JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Occurrence of Perillaldehyde and Other (Suspected) Genotoxic Flavoring Substances in Water-Based Beverages Consumed by Belgian Children

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Cite This: J. Agric. Food Chem. 2023, 71, 18538−18545 Read Online

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ABSTRACT: Despite the extensive use of flavoring substances in food, their monitoring for regulatory purposes is currently limited. This raises public health issues, especially as some compounds are prohibited due to (geno)toxicity. A solvent-assisted flavor evaporation (SAFE) method coupled with GC/MS (SIM) was validated here for diverse water-based beverages. Thirty flavoring substances out of the 38 targeted were validated, showing good analytical performances and confirming the versatility of the SAFE technique. The method was then applied to 94 samples, including fruit juices, iced teas, lemonades, colas, and sports beverages. Overall, seven different flavoring substances of interest were detected in the samples. Perillaldehyde and furan-2(5*H*)-one, two genotoxic flavoring substances, were found at concentrations up to 153 and 143 $\mu g \cdot kg^{-1}$, respectively. Perillaldehyde levels were significantly higher in commercial citrus juices than in freshly squeezed juices. Food control laboratories could use the developed method to face the current growing need to improve flavoring substance monitoring and conduct risk assessments.

KEYWORDS: *flavoring substance monitoring, SAFE, GC/MS, genotoxicity, perillaldehyde, furan-2(5H)-one*

1. INTRODUCTION

"Flavoring" is a general term used to describe a food ingredient used to improve or modify odor and/or taste. Generally added in small amounts, flavorings are extensively used in all food categories. In Europe, they are grouped into specific categories, depending on their origins and chemical compositions. Among the "flavorings", one should distinguish a "flavoring substance" from a "flavoring preparation". The first is a chemically defined substance with flavoring properties (e.g., limonene and menthol). To be designated as natural, a flavoring substance must be obtained from a material of vegetal, animal, or microbiological origin by specific traditional food preparation processes and identified in nature (e.g., purified limonene obtained by steam distillation from oranges).[1] In turn, a "flavoring preparation" is a product other than a flavoring substance, obtained by specific traditional food preparation processes (e.g., distillation, filtration, and peeling), often resulting in complex mixtures of flavoring substances such as plant extracts/oils (e.g., mint extracts and orange essential oils). Other flavoring categories are defined in Regulation (EC) No. 1334/2008, including thermal process flavorings, smoke flavorings, flavor precursors, and other flavorings or mixtures.[1]

According to Regulation (EC) No. 1334/2008, two general conditions exist for using flavorings or food ingredients with flavoring properties. First, they should not pose a safety risk to the consumer's health based on available scientific evidence. Second, their use should not mislead the consumer. Concerning labeling, Regulation (EU) No. 1169/2011 only requires the term "flavoring" (or "smoke flavoring" if applicable) to be present on the label when a flavoring is used.[2] More detailed designations are possible following the requirements of Regulation (EU) No. 1169/2011. As flavorings are often complex mixtures of chemicals (especially flavoring preparations), their entire chemical composition is never provided to the consumer. Moreover, labels do not require quantitative indication and nutrition declaration since flavorings generally comprise only 0.1−2% of the product and do not significantly contribute to energy intake.

Despite being minor ingredients in food, the safety of flavorings is evaluated by the European Food Safety Authority (EFSA), and their use is accordingly regulated by Regulation (EC) No. 1334/2008. Since 2012, all authorized flavoring substances in food—around 2500—have been included in a positive list, also called the Union list. Only a few compounds are subject to maximum levels in specific food categories. In addition to the Union list, Annex III of Regulation (EC) No. 1334/2008 lists 15 naturally present substances (e.g., estragole, menthofuran, and pulegone) forbidden to be added as such to food. Maximum levels for some of these natural substances are also in place for some food categories (e.g., maximum 350 $mg \cdot kg^{-1}$ of pulegone in chewing gum). Interestingly, while they are authorized in the Union list, a few flavoring substances are still in the process of safety evaluation by the EFSA, primarily due to the lack of toxicological data. Other flavoring substances have instead already been removed from the Union list because

Received: August 2, 2023
Revised: October 16, 2023
Accepted: October 20, 2023
Published: November 16, 2023

