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Occurrence of (suspected) genotoxic flavoring substances in Belgian alcohol-free beers

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ABSTRACT

The regulatory landscape of flavorings is evolving, thereby putting pressure on control laboratories to develop analytical methods for a wide range of compounds in various types of food and drinks. In order to improve the monitoring of flavoring substances, a versatile and accurate analytical method using the solvent-assisted flavor evaporation (SAFE) technique coupled to GC–MS(SIM) was developed and validated. Focus was put on authorized flavoring substances requiring specific attention due to a genotoxic concern based on information from European risks assessment reports. Thirty-seven (suspected) genotoxic flavoring substances were analyzed in a selection of ten alcohol-free beers. Five suspected genotoxic compounds (i.e. 1-(2-furyl)-2-propanone, 2-acetylfuran, 2-acetyl-5-methylfuran, 2-acetyl-3,5-dimethylfuran, hex-2-eno-1,4-lactone) as well as two confirmed genotoxic flavoring substances (*p*-mentha-1,8-dien-7-al, 2,4-pentanedione) were identified and quantified among the selected samples. Low concentrations and natural occurrences of the identified compounds suggested that these were not added as such but rather originated from heat-treatments or from plant-based extracts.

1. Introduction

Flavorings can be defined as products not intended to be consumed as such, but to be added to food in order to impart or modify odor and/or taste (European Commission, 2008). Instead, the term aroma is generally used for naturally occurring aromatic substances in food. The use of odorous materials such as plants, spices or balms is as old as mankind, originally used as part of religious ceremonies, rituals, and perfumery (Berger, 2007). Nowadays, thousands of chemically defined flavoring substances exist and are added to a wide variety of food and drinks under specific regulations. The world flavoring market has a turnover of €10–12 billion, of which €3.5 billion is in Europe (IOFI, 2020).

In Europe, Regulation (EC) No 1334/2008 lays down the general provisions on the use of flavorings in food. This includes restrictions of use, such as maximum levels of certain substances for certain food categories (defined in Regulation (EC) No 1333/2008), conditions of use for some source materials, as well as substances which shall not be added as such to food (European Commission, 2008). In parallel, over the past twenty years, the European Food Safety Authority (EFSA) has assessed the risks of flavoring substances by grouping them according to their metabolic and biological behavior into 34 chemical groups and performing flavoring group evaluations (FGEs). Intake levels, absorption, metabolism and toxicity are considered in the risk assessments, with a particular focus on their genotoxic potential. Since 2012, all authorized flavoring substances have been included in a positive list, called the Union list, within Regulation (EC) No 1334/2008. Up to the consolidation of 21st May 2019, 2503 compounds were listed, among which 302 were marked as being in the process of evaluation. α,β-Unsaturated carbonyls are especially of concern due to the presence of structural alerts for genotoxicity (EFSA Panel on Food Additives and Flavourings (FAF), 2019; European Commission, 2008). Until new data were available to overrule their genotoxic concern, these flavoring substances were considered as suspected genotoxic. However, it should be noted that Regulation (EC) No 1334/2008 is not consolidated each time a new EFSA opinion is available. Consequently, the most recent EFSA opinions must be reviewed to discard substances for which the

Abbreviations: AFB, alcohol-free beer; EC, European Commission; EFSA, European Food Safety Authority; EST, external standard; IST, internal standard; SAFE, solvent-assisted flavor evaporation.

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genotoxic concern has already been ruled out. In addition, some substances are already prohibited to be added as such to food because of confirmed genotoxic effects.

Member State authorities are responsible for EU law enforcement. Compared to the monitoring and control of residues of veterinary medicines, pesticides and other contaminants, a general lack of analytical methodology for the analysis of flavoring substances was reported in Europe (European Court of Auditors, 2019). Italy and the Netherlands reported no control on flavoring substances in 2016, while only 22 food samples were tested by Slovenia (European Court of Auditors, 2019). As the majority of flavoring substances were not subject to maximum limits, the few analytical techniques developed for regulation enforcement mainly focused on the fifteen naturally occurring flavoring substances from the Annex III of Regulation (EC) No 1334/2008 for which addition as such to food is prohibited and for which maximum levels may apply (Bousova, Mittendorf, & Senyuva, 2011; Lopez et al., 2015; European Commission, 2008). In this regards, in 2019, Belgium controlled 85 samples, focusing on coumarin, pulegone and theobromine.

Although generally not used as routine methods for law enforcement, numerous analytical techniques exist for the analysis of flavoring substances (mainly focusing on the organoleptic properties). To quickly respond to current and future calls for the monitoring and control of flavoring substances, a versatile and accurate analytical technique should be used with the ability to adapt to several matrices and to almost any target compound. Steam distillation extraction (SDE) is an old but versatile and easy technique which uses special glassware that allows a steam distillate from the sample to be continuously extracted in a small amount of solvent that is enriched during the process (Likens & Nickerson, 1964). The main drawbacks of this technique are artefact formation (Maillard and Strecker reaction products) and compound degradation due to the high temperature (Majcher & Jeleń, 2009; Pozo-Bayón, Guichard, & Cayot, 2006). SDE should thus be avoided with matrices rich in carbohydrates and amino acids. Softer techniques such as static headspace (SH), dynamic headspace (DH), solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) are also widely used. Except for SH, most of these techniques use specific coated fibers/bars which trap and concentrate volatiles from the matrix before their desorption and subsequent analysis, with reduced solvent consumption and possible automation. However, the versatility of these methods is limited as they are not suitable for the isolation of high molecular weight and less volatile compounds (Majcher & Jeleń, 2009). Another technique called solvent-assisted flavor evaporation (SAFE) recovers volatiles by performing a sample distillation under high vacuum (10^{-3} Pa) and low temperature (40 °C) (Engel et al., 1999). This prevents thermal degradation such as lipid oxidation and avoids artefacts that could lead to false positive results. Little extraction discrimination between chemical groups makes the SAFE a versatile technique, that is also suitable for complex matrices (Engel et al., 1999; Wang et al., 2016).

