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

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Abstract

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

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Author Contributions
PT and EO formed the concept and design of this study. Sample preparation, data collection, and data processing were carried out by EO, WL, and KJM. Data were analyzed and interpreted by EO, WL, JFP, and PT. Writing and editing of the manuscript was done by EO, WL, KJM, JFP, and PT.

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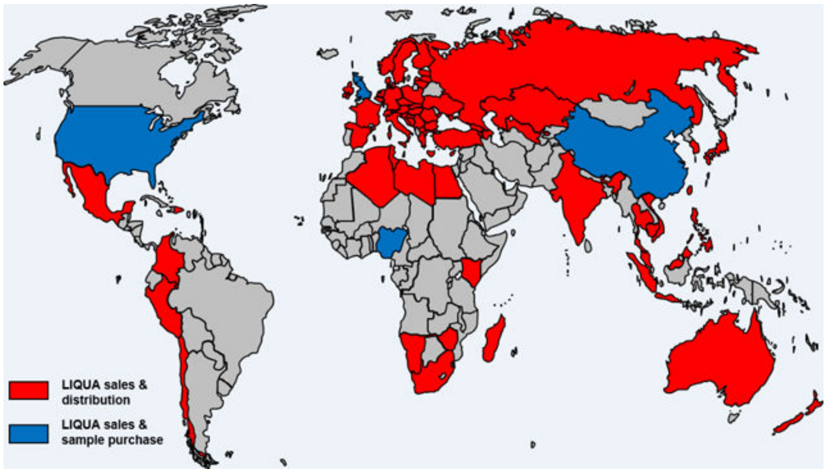
refill fluids often exceeded concentrations permitted in other consumer products. These data support the regulation of flavor chemicals in EC products to reduce their potential for producing both cancer and non-cancer toxicological effects.

Graphical Abstract



Keywords

LIQUA; electronic cigarette; e-liquids; flavor chemicals; flavors; cytotoxicity; carcinogenicity; GC/MS; MTT

INTRODUCTION

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

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

MATERIALS AND METHODS

Product Selection and Collection

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

Evaluation and Quantification of Flavor Chemicals using GC/MS

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

Culturing of mNSC and BEAS-2B Cells

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

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

MTT Cytotoxicity Assay

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

Live Cell Imaging of mNSC

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

Data Analysis

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

RESULTS

Total Flavor Chemical Concentrations and Total Number of Flavor Chemicals

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

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

Individual Flavor Chemical Concentrations in LIQUA Refill Fluids

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

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

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

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

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

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

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

Mixtures of Flavor Chemicals Sometimes Reduced Toxicity

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

Relationship between Cytotoxicity of LIQUA Products and Flavor Chemical Concentration

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

The Margin of Exposure Assessment of Potential Carcinogens in Refill Fluids

Some refill fluid chemicals are known or probable carcinogens. The Margin of Exposure (MOE) approach aids risk managers in prioritization and is used by the FDA and other expert groups to access the cancer risk of food additives.[24–27] The MOE is the ratio of a reference point for an adverse effect to the estimated daily intake or exposure of a chemical in humans. Reference points obtained from experimental or epidemiological data based on dose-response curves include the BenchMark Dose (BMD), the No Observed Adverse Effect Level (NOAEL), or the Low Observed Adverse Effect Level (LOAEL). For MOEs below 10,000, cancer risk needs to be considered. We calculated MOEs for β-myrcene, hydrocoumarin, estragole, and pulegone based on an available BMD that caused a 10% increase in tumor incidence in animal models ($BMDL_{10}$) and NOAELs and a user consumption of 3.4 or 5 mL of fluid/day for a body weight of 60 kg [15,24,28–30] (Table 4). The MOEs for β-myrcene and hydrocoumarin were >10,000 in all samples (Figure 8a and 8b), indicating a low cancer risk. In contrast, some products had pulegone and estragole concentrations that were well below 10,000, meaning there is a cancer risk associated with these products (Figure 8c and 8d). Q Menthol (pulegone) and Two Apple (estragole) had extremely low MOEs.

Comparison of the Dominant Flavor Chemicals in Three Refill Fluid Studies

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

The individual dominant flavor chemicals (not averaged) were compared across our current and two previous studies (Supplemental Figure 4). Twenty-seven dominant flavor chemicals were identified in the present study bringing the total number across our three studies on refill fluids to 37 (Figure 9b).[12,14] Of these, five flavor chemicals (benzyl alcohol, ethyl maltol, menthol, triacetin, and vanillin) were used in at least one product at > 1 mg/mL in all three studies (Figure 9b). Ten dominant chemicals (eugenol, p-menthone, maltol, (3Z)-3-Hexen-1-ol, corylone, ethyl acetate, ethyl butanoate, ethyl vanillin, furaneol, and isoamyl acetate) were in two of the three studies, and 13 (1-hexanol, 4-terpineol, acetylpyrazine, benzaldehyde PG acetal, butyl acetate, carvone, ethyl lactate, ethyl propanoate, hexyl acetate, isobutyl acetate, limonene, methyl anthranilate, γ-decalactone) were found only in the current study. Other chemicals present in only one study of our prior studies at > 1 mg/mL included acetoin, allyl hexanoate, linalool, strawberry glycidate_A and _B,[14] and benzaldehyde, cinnamaldehyde, ethyl cinnamate, and p-anisaldehyde.[12]

