Toxicological Concerns from Inhaled Food Flavorings Found in Electronic (E-) Cigarette Aerosols

A Report from the Environmental Health Investigations Branch

January 2017

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Keywords: *electronic-cigarettes (e-cigarettes), aldehydes, ketones, inhalation toxicity, diacetyl, 2,3-pentanedione, diketones, 2,4-pentanedione, 2,3-hexanedione, 2,3-heptanedione, cinnamaldehyde, pyrazines, inflammation, respiratory irritation.*

Contents

Glossary of Terms

Aerosol: An airborne collection of dust or mist particles that can be inhaled.

Aldehyde: A class of organic chemicals that often has irritating properties. Some are used as flavors.

Carrier liquid: Chemical used to dissolve and deliver nicotine and flavorings in an e-liquid product.

Cinnamaldehyde: A chemical used in cinnamon flavorings and associated with lung irritation.

Diketone: A chemical class that includes flavoring chemicals such as diacetyl, 2,3-pentanedione, and others.

Dosimetry Model: Dosimetry is the study and practice of measuring or estimating the internal dose of a substance in individuals or a population. Dosimetry models provide a link to understanding the relationship between an external exposure such as a chemical and the biological response. They are used in risk assessment for development of occupational exposure limits for inhaled substances.

E-cigarette: A handheld electronic device that vaporizes or aerosolizes a flavored liquid, which the user inhales.

Electronic smoking device: A generic term encompassing all forms of nicotine or other non-nicotine products that heat a carrier liquid to form an aerosol for inhalation.

E-liquid: The liquid that is vaporized or aerosolized and inhaled when an e-cigarette device is used. E-liquids often include nicotine, but some are non-nicotine.

Glycol: An organic chemical used as carrier liquid, commonly propylene glycol or vegetable glycerin.

Inflammation: The release of proteins that cause immune cells to gather in specific areas, letting off damaging oxidizing chemicals in a defense reaction.

Inflammatory response: The release of proteins that cause immune cells to gather in specific areas, releasing damaging oxidizing chemicals in a defense reaction.

Irritation: Overstimulation of tissue often resulting in inflammation, caused by the breakdown by an external agent or chemical of the mucous membranes that, in the case of e-cigarette smoking, protect the respiratory tract.

Ketone: A class of chemical that can be found in many different flavorings.

MouseRD_{50}: The concentration of chemical required to reduce the mouse respiration rate by 50 percent.

Necrosis: Induced and programmed cell death, which can be triggered by environmental factors.

Parts per million (ppm): Unit of measure for concentrations of chemicals in the air or other matrix.

Particulates: Small, distinct solids suspended in a liquid or gas. They are a major component of air pollution and tobacco smoke. Exposure to inhaled particulate matter is associated with development of respiratory and cardiovascular disease.

List of Acronyms

ADI: Acceptable Daily Intakes.

BO: Bronchiolitis obliterans, a severe life-threatening, non-reversible obstructive lung disease in which the small airway branches, known as bronchioles become damaged and inflamed by chemical particles leading to extensive scarring that blocks the airways.

DA: Diacetyl, a chemical flavoring agent associated with a severe respiratory disease known as bronchiolitis obliterans.

FDA: Food and Drug Administration, a federal agency within the U.S. Health and Human Services Agency which is responsible for protecting and promoting public health through the regulation and supervision of food safety, tobacco products, dietary supplements, prescription and over-the-counter pharmaceutical drugs (medications), vaccines, biopharmaceuticals, blood transfusions, medical devices, electromagnetic radiation emitting devices, cosmetics, animal foods and feed, and veterinary products.

ILD: Interstitial lung disease is a general category that includes many different lung conditions characterized by progressive scarring of the lung tissue between and supporting the air sacs. This scarring may cause progressive lung stiffness, eventually affecting the ability to breathe and get enough oxygen into your bloodstream. Once lung scarring occurs, it is generally irreversible.

JECFA: Joint Expert Committee on Food Additives and Contaminants, an international scientific expert committee administered jointly by the Food and Agriculture Organization of the United Nations and the World Health Organization. It was initially formed to evaluate the safety of food additives. Its work now also includes the evaluation of contaminants, naturally occurring toxicants and residues of veterinary drugs in food.

NIOSH: National Institute for Occupational Safety and Health is part of the U.S. Centers for Disease Control and Prevention and is mandated to assure safe and healthful working conditions and to preserve our human resources.

WHO: World Health Organization, a specialized agency of the United Nations that is concerned with international public that works with governments and nations and other partners to prevent infectious diseases and non-communicable diseases like cancer and heart disease.

µg: (Microgram): unit of mass equal to one billionth of a kilogram, one millionth of a gram, or one thousandth of a milligram.

Executive Summary

Electronic smoking devices (e-cigarettes) often contain food flavorings as part of the e-liquid component that is vaporized and inhaled. While food flavorings have a history of safe use when orally ingested, much less is known about this new route of exposure to flavoring chemicals through inhaled aerosols. This paper describes toxicological data for some food flavorings that are commonly used in commercially available e- liquids.

In the course of our review, we found that some e-cigarette aerosols are inflammatory and irritating to lung cells, in a manner that is unrelated to their nicotine content. Toxicological data clearly show that irritant and inflammatory effects occur with inhalation of different diketones, like diacetyl, 2,3-pentanedione, and chemically related substitutes. These chemicals should not be assumed to be safe for chronic inhalation. Cinnamon flavorings clearly induce pro-inflammatory cytokines in the lungs in animal studies that could lead to respiratory disease upon prolonged exposure in humans. The possibility of respiratory toxicity from chemical mixture interactions exists and should be considered and studied.

Introduction

E-cigarettes are in widespread use throughout California (CDPH, 2015). There is a large variety of device designs, flavoring agents, and nicotine content. A solvent "carrier liquid" such as glycerin or propylene glycol is used to deliver the flavorings and nicotine. Many previous assessments focused on the glycerin or propylene glycol solvents used in e-cigarettes and the production of reactive oxidative products (i.e., chemical pollutants that can cause cell damage) formed by heating the carrier liquid to temperatures in excess of 200 C (Wang et al., 2016). Less information is available about the potential health impacts of chronic inhalation of the food flavoring chemicals that vary across different e-liquids. The World Health Organization (WHO) estimated that over 7,000 flavoring combinations existed in 2014, and that number may be an underestimate today (WHO, 2014). One published estimate placed the number of flavorings at 7,764, and 466 distinct types of electronic devices (Barrington-Trimis et al., 2014). A survey of 30 e-liquids confirmed a variety of chemical flavorings and found that most chemicals were present in the 1-4 percent percent range (a very high concentration equal to 10,000 – 40,000 parts per million or ppm) in the liquid (Tierney et al., 2015).

