AMERICAN UNIVERSITY OF BEIRUT

PREDICTING THE TOXICITY OF ELECTRONIC CIGARETTE BASED ON A SYSTEMATIC APPROACH

by
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AN ABSTRACT OF THE THESIS OF

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Title: Predicting the Toxicity of Electronic Cigarette based on a Systematic Approach

Electronic cigarette (ECIG) is marketed as a "safe" alternative to combustible cigarette. This battery-powered device vaporizes a liquid on a heating coil. The liquid is mainly a solution of propylene glycol (PG), vegetable glycerin (VG), with or without nicotine and/or flavors. The number of ECIG users expanded rapidly from 7 million in 2011 to 41 million in 2018. However, many studies analyzed the chemical profile of ECIG liquids and aerosols and reported the detection of some toxicants, carcinogens, and oxidants in ECIG emissions. In an attempt to complement the literature, this thesis focuses on the quantification of reactive oxygen species (ROS) as a generic assessment of ECIG toxicity. The influence of the different ECIG operating parameters, i.e., ECIG device design, liquid composition, battery power output, user puff topography, and inhalation flow rate, on ROS emissions was assessed. In a first study, we compared ROS emissions from a USB stick-like ECIG known as JUUL in two different markets: US and UK, to check any possible differences. In a second study, ROS was measured for the above conditions in a highly customizable device known as sub-ohm ECIGs (coil resistance < 1 Ohm). A fluorescent probe was used to trap and semi-quantify ROS on a microplate reader.

In a second approach to the assessment of the toxicity of ECIG, we attempted to establish a direct correlation between flavoring chemicals and aerosol toxicants. The flavor is considered as a major factor in increasing ECIG acceptance and safety perceptions among youth. Nowadays, more than 15000 flavors are available in the market. Our work focused on the chemical profiling of flavored liquids using non-targeted analysis via gas chromatography-mass spectrometry (GC-MS), and then categorizing the chemical constituents according to their functional groups. This unique approach helps to identify the reactions that might take place under ECIG realistic conditions, thus allowing the prediction of toxicant emissions.

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ABBREVIATIONS

ALVIN	Aerosol lab vaping instrument
ATPs	Alternative tobacco products
BTEX	Benzene, toluene, ethylbenzene, xylene
Cd	Cadmium
CKC	Contextual Knowledge Core
CSTP	Center for the Study of Tobacco Products
DBNBS	3,5-dibromo-4-nitrosobenzene sulfonic acid
DCF	Dichlorofluorescein
DCFH2	2',7'-dichlorodihydrofluorescein
DCFH2-DA	2′,7′-dichlorodihydrofluorescin diacetate
DHE	Dihydrorthidum
DHR-123	Dihydrorhodamine 123
DI	Deionized water
DMPO	5,5-dimethyl-1-pyrroline N-oxide
ECIG	Electronic Cigarette
ENDS	Electronic Nicotine Delivery Systems
EPA	Environmental Protection Agency
ESR	Electron spin resonance
FDA	Food and Drug Administration
FGs	Functional groups
FLARE	FLavor Associated Reactivity Evaluation
HRP	Horseradish peroxidase

HTPs	Heated tobacco products
ICP-MS	Inductively coupled plasma mass spectrometry
JUUL	Juice USB Lighting
LC-ESI–MS/MS	Liquid chromatography-electrospray ionization-tandem-mass spectrometry
LC-MS	Liquid chromatography-mass spectroscopy
LED	Light Emitting Diode
LOD	Limit of detection
LOQ	Limit of quantification
MLSA	Minimum legal sales age
MNP	2-methyl-2-nitrosopropane
Ni	Nickel
NYTS	National Youth Tobacco Survey
P	Power
Pb	Lead
PG	Propylene glycol
POHPAA	p-hydroxyphenylacetic acid
R	Resistance
ROS	Reactive oxygen species
SD	Standard deviation
SPME-GC-MS	Solid-phase micro extraction and gas chromatography-mass spectrometry
TSNAs	Tobacco-specific nitrosamines
USB	Universal serial bus
V	Voltage

VG	Vegetable glycerin
VOCs	Volatile organic compounds
WHO	World Health Organization

CHAPTER I

INTRODUCTION

A. Electronic Cigarettes as alternative tobacco products

A general consensus now exists that smoking tobacco places high health and financial burdens on public health and economy, respectively. Smoking remains the main preventable cause of sickness and unexpected death worldwide. In 2015, a total of 933 million individuals were assessed to be daily smokers leading to around one-tenth of global deaths.[1, 2] Researchers expect the number of smoking-attributable death to reach 7 million by the current year and exceed 8 million yearly by 2030, if the current smoking rate remains the same.[3] Besides, according to statistics from the Action on Smoking and Health, 25% of cancer deaths were caused by smoking.[4] Moreover, an estimation showed that 600,000 deaths around the globe were contributed to second-hand smoking each year, where the majority were women and children.[4] Economy-wise, yearly smoking-attributable health-care costs were around $96 billion during 2000-2004,[5] and this tremendously increased to reach a total of $422 billion in 2012, which is equivalent to 5.7% of global health expenditure.[6]

Consequently, strict and effective policies and measurements were conducted by governments and public health authorities to fight the spread of tobacco and reduce its use. Some of these countermeasures are: tobacco tax upsurges, media campaigns, and authorization of the Framework Convention on Tobacco Control by the World Health Organization (WHO).[7] All these measures resulted in more smokers succeeding in quitting the deadly habit of smoking. As reported by the 2010 National

Actual Smoking Survey, the number of current Korean smokers who have expressed a willingness to quit smoking has reached 60% of the current total smokers in Korea.[8] Similarly, The United States of America put huge efforts to reduce the number of tobacco smokers in the whole population. One of the effective policies was applying the T21 law on all its territories. This law states that the minimum legal sales age (MLSA) for any tobacco product was raised from 18 years to 21 years imposing penalties on violators.[9] In addition, tobacco-free policies were applied on different public and private spaces such as college and university campuses where 84% of these campuses are now tobacco-free.[10] Similarly, tobacco-free airports are around 78% in North America, 44% in Europe, and 18% in Asia of all airports.[11] Applying these policies has proven to reduce smoking rate by 29% in 2011, and it is expected to reach 41% by 2041.[12]

Nevertheless, tobacco use, including smoking is still a big challenge to public health. Tobacco use is usually initiated during adolescence.[13] As stated by the National Survey on Drug use and Health 2012, the average age where youth start smoking is 15.3 years.[14] Moreover, The Executive Summary of the U.S Surgeon General Office report in 2012 declared that 3800 underage youth are initiating smoking on daily basis.[13] The majority of adult smokers (88%) reported attempting smoking before the age of 18 years. [13, 15] Various individual factors influence adolescents' decision of starting smoking such as stress and low self-confidence, in addition to some social factors like having smoker parents or siblings.[16] Moreover, some teenagers imitate adult smokers, believing that this behavior can make them look more mature and independent.[17]

Smokers at different stages of their smoking journey may attempt to quit smoking. They usually try to do so by shifting to alternative tobacco products (ATPs) that are thought to be less toxic, although not risk-free.[18] Several products fall under the definition of ATPs, including smokeless tobacco products (such as chew, snuff, and snus), heated tobacco products (HTPs), and Electronic Cigarette (ECIG) that was recently introduced to the market in 2003.[19] The efficacy of all these products in assisting the quit attempts is not validated, and is out of the scope of the current study.

Although initially advertised as a safer alternative to combustible cigarettes and should help smokers to quit, ECIGs became highly widespread among youth. According to a study published in 2019, ECIG was the most used tobacco product among middle and high school students, where 4.04 million high school students and 840 000 middle school students were consuming different tobacco products.[20, 21] Analysis of data from the National Youth Tobacco Survey (NYTS), which is an annual school-based survey of US middle and high school students, showed that more than 10% of middle and 27% of high school students reported current use of ECIG.[22] A recent study showed that 81% of youth ECIG users initiated vaping due to the myriad of designs and flavors available on the market.[23, 24]

ECIG prevalence increased dramatically in recent years and the number of users expanded rapidly from 7 million in 2011 to 41 million in 2018.[25] The global sales of ECIGs have developed significantly and the worldwide market is assessed to be worth $19.3 billion in 2018 up from $6.9 billion in 2013.[25] As ECIG is getting more popular, researchers from different disciplines are joining forces to advance our understanding of these devices and assess the toxicity profile of their emissions.

In order to slow down the epidemic use of ECIGs,[26] special policies were applied to ECIGs worldwide. In addition to banning some ECIG flavors that appeal to youth,[13] Food and Drug Administration (FDA) prevented selling ECIGs in vending machines unless in adult-only facilities, as well as distributing ECIG free samples including all related parts, starting 2016.[27] Besides, companies are enforced to market ECIGs or other Electronic Nicotine Delivery Systems (ENDS) with warning statement on the package beginning May 2018.[27] ECIG users including minors have the access to buy these electronic devices from online vendors where the majority does not apply sufficient verification steps. Thus, FDA enforced ECIG retailers to check photo ID for consumers under the age of 27.[27] Recently, the FDA finalized a policy banning all flavors (other than tobacco or menthol) for "cartridge-based e-cigarettes".[28]

B. What is an ECIG?

An ECIG is a battery-powered electronic device. Its basic operating principle consists of vaporizing a liquid on an electrically powered coil to produce an inhalable aerosol. The liquid present around the coil will heat up and undergo the process of vaporization as soon as the heater coil is activated during a puff either by pushing a button or triggering an air-flow sensor by inhaling through the mouthpiece. Vapors produced are directed by the air drawn through the device away from the vicinity of the coil, then undergo the process of re-condensation to produce an aerosol mist that visually mimics tobacco smoke as illustrated in Figure 1.[29]