DISCUSSION

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

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

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

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

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

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

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

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

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

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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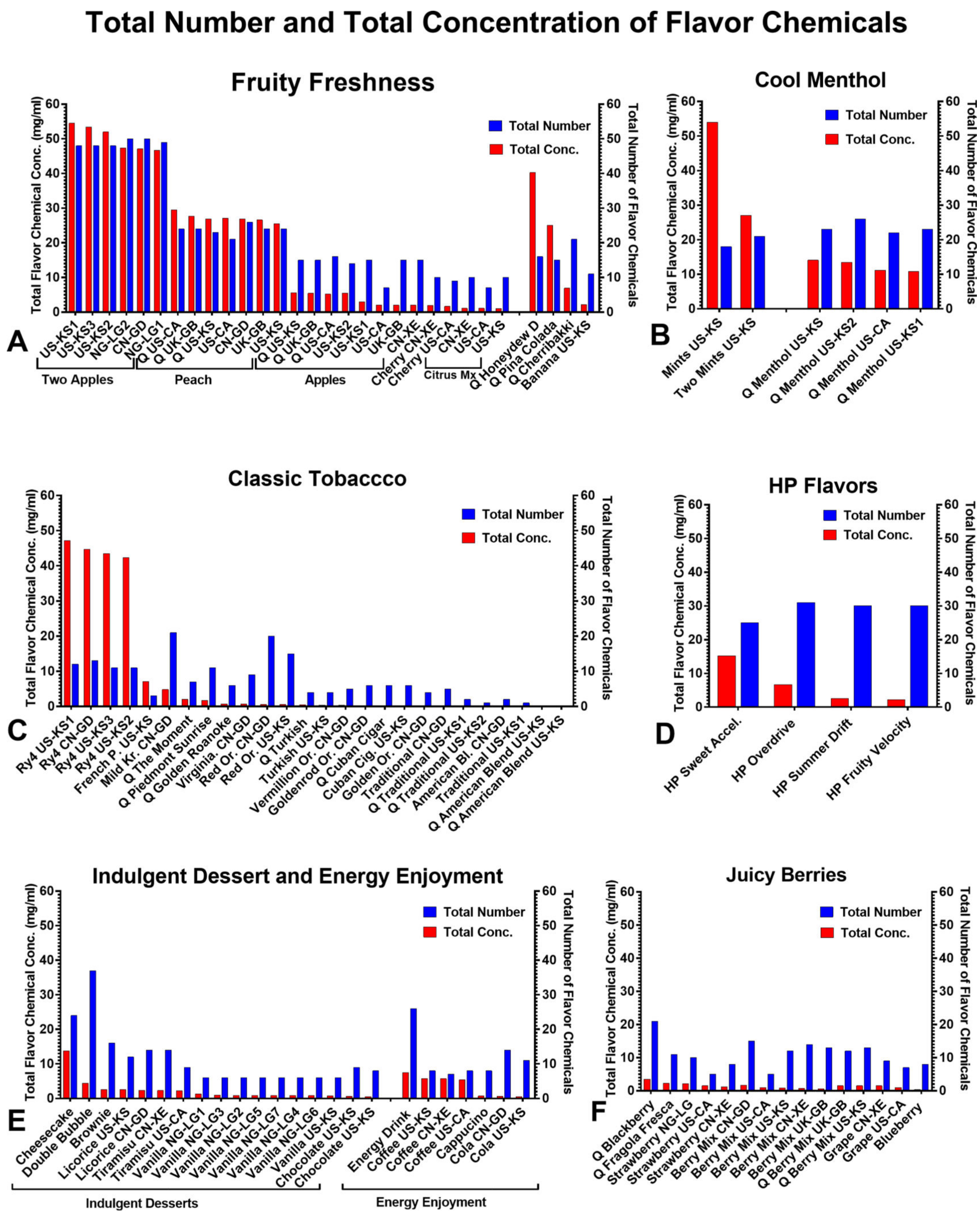

Figure 1.

