Synthetic Cooling Agents in US-marketed E-cigarette Refill Liquids and Disposable E-cigarettes: Chemical Analysis and Risk Assessment.

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Abstract:

Background: Menthol, through its cooling sensory effects, facilitates smoking and tobacco product initiation, which is reflected by the high popularity of mint/menthol-flavored E-cigarettes. More recently, E-cigarette vendors started marketing synthetic cooling agents as additives that impart a cooling effect but lack a characteristic minty odor. Knowledge about content of synthetic coolants in US-marketed E-cigarette products and associated health risks is limited.

Methods: E-liquid vendor sites were searched with the terms “koolada”, “kool/cool”, “ice”, or WS-3/WS-23, denoting individual cooling agents, and relevant refill E-liquids were purchased. “Ice” flavor varieties of Puffbar, the popular disposable E-cigarette brand, were compared with non-“Ice” varieties. E-liquids were characterized, and synthetic coolants quantified using GC/MS. Margin of exposure (MOE), a risk assessment parameter, was calculated to assess the risk associated with synthetic coolant exposure from E-cigarette use.

Results: WS-3 was detected in 24/25 of the identified refill E-liquids (24/25), while almost all Puffbar disposables flavor varieties contained WS-23 (13/14), with 5/14 also containing WS-3, in both “Ice”- and non-“Ice” flavors. Modeling consumption of WS-3 from vaped E-liquids resulted in MOEs below the safe margin of 100 for most daily use scenarios (3-mL:14/25 E-liquids; 5-mL:18/25; 10-mL:20/25). MOEs for WS-23 from 11/13 Puffbar products were ≤100 in all use scenarios.

Conclusions: Synthetic cooling agents (WS-3, WS-23) were present in US-marketed E-cigarettes, at levels that may result in consumer exposures exceeding safety thresholds set by regulatory agencies. Synthetic cooling agents are not only found in mint-or menthol-flavored products, but also in fruit- and candy-flavored products, including popular disposable E-cigarette products such as Puffbar.

Implications: Synthetic cooling agents are widely used in “kool/cool”- and “ice”-flavored E-liquids and in E-liquids without these labels, both as a replacement for menthol or to add cooling ‘notes’ to non-menthol flavors. These agents may be used to bypass regulatory limits on menthol content in tobacco products. Since synthetic cooling agents are odorless, they may not fall under the category of “characterizing flavor”, potentially circumventing regulatory measures based on this concept. Regulators need to consider the additional health risks associated with exposure to synthetic cooling agents.

Introduction

Additives and flavors are added to tobacco products to counteract the harshness, sensory irritation and bitter tastes associated with tobacco and nicotine. In the United States, the Family Smoking Prevention and Tobacco Control Act (FSPTCA) of 2009 authorized the Food and Drug Administration (FDA) to regulate constituents, additives, and flavors in tobacco products. The FSPTCA banned the sales of flavored cigarettes, with the exception of menthol cigarettes. Menthol in tobacco products is well understood to reduce harshness, impart a pleasant minty and cooling flavor and increase palatability [1,2]. By altering the sensory perception and appeal of these products, menthol affects tobacco and nicotine use behaviors such as initiation, dependence, abuse liability and reduces the ability to quit, especially among the youth and young adults [3]. Menthol in tobacco products is also known to increase tobacco use disparities, with 85% of African American smokers using menthol cigarettes [3,4]. The US FDA announced in May 2021 that it will issue a rule that will ban menthol cigarettes [5]. Menthol is also a popular flavor in E-cigarettes, especially since the FDA banned flavored, closed pod systems such as Juul, except for menthol and tobacco flavors [6]. After Juul "Mint" pods were removed, sales of menthol pods increased 6-fold during August 2019 and May 2020 (10.7% to 61.8%) [7,8].

In a recent study we compared menthol concentrations in menthol-flavored pods marketed by Juul in the US, Canada and Europe [9]. In addition to menthol, the European-marketed menthol pods were found to contain the synthetic cooling agent WS-3, while the US and Canadian pods did not [9]. Synthetic cooling agents share the sensory cooling effects of menthol, but lack menthol's strong minty odor [10,11]. These agents were developed beginning in the 1970s, to replace menthol in body care and foods. In the 1970s and 80s, the tobacco industry including R.J. Reynolds and Phillip Morris, carried out consumer tests with cigarettes containing synthetic cooling agents, to impart a "cooling without menthol" experience [10–13]. More recently, an analysis of combustible cigarettes (both menthol and non-menthol) marketed in Germany detected WS-3, indicating that coolants other than menthol are added to tobacco products [14,15]. Currently, there exists a gap in knowledge about the presence and quantities of synthetic cooling agents such as WS-3 and WS-23 in US-marketed E-cigarettes and E-cigarette refill liquids ("E-liquids"). In addition to being added by manufacturers, synthetic cooling agents are also sold for (Do-It-Yourself) DIY users, often under the name "Koolada" [16,17].

We recently reported that some menthol-flavored E-liquids contain unsafe levels of pulegone, a carcinogenic mint flavorant banned by FDA in food, raising concerns about the unregulated addition of mint- and cooling flavorants to E-cigarettes [18,19]. While synthetic cooling

agents such as WS-3 are classified by the Flavor Extracts Manufacturers Associaton (FEMA) as GRAS (Generally Recognized As Safe), this applies only to their intended use in oral care and food products, but not to E-cigarette use resulting in inhalational exposure [20]. The Joint Expert Committee on Food Additives (JEFCA) of the World Health Organization (WHO) established a threshold of concern of 90 µg/day/person for intake of WS-3 and related compounds, a level that is often exceeded by consumers [21]. JECFA established a No-Observed-Adverse-Effect-Level (NOAEL) of 8 mg/kg-bw for WS-3 [21,22]. For WS-23, a NOAEL of 5 mg/kg-bw was determined for oral administration, with higher doses causing kidney and liver lesions [21,23]. A WS-23 inhalation exposure study by an E-liquid manufacturer determined a NOAEL of 29 mg/kg-bw (342 mg/m3) [24]. *In vitro* genotoxicity studies in mammalian cells demonstrated WS-23 to be clastogenic (i.e., inducing disruption or breakages of chromosomes), leading to a call by JECFA for additional toxicological studies to evaluate the safety of WS-23 [21]. Due to their evident toxicity and increased use in E-cigarettes, it is critical to evaluate the potential health risks associated with synthetic coolant exposure in chronic users.