Due to their ancestral use and their application in perfumes, plants and fruits are the most extensively studied matrices. Back in 1833, cinnamaldehyde was the first odorant to be isolated and identified from cinnamon oil (Büttner, 2017). Later, advances in organic chemistry and the development of extraction methods made it possible to study numerous matrices and to discover countless odorants from many different chemical groups (Büttner, 2017). Today, fermented products (e.g., alcoholic beverages, bread, chocolate) and heat-treated food (e.g., meat, fish, coffee) are among the most studied matrices because of their rich aromatic profile.

In this paper, focus was put on alcohol-free beers (AFBs), consumption of which is on the rise. In Belgium, from 2017 to 2019, the population that often drinks AFBs increased from 7 to 27% (Belgian Brewers, 2019), a trend that can be explained by a desire for a healthier lifestyle (e.g., driving safety, alcohol-free events or diet) (Belgian Brewers, 2019). To avoid ethanol in the final product, AFBs are produced using two main methods with the challenge of matching the aromatic profile of alcoholic beers. Pre-processing biological methods such as arrested fermentation, cold contact fermentation or use of special yeast strains limit the ethanol formation but also usually limit the formation of desired aromas. Also, the incomplete reduction of Strecker aldehydes often results in a worty taste (Perpète & Collin, 2000; Pilarski & Gerogiorgis, 2020; Piornos et al., 2020). On the other hand, post-processing methods let the classic fermentation take place and ultimately selectively remove ethanol by physical methods such as thermal or membrane treatments or solvent extraction. Besides being expensive, the main drawback of physical methods is the degradation and/or the removal of interesting aromas along with ethanol (Pilarski & Gerogiorgis, 2020). Moreover, thermal dealcoholization processes could increase the formation of furans (Riu-Aumatell et al., 2014). While various studies are currently ongoing to solve the above-mentioned difficulties, brewers can also directly add flavorings to their AFBs to obtain the desired aromatic profile. Since in most cases no information is given regarding the added compounds, monitoring is therefore important.

The scientific rationale of this research is the development and validation of a multi-method for the simultaneous analysis of several (suspected) genotoxic flavoring substances. Up till now, no multi-methods for (suspected) genotoxic flavorings exist. In addition, the research focused on substances that might be present in AFBs that have not been monitored before. Future validation of this method in other food matrices will support food control laboratories to expand the control on flavorings. Attention on the analytical performance of the SAFE technique was therefore made in the context of a possible use for law enforcement. Furthermore, this method will also generate occurrence data, which is highly desired by health authorities in order to conduct safety evaluations (EFSA, 2012). Additionally, more thorough monitoring will provide a better understanding of the origins of the selected substances.

2. Material and methods

2.1. Selection of compounds

According to the Union list (consolidation of 21st May 2019), 302 compounds were under evaluation. Among these, recent EFSA opinions (up to June 2020), showed that only 53 were still under evaluation due to a genotoxic concern. For these 53 substances, it was verified whether they were commercially available (the case for 29 compounds; retention times and mass fragmentation data for 5 others). In addition, three confirmed genotoxic flavoring substances commercially available that were no longer authorized to be added as such to food in Europe were also included in this study: *p*-mentha-1,8-dien-7-al (perillaldehyde), 2,4-pentanedione and 3-acetyl-2,5-dimethylthiophene. In total, 37 compounds, listed in Table 3, were included in this study.

2.2. Alcohol-free beer samples

Ten popular commercial Belgian AFBs (**A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, **I**, and **J**) from category 14.2 in Regulation (EC) No 1333/2008 were selected (purchased from Belgian supermarkets) and analyzed. Except for beers **E**, **F** and **J**, all other beers mentioned "flavoring(s)" in their ingredients list. Beers **G**, **H**, **I** and **J** were specifically selected because of the presence of *citrus* spp. (citrus, orange, bergamot) in the ingredients, a known source of *p*-mentha-1,8-dien-7-al (perillaldehyde) (Burdock & Fenaroli, 2010). In Belgium, alcohol content of AFBs should be below 0.5% (Arrêté royal concernant la bière du 31 mars 1993, 1993). Samples were purchased in September 2020 and stored in the dark at 20 °C until analysis.

2.3. Reference odorants and chemicals

1-(4-Methoxyphenyl)-4-methyl-1-penten-3-one (homo ethone), 2-acetylfuran, 2-acetylthiophene, 3-acetyl-2,5-dimethylfuran, 3-acetyl-2,5-dimethylthiophene, 4-methyl-2-phenyl-2-pentenal, 4-methyl-5-vinylthiazole, 5-methyl-2-phenyl-2-hexenal, α-damascone, δ-damascone, menthalactone, *n*-decane, *p*-mentha-1,8-dien-7-al and vanillylidene acetone were purchased from Merck (Overijse, Belgium). 1-(2-Furyl)-2-propanone, 2-*sec*-butyl-4,5-dimethyl-3-thiazoline, 2-acetyl-5-methylfuran, 2-hexanoylfuran, 2-butylfuran, 2-butyrylfuran, 2-heptylfuran, 2-pentanoylfuran, 2-pentylfuran, 2-phenylcrotonaldehyde, 2-phenyl-2-pentenal, 3-(2-furyl)-2-methyl-2-propenal, 3-(2-furyl)acrylaldehyde, 4-(2-furyl)-3-buten-2-one, 4,5-dimethyl-2-ethyl-3-thiazoline, 4,5-dimethyl-2-isobutyl-3-thiazoline and 2,4-pentanedione were purchased from Fisher Scientific (Bruxelles, Belgium). 1-(2-Furyl)-3-butanone, 1-(4-methoxyphenyl)-1-penten-3-one (ethone), 2-acetyl-3,5-dimethylfuran, 3-(5-methyl-2-furyl)-2-propenal and hex-2-eno-1,4-lactone were purchased from Chemspace (Riga, Latvia). Structures are given in Table 2.