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

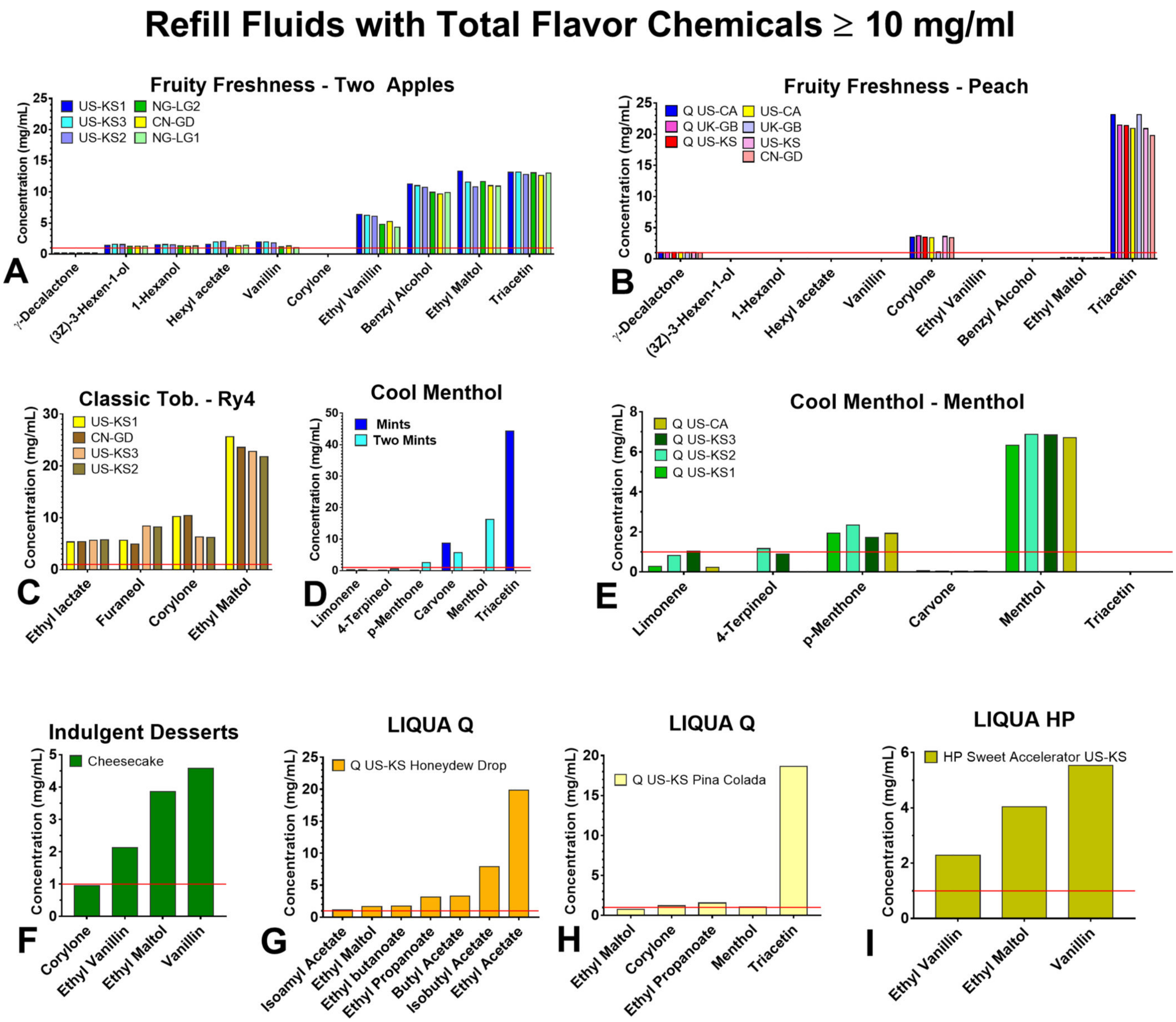

Figure 2.