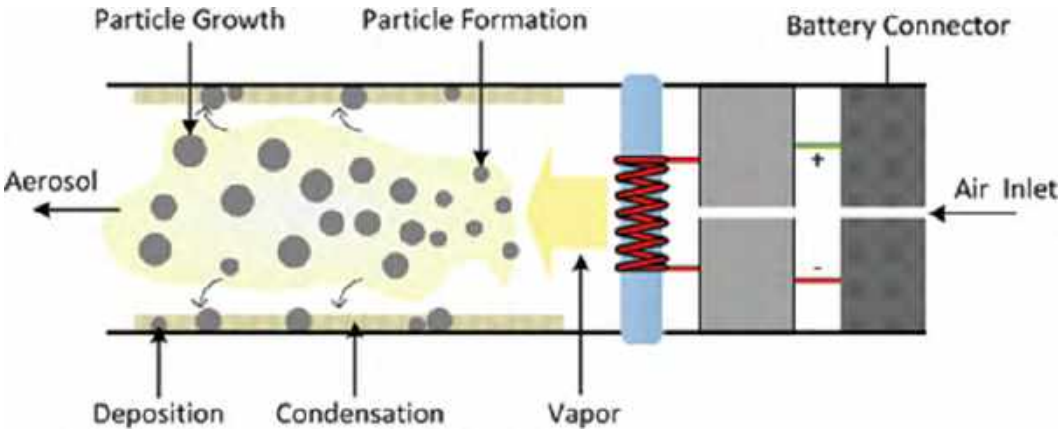

Figure 1. A schematic showing ECIG aerosol generation.[29]

Regardless of ECIGs' design and appearance, these devices generally operate in the same way and share the same basic compartments.[29] ECIGs' main components consist of: a lithium battery, an atomizer, a cartridge or liquid reservoir to hold the ECIG liquid, and a mouthpiece as shown in Figure 2. Some devices have a Light Emitting Diode (LED) indicator light attached to one end of the battery. The battery is responsible for activating the device. The atomizer is made up of a coil and a wick. A short resistance wire is looped around the wicking material that draws ECIG liquid onto the coil. Wicking material can be made up of cotton, bamboo yarn or silica. ECIG coils consist of several types of wires, such as Nichrome, Kanthal, or Stainless Steel, that showed an important catalytic effect on carbonyl emissions according to Saliba et al. [30]

Figure 2. Parts of an electronic cigarette.[31]

C. ECIG liquid composition

Besides the rapid growth and changes in ECIG designs, a huge variability in ECIG liquid composition existed in the market. ECIG liquid is usually composed of propylene glycol (PG) and/or vegetable glycerin (VG), with or without flavorants and nicotine,[32] of variable concentration.[33]. ECIG liquid usually comprises of PG and VG (>90%), and the remaining 10% for flavorants and nicotine.[34] Some ECIGs allow users to customize their device and thus change the liquid constituents, including PG/VG ratio, nicotine concentration, in addition to the type and load of flavorants and additives. Both PG and VG are considered as nicotine and flavorants' carriers to the user's mouth, throat, and/or lungs when vaporized into steam.[35]. PG is considered as a tasteless fluid with low viscosity while VG or glycerol is considered as a naturally sweet flavor of higher viscosity. Based on these physical properties, liquids with high ratio of VG produce larger vapors and offer a slight sweetness. On the other hand, liquids with high ratio of PG cause more "throat hit" and carry flavors more strongly.

Besides, the sensory appeal of ECIG is increased by flavors.[36] ECIG users can purchase liquids based on the desired flavor. Fruit, tobacco, mint/menthol and

candy are the most popular flavor categories.[37] According to a recent study, adolescents were found to prefer vanilla, candy/dessert, and fruit flavors whereas adults mostly preferred non-sweet ECIG liquid flavors like tobacco, fruit, and menthol/mint.[38]

D. ECIG evolution

As ECIG market expanded enormously, different generations of this electronic powered device appeared, with all generations having the same operating principle. The first generation of ECIG devices resembled conventional tobacco cigarettes and was called Cig-a-like, being either disposable or rechargeable. The device has a LED light that looks like a lit tobacco cigarette tip that turns on upon inhalation, permitting one to monitor its usage.[39] But these devices were proven to have a low efficiency in nicotine delivery.[32, 40]

The second generation is referred to as “personal vaporizer”. It looks like a pen or a laser pointer. Using such devices, an “on/off” button should be pressed to activate the heating coil. In addition, second generation devices contain a rechargeable battery and a refillable cartomizer. Thus, user has access to customize the battery power output and liquid composition according to his preference.[39]

The third generation devices are called “Mods”. They operate at very high powers that may reach up to 200W. According to Ohm’s law, power (P) in an electrical circuit in watts is equal to the square of voltage (V) divided by the resistance (R) in ohms: $P = V^2/R$. [33] Since battery power and coil resistance are inversely proportional, the high power output is achieved by a coil of resistance lower than unity. This is why such devices are called sub-ohm devices unlike other ECIG devices operating at higher

resistance which are called supra-ohm devices. Accordingly, reducing resistance rapidly increases the heat of the atomizer coil, vaporizes more liquid, delivers more robust flavor, and develops massive clouds because of the boosted current passing though the coil.[41] This explains why these devices became popular and appealing to youth.

In addition to the features of the third generation, the fourth generation is characterized by a temperature sensor that will allow the user to manually control the temperature. Coil heat temperature is a key parameter in affecting the quantity and the composition of ECIG aerosol.[42] Also, research showed that the higher the temperature, the more nicotine is delivered, and larger amounts of formaldehydes and other toxicants are emitted to the aerosol.[43]

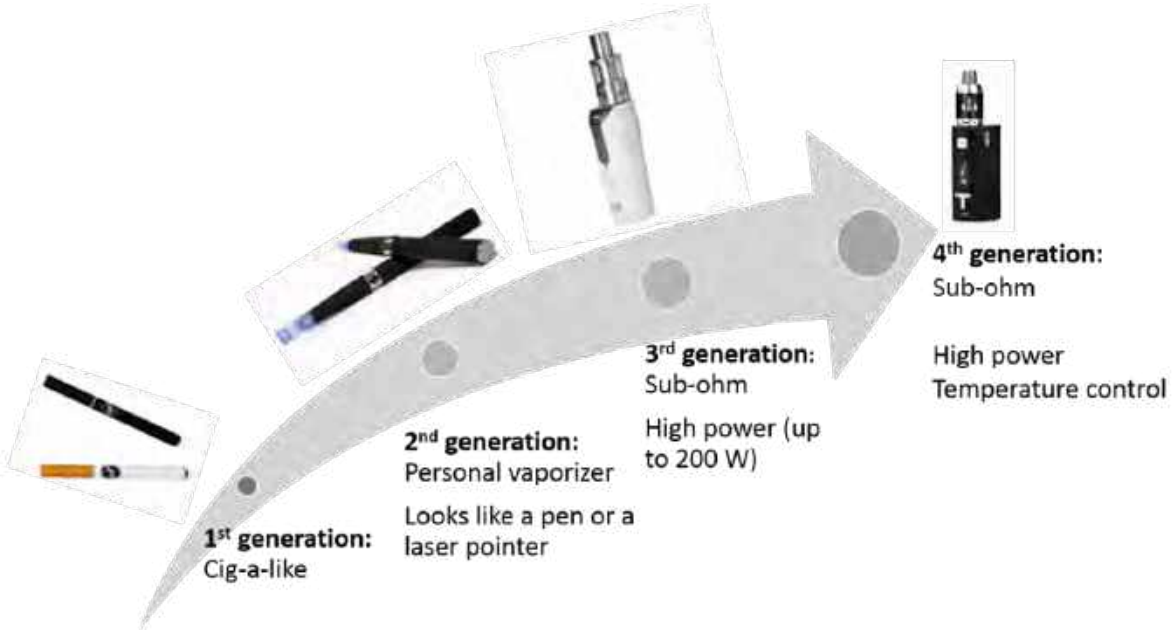

Figure 3. ECIG evolution showing the different generations of devices available in the market.

As ECIG population is increasing dramatically among youth, Juice USB Lighting (JUUL), -a new high-tech generation- was launched to the US market in June 2015 by Pax Labs.[44] JUUL is a flat rectangular ultraportable ECIG device looking like a universal serial bus (USB) flash drive as shown in Figure 4.[44] Unlike other ECIG generations, JUUL is made up of only two components: a USB-rechargeable battery and JUUL pods containing flavored prefilled liquid and heating coil. JUUL pods are considered as a mouthpiece for inhaling aerosols.[44, 45] The pre-filled liquid was found to have a ratio of 30/70 PG/VG with a high nicotine concentration of 60 mg/mL.[45, 46] Talih et al. found that JUUL is a supra-ohm electronic device of 1.6 Ω resistance with a maximum battery power output of 8.1W.[47] Operating at low power and limiting its temperature to 215℃ resulted in generating low levels of carbonyls compared to combustible cigarettes.[47]

Figure 4. JUUL device with JUUL pods.[48]

Nowadays, JUUL is one of the most popular ECIG brands in the US.[45] The annual total sales of ECIG has increased from $775 million in 2015 to $1318 million in 2017. This rapid jump is usually attributed to the rapid growth of JUUL use in the US market.[44] As an evidence, JUUL labs sales increased by 641% from 2016 to 2017 as stated by King et al.[49]. Additionally, JUUL labs sales reached $3.2 million monthly by the end of 2017.[49] This high prevalence of JUUL, especially among youth, is attributed to sleek use and availability in different youth-appealing flavors such as Crème Brulée, Cool Mint, Fruity and many others.[50]

E. Toxicity assessment

Due to the unconditional ECIG prevalence, it entirely caught the attention of scientists and researchers. Their efforts were and still dedicated to assess ECIG safety and toxicity. While vaping, chemical reactions such as thermal degradation and

oxidation might take place.[51]. Thus, ECIG aerosol toxicity is assessed chemically by determining the chemical profile of ECIG aerosols, or biochemically on cell lines and animal studies.

According to the FDA, the main solvents of ECIG liquid: PG and VG are generally recognized as safe food additives but limited in standardized foods.[52] Thus, some reports focused on detecting toxicant contaminants in ECIG liquids. For instance, tobacco-specific nitrosamines (TSNAs) were identified and quantified in ECIG refill liquids by liquid chromatography-mass spectroscopy (LC-MS).[53] TSNAs are considered as strong carcinogens and they are present in tobacco smoke.[53] Besides, benzene, a known human carcinogen, was found in ECIG liquid sample with a quantity higher than the authorized level in pharmaceutical products.[54] Other volatile organic compounds (VOCs) such as toluene, xylene, and ethyl benzene were also detected in the liquids by headspace solid-phase micro extraction and gas chromatography-mass spectrometry (SPME-GC-MS).[54] Researchers suppose that these VOCs result from the usage of hydrocarbon solvents in extracting nicotine or flavors from natural plants.[54] Aldehydes like benzaldehyde and vanillin were detected in some flavored ECIG liquids which may result in respiratory irritation.[55] These contaminants may distill intact to the vapor that will be inhaled by the user.

