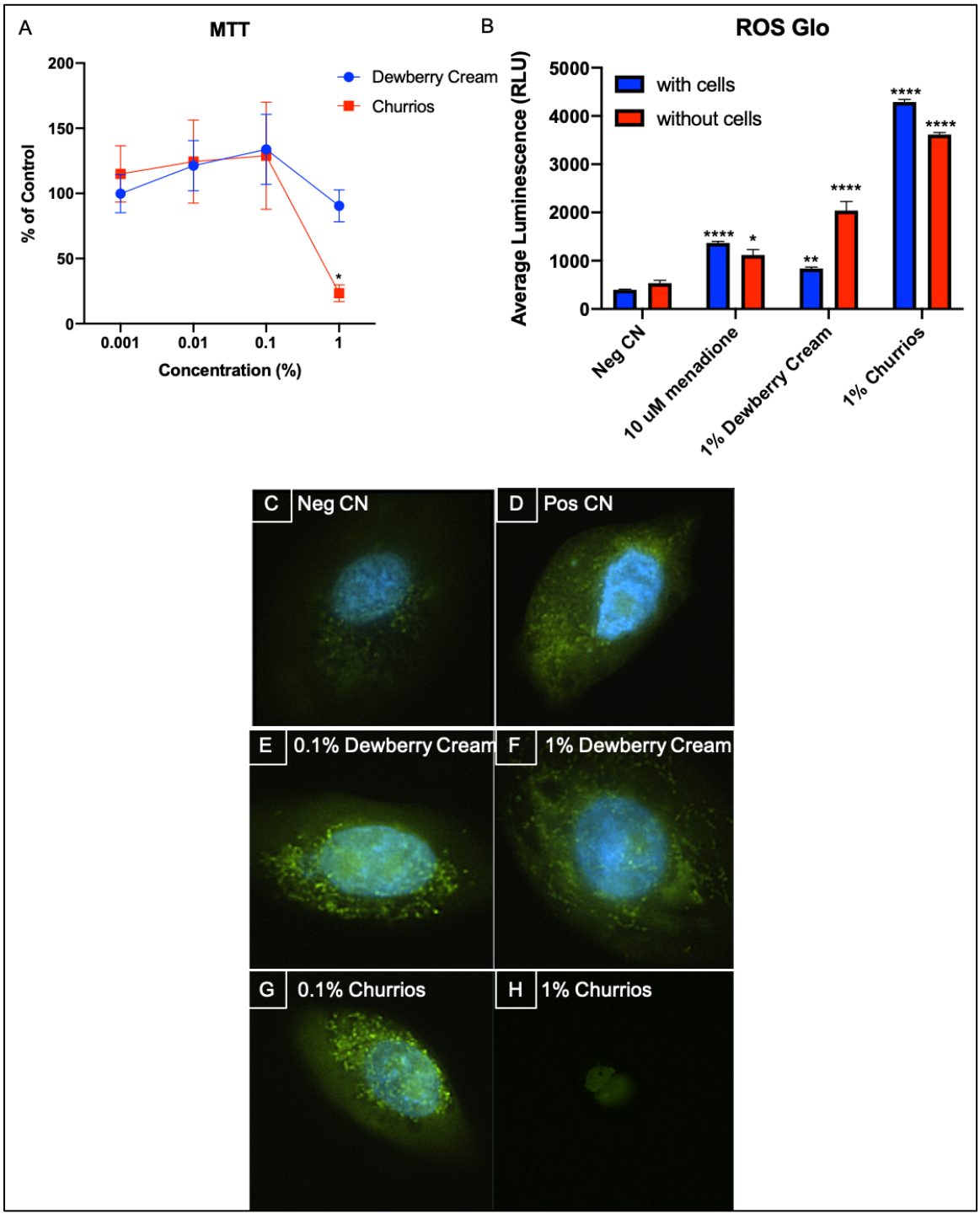
A
MTT
% of Control
200
150
100
50
0
0.001
0.01
0.1
1
Concentration (%)
Dewberry Cream
Churrios
*
B
ROS Glo
Average Luminescence (RLU)
5000
4000
3000
2000
1000
0
with cells
without cells
****
*
**
****
****
****
Neg CN
10 uM menadione
1% Dewberry Cream
1% Churrios
C Neg CN
D Pos CN
E 0.1% Dewberry Cream
F 1% Dewberry Cream
G 0.1% Churrios
H 1% Churrios

Figure 3.2: Cytotoxicity of Keratinocytes exposed to Dewberry Cream and Churrios fluids for 24 hrs. (A) MTT assay. 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. Dewberry Cream is in blue and Churrios is in red. (B) ROS Glo. Experiment was done with (blue) and without (red) cells in wells. The y-axis shows the average luminescence (RLU). The negative control is media only and the positive control is 10 micromolar of menadione. Each bar is the mean ± standard deviation for three independent experiments. (C-H) CellRox. Live cell imaging was done with 40X objective. Representative images are shown. The negative control is media only and the positive control is 50 micromolar of menadione.

Reactive Oxygen Species in Culture Medium and Cells

Extracellular H_2O_2 was measured in the culture medium, culture medium with refill fluids, and culture medium with refill fluids and cells using the ROS-Glo™ assay, in which H_2O_2 concentration is directly proportional to luminescence (Figure 3.2B). The negative control (culture medium only) was slightly luminescent, while the menadione positive control had a significant elevation in luminescence. Addition of Dewberry Cream to the culture medium produced a significant increase in luminescence, which was greater than that observed in the positive control. Addition of Churrios to the medium further increased luminescence beyond that of Dewberry Cream. Addition of cells had little effect on the positive and negative controls. However, addition of cells to the medium containing 1% Dewberry Cream significantly reduced luminescence. In contrast, addition of cells to medium containing 1 % Churrios caused a slight elevation of luminescence.

Reactive oxygen species (ROS) were measured in cells using CellROX™ Green Reagent, which fluoresces upon oxidation by ROS and subsequent binding to DNA. In figures 2C-H, keratinocytes were exposed to 0.1% and 1% concentrations of Dewberry Cream or Churrios for 24 hours then assayed with CellROX™. The negative control was very weakly fluorescent (Figure 3.2C). The positive control, 50 μM menadione, was fluorescent, indicating ROS production (Figure 3.2D). Increased fluorescence of CellROX™ was induced by adding 0.1% and 1% Dewberry Cream or 0.1% Churrios to the culture medium. However, cells exposed to 1% Churrios did not show fluorescence (Figure 3.2A). In all cells showing increased ROS, fluorescence appeared to be localized in the mitochondria.

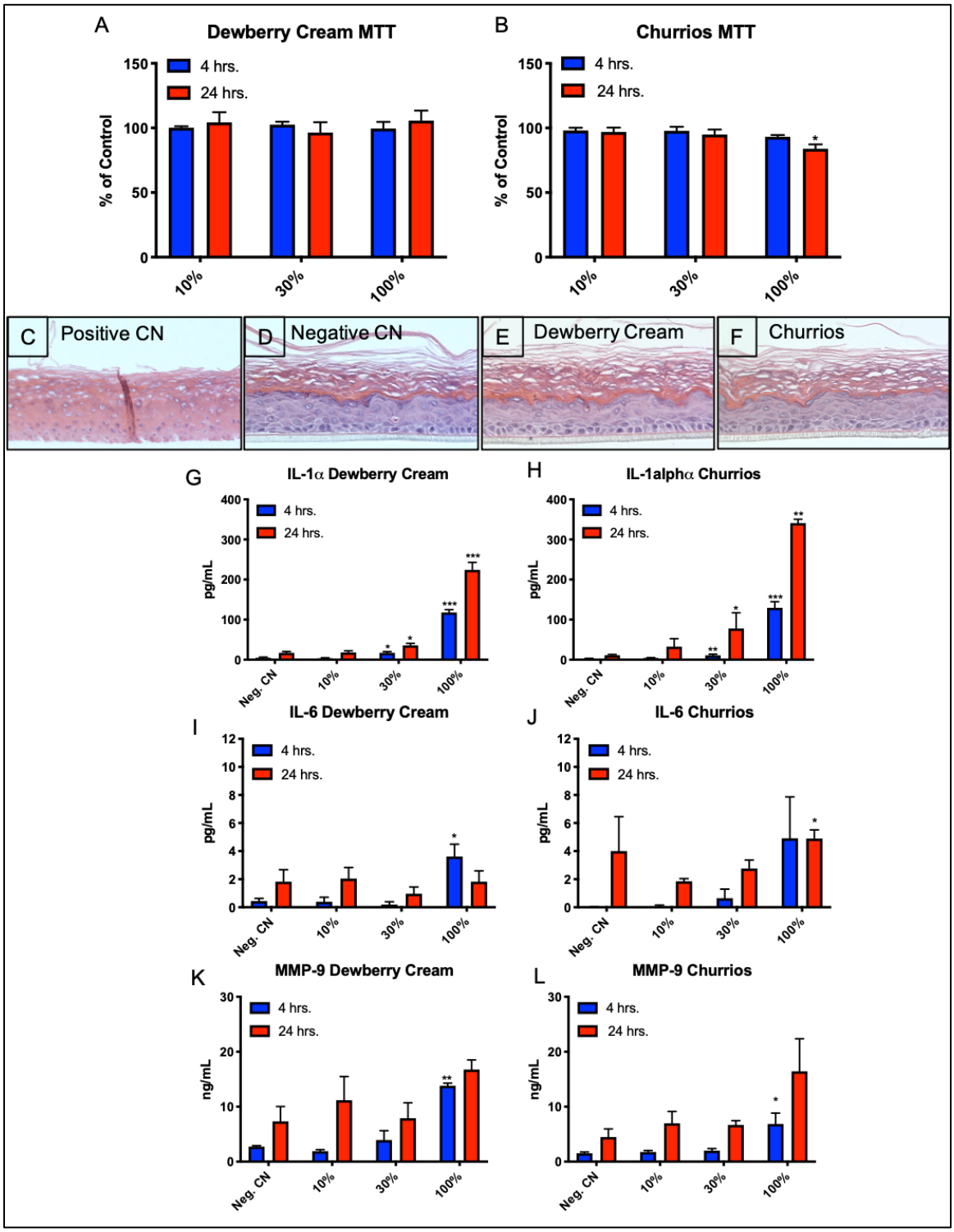
A
Dewberry Cream MTT
4 hrs.
24 hrs.
% of Control
150
100
50
0
10%
30%
100%
B
Churrios MTT
4 hrs.
24 hrs.
% of Control
150
100
50
0
10%
30%
100%
C
Positive CN
D
Negative CN
E
Dewberry Cream
F
Churrios
G
IL-1α Dewberry Cream
4 hrs.
24 hrs.
pg/mL
400
300
200
100
0
Neg. CN
10%
30%
100%
H
IL-1alphα Churrios
4 hrs.
24 hrs.
pg/mL
400
300
200
100
0
Neg. CN
10%
30%
100%
I
IL-6 Dewberry Cream
4 hrs.
24 hrs.
pg/mL
12
10
8
6
4
2
0
Neg. CN
10%
30%
100%
J
IL-6 Churrios
4 hrs.
24 hrs.
pg/mL
12
10
8
6
4
2
0
Neg. CN
10%
30%
100%
K
MMP-9 Dewberry Cream
4 hrs.
24 hrs.
ng/mL
30
20
10
0
Neg. CN
10%
30%
100%
L
MMP-9 Churrios
4 hrs.
24 hrs.
ng/mL
30
20
10
0
Neg. CN
10%
30%
100%

Figure 3.3: EpiDerm™ exposed to Dewberry Cream and Churrios. MTT assay (A and B), histology (C-F), secretion of IL-1alpha (G and H), IL-6 (I and J), and MMP-9 (K and L). The y-axis shows the response of cells in each assay as a percentage of the untreated control for the MTT assay. Each point is the mean ± standard error of the mean for three independent tissues. 4 hr. exposure is in blue and 24 hr. exposure is in red. The y-axis for IL-1α and IL-6 shows secretion of IL-1α and IL-6 in pg/mL. The y-axis for MMP-9 shows secretion of MMP-9 in ng/mL.

Cytotoxicity of Refill Fluids When Tested with EpiDerm™

EpiDerm™ tissues were exposed to 10%, 30%, and 100% concentrations of either Dewberry Cream or Churrios refill fluids for 4 and 24 hours. Tissues were then subjected to the MTT assay to determine if exposures were cytotoxic (Figures 3.3A, B). No significant differences were seen between the untreated control group and the groups treated with Dewberry Cream or with 10 or 30% Churrios. There was a small, but significant, decrease in mitochondrial reductase activity for EpiDerm™ exposed to 100% Churrios for 24 hrs., but this may not be biologically significant as the decrease was very small and did not reach the ISO level of cytotoxicity (< 70% of the control) (Figure 3.3B).

Histology of EpiDerm™ Exposed to Refill Fluids

Histological sections of EpiDerm™ exposed to refill fluids were evaluated microscopically. The positive control (treated with 5% SDS) lacked cellular integrity, did not have discrete cells, had empty gaps in the tissue, and lacked a clear outer cornified layer (Figure 3.3C). The negative control (treated with DPBS) (Figure 3.3D) had a clear stratum corneum, granulosum, spinosum and basal layer. Dewberry Cream and Churrios exposed tissues (Figures 3.3E, F) were similar to the negative control. Distinct layers were observed within the tissues, and cells were intact, unlike the positive control. Tight junctions in the spinosum layer and granules in cytoplasm of the cells in the granulosum layer are clear in all images except for the positive control.

Refill Fluids Induced Secretion of Inflammatory Proteins from EpiDerm™

IL-1α, IL-6 and MMP-9 are markers of an inflammatory response, and their secretion was examined in culture medium of EpiDerm™ treated with Dewberry Cream and Churrios (Figure 3.3G-L). IL-1α was significantly elevated in culture medium of

tissues exposed to 30% and 100% of Dewberry Cream or Churrios. Elevation of Il-1α was first observed at 4 hours of exposure, and it was further elevated at 24 hours.

IL-6 was elevated in culture medium of EpiDerm™ treated with 100% of Dewberry Cream at 4 hours but was not significant in the 24-hour sample (Figure 3.3I). For EpiDerm™ treated with Churrios, IL-6 was elevated in both the 4- and 24-hour samples, but significance was found only in the 24-hour sample (Figure 3.3J).

Secreted matrix metallopeptidase 9 (MMP-9) was elevated by treatment with 100% Dewberry Cream at both 4 and 24 hours, although only the 4-hour treatment showed significance (Figure 3K). Similar results were obtained with 100% Churrios (Figure 3.3L). While the 100% Churrios sample at 24 hours was not significant, it had a p value of 0.063.