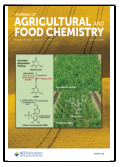
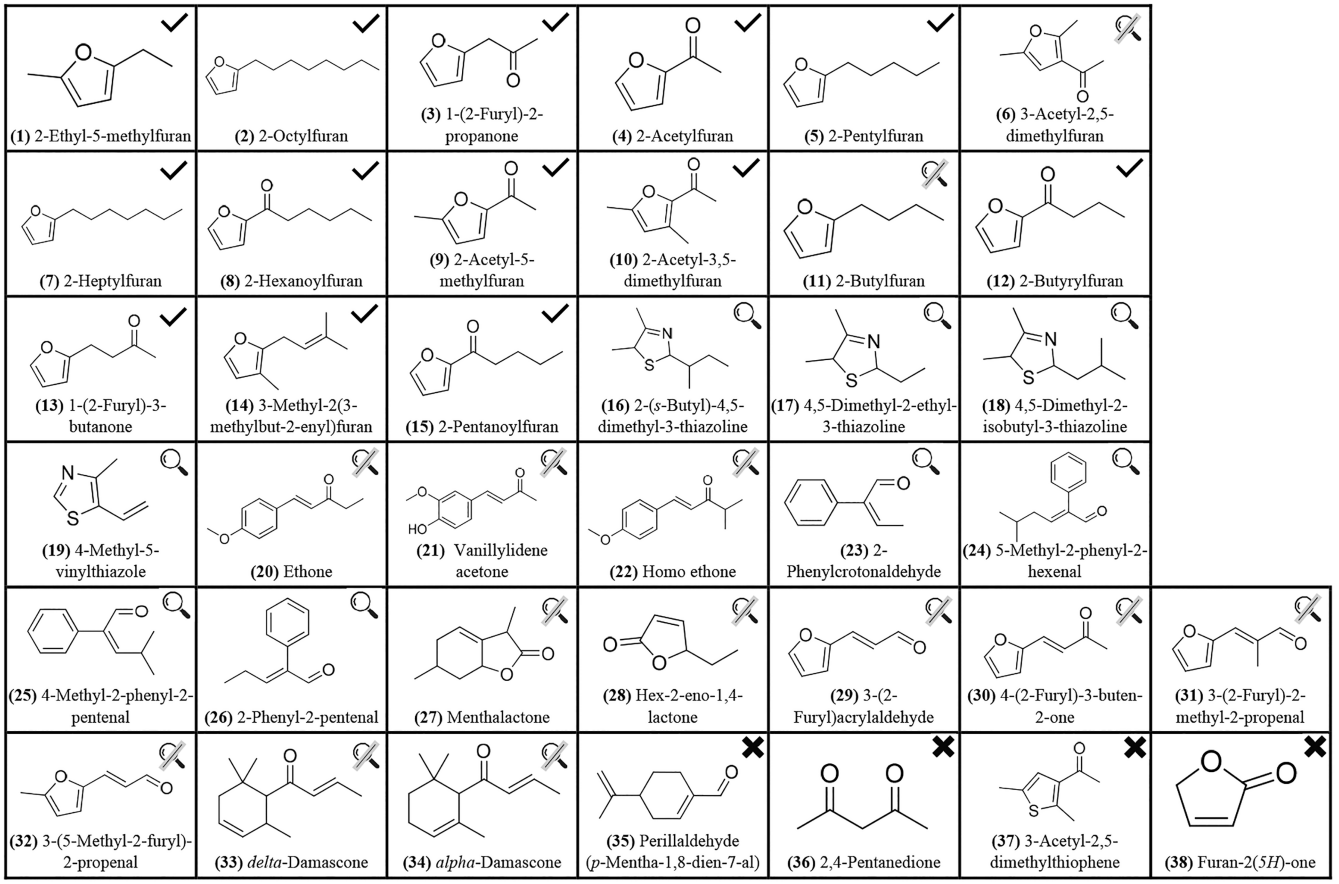


Table 1. Selected Flavoring Substances for Analysis[a]



[a] ✓: Compounds evaluated as safe by EFSA. 🔍: Compounds under evaluation by EFSA. 🔍/: Compounds whose evaluation by EFSA was stopped due to discontinued use by industry. ✖: Compounds evaluated as genotoxic by EFSA. Data updated up to Jan 2023.

they demonstrated (geno)toxic effects. This is the case of *p*-mentha-1,8-dien-7-al (perillaldehyde) and furan-2(5*H*)-one, removed from the list for genotoxicity in 2015 and 2019, respectively. Alpha, beta-unsaturated carbonyls and alpha, beta-unsaturated lactones (after hydrolysis and oxidation) are indeed structural alerts for genotoxicity due to their high reactivity (e.g., nucleophilic addition of DNA).

Member states are responsible for monitoring the consumption and use of flavoring substances in food and beverages. According to Regulation (EC) No. 1334/2008, this monitoring should be risk-based. However, the analysis of flavoring substances for law enforcement needs to be improved as validated methods are not widely implemented in official control laboratories.[3] Current methods for analyzing flavoring substances are often specific to a few volatiles, mainly those from Annex III of Regulation (EC) No. 1334/2008. In 2011, a method using headspace solid-phase microextraction coupled with GC/MS/MS was developed and validated for the simultaneous analysis of coumarin, beta-asarone, estragole, menthofuran, methyleugenol, pulegone, and thujone in (non)alcoholic beverages, semisolid foods (e.g., soups, sauces, and confectionary), and solid foods (e.g., muesli and bakery products).[4] More recently, Lopez et al. developed and validated an extraction procedure using solvent extraction coupled with GC/MS for the simultaneous analysis of 14 volatiles in different types of liquids, semisolid, dry-solid, and fatty-solid foods such as tomato sauce, oat flakes, or cheese. Seven flavoring substances were from Annex III of Regulation (EC) No. 1334/2008.[5]

Over the past 20 years, the solvent-assisted flavor evaporation (SAFE) technique initially developed by Engel et al.[6] has been used to study key odorants in several food matrices. This technique consists of sample distillation under a high vacuum (10^{-3} Pa) and low temperature (40 °C). Due to its nonselective nature, this technique is versatile and enables multianalyte analysis, either quantitatively or semiquantitatively (e.g., screening of odor-active compounds by GC-O/AEDA). Many food matrices have already been analyzed (e.g., coffee, fruits, juices, fishes, and alcoholic beverages) with adaptations to the method made on a case-by-case basis but without a full validation procedure following method development.[7−10]

Recently, a SAFE-GC/MS method was developed and validated for the simultaneous analysis of 29 flavoring substances in alcohol-free beers.[11] Following these initial results, the SAFE method was developed and validated in the present study to analyze 38 (suspected) genotoxic flavorings in different nonalcoholic beverages. Afterward, the method was applied to analyze 94 commercial samples, including fruit juices, iced teas, lemonades, colas, and sports beverages. To the best of our knowledge, this is the first time that the occurrence of (suspected) genotoxic flavoring substances has been studied in such food matrices. As flavoring substances are used or are naturally occurring in numerous food categories, it was

https://doi.org/10.1021/acs.jafc.3c05381
J. Agric. Food Chem. 2023, 71, 18538−18545

expected to identify them in the analyzed food products but with levels hardly predictable due to lacking literature data. Ultimately, this work aimed to provide a validated, accurate multianalyte method for the analysis of flavoring substances with a public health concern and provide more occurrence data to fill in the literature gap of such substances.