A primary concern about the widespread application of food flavoring additives in the liquids used in e-cigarettes is that, while approved food flavorings have been deemed "generally regarded as safe" for ingestion by the U.S. Food and Drug Administration (FDA) or the WHO, very few have undergone adequate toxicity or safety testing for acute or chronic inhalation (Costigan and Meredith, 2015). Similarly, a "toxicological threshold of concern" approach, comparing human exposures with doses causing toxicological effects in animal studies, only focused on systemic toxicity and not on respiratory effects (Costigan and Meredith, 2015). Despite assessments that imply that the inhaled flavorings are toxicologically benign (Public Health England, 2015; Farsalinos, 2015), and widespread public claims that e-cigarette aerosols are essentially harmless when compared with conventional cigarette smoke, numerous published studies show quite clearly that e-cigarette aerosols and e-liquids possess the ability to cause respiratory irritation, inflammation, and toxicity to lung cells (Higham et al., 2016; Scheffler et al., 2015). Inflammatory and irritant effects of e-cigarette aerosols *in vitro* appear to be independent of the presence or absence of nicotine, implying that non-nicotine components are responsible (Higham et al., 2016). A recent study found a positive association between

e-cigarette use and incidence of asthma among high school students in South Korea, after adjusting for conventional cigarette use (Cho and Paik, 2016).

A secondary concern about food flavoring chemicals is that the safety assessments that form the basis of the WHO's Joint Expert Committee on Food Additives and Contaminants (JECFA) acceptable daily intakes (ADIs) are almost entirely founded on the assumption that the daily dose of each chemical stems from dietary intake due to its presence in specific foods. These assessments never envisaged that a new route of exposure would arise in the form of inhaled aerosols of these chemicals. Thus, the ADI's calculated by the WHO/JECFA are obsolete for the purposes of determining safety from inhalation of food flavorings via e-cigarettes. Consequently, food flavorings, previously considered benign, may in fact result in both systemic and local (pulmonary) toxicities that are likely to have been, until now, unanticipated and therefore undescribed.

This paper aims to review and summarize what is currently known about the inhalation toxicity of some of the major flavorings found in e-cigarettes, using publicly available databases, including PubMed and Toxnet, to identify peer-reviewed articles on the inhalation toxicity of selected food flavorings reported to be found in e-cigarette liquids. The paper is not intended to be a comprehensive review of all toxicology or epidemiology data on all potential flavoring chemicals. Exposure and risk scenarios have been constructed to allow for a dose comparison with occupational standards and observed toxicological effects in experimental animal models.

Acute Inhalation Toxicity of Food Flavorings

Aldehydes

Many aldehydes have specific fragrances or flavors associated with them, and are used as food additives and in e-liquids used in e-cigarettes (Tierney et al., 2015). Nearly all known aldehydes cause some degree of mucus membrane irritation with resulting inflammation when inhaled at sufficient concentrations. Many aldehyde, alcohol, and ketone flavorings are simple chemical modifications of one another. Other flavorings may have an entirely different chemical structure. For example, Figure 1 shows the similarity of chemical structures for cinnamaldehyde (known to cause lung irritation if inhaled), and raspberry ketone, about which much less is known. Other compounds, such as 2-heptanone, display a different chemistry. Due to similarities in structure, it

is plausible that toxicological effects may be shared across groups of flavorings, allowing for generalizations to be made regarding their potential to cause lung irritation.

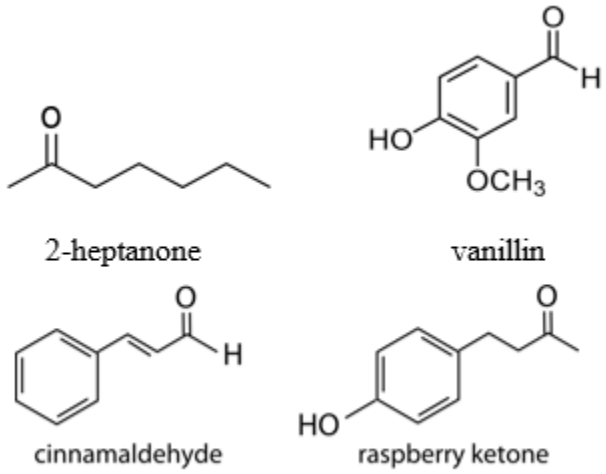

Figure 1. Chemical structures of 2-heptanone, vanillin, cinnamaldehyde and raspberry ketone.

To better understand the relative toxicity of inhaled flavorings, it is necessary to determine their relative irritancy and their ability to cause local tissue inflammation. Human data are sparse, so animal data are relied upon. One approach to characterizing irritancy is the estimation of the mouse RD_{50} (i.e., the concentration of a chemical required to reduce the mouse respiration rate by 50 percent). Steinhagen and Barrow reported on the irritant properties of inhaled food additive aldehydes in mice, using the RD_{50} as a ranking metric (Appendix A, Table A1; Figure 2). This metric of the potency of respiratory irritants has been successfully correlated with irritant thresholds in occupational and general population settings (Alarie 1986; Alarie et al., 1995; Kuwabara et al., 2007). The RD_{50} has been shown to be predictable using common physical parameters ($LogK_{ow}$, and $LogK_{aw}$) for chemicals that otherwise have poor toxicological data sets (ECETOC, 2006; Appendix B). Multiplying the RD_{50} by 0.03 is often used to approximate the threshold for human irritation in occupational settings (ECETOC, 2006; Dalton, 2006). Several flavorings that would qualify as “moderate” irritants (those causing an RD_{50} at less than 1000 ppm) are found in e-cigarette liquids (Tierny et al., 2015). Some strong irritants, including acrolein and formaldehyde, while not added as flavorings, are still formed in e-cigarette aerosols under heating conditions, as described by Chen (2016). The role of specific flavorings in the formation of these aldehydes has not been studied. Acrolein is an example of a

severe respiratory irritant. It was found in trace levels in e-cigarette aerosols, and caused severe respiratory irritation and death in rats exposed to a relatively low concentration of 8 ppm for four hours (Ballantyne et al., 1989).

Figure 2 illustrates the range of irritancy potency among various aldehydes, many of which are used as food additives and many are relatively mild in irritation potency. The most severe irritants are unlikely to be added to e-liquids, but some of those in the moderate category, including benzaldehyde and 2-furaldehyde have been reported to occur (Tierney et al., 2015).