Effect of Exposure Protocol on Responses

Prior to evaluating individual flavor chemicals, EpiDerm™ was treated with PG containing 5.2 mg/mL of ethyl maltol using one of three protocols: (1) one-time exposure to 30 µl of PG/ethyl maltol, (2) two-time exposure to 30 µl of PG/ethyl maltol with exposures done 2 hours apart, and (3) one-time exposure to 60 µl of PG/ethyl maltol (Figures 3.4A-D). In each protocol, the endpoints were evaluated 4 hours after exposure. None of the exposure protocols produced an effect in the MTT or the LDH assay (Figures 3.4A, B), indicating treatments were not cytotoxic and were not killing the tissues. IL-1α and MMP-9 were elevated in all three exposure protocols. Elevation was significant for each protocol in the IL-1α assay. Significance was not reached in the MMP-9 assay for any group, although MMP-9 was increased. We used the first protocol

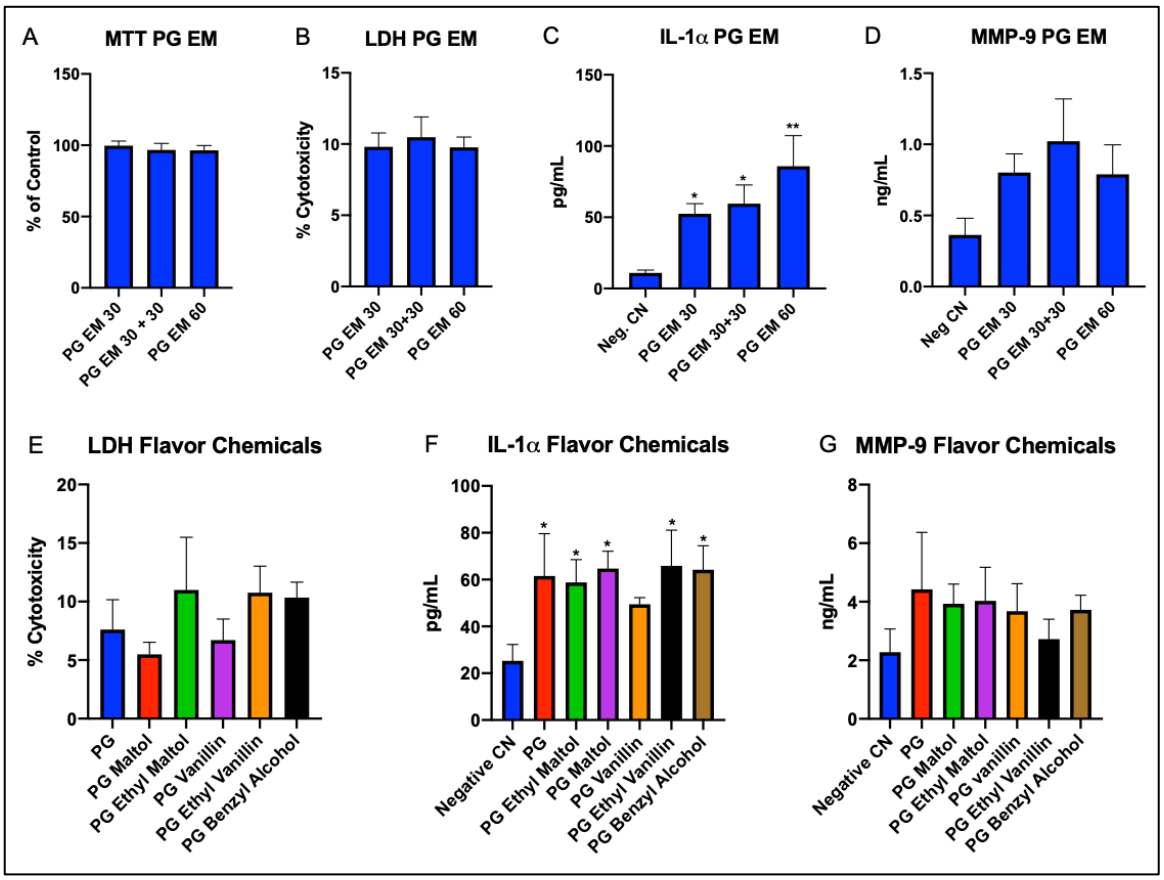

Figure 3.4: EpiDerm™ exposed to lab made refill fluids for 4 hrs. Varying concentrations of PG ethyl maltol were exposed to EpiDerm™ with different protocols (1: 30 µl, 2: 30 µl + 30 µl, and 3: 60 µl) for 4 hrs. total. MTT assay (A), percent cytotoxicity calculated by secretion of LDH (B), IL-1α (C), MMP-9 (D). Lab made refill fluids exposed to EpiDerm™ with protocol 2 for 4 hrs. total. Secretion of LDH (E), IL-1α (F), and MMP-9 (G). Each bar is the mean ± standard error of the mean for three independent experiments. Data was transformed using the box cox transformation and statistical analysis was done with one-way ANOVA comparing treatment to negative control. The y-axis shows the response of cells in each assay as a percentage of the untreated control for the MTT assay. The y-axis for IL-1α shows secretion of IL-1α in pg/mL. The y-axis for MMP-9 shows secretion of MMP-9 in ng/mL.

for subsequent exposures with authentic standards since it gave significance, and it follows the EpiDerm™ validated SIT protocol.

Exposure of EpiDerm™ to Flavor Chemicals

To determine if PG or specific flavor chemicals in Dewberry Cream and Churrios induced cell death or inflammatory responses, EpiDerm™ tissues were exposed for 4 hours to either PG or PG containing a pure flavor chemical using exposure protocol #1 (Figure 3.4E-G). While PG and each flavor chemical produced an increase in LDH release, the responses in each group were relatively low (~ 10% greater than the control) and were therefore treatments were interpreted to have little cytotoxicity. In contrast, all treatment groups, except PG vanillin, produced a significant increase in IL-1α secretion (Figure 3.4F). The p value for PG/vanillin was close to significant (0.08). MMP-9 was elevated in all treatment groups; however, none of the groups were significant compared to the negative control (Figure 3.4G).

Exposure of EpiDerm™ to ECEAR

Experiments were done to determine if ECEAR, which builds up on surfaces where vaping occurs, could affect EpiDerm™ (Figure 3.5A-F). Exposures were done for both 4 and 24 hours using either ECEAR or ECEAR extracts. ECEAR treatments did not produce effects in the MTT or LDH assay when Dewberry Cream was used to create ECEAR (Figure 3.5A, C). Similar results were obtained with ECEAR made from Churrios, except that there was a small (10%) (non-significant) increase in LDH activity in EpiDerm™ samples treated with ECEAR extracts for 24 hours (Figure 3.5D). IL-1α secretion was increased in both Dewberry Cream and Churrios ECEAR extract samples

treated for 4 and 24 hours, and significance was observed in the 24-hour Churrios extract (Figure 3.5E, F).

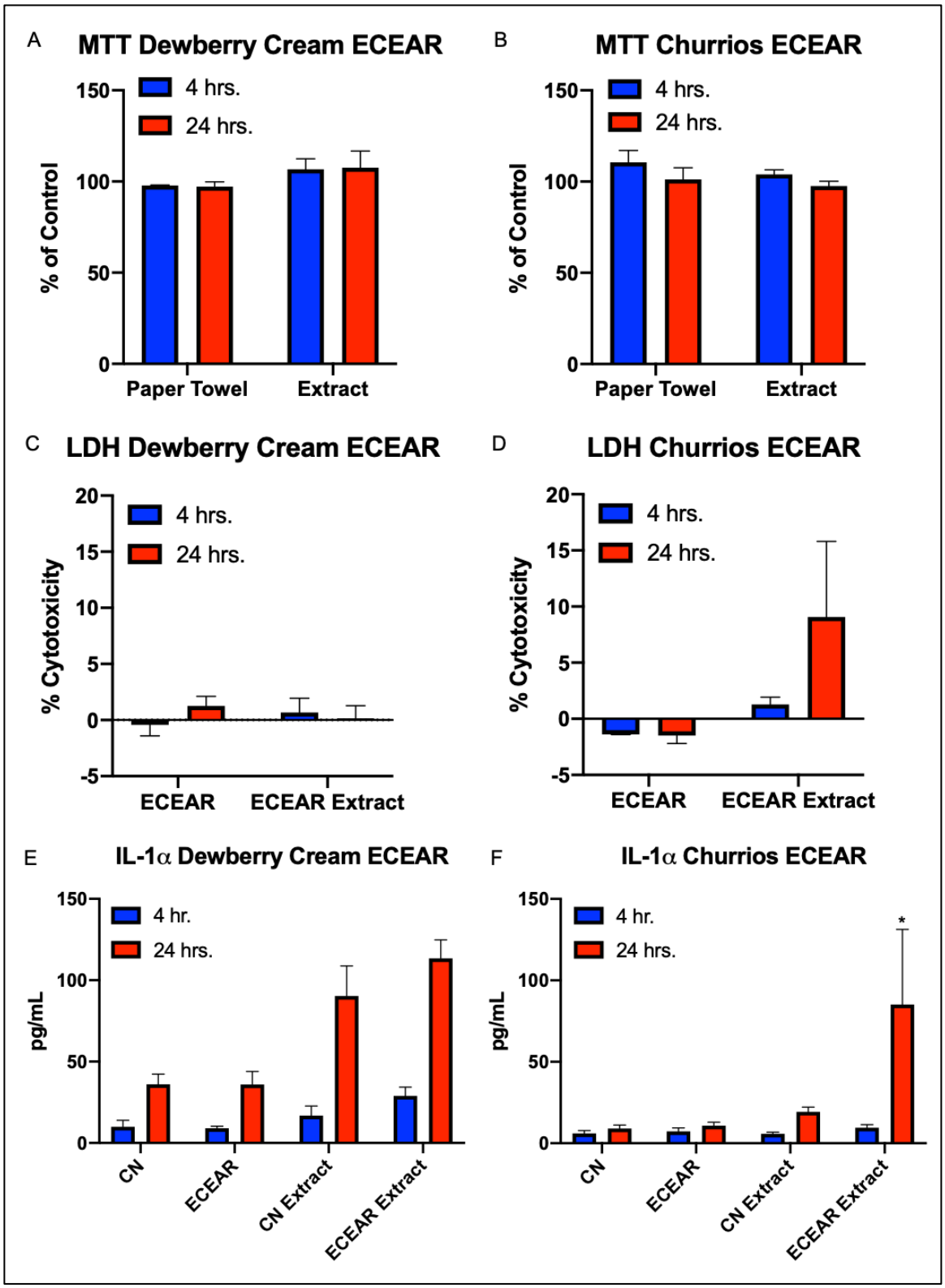
A
MTT Dewberry Cream ECEAR
4 hrs.
24 hrs.
% of Control
150
100
50
0
Paper Towel
Extract
B
MTT Churrios ECEAR
4 hrs.
24 hrs.
% of Control
150
100
50
0
Paper Towel
Extract
C
LDH Dewberry Cream ECEAR
4 hrs.
24 hrs.
% Cytotoxicity
20
15
10
5
0
-5
ECEAR
ECEAR Extract
D
LDH Churrios ECEAR
4 hrs.
24 hrs.
% Cytotoxicity
20
15
10
5
0
-5
ECEAR
ECEAR Extract
E
IL-1α Dewberry Cream ECEAR
4 hr.
24 hrs.
pg/mL
150
100
50
0
CN
ECEAR
CN Extract
ECEAR Extract
F
IL-1α Churrios ECEAR
4 hr.
24 hrs.
*
pg/mL
150
100
50
0
CN
ECEAR
CN Extract
ECEAR Extract

Figure 3.5: EpiDerm™ exposed to ECEAR paper towel and ECEAR paper towel extract for 4 and 24 hrs. MTT (A and B), secretion of IL-1α (C and D), and percent cytotoxicity calculated by secretion of LDH (E and F). Each bar is the mean ± standard deviation for three independent experiments. Data was transformed using the box cox transformation and statistical analysis was done with one-way ANOVA comparing treatment to negative control. The y-axis shows the response of cells in each assay as a percentage of the untreated control for the MTT assay. The y-axis for IL-1α shows secretion of IL-1α in pg/mL. The y-axis for MMP-9 shows secretion of MMP-9 in ng/mL.

DISCUSSION

We investigated the response of human skin models to EC refill fluids and ECEAR. Dermal exposure is of interest since the skin of EC users comes in contact with refill fluids through leakage and spills and with ECEAR though direct touching of ECEAR on surfaces. Non-users may also be passively exposed via the skin when occupying indoor environments where ECEAR has been deposited. To our knowledge, this is the first study to test cellular responses of keratinocytes and/or EpiDerm™ tissues to refill fluids, their flavor chemicals, and their ECEAR. Both extracellular and intracellular ROS and secretion of inflammatory cytokines and MMP-9 increased in keratinocytes treated with the two refill fluids. Both refill fluids also induced secretion of inflammatory biomarkers from EpiDerm™. The release of inflammatory markers from EpiDerm™ was induced by PG, but not flavor chemicals. At concentrations that were not cytotoxic in the MTT and LDH assay, Churrios ECEAR extract elevated IL-1α secretion from EpiDerm™.

Except for a small difference in total flavor chemical concentrations, our flavor chemical analysis of Dewberry Cream was in good agreement with prior studies (Hua et al., 2019; Khachatoorian et al., 2020). The small discrepancy is probably due to variations in manufacturing. Dewberry Cream was not cytotoxic to keratinocytes at any concentration tested up to 1%. However, Hua et al. showed a decrease in mitochondrial reductase activity in the MTT assay using mouse neural stem cells (mNSC) at 1% refill fluid concentration (Hua et al., 2019). These differences between studies could be related to the different cell types that were used (mNSC versus human keratinocytes).

Churrios has not been analyzed previously; however, many other cinnamon-flavored refill fluids have been evaluated for cinnamaldehyde concentration, cytotoxicity,

and cellular effects (Behar et al., 2016; Wavreil and Heggland, 2019; Clapp et al., 2019; Fetterman et al., 2018). Cinnamon flavored refill fluids are among the most toxic of the many that have been tested in the MTT assay (Behar et al., 2016). Their toxicity has been attributed to cinnamaldehyde, which produces an IC_{50} at 0.009 mg/mL when tested with human embryonic stem cells and human pulmonary fibroblasts (Behar et al., 2016). Cinnamaldehyde concentrations vary in cinnamon-flavored refill fluids (Behar et al., 2016), and Churrios contained a relatively low concentration of cinnamaldehyde (0.3 mg/mL), yet produced toxicity at 1% refill fluid concentration. Churrios also contained benzyl alcohol, which was not present in Dewberry Cream, and may have contributed to Churrios' cytotoxicity. Benzyl alcohol was cytotoxic to mNSC and human lung epithelial cells (BEAS-2B) in the MTT assay at concentrations < 1 mg/mL (Hua et al., 2019), which is considerably lower than the concentration we detected in Churrios refill fluid (4.2 mg/mL).

In the culture medium, H_2O_2 was affected by both refill fluids and keratinocytes. We showed that both Dewberry Cream and Churrios increased H_2O_2 levels in culture medium. This increase in H_2O_2 has been shown previously with E-liquids and flavoring agents increasing the levels of ROS in cell-free systems (Lerner et al., 2015; Muthumalage et al., 2018), and this increase was dependent on the flavor chemicals (Bitzer et al, 2018; Lerner et al., 2015). Nicotine lowered the levels of H_2O_2, while flavor chemicals increased H_2O_2. However, the decrease in H_2O_2 in the Dewberry Cream-containing culture medium with keratinocytes could be due to the release of antioxidants and/or the ability of aquaporin-3 to transport H_2O_2 into the cell for breakdown (Miller et al., 2010). This idea is supported by our observation that cells exposed to Dewberry Cream are still metabolically active, as shown in the MTT assay. In contrast, media

containing Churrios had highly elevated levels of H_2O_2, which were not reduced by the addition of cells. This may be due to the reduced metabolic activity (MTT assay) of cells exposed to Churrios, which in turn have reduced the antioxidizing capacity of the cells.

Both refill fluids increased intracellular ROS in keratinocytes as shown by CellROX™ imaging. The 0.1% and 1% Dewberry Cream and the 0.1% Churrios exposed cells had mitochondrial fluorescence, indicating the presence of ROS. The lack of fluorescence in cells exposed to 1% Churrios was likely due to their decrease in mitochondrial activity, as shown in the MTT assay. Dewberry Cream and Churrios refill fluids induced oxidative stress in human keratinocytes, similar to the increase in ROS observed in osteoblast-like MG-63 cells treated with cinnamon flavored refill fluids (Wavreil and Heggland, 2019). This increase in ROS can cause damage to cells and increase oxidation of DNA, lipids, and proteins. Mitochondrial protein oxidation occurred in mNSC exposed to EC liquids or aerosols in vitro (Zahedi et al., 2019), and superoxide was generated in menthol treated BEAS-2B cells in submerged cultures (Nair et al., 2020). In humans, urinary biomarkers of oxidative stress, 8-OHdG and 8-isoprostane, increased in EC users when compared to non-users (Sakamaki-Ching et al., 2020; Singh 2019). Finally, oxidative stress and inflammation in the serum of nonsmoking (smoking-naïve) subjects was studied in response to acute EC aerosol inhalation, and ICAM (I-cell adhesion molecule), endothelial ROS, and CRP (C-reactive protein) were increased, while nitric oxide was decreased (Chatterjee 2019).

Nicotine absorption and permeation has also been studied in skin. Previous work done on skin absorption of nicotine estimate about 3.04 mg transdermal nicotine absorption after the contamination of an area of 100 cm^2 (area of half palm) (Maina et al., 2016) and even a short contact time (10 min) produces transdermal absorption of

nicotine (Maina et al., 2017). Other *in vitro* permeation studies have modeled nicotine exposure and absorption estimating up to 100mg of absorption (Frasch and Barbero, 2016). Recently, the JUUL has been reported to contain more than 60 mg/mL of nicotine (Omaiye et al, 2019), while the nicotine content in skin absorption studies has been 25 mg/mL. This is a concern because the concentration of nicotine is increasing with newer generations of ECs.

Increases in oxidative stress often leads to an inflammatory response in cells exposed to refill fluids or EC aerosols (Lerner et al., 2016; Nair et al., 2020). Markers of an inflammatory response were released from EpiDerm™ when exposed to Dewberry Cream and Churrios, although there was no signs of histological damage or responses in the MTT and LDH assay. When we exposed Epiderm™ to lab made refill fluids, our data showed that PG specifically caused an increase in the secretion of MMP-9 and a significant increase in IL-1α from the EpiDerm™. This is in agreement with work on gingival epithelial cells exposed to PG/VG, which increased secretion of inflammatory markers (IL-6, IL-8, and MMP-9) (Beklen and Uckan, 2020). PG is a recognized allergen (Jacob et al., 2018) that can cause ocular and upper airway irritation (Wieslander et al., 2001; National Academies of Sciences, Engineering, and Medicine, 2018). PG is also a skin penetration enhancer (Lane, 2013) and could facilitate the delivery of refill fluid components, such as nicotine and flavor chemicals, to the skin, although this was not measured in our study .

Cigarette smoking and thirdhand smoke (THS) also affect the skin. Cigarette smoke causes pre-mature aging of skin and reduces wound healing in humans (Frances, 1998; Silverstein, 1992; Martins-Green et al., 2014). THS, tobacco residue left behind by smoking, is analogous to ECEAR. Exposure to THS and TSNAs produced by

THS increased circulating inflammatory cytokines, such as TNF-a, IL1-a, and GMPSF (Chen et al., 2018). When studying oxidative stress, THS increased levels of malondialdehyde, a marker for lipid peroxidation, and 8-OH-dG, a major oxidation product in DNA in mice (Jacob et al., 2017). This suggests that THS causes an increase in inflammatory markers and oxidative DNA damage. Although there are not many studies on the skin and EC use, it is probable that EC users experience similar affects to the skin as cigarette smokers.