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. Characterization of beers

The beers were characterized by their ethanol content, color, bitterness and pH following Analytica EBC methods 9.2.6, 9.6, 9.8, and 9.35, respectively (Brewers of Europe, 2020). Density was also measured using a density meter (DM4500; Anton Paar GmbH, Graz, Austria).

2.5. Isolation of volatiles

Degassed samples (50 mL) were spiked with 150 μL of 2-acetylthiophene solution (8 mg/L) as internal standard (IST). Samples were then extracted with bidistilled dichloromethane (1 × 75 mL) for 20 min. After centrifugation (20 min at 2264 *g*) of the resulting emulsion, the aqueous phase was discarded and the remaining organic phase was dried over anhydrous sodium sulfate. Non-volatile compounds were then separated by high-vacuum distillation using the SAFE system (Glasblaeserei Bahr, Manching, Germany) (Engel et al., 1999). The conditions for the SAFE analyses were the following: water bath temperature 40 °C, pressure below 10^{-3} Pa and the apparatus body at 30 °C. The distillate was continously recovered in the liquid-nitrogen-cooled SAFE flask for 15 min distillation, followed by an extraction with distilled water (3 × 25 mL) to remove any residual alcohol. The extract was dried over anhydrous sodium sulfate. To measure absolute recoveries, 25 μL of decane solution (250 mg/L) were spiked as an external standard (EST) before concentration to 500 μL in a Kuderna-Danish apparatus at 45 °C. In order to ensure maximum stability, extracts were stored at −80 °C until analysis by GC–EI-MS.

2.6. Gas chromatography–mass spectrometry

SAFE extracts were analyzed with a wall-coated open tubular apolar capillary column (CP-Sil 5 CB, 50 m × 0.32 mm i.d., 1.2 μm film thickness) on an Agilent 7890B gas chromatograph. Injections (1 μL) were carried out at 250 °C in splitless mode. The carrier gas was helium and the pressure was set at 65 kPa. The oven temperature was programmed to rise from 36 °C 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. The column was connected to a single quadrupole mass spectrometer (Agilent 5977B MSD) operating in selected ion monitoring (SIM) mode with electron ionization at 70 eV. Full-scan (FS) chromatograms (*m/z* 40–380) were also recorded on separate runs for possible qualitative *retro*-analysis. Data were recorded and analyzed with Agilent OpenLab software (version 2.1).

2.7. Quantification of flavoring substances

Standard addition technique was used to quantify analytes. A mix containing analytical standards in dichloromethane was prepared and used to spike four times the sample (10; 25; 50; 75 μg/L). In addition, to ensure a correction for the injected volume into the chromatograh and to compensate for the possible variation of the detector sensitivity, an internal standard (IST, 2-acetylthiophene) was spiked in the sample at a constant concentration (24 μg/L). The concentration of an analyte X in the sample was obtained using the following equation: X concentration (in μg/L) = IST concentration (in μg/L) × (X area / IST area) × (IST response coefficient / X response coefficient) × (IST absolute recovery / X absolute recovery).

Because standard addition was a time consuming experiment, it was performed on three different samples. For each compound, standard addition slope values were then statistically compared (*t*-test, 95% confidence) to determine if matrix effects were similar between samples. Standard addition slopes from a reference sample could be reused for other samples which would not undergo standard addition.

2.8. Validation of the method

The developed method was validated in house in terms of linearity, matrix effects, intra- and interday repeatability, limit of detection (LOD) and quantification (LOQ), selectivity and apparent recoveries. “Beer A” was used as the matrix for validation. The spiked levels were chosen in accordance with the expected concentrations of flavoring substances in the targeted matrix. Coefficients of variation on the intraday repeatability ($CV_{intra\text{-}r}$) and interday repeatability ($CV_{inter\text{-}r}$) were evaluated using Horwitz statistical analysis, based on triplicate experiments, performed three times on different days. The measurement of uncertainties (MU) was assessed through $CV_{inter\text{-}r}$ (ISO, 2008).

2.9. Quality control

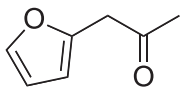
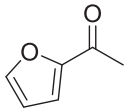
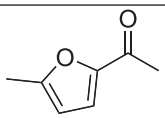
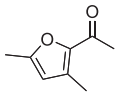
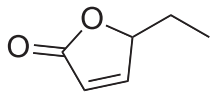
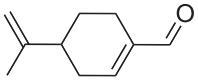
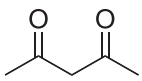
Standards at concentrations corresponding to the LOQ were spiked in solvent and taken as control for sensitivity of the GC–MS. The mass spectrometer was tuned twice a week and pure solvent was regularly injected to ensure the absence of cross-contamination between injections. Compound stability was monitored by comparing chromatograms of fresh standard solutions to those stored at –80 °C and –20 °C. Peak areas were identical for both storage temperatures. The autosampler syringe was rinsed with methanol and dichloromethane between