Furthermore, many researchers identified the presence of toxic and carcinogen chemicals in ECIG aerosols such as carbonyls, metals, reactive oxygen species (ROS), and VOCs resulting from chemical transformations upon ECIG activation.[43, 56, 57] A recent study published in May 2020 mentioned that carbonyls emission depends on: vaping topography, battery power output, ECIG liquid composition, ECIG construction and wick material.[57] The formation of toxic carbonyls increases as the puff duration

and battery power output increase.[41, 56] Also, PG-based ECIG liquids were found to produce more carbonyls compared to VG-based ECIG liquids.[43] Some of these carbonyls like acetaldehyde and formaldehyde are classified as probable human carcinogens according to the U.S. Environmental Protection Agency (EPA).[43] Moreover, different metals were detected in ECIG aerosols such as Cadmium (Cd), Nickel (Ni), and Lead (Pb).[58] These metals were quantified using inductively coupled plasma mass spectrometry (ICP-MS). A study showed that some ECIG aerosols contain Ni and Pb in equal or higher concentrations compared to conventional cigarettes.[59] Long exposure to Ni from ECIGs may cause pulmonary diseases.[60] Besides, some toxic VOCs such as benzene, toluene, isoprene, and acrylonitrile were detected in some ECIG aerosols.[61, 62] Such organic compounds might be detected by thermal desorption and head-space coupled to GC-MS machines.[54] In addition to all these toxicants, ECIGs were shown to produce ROS which might result in DNA damage and cell death in human umbilical vein endothelial cells.[63, 64] All chemicals and toxicants detected in ECIG aerosols are produced from chemical transformations of the ECIG liquid constituents. These chemical transformations include thermal degradation, radical generation/addition reactions, and adduct formation.

In an attempt to complement the literature, this thesis focuses on the quantification of ROS as a generic assessment of ECIG toxicity by measuring the fluorescence intensity on a microplate reader. The influence of the different ECIG operating parameters, like ECIG device design, liquid composition (PG/VG ratio and nicotine concentration), battery power output, user puff topography (puff duration), and inhalation flow rate, on ROS emissions will be assessed. In a first study, we compared ROS emissions from JUUL devices in two different markets: US and UK, to check any

possible differences. In another separate study, ROS was measured for the above conditions in a highly customizable device sub-ohm ECIG. A fluorescent probe was used to trap and semi-quantify ROS on a microplate reader.

In a second approach to assessing the toxicity of ECIG, we tried to establish a direct correlation between flavoring chemicals and aerosol toxicity. Our work focused on the chemical profiling of flavored liquids using non-targeted analysis on GC-MS by applying the simple approach called “Dilute and Shoot” in order to minimize sample preparation as much as possible, and then categorizing the chemical constituents according to their functional groups by using an open-source software. This unique approach helps to identify the reactions that might take place under ECIG realistic conditions, thus allowing the prediction of toxicant emissions. A follow-up work to this project will be to empirically validate the reactivity predictions.

CHAPTER II

REACTIVE OXYGEN SPECIES EMISSIONS IN ECIG AEROSOLS

A. Introduction

1. Background

Reactive Oxygen Species (ROS) is a collective term of a general category of oxygen-based reactive chemical species that includes free radicals such as superoxide radical ($O_2^{\bullet -}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical (•OH), peroxyl radical (ROO•) and alkoxyl radical (RO•), as well as some non-radicals species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl).[65-67] Compared to other ROS species, H_2O_2 is considered relatively stable with lesser reactivity.[64]

The ROS can be generated endogenously by the cells or exogenously by pollutants, heavy metals, and tobacco smoke. Similarly, reported evidence showed that ECIG aerosols could be a source of exogenous ROS emissions.[65, 66, 68] Endogenous generation of ROS is essential for life since these species are involved in different kinds of biological functions such as modulating blood pressure, regulating cellular growth, and controlling immune system.[69] ROS are produced via enzymatic and non-enzymatic processes in mammalian cells.[67] Catalyzed electron transfer reactions and electron transport chain reactions are considered as endogenous sources of ROS formation.[70, 71] Human cells control all the endogenous production forms of ROS. Thus, under ordinary conditions, there is a balance of oxidant levels and antioxidants in the human body. Once ROS levels increase in the body from exogenous sources,

specific scavenging anti-oxidants will be generated.[72] This imbalance will result in oxidative stress that contributes to cell and tissue damage.[68] Besides, oxidative stress was linked to numerous neurological diseases such as Alzheimer's disease, Parkinson's disease, and depression, [73-75] as well as some respiratory diseases like chronic obstructive pulmonary disease and asthma.[76, 77]

Several reports in the literature assessed ROS emissions in ECIG aerosols. In one study, researchers quantified ROS generated from ECIG and combustible cigarettes by acellular fluorescent assay. Consequently, Blu ECIG (second generation) was found to emit similar ROS levels compared to Marlboro and Kentucky combustible cigarettes.[78] Another study detected the presence of ROS in ECIG emissions by two complementary cellular and acellular approaches.[64] This report confirmed that ROS emissions are impacted by ECIG liquid flavors,[64] as tobacco flavor generated less than one third of ROS emitted from fruit flavor. This was justified by the presence of more flavor chemicals in the fruity flavored ECIG liquid that increased the generation of free radicals.[64] This study found that a direct correlation exists between ROS levels and battery voltage output with higher levels of ROS detected at higher voltages.[64] A study from our group assessing ROS emissions from sub-ohm and supra-ohm ECIGs found that power per coil surface area (W/m^2) is a better descriptor of ROS emissions covering all relevant parameters.[79]

ROS can be detected either by measuring total ROS [66] or specific species such as examining hydroxyl and superoxide radicals by spin trapping.[80] In this project we aimed to detect total ROS emissions from sub-ohm ECIG and JUUL.

2. ROS detection methods and probes

Several methods were used to detect ROS emissions from ECIG. In a recent report, an acellular Trolox-based MS method was used to quantify the total ROS. After collecting ECIG emissions using Trolox as a trapping reagent, ROS was measured using liquid chromatography-electrospray ionization-tandem-mass spectrometry (LC-ESI–MS/MS). The same study reported using electron spin resonance (ESR) spin trapping technique to detect short-lived radicals. They are quantified by being transformed to long-lived radicals known as spin adduct after the addition of a spin trap. The two methods were shown to be complimentary and interference-free.[64] Alternative techniques to detect ROS use different types of probe molecules such as spectrophotometric and fluorescent probes.[81] Examples of spectrophotometric probes include 3,5-dibromo-4-nitrosobenzene sulfonic acid (DBNBS), 2-methyl-2-nitrosopropane (MNP), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO). These probes are described with much lower sensitivity compared to fluorescent probes. Indeed, fluorescence method turned to be the most used method for ROS detection. Scoping the literature, several fluorescent probes were used in order to detect ROS like 2′,7′-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA), p-hydroxyphenylacetic acid (POHPAA), dihydrorhodamine 123 (DHR-123), dihydrorthidum (DHE) and many others as mentioned by Zhao and Hopke.[64, 66] A wide range of instruments can be used to measure fluorescence such as: cytometer, fluorimeter, microplate reader or a microscope.[81]

3. $DCFH_2$-DA Probe

Due to its stability, high sensitivity, reproducibility, and selectivity, $DCFH_2$-DA has been commonly used for ROS detection.[66, 82] This probe was introduced in 1965. It was firstly used in cell-free systems for H_2O_2, hydroperoxides and lipid hydroperoxides detection.[83-86] Afterwards, it was widely used to detect ROS produced from intracellular processes.[81] One of its special characteristics is being a positive fluorogenic probe.[81, 87] This means that initially $DCFH_2$-DA is non-fluorescent, however, after being exposed to oxidants, DCF (Dichlorofluorescein, the final product) shows a good fluorescence, unlike ratiometric probes where initial probe is fluorescent not the product (at a given wavelength).[87]

As a first step, $DCFH_2$-DA should be deacetylated as shown in figure 5, either by chemical activation with a strong base such as NaOH, or by a specific esterase.[82] The deacetylated product is called 2',7'-dichlorodihydrofluorescein ($DCFH_2$).[82]

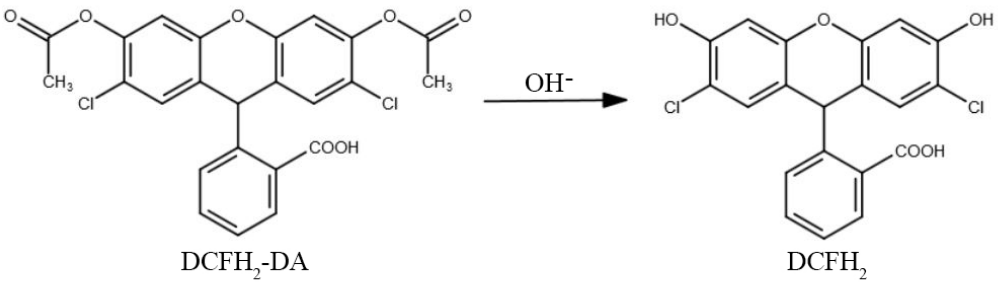

Figure 5. $DCFH_2$-DA Deacytelation.

In the second step, $DCFH_2$ undergoes two consecutive one-electron oxidation reactions to produce fluorescent DCF as shown in Figure 6.[82] Noting that $DCFH_2$-DA and $DCFH_2$ are two non-fluorescent colorless chemicals. In contrast, DCF is

characterized with a yellow color of significant fluorescence where the excitation intensity wavelength ranges between 485-500 nm.[82]

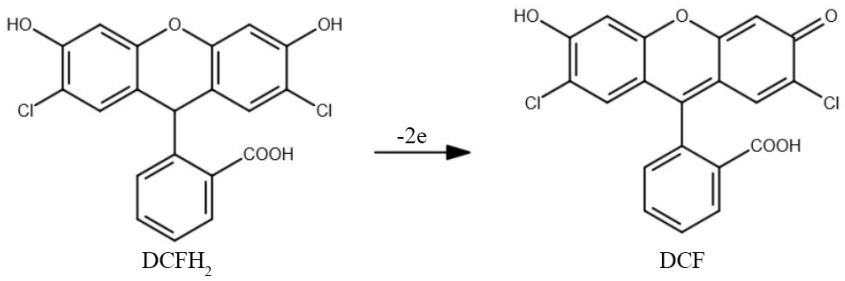

Figure 6. $DCFH_2$ oxidation.