In the present study, we determined the levels of synthetic cooling agents (WS-3, WS-23) as well as three important peppermint- and spearmint flavor chemicals (menthol and menthone, and carvone, respectively) in US-marketed refill E-liquids and in disposable E-cigarettes of the brand Puffbar, one of the leaders in this rapidly growing category especially popular among middle and high school students [7,8]. We also assessed the health risk associated with synthetic coolant exposure via E-cigarette use by calculating the Margin of Exposure (MOE), a risk assessment parameter, for a range of product use scenarios modeling low, moderate and frequent E-cigarette use.

Materials and Methods:

Analysis of synthetic cooling agents and other flavor content in US-marketed E-cigarette liquids and their aerosols:

E-liquid selection

E-cigarette vendor websites keyword searches for “koolada”, “kool”, “WS-3”, and “WS-23” were carried out to identify E-liquids potentially containing synthetic coolants, and identified products were purchased directly from vendors in 2019 and 2020; this also included one product (“Agent Cool”) sold as a concentrate for do-it-yourself (DIY) users. In addition, previously purchased menthol/mint/spearmint/wintergreen E-liquids (2019) were re-analyzed for synthetic coolant presence. In early 2020, all available flavors of “Puffbar” were purchased from puffecig.com due to the frequent occurrence of the word “cool” in Puff bar flavor names (see Table 1 and 2 for an overview).

E-liquid chemical analysis

E-liquids were analyzed using an established GC-MS method and selected compounds were quantified using an established GC-FID method [25]. To do so, commercially available standards of N-Ethyl-p-menthane-3-carboxamide, WS-3 (99%; CAS No. 39711-79-0), (S)-nicotine (>99%; CAS No. 54-11-5), (DL)-menthol (99%; 89-78-1), (D)-carvone (>96%; CAS No. 2244-16-8; all Sigma-Aldrich, St. Louis, MO), 2-isopropyl-N,2,3-trimethylbutyramide, WS-23 (>98%; CAS No. 51115-67-4, TCI, Portland, OR), and (L)-menthone (97%; CAS No. 14073-97-3; Alfa Aesar, Haverhill, MA) were used to construct calibration curves in the relevant concentration range. In brief, samples were diluted in 1mL of methanol [JT Baker, Center Valley, PA] containing 1 g/L of 1,4-dioxane [Alfa Aesar] as internal standard (IS) and injected; the detailed analytical methods have been described previously [25].

Coolant carryover analysis

To assess the carryover of the synthetic coolants WS-3 and WS-23 from refill E-liquids to E-cigarette-generated aerosol, the Suorin iShare refillable pod E-cigarette device was used (3.7 V, 1.8 Ohm, resulting max. power 7.6 W), which was attached via a custom-3D printer connector to a previously described house-built vaping machine [26]. In brief, the device is controlled by a programmable Arduino board that activates a micro-diaphragm pump at an operator-defined puff length, inter-puff break, and number of puffs. A microneedle valve in combination with a flow meter [Omega, Norwalk, CT] is used to control the flow rate. Generated aerosol was trapped by a series of liquid nitrogen-chilled cold-finger traps. Carryover was

determined in triplicate (three separate cartridges), and percent carryover was calculated as coolant content in trapped aerosol over coolant content of consumed liquid. A total of 9 carryover experiments were performed (7 in triplicate, 2 singular; n=23), with the following puffing regimen: 20 puffs, 2L/min flow rate, 2.4s puff length, 80mL puff volume, 30s inter-puff break. E-liquids for carryover experiments were chosen based on type of synthetic coolant present (WS-3, WS-23), and based on high, average, or low synthetic coolant and/or nicotine content (see Table 1). After 20 puffs were collected for each experiment, the traps were allowed to thaw and the captured material was taken up in 1mL of methanol containing 1 g/L of 1,4-dioxane as IS and injected into the GC/FID.

Margin of Exposure calculation for synthetic cooling agents:

The Margin of Exposure (MOE) is defined as the ratio of the highest test dose that causes no-observed toxicity in animals and the average human dietary intake, and MOE is considered the preferred option for risk assessment of toxic compounds by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [27,28]. MOEs for non-genotoxic additives exceeding 100 indicate a low health risk for human organ toxicity [29,30]. Additives with MOEs below the safety threshold of 100 are required to be prioritized for risk mitigation. MOEs for WS-3 and WS-23- containing E-liquids were calculated using the following formula, as described previously:[18]

$$MOE = \frac{\textbf{NOAEL for synthetic coolant}}{\textbf{Estimated Exposure Dose (EED)}}$$

Where NOAEL is the No-Observed-Adverse-Effect-Level, a dose determined as the threshold producing a toxic effect in animal oral toxicity studies. NOAELs for WS-3 and WS-23 have been determined to be 8 mg/kg-bw/day and 5 mg/kg-bw/day, respectively [22,23,31]. Daily Estimated Exposure Doses (EED) were determined from WS-3 and WS-23 concentrations in the E-liquids and their estimated average daily amount consumed. The nicotine amounts in the majority of refill E-liquids ranged between 0.1 – 1.0%, and user surveys found consumption volumes for such low nicotine E-cigarette liquids to be 9 mL/day, ranging from 1.7-17.7 mL/day) [19,32,33]. To cover a wide range of user behviours, MOEs for these refill E-liquids were calculated for consumption volumes of 1, 3, 5, 10 and 15 mL/day. For nicotine salt-containing refill liquids (nicotine content >3%), MOEs were calculated for 1, 3, 5 mL/day to account for the higher nicotine content. Puffbar E-liquid MOEs were calculated for consumption amounts of half a disposable bar/device, 1 Puffbar/device, and 2 Puffbars/devices. These amounts can be considered equivalent to volumes consumed by light, moderate, and heavy users of 3-5%

nicotine salt-containing E-cigarettes (Puffbar volume is 1.3 mL). For the calculations, a user body weight of 60 kg was assumed; to convert E-cigarette liquid consumption from volume in mL/day to g/day, the densities of propylene glycol and glycerol (1.04 and 1.26 g/cm^3, respectively) were taken into consideration.