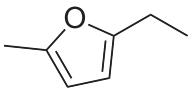
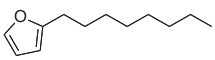
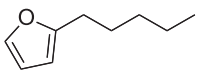
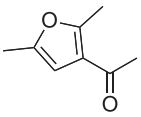
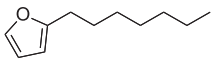
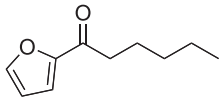
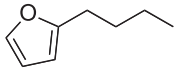
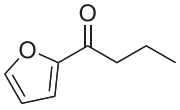
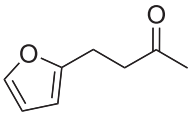
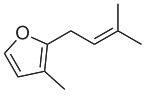
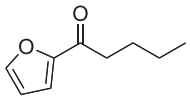
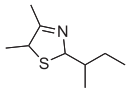
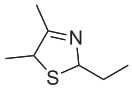
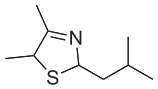
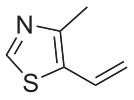
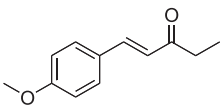
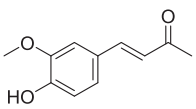
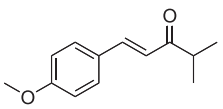
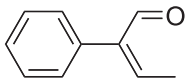
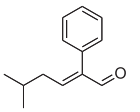
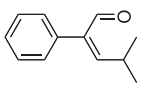
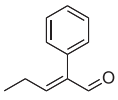
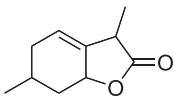
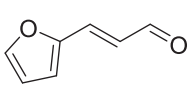
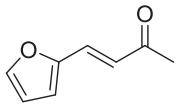
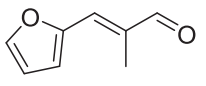
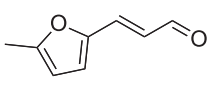
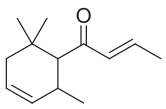
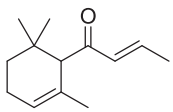
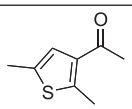
Table 1
Characteristics and pre/post-processing methods of the 10 analyzed Belgian AFBs.

Parameter	A	B	C	D	E	F	G	H	I	J
Ethanol (% *v/v*)	<0.01	0.01	0.07	0.10	0.40	0.50	<0.01	0.02	0.04	0.70
Real extract (% *w/w*)	9.14	8.13	4.83	3.83	6.91	3.62	8.27	5.46	6.91	3.99
Color (EBC)	11.5	7.0	7.5	8.6	4.6	7.1	24.7*	21.7*	19.0*	6.9
Bitterness (°BU)	20.6	17.6	15.2	12.6	20.0	15.1	6.0	5.4	10.2	6.7
pH	4.2	3.9	4.3	4.2	3.2	4.2	3.1	3.6	3.7	3.4
Pre/post-processing method	dealc.	dealc.	dealc.	dealc.	s.y.	n/a	dealc.	n/a	n/a	s.y.

* = Color measurements biased due to fruit content; s.y. = special yeasts; dealc. = dealcoholization (unspecified type); n/a = not available.

Table 2
(a) Identified or (b) undetected suspected and confirmed* genotoxic flavoring substances, with monitored *m/z* values.

 **(3)** 1-(2-Furyl)-propan-2-one 53, 81, 124	 **(4)** 2-Acetylfuran 43, 95, 110	 **(9)** 2-Acetyl-5-methylfuran 43, 53, 109, 124	 **(10)** 2-Acetyl-3,5-dimethylfuran 67, 123, 138	 **(28)** Hex-2-eno-1,4-lactone 55, 83, 112
 **(35)** *p*-Mentha-1,8-dien-7-al* 68, 79, 107, 150	 **(36)** 2,4-Pentandione* 43, 85, 100	(a)		

 **(1)** 2-Ethyl-5-methylfuran 95, 110	 **(2)** 2-Octylfuran 53, 81, 123, 180	 **(5)** 2-Pentylfuran 53, 81, 138	 **(6)** 3-Acetyl-2,5-dimethylfuran 81, 123, 138	 **(7)** 2-Heptylfuran 81, 95, 109, 166
 **(8)** 2-Hexanoylfuran 95, 110, 123	 **(11)** 2-Butylfuran 53, 81, 124	 **(12)** 2-Butyrylfuran 95, 110, 138	 **(13)** 1-(2-Furyl)-3-butanone 81, 95, 123, 138	 **(14)** 3-Methyl-2(3-methylbut-2-enyl)furan 91, 107, 135, 150
 **(15)** 2-Pentanoylfuran 95, 110, 123	 **(16)** 2-(*sec*-Butyl)-4,5-dimethyl-3-thiazoline 87, 100, 114, 171	 **(17)** 4,5-Dimethyl-2-ethyl-3-thiazoline 102, 114, 143	 **(18)** 4,5-Dimethyl-2-isobutyl-3-thiazoline 87, 114, 138, 171	 **(19)** 4-Methyl-5-vinylthiazole 58, 97, 125
 **(20)** Ethone 133, 161, 190	 **(21)** Vanillylidene acetone 117, 145, 177, 192	 **(22)** Homo ethone 133, 161, 204	 **(23)** 2-Phenylcrotonaldehyde 91, 115, 117, 146	 **(24)** 5-Methyl-2-phenyl-2-hexenal 91, 104, 117, 188
 **(25)** 4-Methyl-2-phenyl-2-pentenal 103, 131, 159, 174	 **(26)** 2-Phenyl-2-pentenal 91, 117, 131, 160	 **(27)** Menthalactone 95, 109, 137, 166	 **(29)** 3-(2-Furyl)acrylaldehyde 65, 94, 122	 **(30)** 4-(2-Furyl)-3-buten-2-one 65, 94, 121, 136
 **(31)** 3-(2-Furyl)-2-methyl-2-propenal 68, 79, 108, 136	 **(32)** 3-(5-Methyl-2-furyl)-2-propenal 94, 107, 121, 136	 **(33)** *δ*-Damascone 69, 81, 123, 192	 **(34)** *α*-Damascone 69, 81, 123, 192	 **(37)** 3-Acetyl-2,5-dimethylthiophene* 111, 139, 154 (b)

each injection. Compounds were identified using retention times and abundance ratios between ions in comparison with the injected analytical standards, according to European recommendations (European Commission, 2002). As compounds **(1)**, **(2)** and **(14)** were not commercially available, they were monitored using information available on the National Institute of Standards and Technology (NIST) 14 version 2.2 database. Note that purity of standards **(22, 25)** was unknown so that they were only used to determine their retention index and mass spectrum.