DCF existing form is highly pH dependent. In a pH < 5, the lactonic form of DCF obtained by ring closure dominates. This form is not totally conjugated, thus it's not fluorescent. Figure 7 shows the lactonic structure.[88]

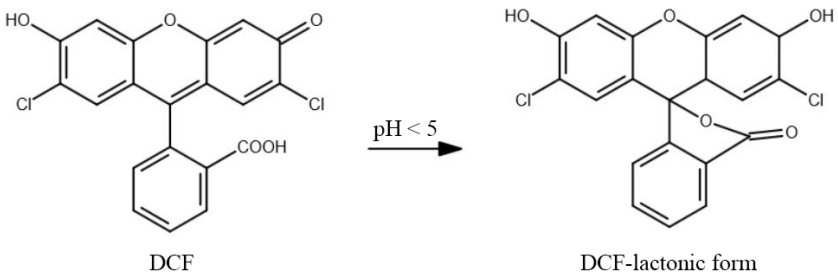

Figure 7. DCF Lactonic form.

However, at basic pH (pH > 5), the dominant form will be an open structure totally conjugated, characterized with a maximum absorption around 500 nm as shown

in Figure 8.[88] In order to maintain a basic pH, a phosphate buffer solution is added.[89]

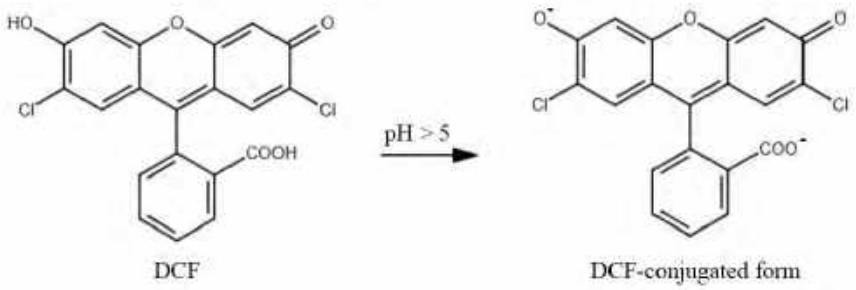

Figure 8. DCF-conjugated form.

In this project, an optimized method in our lab using DCFH-DA probe was used in order to quantify ROS emissions.[79] The method was used as a generic assessment of ECIG toxicity emitted from JUUL and sub-ohm ECIGs in two separate studies as mentioned in the previous chapter.

B. Experimental Work

1. Materials

All the used chemicals such as ethanol, PG (99.5%), VG (99-101%), deionized water (DI), pure nicotine, horseradish peroxidase (HRP) (52 units/mg), potassium phosphate monobasic, and dibasic were acquired from Sigma-Aldrich. Moreover 2',7'-dichlorofluorescin diacetate (DCFH-DA) was procured from Molecular Probes (product code D399). Finally, Quartz filters (ADVENTEC, QR-100.47 mm) were purchased from the Pall Corporation. A Sub-Ohm MiniBox ECIG device (Kangertech), and stainless steel coils were procured from online retailers.

2. Preparation of DCFH Probe Solution

The solution of DCFH-DA (125 μM) was prepared by dissolving DCFH-DA in ethanol. Sodium Hydroxide (NaOH) aqueous solution (0.01 M, 40 mL) was used to deacetylate 10 mL of DCFH-DA solution. Then, it was followed by wrapping the activated DCFH formed solution by aluminum foil and keeping it in pure darkness for 30 minutes. A phosphate buffer solution (200 mL) of pH 7.1, produced by combining both monobasic and dibasic potassium phosphate to obtain a concentration of 0.25 mM, was mixed with 50 mL of the prepared DCFH solution. Then, 2.4 mg of Horseradish Peroxidase (0.5units/mL) were added for the amplification of the fluorescence signal. The final DCFH working solution had a concentration of 5 μM and a volume 250 mL. In order to express ROS equivalents, a linear calibration curve was constructed ranging from 1×10^{-7} to 10^{-6} M using H_2O_2.[79] The calibration curve showed good linearity with $R^2 = 0.9936$. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard deviation (SD) of replicates of a low concentration 0.5×10^{-7} M by applying these two equations: LOD = 3SD/m and LOQ = 10 SD/m, where m stands to the slope of the calibration curve. LOD and LOQ were 0.14 x 10^{-7} M, and 0.48 x 10^{-7} M of H_2O_2 respectively.[79] This method was optimized by Haddad et al. in 2018.[79] They tested different experimental conditions in order to minimize photo-oxidation and auto-oxidation of the probe used, noting that these processes start to occur after deacetylation of $DCFH_2$-DA.[81] The optimized probe solution had the following conditions: concentration of 5 μM, storage temperature of 4℃, and finally mixing time of 30 minutes.

3. Fluorescence Measurement

Fluorescence was read on a SpectraMax M5 microplate reader (96-well multiple plate reader) acting as a fluorimeter, as in Haddad, et al.[79] Excitation and emission wavelengths were 490 and 510 nm, respectively.

4. Aerosol generation and sampling

As shown in Figure 9, JUUL and sub-ohm ECIGs aerosols were generated using the custom-designed aerosol lab vaping instrument (ALVIN).[90] The produced aerosol was trapped on a 47-mm quartz filter. Afterwards, the amount of total particulate matter was measured gravimetrically by weighing the filter pad and holder before and after sampling sessions. The filter was then soaked in DCFH solution and shaked for 30 min for quantitation of ROS on a microplate reader.

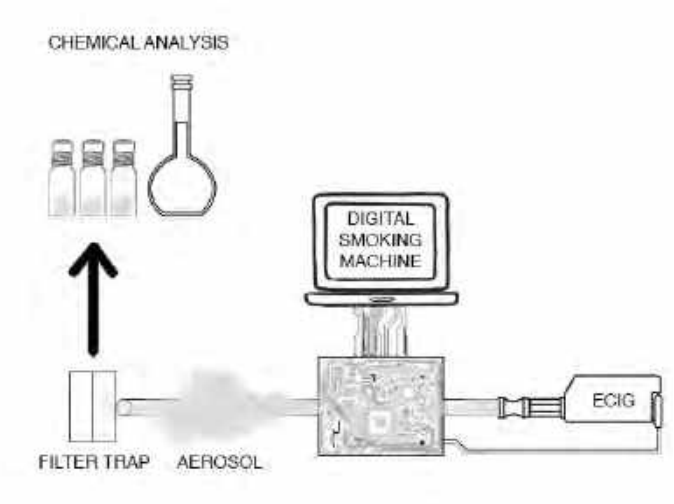

Figure 9. Aerosol generation and sampling.

5. Statistical Analysis

The multiple linear regression analysis in SPSS was applied in order to check if the tested ECIG operating parameters: ECIG device design, liquid composition, battery power output, nicotine concentration, inhalation flow rate, and puff duration had a significant effect on ROS emissions. All results with *p*-values less than 0.05 were significant.

6. Study Design

a. JUUL Study

JUUL was launched in UK market in 2018 with nicotine concentration in ECIG liquids limited to 20 mg/mL to abide with the EU regulations,[91] which is nearly one-third of the nicotine concentration of JUUL in the US. We wanted to assess the design modification that could have been made on the JUUL device between the two markets. This modification is supposed to affect toxicant emissions especially ROS. We compared ROS emissions of tobacco flavored JUUL pods procured in the US and the UK. The used vaping instrument has been automated to generate aerosol based on the following conditions: 15 puffs of 4 sec duration, 10 sec inter-puff interval and 1 L/min flow rate.[46] Analyses were performed in triplicate, using a new pod for each puffing session.

b. Sub-ohm ECIG

In the second ROS study, we analyzed ROS emissions from a sub-ohm ECIG operating at different conditions of power, puff duration, PG/VG ratio, puffing flow rate, and nicotine concentration. We wanted to assess the impact of these operating

parameters on ROS emissions. The conditions of power (15, 30, and 45 W), puff duration (1, 2, and 4 seconds), and PG/VG ratio (100/0, 50/50, and 0/100) were cross-randomized while flow rate and nicotine concentration were kept constant at 8 L/min and 15 mg/mL respectively. A separate set of experiments was designed to study the effect of flow rate (5, 8, and 12 L/min) and nicotine concentration (10, 15, and 30 mg/mL) at the following fixed conditions: PG/VG: 30/70, power: 30W, and puff duration: 4 seconds. The total number of combinations tested was 99. A new coil was used for each combination of conditions, and each combination was tested 3 times. Before sampling, conditioning of the ECIG device was performed by filling the reservoir with liquid and letting it to sit vertically for 1 hour to ensure the saturation of the wick. Conditioning puffs were done with a duration of 4 seconds and a velocity of 16.7mL/s. Before puffing, the remaining liquid in the reservoir was removed and replaced with fresh liquid.[92] Each vaping session consisted of 15 puffs.

C. Results and Discussion

1. JUUL Study

The results showed that JUUL US emitted 1.01 nmol ROS/15 puffs, greater than ROS emitted from JUUL UK (0.13 nmol/15 puffs).[46] These ROS emissions were not significantly different across the two market devices ($p > 0.05$). However, these values were considerably lower than ROS emitted by a single combustible cigarette (36-416 nmol/cigarette).[66, 93] Both devices delivered a voltage of approximately 1.1 V during puffing, and provided a very low power of 0.69 W. [46] This might explain the low levels of ROS emitted.

2. Sub-ohm ECIG

a. Effect of puff duration, power and PG/VG ratio

Table 1 summarizes the data obtained in this study. Results analysis showed that as power and puff duration increase, ROS emissions increase, with no specific pattern observed for PG/VG ratio. To validate these observations, the multiple linear regression analysis in SPSS was applied. As a result, both power and puff duration gave significant *p*-values of 0.013 and 0.003 respectively (significant at $p < 0.05$). Unlike the previous two operating parameters, varying PG/VG ratio did not show any significant effect with *p*-value at 0.217 ($p > 0.05$).