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

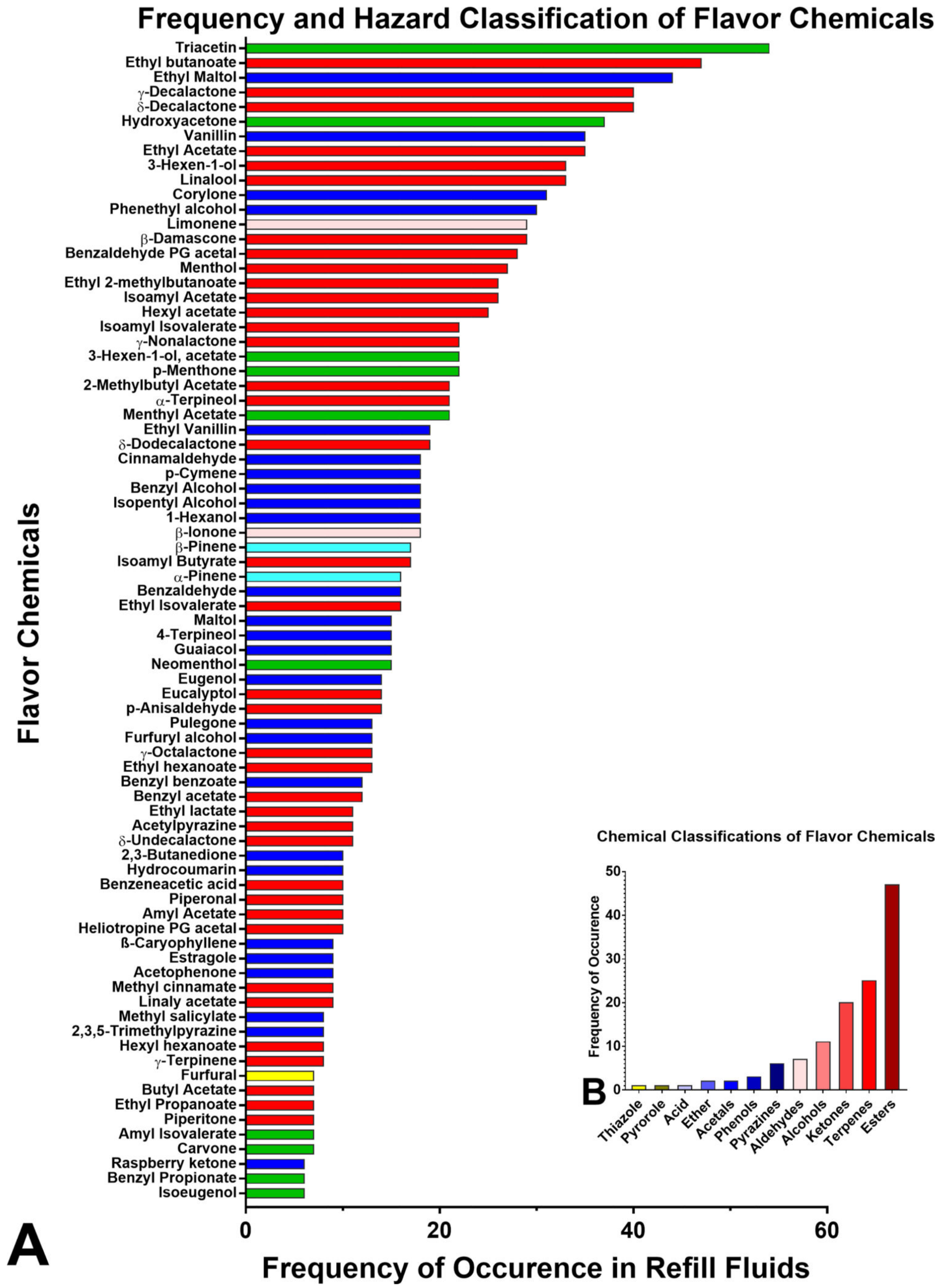

Figure 3.

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

= irritant and dangerous to the environment, cyan = harmful and dangerous to the environment. light yellow = toxic and dangerous to the environment. (b) The chemical classes of the flavor chemicals (x-axis) are plotted versus the frequency of occurrence of each class of flavor chemicals (y-axis).