Results

Chemical analysis of synthetic cooling agents, nicotine and other flavorants in US-marketed E-cigarette refill liquids, concentrates and their aerosols:

Using our search criteria, 25 E-cigarette refill liquids and 1 concentrate from 19 different brands were identified and their contents of synthetic coolants, mint-related compounds and nicotine quantified by GC/MS. Of the analyzed liquids, 21 contained free-base nicotine and 4 nicotine salts. 24 of the 25 E-liquids contained WS-3, with amounts varying from 150 µg/g (Popsicle USA) to 14,650 µg/g (Arctic Rush) (Table 1). One E-liquid contained WS-23 exclusively (Dillinger), at a concentration of 1,860 µg/g (Table 1). The concentrate contained both WS-3 and WS-23, with concentrations of 8,840 µg/g and 124,060 µg/g, respectively (Table 1).

To verify whether WS-3 and WS-23 carries over into aerosol and reaches the user during E-cigarette use, vaping experiments using an established method were carried out [26]. Carryover was determined for WS-3 with 8 different refill E-liquids (n=22) and carryover rates ranged from 91-109% (vs. neat E-liquid content), with an overall average carryover of 105% (s.d. 6.4%; n=20; Table 1). WS-23 was only detected in 1 refill E-liquid, and carryover ranged from 87-105% (vs. neat E-liquid content), with an overall average carryover of 99% (s.d. 9.8%; n=3; Table 1).

In addition to WS-3 and WS-23, menthol, menthone, carvone, and nicotine were also quantified. Interestingly, 13 of the 25 refill E-liquids contained varying amounts of menthol, ranging from 330 – 23,490 µg/g (Table 1). Menthone and Carvone were present in 6 of the 25 E-liquids, ranging from 30 - 25,450 µg/g for menthone and 10 – 6830 µg/g for carvone (Table 1). There were also four refill E-liquids that contained menthol, menthone, carvone and WS-3, with total combined amounts for these compounds ranging from 4,940 – 46,150 µg/g (Table 1). Nicotine concentrations in 20/21 free-base nicotine containing E-liquids were below 1% (1.41 – 9.97 mg/g), except for Clove+Cool Hit (17.91 mg/g; Table 1). Nicotine contents in nicotine-salts containing E-liquids ranged from 28.74 – 44.6 mg/g (Table 1). Taken together, these results suggest that synthetic coolants are being added to US-marketed E-liquids in a variety of marketed flavors (see Table 1), including in those already containing the “natural” coolant menthol.

Chemical analysis of synthetic cooling agents and menthol in US-marketed disposable E-cigarettes:

Following the withdrawal of the popular flavored Juul cartridges from the US market, disposable E-cigarette products of the brand “Puffbar” brand have become increasingly popular among US youth and young adults [7,8]. Several Puffbar flavors had ‘Ice’ in their marketed names, suggesting they may contain synthetic cooling agents. Of the 14 Puffbar flavors analyzed, 13 contained varying amounts of WS-23, ranging from 1,610 – 36,660 µg/g (Table 2). WS-3 was present in 5/14 flavors with concentrations ranging from 1,880 – 10,770 µg/g (Table 2). Menthol was present in 7/14 liquids analyzed with amounts ranging from 620 – 3,680 µg/g (Table 2). All WS-3 and menthol containing Puffbar flavors contained significant amounts of WS-23, with 3/14 flavors containing all three compounds. The total combined concentrations of all three compounds ranged between 8,830 – 48,050 µg/gram (Table 2). Puffbar liquids containing only WS-3 and WS-23 had a total combined synthetic coolants content ranging from 5,150 – 47,430 µg/g (Table 2). The nicotine concentrations in the Puffbar E-liquids ranged from 29.4 – 40.7 mg/g.

Margin of Exposure calculations for synthetic coolants in US-marketed refill E-liquids and disposable E-cigarettes:

Toxicological studies have confirmed significant organ toxicity of orally administered WS-3 and WS-23: JECFA determined NOAEL of 8 and 5 mg/kg-bw, respectively [23]. Some cellular studies observed that WS-23 has clastogenic properties [21]. To assess the risk associated with daily intake of WS-3 and WS-23 from E-cigarette use, we calculated the margin of exposure (MOE) resulting from the daily consumption of a liquid volume range covering low, moderate and high level use scenarios (1, 3, 5, 10, 15 mL/day for free-base nicotine refill liquids; 1,3,5 mL/day for nicotine-salts refill liquids; 0.5, 1 or 2 Puffbars/day for disposables). For organ toxicity, a safety margin MOE of 100 or higher is considered low risk, while MOEs lower than 100 require prioritization by FDA for risk mitigation for a given additive [29,30].

For the E-liquid with the highest WS-3 concentration (Arctic Rush), MOEs ranged from 32 (1-mL/day) to 2 (15-mL/day; Table 3 and Figure 1), and are thereby significantly lower than the recommended safety margin of 100. Except for this flavor, MOEs for consumption of 1ml of all other analyzed refill E-liquids exceeded the threshold of 100. For consumption volumes of 3 mL/day, 13/24 WS-3 containing refill E-liquids had a MOE of ≤100, ranging from 87 - 9. Similarly, 18/24 WS-3 containing refill E-liquids had an MOE of ≤100, ranging from 96 - 6, for consumption of 5 mL E-liquid/day. For 10 and 15 mL/day, MOEs were calculated only for the 20 free-base nicotine containing E-liquids: For 10 ml/day, 16/20 E-liquids yielded an MOE below 100, ranging from 96 – 3, and for 15 ml/day, 19/20 E-liquids yielded an MOE below 100, ranging

from 96 - 2 (Table 3 and Figure 1). Depending on consumption rates, MOEs for the one WS-23-containing refill E-liquid ranged from 135 - 9, with MOE <100 in all assessed scenarios but 1mL/day consumption (Table 3 and Figure 1). The tested concentrate ("Agent Cool") contained both WS-3 and WS-23. With the vendor recommending to add 1-2 drops of concentrate per pod, we conservatively estimated MOEs for a resulting dilution range of 1 – 3%. Resulting MOEs for WS-23 when added at 1-3% to E-liquids were <100 at all consumption rates, except for the lowest consumption rate (1mL/day) at 1% dilution. WS-3 content of the concentrate was approx. 14-fold lower than WS-23, and the resulting MOEs for WS-3 were >100 at all consumption and dilution rates. Taken together, these results indicate that users of synthetic coolants flavored E-liquids are exposed to WS-3 and WS-23 levels that can potentially pose a health risk upon long-term use.