2. MATERIALS AND METHODS

2.1. Selection of Flavoring Substances. The selection was made based on our previous work.[11] Among the authorized flavoring substances in Europe, compounds under safety evaluation (primarily due to genotoxic concerns) were retrieved by their attributed footnotes within Regulation (EC) No. 1334/2008. This information was consolidated with the relevant EFSA opinions. The identified flavoring substances under safety evaluation were selected, and the standard commercial availability was verified. In addition, four flavoring substances forbidden due to genotoxicity were also selected for monitoring, i.e., perillaldehyde, 2,4-pentanedione, 3-acetyl-2,5-dimethylthiophene, and furan-2(5*H*)-one.

2.2. Selection of Samples. The sampling scheme focused on the food consumption habits of children as they are a sensitive population. The most recent Belgian Food Consumption Survey was used to identify which beverages were the most consumed by children between 3 and 9 years old.[12] Samples were distributed within the identified food categories proportionally to their frequency of consumption and their daily mean consumption per body weight. Furthermore, they were sampled among national and distributor brands to provide a broad food survey. The sampling scheme also considered product labeling information. Some products were chosen for their absence of flavorings (according to the definitions of Regulation (EC) No. 1334/2008), while other similar products were selected because of the presence of the term "flavoring(s)" (or a more detailed description) on the label. Additionally, special attention was given to samples that may contain the genotoxic flavoring substance perillaldehyde (i.e., citrus-containing products). Two freshly squeezed citrus juices (handmade) were also added for comparison with supermarket products. The sampling campaign was done continuously from March 2021 to February 2022 in accordance with laboratory capabilities to avoid any analysis after the food product's expiration date. Finally, photographs of food products were taken after purchasing, and the ingredient list was carefully transcribed. Supporting Information 1 describes all samples with relevant labeling information.

2.3. Reference Flavoring Substances and Chemicals. 1-(4-Methoxyphenyl)-4-methyl-1-penten-3-one (**22**, homo ethone), 2-acetylfuran (**4**), 2-acetylthiophene, 3-acetyl-2,5-dimethylfuran (**6**), 3-acetyl-2,5-dimethylthiophene (**37**), 4-methyl-2-phenyl-2-pentenal (**25**), 4-methyl-5-vinylthiazole (**19**), 5-methyl-2-phenyl-2-hexenal (**24**), *α*-damascone (**34**), *δ*-damascone (**33**), menthalactone (**27**), *n*-decane, perillaldehyde (**35**, *p*-mentha-1,8-dien-7-al), and vanillylidene acetone (**21**) were purchased from Merck (Overijse, Belgium). 1-(2-Furyl)-2-propanone (**3**), 2-(*s*-butyl)-4,5-dimethyl-3-thiazoline (**16**), 2-acetyl-5-methylfuran (**9**), 2-hexanoylfuran (**8**), 2-butylfuran (**11**), 2-butyrylfuran (**12**), 2-heptylfuran (**7**), 2-pentanoylfuran (**15**), 2-pentylfuran (**5**), 2-phenylcrotonaldehyde (**23**), 2-phenyl-2-pentenal (**26**), 3-(2-furyl)-2-methyl-2-propenal (**31**), 3-(2-furyl)acrylaldehyde (**29**), 4-(2-furyl)-3-buten-2-one (**30**), 4,5-dimethyl-2-ethyl-3-thiazoline (**17**), 4,5-dimethyl-2-isobutyl-3-thiazoline (**18**), 2,4-pentanedione (**36**), and furan-2-(5*H*)-one (**38**) were purchased from Fisher Scientific (Bruxelles, Belgium). 1-(2-Furyl)-3-butanone (**13**), 1-(4-methoxyphenyl)-1-penten-3-one (**20**, ethone), 2-acetyl-3,5-dimethylfuran (**10**), 3-(5-methyl-2-furyl)-2-propenal (**32**), and hex-2-eno-1,4-lactone (**28**) were purchased from Chemspace (Riga, Latvia). Structures and numbering are given in Table 1. Dichloromethane (>99.8%) and ethanol absolute (99%) were purchased from VWR International (Leuven, Belgium). Anhydrous sodium sulfate was purchased from Merck (Overijse, Belgium).

2.4. Isolation of Flavoring Substances. The procedure previously developed for alcohol-free beers was used here.[11] Homogenized (vigorously agitated) samples (50 g) were spiked with 150 μL of 2-acetylthiophene solution (8 mg·L^{-1}) as an internal standard (IST). Samples were then extracted with bidistilled dichloromethane (1 × 75 mL) for 20 min. The aqueous phase was discarded if no emulsion appeared, and the remaining organic phase was dried over anhydrous sodium sulfate. Otherwise, the resulting emulsion was centrifuged (20 min at 2264*g*) to separate the two phases. The SAFE system (Glasblaeserei Bahr, Manching, Germany) was used to separate by high-vacuum distillation the nonvolatile compounds from the organic phase.[6] The SAFE conditions were as follows: water bath temperature 40 °C, pressure below 10^{-3} Pa, and the apparatus body at 30 °C. The distillate was continuously recovered in the liquid-nitrogen-cooled SAFE flask for 15 min distillation. Once returned to room temperature, the extract was dried over anhydrous sodium sulfate. Additionally, 25 μL of decane solution (250 mg·L^{-1}) was spiked as an external standard (EST). The extract was then concentrated to 500 μL in a Kuderna-Danish apparatus at 45 °C. To ensure maximum stability, extracts were stored at −80 °C until analysis by GC-EI-MS.[11]