3. Results and discussion

3.1. Selection of the flavoring substances

The objective of the selection was to identify all flavoring substances currently authorized on the market with a genotoxic concern. As a starting point, substances with a footnote (indicating that their evaluation was still pending) were retrieved from the Union List (Fig. 1) from Regulation (EC) 1334/2008 (consolidation from 21st May 2019). Then, to identify the nature of the pending evaluation and since the consolidation of the Union List was delayed from EFSA's Flavoring Group Evaluations (FGEs), an exhaustive revision of relevant FGEs (up to June

Table 3
Concentrations (μg/L) of suspected and confirmed* genotoxic flavoring substances among 10 Belgian alcohol-free beers.

Category	N°	FL-no	Compound	Odor (Burdock & Fenaroli, 2010; The Good Scents Company, 2018)	CAS n°	RI (CP-Sil 5 CB)	Abs. recov. (%)	A	B	C	D	E	F	G	H	I	J
Furan-substituted	1	13.125	2-Ethyl-5-methylfuran	Fresh/gassy/burnt	1703-52-2	791[a]	n/a	–	–	–	–	–	–	–	–	–	–
	2	13.162	2-Octylfuran	?	4179-38-8	1283[a]	n/a	–	–	–	–	–	–	–	–	–	–
	3	13.045	1-(2-Furyl)-2-propanone	Herbal/radish/caramel	6975-60-6	925	89	–	–	–	–	n.q.	–	–	–	–	n.q.
	4	13.054	2-Acetylfuran	Coffee/caramel/sweet	1192-62-7	887	88	8.1 ± 1.4	5.7 ± 1.0	7.6 ± 1.4	6.6 ± 1.2	23.3 ± 4.2	6.8 ± 1.2	8.1 ± 1.4	6.4 ± 1.1	9.1 ± 1.6	8.0 ± 1.4
	5	13.059	2-Pentylfuran	Fruity/caramel	3777-69-3	981	75	–	–	–	–	–	–	–	–	–	–
	6	13.066	3-Acetyl-2,5-dimethylfuran	Roasted/nutty/almond	10599-70-9	1062	84	–	–	–	–	–	–	–	–	–	–
	7	13.069	2-Heptylfuran	Coffee/nutty/herbal	3777-71-7	1184	70	–	–	–	–	–	–	–	–	–	–
	8	13.070	2-Hexanoylfuran	Apricot/peach/fruity	14360-50-0	1281	87	–	–	–	–	–	–	–	–	–	–
	9	13.083	2-Acetyl-5-methylfuran	Nutty/cocoa/toasted	1193-79-9	1011	90	–	–	–	–	0.9 ± 0.1	–	–	–	–	–
	10	13.101	2-Acetyl-3,5-dimethylfuran	Sweet/balsam	22940-86-9	1072	86	0.08 ± 0.01[b]	–	–	–	–	–	–	–	–	–
	11	13.103	2-Butylfuran	Spicy	4466-24-4	882	70	–	–	–	–	–	–	–	–	–	–
	12	13.105	2-Butyrylfuran	Balsam/fruity	4208-57-5	1075	93	–	–	–	–	–	–	–	–	–	–
	13	13.106	1-(2-Furyl)-3-butanone	Spicy/caramel	699-17-2	1043	83	–	–	–	–	–	–	–	–	–	–
	14	13.138	3-Methyl-2(3-methylbut-2-enyl)furan	Caramel	15186-51-3	1089[a]	n/a	–	–	–	–	–	–	–	–	–	–
	15	13.148	2-Pentanoylfuran	Sweet/caramel/fruity	3194-17-0	1178	92	–	–	–	–	–	–	–	–	–	–
Thiazolines	16	15.029	2-(*sec*-Butyl)-4,5-dimethyl-3-thiazoline	Meaty/spicy/vegetable	65894-82-8	1222;1238;1240	73	–	–	–	–	–	–	–	–	–	–
	17	15.030	4,5-Dimethyl-2-ethyl-3-thiazoline	Meaty/vegetable	76788-46-0	1061;1074	69	–	–	–	–	–	–	–	–	–	–
	18	15.032	4,5-Dimethyl-2-isobutyl-3-thiazoline	Meaty/spicy/herbal	65894-83-9	1215;1221	61	–	–	–	–	–	–	–	–	–	–
Thiazole	19	15.130	4-Methyl-5-vinylthiazole	Nutty/cocoa	1759-28-0	1010	83	–	–	–	–	–	–	–	–	–	–
Methoxyphenyl -ketones	20	07.030	Ethone	Butter/fruity/burnt	104-27-8	1783	36	–	–	–	–	–	–	–	–	–	–
	21	07.046	Vanillylidene acetone	Sweet/balsam/vanilla/spicy	1080-12-2	1795	9	–	–	–	–	–	–	–	–	–	–
	22	07.049	Homo ethone	Butter/fruity/caramel	103-13-9	1783	n/a	–	–	–	–	–	–	–	–	–	–
Phenyl-α,β-unsaturated aldehydes	23	05.062	2-Phenylcrotonaldehyde	Floral/woody/cocoa/nutty	4411-89-6	1246	81	–	–	–	–	–	–	–	–	–	–
	24	05.099	5-Methyl-2-phenyl-2-hexenal	Cocoa	21834-92-4	1474	82	–	–	–	–	–	–	–	–	–	–
	25	05.100	4-Methyl-2-phenyl-2-pentenal	Cocoa	26643-91-4	1357	n/a	–	–	–	–	–	–	–	–	–	–
	26	05.175	2-Phenyl-2-pentenal	Floral/spicy/radish/honey	3491-63-2	1327	81	–	–	–	–	–	–	–	–	–	–
Furanones	27	10.036	Menthalactone	Sweet/coconut/vanilla/bread	13341-72-5	1474	95	–	–	–	–	–	–	–	–	–	–
	28	10.046	Hex-2-eno-1,4-lactone	Spicy	2407-43-4	997	100	–	–	1.3 ± 0.2	–	5.6 ± 0.7	1.3 ± 0.2	2.3 ± 0.3	–	–	2.5 ± 0.3

(*continued on next page*)

Table 3 (*continued*)