Next, we calculated the MOEs for synthetic coolants for daily consumption of Puffbar devices. For the Puffbar flavor with the highest content of both WS-3 and WS-23 (Cool Mint), the MOEs for consumption of 0.5 – 2 devices/day ranged from 57 -14 for WS-3, and 11 – 2 for WS-23, values that are significantly lower than the safety margin threshold of 100 (Table 4 and Figure 2). For all other flavors, WS-23 MOEs were similarly <100 for all use scenarios, with the exception of Café Latte (0.5 device/day) and Menthol (0.5 and 1 device/day). MOEs for WS-3 were lower than the safety threshold of 100 at the highest estimated consumption rate (2 devices/day) for all five WS-3 containing Puffbar flavors. MOEs for WS-3 were also <100 for Cucumber and Menthol flavors at 1 pod/day consumption rate. These results indicate that users of Puffbar disposable E-cigarettes are exposed to levels of synthetic coolants that are considered to pose organ toxicity risks, especially from WS-23 exposure.

Discussion:

We detected the presence of synthetic coolants (WS-3 and WS-23) in US-marketed E-cigarette refill liquids and almost all tested flavors of the popular disposable E-cigarette brand, Puffbar. Using a relatively low-power Juul-like vaping device, we demonstrate that these coolants can efficiently be aerosolized and carried over to reach user airways. Margins of Exposure (MOE) were calculated to assess the health risks associated with exposure to these coolants from E-cigarette use, a parameter used by FDA and other global regulatory agencies to regulate the safe use of food additives with carcinogenic and organotoxic effects. These calculations suggests that daily intake of WS-3 or WS-23 by E-cigarette users exceed levels that would be considered acceptable for consumption by FDA and European Flavour Association (EFFA).

Natural cooling agents such as menthol provide a cooling sensation by activating cold-sensitive trigeminal nerve fibers innervating the upper respiratory tract and oral cavity. These sensory neurons express TRPM8 ion channels, the receptor for menthol that is also activated by cooling [34,35]. Menthol also has counterirritant, antitussive and analgesic properties and inhibits respiratory irritation responses to tobacco smoke and its various irritant constituents through a TRPM8 dependent mechanism in rodent models [36,37]. Menthol, through its actions on TRPM8, attenuated the aversive oral effects of nicotine in mice and increased nicotine intake [38]. The half-maximal concentrations of menthol to activate human TRPM8 is 4 µM [39,40]. This concentration is known to be exceed after inhalation of menthol cigarette smoke [41]. A menthol concentration of 0.65 µM (16 ppm), was sufficient to reduce sensory irritation by cigarette smoke exposure in mice, illustrating the strong counterirritant properties of menthol even at low concentrations [36]. In E-cigarette users, menthol is transferred to the airways with high efficiency, where menthol concentrations per puff are comparable to concentrations in puffs generated from menthol cigarettes [9,42]. Previous analytical studies demonstrated that menthol concentrations in marketed E-liquids range from 2 – 68,000 µg/mL (13 µM-435 mM) [43]. In the present study, menthol levels in E-cigarette refill liquids and Puffbar devices ranged from 330 – 23,490 µg/g (~2.4-173 mM) and 620 – 3,680 µg/g (~4.6-27 mM), respectively. These levels are sufficient to reduce sensory perception of irritation and harshness elicited by nicotine in humans [44]. Similar to menthol, synthetic cooling agents (including WS-3 and WS-23) also activate TRPM8 with increased efficacy (WS-3) and with comparable half-maximal concentrations (WS-3: 2-4 µM; WS-23: 44 µM) [39,45,46]. Several of the refill E-liquids analyzed in this study contained WS-3 at levels comparable to menthol levels in mint- and menthol-flavored E-liquids

(150-14,650 µg/g; ~816 µM-80 mM), with some containing synthetic coolants in addition to "natural" cooling agents. The concentrations of WS-3 in several Puffbar E-liquids, up to 1% (1,880 – 10,770 µg/g; 10-59 mM), are similar to menthol concentrations in liquids of Juul, the market-leading pod E-cigarette system in the US [9,42]. In addition, the majority of the Puffbar E-liquids analyzed in this study contained WS-23 ranging between 1 – 3.7% (up to 250 mM), which is sufficient to mediate TRPM8-dependent counterirritant effects. Carryover of synthetic cooling agents from E-liquids into the vapor was highly efficient, approximating 100%, as previously demonstrated for menthol (Table 1) [42]

Our risk assessment analysis suggests that users of E-cigarettes containing synthetic cooling agents are exposed to amounts of WS-3 and WS-23 that exceed the amounts considered acceptable by regulatory bodies for intake from food. Detected amounts were much higher than the thresholds set by JECFA for human intake of flavor additives of this particular class of chemicals (aliphatic and aromatic amines and amides, FEMA Nos 1594-1601, 1606 and 1613) at 90 µg/person/day, either added as individual flavoring or combined total [21]. Even consumption of just 1 mL of WS-3 containing E-cigarette liquid (150-14650 µg/g) results in exposures exceeding JECFA's safety threshold by ~2 – 180 fold. Vaping of 1 disposable Puffbar E-cigarette containing synthetic cooling agents would expose a user to amounts of WS-3 (1,880-10770 µg/g) that exceed the threshold of concern by ~29 – 176 fold, or ~26 – 598 fold for WS-23 (3,940-36660 µg/g). There were 5 Puffbar varieties that contained both WS-3 and WS-23, and when added together, would expose a user vaping 1 Puffbar device to ~126 – 774 fold the recommended amount of amides based on JECFA recommendations. These results strongly suggest that E-cigarette users are exposed to synthetic coolant amounts which would be considered unsafe when consumed in food, raising concerns for health risks upon chronic exposure.

Route to route extrapolation significance

The available chemical toxicity margins for WS-3 and WS-23, such as provided by JECFA, are derived from toxicological studies in animals in which coolants were administered orally [21]. Calculating Margins of Exposure (MOE) for inhalation of a compound based on past oral toxicity studies requires some caution. For human risk assessment and to calculate risk assessment parameters from exposure to these chemicals through various routes, it is routine practice for US and International regulatory agencies (US Environmental Protection Agency, EPA; European Chemical Agency, ECHA; UK's Interdepartmental Group on Health Risks from Chemicals, IGHRC) to employ route-to-route (R2R) extrapolation [47,48]. Regulatory agencies

agree that the absorption efficiency for chemicals by the respiratory system is higher than the efficiency of the digestive tract and usually, an oral-to-inhalation ratio of 2 is applied for route-to-route extrapolation, reflecting the higher absorption of toxicants following inhalation [47–49]. In this study, R2R extrapolation was not used, however, if applied, the MOE values would be even lower, reflecting an even higher risk associated with inhalation of these two synthetic cooling agents at various daily consumption volumes. This would suggest even greater urgency for regulatory measures mitigating the risk from WS-3 and WS-23 exposures in E-cigarette users.