In conclusion, this study provides evidence that EC use adversely effects human skin. Exposure to refill fluids (due to leakage or spills) and/or contact with ECEAR can increase oxidative stress and release of inflammatory proteins. Our study focused on two popular refill fluids, containing mostly sweet flavors with a relatively low nicotine concentration, available online and in shops. Churrios was more cytotoxic to keratinocytes than Dewberry Cream and both fluids increased extracellular and intracellular ROS. Although refill fluids contain many chemicals that may cause irritation and inflammation, our work demonstrated a clear increase in inflammatory marker secretion upon ALI exposure of 3D EpiDerm™ tissues to PG. EC users and employees at vape shops should be aware that harm could be caused by handling refill fluids and leaky ECs or by touching ECEAR, which is found on all surfaces in vape shops (Khachatoorian et al., 2019). With the world-wide increase in EC use, it is important to expand future research on dermal exposure to include additional refill fluids, nicotine concentrations, and flavor chemicals.

Our study has several limitations. There are many refill fluids with higher flavor chemical and nicotine concentrations that could be evaluated in future studies. We did not quantify the flavor chemicals or nicotine in the participant's exhale; therefore we do

not know if the participant was a mouth or lung inhaler, which would affect ECEAR deposition (Khachatoorian et al., 2020). Other potential toxicants in ECEAR include metals and reaction products, such as nickel, formaldehyde, and acetaldehyde, and these could also affect the skin. The concentrations of flavor chemicals and nicotine in the ECEAR we used were relatively low. Higher concentrations of ECEAR may produce stronger effects.

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Chapter 4

Identification and Quantification of Electronic Cigarette Exhaled Aerosol Residue Chemicals in Field Sites

Khachatoorian et al.

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ABSTRACT

Background: Electronic cigarette (EC) users may exhale large clouds of aerosol that can settle on indoor surfaces forming ECEAR (EC exhaled aerosol residue). Little is known about the chemical composition or buildup of this residue.

Objective: Our objective was to identify and quantify ECEAR chemicals in two field sites: an EC user's living room and a multi-user EC vape shop.

Methods: We examined the buildup of ECEAR in commonly used materials (cotton, polyester, or terrycloth towel) placed inside the field sites. Materials were subjected to different lengths of exposure. Nicotine, nicotine alkaloids, and tobacco-specific nitrosamines (TSNAs) were identified and quantified in unexposed controls and field site samples using analytical chemical techniques.

Results: Nicotine and nicotine alkaloids were detected in materials inside the EC user's living room. Concentrations of ECEAR chemicals remained relatively constant over the first 5 months, suggesting some removal of the chemicals by air flow in the room approximating a steady state. ECEAR chemicals were detected in materials inside the vape shop after 6 hours of exposure and levels continually increased over a month. By 1 month, the nicotine in the vape shop was 60 times higher than in the EC user's living room. ECEAR chemical concentrations varied in different locations in the vape shop. Control fabrics had either no detectable or very low concentrations of chemicals.

Conclusions: In both field sites, chemicals from exhaled EC aerosols were deposited on indoor surfaces and accumulated over time forming ECEAR. Non-smokers, EC users, and employees of vape shops should be aware of this potential environmental hazard.

1. Introduction

When electronic cigarette (EC) users exhale, the chemicals that are in the aerosol (nicotine, flavorings and solvents) settle on indoor surfaces where they accumulate, and form EC exhaled aerosol residue (ECEAR). In a previous study, we showed that nicotine, other alkaloids, and tobacco specific nitrosamines (TSNAs) were transferred from a vape shop where they originated to an adjacent business, in a multiple-tenant retail building, where they formed ECEAR (Khachatoorian et al., 2018). Passive exposure to EC aerosols and ECEAR can occur through inhalation, dermal absorption or ingestion even after EC use has stopped.

The components of inhaled EC aerosols include nicotine, propylene glycol, glycerol, metals, particulate matter, ultra-fine particles, flavor chemicals, and volatile organic chemicals (VOCs) (Goniewicz et al., 2014; Hutzler et al., 2014; Kosmider et al., 2014; Logue et al., 2017; Schripp et al., 2013; Sleiman et al., 2016; Williams et al., 2017; 2013). The process of oxidation and/or dehydration of propylene glycol and glycerol creates several additional chemicals, such as formaldehyde, acetaldehyde and acrolein, which have also been reported in EC aerosols (Flora et al., 2016; Goniewicz et al., 2014; Jensen et al., 2015). TSNAs, some of which are known carcinogens, such as (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitosnornicotine (NNN)), are present in both some refill fluids (Kim and Shin, 2013) and EC aerosols (Goniewicz et al., 2014; McAuley et al., 2012).

While less is known about the composition of exhaled EC aerosol or secondhand aerosol, several studies have found glycerol, propylene glycol and nicotine, as well as $PM_{2.5}$ particles and ultrafine particles in exhaled aerosol generated in laboratory settings, vaping conventions, and homes (Fernández et al., 2015; National Academies of

Sciences, Engineering, and Medicine et al., 2018). Modeling exposures of participants using ECs inside a vaping bar indicated that ECs contributed to changes in formaldehyde, acetaldehyde, and acrolein levels, which exceeded governmental health hazard reference level limits (Logue et al., 2017). In general, the studies on exhaled EC aerosol have found that indoor air quality deteriorates in situations where vaping occurs.

However, there is very little information on the composition of ECEAR that is deposited on indoor surfaces where ECs are used. In a vape shop where ECEAR was evaluated with surface wipes, nicotine was not detected. In our opinion, this is probably because this particular shop had extremely thorough cleaning procedures, such as cleaning floors, counters, displays, and the bar each night with cleaning agents and bleach, and also had ventilation that included many air supply vents and an exhaust fan that was used during open hours (Zwack et al., 2017). In a laboratory study in which EC users exhaled into a chamber, surface wipes of nicotine were not significantly higher than baseline concentrations (Liu et al., 2017). However, a second laboratory study did find nicotine deposition on surface wipes from an exposure chamber (Goniewicz and Lee, 2015). A pilot study using surface wipes showed no significant differences in the amount of nicotine in EC users' and non-users' homes (Bush and Goniewicz, 2015).

The limited and inconsistent data on ECEAR and the lack of data from field sites indicate a clear need for more information on this topic. The purpose of this study was to determine if ECEAR chemicals could be detected and quantified in two field sites, a living room inside an EC user's home and a typical vape shop with active EC use.

2. Material and methods

2.1 Collection of Fabrics-Living Room Field Site

The field site was a 187.5 ft^2 (47.78 m^3) living room inside a 4-bedroom family home in Riverside, CA built in 2005. The room was at the west side of the first floor adjacent to a guest bathroom and a bedroom. The room had adequate ventilation including a door into an adjacent bedroom, the front door around the corner, two windows, and a vent providing central cooling and forced air heating. The desk where fabrics were hung was next to a window.

Cotton and polyester fabrics (Jo-Anne Fabric and Craft Stores, Riverside, CA, USA) were placed in the field site for different exposure times. The area of 1 gram of cotton and polyester is 43.18 cm^2 and 41.91cm^2, respectively. Fabrics in the living room field site were collected at the end of each month after 1, 2, 3, 4, 5, and 6 months of exposure. Control fabrics were placed inside a non-smoker's home for the same amount of time. After exposure, samples were placed in Ziploc® bags and brought to lab on dry ice and either extracted immediately or stored at -80°C in heat sealed, cut to size Mylar bags (ULINE, Pleasant Prairie, Wisconsin, USA). The EC user reported that there was no cigarette smoking inside the house.

2.2 Collection of Fabrics-Vape Shop Field Site

The vape shop was located in San Gabriel, California on a busy street with mostly car traffic. A glass supply store was adjacent to the shop. The vape shop was approximately 600 ft^2 (317.9 m^3). The store had glass windows facing the street and one entrance. The city of San Gabriel, CA, has prohibited smoking at and within a 25-foot buffer zone of all indoor and outdoor public places, which includes vape shops

(http://www.ci.south-pasadena.ca.us/index.aspx?page=375, August 2018). However, vaping is allowed in vape shops. The vape shop we recruited specifically mentioned that they do not support the use of cigarettes and do not allow their use inside the store. Fig. 3.1A shows the layout of the vape shop. The shop lacks a ventilation system and does not have any system to replenish clean air. The only air circulation was an A/C unit located on the wall of the lounge area (Innova 18,000 BTU split type air conditioner). At its lowest and highest setting, the air conditioner circulated air at 5.7 and 6 cubic feet per hour, respectively. The door to the manager's office remained closed during business hours. The entrance to the shop was only used when customers entered and exited. The store hours were Mondays to Sundays from 11:00 am to 12:00 am. In the evenings, there were usually 5-15 customers vaping in the shop.

Terrycloth towels (Jo-Anne Fabric and Craft Stores, Riverside, CA, USA) were placed in the field site for different exposure times. The area of 1 gram of terrycloth towel is 26.125 cm^2. Fabrics in the vape shop were collected after 6, 7, 18, 24, 48 hours, 1 week and 1 month. Control fabrics were placed outside the vape shop for the same amount of time as the indoor fabrics. After exposure, samples were placed in Ziploc® bags and brought to lab on dry ice and either extracted immediately or stored at -80°C in heat sealed, cut to size Mylar bags (ULINE, Pleasant Prairie, Wisconsin, USA).

2.3 Extraction of Nicotine and its Derivatives in ECEAR Fabrics

ECEAR was extracted from control and exposed samples of cotton, polyester, or terrycloth. Fabrics were cut into small pieces and immersed in cell culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) as described in (Bahl et al., 2016; Sarker et al., 2014, Khachatoorian et al., 2018) at a

concentration of 0.05 g of fabric/ml of medium. The extracts were aliquoted into 1.5 ml vials for storage at −80 °C and later shipment on dry ice to the Clinical Pharmacology Laboratory at the University of California at San Francisco.

2.4 Chemical Analysis of Nicotine and its Derivatives in ECEAR Fabrics

The quantification of nicotine, nicotine derivatives, and TSNAs was done by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) at the University of California, San Francisco as described previously in detail (Bahl et al., 2014; Hang et al., 2013; Whitehead et al., 2015). The limits of quantification for each chemical was as follows: nicotine = 2 ng/ml, cotinine = 1 ng/ml, n-formylnornicotine = 1 ng/ml, myosmine = 1 ng/ml, NNA (1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal) = 1 ng/ml.

3. Results

3.1 Living Room Field Site

Fig. 4.1 shows the six sets of cotton and polyester fabrics hung across the bottom shelf of a bookcase above the computer. The individual started using an Innokin iTaste MVP with an Aspire Nautilus tank in July and changed to a Wotofo ZNA 30 clone by A-mod Technology Co., LTD with an Aspire Nautilus tank in late September. Usage was at 1.9 ohms, 4.0 volts and 8.4 watts. Refill fluids used over the 6-month period are shown in Appendix B S1. All refill fluids had a nicotine concentration of 6mg/mL. We asked the user to log the number of puffs when using the EC. The average puff count per day (Fig. 4.1B) was similar each month; however, the Total Puff count per month (Fig. 4.1C) was lower during month 6 when the desk was only used for 2 weeks. The user puffed about 3 hours a day and averaged about 15 days of puffing per month.

3.2 Nicotine and Other Alkaloids Detected in Living Room ECEAR Samples

Nicotine and other minor alkaloids were detected in ECEAR extracts of cotton and polyester from the home of an individual EC user (Fig. 4.1 D-G). Nicotine was the most abundant marker of ECEAR contamination, and its highest concentration (3 months) was 5,100 ng/gram of cotton fabric (Fig. 4.1D). During other months, nicotine concentrations varied between 2000 and 3000 ng of nicotine/g of cotton, while nicotine was only detectable in polyester samples after 5 and 6 months of exposure. Cotinine was detected in cotton samples during all months and on polyester during the 1st, 3rd, and 4th month (Fig. 4.1E). N-Formylnornicotine was detected on both cotton and polyester samples for all exposure times (Fig. 4.11F). The quantities of N-formyl-nornicotine were

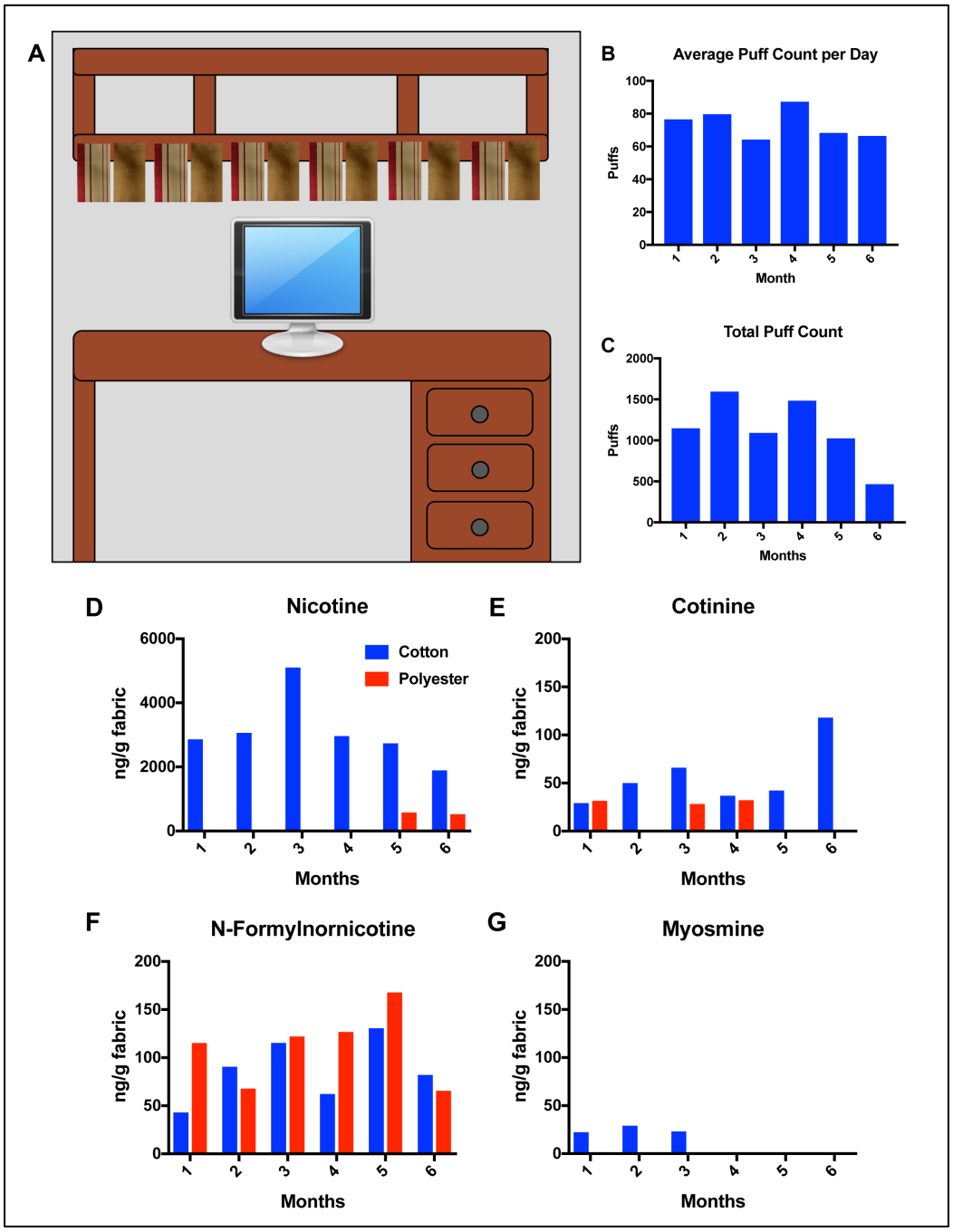

A
B
Average Puff Count per Day
Puffs
Month
C
Total Puff Count
Puffs
Months
D
Nicotine
Cotton
Polyester
ng/g fabric
Months
E
Cotinine
ng/g fabric
Months
F
N-Formylnornicotine
ng/g fabric
Months
G
Myosmine
ng/g fabric
Months

Figure 4.1: Living room field site. (A) Schematic of six sets of cotton and polyester fabrics hung from a shelf on a desk where EC were used. (B) Average puff count/day for each month over 6 months. The average number of days that the user puffed was 15 days each month. (C) Total puff count for each month over 6 months. Concentrations of nicotine (D), cotinine (E), n-formylnornicotine (F), and myosmine (G) extracted from cotton and polyester fabrics hung inside the living room of an EC user. The scale for Figure D is 0-6000 ng/gram of fabric and the scale for Figure E-G is 0-200 ng/gram of fabric. Cotton – blue. Polyester – red.

similar for both types of fabric, unlike the results with nicotine. Myosmine was only detected in the first 3 months of exposures on cotton fabric (Fig. 4.1G).