2.5. Gas Chromatography/Mass Spectrometry. The SAFE extracts were analyzed by GC/MS using a temperature program previously developed.[11] A wall-coated open tubular apolar capillary column (CP-Sil 5 CB, 50 × 0.32 mm inner diameter, 1.2 μm film thickness) was used on an Agilent 7890B gas chromatograph. Extracts (1 μL) were injected in splitless mode (250 °C front inlet). The carrier gas was helium, and the pressure was set at 65 kPa. The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C (held for 30 min) at 3 °C/min. A single quadrupole mass spectrometer (Agilent 5977B MSD) was used for detection of the analytes in selected ion monitoring (SIM) mode with electron ionization 70 eV. Extracts were also analyzed in full-scan (m/z 40−380) for potential subsequent qualitative analysis. Data were recorded and analyzed with Agilent OpenLab software (version 2.1). Retention indices and major ions are listed for all compounds in Supporting Information 2.

2.6. Quantification of Flavoring Substances. This study did not use stable isotope dilution (SIDA) because isotopically labeled standards were not commercially available for all compounds included in the method. To ensure an accurate correction for extraction losses and variation of the detector sensitivity, quantifications were made by standard addition (4 spikes) with an internal standard (2-acetylthiophene). The concentration of an analyte X in the sample was determined with the following equation: [X] (in μg·kg^{-1}) = [IST] (in μg·kg^{-1}) × (X area/IST area) × (IST response coefficient/X response coefficient) × (IST absolute recovery/X absolute recovery). Similar samples were grouped and quantified based on the standard addition slopes of their respective similar reference sample (e.g., reference juice; reference cola).

2.7. Validation. An in-house validation of the method was done in terms of linearity, compound stability, matrix effects, repeatability, within-lab reproducibility, limit of detection (LOD) and quantification (LOQ), selectivity, and recoveries. Samples were grouped based on food matrix similarities. The fortification levels were based on the expected concentrations of flavoring substances in the targeted matrix. Reapeatability and within-lab reproducibility were evaluated based on ISO 5725 and ISO 11843 standards.[13] Repeatability and within-lab reproducibility coeficents of variation were determined (Horwitz statistical analysis) by analyzing samples, in triplicate, on three different days. Relative standard deviations (RSD) were then calculated. Measurement uncertainty (MU) was assessed by taking two times the RSD for within-lab reproducibility.[14,15] Validation results are provided in Supporting Information 3.

2.8. Quality Control. For each batch, the sensitivity of the analytical instrumentation was verified by injecting standards at concentrations corresponding to their LOQ, and the signal-to-noise ratio should be at least 10 for each compound. The absence of standard carryover was ensured during GC method development and was verified by injecting solvent blank between batches. According to European recommendations, compounds were identified based on retention times and ion relative intensities compared to the injected

https://doi.org/10.1021/acs.jafc.3c05381
J. Agric. Food Chem. 2023, 71, 18538−18545

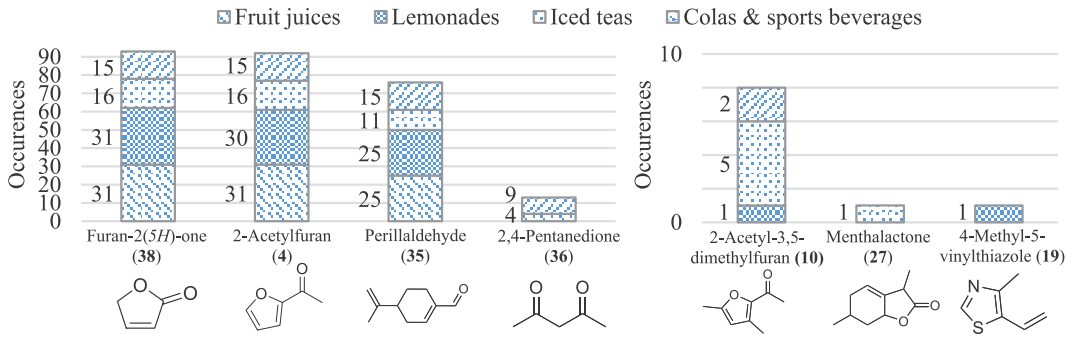


Figure 1. Number of occurrences of selected flavoring substances among the 94 samples.

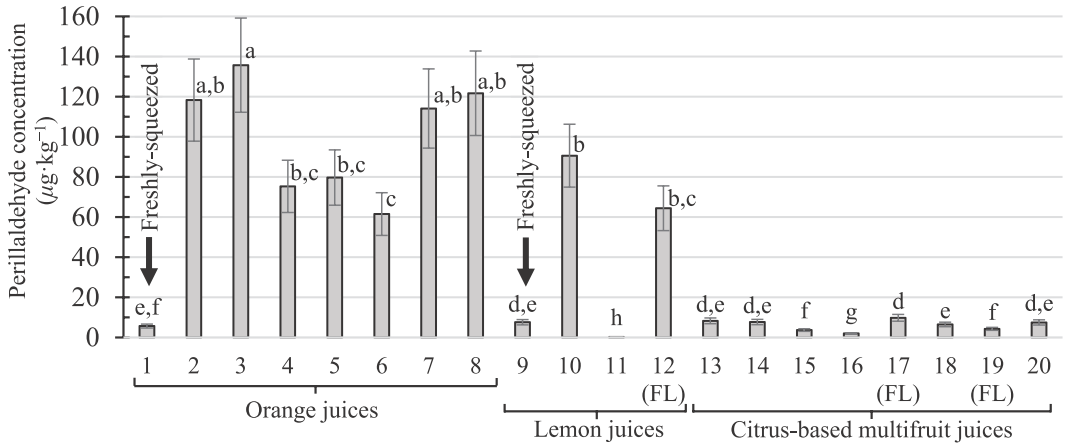


Figure 2. Perillaldehyde levels in orange juices (1−8), lemon juices (9−12), and citrus-based multifruit juices (13−20). "(FL)" stands for the mention of "flavoring(s)" (or more detailed) on the product labeling. Error bars represent confidence intervals at 95%. Significantly different results are shown with a distinct letter, the letter "a" being the highest value of the series.