Recently, an e-vapor product company (RLX technology, China) has conducted a nose-only acute and sub-acute inhalation study for WS-23 (28-day exposure at doses of 19 and 29 mg/kg-bw/day) [24]. Whole-body plethysmography was conducted after 28 days of repeated exposure to measure various parameters of pulmonary function. Compared to control and solvent groups, mice exposed to WS-23 demonstrated significant changes in various parameters, such as time of expiratory (Te), peak expiratory flow (PEF), relaxation time (RT), minute volume (MV), respiratory rate (F), end-inspiration pause (EIP), end-expiratory pause (EEP). These results indicate that WS-23 may alter pulmonary function of users of WS-23 containing E-cigarettes. However, the study reports no remarkable histopathological changes in either the respiratory organs (nose, throat, trachea and lungs) or other visceral organs (liver, kidney, heart etc.). It has to be noted that the histopathological analysis was not based on a quantitative histopathologic scoring of the organ tissue lesions but based on visual examination of the H&E stained sections. The absence of histopathological evidence for organ toxicity contradicts prior WS-23 oral toxicity studies in rats that reported kidney lesions and hepatic toxicity starting at 10 mg/kg-bW/day (NOAEL of 5 mg/kg-bW/day) [21,22]. The discrepancy between these observations could be due to the different routes of exposure (oral vs. inhalation), or more importantly, may have resulted from the different exposure durations (14-weeks oral dosing vs. 4-weeks inhalational exposure).

In vitro genotoxicity studies of WS-23 in mammalian cells showed evidence for clastogenicity [21]. Clastogenicity was observed only in the presence of metabolic activation, indicating that the effect is mediated by the formation of a reactive metabolite. Due to the increased exposure to WS-23 from use of E-cigarettes, it is imperative that more studies are conducted to understand the mechanisms of their metabolism and further identify WS-23's reactive clastogenic metabolites.

Previous toxicological studies have examined the toxicological effects of synthetic cooling agents, including WS-3 and WS-23, in isolation, and not in mixtures. As demonstrated in

this study, refill E-cigarette liquids and popular disposable E-cigarettes contain mixtures of natural and synthetic cooling agents. Inhalation toxicity studies are required to examine the effects of acute, sub-acute and chronic exposures to mixtures of these chemicals and marketed E-cigarettes that contain such mixtures. Another limitation in assessing the risk associated from use of these E-cigarettes is the rapidly evolving product variety and the dynamic nature of their composition. The products analyzed in this study were purchased in the last 1-2 years. More recent marketed products may contain different compositions. Hence, a continuous monitoring of new E-cigarette products by determining their chemical compositions for presence of potentially toxic flavorant levels is critical for both regulatory and risk assessment purposes.

In Puffbar disposable products, synthetic cooling agents were not only found in mint/menthol-flavored, or “cool”- or “ice”-labelled E-liquids, but in almost all products tested (13/14), suggesting that a cooling effect is preferred by consumers. Cooling agents were added to fruit-flavored E-liquids named “Orange Ice” and “Blueberry Ice”, but also “Strawberry Banana” and “Lychee”, without the “Ice” label, and in “Cucumber” and “OMG” flavors. This illustrates the utility of odorless synthetic cooling agents to add a cooling effect to flavors that likely would not be favored by users when combined with menthol, due to the incompatible minty odor the “natural” coolant menthol would add. Synthetic cooling agents may allow tolerance of more intense flavor combinations that would otherwise be irritating.

The fact that synthetic coolants are odorless also raises another important question: Legislation such as the FSPTCA or the European Tobacco Product Directive (TPD) [1,2,50,51] banned “characterizing” flavorants from combustible tobacco products (with exemption for menthol in the US), yet is unclear how synthetic coolants lacking a characterizing flavor would be treated. A replacement of the cooling compound menthol (with a minty characterizing flavor) with a synthetic coolants (lacking characterizing flavor) could be a strategy to circumvent a a menthol ban. Policy makers should consider this when implementing menthol bans, such as both Canada and Germany have done in respective regulations that either maintain a positive list of compounds permitted as additives in tobacco products (Canada), or specifically ban a whole class of compounds, including all natural and synthetic derivatives of menthol and other cooling agents (Germany) [52,53]

Conclusions:

Synthetic cooling agents are added to US-marketed E-cigarettes in a wide range of amounts that are comparable and relative to menthol amounts in flavored E-cigarettes. Synthetic coolants were found not only in mint- and menthol-flavored products, but also in fruit-, dessert- and sweet-flavored E-cigarettes. These coolants are added to refill liquids, E-cigarette liquid diluents and popular disposable E-cigarettes such as Puffbar, at levels that pose potential health risks to users, thereby requiring for these products to be prioritized for risk mitigation measures.

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Declaration of Interests:

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Author contributions were as follows: SVJ, HCE and SEJ conceptualized and designed the study; DGT, LD, and HCE acquired and analyzed the chemical analytical data; SVJ analyzed and interpreted the data for risk analysis calculations; SVJ, SEJ and HCE drafted the manuscript; SVJ, HCE, PTA, JBZ, and SEJ provided supervision; JBZ critically revised the manuscript for important intellectual content.

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Figure Legends

Figure 1: Margins of Exposure (MOE) for synthetic cooling agents from E-Cigarette refill liquids and coolant concentrate. MOEs are plotted for representative WS-3 (left) and WS-23 (right) -containing E-liquids and diluents with high, intermediate and low coolant concentrations, for daily consumption of 1, 3, 5, 10 and 15 ml E-liquid. No Observed Adverse Effect Levels (NOAEL) determined by JECFA in animal studies of organ toxicity upon oral administration were used for MOE calculations. A MOE of <100 (dashed line) signals a safety concern and requires regulatory mitigation. For WS-23 concentrate (Agent Cool, TBD Liquids), MOEs were calculated for dilutions within the range of manufacturer-recommendations to 1 and 3%. MOEs of the oral average daily intake of US consumers (US-OADI) determined by JECFA are displayed for comparison.