Table 1. Levels of ROS at all conditions of puff duration, PG/VG ratio, power, nicotine concentration and flow rate.

Power (W)	PG/VG ratio	Puff Duration (s)	Nicotine Concentration (mg/mL)	Flow Rate (L/min)	Average of ROS (nmol/15 puffs)	Standard Deviation (nmol/15 puffs)
15	0/100	1	15	8	0.00	0.00
15	0/100	2	15	8	2.15	0.53
15	0/100	4	15	8	2.48	0.26
30	0/100	1	15	8	1.08	0.39
30	0/100	2	15	8	1.13	0.92
30	0/100	4	15	8	0.94	1.61
45	0/100	1	15	8	1.14	0.82
45	0/100	2	15	8	2.78	1.87
45	0/100	4	15	8	30.10	12.77
15	30/70	1	15	8	0.00	0.00
15	30/70	2	15	8	0.26	0.37
15	30/70	4	15	8	1.47	0.58
30	30/70	1	15	8	0.77	0.03
30	30/70	2	15	8	2.47	0.22
30	30/70	4	15	8	4.45	0.92
45	30/70	1	15	8	3.20	0.61

45	30/70	2	15	8	10.85	0.01
45	30/70	4	15	8	81.41	1.83
15	50/50	1	15	8	0.00	0.00
15	50/50	2	15	8	1.11	0.08
30	50/50	1	15	8	1.46	0.36
30	50/50	2	15	8	6.79	1.83
30	50/50	4	15	8	15.12	14.03
45	50/50	1	15	8	2.46	0.41
45	50/50	2	15	8	9.64	3.48
45	50/50	4	15	8	73.90	19.58
30	30/70	4	10	8	21.32	8.65
30	30/70	4	15	8	15.35	2.10
30	30/70	4	30	8	47.62	48.42
30	30/70	4	15	5	16.50	5.13
30	30/70	4	15	8	33.39	11.74
30	30/70	4	15	12	37.35	22.10
Combustible cigarette					36-416	X

The following bar graph is drawn to facilitate the visualization of the 81 cross-randomized conditions. This graph clearly showed that ROS levels emitted from 15 puffs using sub-ohm ECIG can reach combustible cigarette-like levels (36-416 nmol/cigarette). [66, 93] This is achievable at high power (30 and 45 W) and long puff duration (4 seconds).

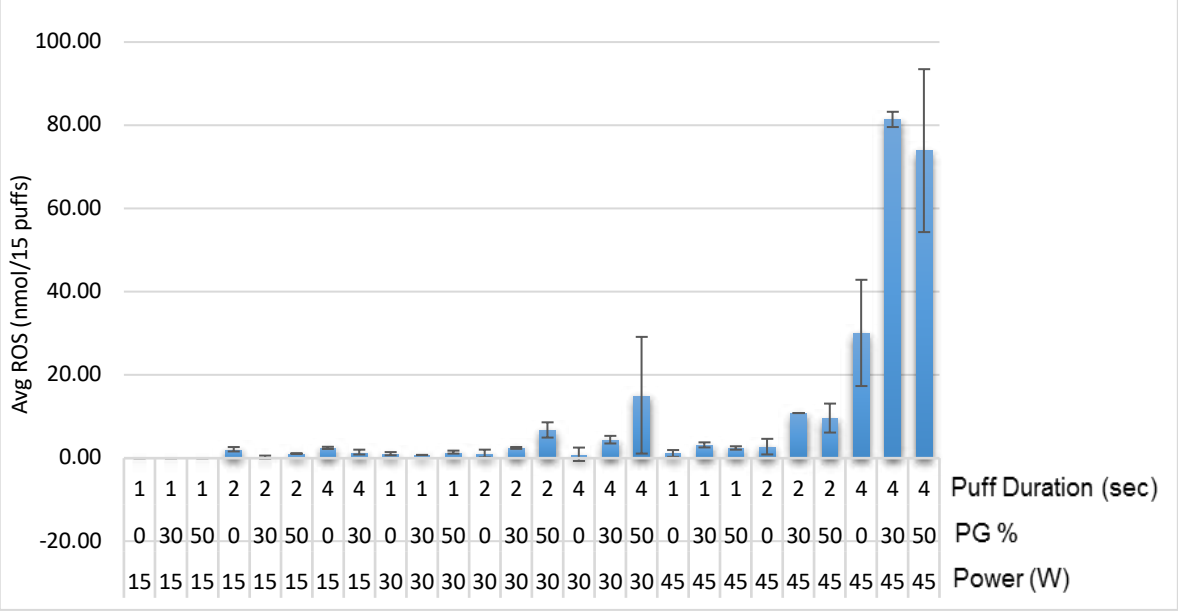

Figure 10. ROS emissions as a function of the randomized conditions of puff duration, power and PG/VG ratio.

The literature showed that wire temperature is directly proportional to battery power. Thus, increasing the battery power output, improves ECIG liquid vaporization, increases ECIG emissions, and consequently drives more chemical reactions to occur including ROS generation.[94] Moreover, as per Talih et al., longer puff duration means longer heating process, i.e., longer reaction time. Hence, longer puffs would possibly result in higher nicotine and other toxicants yields than shorter puffs.[90]

b. Effect of nicotine concentration and flow rate

As mentioned in our study design, the effect of nicotine concentration and flow rate on ROS emissions was assessed by varying only these 2 parameters in two different sets of experiment, while maintaining all other parameters unchanged. Nicotine concentration and flow rate showed no significant correlation with ROS emissions (*p*-values of 0.267 and 0.265 respectively). Results are shown in figures 11 and 12.

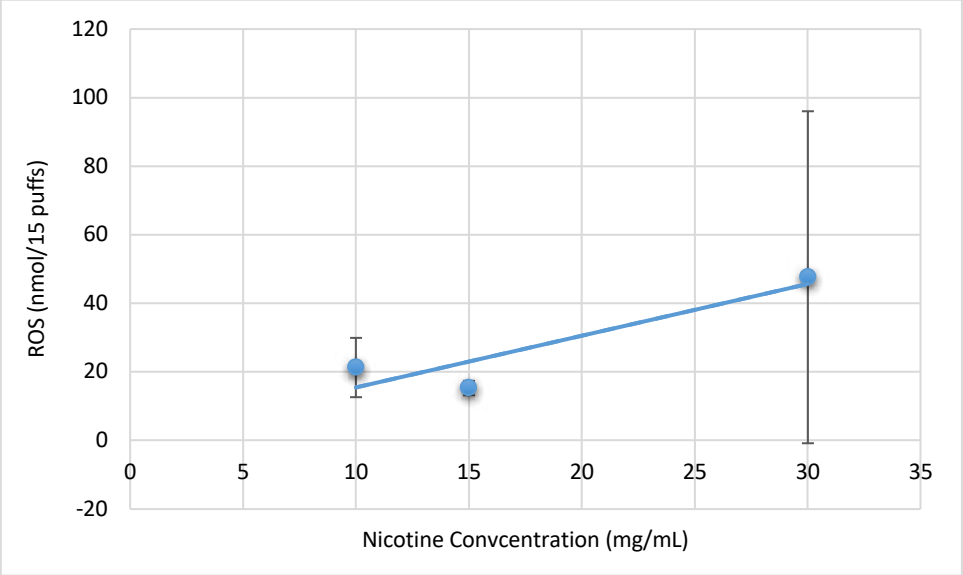

Figure 11. Average of ROS emissions as a function of nicotine concentration.

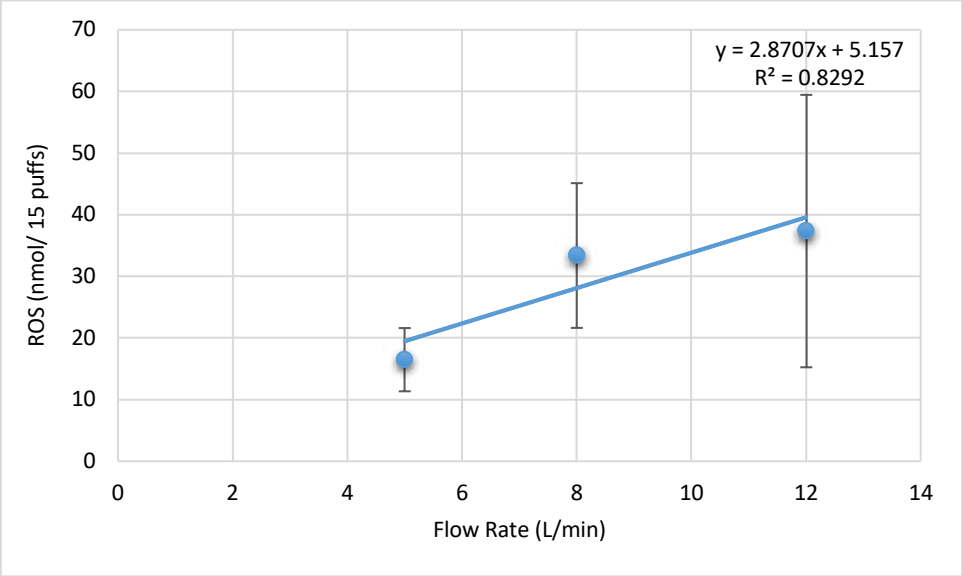

Figure 12. Average of ROS emissions as a function of flow rate.

D. Conclusion

Our results showed that JUUL devices emit very low levels of ROS compared to combustible cigarettes, with no significant difference between JUUL from US and UK markets. For the sub-ohm ECIG, we were able to detect ROS levels in ECIG aerosols even in the absence of flavoring chemicals. ROS emissions were significantly correlated with only two tested parameters out of five: power and puff duration, where nicotine concentration, PG/VG ratio, and flow rate did not show any significant effect on ROS emissions.