analytical standards.[16] The NIST 2014 (2.2 version) spectral database was used for the qualitative monitoring of compounds **1**, **2**, and **14** as not being commercially available.

2.9. Statistical Analysis. The measurement of uncertainty (MU) was determined, as described in Section 2.7. Afterward, the MU was considered for the interpretation of the results of the samples. On the graphs, it is shown as error bars representing the confidence intervals at 95%. Significantly different results are shown with a distinct letter, the letter "a" being the highest value of the series. No overlapping error bars indicate a significant difference. In that case, samples are marked with a different letter. Data were processed using Excel 2021 (Microsoft Corporation).

3. RESULTS AND DISCUSSION

3.1. Selection of Flavoring Substances. According to the consolidation of Regulation (EC) No. 1334/2008 of 26th September 2022, among 2479 authorized flavoring substances, the safety evaluation was still pending for 250 compounds (substances with a footnote). Pending evaluations were primarily due to genotoxic concerns, requiring additional data before a conclusion could be drawn. EFSA evaluations were also checked for consolidation of the data, as described in our previous work.[11] This revealed that only 12 flavoring substances were still under genotoxicity evaluations by the EFSA. These are stipulated here as suspected genotoxic flavoring substances. Analytical standards were available for eight of them. Because Regulation (EC) No. 1334/2008 consolidations and EFSA opinions were continuously updated during this work, the analysis was initiated on a broader selection of compounds. All of these substances were still maintained in the current method for scientific knowledge. In addition, four confirmed genotoxic flavoring substances that were no longer authorized to be added as such to food in Europe were also included in this study: perillaldehyde (**35**, *p*-mentha-1,8-dien-7-al), 2,4-pentanedione (**36**), 3-acetyl-2,5-dimethylthiophene (**37**) and furan-2(5*H*)-one (**38**). In total, 38 compounds were selected for analysis and are listed in Table 1 with their current safety evaluation status. Except for ethone (**20**) and homo ethone (**22**), all selected compounds have already been identified in nature.[17−19]

3.2. Sample Analysis. The most recent Belgian Food Consumption Survey was used to identify which beverages were the most consumed by children between 3 and 9 years old.[12] In total, 94 samples were distributed among these categories proportionally to their consumption. Accordingly, 33 fruit juices, 31 lemonades, 14 iced teas, 11 colas, and 5 sports beverages were selected (purchased from Belgian supermarkets, including national and distributor brands) and analyzed.

Among the 38 flavoring substances monitored, seven different flavoring substances of interest were found among all samples combined, for a total of 284 occurrences (Figure 1). Furan-2(5*H*)-one (up to 143 $\mu g \cdot kg^{-1}$) was the most commonly found compound followed by 2-acetylfuran (up to 41 $\mu g \cdot kg^{-1}$) and perillaldehyde (up to 153 $\mu g \cdot kg^{-1}$). Fewer occurrences and lower levels were reported for 2,4-pentanedione (up to 20 $\mu g \cdot kg^{-1}$), 2-acetyl-3,5-dimethylfuran (up to 2 $\mu g \cdot kg^{-1}$), menthalactone (9 $\mu g \cdot kg^{-1}$), and 4-methyl-5-vinylthiazole (up to 15 $\mu g \cdot kg^{-1}$). Among these compounds, as

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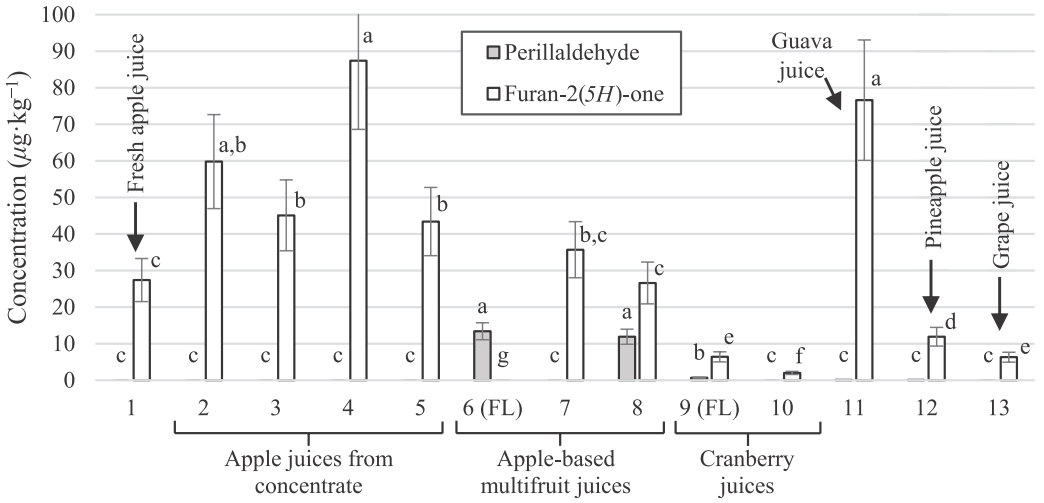