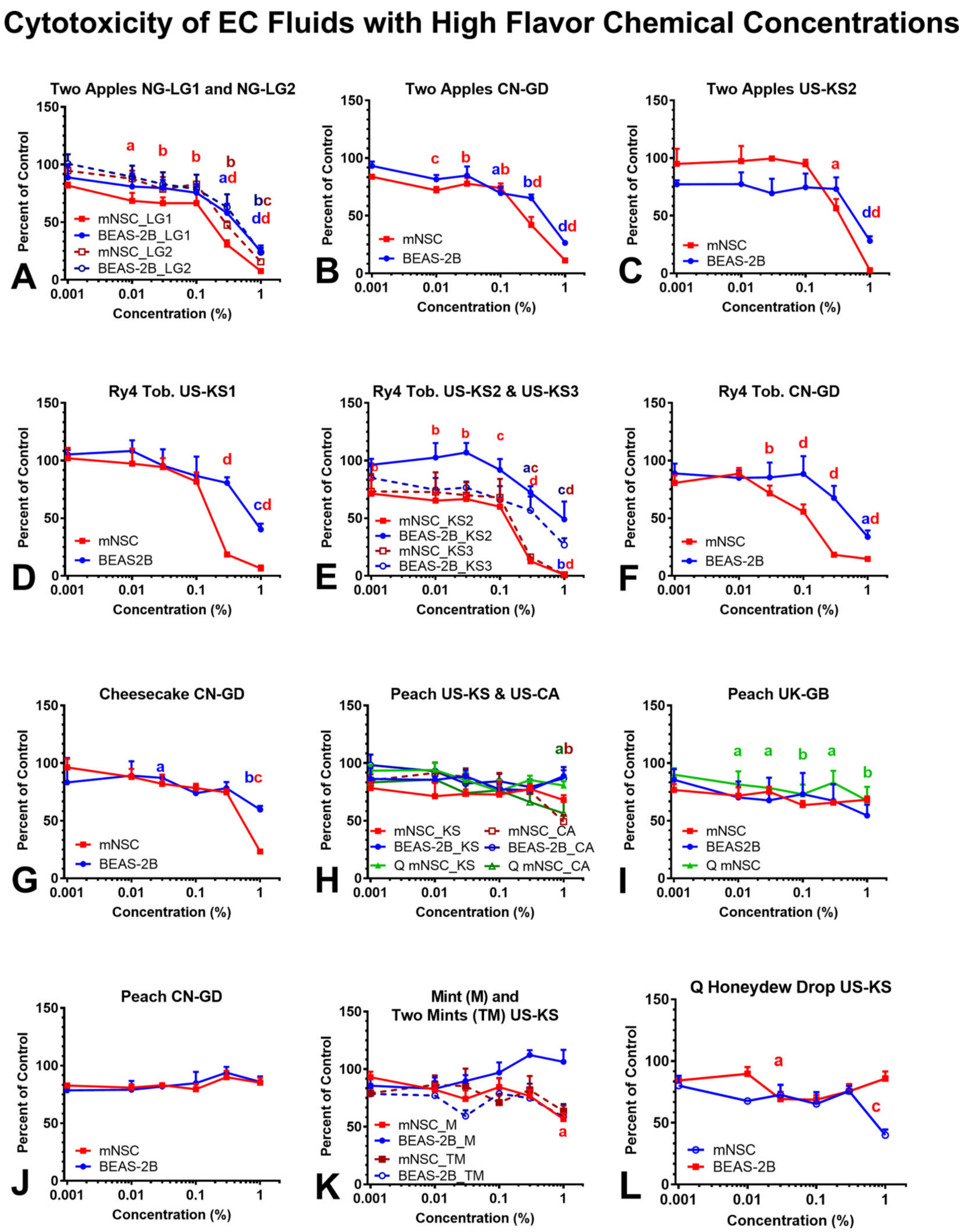

Figure 4.

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

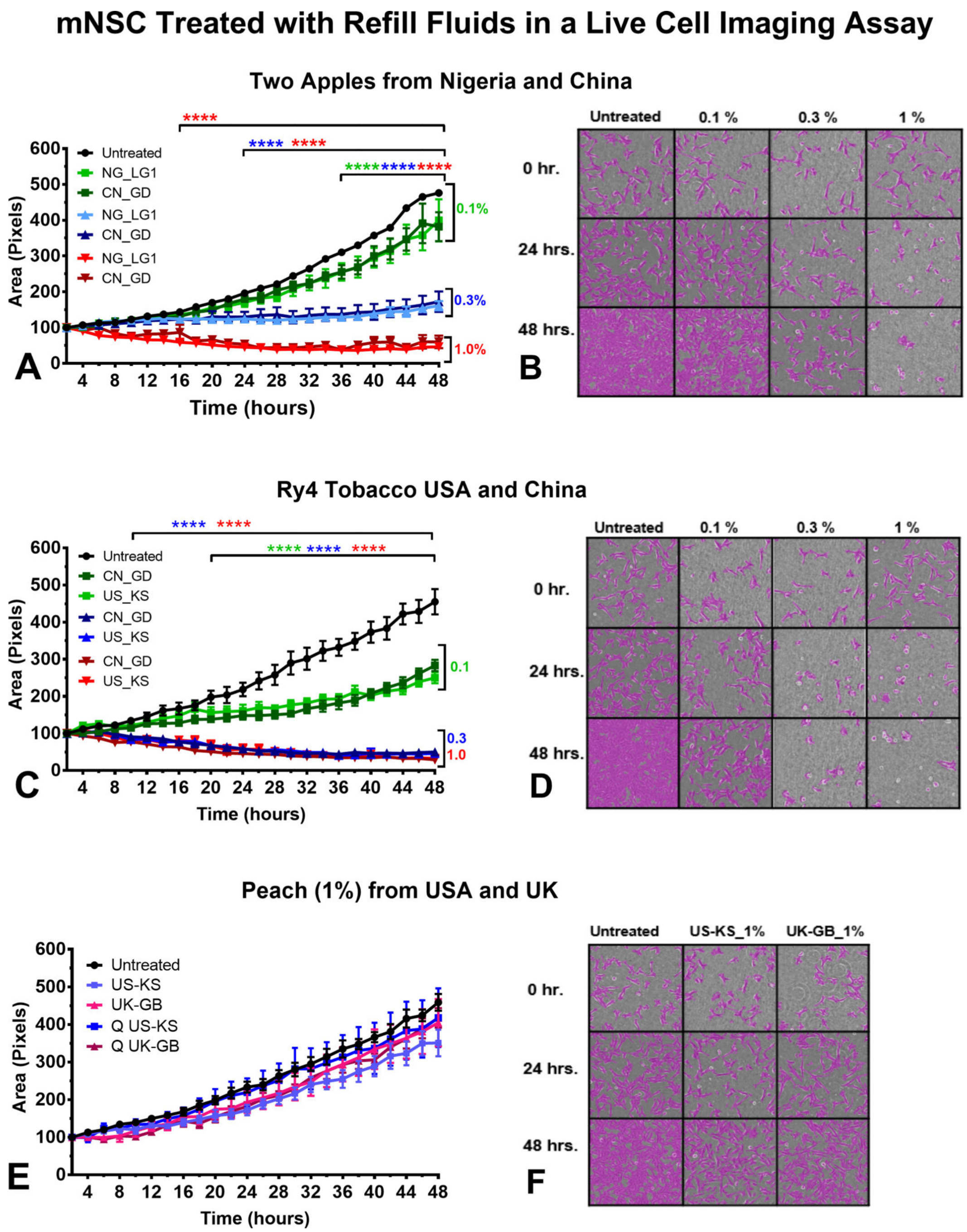

Figure 5.