Category	N°	FL-no	Compound	Odor (Burdock & Fenaroli, 2010; The Good Scents Company, 2018)	CAS n°	RI (CP-Sil 5 CB)	Abs. recov. (%)	A	B	C	D	E	F	G	H	I	J
Furyl-aldehydes	29	13.034	3-(2-Furyl)acrylaldehyde	Spicy/herbal	623-30-3	1081	62	–	–	–	–	–	–	–	–	–	–
	30	13.044	4-(2-Furyl)-3-buten-2-one	Sweet/spicy/balsam/vanilla	623-15-4	1158	69	–	–	–	–	–	–	–	–	–	–
	31	13.046	3-(2-Furyl)-2-methyl-2-propenal	Spicy/cinnamon/cassia/woody	874-66-8	1167	60	–	–	–	–	–	–	–	–	–	–
	32	13.150	3-(5-Methyl-2-furyl)-2-propenal	Sweet/spicy	5555-90-8	1202	58	–	–	–	–	–	–	–	–	–	–
Miscellaneous	33	07.130	δ-Damascone	Fruity/rose/cassis	57378-68-4	1354	85	–	–	–	–	–	–	–	–	–	–
	34	07.134	α-Damascone	Fruity/floral	43052-87-5	1378	83	–	–	–	–	–	–	–	–	–	–
	35	05.117	*p*-Mentha-1,8-dien-7-al*	Spicy/herbal/orange/citrus	2111-75-3	1257	78	–	–	–	–	–	–	45.4 ± 6.7	23.2 ± 3.4	–	–
	36	07.191	2,4-Pentanedione*	Pleasant	123-54-6	760	73	–	–	–	–	6.3 ± 1.3	–	–	–	1.3 ± 0.3	2.4 ± 0.5
	37	15.024	3-Acetyl-2,5-dimethylthiophene*	Burnt/roasted/nutty/almond	2530-10-1	1232	92	–	–	–	–	–	–	–	–	–	–

n/a = not applicable (pure analytical standard not available); – = not detected (mean LOD = 0.04 µg/L); n.q. = not quantifiable (mean LOQ = 0.12 µg/L); [a] = RI from NIST database; [b] = LOQ of compound 10 is 0.02 µg/L. Compounds 5,7,11,16,17,18,20,21 were semi-quantitatively analyzed.

2020) enabled us to select authorized compounds for which genotoxicity was still under evaluation by EFSA (#53). As indicated in Fig. 1, the majority of the substances with a footnote were out of the scope of this work (#249). In fact, these substances either have been cleared of genotoxic concern by EFSA or will be deleted from the Union list in the upcoming consolidations. Note that a deletion of a flavoring substance from the Union list was not necessarily based on evidence for genotoxicity. The applicant for a flavoring substance could in fact communicate to the European Commission (EC) that its substance was no longer supported, which interrupted EFSA's evaluation and ultimately led to the deletion of the substance in the Union List consolidations. Moreover, the EC could delete a substance from the Union list if requested additional scientific data were not submitted before a certain deadline.

Among the fifty-three suspected genotoxic flavoring substances identified, thirty-four were further selected because they were easily available (or with known retention times and mass spectra). In addition to suspected genotoxic flavoring substances, three known genotoxic compounds banned from being added as such were also monitored: *p*-mentha-1,8-dien-7-al (**35**), 2,4-pentanedione (**36**) and 3-acetyl-2,5-dimethylthiophene (**37**). Table 3 lists all target flavoring substances.

Except for ethone (**20**) and homo ethone (**22**), all retained compounds have already been identified in nature (Burdock and Fenaroli, 2010; The Good Scents Company, 2018). Either as plant-derived substances (e.g., *p*-mentha-1,8-dien-7-al) or formed during food heat-processes as a result of non-enzymatic reactions such as the Maillard reaction (e.g., furans, furanones, thiazoles) or the Strecker degradation followed by an aldolization under heat conditions (e.g., phenyl α,β-unsaturated aldehydes) (Büttner, 2017). As indicated in Table 3, a large range of odors was covered by the selected substances, including caramel, roasted, cocoa, fruity, spicy, herbal and floral odors.

3.2. Updates on EFSA opinions after June 2020 and Union list consolidation

The selection of the compounds was based on the Union List consolidated version from 21st May 2019 and EFSA opinions up to June 2020. Later Union list consolidations, EFSA opinions and additional information from the European flavor industry concerning no longer supported substances were not taken into account for the selection. A consolidation of the Union list was published on 3rd December 2020, where α- and δ-damascone were deleted from the Union list as the applicant for these substances did not provide the EC with the requested additional scientific data before the deadline. Concerning EFSA opinions, FGE.13Rev3 and FGE.67Rev3 were published on 3rd February 2021 and ruled out genotoxic concern for 14 furan-substituted substances. Among them, 13 were included in our study: 2-ethyl-5-methylfuran (**1**), 2-octylfuran (**2**), 1-(2-furyl)-2-propanone (**3**), 2-acetylfuran (**4**), 2-pentylfuran (**5**), 2-heptylfuran (**7**), 2-hexanoylfuran (**8**), 2-acetyl-5-methylfuran (**9**), 2-acetyl-3,5-dimethylfuran (**10**), 2-butyrylfuran (**12**), 1-(2-furyl)-3-butanone (**13**), 3-methyl-2(3-methylbut-2-enyl) furan (**14**), and 2-pentanoylfuran (**15**). Additionally, during this work, the European flavor industry (represented by EFFA) decided to no longer support several selected substances under evaluation, which will then be deleted in future Union list consolidations without any further toxicological investigation. For scientific knowledge, all these substances were still monitored here.