Figure 2: Margins of Exposure (MOE) for synthetic cooling agents from Puffbar-branded disposable E-cigarettes. MOEs are plotted for exposures from Puffbar devices with flavored E-liquids containing both WS-3 and WS-23, at high (Cool mint), intermediate (Blueberry ice) or low (Menthol) concentrations, separated for each coolant (left: WS-3, right: WS-23), calculated for daily consumption of ½ Puffbar (0.65 mL), 1 Puffbar (1.3 mL) and 2 bars (2.6 mL). JECFA-determined NOAEL values were used for MOE calculations. MOEs of the oral average daily intake of US consumers (US-OADI) determined by JECFA are displayed for comparison.

		Flavor Concentration ± s.d. (µg/g)					Nicotine concentration ± s.d. (mg/g)	PG/VG ratio	Synthetic coolant carryover ± s.d. (%)
	E-cigarette Flavor (Manufacturer)	**WS-3**	**WS-23**	**Menthol**	**Methone**	**Carvone**			
1	Arctic Rush (Central Vapors)	14650 ± 230					9.2 ± 0.09	50/50	106 ± 4
2	Frzn Mango (BLVK)	2450 ± 00		3640 ± 10			5.08 ± 0.03	30/70	
3	Nuclear White Menthol (Vapor HQ)	2440 ± 10		23490 ± 130	310 ± 20	190 ± 70	9.88 ± 0.02	50/50	104 ± 4
4	Rich Girl (Stunna)	2260 ± 30					3.31 ± 0.06	70/30	107 ± 11
5	Frozen Berries (Shinjin Vapor)	2220 ± 20		2320 ± 20			3.36 ± 0.05	30/70	
6	Frozen Limeade (Shinjin Vapor)	1540 ± 20		3470 ± 50			3.38 ± 0.04	30/70	
7	Cool Lime (Chill)	2110 ± 60					1.63 ± 0.05	30/70	
8	Tropicool (Chill)	2040 ± 40					3.02 ± 0.07	30/70	91[a]
9	Kool Cali (Chill)	1690 ± 20					4.20 ± 0.06	40/60	
10	Sakura 3 mg Nic (Sugoi)	1090 ± 20		910 ± 10			2.32 ± 0.03	30/70	105 ± 2
11	Coconut Ice (EC Blend)	2240 ± 10					1.41 ± 0.02	60/40	
12	Clove + Cool Hit (EC Blend)	1600 ± 20					17.91 ± 0.23	50/50	
13	Popsicle USA (EC Blend)	150 ± 10				10 ± 1		60/40	
14	Frozen (FJ's)	1550 ± 50						30/70	
15	Wintergreen Menthol (VaporHQ)	870 ± 20		4850 ± 30			9.72 ± 0.08	50/50	
16	Icy Menthol (Vista Vapors)	560 ± 10		9240 ± 90	1320± 50	2890 ± 10	9.80 ± 0.02	50/50	
17	Ice Dragon (ELD Flavors)	410 ± 30					2.75 ± 0.01	20/80	
18	Wintermint (Gothic Vapor)	330 ± 50		1110 ± 190		510 ± 10	9.03 ± 0.84	50/50	
19	Climate Change (What Juice)	290 ± 10		330 ± 10			9.48 ± 0.12	30/70	
20	Arctic (Vista Select)	280 ± 20		3470 ± 10	30 ± 10	930 ± 70	6.59 ± 0.06	30/70	
21	Dillinger (NastyJuice X Kilo)		1860 ± 40		280 ± 10		4.29 ± 0.07	30/70	99 ± 10
22	Kool Peach (Salty Man)	990 ± 20					46.4 ± 1.09	40/60	109 ± 1
23	Sakura Nic Salts 35 mg (Sugoi)	1170 ± 30		1170 ± 20			28.74 ± 0.43	50/50	
24	Sakura Nic Salts 50 mg (Sugoi)	1140 ± 60		1140 ± 90	14110 ± 670		39.19 ± 2.20	50/50	97[a]
25	Unicorn Nicotine Salt Spearmint (BLVK)	2950 ± 20		10920 ± 160	25450 ± 600	6830 ± 40	41.36 ± 0.33	40/60	107 ± 8
26	Agent Cool Super Concentrate (TBD Liquids)	8840 ± 130	124060 ± 720						

WS-3

WS-23	Concentrate

Table 1: Chemical concentrations for menthol, menthone, carvone, synthetic cooling agents (WS-3 and WS-23) and solvent chemicals in US-marketed E-cigarette refill liquids. Carryover rates for synthetic coolants are provided for select refill liquids. [a]single experiment.

	Puffbar flavors	Flavor Concentration ± s.d. (µg/g)			Nicotine ± s.d. (mg/g)
		WS-3	WS-23	Menthol	
1	Cool Mint	10770 ± 210	36660 ± 530	620 ± 50	32.79 ± 0.38
2	Café Latte		2300 ± 30	1500 ± 50	36.56 ± 0.30
3	Orange Ice	1750 ± 20	22950 ± 450		37.61 ± 0.76
4	Pomegranate				29.37 ± 0.47
5	Lush Ice		25830 ± 450	2250 ± 190	32.26 ± 0.53
6	Melon Ice		8810 ± 60		32.81 ± 0.29
7	Cucumber	3770 ± 20	3940 ± 10		31.67 ± 0.15
8	Lychee		26240 ± 240		33.22 ± 0.27
9	Menthol	3540 ± 30	1610 ± 1950	3680 ± 550	33.25 ± 0.22
10	Blueberry Ice	1880 ± 20	14120 ± 210	2300 ± 40	32.71 ± 0.45
11	Strawberry Banana		5420 ± 90		32.12 ± 0.52
12	OMG		7390 ± 330	1330 ± 40	34.01 ± 1.35
13	Peach Ice		11540 ± 270		33.11 ± 0.63
14	Banana Ice		13110 ± 50	2040 ± 110	40.66 ± 0.22

WS-3
WS-23
Menthol

Table 2: Chemical concentrations for synthetic cooling agents (WS-3 and WS-23), menthol and menthol in various flavors of US-marketed disposable E-cigarettes, Puffbars.