3.3 Vape Shop Field Site

Terrycloth towels were placed on the display case towards the front of the shop, on the lounge table between the two couches, and hung on the chalkboard towards the back of the store (red arrows in Fig. 4.2A). Control samples were hung up outside on the window of the vape shop facing the street. Appendix B Table S2 lists the day of the week, length of exposure, and placement of each sample inside the vape shop. The display area was composed of three separate display cases extending from the front to the back of the store. Display cases contained different types of ECs, batteries, tanks, and mouth pieces. Refill fluids were stored on the wall near the display cases. The chalkboard was located towards the back of the store near the manager's office. There were usually two employees working at any given time of the day, and the manager was usually in his office.

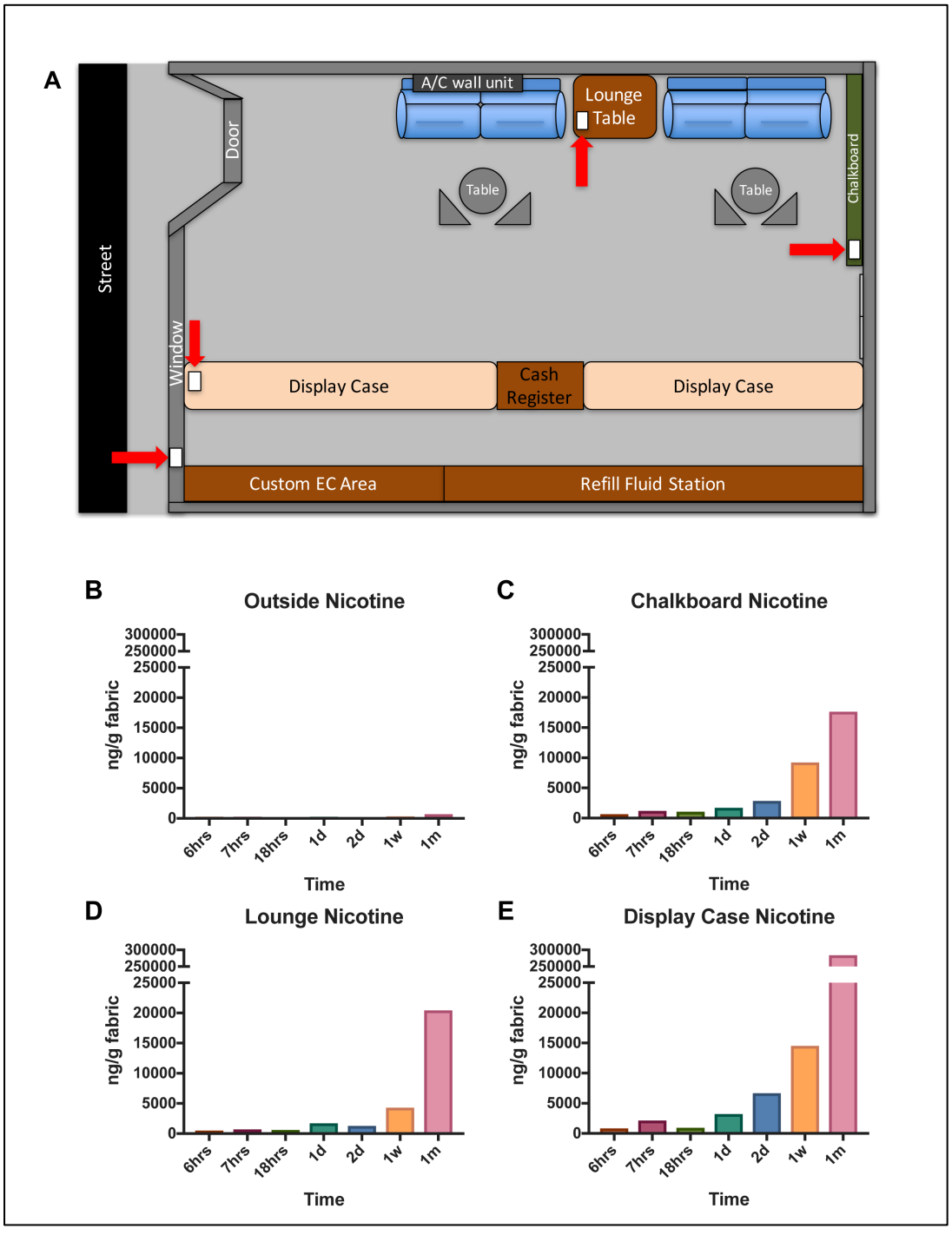
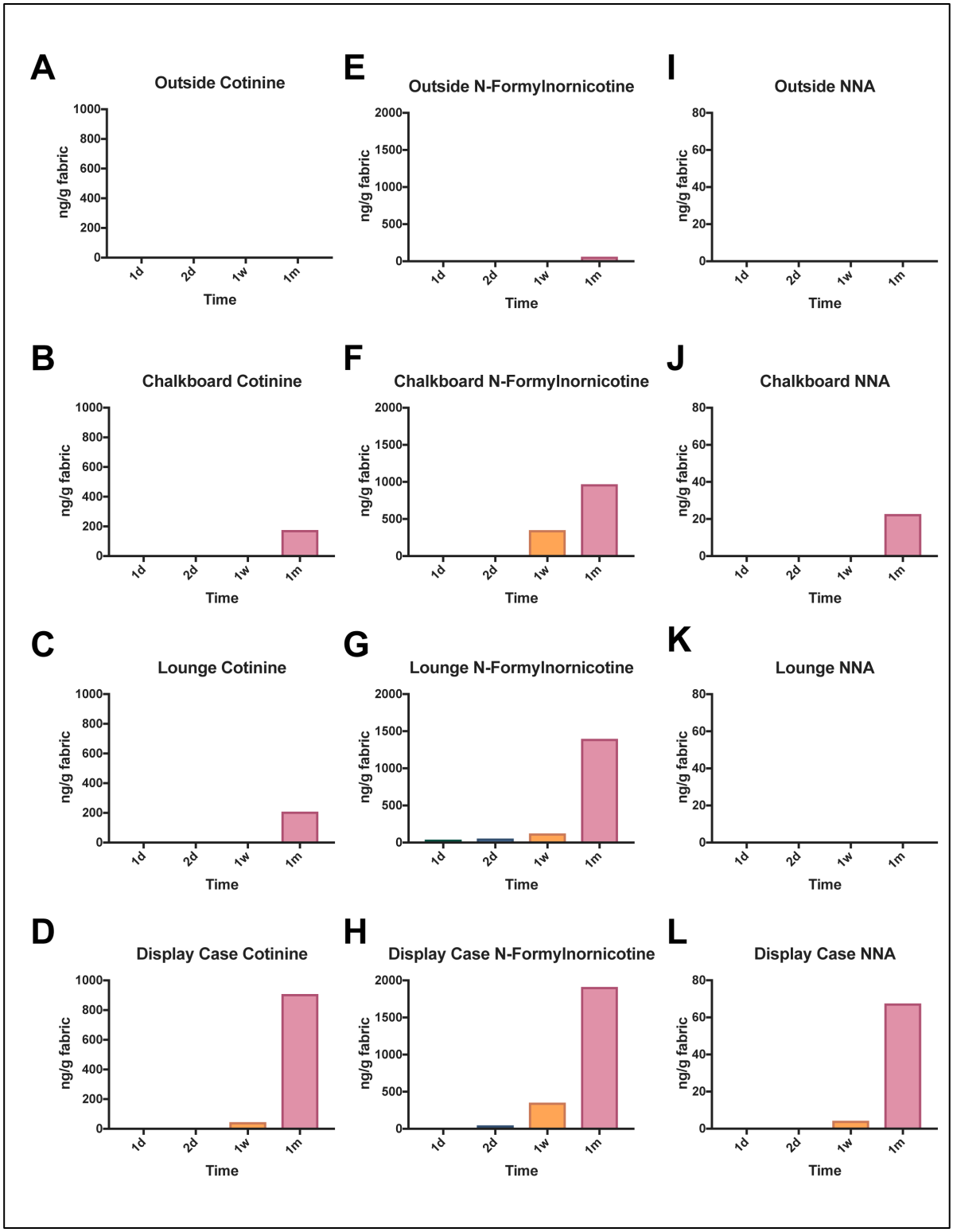


Figure 4.2: Vape shop field site. (A) Schematic of the vape shop field site showing placement of terrycloth towels (red arrows). (B-E) Concentrations of nicotine extracted from terrycloth towel that was placed inside the vape shop. The scale is from 0-300,000 ng/gram.

3.4 Nicotine Detected in Vape Shop Field Site ECEAR Samples

Outside control samples had very low levels of nicotine after exposure for 6 hrs, 7 hrs, 1 day, 1 week and 1 month. The latter had the highest nicotine at 719 ng/gram of fabric (Fig. 4.2B). Samples placed towards the back of the vape shop on the chalkboard had increasing amounts of nicotine as exposure times increased. The 6 hr. exposure had 649 ng of nicotine/gram of terrycloth towel and reached 17,655 ng of nicotine/gram of terrycloth towel at 1 month of exposure (Fig. 4.2C). The fabrics exposed in the lounge area had a similar result starting at 516 ng of nicotine/gram of terrycloth towel at 6 hrs. of exposure and reaching 20,447 ng of nicotine/gram of terrycloth towel at 1 month of exposure (Fig. 4.2D). The terrycloth towel placed at the display case toward the front of the vape shop had the highest nicotine concentration starting at 839 ng of nicotine/gram of terrycloth towel at 6 hrs. of exposure and increasing to 283,775 ng of nicotine/gram of terrycloth towel at 1 month of exposure (Fig. 4.2E).


A
Outside Cotinine
ng/g fabric
1000
800
600
400
200
0
1d
2d
1w
1m
Time
E
Outside N-Formylnornicotine
ng/g fabric
2000
1500
1000
500
0
1d
2d
1w
1m
Time
I
Outside NNA
ng/g fabric
80
60
40
20
0
1d
2d
1w
1m
Time
B
Chalkboard Cotinine
ng/g fabric
1000
800
600
400
200
0
1d
2d
1w
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Time
F
Chalkboard N-Formylnornicotine
ng/g fabric
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1d
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Time
J
Chalkboard NNA
ng/g fabric
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C
Lounge Cotinine
ng/g fabric
1000
800
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200
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1d
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G
Lounge N-Formylnornicotine
ng/g fabric
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D
Display Case Cotinine
ng/g fabric
1000
800
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1d
2d
1w
1m
Time
H
Display Case N-Formylnornicotine
ng/g fabric
2000
1500
1000
500
0
1d
2d
1w
1m
Time
L
Display Case NNA
ng/g fabric
80
60
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1d
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Figure 4.3: Concentrations of cotinine, n-formylnornicotine, and NNA in terrycloth towels from the vape shop field site. Terrycloth placed outside (A, E, I), on the chalkboard (B, F, J), in the lounge (C, G, K), and on the display case (D, H, L) inside the vape shop had detectable and quantifiable levels of nicotine related alkaloids and a TSNA, NNA. The scale for Figure A-D is 0-1000 ng/gram, E-H is 0-2000 ng/gram, and I-L is 0-80 ng/gram.

3.5 Cotinine, N-Formylnornicotine and NNA Detected in Vape Shop Field Site ECEAR Samples

Cotinine was not detected in outside control samples (Fig. 4.3A), while the chalkboard and lounge areas had detectable levels at 1 month (175 and 209 ng of cotinine/gram of terrycloth towel, respectively) (Fig. 4.3B-D). N-Formylnornicotine was detected at very low levels in the 1 month outside control fabric (Fig. 4.3E) but was detected in the 1-day lounge sample (40 ng/gram of fabric). Its concentration increased in later samples, reaching a high of 1,912 ng/gram of fabric for the display case sample (Fig. 4.3F-H). 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA) was not detected in outside control fabrics (Fig. 4.3I) nor in the lounge fabrics (Fig. 4.3K) but was present in the 1-month chalkboard sample at 22 ng/gram of terrycloth and the display case sample at 67 ng/gram of terrycloth (Fig. 4.3J, L).

4. Discussion

This is the first study to compare ECEAR inside a vape shop and an EC user's living room. Nicotine and related chemicals were detected in both sites; however, chemical concentrations were higher in the vape shop where they increased over time. Our sampling method allowed us to evaluate the buildup of ECEAR. The kinetics of chemical deposition were different in the two sites, with the ECEAR chemicals showing steady state concentrations in the living room, while the vape shop chemicals continually increased in concentration. This difference may be related to the shorter length of time over which vape shop samples were collected or due to better ventilation in the living room field site.

Although it has been reported that EC users retain 94% of the inhaled nicotine (St Helen et al., 2016), we were able to detect a buildup of nicotine in the living room field site where there was a single EC user. During months 1-5, nicotine concentration in ECEAR did not increase with increased EC use, probably because air circulation in this site removed much of the exhaled vapor before it settled, creating a steady state concentration. In the 6^{th} month, the user puffed only 7 days, instead of the usual 15, and the concentration of nicotine was half the amount detected in the prior months. While most chemicals in the living room did not increase in concentration over time, cotinine had a higher concentration at 6 months when there was less puffing. This may have occurred because of oxidation of nicotine in the ECEAR during aging (Goniewicz et al., 2014; Sleiman et al., 2010b). Although a higher ventilation rate results in faster nicotine reduction in indoor air (Rostami et al., 2018), our data show that in a modern home with central air conditioning, nicotine in exhaled EC aerosol accumulates on surfaces where it is likely converted into other alkaloids during aging.

In the living room, nicotine was detected in every cotton sample in contrast to polyester which had no detectable nicotine until the last 2 months of sampling. Others have shown that nicotine is readily removed from cotton but not from polyester (Bahl et al., 2014; Destaillats et al., 2006). This preferential removal from cotton was not observed for all chemicals. For example, n-formylnornicotine was extracted equally efficiently from both cotton and polyester. This may be due to the higher polarity and solubility of N-formylnornicotine, which allows for it to readily extract from a non-polar, hydrophobic material such as polyester. Nicotine has significant lipophilicity and may stay adsorbed on the polyester and not be readily extracted by the aqueous media. These data suggest that re-emission of nicotine into the air can occur more readily from cotton than from polyester, but this does not necessarily hold for all ECEAR chemicals.

In contrast to the living room, the concentration of ECEAR chemicals increased over time inside the vape shop. This could be due to the large amount of aerosol produced by multiple users and the limited circulation of air in the shop. Maximum nicotine levels in the vape shop were about 60 times higher than those in the living room field site (~300,000 ng/gram of fabric in the vape shop versus ~ 5,000 ng/gram of fabric in the living room). The continual buildup of ECEAR chemicals in the vape shop would likely have continued if fabrics were left for periods longer than 1 month. If accumulation of nicotine continued at the same rate over 1 year, its concentration in ECEAR would be approximately 3.6 mg/gram of fabric.

The concentration of ECEAR chemicals varied in the three sampling locations in the vape shop. Highest concentrations were found in the display case samples, which were located in the area of highest vaping by both the customers and employees.

Concentrations in the other two sites were about 15 times lower than near the display case. These data show that ECEAR is not evenly distributed within the vape shop and that even in a relatively small field site with poor ventilation, concentrations of nicotine varied significantly. The nicotine accumulation in the vape shop was due to vaping since the outside (control) fabrics contained either no or very low concentrations of nicotine and other ECEAR chemicals.

NNA, a TSNA, was detected in ECEAR and is of interest because it can form from nicotine in the environment (Jacob et al., 2017). NNA is rarely found in tobacco or tobacco smoke, probably because it is too unstable to survive the relatively harsh conditions of tobacco curing and combustion. But under milder conditions, nicotine on surfaces can react with nitrous acid in the environment to form NNA, as well as NNK and NNN (Sleiman et al., 2010a). Our data suggest that chemical reactions occur in ECEAR leading to the formation of TSNAs, such as NNA.

ECEAR concentrations can be affected by ventilation and cleanliness. The vape shop in our study did not have a dedicated exhaust and good ventilation which most likely lead to the continual buildup of ECEAR. In contrast, surface wipes from a vape shop with daily cleaning and air circulation contained no nicotine, although the limit of quantification was not given in the study (Zwack et al., 2017). This particular vape shop shows that ECEAR levels can be reduced by ventilation and cleaning procedures. To mitigate the buildup of ECEAR, regulatory agencies could require specific air exchanges in vape shops to clear exhaled aerosol and reduce ECEAR levels. Currently, the American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) has no standard or guideline for air exchange inside of vape shops (American Society of

Heating, 2016), although there are standards for retail stores and cocktail bars. Owners could also establish daily cleaning protocols to further limit the accumulation of ECEAR.