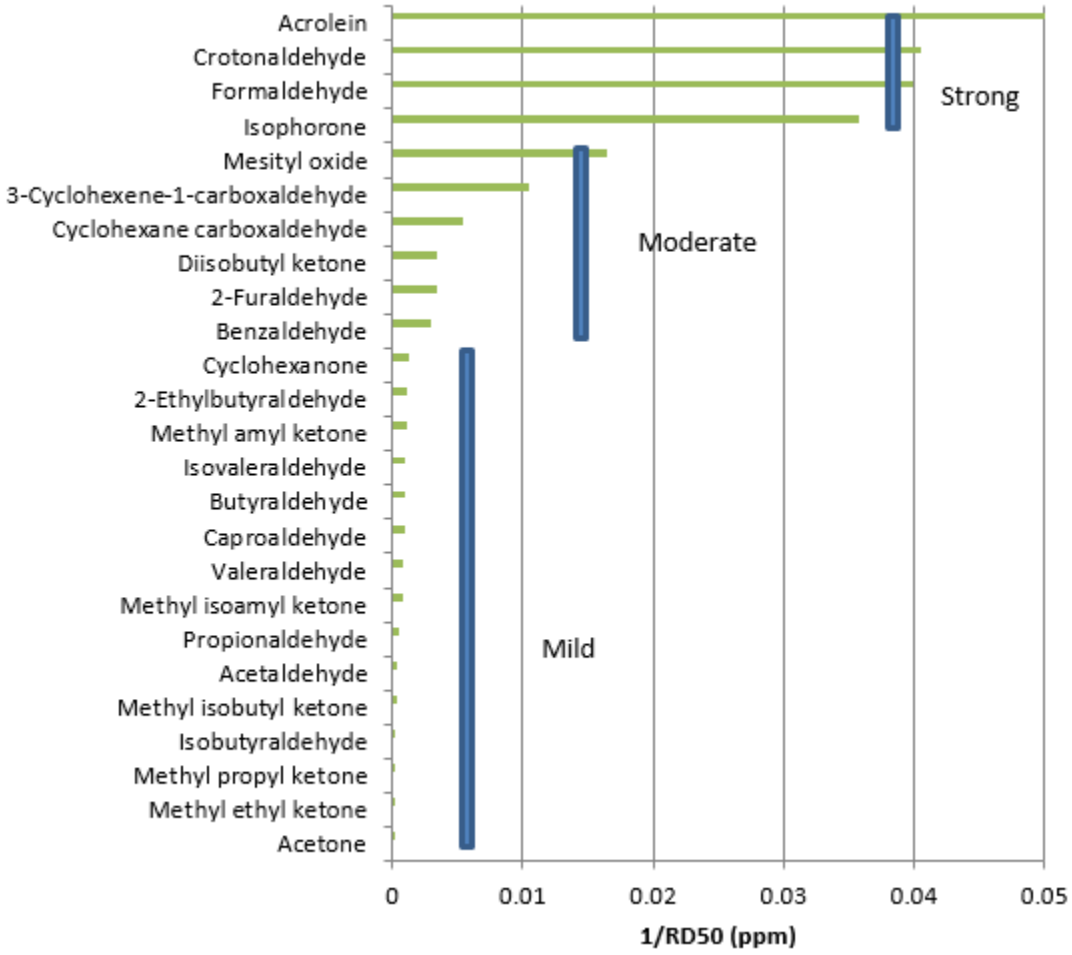

Figure 2. Relative irritancy (RD_{50}) of various aldehydes and ketones used as flavoring chemicals, and potentially occurring in e-cigarette aerosols (data from Steinhagen and Barrow, 1984; DeCeaurriz et al., 1984). Crotonaldehyde, formaldehyde, and acrolein are shown as 1/8 actual value for graphic compatibility. Data expressed as reciprocal of RD_{50} for visualization of potency.

Cinnamaldehyde

Cinnamaldehyde, a chemical used in cinnamon flavorings, is a flavoring aldehyde with irritating properties. The chemicals in Cinnamon Ceylon e-juice were identified and tested for cytotoxicity (i.e., how toxic these chemicals are to cells) (Behar et al., 2014). In their study, Behar and colleagues found that dipropylene glycol (a chemical found in some e-liquids) and vanillin (used in vanilla flavorings) were cytotoxic only at high doses, while cinnamaldehyde and 2-methoxycinnamaldehyde (another cinnamon flavoring agent) were cytotoxic at doses found in e-cigarette refill fluids. The dental literature also has reports of adverse reactions to cinnamaldehyde, and one case report links heavy use of cinnamon-flavored gum to the development of squamous cell carcinoma on the tongue (Westra et al., 1998). In other studies, cinnamaldehyde and 2-methoxycinnamaldehyde inhibited a protein involved in controlling immunity and inflammatory responses (Reddy et al., 2004). A flavor called "Cinnamon-cookies" was similarly found to be among the most cytotoxic e-liquid flavor on heart cells in vitro (Farsalinos et al., 2013).

Behar and colleagues also reported that human embryonic stem cells were sensitive to low concentrations of cinnamaldehyde, suggesting that pregnant women should be cautious using these products (Behar et al., 2014).

Benzaldehyde

Benzaldehyde is another well-known food flavoring chemical, which has some limited inhalation toxicity data available. Laham and colleagues found that 14 days of inhalation of benzaldehyde at 500, 750, or 1000 ppm in rats resulted in clinical neurological signs at all doses, and nasal epithelial metaplasia in males. No threshold for adverse effects was established in their study (Laham et al., 1991). Similarly, the inhalation toxicity of furfural (2-furaldehyde) was studied in rats exposed for 28 days for 6 hours/day, 5 days/week (Arts et al., 2004). While systemic measures of toxicity were seen only at the higher concentrations of 320 and 640 mg/m^3, nasal histological lesions were observed at the lowest dose tested (20 mg/m^3; 5 ppm).
It should be noted that, in addition to some flavorings being themselves aldehydes, one recent study found that flavorings in e-cigarette aerosols can break down to form considerable levels of toxic aldehydes, including formaldehyde, acetaldehyde, acrolein, and glyoxal depending on the concentration and type of flavoring (Kylytov and Samburova, 2017).

Ketones

Diacetyl

The alpha-diketones are a group of substances commonly used as flavoring chemicals in the food industry. Diacetyl, the best known of these, has been associated with a severe respiratory disease, bronchiolitis obliterans (BO), in workers producing popcorn flavoring (NIOSH, 1986). In 2000, eight workers were diagnosed with BO after working in an artificial butter flavoring manufacturing plant, which used diacetyl (Curwin et al., 2015; Van Rooy et al., 2007; Hubbs et al., 2004). Flavoring workers are nearly three times more likely than the general population to have severe airways obstruction (Kim et al., 2010). As a result of its association with this reported respiratory disease, diacetyl has become comparatively well-studied in rats and mice for its inhalation toxicity (Hubbs et al., 2008; Hubbs et al., 2012; Morgan et al., 2008). While the precise mechanisms of action of diketones on the lung epithelium are not completely known, the similar pathologies imply that close structural analogs such as 2,3-pentanedione, 2,3-hexanedione, and 2,3-heptanedione, all food flavorings, may act on the lung in ways similar to diacetyl. Dr. Ann Hubbs, in a presentation to the National Institute of Occupational Safety and Health (NIOSH) described the likely mode of action of diacetyl and 2,3-pentanedione, through formation of localized reactive intermediate metabolites and cross-linking of proteins (NIOSH, 2011).

Inhalation studies in rodents conducted by NIOSH and the National Institute of Environmental Health Sciences indicated similarities in the pulmonary pathology of 2,3-pentanedione and diacetyl (Morgan et al., 2008; Hubbs et al., 2008). Data from these studies suggest that chronic exposure to either 2,3-pentanedione or diacetyl can cause fibrous scarring of lungs in rats. Andersen et al., (2013) found that all four of the diketones (2,3-pentanedione, 2,3-hexanedione, 2,4-hexanedione, and 2,3-heptanedione) caused rapid growth of lymphocytes in the lungs of exposed mice, indicating the potential for development of hypersensitivity and inflammation across this chemical group (Andersen et al., 2013). The precise mechanism for the formation of obstructive lung disease from the diketones is not known.