CHAPTER III

FLAVORS' CONTRIBUTION TO ECIG TOXICITY

A. Introduction

Most ECIG users say that flavor is a key factor in the initiation and continued use.[95] Flavors are reported to give pleasantness and reward, and perception of ECIG safety, thus prolonging use among smoking-naïve individuals.[96-98] Flavors are also reported to affect smokers' decision to switch to ECIGs,[99] and as such, may assist in reducing tobacco smoking. At the same time, the uncountable flavored liquids on the market pose a daunting public health challenge to regulatory bodies because the toxicity profiles of the growing number of aerosolized liquids are largely unknown.[23, 100]

The contribution of flavors to aerosol toxicity was recognized several years ago.[101] This contribution can be due to toxic ingredients initially present in the flavored liquid, or toxicants formed when the parent liquid is heated and vaporized.[102-104] Some reports in the literature have found that flavors are a dominant source of toxic aldehyde emissions.[105, 106] Others found that flavors affect the emission of radicals and ROS.[107, 108] Only a small number of studies built a direct correlation between specific flavor chemicals and aerosol toxicants.[109-111]

In general, studies that analyze toxicant emissions in ECIG aerosols usually target toxicants commonly found in combustible cigarette smoke.[58] Only one study performed a non-targeted screening of ECIG aerosols to check for other toxicants.[112] In contrast, this screening or chemical profiling is more commonly done on flavored ECIG liquids.[55, 113-116] Knowing the chemical constituents of flavored liquids is of paramount importance as it may give an insight into the toxicity of the generated

aerosols based on the transfer efficiency of these chemicals and their reactivity under ECIG conditions.[104, 117, 118] However, there has never been a guide on what to look for in the generated aerosols. This study proposes a map that can be used to guide researchers to focus future assessments of toxicants in the corresponding aerosols of flavored ECIG liquids. To construct it, we performed a meta-synthesis of the published literature to identify all reported chemical compounds in flavored ECIG liquids. Next, we classified these compounds into chemical classes based on their functional groups (FGs) to predict their possible chemical reactivity upon ECIG activation. Hence, a correlation of flavor chemical reactivity with aerosol toxicant formation could be built, and this is presented in a conceptual framework: FLavor Associated Reactivity Evaluation (FLARE) framework.

In part C of this chapter, we present our work to validate the first step of FLARE, and to confirm the feasibility of ECIG liquid screening methods published in the literature. This included by conducting an experiment on Top 10 flavored ECIG liquids in the US market by applying the "Dilute and Shoot" approach using GC-MS.[115]. Even though the proposed framework FLARE can be applied to any flavored ECIG liquid, applying it to the most popular ones will be more significant.

B. Literature Review

1. Methodology

a. Search Method

A literature search on "PubMed" database with no time restriction was conducted in November 2019, using the following terms: (flavor OR flavour OR flavoring OR flavouring OR flavored OR flavoured OR flavorant OR flavourant) AND

(electronic cigarette OR e-cigarette OR e-cig OR e-liquid OR electronic nicotine delivery system OR ENDS OR vape OR e-hookah). The only limits applied were: published online and in English.

b. Inclusion Criteria

Studies were included in the meta-synthesis if: 1) they reported a general chemical profiling of flavored liquids, and 2) clearly stated the commercial names of these liquids. These names are needed for the graphical representation of the data. To determine eligibility, two reviewers independently examined the title and abstract of each reference from the literature. Studies that did not meet the inclusion criteria upon review of titles and abstracts were excluded. The papers that did meet the study requirements were collected and stored in a shared folder. The two reviewers read all of the texts of the reports included and further removed irrelevant articles. Each reviewer extracted information from the text of the remaining articles, and they cross-validated the data they found.

c. Network representation of the results

To visualize the most common FGs of the identified chemical compounds in ECIG liquids, we used the open-source Gephi software (version 0.9.2). The extracted information from the included articles was compiled in a Microsoft Excel file, which included article title, commercial names of the studied liquids, identified chemical compounds, chemical structures, and determined functional groups. Each liquid, chemical, and the functional group was given a unique code to be used later in the input to the Gephi software. The data were sorted in the following hierarchy: commercial

name of flavored liquid, name of the chemical compound, and chemical class according to FGs. The FGs were determined after careful examination of the chemical structure of the identified compound. These FGs were verified by an online tool (ACE functional group finder).[119] It should be noted that one chemical could have two or more FGs, and thus linked in the *étoile* to more than one chemical class. The three categories were taken as nodes connected by undirected connectors: flavored liquids are linked to chemical compounds, and the latter are linked to chemical classes—the size of a spherical node changes with the number of connections it has to the other nodes.

2. Results

a. Included Studies

After removing duplicates and non-English articles, the search retrieved 868 items. Studies were included in the meta-synthesis if they matched the inclusion criteria. Upon review of the article titles and abstracts, 811 articles were excluded due to irrelevance to the study aims. A review of the full text of the remaining 57 articles removed 46 items that were excluded due to a lack of relevance/failure to meet the inclusion criteria. Thus, a total of 11 articles were included in the meta-synthesis. Figure 13 illustrates the steps that accompanied the selection process.

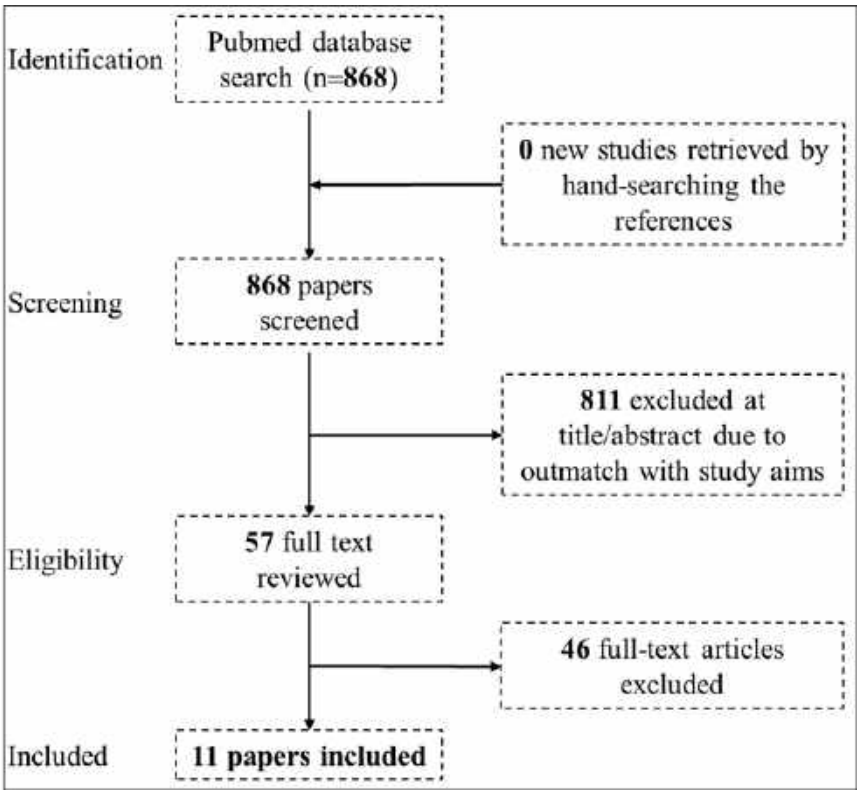

Figure 13. Flow chart showing the process of literature selection.

b. Classification of Flavor Chemicals

The data were extracted from the included 11 articles.[55, 104, 107, 113-115, 117, 120-123] Analysis of the data gave a total of 189 flavored liquids containing some combination of 173 chemical compounds (8.4 ± 9.5 chemical compounds per liquid). All chemicals are shown in the table below sorted in alphabetical order.

Table 2: *Chemical class étoile* chemicals

Chemicals	
1,4-Cineole	Ethyl Salicylate
1-Amylalcohol	Ethyl vanillin
1-Ethenyloxy pentane	Ethyl-methyl-hydroxymethyl amine
1-Pentanol	Eucalyptol
2,3,5,6-Tetramethylpyrazine	Eugenol

2,3,5-Trimethylpyrazine	Fenchol
2,3-Butanedione	Furaneol
2,3-Pentanedione	Furfural
2,5-Dimethylpyrazine	Furfuryl alcohol
2,6-Dimethyl-1,8-naphthyridine	Geranyl propanoate
2,6-Dimethyl-4-propyl pyridine	Guaiacol
2,6-Dimethylpyridine	Hedione
2-Acetylpyridine	Heliotropine
2-Ethoxy-1-(3' pyridyl) ethylene	Hemineurine
2-Isopropyl-4-methylthiazole	Hexanol
2-Isopropyl-5-metylohex-2-enal	Hexyl 2-methylbutyrate
2-Methoxy-4-propylphenol	Hexyl acetate
2-Methylbutyl acetate	Hexyl hexanoate
2-Methylbutyrate	Hydrocoumarin
2-Methylpyrazine	Hydroxyacetone
3-Methyl-1-phenyl-1H-pyrazole	Hydroxydihydrocarvone
3-Methylcoumarin	Isoamyl butyrate
3-Methylcyclopentane-1,2-dione	Isoamyl isovalerate
4-(4-Hydroxyphenyl)butan-2-one	Isoamyl phenylacetate
4,4-Dimethyloxolan-2-one	Isobutyl acetate
4-Methoxy benzaldehyde	Isopentyl acetate
4-Methylacetophenone	Isopentyl alcohol
5-Methylfurfural	Isopulegol
6-Methyl-5-hepten-2-one	Isosafroeugenol
6-Methylcoumarin	Limonene
7-Methylcoumarin	Linalool
Acetaldehyde hexamethylenehydrazone	Linalool oxide
Acetoin	Linalyl acetate
Acetophenone	L-menthyl acetate
Acetylpyrazine	Luminol
Acetylpyrrole	Maltol
Allyl cyclohexanepropionate	Melonal
Allyl hexanoate	Menthol
Amyl acetate	Menthone
Amyl butyrate	Methyl 3-hydroxyhexanoate
Anethole	Methyl anthranilate
Anisyl acetate	Methyl cinnamate
Benzaldehyde	Methyl cyclopentenolone
Benzyl acetate	Methyl heptenone
Benzyl Alcohol	Methyl salicylate