Nicotine was the dominant chemical in our field site ECEAR samples. This is of concern because nicotine is water and lipid soluble, and readily permeates into skin (Maina et al., 2017; 2016), where it can accumulate and be released into the blood after exposure (Bekö et al., 2017). Nicotine can cause dermatitis, irritation, and skin sensitization in humans (Berlin et al., 2014; Berner et al., 1990; Bircher et al., 1991; Gourlay et al., 1999; Greenland et al., 1998). Patients using transdermal nicotine therapy have reported erythema, rash, pruritis, irritation and edema (Gourlay et al., 1999), and in several studies of nicotine patches, significant increases in dizziness, nausea and sleep disorders were reported (Greenland et al., 1998). It is interesting that the main symptoms associated with patch users are all symptoms frequently reported by EC users in online websites (Hua and Talbot, 2016). Although ECEAR uptake through the skin and other routes has not yet been analyzed, the data with patches and EC user reported health effects are consistent with the idea that ECEAR could cause skin and systemic responses. It will be important in future studies to determine how much exposure and uptake of EC chemicals occur in both EC users and non-users in vape shops or other indoor environments where EC are used. Such uptake could be due to both the aerosol and ECEAR.

Indoor settings where ECs are used could cause involuntary inhalation, ingestion, or dermal uptake of EC aerosols and ECEAR chemicals. This is especially true for employees and customers inside a vape shop and family members inside a home where ECs are used. Most worrisome is the exposure that toddlers and small

children would receive in a home with ECEAR due to their frequent mouthing of fabrics and touching surfaces and floors. The FDA currently prohibits the sale of EC products to minors under the age of 18, but there are no stringent regulations concerning the age limit of patrons inside vape shops (Food and Drug Administration, HHS, 2016). California business and professions code #22962C prohibits anyone under the age of 18 from entering a vape shop unless accompanied by a parent or guardian, but there is no "enforcement" agency for this law (http://leginfo.legislature.ca.gov, September 2018). Federal and state governmental agencies may consider tightening and restricting access to vape shops to adults 21 and over only.

In the vape shop, we detected the equivalent of approximately 108 mg of nicotine/m^2 after 1 month of accumulation. This is considerably higher than the nicotine concentration found in surface wipes from a smokers living room (73 μg/m^2) (Matt et al., 2004). Even in our living room field site, the concentration of nicotine (1,181 μg/m^2) in the 3-month cotton sample was considerably higher than in the smoker's home. Our data may be higher since we left fabric in the field sites, while wipes were taken in the smoker's home.

Our study was based on one home and one vape shop. Other field sites may produce different concentrations of chemicals in ECEAR depending on the amount of use, the type of EC, its nicotine content, topography of the users, cleaning protocols, and ventilation (Zwack et al., 2017; Maloney et al., 2016). In addition, our samples were collected over a relatively short period of time. Most indoor facilities and homes accumulate ECEAR over much longer times, suggesting that concentrations of nicotine and TSNAs could be higher than reported in our study.

5. Conclusion

In summary, this study shows that nicotine and other chemicals in exhaled EC aerosol can build up on indoor surfaces where they could result in exposure of EC users and non-smokers through dermal contact, inhalation, or ingestion. Further research on ECEAR deposition is needed to provide a broader perspective on ECEAR levels in indoor environments. Employees, whether they are EC users or not, who work long hours inside vape shops are exposed to ECEAR and EC aerosols, which may be an occupational health hazard. Given the accumulation of nicotine and related chemicals in ECEAR, it is important for government agencies to look closely into age restrictions inside vape shops and the health effects of ECEAR on exposed populations.

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Chapter 5

Electronic Cigarette Chemicals Transfer from a Vape Shop to a Nearby Business in a Multiple-Tenant Retail Building

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Tobacco Control 2019; **28:519**-525.

ABSTRACT

Background: Electronic cigarettes (ECs) are nicotine delivery devices that produce aerosol without combustion of tobacco; therefore, they do not produce sidestream smoke. Nevertheless, many users exhale large clouds of aerosol that can result in passive exposure of non-users. Analogous to thirdhand cigarette smoke, the exhaled aerosol also settles on indoor surfaces where it can produce a residue. We refer to this residue as EC exhaled aerosol residue (ECEAR). Our objective was to determine if exhaled EC aerosol transferred from a vape shop in a multiple-tenant retail building, where it was produced, to a nearby business (field site) where it could deposit as ECEAR.

Methods: We examined the buildup of ECEAR in commonly used materials (cotton towel and paper towels) placed inside the field site across from the vape shop. Materials were subjected to short-term (days) and long-term (months) exposures. Nicotine, other alkaloids, and tobacco-specific nitrosamines (TSNAs) were identified and quantified in unexposed controls and field site samples using analytical chemical techniques.

Results: Nicotine and other alkaloids were detected after 1 day of exposure in the field site, and these chemicals generally increased as exposure times increased. TSNAs, which have been linked to carcinogenesis, were also detected in short and long-term exposed samples from the field site.

Conclusions: In a multiple-tenant retail building, chemicals in EC aerosol traveled from a vape shop into an adjacent business where they deposited forming ECEAR. Regulatory agencies and tenants occupying such buildings should be aware of this potential environmental hazard.

INTRODUCTION

Electronic cigarettes (ECs) deliver nicotine in an aerosol that is produced by heating a fluid containing a solvent, e.g., 1,2-propanediol (propylene glycol, PG) and/or glycerol (vegetable glycerin, VG), nicotine, and flavor chemicals [1-4]. EC aerosols also contain volatile organic compounds (VOCs), including carbonyls, and metals [5-10]. EC users may exhale large quantities of aerosol that contains nicotine [11-14] and the exhaled aerosol forms a residue on indoor surfaces [15,16], in much the same way that thirdhand smoke (THS) forms indoors after secondhand smoke has settled [17,18]. We refer to EC exhaled aerosol residue as ECEAR. Both the exhaled aerosol and ECEAR can result in passive exposure of non-users, and these exposures are a growing environmental and health concern.

Several toxicants including formaldehyde, acetaldehyde and acrolein are created by the oxidation and/or dehydration of VG or PG, and have been reported in EC aerosol [5,19,20]. Significant amounts of 1,2-propanediol, glycerin, nicotine and $PM_{2.5}$ particles were present indoors during 2 hours of vaping [21]. Moreover, an indoor air quality study showed that a large room with active EC users contained $PM_{2.5}$ at concentrations that were higher than in hookah cafes and bars that allow cigarette smoking [22]. Studies done inside the homes of EC users showed airborne nicotine levels of 0.13μg/m^3, in contrast to non-smokers' homes which had 0.02 μg/m^3 [23]. In the same study, salivary cotinine concentrations were significantly higher in non-smokers living in a home where ECs were used than in non-smokers living in non-smokers' homes. Because EC users as well as bystanders who do not use ECs can be exposed to the chemicals and particles in suspended EC aerosols, the International Union Against Tuberculosis and Lung Disease has recommended that EC should not be used in public places,

workplaces, or on public transportation [24]. A surface sampling study of indoor environments where vaping occurred showed that ECEAR contained nicotine, although concentrations were not as high as in the homes of smokers [15]. Passive exposure to nicotine and other chemicals in ECEAR could occur through dermal absorption, ingestion, or the inhalation of re-emitted chemicals.

In vape shops, multiple EC users exhale aerosols that could potentially move through the heating, ventilating, and air conditioning system (HVAC) to adjacent businesses. The purpose of this study was to determine if tobacco specific chemicals in the aerosol from an active vape shop transferred into a nearby business in a multiple-tenant retail building (mall) by examining deposition of nicotine and nicotine derivatives in the nearby business.

METHODS

Collection of Fabrics

Cotton towels (Easydry, easydry.com), paper towels (Bounty®, Stater Bros. Riverside, CA, USA), terrycloth towels (Jo-Anne Fabric and Craft Stores, Riverside, CA, USA), and two air filters [3M high performance 20x25x1 Filtrete™ air filter (amazon.com) and Rabbit Air Classic BioGS Replacement HEPA filter (amazon.com)] were placed in the field site for short or long-term exposures. The area of 1 gram of each fabric is: terrycloth towel 5.5 cm x 4.75 cm (26.125 cm^2), air filter 10.5 cm x 9.5 cm (99.75 cm^2), paper towel 15.5 cm x 13 mm (201.5 cm^2), and cotton towel 13.5 cm x 12.5 cm (168.75 cm^2). Short-term exposure samples were collected after 1 (24 hrs.), 4 (96 hrs.) and 8 days (192 hrs.), while long-term exposure samples were collected after 1, 2 and 3 months. Mall control fabrics (terrycloth towel) were exposed in a hallway on a separate HVAC system outside the field site for 1 day (24 hrs.), 3 days (72 hrs.), and 1 week. Additional control samples of fabrics (terrycloth towels) were collected from a non-smoker home in the same community after exposure for 1 day (24 hrs.), 4 (96 hrs.) days, and 1 week. Control samples inside the non-smoker home were placed inside the front room of the house and in the garage. Unexposed samples of each type of fabric (cotton towels, paper towels, terrycloth towels, and both air filters) were also used as controls. After exposure, samples were placed in Ziploc® bags and/or envelops and either extracted immediately or stored at -80°C in heat sealed, cut to size, Mylar bags (ULINE, Pleasant Prairie, Wisconsin, USA).

Extraction of Nicotine, Other Alkaloids, and Tobacco-Specific Nitrosamines (TSNAs) from ECEAR Fabrics

ECEAR was extracted from control (unexposed) and exposed samples of cotton towel, paper towel, terrycloth towel, and air filters. Fabrics were cut into small pieces and immersed into cell culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 5% horse serum (Invitrogen, Carlsbad, CA), 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, California) [25,26] at a concentration of 0.05 g of fabric /ml of medium. This mix was agitated in 50 ml Falcon tubes on a rocker for 1 h at room temperature [27]. The contents of the tubes were extruded through a 30 ml plastic syringe (Sigma-Aldrich, St. Louis, MO) to obtain only the medium. The extracts were then filtered using 0.22 μm sterile filters (Pall Corporation, Port Washington, NY) and aliquoted into 1.5 ml vials for storage at −80 °C and later shipment on dry ice to the Clinical Pharmacology Laboratory at the University of California at San Francisco.

Chemical Analysis of ECEAR Fabrics for Nicotine, Other Alkaloids, and TSNAs

The quantification of nicotine, nicotine derivatives, and TSNAs was done by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) at the University of California San Francisco. Sample prep has been described previously [27-29]. The LC-MS/MS analysis, including the method for determining the LOQ, is described in Whitehead et al. [27-29]. The limits of quantification for each chemical was as follows: nicotine = 2 ng/ml, cotinine = 1ng/ml, n-formylnornicotine = 1, bipyridine = 1 ng/ml,

nicotelline = 0.2 ng/ml, myosmine = 1 ng/ml, NAB (N-nitrosoanatabine) = 1 ng/ml, NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) = 0.1 ng/ml, NNN (N'-nitrosonornicotine) = 0.1 ng/ml, NAT (N-nitrosoanatabine) = 0.2 ng/ml, NNA (1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal) = 1 ng/ml.

RESULTS

Field Site

The field site is located on the basement floor of a two-story mall in a metropolitan area. The commercial building is over 50 years old. The field site for this study included the three basement suites shown in Figure 5.1. Suite #1 was an actively operated shop, and the site where the cotton fabrics, paper towels, and filter samples were placed for collection of ECEAR. The Filtrete™ air filter was placed in the return vent towards the back of Suite #1 while the Rabbit air filter was placed in the middle of the suite. Suite #3 is an active vape shop that blends and manufactures refill fluids which they sell in the store and online. The vape shop allows vaping inside the shop and has a bar and lounge where customers can try out ECs or refill fluids. Suite #2 is a shop that was seldom used during the course of our study. Approximate dimensions of Suite #1, Suite #2, and Suite #3 were 398 ft^2 (37 m^2), 405 ft^2 (37 m^2), 311 ft^2 (28 m^2), respectively.

The air intake to the building is from an adjacent alley. The HVAC system for the suites is a gas forced air furnace located in a mechanical room in back of Suite #1 (Figure 5.1). All suites have supply ducts in the floor, but only suite #1 has a return vent, which draws air from all three suites into the mechanical room. Each suite also contains storefront screen partitions that allow the air from suites #2 and 3 to enter the return vent in suite #1. Air pulled through Suite #1 was recirculated to the three Suites through the air supply ducts in the floor. There are no dedicated exhaust systems for any of the suites.

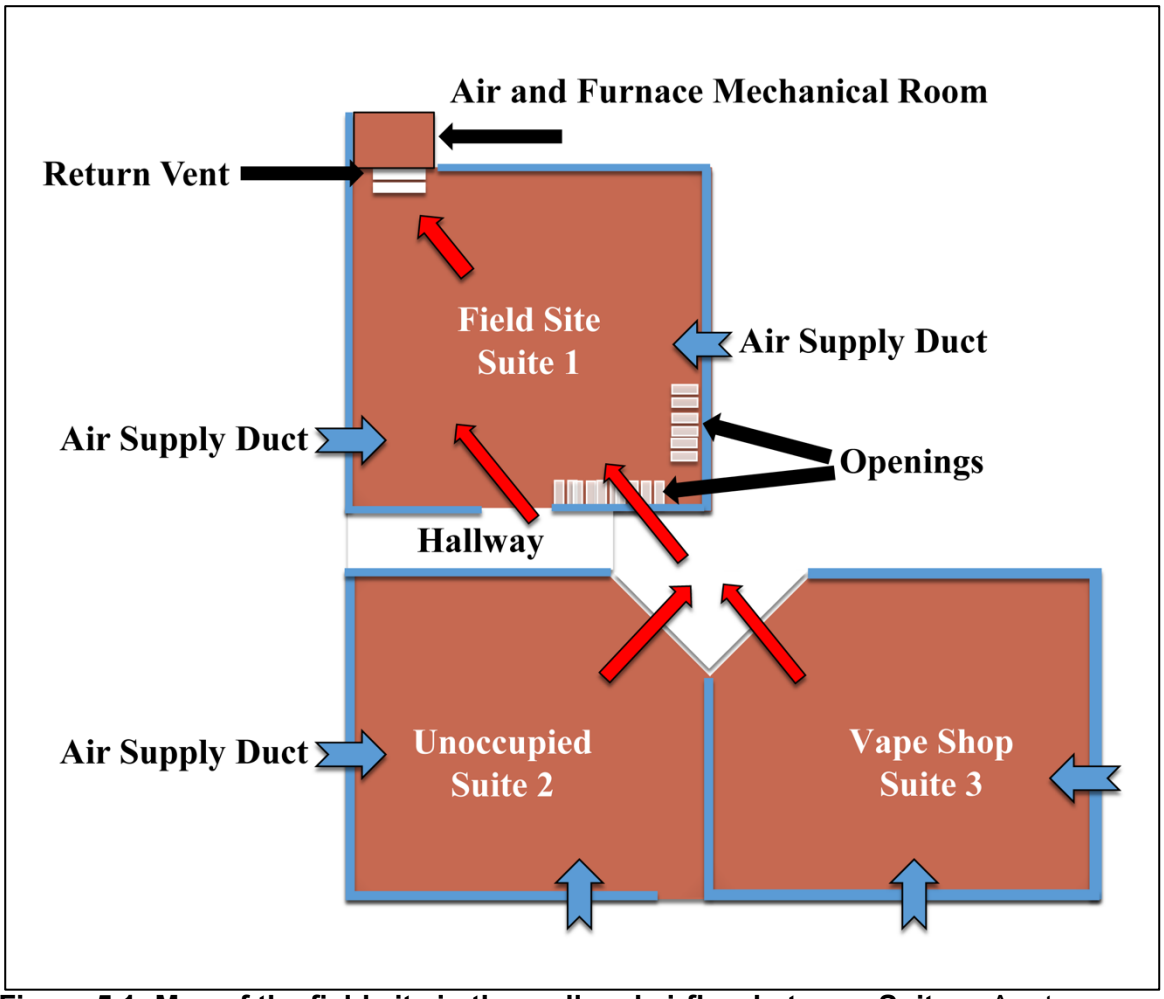

Figure 5.1: Map of the field site in the mall and airflow between Suites. A return vent, located at the back of Suite #1, intakes air (red arrows) from the surrounding spaces creating negative pressure inside Suite #1. Air can enter Suite #1 through the door and also through openings in the upper portion of the front and side walls. Air is redistributed to the three Suites through vents (blue arrows) from the air and furnace mechanical room located in the back of Suite #1.

The furnace in the mechanical room was produced by International Comfort Products Corporation (Louisburg, TN.) and the manufacturer's model number was GNE150J20A. Air flow through the field site was approximately 1401 to 1709 cubic feet per minute. The air filter in the return vent was a 3M Filtrete ™ healthy living air filter (20 inches (L) x 25 inches (W) x 1.0 inches (D)), which was made of polypropylene. The

Cotton Towel			**Paper Towel**		
	Sample	**Exposure Date**		**Sample**	**Exposure Date**
Short Term	SF 1D	March 5, 2014	**Short Term**	SF 1D	March 5, 2014
	SF 1D	March 15, 2015		SF 4D	March 1, 2014 – March 5, 2014
	SF 4D	March 1, 2014 – March 5, 2014		SF 4D	March 15, 2014 – March 19, 2014
	SF 10D	January 27, 2015 – February 6, 2015		SF 8D	March 7, 2014 – March 15, 2014
Long Term	SF 28D	January 27, 2015 – February 24, 2015	**Long Term**	SF 18D	February 6, 2015 – February 24, 2015
	SF 35D	January 31, 2015 – March 7, 2015		SF 28D	January 27, 2015 – February 24, 2015
	SF 31D	March 7, 2015 – April 7, 2015		SF 30D	March 15, 2014 – April 15, 2014
	SF 31D	March 7, 2015 – April 7, 2015		RV 59D	February 2014 – April 2014
	SF 74D	October 1, 2014 – December 13, 2014		SF 61D	May 2014 – July 2014
	Filter A 181D	February 2014 – August 2014		RV 61D	May 2014 – July 2014
	Filter B 365D	April 2014 – April 2015		RV 74D	October 1, 2014 – December 13, 2014
				SF 75D	August 1, 2014 – October 15, 2014

Table 5.1: Samples and exposure dates

basement also had a locked thermostat in the hall that controlled the furnace in the mechanical room.