In toxicological studies of rodents, the term BO has been used to refer to different types of pulmonary fibrosis (a type of irreversible and progressive lung disease). In the study by Hubbs et al., (2012) inhalation of butter flavoring, or vapors of diacetyl alone, caused death of airway

epithelial cells in exposed rats and mice. Damage to airway epithelium was a critical finding as this is believed to be the cause of BO.

An inhalation dosimetry model was developed which showed that diacetyl uptake during short-term exposures was predominantly in the upper airway in rodents, whereas flavorings-related lung disease in workers predominantly affected the deep lung. As rodents are poor models for characterizing the impact of inhaled irritants or particulates in humans due to their different upper respiratory architecture, it may be that humans, breathing through their mouths, would experience higher doses of deposited chemical flavorings deeper in the lung than rats or mice. Farsalinos, (2015) commented on recent data showing diacetyl as a common analyte in e-liquids, concluding that, while there is no reason to include diacetyl or related molecules in e-liquids, the risk posed should be miniscule in comparison with traditional cigarettes, since the concentrations are far lower in e-cigarette vapor than in cigarette smoke (Farsalinos, 2015). However, while BO is not commonly seen in smokers, there is evidence that diacetyl, which is added to some tobacco and occurs in tobacco smoke, may contribute to a more generalized respiratory bronchiolitis seen in many smokers (Egilman and Schilling, 2014). Fourteen commercial cigarette brands and one reference cigarette released 301–433 μg of diacetyl per cigarette (Egilman and Schilling, 2014). Peak exposures to smokers were not measured or estimated, but almost all smokers have been found to have some degree of respiratory bronchiolitis interstitial lung disease, and some develop other interstitial lung disease as well. Smoking enhances expression of an enzyme that catalyzes the conversion of arginine to citrulline in the bronchial mucosa and alveolar region, generating citrullinated proteins, which in turn can generate antibodies that can cause inflammation and local tissue damage.

NIOSH researchers found peak exposures of 4 and 13 million molecules of diacetyl per cubic foot in the breathing zone of quality control workers while they were opening freshly popped microwave popcorn (Egilman and Schilling, 2014). Diacetyl, inhaled as a result of exposure to microwave popcorn flavoring, tobacco smoke or e-cigarette vapor, may enhance formation of citrulline antigens that result in antibody production and thus play a role in the pathogenesis of interstitial lung disease and bronchiolitis. However, this might only occur in people who are genetically predisposed to these effects.

Figure 3 shows that diacetyl concentrations in e-cigarette liquid, as reported by Allen et al., (2015) result in adjusted one-hour average air concentrations that form a range from below

occupational standards to significantly above occupational standards. The range of diacetyl is more likely to be a dichotomous rather than a continuous variable due to the fact that it is not a necessary component of flavorings that are not buttery by nature. A risk assessment scenario is presented at the end of this paper to illustrate the potential for high doses and concentrations of diacetyl.

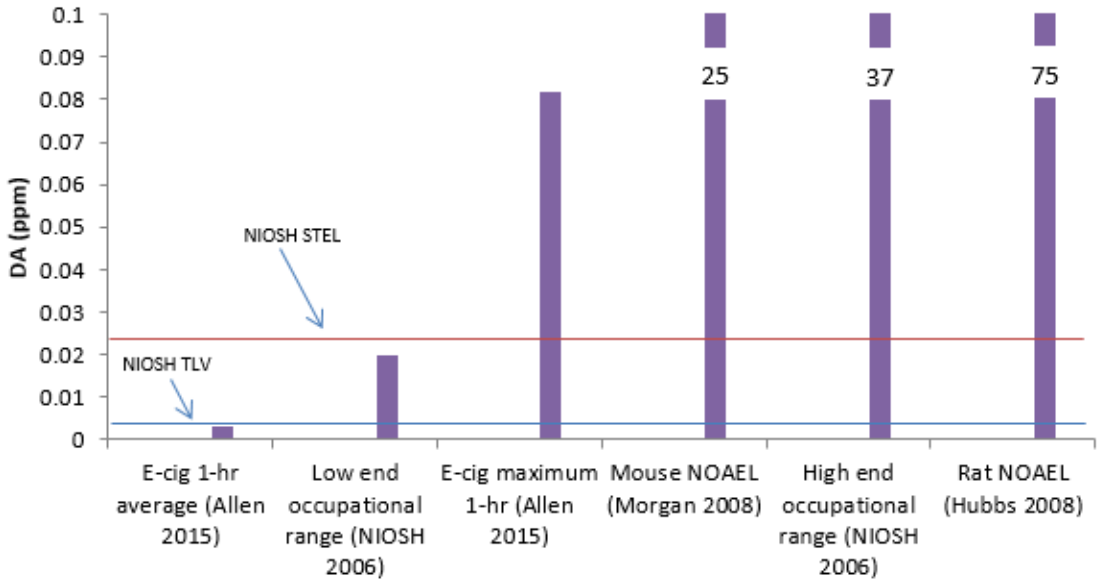

Figure 3. Comparison of Reported Diacetyl Concentrations and Workplace Standards

For diacetyl, computational physiologically-based pharmacokinetic models have been developed that indicate that lightly exercising workers have much higher concentrations of diacetyl in bronchioles than do rats. Table 1 shows that, as with diacetyl, 2,3-pentanedione exerts respiratory epithelial cytotoxicity in the nasal region (T2), with somewhat less impact in the tracheal region (T3) (Hubbs et al., 2012). The deeper lung region (T4) was largely unaffected in rats.

Table 1. Nasal pathology (necrotizing rhinitis) in rats inhaling 2,3-pentanedione or diacetyl

Exposure	Concentration (ppm)	T2	T3	T4
Air	NA	0 (0/9)	0 (0/9)	0 (0/9)
Diacetyl	240.3	2.33 (3/6)	2.67 (3/6)	0 (0/6)
2,3-Pentanedione	111.7	0 (0/6)	0 (0/6)	0 (0/6)
	241.2	4.00 (5/6)	0 (0/5)	0 (0/6)
	318.4	6.17 (6/6)	1.60 (1/5)	0.83 (1/6)
	354.2	5.50 (6/6)	4.17 (4/6)	0 (0/6)

From Hubbs et al., 2012

In their study, Hubbs and colleagues (Hubbs et al., 2012) found respiratory toxicity, olfactory neurotoxicity, and central neurotoxicity for diketone flavoring agents classified as "generally recognized as safe" under conditions of normal use when consumed in food. This study, as with previous diacetyl studies, is a reminder that a chemical with a long history of being eaten without any evidence of toxicity can still be found to be harmful to the respiratory tract. Their study suggests that shared chemical structural similarities of the smaller diketones may be related to their toxicity when inhaled. The direct effect of the diketone flavoring agents and the ability of the diketones to modify proteins and nucleic acids are features consistent with the direct cytotoxicity of diacetyl and 2,3-pentanedione. This study also provides insights into the role of metabolism in the pathogenesis of injury to the olfactory neuroepithelium and brain of 2,3-pentanedione -exposed rats. Selective toxicity of 2,3-pentanedione was reported to occur to olfactory neurons in the neuroepithelium (Hubbs et al., 2012).