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

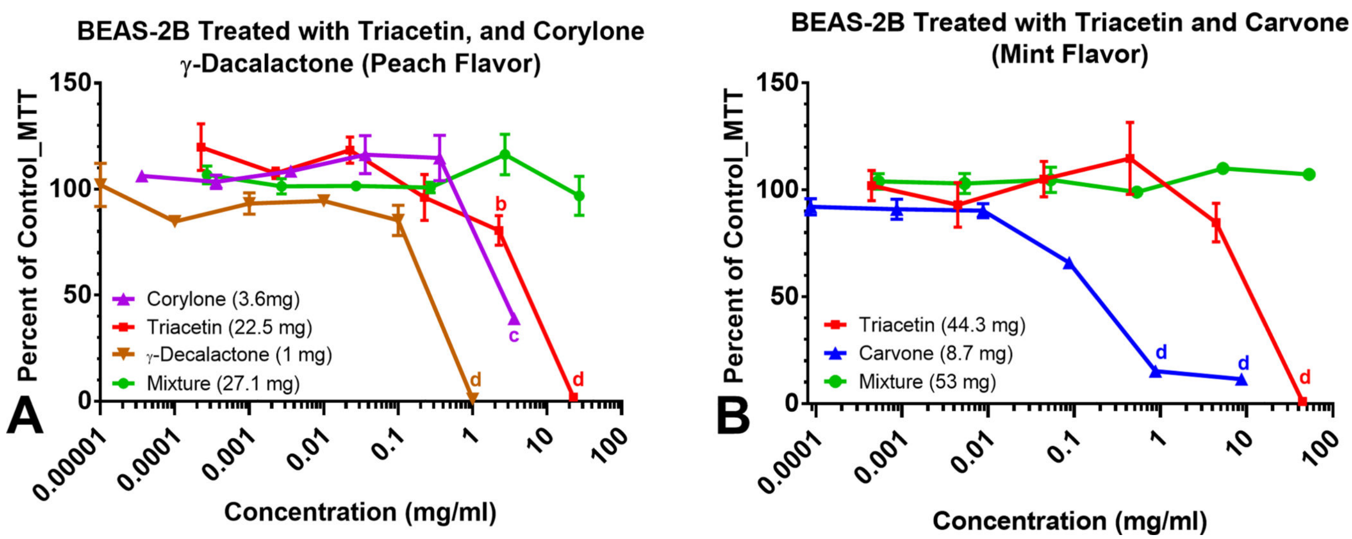

Figure 6.