Table 5.1 summarizes the types of samples collected from the field site (Suite #1) and their exposure dates. All short-term and some long-term samples were exposed at the front top openings of Suite #1 in the field site, while some long-term samples were exposed at the back near the return vent (Figure 5.1). The 3M Filtrete™ is labeled as Filter A while the Rabbit air filter is labeled as Filter B.

Nicotine, Other Alkaloids, and TSNAs Detected in Suite #1 ECEAR Samples

Nicotine, other alkaloids, and TSNAs were detected in ECEAR extracts of cotton towels (Figure 5.2) and paper towels (Figure 5.3) from Suite #1. Nicotine was the most abundant marker of EC aerosol contamination in Suite #1 (Figures 5.2A-B and 3A-B) (highest concentration = 23,260 ng/g of fabric). Nicotine was present in extracts of both short and long-term samples of cotton towel, paper towel, and terrycloth towel, and its concentration generally increased with exposure time (Figure 5.2A, B and 5.3A, B). Even samples exposed for only 1 day had detectable amounts of nicotine, e.g., paper towel exposed 1 day had 154 ng of nicotine/g of paper towel. Both air filter A and air filter B had high concentrations of nicotine (Figure 5.2B). Control samples of paper towels and terrycloth towels exposed both in the home of a non-smoker and in the mall had no detectable nicotine except for a low level (107 ng/g and 93 ng/g) in two samples (Appendix C Table S1).

Several tobacco alkaloids (cotinine, nicotelline, N-formylnornicotine, 2,3'-bipyridine, and myosmine) were found in ECEAR extracts from Suite #1, and their

concentrations generally increased as exposure time increased (Figures 2C-L, 3C-L). The alkaloids were found more frequently in paper towel extracts than in cotton towel, and their concentrations were generally higher in paper towel samples. The air filters appeared to trap nicotine and the alkaloids, except for 2,3' bipyridine and myosmine, which were not detected in the filter samples.

TSNAs were found in many of the paper towel extracts and in some of the terrycloth towel samples (Figures 5.2M-T and 5.3M-T). When TSNAs were found in the frequency with which chemicals appeared in paper and cotton samples is given in Appendix C Table S2. Almost all samples contained nicotine. Cotinine, nicotelline, NNK, and NNN were detected more frequently in paper than in cotton. N-Formylnornicotine, 2,3'-bipyridine, myosmine, NAT and NAB appeared with about equal frequency in cotton and paper. Both air filters contained nicotine, cotinine, nicotelline, and n-formylnornicotine. However, they did not contain 2,3 bipyridine, NAT, and NAB. Air filter A did contain myosmine, NNK, and NNN, while air filter B did not.

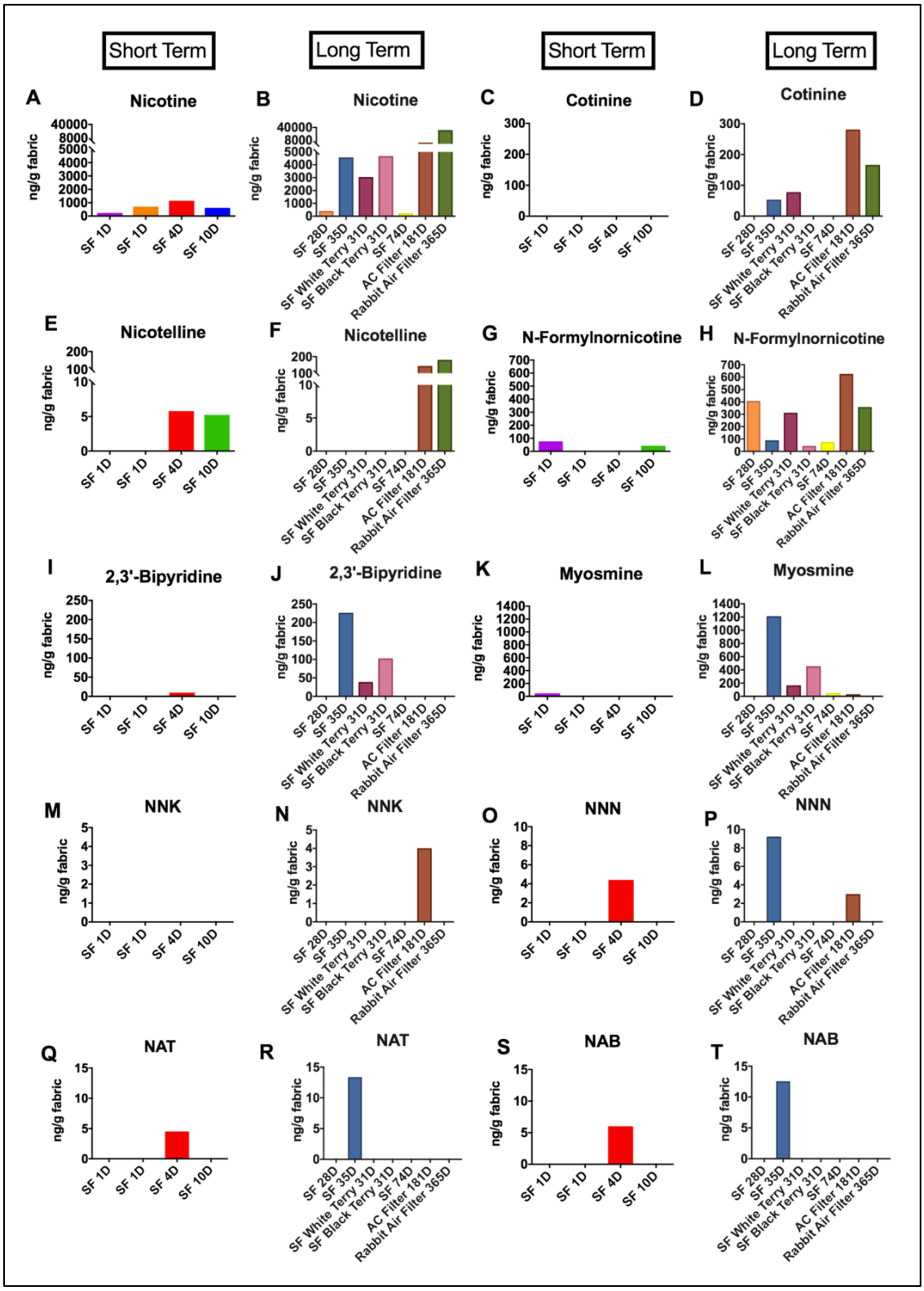
Short Term
Long Term
Short Term
Long Term
A
Nicotine
B
Nicotine
C
Cotinine
D
Cotinine
E
Nicotelline
F
Nicotelline
G
N-Formylnornicotine
H
N-Formylnornicotine
I
2,3'-Bipyridine
J
2,3'-Bipyridine
K
Myosmine
L
Myosmine
M
NNK
N
NNK
O
NNN
P
NNN
Q
NAT
R
NAT
S
NAB
T
NAB
ng/g fabric
SF 1D
SF 1D
SF 4D
SF 10D
SF 28D
SF 35D
SF White Terry 31D
SF Black Terry 31D
SF 74D
AC Filter 181D
Rabbit Air Filter 365D

Figure 5.2: Concentrations of nicotine, nicotine alkaloids, and TSNAs in cotton towels and air filters from the field site. Short term vs. long term samples are shown for each sample collected from the field site. Nicotine (A-B), cotinine (C-D), nicotelline (E-F), n-formylnornicotine (G-H), 2,3'-bipyridine (I-J), myosmine (K-L), NNK (M-N), NNN (O-P), NAT (Q-R), NAB (S-T). SF = Store Front. RV = Return Vent. Air Filter A =3M Filtrete™ air filter. Air Filter B = Rabbit Air HEPA filter.

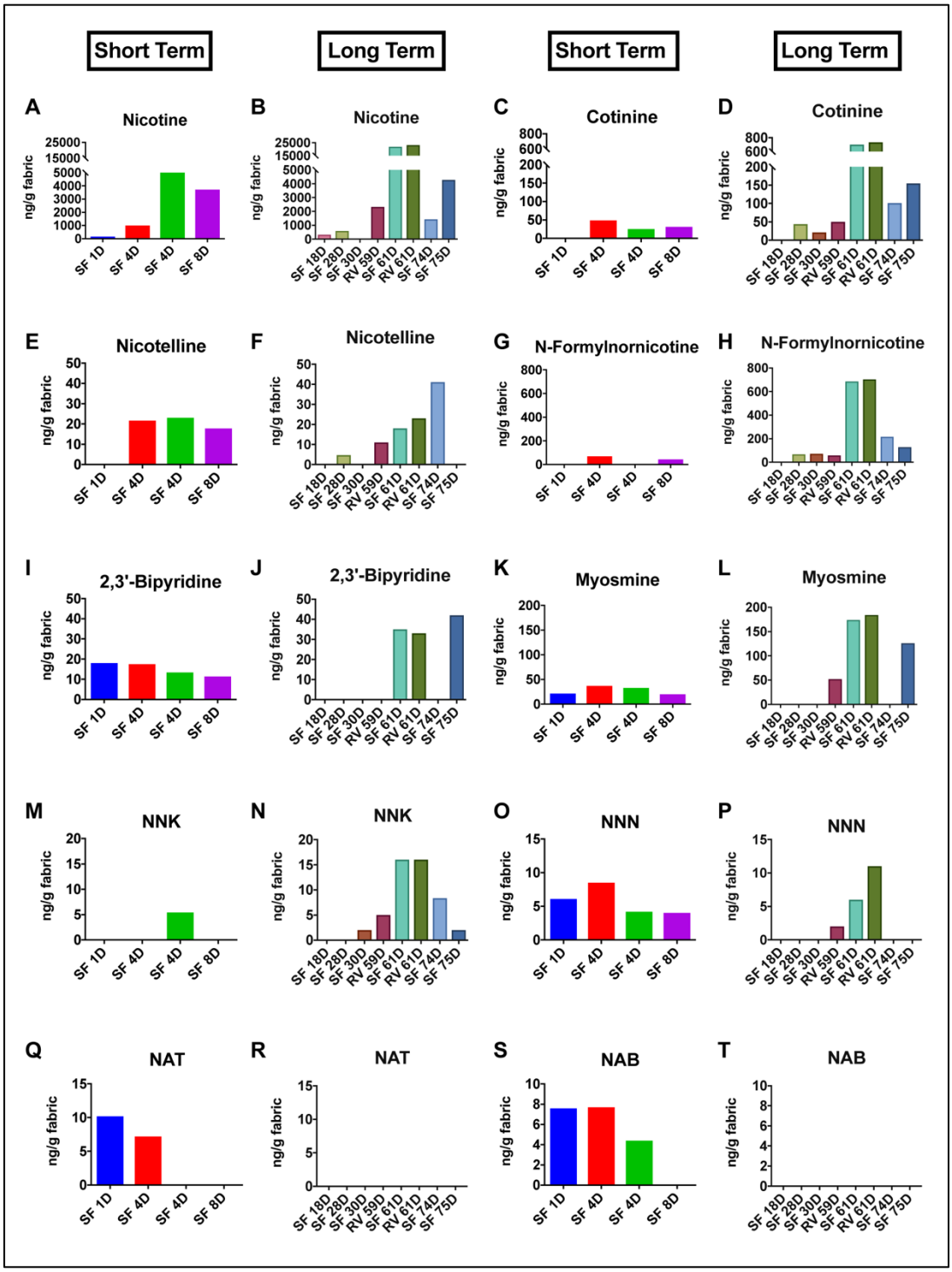
Short Term
Long Term
Short Term
Long Term
A
Nicotine
B
Nicotine
C
Cotinine
D
Cotinine
E
Nicotelline
F
Nicotelline
G
N-Formylnornicotine
H
N-Formylnornicotine
I
2,3'-Bipyridine
J
2,3'-Bipyridine
K
Myosmine
L
Myosmine
M
NNK
N
NNK
O
NNN
P
NNN
Q
NAT
R
NAT
S
NAB
T
NAB
ng/g fabric
SF 1D
SF 4D
SF 4D
SF 8D
SF 18D
SF 28D
SF 30D
RV 59D
SF 61D
RV 61D
SF 74D
SF 75D

Figure 5.3: Concentrations of nicotine, nicotine alkaloids and TSNAs in paper towels from the field site. Short term vs. long term samples are shown side by side for each sample collected from the field site. Nicotine (A-B), cotinine (C-D), nicotelline (E-F), n-formylnornicotine (G-H), 2,3'-bipyridine (I-J), myosmine (K-L), NNK (M-N), NNN (O-P), NAT (Q-R), NAB (S-T). SF = Store Front.

DISCUSSION

Our results demonstrate that EC aerosols generated in a vape shop can travel into a nearby business where they deposit on surfaces forming ECEAR. In our field site, this likely occurred because air circulated from Suites #2 and #3 (vape shop) to Suite #1 where samples were collected. The ECEAR in Suite #1 contained nicotine, other alkaloids, and nitrosamines, consistent with it originating in the vape shop. According to the American Society of Heating, Refrigerating and Air-Conditioning Engineers, Inc. (ASHRAE), environmental tobacco smoke (ETS) includes emissions produced by electronic smoking devices [30] and air should not recirculate or transfer from an ETS area to an ETS free area (5.17.5). The transfer of exhaled EC aerosol to Suite #1 may have been avoided or reduced if each suite had its own air distribution system. Since many types of HVAC systems are currently in buildings worldwide, the possibility of transfer of EC chemicals to businesses near vape shops should be addressed in building codes and regulated.

Our conclusion regarding the transfer of exhaled EC aerosol to a nearby business is based mainly on finding chemicals known to be present in EC aerosol in Suite #1, but not in the mall away from Suite #1 or in the control home. Nicotine and other alkaloids have been found in EC liquids and aerosols [31] and in exhaled EC aerosol [11]. The chemical markers did not come from cigarette smoke as smoking had been banned in the mall since 2009 (Public Act 188, www.michigan.gov). TSNAs have been reported in both refill fluids [32] and EC aerosols [5], and those found in the field site may have originated in the aerosol and/or formed after deposition in Suite #1 through interconversion of nicotine, as described previously for THS [33]. Laboratory

studies have shown that 93-99% of the inhaled nicotine in EC aerosol is retained in the lungs by EC users [14,34], but the extent of nicotine exhalation depends on the user's propensity to produce clouds of aerosol. In our real-world study, nicotine generated by vape shop occupants reached Suite #1 and contributed to ECEAR.

The 3M Filtrete ™ air filter and Rabbit Air HEPA filter, which were in the field site for 6 months and 1 year, respectively, picked up nicotine, cotinine, nicotelline, and n-formylnornicotine, and the 3M Filtrete ™ also trapped NNK and NNN. Concentrations of the chemical markers in these filters were likely higher than in the paper towel and terrycloth towel samples due to their longer exposure period. These data suggest that filtering air helps reduce exposure to nicotine and it alkaloids, but several chemicals (2,3' bipyridine and myosmine) were not trapped in either filter. Our study did not determine the efficiency with which these filters removed the chemical markers; however, ECEAR clearly collected on our cotton and paper samples before air circulated through the filters.

ECEAR chemicals in Suite #1 could be internalized passively through dermal contact and/or inhalation. Ingestion could also occur if a toddler mouthed fabrics or surfaces in Suite #1. Nicotine, the most abundant chemical we detected in ECEAR, is water and lipid soluble, and it readily permeated an in vitro skin model exposed to refill fluids containing nicotine, even when the period of dermal contact with refill fluids was short (10 minutes) [35,36]. Research into dermal uptake of nicotine from contact with ECEAR in realistic scenarios, such as the one in this study, is needed. Volatile organic compounds (VOCs), which are present in EC aerosol [8,37] and were found in ECEAR [38], could be passively inhaled. Acrolein and formaldehyde have been reported in EC aerosols [39] and likely contributed to ECEAR, although we did not analyze them in this

study. Inhalation of these and related VOCs would be a concern given their known toxicity [40-41].