3.3. Optimization of the extraction technique

After reviewing the advantages and drawbacks of the different analytical methods available, the SAFE technique was retained because of its soft and exhaustive character of extraction and its versatility. These were essential characteristics to tackle the great compound diversity. Future method developments would easily be possible for complex matrices. An exhaustive extraction was also valuable for *retro*-analysis of other potential volatiles of concern. Moreover, the SAFE technique led to

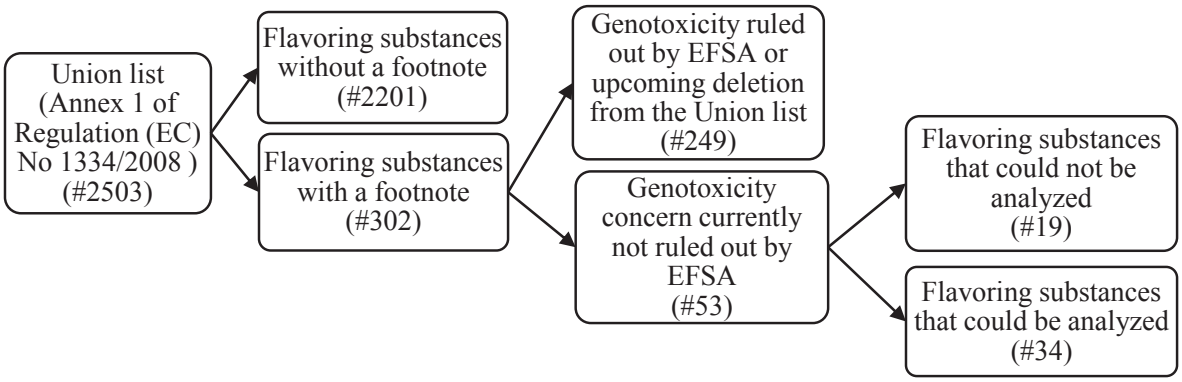


Fig. 1. Selection strategy of the suspected genotoxic flavoring substances.

a final liquid extract that could be stored and injected multiple times. This attribute was very useful in analyzing the same extract with multiple GC programs (SIM and FS for *retro*-analysis) and reinjecting the diluted extract if peak saturation occurred. This contrasted with equilibrium-based techniques where one extraction led to only one injection.

Liquid samples like AFBs could be extracted with an organic solvent before or after SAFE (Engel et al., 1999; Majcher & Jeleń, 2009; Piornos et al., 2020; Uselmann & Schieberle, 2015). Both approaches were tested and compared.

Although performing the SAFE directly on the beer provided absolute recoveries above 80% for most of the compounds, some flavoring substances were not recovered at all, including 2-pentylfuran (**5**), 2-heptylfuran (**7**), 2-butylfuran (**11**) and thiazolines **16**, **17**, **18** (Fig. 2). Instead, when the samples were extracted with dichloromethane before the SAFE, similar recoveries were obtained, but substances that were not extracted with the previous approach, were also extracted this time. In addition, the vacuum distillation of the organic extract was much faster than that carried out directly on aqueous samples (15 min *vs* 60 min). Nonetheless, recoveries of ethone (**20**) and vanillylidene acetone (**21**), two very apolar compounds ($\log P_{ow}$ of 2.65 and 2.03 respectively), remained low.

3.4. Optimization of the GC–MS(SIM) method

A CP-Sil 5 CB column was chosen for its apolar nature and good separation of volatiles which mostly contain apolar structures. After individual injection of commercial standards, two separate SIM programs needed to be developed to quantify coeluting compounds. Commercial standards of compounds **16**, **17**, **18** showed the presence of isomers for each of them. Dedicated time segments were optimized to take into account all structures.

3.5. Quantification of the flavoring substances

Stable isotope dilution assay (SIDA) is generally advised for quantification of complex matrices. However, this could not be applied for this study due to the absence of commercially available isotopically labelled standards for all compounds. Alternatively, they could be synthesized but this would be very expensive, certainly because they need to be of sufficient (and known) purity in order to allow correct quantitation. Instead, standard addition was used as it provides the same degree of precision as SIDA (Pozo-Bayón, Guichard, & Cayot, 2006; Schieberle & Molyneux, 2012). As for SIDA, matrix effects and analytes losses during the extraction were taken into account by performing repeatedly the same extraction with increasing amounts of standards spiked to the sample. Standard addition has been performed for years on several matrices including orange juice (Lum et al., 1990), baked matrices (Pozo-Bayón, Guichard, & Cayot, 2006), wine (Bailly et al., 2009), hop and beer (Cibaka et al., 2017; Jerkovic & Collin, 2007). The analyte concentration in the sample was obtained by linear regression, as long as responses were within the linear range of the detector. Quantitative results are presented in Table 3.

3.6. Validation

3.6.1. Linearity and matrix effects

Linearity was assessed at 6 concentration levels (i.e. 0.25; 2.5; 5; 7.5; 10 and 15 mg/L). An external standard (*n*-decane) spiked at a constant concentration in each vial (12.5 mg/L) was used to compensate the error in the injected volume. The relative response of each compound was plotted against relative concentration levels and submitted to a Mandel's Fitting test ($R^2 > 0.99$) (Funk et al., 2006). All responses were linear within the concentration range. Standard addition inherently took into account matrix effects. Performed on three different samples (**A**, **B**, **D**), it showed similar relative standard addition slopes for most of the compounds (at 95% confidence level). This indicated that matrix effects were similar between samples so that the relative standard addition slopes from a reference sample (**A**) could be used for the other AFBs. However, substances **5**, **7**, and **11** (three furan-substituted substances), and **16**, **17**, and **18** (three thiazolines) showed non-linear responses. No clear explanation could be found on their difficulty of analysis. Additionally, despite linear responses of compounds **20** and **21**, relative standard addition slopes differed from sample-to-sample.

3.6.2. Selectivity and limits of detection (LOD) and quantification (LOQ)

High selectivity was achieved using a combination of a L/L extraction on the sample followed by a SAFE and GC–MS(SIM). Due to the exhaustive extraction nature of the SAFE technique, many untargeted compounds were visible in GC–MS(full-scan) chromatograms. The use of selected ion monitoring (SIM) was essential to increase sensitivity and selectivity. The targeted flavoring substances could be quantified using the SIM *m/z* values given in Table 2.