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

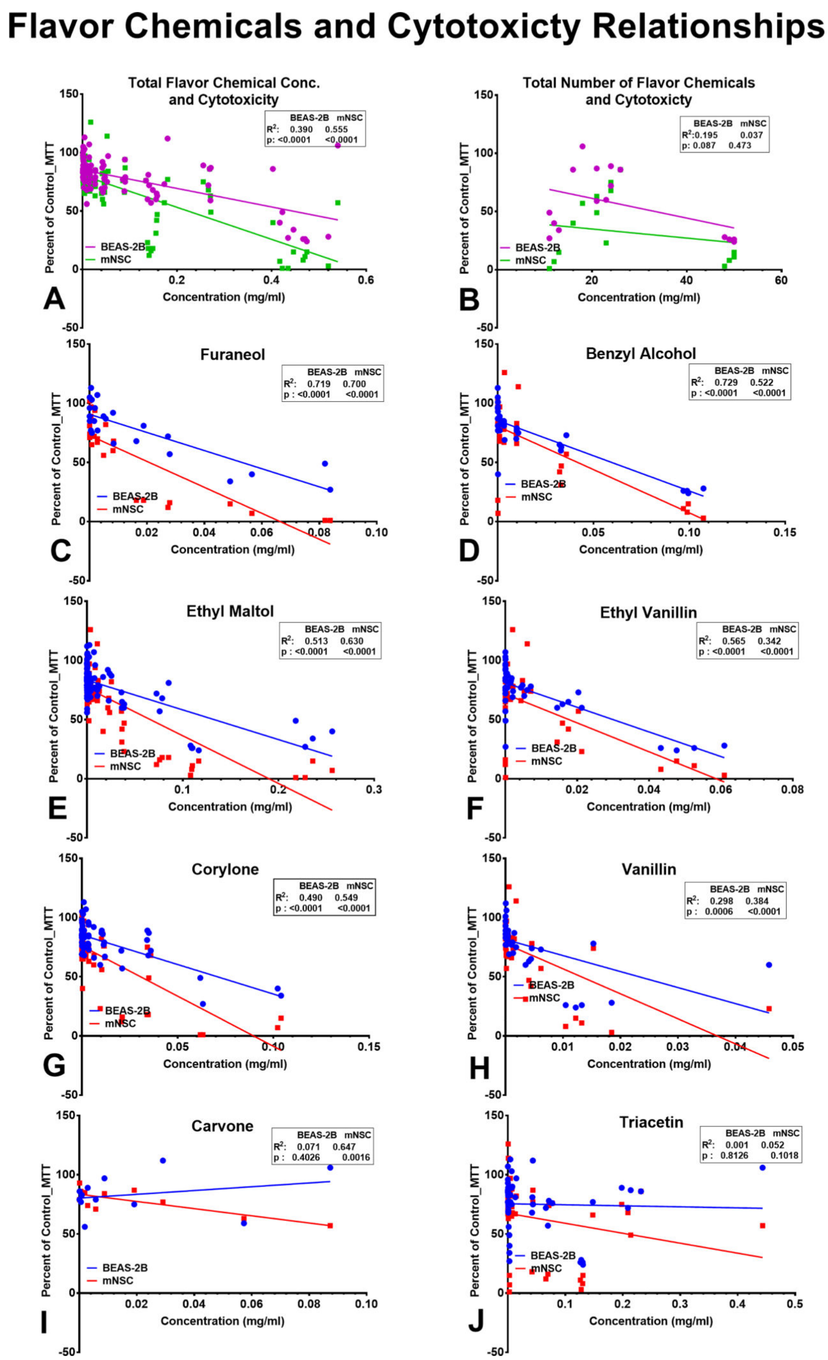

Figure 7.

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

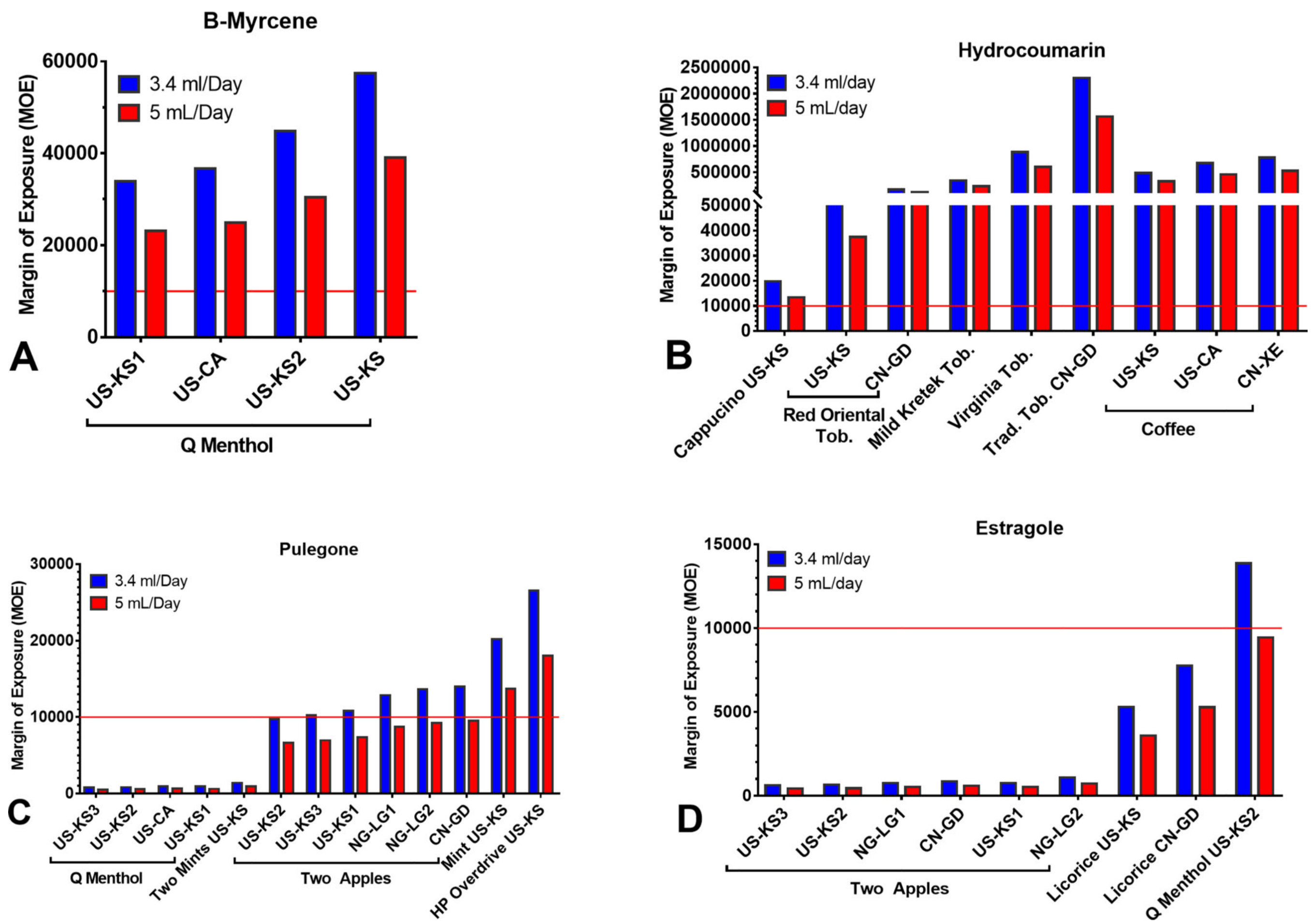

Figure 8.