	E-cigarette Flavor (Manufacturer)	Flavor Concentration (µg/g)		MOE for Daily E-Liquid (mL/day) Amount-Consumed				
		WS3	WS23	1 mL	3 mL	5 mL	10 mL	15 mL
1	Arctic Rush (Central Vapors)	14650		28	9	6	3	2
2	Frzn Mango (BLVK)	2450		164	55	33	16	11
3	Nuclear White Menthol (Vapor HQ)	2440		171	57	34	17	11
4	Rich Girl (Stunna)	2260		192	64	38	19	13
5	Frozen Berries (Shinjin Vapor)	2220		181	60	36	18	12
6	Frozen Limeade (Shinjin Vapor)	1540		261	87	52	26	17
7	Cool Lime (Chill)	2110		190	63	38	19	13
8	Tropicool (Chill)	2040		197	66	39	20	13
9	Kool Cali (Chill)	1690		242	81	48	24	16
10	Sakura 3 mg Nic (Sugoi)	1090		368	123	74	37	25
11	Coconut Ice (EC Blend)	2240		190	63	38	19	13
12	Clove + Cool Hit (EC Blend)	1600		261	87	52	26	17
13	Popsicle USA (EC Blend)	150		2834	945	567	283	189
14	Frozen (FJ's)	1550		259	86	52	26	17
15	Wintergreen Menthol (VaporHQ)	870		479	160	96	48	32
16	Icy Menthol (Vista Vapors)	690		604	201	121	60	40
17	Ice Dragon (ELD Flavors)	410		961	320	192	96	64
18	Wintermint (Gothic Vapor)	330		1263	421	253	126	84
19	Climate Change (What Juice)	290		1384	461	277	138	92
20	Arctic (Vista Select)	280		1433	478	287	143	96
21	Dillinger (NastyJuice X Kilo)		1860	135	45	27	13	9
22	Kool Peach (Salty Man)	990		413	138	83		
23	Sakura Nic Salts 35 mg (Sugoi)	1170		356	119	71		
24	Sakura Nic Salts 50 mg (Sugoi)	1140		366	122	73		
25	Unicorn Nicotine Salt Spearmint (BLVK)	2950		139	46	28		
26	Agent Cool Super Concentrate (TBD Liquids) (recommended use at 1-3%)	8840 (88.4-265.2)	124060 (1241-3722)					
	1% Dilution	88.4		5221	1740	1044	522	348
	3% Dilution	265.2		1740	580	348	174	116
	1% Dilution		1241	232	77	46	23	15
	3% Dilution		3722	78	26	16	8	5

MOE > 100 (Risk Mitigation need not be prioritized)	WS-3
MOE < 100 (Risk Mitigation to be Prioritized)	WS-23
	Concentrate

Table 3: Predicted MOE for Synthetic Cooling Agents Containing US-marketed E-Cigarette Liquids Used in Vape Tanks and Vape Mod Devices.

	Puff Bars Flavors	Flavor Concentration (µg/g)		WS-3 MOE for Daily E-Liquid Amount-Consumed (pods/day)				WS-23 MOE for Daily E-Liquid Amount-Consumed (pods/day)		
		WS-3	**WS-23**	**1/2 Pod**	**1 Pod**	**2 Pods**		**1/2 Pod**	**1 Pod**	**2 Pods**
1	Cool Mint	10770	36660	57	29	14		11	5	3
2	Café Latte		2300					168	84	42
3	Orange Ice	1750	22950	353	177	88		17	8	4
4	Pomegranate									
5	Lush Ice		25830					15	7	4
6	Melon Ice		8810					44	22	11
7	Cucumber	3770	3940	164	82	41		98	49	25
8	Lychee		26240					15	7	4
9	Menthol	3540	1610	175	87	44		240	120	60
10	Blueberry Ice	1880	14120	329	164	82		27	14	7
11	Strawberry Banana		5420					71	36	18
12	OMG		7390					52	26	13
13	Peach Ice		11540					33	17	8
14	Banana Ice		13110					29	15	7

MOE > 100 (Risk Mitigation need not be prioritized)	WS-3
MOE < 100 (Risk Mitigation to be Prioritized)	WS-23

Table 4: Predicted MOE for Synthetic Cooling Agents Containing Popular Disposable E-Cigarettes, Puffbar.