While EC refill fluids can produce cytotoxic effects in vitro [42-44] and adverse health effects have been reported in EC users [45-47], the effects of exposure to ECEAR chemicals on human health are not yet known. The concentrations of these chemicals are likely much higher in the vape shop, but those chemicals reaching Suite #1 could build up over time. After 35 days in the field site, a cotton towel collected 4.571 μg of nicotine. If a toddler sucked on 0.3 m^2 or about 1 $foot^2$ of cotton fabric from Suite #1, they would be exposed to 81.26 μg of nicotine. Surface wipes measuring indirect exposure to cigarette smoke inside households where smokers only used cigarettes outside the home contained about 3.2 μg of nicotine/ per 0.3 m^2 at the average of the mean nicotine level per household [48], indicating greater transfer of nicotine from the vape shop to Suite #1 than to the household with indirect cigarette smoke exposure.

Others have shown that secondhand tobacco smoke also settles on indoor surfaces and forms THS [18,48]. While the health effects of THS are not fully known, it does have toxicity both in vitro and in mice [25,28,49,50]. Further monitoring of ECEAR and its health effects would be important in the future.

The data collected in this study pertains to a specific commercial vape shop and may not be generalizable to multiple-tenant residences or to vape shops in malls with alternative HVAC systems. ECEAR was not measured on all surface in the field site, and extraction may vary with different surfaces.

Store owners and tenants of malls should be aware that EC aerosols from nearby vape shops can enter their units and deposit as ECEAR. Chemicals in ECEAR

include nicotine, minor alkaloids, and TSNAs, the latter of which are potential carcinogens. Passive and unwanted exposure to these chemicals could occur through dermal contact, ingestion, or inhalation. The identification of chemicals, their concentrations, and their secondary products in ECEAR will be a necessary first step towards understanding the health and environmental effects of ECEAR. Building codes will need to be developed and enforced to protect those who do not wish to be exposed to ECEAR. Vape shop air quality is not currently regulated, nor has it been thoroughly studied. Regulatory agencies should exercise authority over malls to ensure that employees and tenants do not receive unwanted exposure to EC aerosol and its residue.

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Chapter 6: Conclusion

ECEAR was identified, quantified, and its toxicity was examined with 2D cells and 3D skin tissues. We started with the identification of flavor chemicals and nicotine in refill fluids, aerosols, and exhale. We also studied the transfer efficiency of chemicals from refill fluid to aerosol and determined that transfer efficiency was complex and variable for flavor chemicals and nicotine. When studying topography, puff duration and volume also played a role in the transfer of chemicals. Our results showed that flavor chemicals have a higher transfer efficiency when the puff duration and puff volume increased. However, cinnamaldehyde and eugenol had a constant transfer efficiency when a standard pump head was used. Nicotine did not depend on topography to transfer; it had a constant transfer rate due to its higher vapor pressure compared to the flavor chemicals. Participants had two types of topographies in our exhale study: mouth and lung inhalation. The mouth inhalers contributed more to ECEAR, while the lung inhalers retained more chemicals. Therefore, user topography affects not only the user but also non-users.

Since the skin is one of the first points of contact to refill fluids and ECEAR, it was important to test for cytotoxicity. Our study focused on two popular refill fluids, that contained mostly sweet flavors with a low nicotine concentration, available online and in shops. Both refill fluids, Dewberry Cream and Churrios, induced ROS production in human keratinocytes, while only Churrios at 1% concentration had an effect in the MTT assay. Both refill fluids also increased ROS inside mitochondria in keratinocytes. Human 3D EpiDerm™ tissues were also exposed to both refill fluids, and inflammatory marker secretions (IL-1α, IL-6, and MMP-9) increased after 4 and 24 hrs. Our work also

demonstrated that PG alone increased IL-1α secretion and that flavor chemicals did not cause additional increase. Finally, Churrios ECEAR extract increased in IL-1α secretion from EpiDerm™. Exposure of skin to refill fluids due to leakage or spills can increase oxidative stress and release of inflammatory proteins. EC users and employees at vape shops should be aware of the harm that could be caused by exposures to refill fluids, whether they be from handling refill fluids or from leaky pods. The accumulation of exhaled aerosol resulting in ECEAR could also contribute to dermal contact and inflammation.

To identify ECEAR in field sites, we placed fabrics (cotton) inside a vape shop and an EC user's living room. In the vape shop, we detected approximately 108 mg of nicotine/m^2 after 1 month of accumulation. In our living room field site, the concentration of nicotine was 1,181 μg/m^2 in a 3-month cotton sample. This study shows that nicotine and other chemicals in exhaled EC aerosol builds up on indoor surfaces where they result in exposure of EC users and non-smokers through dermal contact, inhalation, or ingestion. Employees, whether they are EC users or not, who work long hours inside vape shops are exposed to ECEAR and EC aerosols, which may be an occupational health hazard.

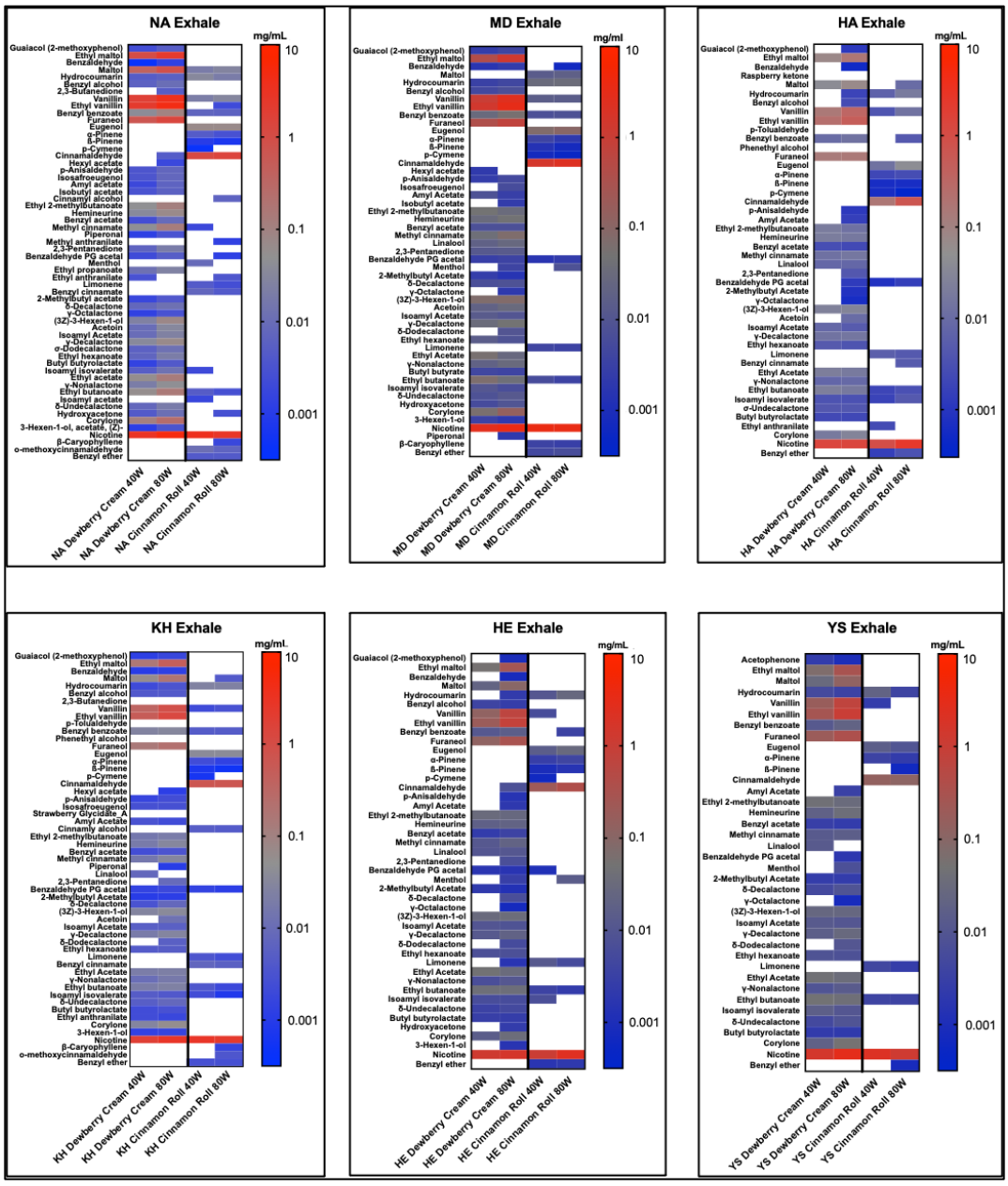
Lastly, we were able to identify ECEAR inside a business located next to a vape shop meaning that EC aerosol can travel to nearby spaces where the chemicals in the aerosol deposit and leave a residue that could potentially harm health. Cotton and paper towel fabrics were placed throughout the field site, and nicotine was detected after 1 day of exposure. After 35 days in the field site, a cotton towel collected 4.571 μg of nicotine and 35.12 ng of TSNAs /gram of towel, respectively. If a toddler sucked on 1 m^2 of this fabric from the field site, they would be exposed to 2.5 mg of nicotine and 19.5 μg of

TSNAs. This work shows that vape shops need to have dedicated exhausts and their own HVAC system so as not to allow the movement of aerosol from the vape shop to surrounding spaces.

In this dissertation I was able to show how the use of EC can progress and lead to the deposition of chemicals from the exhaled aerosol. EC users' topography influences how much flavor chemicals and nicotine they retain or contribute to ECEAR. Mouth inhalers retain less and contribute more while lung inhalers retain more and contribute less. EC users are also exposing themselves to refill fluids and ECEAR which cause oxidative stress and inflammatory marker release in the skin. ECEAR is detectable and quantifiable wherever ECs are used. Lastly, vape shops are a source of aerosol and movement of aerosols leads to residue buildup in adjacent spaces.

Appendix A: Supporting Information for Chapter 2

Figure S1: Heatmaps of participant's exhale with "Dewberry Cream" #518 and "Cinnamon roll" #537 used at 40 and 80 watts.



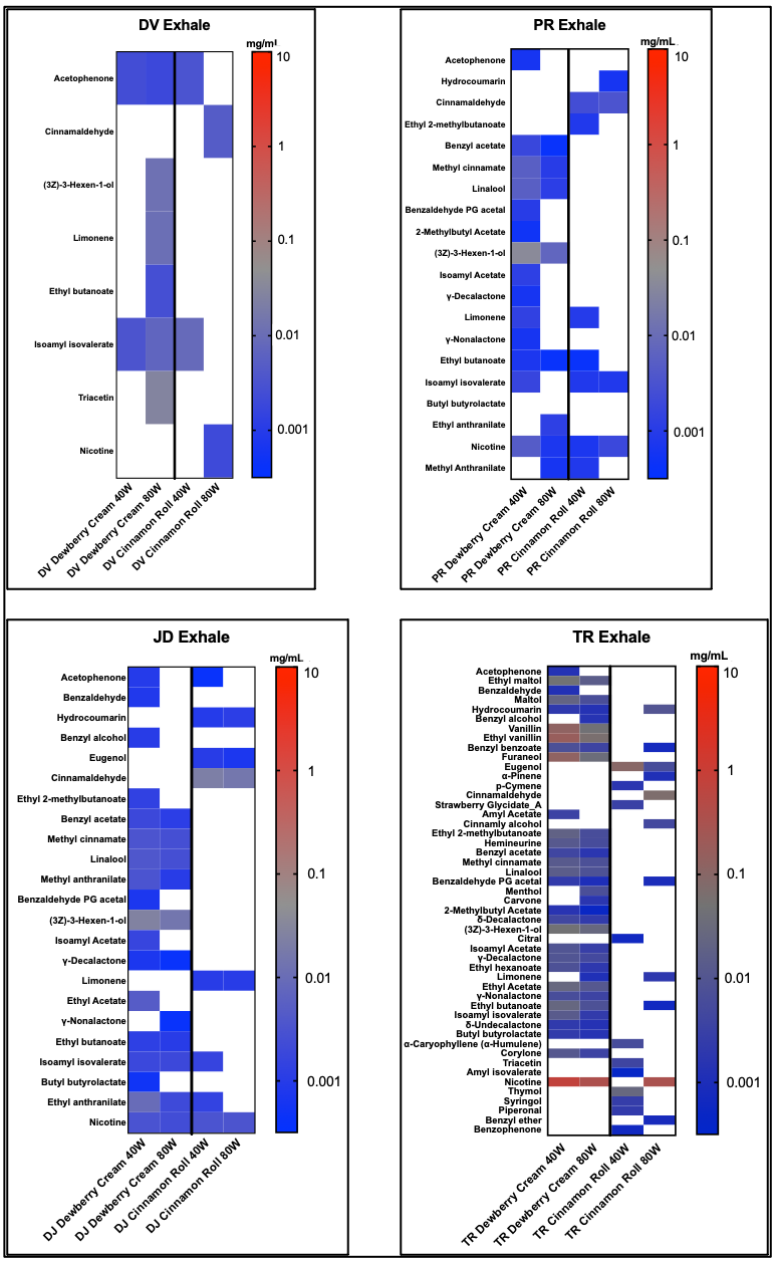

DV Exhale
mg/ml
10
1
0.1
0.01
0.001
Acetophenone
Cinnamaldehyde
(3Z)-3-Hexen-1-ol
Limonene
Ethyl butanoate
Isoamyl isovalerate
Triacetin
Nicotine
DV Dewberry Cream 40W
DV Dewberry Cream 80W
DV Cinnamon Roll 40W
DV Cinnamon Roll 80W
PR Exhale
mg/mL
Acetophenone
Hydrocoumarin
Cinnamaldehyde
Ethyl 2-methylbutanoate
Benzyl acetate
Methyl cinnamate
Linalool
Benzaldehyde PG acetal
2-Methylbutyl Acetate
(3Z)-3-Hexen-1-ol
Isoamyl Acetate
γ-Decalactone
Limonene
γ-Nonalactone
Ethyl butanoate
Isoamyl isovalerate
Butyl butyrolactate
Ethyl anthranilate
Nicotine
Methyl Anthranilate
PR Dewberry Cream 40W
PR Dewberry Cream 80W
PR Cinnamon Roll 40W
PR Cinnamon Roll 80W
JD Exhale
mg/mL
Acetophenone
Benzaldehyde
Hydrocoumarin
Benzyl alcohol
Eugenol
Cinnamaldehyde
Ethyl 2-methylbutanoate
Benzyl acetate
Methyl cinnamate
Linalool
Methyl anthranilate
Benzaldehyde PG acetal
(3Z)-3-Hexen-1-ol
Isoamyl Acetate
γ-Decalactone
Limonene
Ethyl Acetate
γ-Nonalactone
Ethyl butanoate
Isoamyl isovalerate
Butyl butyrolactate
Ethyl anthranilate
Nicotine
DJ Dewberry Cream 40W
DJ Dewberry Cream 80W
DJ Cinnamon Roll 40W
DJ Cinnamon Roll 80W
TR Exhale
mg/mL
Acetophenone
Ethyl maltol
Benzaldehyde
Maltol
Hydrocoumarin
Benzyl alcohol
Vanillin
Ethyl vanillin
Benzyl benzoate
Furaneol
Eugenol
α-Pinene
p-Cymene
Cinnamaldehyde
Strawberry Glycidate_A
Amyl Acetate
Cinnamyl alcohol
Ethyl 2-methylbutanoate
Hemineurine
Benzyl acetate
Methyl cinnamate
Linalool
Benzaldehyde PG acetal
Menthol
Carvone
2-Methylbutyl Acetate
δ-Decalactone
(3Z)-3-Hexen-1-ol
Citral
Isoamyl Acetate
γ-Decalactone
Ethyl hexanoate
Limonene
Ethyl Acetate
γ-Nonalactone
Ethyl butanoate
Isoamyl isovalerate
δ-Undecalactone
Butyl butyrolactate
α-Caryophyllene (α-Humulene)
Corylone
Triacetin
Amyl isovalerate
Nicotine
Thymol
Syringol
Piperonal
Benzyl ether
Benzophenone
TR Dewberry Cream 40W
TR Dewberry Cream 80W
TR Cinnamon Roll 40W
TR Cinnamon Roll 80W

Table S1: Participant's control (CN) exhale.