Morgan and colleagues reported inflammatory responses in the lungs of mice even at the lowest concentration of DA tested (Morgan et al., 2008, Table 2). Occupational studies have shown that excessive exposure to diacetyl in manufacturing settings is associated with impaired lung function (Lockey et al., 2008) (Table 3). In their study, lung function deficits prior to the use of personal protection (respirator "Pre-PAPR") were much more significant than that accounted for by smoking.

Table 2. Mouse Diacetyl Inhalation Toxicity Study Lung Pathology

Lesion	Control	31.7 ppm	63.4 ppm	126.8 ppm
Peribronchial Lymphocytic Inflammation (at 6 weeks)	0/5[1]	**3/5**	**5/5**	**5/5**
Bronchial Epithelial Atrophy (at 6 weeks)	0/5	0/5	1/5	5/5
Bronchial Epithelial Regeneration (at 6 weeks)	0/5	0/5	0/5	5/5
Peribronchiolar Lymphocytic Inflammation (at 6 weeks)	2/5	0/5	1/5	3/5
Peribronchial Lymphocytic Inflammation (at 12 weeks)	0/5	**2/5**	**4/5**	**5/5**
Bronchial Epithelial Atrophy (at 12 weeks)	0/5	0/5	0/5	5/5
Bronchial Epithelial Regeneration (at 12 weeks)	0/5	0/5	0/5	5/5
Peribronchiolar Lymphocytic Inflammation (at 12 weeks)	0/5	0/5	0/5	3/5

[1] fractions show number of animals affected/number of animals examined

Table 3. Odds ratios of Impaired Lung Function Results in Diacetyl Manufacturing Workers (n = 384) (Lockey et al., 2008)

Variable	Odds Ratio	95 percent CI
Pack Years	1.6	1.3-2.0
Current Smoker	1.4	0.5-4.0
BMI	1.0	0.9-1.1
Pre-PAPR mixer	8.2	2.3-30.0
PAPR mixer	3.2	0.6-18.7

All concentrations of diacetyl and 2,3-pentanedione above 60 ppm are toxic to cultured cells (Zaccone et al., 2015). At 25 ppm, exposure to these flavoring chemicals did not result in cell death; however cellular sodium transport was reduced immediately after a six-hour exposure. Thus, reductions in sodium ion transport may be a component of the BO lung disease caused by inhalation of the diketone family of flavorings.

2,3-Pentanedione

Due to emerging concerns about diacetyl, manufacturers of foods and e-cigarettes are now, in some cases, producing and working with alternative flavors using alpha-diketone substitutes such as: 2,3-pentanedione, 2,3-hexanedione, and 2,3-heptanedione (Curwin et al., 2015).
According to Morgan et al., (2012), consumption of the low levels of 2,3-pentanedione typically present in food products has not been reported to cause adverse health effects. However, workers in the food and flavoring industries may be exposed to potentially toxic concentrations of 2,3-pentanedione vapors. Currently, there are no occupational exposure limits for 2,3-pentanedione.
Male and female Wistar-Han rats and B6C3F1 mice were exposed to 0, 50, 100, or 200 ppm 2,3-pentanedione, for six hours/day, five days/week for up to two weeks (Morgan et al., 2008). Lung inflammation was measured after 1, 3, 5, and 10 exposures, and tissues were examined after 12 exposures. Various inflammatory markers were increased 2- to 9-fold in rats exposed for 5 and 10 days to 200 ppm. In mice, the response was less than in rats, with only fibrinogen increased after five exposures to 200 ppm. The epithelium lining the respiratory tract was the site of toxicity in all mice and rats exposed to 200 ppm. Significantly, 2,3-pentanedione also caused fibrotic airway lesions in rats. These changes observed in rats raise concerns that 2,3-pentanedione inhalation may cause BO in exposed humans.
Morgan and colleagues hypothesized that the development of bronchial fibrosis in rats was probably the result of necrosis of both the bronchial epithelium and the underlying basement membrane upon direct contact with 2,3-pentanedione, thereby exposing and activating the connective tissue and leading to a fibroblastic (scar tissue forming) response.
In a more recent study on 2,3-pentanedione in rats, fibrotic lesions in the lung following 200 ppm 2,3-pentanedione exposure for six hours/day, five days/week for two weeks, were found to increase expression of genes linked to inflammatory responses (Morgan et al., 2015). In their study, all five rats treated with 2,3-pentanedione had bronchial inflammation and fibrosis.
Rats inhaling 2,3-pentanedione developed inflammation in the nasal, tracheal, and bronchial regions, comparable to diacetyl-induced injury. To investigate delayed toxicity, additional rats inhaled 318 ppm 2,3-pentanedione for six hours which resulted in cell death in the respiratory epithelial and loss of olfactory neurons. Cellular damage continued to progress 12 to 14 hours after exposure. An additional group of rats inhaling 270 ppm 2,3-pentanedione for six hours,

showed increased expression of IL-6, and nitric oxide synthase-2 enzyme and decreased expression of vascular endothelial growth factor A in the brain. The authors concluded that 2,3-pentanedione is a respiratory hazard that can also alter inflammation and gene expression in the brain (Morgan et al., 2015).

In the study by Hubbs et al., (2012), it was found that short-term 2,3-pentanedione inhalation in rats has respiratory toxicity that is comparable to diacetyl. However, it also causes cell death in the olfactory neuroepithelium (where our sense of smell is located), olfactory nerves, and upregulation of enzymes and molecules involved in inflammation, including brain IL-6, inducible nitric oxide synthase (Nos)-2, and claudin-1 transcripts.

In the study published by Behar et al., 2014, recent *in vitro* studies of cytotoxicity suggest that e-liquid products differ in their potential to adversely affect health. In a prior *in vitro* screen, e-liquids varied widely in their cytotoxicity when tested with human embryonic stem cells (hESC), mouse neural stem cells, and human lung cells (Bahl et al., 2012). The stem cells were generally more sensitive to e-liquids than differentiated adult lung cells. The same study also showed that the flavoring chemicals and their concentrations varied among e-liquids of the same flavor both within and between manufacturers. In addition, the cytotoxicity of e-liquids correlated with the number and concentration of chemicals used for flavoring.

It is worth noting that a number of flavoring chemicals have skin sensitizing (allergenic) potential (Table 4). Although the relationship between skin allergenicity and respiratory allergenicity is not well established, it remains possible that some skin sensitizing compounds could induce respiratory allergy via an atopic mechanism.

Table 4. Some commonly found alcohols used as flavorings, and irritant/sensitizing properties.

Compound	Respiratory irritant	Skin sensitizer	Reference
Anis alcohol	++	Yes	Fisher Scientific MSDS
Eugenol	+++	Yes	Fisher Scientific MSDS
Linalool	+	Yes	Sigma Aldrich MSDS
Coumarin	+	Yes	Vigon MSDS

Table 5 shows an exposure scenario for someone using an e-cigarette with 300 puffs/day, and a total volume of 5 mL/day of an e-liquid containing diacetyl. Similar assessments can be

constructed using any flavoring chemical, provided that occupational limits and ADIs are available.