Figure 1

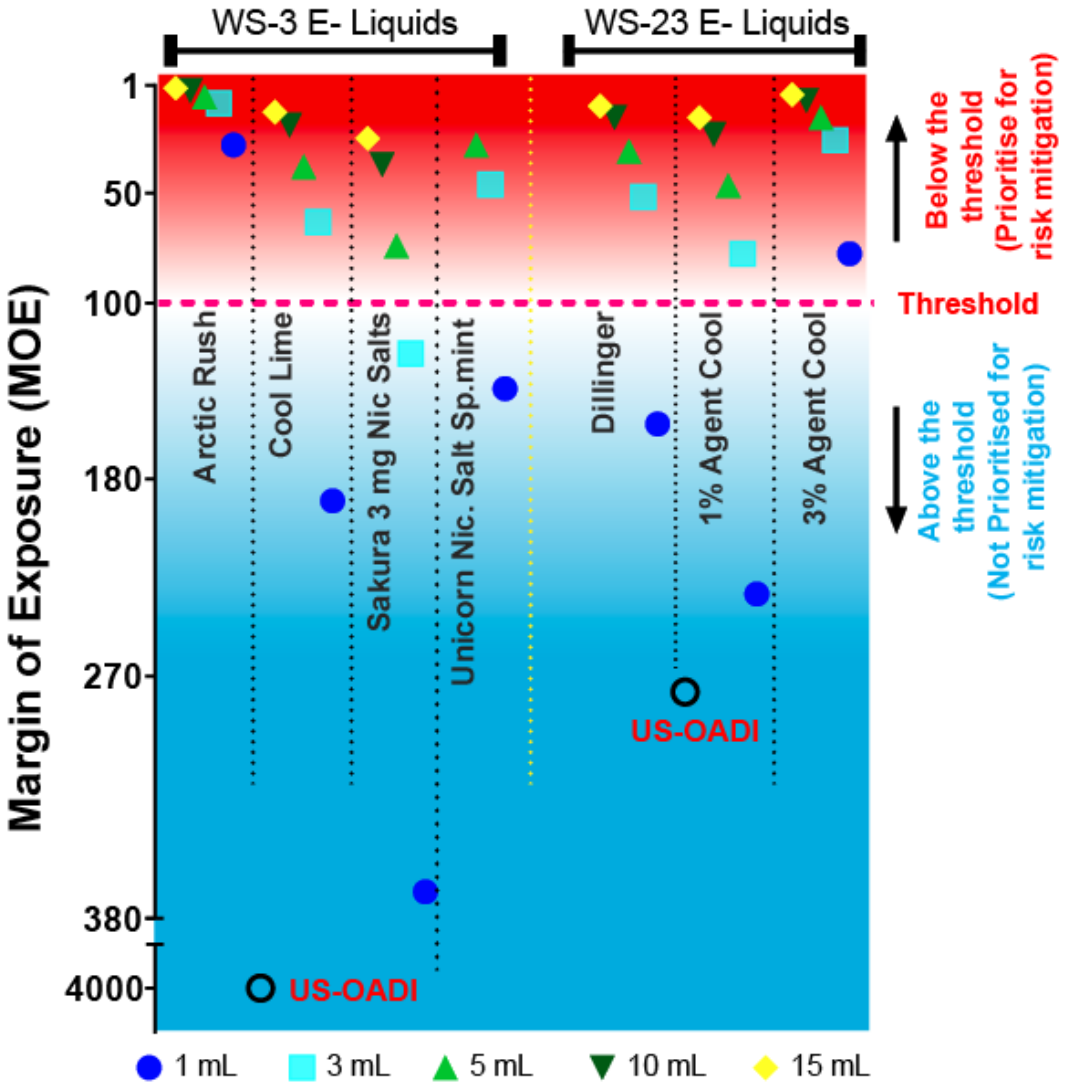
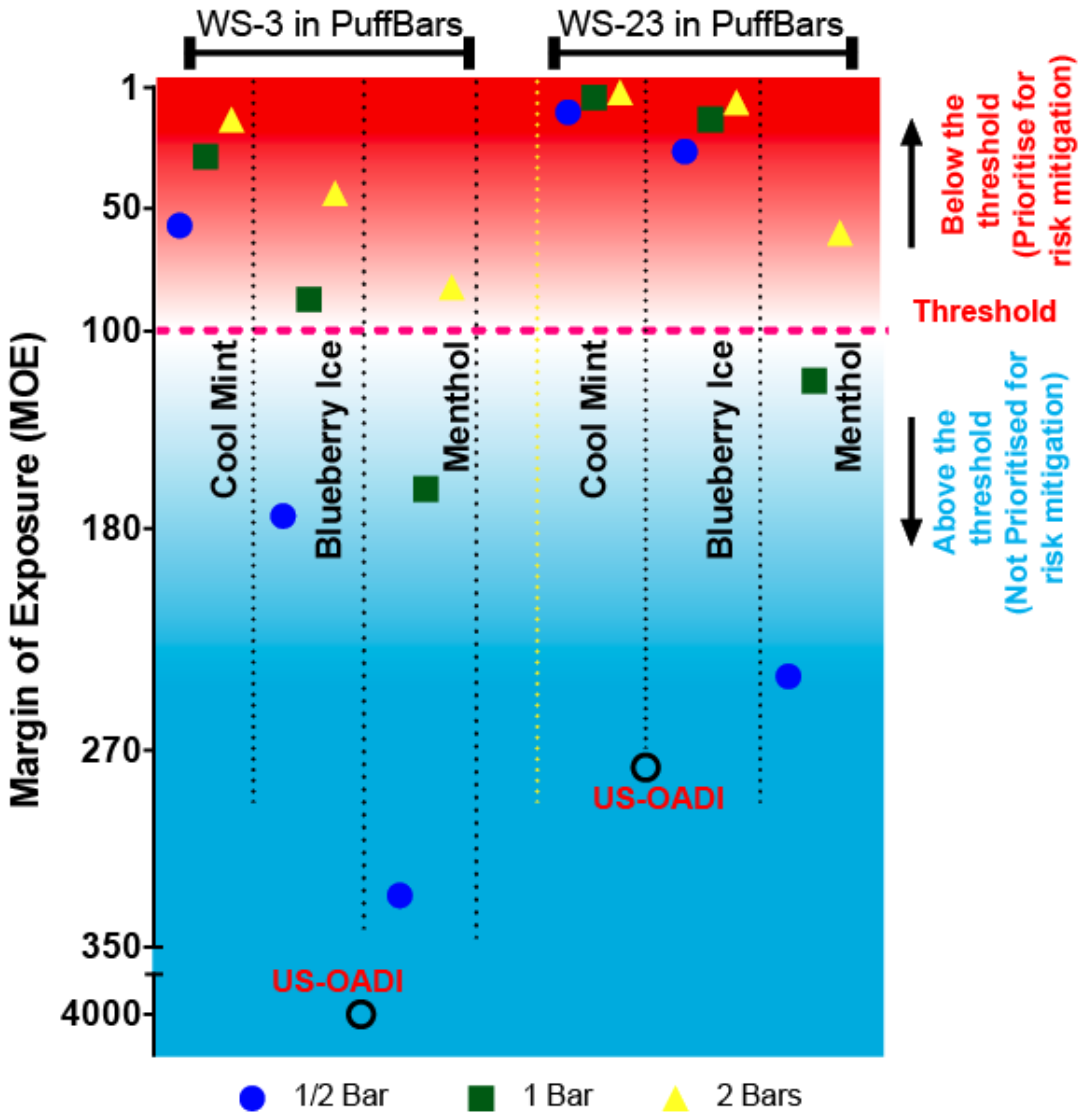

WS-3 E- Liquids
WS-23 E- Liquids
Margin of Exposure (MOE)
1
50
100
180
270
380
4000
Arctic Rush
Cool Lime
Sakura 3 mg Nic Salts
Unicorn Nic. Salt Sp.mint
Dillinger
1% Agent Cool
3% Agent Cool
US-OADI
US-OADI
Threshold
Below the threshold (Prioritise for risk mitigation)
Above the threshold (Not Prioritised for risk mitigation)
1 mL
3 mL
5 mL
10 mL
15 mL

Figure 2


WS-3 in PuffBars
WS-23 in PuffBars
Margin of Exposure (MOE)
1
50
100
180
270
350
4000
Cool Mint
Blueberry Ice
Menthol
Cool Mint
Blueberry Ice
Menthol
US-OADI
US-OADI
Threshold
Below the threshold (Prioritise for risk mitigation)
Above the threshold (Not Prioritised for risk mitigation)
1/2 Bar
1 Bar
2 Bars