Figure 3. Perillaldehyde and furan-2(5*H*)-one levels in apple juices (1−5), apple-based multifruit juices (6−8), and other noncitrus fruit juices (9−13). "(FL)" stands for the mention of "flavoring(s)" (or more detailed) on the product labeling. Error bars represent confidence intervals at 95%. Significantly different results are shown with a distinct letter, the letter "a" being the highest value of the series.

indicated in Table 1, the genotoxic concern for 2-acetylfuran and 2-acetyl-3,5-dimethylfuran was ruled out within the time frame of this work.[20] Menthalactone was instead no longer authorized to be added to food as industry discontinued its use. This stopped its safety evaluation and led to its removal from the Union list. The only occurrence of menthalactone was nonsurprisingly in a sample of a mint-flavored ice tea, mint being a natural source of menthalactone. The following sections will discuss (suspected) genotoxic flavoring substances in different food subgroups. Additionally, it is worth noting that substances 1-(2-furyl)-2-propanone, 2-acetyl-5-methylfuran, 2-acetyl-3,5-dimethylfuran, and hex-2-eno-1,4-lactone were not found in the selected samples, in contrast to their identification in the previously analyzed alcohol-free beers.[11]

3.2.1. Citrus and Citrus-Based Juices. Twenty citrus and citrus-based juices were analyzed, including one freshly squeezed orange juice and one freshly squeezed lemon juice. The perillaldehyde content was particularly interesting due to its genotoxicity and recent deletion from the Union list.[21] Perillaldehyde is a terpenoid aldehyde naturally occurring in several plants, mainly in *Perilla* spp. and *Citrus* spp. (mainly peel). It has a powerful fatty, spicy, woody, and citrus odor (threshold of 30−62 $\mu g \cdot kg^{-1}$).[18] The perillaldehyde content of the freshly squeezed orange juice (5.7 $\mu g \cdot kg^{-1}$) was significantly lower than in commercial orange juices (either in pure juices or from concentrate), up to 136 $\mu g \cdot kg^{-1}$ (Figure 2). The same trend also occurred in lemon juices, where only 7.6 $\mu g \cdot kg^{-1}$ was observed for the freshly squeezed lemon juice compared to higher levels in some commercial lemon juices (up to 90 $\mu g \cdot kg^{-1}$). Terpenes are known to be found at higher levels in mechanically squeezed orange juices due to the higher incorporation of peel oils into the juice.[22] The results presented here confirm this trend for perillaldehyde. In the same way, the analysis of full-scan chromatograms showed much higher levels of terpenes and terpenoids in industrial juices (58 vs 12 $mg \cdot kg^{-1}$ of limonene; 1 vs 0.09 $mg \cdot kg^{-1}$ linalool, in IST equivalent). As a natural constituent of the citrus essential oil, perillaldehyde levels can be impacted by the citrus cultivar, fruit maturity, or juicing process.[22] In citrus-based multifruit juices in which other ingredients dilute the citrus content, perillaldehyde levels logically dropped to 1.5−12.9 $\mu g \cdot kg^{-1}$.

Furan-2(5*H*)-one (fruity, smoky, buttery odor), forbidden as a flavoring substance in 2019 due to genotoxicity,[23,24] was present in all fruit juices (except sample 20) with high variability, ranging from 1.6 to 31 $\mu g \cdot kg^{-1}$. Interestingly, among citrus juices, the highest level of furan-2(5*H*)-one was found in the freshly squeezed orange juice (17.6 $\mu g \cdot kg^{-1}$), while levels in commercial orange juices ranged from 2.3 to 5.7 $\mu g \cdot kg^{-1}$. Furan-2(5*H*)-one has been reported to be found in heated products such as coffee, roasted hazelnut, and cooked fish.[18,25] Its relatively higher volatility (retention index on CP-Sil 5CB = 864) could suggest a loss during industrial food processes, probably explaining the significant variability between similar samples and fresh versus commercial orange juices.

Neither 2,4-pentanedione nor 4-methyl-5-vinylthiazole was found in citrus and citrus-based juices.

3.2.2. Apple, Apple-Based Fruit Juices, and Other Noncitrus Fruit Juices. Thirteen samples were analyzed in this subgroup. Perillaldehyde was unsurprisingly absent from all apple juices but was evidenced at low levels in two apple-based multifruit juices (11.9−13.4 $\mu g \cdot kg^{-1}$), flavored and nonflavored (Figure 3). Traces of perillaldehyde were also shown in one cranberry juice (0.7 $\mu g \cdot kg^{-1}$), one guava juice (0.1 $\mu g \cdot kg^{-1}$), and one pineapple juice (0.1 $\mu g \cdot kg^{-1}$).

Compared to citrus juices, higher furan-2(5*H*)-one levels were reported in apple juices (27−87 $\mu g \cdot kg^{-1}$), yet with again high variability among samples (Figure 3). The commercial fresh apple juice had, this time, the lowest furan-2(5*H*)-one level (27 $\mu g \cdot kg^{-1}$). Among other samples, guava juice had the highest level of furan-2(5*H*)-one (76.6 $\mu g \cdot kg^{-1}$).