Benzyl Benzoate	Neomenthol
Benzyl butyrate	Nerol
Benzyl cinnamate	Nerol acetate
Benzyl propanoate	p-Anisalaldehyde
Butyl butyrate	p-Cymene
Butyl butyrolactate	Phenenthyl alcohol
Caffeine	Phenenthyl isovalerate
Camfor	Piperitone
Carvone	Piperonal
Cinnamaldehyde	p-Tolualdehyde
Cinnamyl alcohol	Pulegone
Cinnamyl isovalerate	Pyridine
Cis-3-Hexenal	Styralyl acetate
Cis-3-Hexenol	Tetrahydrolinalool
Cis-3-Hexenylacetate	Theaspirane
Cis-3-Hexenylvalerate	Thymol
Cis-Limonene oxide	Trans-2-Hexenol
Citral	Triacetin
Citronellol	Vanillin
Cocal	α,α-Dimethylphenethyl butyrate
Corylone	α-Ionone
Coumarin	α-Pinene
Decanal	α-Terpineol
Diethyl succinate	β-Damascone
Estragole	β-Ionone
Ethanol	β-Myrcene
Ethyl 2-methyl butanoate	β-Pinene
Ethyl 3-methyl-3-phenyloxirane-2-carboxylate	γ-Butyrolactone
Ethyl acetate	γ-Decalactone
Ethyl acetoacetate	γ-Dodecalactone
Ethyl anthranilate	γ-Hexalactone
Ethyl butanoate	γ-Ionone
Ethyl caproate	γ-Nonalactone
Ethyl caprylate	γ-Octalactone
Ethyl cinnamate	γ-Terpinene
Ethyl heptanoate	γ-Undecalactone
Ethyl hexanoate	γ-Valerolactone
Ethyl isovalerate	δ-Decalactone
Ethyl lactate	δ-Dodecalactone
Ethyl maltol	δ-Tetradecalactone

Ethyl phenylacetate	δ-Undecalactone
Ethyl propanoate	

Figure 14 shows the Gephi diagram, or the *chemical class étoile*, of the collected data with the 173 distinct chemical compounds detected. The sphere in the center of the diagram is a virtual sphere that represents the general category of flavored ECIG liquids. Analysis of the structures of these chemical compounds allowed their classification into 22 chemical classes: alcohol, aldehyde, alkene, amide, amine, aryl (or aromatics), diketone, epoxide, ester, ether, furan, hydrazone, imidazole, ketone, lactone, phenol, pyrazine, pyrazole, pyridine, pyrimidine, pyrrole, and thiazole. This representation shows that, in principle, a large number of flavored ECIG liquids and their corresponding chemical ingredients could be reduced to a manageable number of chemical classes. Some of the studies included in this review mentioned the frequency of chemical classes present in their sample set.[55, 104, 113, 123]

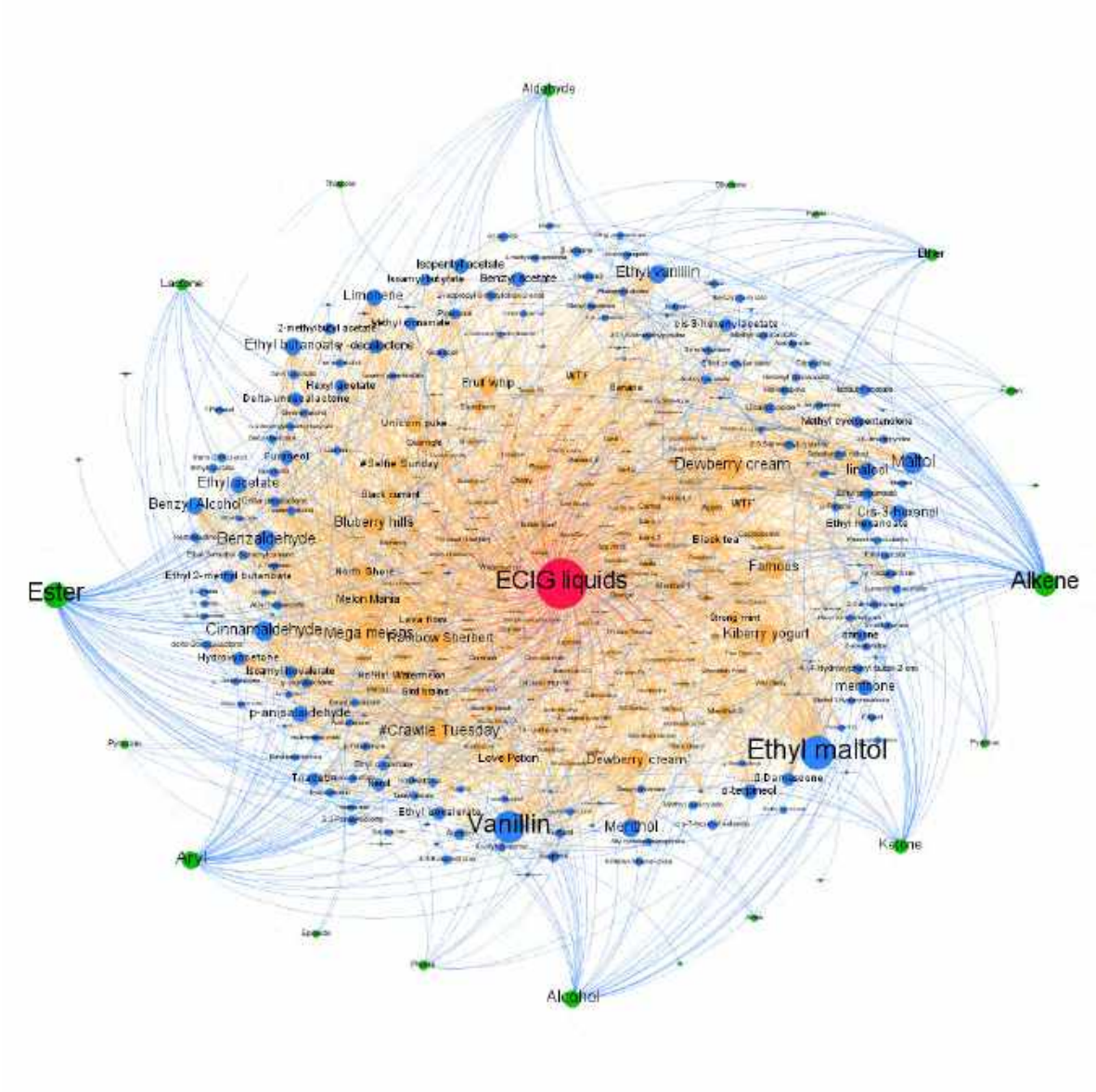

Figure 14. A *chemical class étoile* of 173 chemicals found in 189 flavored liquids and their classification into 22 chemical classes. Color code: red = general category of ECIG liquids, orange = commercial flavored liquid, blue = chemical compound, green = chemical class.

The *étoile* shows that some chemical compounds are common among the tested flavored ECIG liquids, like ethyl maltol (n = 89, 47%), vanillin (n = 69, 37%), menthol (n = 54, 29%), ethyl vanillin (n = 43, 23%), linalool (n = 43, 23%), benzaldehyde (n =

41, 22%), benzyl alcohol (n = 39, 21%), maltol (n = 38, 20%), cinnamaldehyde (n = 37, 20%), ethyl butanoate (n = 35, 19%), and hydroxyacetone (n = 31, 16%). This observation is in agreement with a recent report that analyzed flavor ingredients in ECIG liquids marketed in the Netherlands using the information provided by the manufacturers.[124]

3. Discussion

a. Reactivity of Chemical Classes

The *étoile* shows that ester (n = 58, 33%) and alkene (n = 57, 33%) are the most frequent chemical classes, followed by aryl (n = 41, 23%), alcohol (n = 31, 18%), ketone (n = 25, 14%), aldehyde (n = 17, 10%), and lactone (n = 15, 9%). Considering the ECIG atomizer as a pyrolysis reactor operating under an oxygen-containing atmosphere,[30, 125] the reactions taking place during ECIG use can be classified as oxidation, thermal degradation, radical generation/addition reactions, and adduct formation. Under such conditions, esters are prone to break down into carboxylic acids and small alkenes.[126] Ethyl butanoate, for example, will decompose to give ethylene and butanoic acid. Alkenes may undergo oxidation to give diols, carbonyls or peroxides.[127, 128] The aryl class in our classification includes chemicals that have a benzene ring next to a functional group (except in the case of *p*-cymene). The aryl class comprises chemicals like benzyl alcohol, benzaldehyde, cinnamaldehyde, and other related chemicals that can oxidize to carboxylic acids followed by decarboxylation to give benzene, toluene, ethylbenzene, xylene (BTEX) and styrene.[129] Alcohols under thermal conditions undergo oxidation via radical intermediates to give carbonyl compounds and/or carboxylic acids.[130] Lactones exhibit transformations similar to

esters.[131] Aldehydes can react with ECIG carriers (PG and VG) to give hemiacetals,[132] or oxidize to give carboxylic acids.[133] Ketones can undergo Baeyer-Villiger oxidation to give esters or lactones.[134] In summary, under ECIG described conditions, predominant chemical classes are predicted to produce mainly carbonyls, carboxylic acids, alkenes, and volatile organic compounds like BTEX (Table 3).[105, 135-137]

Table 3: Summary of predicted chemical transformations of flavor chemical compounds identified in the literature.