Table 5. Risk Characterization for Diacetyl Exposure

Parameter	Value	Reference
Inhalation Exposure vs Occupational Standards		
Puffs/day	300	Online Vaping Websites
Inhalation rate (m^3/day)	20	Cal/EPA 2006
Diacetyl in liquid (percent)	1-4*	Tierney et al., 2014
E-liquid consumed (mL/day)	5	Online vaping websites
Diacetyl available for consumption (mg/d)	up to 200	Assuming 4 percent Diacetyl
Hours of use/day	10	Assumed – no data
Puffs/hour	30	Averaged from puffs/day
mg/hour	20	Averaged
15 min equivalent (TWA) mg/m^3	48 (13.3 ppm)	Assuming half of time not vaping
8 hr equivalent (TWA) mg/m^3	24 (6.7 ppm)	Averaged over the day
Difference from NIOSH values (25 ppb – 15 min)	532x	Assuming 100 percent DA vaped is absorbed
Difference from NIOSH values (5 ppb – 8 hr)	1340x	Assuming 100 percent DA vaped is absorbed
Systemic dose vs ADI		
Absorption percent	100	Assumed worst case
Body weight (kg)	60	Cal/EPA
Daily dose (mg/kg/d)	3.3	Calculated
percent ADI (0.9 mg/kg/d)	367	Clark and Winter, 2015

- Applies only if diacetyl is used as a flavoring chemical in the e-liquid

The risk characterization above for diacetyl uses assumptions of diacetyl concentration that may be greater than typically encountered. The assessment assumes the e-liquid product contains diacetyl by design as a principal flavoring chemical and demonstrates that under these conditions, the amount of exposure to diacetyl greatly exceeds occupational standards for short term or eight-hour durations. Even if the absorption of diacetyl is 10 percent and the diacetyl concentration in the e-liquid is 1 percent, the excess exposure over the NIOSH eight-hour TWA is still 33-fold. The exposure assessment is illustrative of the potentially high doses and concentrations that can be experienced when using e-cigarettes. This carries implications for not

only local respiratory tract inflammatory responses, but also systemic doses of chemicals that were not originally envisioned to be inhaled to such an extent.

Pyrazines

The pyrazines are a large family of chemicals found naturally in bell peppers, and synthesized derivatives are added as flavorings. There is very little inhalation data on these compounds that would allow an estimation of the respiratory irritancy. A recent study found that respiratory epithelium cellular characteristics were altered by the chocolate flavoring chemical 2,5-dimethylpyrazine in mouse tracheal cells through a specific receptor protein (Sherwood and Boitano, 2016). The authors of this study suggested that long-term effects of this chemical exposure could include suppression of immune mechanisms through reduced mucous clearance of the airways. The olfactory epithelium appears to possess a specific protein receptor that binds pyrazines found in rodents and bovines (Pevsner et al., 1985). The purpose of this receptor is not well understood. More information is needed to understand the possible respiratory effects of pyrazines.

Consideration of Chemical Mixtures

Toxicology data is usually available for individual chemicals when submitted by chemical manufacturers to governmental regulatory agencies for product registration and evaluation (e.g., pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), other chemicals under the Toxic Substances Control Act (TSCA), and for health effects tracking (e.g., chemical toxicology test results under Section E of TSCA). These data are maintained by the U.S. Environmental Protection Agency (EPA) at the federal level and in some states (for example, California Department of Pesticide Regulation also requires data submission by pesticide manufacturers applying for product registration in California). The data submitted by chemical manufacturers are primarily the results from toxicity testing in laboratory animals and other assays conducted in accordance with guidelines published by EPA.

People are exposed to combinations of multiple chemicals from a variety of sources such as the workplace, food and drinking water, consumer and household products, personal care products,

cigarette smoke, e-cigarettes, pharmaceuticals, and others (De Rosa et al., 2004). Such exposures will likely result in concurrent and/or sequential exposure to more than one chemical on a regular basis. The assessment of the health implications of multiple chemical exposures is further complicated by the fact that the patterns of exposure in everyday life are dramatically different from those typically studied in toxicological testing and research studies. Not only are humans subject to chemical exposures but the chemicals *enter* the body through multiple pathways. Analyses of people's urine and blood from national and local biomonitoring efforts show that nearly 100 percent of the people tested have measurable chemicals, such as pesticides, in their bodies that are either transient (eliminated relative quickly) or persistent (stored for longer periods in body fat) (CDC, 2009). There is no debate that people are exposed to mixtures of chemicals from the environment and in consumer products, and therefore it is reasonable to conclude that consumers are also exposed to a significant number of combinations of chemicals emitted from e-cigarettes.

Despite this knowledge, the vast majority of experimental toxicology data on chemicals used in commerce are for a single chemical only and not for a combination of chemicals. In other words, generally, only one chemical at a time is tested for toxicity in any study. In an attempt to compensate for the limitations in single chemical toxicity testing, government agencies have focused on understanding the mechanisms of action and interaction of the components of the mixture in an attempt to combine assessment efforts for structurally related chemicals. Paradoxically, epidemiological studies generally involve exposures to complex mixtures or exposures to chemicals from several sources and therefore it is often difficult to ascertain the association of risk for adverse health outcomes from any single chemical based on such studies. Individual chemicals can possess comparable mechanisms of causing toxicity and therefore exposures to mixtures of these "like" chemicals present a greater potential of harm than an individual chemical acting alone. There is evidence that one chemical in a mixture can modulate certain toxicities of another chemical in the same mixture (Cedergreen 2014; COT 2002; Seed *et al*., 1995). For example, some chemical carcinogens require metabolism to be activated and there are many chemicals known to promote this activation in animals and humans, even if these "promoters" are not carcinogens themselves. Cigarette smoke is a classic example of a highly complex mixture of both carcinogens and tumor promoters, with combined cancer causing potency higher than that expected from adding individual chemicals together (Fowles and

Dybing, 2003). Asbestos exposure along with exposure to cigarette smoke further enhances the lung cancer causing potency of each other in humans. When combined exposures occur, the relative risk for lung cancer increases by more than the sum of the two carcinogens alone, by as much as ten times (IARC 2004). Therefore, two toxic chemicals acting in combination might increase toxicity in an additive (1 + 1 = 2), synergistic (1 + 1 > 2), or antagonistic manner (1 + 1 < 2). Another type of interaction occurs when an effect of one substance is increased by exposure to a second substance, even though the second substance does not cause that effect by itself; this is called potentiation (CDPH, 2008). There may also be no change in the magnitude or type of toxic effect(s) observed when two or more chemicals are mixed together.

The outcome of mixing chemicals together is difficult to predict with the limitations in capability and throughput of the currently available toxicity testing methods. The lack of information and knowledge about the behavior and toxicity of chemical mixtures in humans is extremely important to acknowledge. Based on the reasons stated above, in the absence of data showing no change in effect or an antagonistic effect, health risk assessments based only on single chemical exposures when multiple chemicals are present, especially chemicals with similar mechanisms of toxicity and toxicity traits (for example aldehydes) are likely going to underestimate health risks to an individual or population.