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

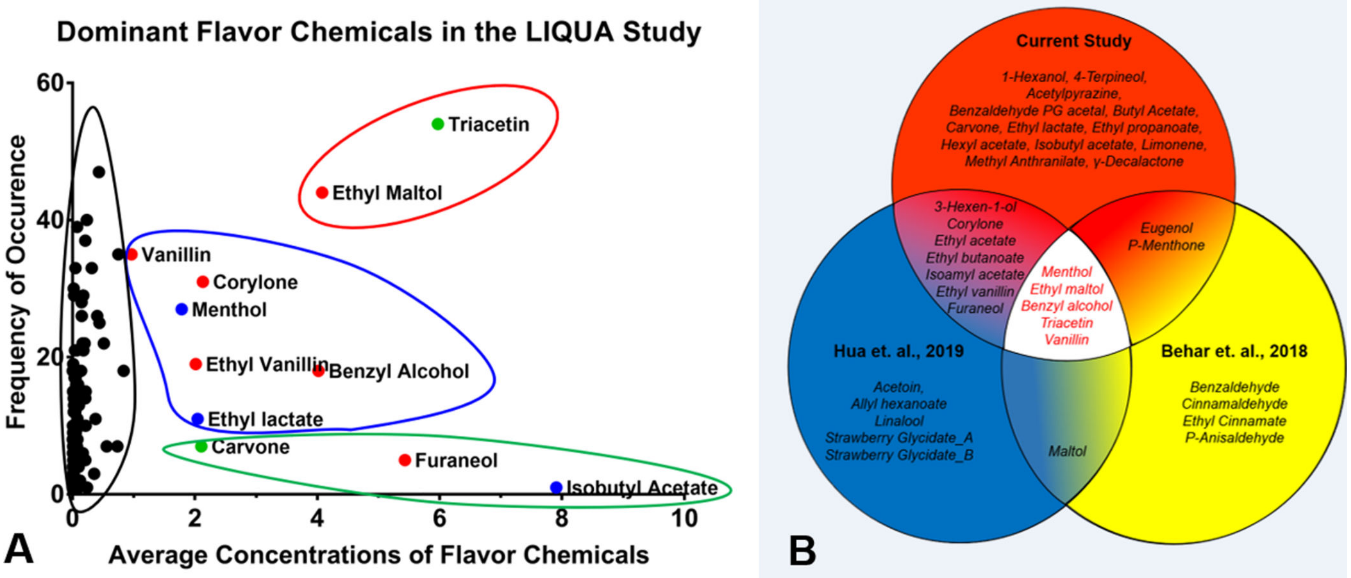

Figure 9.

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

Table 1.

LIQUA EC Refill Fluids and Their Respective Flavor Categories

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

Table 2.

IC_{70}s and IC_{50}s for Cytotoxic Refill Fluids

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

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

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

Table 3.

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

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

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

Table 4:

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

	Carcinogen/Food Additive	Daily Consumption 3.4 mL	5 mL	Reference Point	Study Ref.
Q Menthol US-KS1	B myrcene	33916	23063	64 mg/kg bw/day ($BMDL_{10}$)[2]	FDA, 2018[24]
Q Menthol US-CA		36669	24935		
Q Menthol US-KS2		44818	30476		
Q Menthol US-KS		57476	39084		
Cappuccino US-KS	Hydrocoumarin	19732	13418	150 mg/kg bw/day (NOAEL)[3]	NTP, 1993[26]
Coffee US-KS		483481	328767		
Coffee US-CA		674410	458599		
Coffee CN-XE		778547	529412		
Red Oriental CN-GD		55118	37480		
Red Oriental CN-GD		169412	115200		
Mild Kretek CN-GD		344894	234528		
Virginia CN-GD		882353	600000		
Traditional CN-GD		2301790	1565217		
Q Menthol US-KS	Pulegone	788	536	13.39 mg/kg bw/day (NOAEL)[3]	FDA, 2018[24]
Q Menthol US-KS2		837	569		
Q Menthol US-KS1		918	624		
Q Menthol US-CA		938	638		
Two Mints US-KS		1413	961		
Mints US-KS		20196	13733		
Two Apple US-KS2		9724	6612		
Two Apple US-KS3		10251	6971		
Two Apples US-KS1		10839	7371		
Two Apples NG-LG1		12877	8756		
Two Apples NG-LG2		13619	9261		
Two Apples CN-GD		13982	9508		
HP Overdrive US-KS		26550	18054		
Two Apple US-KS3	Estragole	637	433	3.3 mg/kg bw/day ($BMDL_{10}$)[2]	van den Berg, 2014[25]
Two Apple US-KS2		668	454		
Two Apples NG-LG1		764	520		
Two Apples CN-GD		863	587		
Two Apples US-KS1		769	523		
Two Apples NG-LG2		1086	739		
Licorice US-KS		5294	3600		
Licorice CN-GD		7765	5280		
Q Menthol US-KS2		13866	9429		

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

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

[3] NOAEL = No Observed adverse Effect Level.