Esters	--->	carboxylic acids + alkenes
Alkenes	--->	diols + carbonyls + peroxides
Aryls	--->	BTEX + styrene
Alcohols	--->	carbonyls + carboxylic acids
Lactones	--->	carboxylic acids + alkenes
Aldehydes	--->	hemiacetals + carboxylic acids
Ketones	--->	esters + lactones

Other less frequent chemical classes, like epoxide and hydrazine, could be highly reactive under ECIG pyrolysis conditions. Also, the presence of more than one FG in a chemical and the presence of multiple components in the same reaction medium can lead to more than the accounted products through secondary reactions. Moreover, as the current ECIGs contain nicotine salts and have low-pH liquids, this may affect the outcome of the depicted chemical reactions. Nevertheless, predicting the primary

reaction pathways that may take place in flavored ECIG can help guide future research on the assessment of the ECIG toxicant profile. Explicitly, assessing the most frequent and abundant toxicants in the aerosols while taking into consideration the impact of ECIG operating parameters (e.g., power output and puff duration). An antecedent to this approach can be traced back to the pioneering work of Uchiyama on carbonyl emissions from the thermal degradation and oxidation of PG and VG in ECIG liquid.[51, 138]

b. The FLARE Framework

The preceding discussion allowed us to propose FLARE as a conceptual framework that consists of three main steps to facilitate the assessment of toxicants in ECIG emissions (Figure 15). In step I, the chemicals in the flavored liquid are identified. In step II, the identified compounds are classified based on FGs into chemical classes. In step III, the chemical reactivity is predicted, and the possible aerosol toxicants are identified; these toxicants then can be quantified by targeted analysis. Using these three steps reduces the numerous flavor chemicals in a given liquid to a smaller group of chemical classes with a handful of transformations whose products can be predicted and monitored. According to FLARE, chemical compounds in a flavored liquid may distill intact to the aerosol with their contribution to toxicity assessed according to their emission levels and exposure limits. The toxicity of these chemical compounds in ECIG aerosols is determined based on their reported toxicity and the estimated user exposure. For example, benzaldehyde, commonly found in cherry flavored ECIG liquids, is a known irritant to respiratory airways as induced from animal and exposure studies.[103] Or, these chemical compounds contribute to the toxicity of the aerosol by reacting to produce products that are more or less toxic than

their parent compounds.[102, 110] The FLARE framework is a useful tool for researchers to assess the formation of toxicants in ECIG aerosol in a more targeted and efficient manner as compared to existing approaches. Currently, to assess the toxicant profile of the aerosol, one has to either perform a non-targeted analysis (screening) of the aerosol or conduct a multi-targeted analysis of all possible toxicants, usually guided by the tobacco cigarette literature.[112] Conversely, FLARE avoids the drawbacks of the general screening of aerosols, like missing nonvolatile compounds and those with low molecular weights (e.g., carbonyls), and can economize the efforts of targeted analysis as it predicts what analytes to look for. Also, the predicted reactivity in FLARE can inform the design of clinical trials so that they can avoid flavored liquids that are expected to emit highly toxic compounds, and it may aid in the development of specific biomarkers of exposure to certain flavors. In total, FLARE helps in developing knowledge regarding flavor-induced toxic emissions.

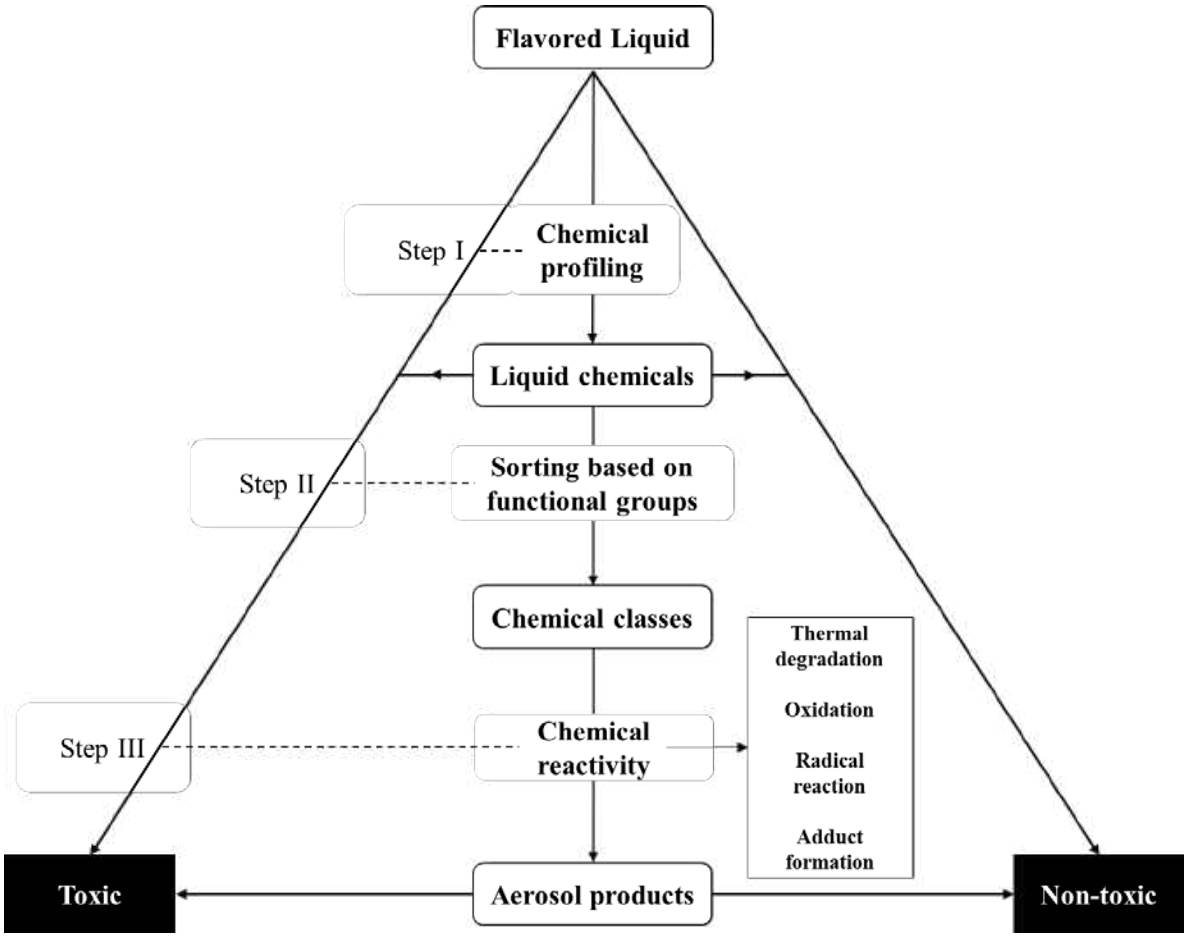

Figure 15. FLavor Associated Reactivity Evaluation (FLARE) framework for predicting and monitoring toxic emissions in ECIG aerosol.

c. Limitations

This work has several limitations that may seemingly restrict its generalizability. Due to the restriction to have commercial names of the flavored liquids clearly stated in the report, necessary for our graphical presentation, we may have missed some important chemicals in the excluded reports. However, the idea behind our approach is not to have a comprehensive list of all identified chemicals, but to build correlations between liquid chemicals and aerosol toxicants. Also, chemicals that have more than one FG may present a challenge to our predictions due to competing

reactivities of the different FGs, or detrimental effect of one FG on the reactivity of the other. This structure-specificity of FG reactivity is to be assessed as we strive to validate our approach empirically. Besides, the influence of ECIG operating parameters on the formation of toxicants should be taken into consideration. To be noted, our reactivity prediction highlights the possible detection of toxicants under specific conditions to be determined experimentally and does not claim that these toxicants are to be found under all conditions.

C. ECIG Liquid Screening

1. Methodology

a. Top 10 ECIG liquids selection

With the help of the Contextual Knowledge Core (CKC) of the Center for the Study of Tobacco Products (CSTP), we constructed and purchased a list of flavored liquids of top 10 brand/flavor combinations among users as shown in table 4. This list was constructed using the following methods: concept mapping, surveillance of online data sources like YouTube, ECIG forums, and ECIG online retail sites to ensure that our information about ECIG is up-to-date (CKC 2019).

Table 4: Top 10 ECIG liquids.

Brand	Flavor
Lemon Twist	Pink Punch Lemonade
Naked	Lava Flow
Naked	Brain freeze
Naked	Hawaiian pog
Keep it 100	Blue Slushie
Cutt wood	Unicorn Milk
Vapetasia	Killer Kustard Lemon
Jam Monster	Strawberry

Sic Boy Black Note	Praline Vanilla Ice Cream Virginia

b. Sample preparation

As per Kubica et al., samples were prepared on the basis of "Dilute and Shoot" approach in order to minimize sample preparation.[115] Thus, 60µL of each liquid were dissolved in 935µL of isopropanol with the addition of 5µL of Quinoline (5 ppm) acting as an internal standard to check the response of the machine. Afterwards, flavored ECIG liquids were directly injected in the GC-MS system. By referring to the NIST library, chemical profile of the top 10 ECIG liquids can be assessed.

2. Results and Discussion

By analyzing the 10 GC-MS spectra, an average of 23 chemicals/liquid was obtained. Of the identified chemicals, 60% were already present in the *chemical class étoile.* As a conclusion, step I of the FLARE framework has been validated to be easily applied based on the "Dilute and Shoot" method.

D. Conclusion

In this project, we constructed a guiding map for researchers to focus their future research of toxicants present in ECIG aerosols. First, we performed a meta-synthesis of the published literature that started with 868 articles to be screened and ended with 11 relevant articles. We then classified the 173 chemicals collected from the relevant articles based to different chemical classes. In order to clearly visualize this classification, we explored the Gephi Software that gave us the opportunity to draw the *chemical class étoile.* Hence, we were able to build a correlation of flavor chemical

reactivity with aerosol toxicant formation. This map was presented in our conceptual framework (FLARE). Finally, we validated the first step of this framework and confirmed the feasibility of ECIG liquid screening methods published in the literature.

CHAPTER IV

CONCLUSION

As a generic assessment of ECIG toxicity, ROS emission was assessed in JUUL and sub-ohm ECIG devices. First, we compared ROS emitted from JUUL US to JUUL UK and the results showed that both versions of the device emit ROS much lower compared to tobacco combustible cigarettes with no significant difference among them. Afterwards, a mini-box sub-ohm ECIG was used to test the effect of different ECIG operating parameters on ROS emissions in the absence of flavors. As a result, ROS emissions were found to be correlated with battery power output and puff duration ($p < 0.05$), unlike nicotine concentration, flow rate, and PG/VG ratio.

In a second approach to assess toxicity from ECIG, we highlighted the importance of categorizing flavor ingredients into a few chemical classes and correlating their chemical reactivity with toxicant formation in the aerosols. This work will be corroborated with empirical validation of some of the predicted reaction pathways of flavor chemicals. For example, the future work will focus on the transformation of alcohol and aryl FGs, under different ECIG operating conditions, to give carbonyl and BTEX emissions, respectively. Our database will be made available online for other researchers to identify aerosol toxicants to be studied and to allow researchers to understand the possible provenances of their analytical discoveries.

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