Some health risk assessments have used rudimentary approaches to account for additive effects of chemicals with similar toxicity traits, or in a more refined way, by adding the effects of chemicals with similar mechanisms for causing toxicity (for example, organophosphorus insecticides that inhibit red blood cell cholinesterase activity) (EPA, 2006). EPA has developed toxicity equivalence factors (TEFs) for structurally related chemicals such as the chlorinated dioxins and furans (EPA, 2010). To our knowledge, EPA has not developed cumulative risk assessment guidelines or TEFs for local irritant or systemic effects of VOCs and other chemical constituents of e-cigarette aerosols.

As discussed previously, aldehydes constitute a group of highly biologically reactive organic compounds. Human exposure to aldehydes primarily occurs from environmental sources such as polluted air, consumer products, and tobacco smoke. All aldehydes include a carbonyl functional group, and are sub-grouped according to other structural similarities such as the length of the carbon chain and saturation of the carbon atoms. Under physiological conditions, aldehydes are

highly reactive with the amino groups of proteins and the nucleophilic portions of DNA and RNA forming covalent adducts in these cellular macromolecules in living organisms (LoPachin and Gavin 2014; O'Brien *et al*., 2005; Fantl *et al*., 1982). Because aldehydes appear to share a common mechanism of toxicity, individual aldehyde chemicals in a mixture can interact, either additively or synergistically, to produce or enhance toxicity greater than the single aldehyde chemical exposure (EPA, 2008). For example, *in vitro* studies with human and rat nasal epithelial cells demonstrated that the combined toxicity of formaldehyde and acrolein was additive (Cassee *et al*., 1996).

With respect to the toxicity traits of aldehydes, the most prominent effect is respiratory irritation, which could exacerbate pre-existing asthma or other respiratory illness in consumers. All e-cigarette liquids have the ability to form multiple aldehydes. The simultaneous exposure of humans to acetaldehyde and other upper-respiratory-tract toxicants, such as acrolein, formaldehyde, and ozone can lead to additive or synergistic effects, particularly sensory irritation, and possibly toxic effects on the cells lining the nasal cavity (HEI, 2007). It is also important to note that the toxicity databases for the majority of the aldehydes are either totally absent or partially incomplete and there are numerous data gaps for the above-mentioned toxicity traits, including but not limited to carcinogenicity. These data gaps are an important consideration when evaluating the overall toxicity of the mixture of aldehydes in these products.

Conclusions

Flavoring chemicals in e-liquids and e-cigarette aerosols, with notable exceptions, are largely unstudied for potential chronic inhalation toxicity. Several diketones and some aldehydes have significant inhalation toxicity, causing a range of pulmonary impairments including irritant and inflammatory effects. Diacetyl is among the most studied flavoring compounds, and has been demonstrated to be present in many e-liquids. The fact that similar irritant and inflammatory effects occur with different diketones, leads to the conclusion that diacetyl and similar diketone substitutes should not be assumed to be safe for inhalation without specific supporting toxicological data and the establishment of a clear threshold for respiratory effects. The potential for additive or super-additive effects with mixtures of chemical flavorings is largely unexplored.

In order to help with this problem, it may be useful to establish a mechanism to classify and categorize the flavoring chemicals for their potential respiratory irritancy whether or not specific respiratory irritation data exist for each individual chemical. The use of the calculated RD_{50} based on physical properties of the individual chemical, as presented in this paper, may be one way to accomplish this.

Principal Conclusions and Recommendations

Findings	Comments/Recommendation
Diacetyl and similar diketone flavors, including 2,3-pentanedione, 2,3-hexanedione, and 2,3-heptanedione, cause respiratory pathology	***Diketones should be assumed to represent a significant respiratory toxicity risk to regular e-cigarette users.*** ***While the concentrations of diacetyl may be less than in cigarette smoke, this does not mean that chronic diacetyl inhalation from e-cigarettes is safe.***
Cinnamaldehyde and derivatives such as methylcinnamate are allergens, cytotoxic, and are respiratory irritants.	***Cinnamaldehyde and derivatives use in e-liquids should be restricted and an acceptable level should be derived.***
There is evidence that many e-liquids contain aldehydes, such as benzaldehyde, that are moderate respiratory irritants. Recent evidence shows that flavorings can break down into more toxic aldehydes.	***Chronic inhalation of irritants may damage the respiratory tract upon prolonged exposure.***
The existing toxicological evaluations of food flavorings, in general, are dated and do not constitute a robust data set for the evaluation of possible systemic effects. ***Pyrazines have a variety of chemical structures and biological effects. These have not been adequately studied for inhalation effects.*** ***It is not currently known if or how mixtures of various flavorings work independently or together in causing inflammation or irritation of the respiratory epithelium.***	***More research is needed to determine what, if any, relevant effects on addiction or respiratory toxicity exist with pyrazines.*** ***It should be assumed that individual toxicological effects are, at least, additive.***

Acknowledgements

The authors would like to acknowledge April Roeseler, Elizabeth Andersen-Rodgers, and Liz Hendrix of the California Department of Public Health's Tobacco Control Program for

technical input and funding support for this review. The authors would further like to acknowledge Dr. John Talarico, Chief Environmental Health Investigations Branch, for editorial comments and technical reviews.

Competing interests

None.

Disclaimer

Any views or opinions in this paper are solely those of the authors and do not necessarily reflect the policies or official views of the California Department of Public Health.

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Appendix A. respiratory irritancy of some flavoring aldehydes and ketones

Table A1. Decrease in respiratory rate of mice in response to specific aldehydes and ketones (Steinhagen and Barrow, 1984; DeCeaurriz et al., 1984.)

Chemical	Used as a Flavoring Agent?	RD50 value (ppm)	Irritant level	ADI
Acrolein	No	1.03 – 1.41	Strong	--
Formaldehyde	No	3.2 – 4.9	Strong	--
Crotonaldehyde[1]	No*	3.53 – 4.88	Strong	--
Isophorone	Yes	28	Strong	2 µg/kg/day[2]
Mesityl oxide	Yes	61	Moderate	--
3-cyclohexane-1-carboxaldehyde	Yes	59 – 95	Moderate	None
Cyclohexane carboxaldehyde	?	163 - 186	Moderate	None
Diisobutyl ketone	Yes	287	Moderate	--
2-Furaldehyde	Yes	234 - 287	Moderate	0.5 mg/kg/d
Benzaldehyde	Yes	333 – 394	Moderate	5 mg/kg/d
Cyclohexanone	?	756	Mild	No limits
2-Ethylbutyraldehyde	?	853	Mild	No limits
Methyl amyl ketone	Yes	895	Mild	No limits
Isovaleraldehyde	Yes	757 – 1008	Mild	No limits
Valeraldehyde	Yes	1121 – 1190	Mild	No limits
Caproaldehyde	Yes	1029 – 1116	Mild	No limits
Butyraldehyde	Yes	1015 - 1532	Mild	No limits
Methyl isoamyl ketone	Yes	1222	Mild	No limits
Propionaldehyde	Yes	2052 - 2078	Mild	No limits
Acetaldehyde	Yes	2845 – 2932	Mild	No limits
Methyl isobutyl ketone	Yes	3195	Mild	No limits
Isobutyraldehyde	Yes	3016 - 4167	Mild	No limits
Methyl propyl ketone	Yes	5915	Mild/non	No limits
Methyl ethyl ketone	?	10745	Mild/non	No limits
Acetone	?	23480	Mild/non	No limits

1) IARC monograph 1993;
2) USEPA Water Quality Criteria for isophorone, 1980

Table 1 places various aldehydes and ketones into 3 categories based on their published RD_{50} potency. The categories in Table 1 are: Mild > 500 ppm > Moderate > 50 ppm > Strong. The relationship is also shown in Figure 2.