Traces of 2,4-pentanedione were also found in two apple juices (0.4−2.9 $\mu g \cdot kg^{-1}$) and one cranberry juice (0.4 $\mu g \cdot kg^{-1}$). This compound was previously only identified in a few products, such as cooked meat or mango.[25,26] The use of 2,4-pentanedione was forbidden in 2005 after genotoxicity was demonstrated.[27,28]

3.2.3. Lemonades. Thirty-one lemonades were analyzed. In line with our previous results, perillaldehyde was found in all citrus-based lemonades, and its content varied according to the content of "citrus-related" ingredients (i.e., citrus or citrus

https://doi.org/10.1021/acs.jafc.3c05381
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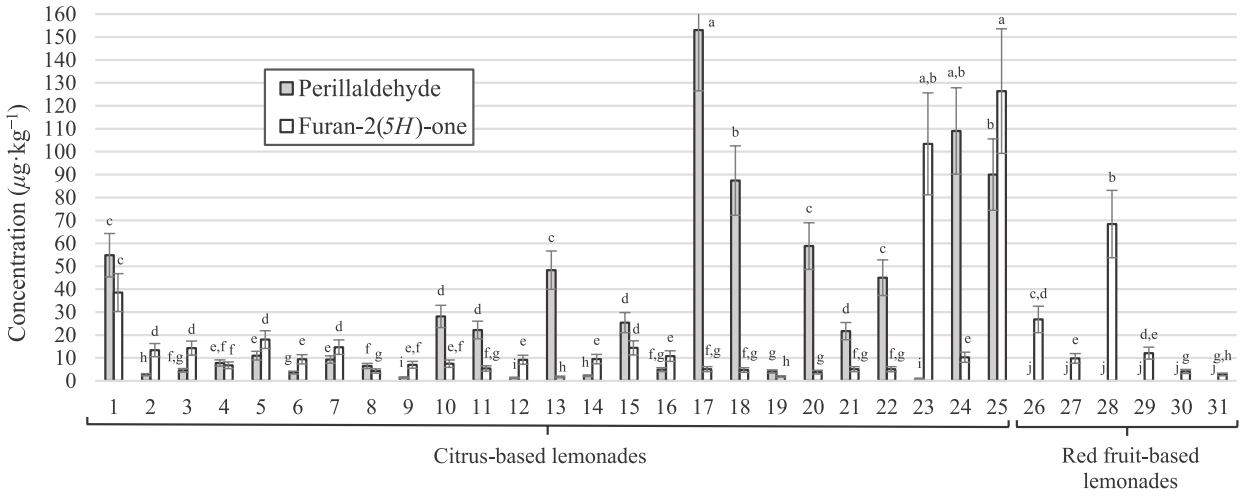

Figure 4. Perillaldehyde and furan-2(5*H*)-one levels in lemonades. All samples were flavored. Error bars represent confidence intervals at 95%. Significantly different results are shown with a distinct letter, the letter "a" being the highest value of the series.

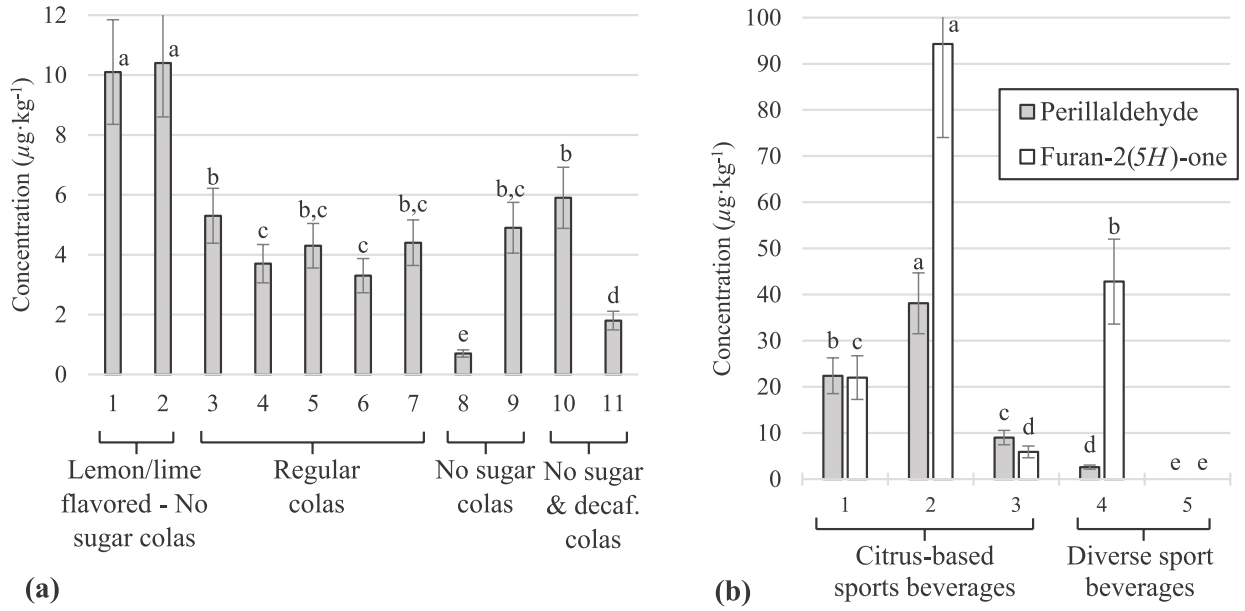

Figure 5. (a) Perillaldehyde levels in colas and (b) perillaldehyde and furan-2(5*H*)-one levels in sports beverages. All samples were flavored. Error bars represent confidence intervals at 95%. Significantly different results are shown with a distinct letter, the letter "a" being the highest value of the series.

flavorings). However, this relationship was difficult to establish (Figure 4). A dilution of its own of the "citrus-related" ingredient was insufficient to explain the variation magnitude. For instance, similar flavored samples differing only by a few percentages in their citrus juice content ranged from 4.6 to 54.8 $\mu g \cdot kg^{-1}$ perillaldehyde content. Therefore, the nature of the citrus flavorings used is probably the key factor (e.g., extraction techniques and citrus variety) to explain the variability of these concentrations. The highest level of perillaldehyde (153 $\mu g \cdot kg^{-1}$) was found in sample no. 17, containing only 10% of citrus juice (from concentrate) in addition to other flavorings. Interestingly, several samples containing citrus-related ingredients showed low perillaldehyde levels (i.e., 0.1 $\mu g \cdot kg^{-1}$). Logically, no perillaldehyde was found in the six red-fruit-based lemonades analyzed.