	DV CN Exhale	YS CN Exhale	HA CN Exhale	HE CN Exhale	KH CN Exhale	TR CN Exhale	MD CN Exhale	DJ CN Exhale	PR CN Exhale	NA CN Exhale
Compound Name										
2,3-Butanedione	0	0	0	0	0	0	0	0	0	0
Ethyl Acetate	0	0	0	0	0	0	0	0	0	0
Hydroxyacetone	0	0	0	0	0	0	0	0	0	0
2,3-Pentanedione	0	0	0	0	0	0	0	0	0	0
Ethyl Propanoate	0	0	0	0	0	0	0	0	0	0
Acetoin	0	0	0	0	0	0	0	0	0	0
Ethyl Isobutyrate	0	0	0	0	0	0	0	0	0	0
Isopentyl Alcohol	0	0	0	0	0	0	0	0	0	0
Isobutyl Acetate	0	0	0	0	0	0	0	0	0	0
Methyl 2-methylbutyrate	0	0	0	0	0	0	0	0	0	0
1-Pentanol	0	0	0	0	0	0	0	0	0	0
2,3-Hexanedione	0	0	0	0	0	0	0	0	0	0
Ethyl butanoate	0	0	0	0	0	0	0	0	0	0
Hexanal	0	0	0	0	0	0	0	0	0	0
Butyl Acetate	0	0	0	0	0	0	0	0	0	0
Ethyl lactate	0	0	0	0	0	0	0	0	0	0
Ethyl 2-methylbutanoate	0	0	0	0	0	0	0	0	0	0
Ethyl Isovalerate	0	0	0	0	0	0	0	0	0	0
Furfural	0	0	0	0	0	0	0	0	0	0
Isoamyl Acetate	0	0	0	0	0	0	0	0	0	0
2-Methylbutyl Acetate	0	0	0	0	0	0	0	0	0	0

(3Z)-3-Hexen-1-ol	0	0	0	0	0	0	0	0	0	0
2-Hexen-1-ol, (E)-	0	0	0	0	0	0	0	0	0	0
1-Hexanol	0	0	0	0	0	0	0	0	0	0
Furfuryl alcohol	0	0	0	0	0	0	0	0	0	0
Amyl Acetate	0	0	0	0	0	0	0	0	0	0
2,5-dimethylpyrazine	0	0	0	0	0	0	0	0	0	0
(3Z)-3-Hexenyl Formate	0	0	0	0	0	0	0	0	0	0
α-Pinene	0	0	0	0	0	0	0	0	0	0
2,3-dimethylpyrazine	0	0	0	0	0	0	0	0	0	0
Ethyl 3-hydroxybutyrate	0	0	0	0	0	0	0	0	0	0
Isoamyl Propionate	0	0	0	0	0	0	0	0	0	0
2-Methoxy-3-methylpyrazine	0	0	0	0	0	0	0	0	0	0
β-Myrcene	0	0	0	0	0	0	0	0	0	0
β-Pinene	0	0	0	0	0	0	0	0	0	0
Butyl butyrate	0	0	0	0	0	0	0	0	0	0
Ethyl hexanoate	0	0	0	0	0	0	0	0	0	0
Benzaldehyde	0	0	0	0	0	0	0	0	0	0
Pentyl Propanoate	0	0	0	0	0	0	0	0	0	0
6-Methyl-5-heptene-2-one	0	0	0	0	0	0	0	0	0	0
3-Hexen-1-ol, acetate, (Z)-	0	0	0	0	0	0	0	0	0	0
2,3,5-Trimethylpyrazine	0	0	0	0	0	0	0	0	0	0
Hexyl acetate	0	0	0	0	0	0	0	0	0	0
2-ethyl-3-methylpyrazine	0	0	0	0	0	0	0	0	0	0
1,4-Cineol	0	0	0	0	0	0	0	0	0	0
Limonene	0	0	0	0	0	0	0	0	0	0
p-Cymene	0	0	0	0	0	0	0	0	0	0

γ-Pentalactone	0	0	0	0	0	0	0	0	0	0
Eucalyptol	0	0	0	0	0	0	0	0	0	0
γ-Terpinene	0	0	0	0	0	0	0	0	0	0
Dimethyl butanedioate	0	0	0	0	0	0	0	0	0	0
Acetylpyrazine	0	0	0	0	0	0	0	0	0	0
Isoamyl Butyrate	0	0	0	0	0	0	0	0	0	0
Corylone	0	0	0	0	0	0	0	0	0	0
Ally hexanoate	0	0	0	0	0	0	0	0	0	0
Benzeneacetaldehyde	0	0	0	0	0	0	0	0	0	0
Benzyl Alcohol	0	0	0	0	0	0	0	0	0	0
Amyl Butyrate	0	0	0	0	0	0	0	0	0	0
2,3,5,6-Tetramethylpyrazine	0	0	0	0	0	0	0	0	0	0
Ethyl Heptanoate	0	0	0	0	0	0	0	0	0	0
cis-Linalool oxide	0	0	0	0	0	0	0	0	0	0
Furaneol	0	0	0	0	0	0	0	0	0	0
Isoamyl Isovalerate	0	0	0	0	0	0	0	0	0	0
Amyl Isovalerate	0	0	0	0	0	0	0	0	0	0
trans-Linalool Oxide	0	0	0	0	0	0	0	0	0	0
2-Nonanone	0	0	0	0	0	0	0	0	0	0
Acetophenone	0	0	0	0	0	0.3	0	0	0	0.2
Linalool	0	0	0	0	0	0	0	0	0	0
2-Acetylpyrrole	0	0	0	0	0	0	0	0	0	0
p-Tolualdehyde	0	0	0	0	0	0	0	0	0	0
Guaiacol (2-methoxyphenol)	0	0	0	0	0	0	0	0	0	0
2-Methylbenzofuran	0	0	0	0	0	0	0	0	0	0
1,2-Dihydrolinalool	0	0	0	0	0	0	0	0	0	0
cis-Limonene oxide	0	0	0	0	0	0	0	0	0	0

Fenchol	0	0	0	0	0	0	0	0	0	0
trans-Limonene oxide	0	0	0	0	0	0	0	0	0	0
Maltol	0	0	0	0	0	0	0	0	0	0
Phenethyl alcohol	0	0	0	0	0	0	0	0	0	0
β-Citronellal	0	0	0	0	0	0	0	0	0	0
Isopulegol	0	0	0	0	0	0	0	0	0	0
4-methylbenzyl alcohol	0	0	0	0	0	0	0	0	0	0
Benzyl acetate	0	0	0	0	0	0	0	0	0	0
Ethyl octanoate	0	0	0	0	0	0	0	0	0	0
2-Hydroxy-3,5,5-trimethyl-cyclohex-2-en	0	0	0	0	0	0	0	0	0	0
p-Menthone	0	0	0	0	0	0	0	0	0	0
Ethyl Benzoate	0	0	0	0	0	0	0	0	0	0
Neomenthol	0	0	0	0	0	0	0	0	0	0
Methyl phenylacetate	0	0	0	0	0	0	0	0	0	0
4-Terpineol	0	0	0	0	0	0	0	0	0	0
Menthol	0	0	0	0	0	1.4	0	0	0	0
Styralyl Acetate	0	0	0	0	0	0	0	0	0	0
Benzylacetaldehyde	0	0	0	0	0	0	0	0	0	0
Methyl 2-Octynate	0	0	0	0	0	0	0	0	0	0
Estragole (4-allyanisole)	0	0	0	0	0	0	0	0	0	0
3'-Methylacetophenone	0	0	0	0	0	0	0	0	0	0
α-Terpineol	0	0	0	0	0	0	0	0	0	0
Hexyl 2-Methylbutyrate	0	0	0	0	0	0	0	0	0	0
Methyl salicylate	0	0	0	0	0	0	0	0	0	0
γ-Heptalactone	0	0	0	0	0	0	0	0	0	0
Linaly acetate	0	0	0	0	0	0	0	0	0	0
Ethyl Maltol	0	0	0	0	0	0	0	0	0	0

Benzeneacetic acid, ethyl ester	0	0	0	0	0	0	0	0	0	0
trans-Geraniol	0	0	0	0	0	0	0	0	0	0
Benzyl Propionate	0	0	0	0	0	0	0	0	0	0
Pulegone	0	0	0	0	0	0	0	0	0	0
Ethyl nonanoate	0	0	0	0	0	0	0	0	0	0
Carvone	0	0	0	0	0	0	0	0	0	0
Menthyl Acetate	0	0	0	0	0	0	0	0	0	0
Benzaldehyde PG acetal	0	0	0	0	0	0	0	0	0	0
Citral	0	0	0	0	0	0	0	0	0	0
Ethyl salicylate	0	0	0	0	0	0	0	0	0	0
Piperitone	0	0	0	0	0	0	0	0	0	0
p-Anisaldehyde	0	0	0	0	0	0	0	0	0	0
Linaly propionate	0	0	0	0	0	0	0	0	0	0
Cinnamaldehyde, (E)-	0	0	0	0	0	0	0	0	0	0
Thymol	0	0	0	0	0	0	0	0	0	0
γ-Octalactone	0	0	0	0	0	0	0	0	0	0
Hemineurine	0	0	0	0	0	0	0	0	0	0
Nerol acetate	0	0	0	0	0	0	0	0	0	0
Butyl butyrolactate	0	0	0	0	0	0	0	0	0	0
Benzyl Butyrate	0	0	0	0	0	0	0	0	0	0
Cinnamly alchohol	0	0	0	0	0	0	0	0	0	0
Triacetin	0	0	0	0	0	0	0	0	0	0
Geraniol Acetate	0	0	0	0	0	0	0	0	0	0
Hexyl hexanoate	0	0	0	0	0	0	0	0	0	0
Ethyl decanoate	0	0	0	0	0	0	0	0	0	0
Nicotine	0	0.2	0	0.7	0	1.6	0	0.7	0	0
Eugenol	0	0	0	0	0	0	0	0	0	0
Syringol	0	0	0	0	0	0	0	0	0	0

Methyl Anthranilate	0	0	0	0	0	0	0	0	0	0
Piperonal	0	0	0	0	0	0	0	0	0	0
Eugenol methyl ether	0	0	0	0	0	0	0	0	0	0
Methyl cinnamate	0	0	0	0	0	0	0	0	0	0
α-Damascone	0	0	0	0	0	0	0	0	0	0
Ethyl Benzolyformate	0	0	0	0	0	0	0	0	0	0
Citronellyl Propionate	0	0	0	0	0	0	0	0	0	0
β-Caryophyllene	0	0	0	0	0	0	0	0	0	0
γ-Nonalactone	0	0	0	0	0	0	0	0	0	0
Methyl N-methylanthranilate	0	0	0	0	0	0	0	0	0	0
β-Damascone	0	0	0	0	0	0	0	0	0	0
Aromadendrene	0	0	0	0	0	0	0	0	0	0
α-Ionone	0	0	0	0	0	0	0	0	0	0
Ethyl anthranilate	0	0	0	0	0	0	0	0	0	0
Strawberry Glycidate_A	0	0	0	0	0	0	0	0	0	0
Cinnamyl Acetate	0	0	0	0	0	0	0	0	0	0
Hydrocoumarin	0	0	0	0	0	0	0	0.6	0	0
α-Caryophyllene (α-Humulene)	0	0	0	0	0	0	0	0	0	0
Vanillin	0	0	0	0	0	0	0	0	0	0
Ethyl Cinnamate	0	0	0	0	0	0	0	0	0	0
Benzyl dimethylcarbinyl butyrate	0	0	0	0	0	0	0	0	0	0
Isopentyl Phenylacetate	0	0	0	0	0	0	0	0	0	0
Isoeugenol methyl ether	0	0	0	0	0	0	0	0	0	0
(E)-β-Ionone	0	0	0	0	0	0	0	0	0	0

Ethyl Vanillin	0	0	0	0	0	0	0	0	0	0
Coumarin	0	0	0	0	0	0	0	0	0	0
Myristicin	0	0	0	0	0	0	0	0	0	0
γ-Decalactone	0	0	0	0	0	0	0	0	0	0
Acetyleugenol	0	0	0	0	0	0	0	0	0	0
Raspberry Ketone methyl ether	0	0	0	0	0	0	0	0	0	0
Hexyl octanoate	0	0	0	0	0	0	0	0	0	0
Strawberry Glycidate_B	0	0	0	0	0	0	0	0	0	0
Isosafroeugenol	0	0	0	0	0	0	0	0	0	0
Ethyl Laurate	0	0	0	0	0	0	0	0	0	0
δ-Decalactone	0	0	0	0	0	0	0	0	0	0
o-methoxycinnamaldehyde	0	0	0	0	0	0	0	0	0	0
δ-Undecalactone	0	0	0	0	0	0	0	0	0	0
Raspberry ketone	0	0	0	0	0	0	0	0	0	0
Coumarin, 6-methyl	0	0	0	0	0	0	0	0	0	0
Heliotropine proplyene gycol acetal	0	0	0	0	0	0	0	0	0	0
Benzyl ether	0	0	0	0	0	0	0	0	0	0
Benzophenone	0	0	0	0	0	0	0	0	0	0
Gingerone	0	0	0	0	0	0	0	0	0	0
δ-Dodecalactone	0	0	0	0	0	0	0	0	0	0
Benzyl Benzoate	0	0	0	0	0	0	0	0	0	0
Benzyl Benzeneacetate	0	0	0	0	0	0	0	0	0	0
Benzoin Ethyl Ether	0	0	0	0	0	0	0	0	0	0
Caffeine	0	0	0	0	0	0	0	0	0	0
Benzyl cinnamate	0	0	0	0	0	0	0	0	0	0

total µg	0	0.2	0	0.7	0	3.3	0	1.3	0	0.2
total mg	0	0.0002	0	0.0007	0	0.0033	0	0.0013	0	0.0002

Appendix B: Supporting Information for Chapter 4

Table S1: Refill fluid brands and flavor name

Brand	**Flavor**
Ballistic Vapes	The Tsunami
Uncle Junk's Genius Juice	Honey Do
Cuttwood	Unicorns Milk
Cosmic Fog	Kryptonite
Cosmic Fog	Milk and Honey
Lucid Liquids	Love Potion
Lost Art Liquids	Unicorn Puke
Villain Vapors	Pair of deuces
Buckshot	m80
OMG	WTF
Eliqcube	Pink Starburst
Mastermind Elixers	Snake Oil
Mastermind Elixers	DJs Locker
Halcyon	Apache
Uncle Junk's Genius Juice	John Wayne
Uncle Junk's Genius Juice	Monica's Eyes
Lucid Liquids	Love Poison
Halcyon	Dragon Chi
Gemini	Koi
Kilo	Dewberry Crème
Holdfast	Mutiny

Table S2: Samples placed inside vape shop field site

Sample #	Day of the Week	Time In	Time Out	Total hrs.	Placement
1	Saturday	12:30 PM	6:30 PM	6.00	Display Case
2	Saturday	12:30 PM	6:30 PM	6.00	Lounge
3	Saturday	12:30 PM	6:30 PM	6.00	Chalkboard
4	Saturday	12:30 PM	6:30 PM	6.00	Outside
5	Saturday	11:30 AM	6:30 PM	7.00	Display Case
6	Saturday	11:30 AM	6:30 PM	7.00	Lounge
7	Saturday	11:30 AM	6:30 PM	7.00	Chalkboard
8	Saturday	11:30 AM	6:30 PM	7.00	Outside
9	Saturday - Sunday	7:30 PM	12:30 PM	18.00	Display Case
10	Saturday - Sunday	7:30 PM	12:30 PM	18.00	Lounge
11	Saturday - Sunday	7:30 PM	12:30 PM	18.00	Chalkboard
12	Saturday - Sunday	7:30 PM	12:30 PM	18.00	Outside
13	Friday - Saturday	6:30 PM	6:30 PM	24.00	Display Case
14	Friday - Saturday	6:30 PM	6:30 PM	24.00	Lounge
15	Friday - Saturday	6:30 PM	6:30 PM	24.00	Chalkboard
16	Friday - Saturday	6:30 PM	6:30 PM	24.00	Outside
17	Friday - Sunday	6:30 PM	6:30 PM	48.00	Display Case
18	Friday - Sunday	6:30 PM	6:30 PM	48.00	Lounge
19	Friday - Sunday	6:30 PM	6:30 PM	48.00	Chalkboard
20	Friday - Sunday	6:30 PM	6:30 PM	48.00	Outside
21	Saturday - Saturday	7:00 PM	7:00 PM	1 w	Display Case
22	Saturday - Saturday	7:00 PM	7:00 PM	1 w	Lounge
23	Saturday - Saturday	7:00 PM	7:00 PM	1 w	Chalkboard

24	Saturday - Saturday	7:00 PM	7:00 PM	1 w	Outside
25	Saturday - Saturday	7:30 PM	7:30 PM	1 m	Display Case
26	Saturday - Saturday	7:30 PM	7:30 PM	1 m	Lounge
27	Saturday - Saturday	7:30 PM	7:30 PM	1 m	Chalkboard
28	Saturday - Saturday	7:30 PM	7:30 PM	1 m	Outside

Appendix C: Supporting Information for Chapter 5

Table S1: Controls

Sample	Nicotine	Cotinine	Nicotelline	N-Formylnornicotine	Bipyridine	Myosamine	NNK	NNN	NAT	NAB
Unexposed Paper Towel	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Unexposed Cotton Towel	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Unexposed White Terry	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Unexposed Black Terry	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1M Exposed White Terry	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1M Exposed Black Terry	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Unexposed Filtrete ™ air filter	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Unexposed Rabbit air filter	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1D A mini mall	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1D B mini mall	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
3D A mini mall	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

3D B mini mall	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7D A mini mall	107.8	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7D B mini mall	93.7	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1D indoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1D outdoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
4D indoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
4D outdoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7D indoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7D outdoor house	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

Table S2: Frequency of alkaloids in cotton towels and paper towels.

	Nicotine	Cotinine	Nicotelline	N-Formylnornicotine	2,3'-Bipyridine	Myosmine	NNK	NNN	NAT	NAB
Cotton	100%	22%	22%	78%	44%	56%	0%	22%	22%	22%
Paper	92%	83%	67%	75%	58%	67%	58%	58%	17%	25%