Appendix B. Calculation of RD50 values from partition coefficient data

The ECETOC Task Force in 2006 studied the relationship between log Kaw and log Kow, and log RD50, using 75 observed RD50 values for 58 volatile organic substances (Alarie *et al*, 1995) and log Kaw and log Kow values derived from the USEPA EpiSuite program (US-EPA, 2000).

The following linear relationship was used, in which the regression coefficients b0, b1 and b2 were estimated by multiple regression:

log RD50 = b0 + b1 x log Kow + b2 x log Kaw

Residual variance = 0.1559

Degrees of freedom = 72

Variance explained = 0.749 (74.9 percent)

b0 = 6.346 Student t for b0 = 25.89

b1 = –0.8333 Student t for b1 = –14.47

b2 = 0.7139 Student t for b2 = 11.22

Appendix C. Public health policy implications of e-liquid flavorings

A statement from the California Tobacco Control Program

Flavored electronic cigarettes and e-liquids pose a threat to the public's health on two fronts. First, evidence suggests that flavors play a role in the uptake of tobacco products by teens, including electronic cigarettes because they are appealing and they mask the harsh taste of tobacco (Ambrose et al., 2015; King et al., 2014; Kostygina et al., 2014; Miech et al., 2015). The earlier a person begins to use tobacco products, the more likely that individual is to develop a severe addiction to nicotine which has lifelong health consequences. Second, e-liquid flavors such as "cherry," "grape," "apple," "peach," and "berry," "Jolly Rancher," and "Kool-Aid" are concoctions of chemicals, some of which are toxic to the respiratory system.

This paper summarizes findings from more than 30 research studies examining the toxicity of chemicals commonly used to produce more than 7,000 unique electronic cigarette flavors widely marketed to the public (Zhu et al., 2014). The impact of flavoring chemicals used in electronic cigarettes on individual and public health is a rapidly evolving area of research and science; however, this review makes it clear that flavoring chemicals classified as "generally regarded as safe" for ingestion by the FDA and WHO/JECFA may not be safe when inhaled. While the toxicity of these chemicals to the respiratory system varies, several flavoring chemicals commonly used in e-liquids are known to cause respiratory irritation, inflammation, or allergic reactions. Despite the need for more research and monitoring to fully understand the short and long-term health implications of respiratory exposure to flavoring chemicals; effects of acute versus chronic exposure to these chemicals; and how voltage output from electronic cigarettes increases the toxicity, there is sufficient evidence for public health action.

Public health educational and policy strategies to protect the public from harm caused by respiratory exposure to flavoring chemicals may be viewed on a continuum from non-restrictive to increasingly restrictive. At the non-restrictive end of the continuum would be efforts such as public education campaigns to raise awareness about the toxicity of various flavoring chemicals used in e-liquids. An example at the restrictive end of the continuum would be a ban on the sale of flavored e-liquids, similar to the FDA's ban on the use of characterizing flavors in combustible cigarettes (FDA, 2009).

As a result of its authority under federal law known as the Family Smoking Prevention Tobacco Control Act of 2009 (Tobacco Control Act), the FDA published its final regulations deeming electronic cigarettes as a tobacco product on May 10, 2016. Among other things, the final deeming regulations requires e-cigarette and e-liquid manufacturers to disclose ingredients, substances, compounds and additives used in the production of these products to the FDA beginning on February 8, 2017, and disclosure of harmful and potentially harmful constituents is to begin on August 8, 2019. Additionally, a single health warning related to the addictiveness of nicotine will be required on packages and advertisements for e-cigarette products and e-liquids beginning on August 8, 2018, (Consortium TCL, 2016).

From the conclusions presented in this research review, it is evident that at the most basic level, consumers need information about the toxicity of various flavoring chemicals used in e-liquids. Standardized labeling and ingredient disclosure (including accurate concentrations) of the chemicals used in e-liquids is an appropriate strategy for informing consumers. Increasingly, researchers are also calling for restrictions on the use of certain flavoring chemicals, limiting the level or mixture of some chemicals, the imposition of quality control manufacturing standards, and additional health warning messages (Tierney et al., 2015; Hahn et al., 2014).

The Tobacco Control Act carved out adoption of tobacco product standards, labeling, and establishment of good manufacturing standards for the federal government, explicitly prohibiting state and local governments from adopting policies that are "different from, or in addition to" FDA standards related to "tobacco product standards, premarket review, adulteration, misbranding, labeling, registration, good manufacturing standards, or modified risk tobacco products." (Consortium TCL, 2016; Wellington, 2016) Therefore, the role of state and local public health agencies related to these matters is in conducting and participating in research and surveillance efforts that will inform decision-making and the federal rules process to ensure that policies for electronic cigarette and e-liquid labeling, manufacturing standards, and health warnings adequately maintain and protect the public's health.

While the Tobacco Control Act preempts state and local tobacco control policy activities in some areas, Congress explicitly preserved the authority of state or local governments to regulate or prohibit the sale or distribution of tobacco products. Such efforts includes restricting or prohibiting the sale flavored tobacco products, licensing retailers, enacting smoke-free laws, and

raising the minimum legal age of tobacco sales above 18 years of age (Consortium TCL, 2016; Wellington, 2016). Nationally, New York City, the City of Providence, and the City of Chicago all adopted local ordinances restricting the sales of flavored tobacco products which withstood legal challenges (Wellington, 2016). In California, six jurisdictions have enacted local ordinances restricting the sale of flavored tobacco products. These are Berkeley, Hayward, El Cerrito, Sonoma City, Manhattan Beach, and Santa Clara County.

Public health seeks to protect and improve the health of populations from illness, disease, and injury, using research and surveillance systems to detect and control diseases and injuries and to develop educational and policy efforts aimed at maintaining and protecting the public's health (CDC Foundation, 2016; APHL 2016). This research review on the toxicity of flavoring chemicals highlights a continued strong public health interest in regulating electronic cigarettes and the need for:

1. Further research and surveillance concerning the toxicity, manufacturing, packaging, use and sale of e-cigarettes and e-liquids;
2. State and local public health agency engagement in promoting stronger federal regulation for ingredient labeling, health warnings, and manufacturing and quality control standards for e-cigarettes and e-liquids;
3. Consumer and public education that raises awareness about the toxicity of flavoring chemicals to the respiratory system; and
4. State and local policy strategies that regulate the sale of flavored electronic cigarettes and e-liquids to protect the public's health from illness, disease, and injury.