As for citrus and apple-based beverages, high variability in the furan-2(5*H*)-one level was observed among lemonades, with concentrations ranging from 1.7 to 126.0 $\mu g \cdot kg^{-1}$ (Figure 4). This compound was also found in red fruit-based lemonades up to 68.4 $\mu g \cdot kg^{-1}$ (Figure 4).

On the other hand, 4-methyl-5-vinylthiazole was evidenced only in one lemonade sample (15 $\mu g \cdot kg^{-1}$) containing concentrate juices of orange, apple, passion fruit, mango, and a flavoring.

3.2.4. Iced Teas. Among the 14 selected ice teas (all flavored except one), perillaldehyde was found at low levels, both in citrus-flavored samples (3.7−13.3 $\mu g \cdot kg^{-1}$) and in the three green ice teas (0.4−9.1 $\mu g \cdot kg^{-1}$). It was, in turn, absent in all peach ice teas.

Furan-2(5*H*)-one occurred at similar levels in all samples (10−22 $\mu g \cdot kg^{-1}$), except for ice tea no. 1 (71 $\mu g \cdot kg^{-1}$) and no. 4 (143 $\mu g \cdot kg^{-1}$). The latter is a sugar-free equivalent (same brand) of ice tea no. 3 containing only 23 $\mu g \cdot kg^{-1}$.

https://doi.org/10.1021/acs.jafc.3c05381
J. Agric. Food Chem. 2023, 71, 18538−18545

3.2.5. Colas and Sports Beverages. Five regular and six "no sugar" colas (including two citrus-flavored and two decaffeinated) were investigated here (Figure 5a). Perillaldehyde was, as expected, evidenced in both lemon/lime-flavored colas (10 $\mu g \cdot kg^{-1}$), but surprisingly also in all other samples (0.7−6 $\mu g \cdot kg^{-1}$). This might indicate that "natural flavorings (plant extracts)" products included citrus-related extracts or other perillaldehyde-containing plants.

Next, the presence of caramel in cola products suggested high Maillard-related compound levels, but it was interestingly not the case for the selected analytes. 2-Acetylfuran and furan-2(5*H*)-one were found in very similar ranges between all colas, with respective levels between 19−41 $\mu g \cdot kg^{-1}$ and 5−10 $\mu g \cdot kg^{-1}$. Low levels of 2,4-pentanedione (1−8 $\mu g \cdot kg^{-1}$) were found in almost all products except for one sugar-free lemon-flavored colas (no. 1), which reached 20 $\mu g \cdot kg^{-1}$. Globally, no significant differences were observed among classical, no-sugar, and decaffeinated colas.

Furan-2(5*H*)-one (<LOD, 94 $\mu g \cdot kg^{-1}$) and perillaldehyde (<LOD, 38 $\mu g \cdot kg^{-1}$) were also found in four out of five sports beverages here analyzed (Figure 5b), with again higher levels of the former in the citrus-based samples (>9 $\mu g \cdot kg^{-1}$ in 1−3 against <5.9 $\mu g \cdot kg^{-1}$ in 4 and 5).

In conclusion, this work used the solvent-assisted flavor evaporation technique (SAFE) coupled with GC/MS (SIM) to investigate the occurrence of 38 (suspected) genotoxic flavoring substances in 94 water-based beverages consumed by children, with broad sample diversity. The method was successfully validated for the quantitative analysis of 30 flavoring substances, while a qualitative screening was done for the eight nonvalidated compounds. This work confirmed the suitability of the SAFE technique for multianalyte analysis. Food control laboratories could use its versatile character for law enforcement to quickly adapt the method to diverse food matrices and other relevant flavoring substances.

Seven different flavoring substances were detected among 94 samples, including two genotoxic flavoring substances: perillaldehyde (76 occurrences) and furan-2(5*H*)-one (93 occurrences) up to 153 $\mu g \cdot kg^{-1}$ (in a lemonade) and 143 $\mu g \cdot kg^{-1}$ (in an iced tea), respectively. In colas and sports beverages, levels were relatively lower for both perillaldehyde (maximum 38 $\mu g \cdot kg^{-1}$) and furan-2(5*H*)-one (maximum 94 $\mu g \cdot kg^{-1}$). Nonetheless, these results are concerning as genotoxic effects are known to occur at very low doses (i.e., TTC: 0.0025 $\mu g/kg$ bw per day).[29] On the other hand, contrary to food additives listed on the food labeling, very limited flavoring information is present on the labeling. The multimethod and results presented here allowed for the identification of relevant future samples and matrices for further studies, which are needed to perform a comprehensive flavoring substance risk assessment. In addition, the sampling scheme of this work showed how similar industrial products can be distinguished according to flavoring substances and processes. Nonetheless, an unexpectedly high variability was observed among the same beverage type. Further investigation should be performed to determine the nature of this variability. In this sense, for perillaldehyde, the initial content in the raw material (fruit cultivar and maturity) and the juicing process should be further studied. While for furan-2(5*H*)-one, its possible formation under heat conditions should drive further research concerning thermal processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c05381.

Analyzed samples with information present on the product labeling (Table S1); mass spectral data and retention indices of monitored compounds (Table S2); method validation results (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract RT 19/7 Flavouran.

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