Among the different approaches for the determination of the LOD and LOQ, the signal-to-noise approach was used to estimate practical values for these two parameters. For each analyte, a comparison of the measured signal from the sample with known low concentrations (lowest standard addition spike when the compound was originally absent from the sample) was made with those of blank samples. The LOD and LOQ were defined as the concentration in the sample giving a S/N ratio of 3 and 10 respectively (ICH, 1996). Low LOD and LOQ values were obtained with an average of 0.04 μg/L and 0.12 μg/L, respectively. This was particularly important to ensure the absence of prohibited compounds. Calculated LOD and LOQ values were confirmed by injection of standards at very low concentration (10 μg/L). LOD and LOQ values are detailed for each analyte in the Supplementary Material.

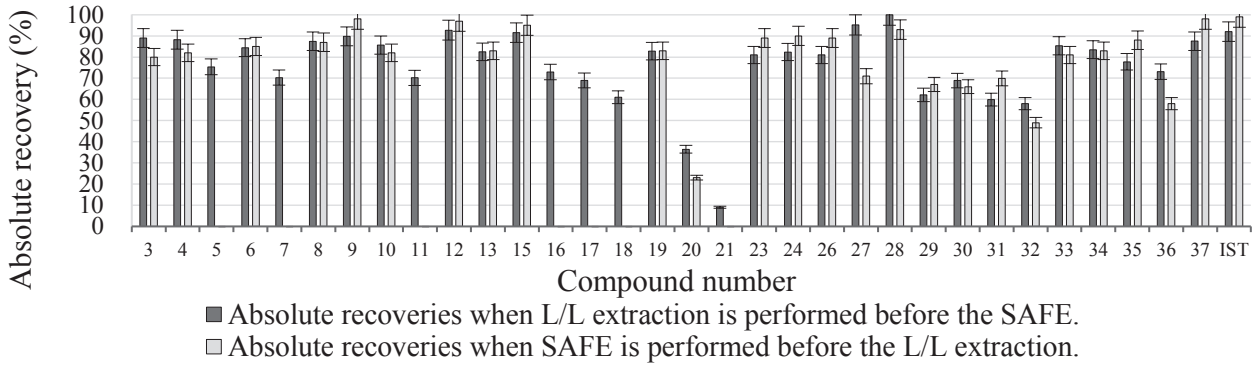


Fig. 2. Comparison of absolute recoveries between the two tested procedures.

3.6.3. Precision, measurement uncertainty and recoveries

Absolute recoveries were assessed with standard addition and an external calibration, both with an external standard (EST, *n*-decane) using the following equation: X absolute recovery (%) = slope from standard addition of X relative to the EST × (1/slope from calibration of X relative to the EST) × (1/concentration factor). Details on recoveries are mentioned in *Section 3.2*. Although compounds **5**, **7**, **11**, **16**, **17**, and **18** were better recovered with the optimized SAFE extraction, they did not pass the validation criteria as their standard addition slopes were not linear. They were thus semi-quantitatively analyzed. Compounds **20** and **21** could also only be semi-quantitavely analyzed, due to poor recoveries in addition to variation of their standard addition slopes from sample-to-sample. For all the remaining compounds (#29), coefficients of variation on intra- and interday repeatability were respectively below 10% and 13%. The measurement of uncertainies ranged between 6–26%.

3.6.4. Apparent recoveries

Apparent recoveries were calculated based on spiked samples of triplicate analysis performed three times on different days. Apparent recoveries were 101–123%.

3.7. Sample analysis

The method was applied on 10 popular Belgian AFBs, that were first characterized. Results are presented in Table 1.

Note that beers **A**, **B**, **C**, **D** and **F** were AFBs equivalent to their respective traditional pale lager beers from the same brand. Based on ethanol content, two groups can be established. The first one consists of beers that underwent post-processing dealcoholization, showing a very limited ethanol content (**A**, **B**, **C**, **D**, **G**, and probably **H**, **I**). The second group comprises beers (**E**, **F**, and **J**) brewed with special yeast or by using cold contact fermentation (CCF). In this group, ethanol content is close to the 0.5% legal limit (or above for **J**). Nonetheless, some yeasts strains and CCF can lead to an ethanol content similar to that of dealcoholized AFBs which makes it difficult to identify the applied brewing process (Bellut & Arendt, 2019; Pilarski & Gerogiorgis, 2020).

Among the ten Belgian AFBs, 5 different suspected genotoxic flavoring substances were identified as well as 2 genotoxic flavoring substances Table 2. Quantitative results are presented in Table 3.

Unsurprisingly for products containing heat-treated ingredients, malts here, furan-substituted substances were identified in AFBs, in both flavored and unflavored products. More specifically 2-acetylfuran was found in all samples at very similar concentrations (average = 7 μg/L), except in beer **E** where it reached a maximum of 23 μg/L, which was still much lower than the 10 mg/L odor threshold (Burdock & Fenaroli, 2010). 2-Acetylfuran content did not significantly differ between dealcoholized beer and beers brewed with special yeasts ($p = 0.44$). In fact, the highest 2-acetylfuran level was found in beer **E** brewed with a special yeast. Breweries did not provide information on the type of dealcoholization. If thermal dealcoholization was performed, no increase in furan-substituted substances level was observed. Note that no correlation was found between color and 2-acetylfuran content. Two other furan-substituted substances were found at lower levels: 0.9 μg/L of 2-acetyl-5-methylfuran (**9**) in beer **E** and 0.1 μg/L of 2-acetyl-3,5-dimethylfuran (**10**) in beer **A**. 1-(2-Furyl)-2-propanone (**3**) was found only at trace levels in samples beer E and beer J. Literature showed comparable or higher levels of similar furan-substituted substances in canned food, varying from 3 to 67 μg/kg (Fromberg et al., 2014). The furanone hex-2-eno-1,4-lactone (**28**) was found in half of the samples at similar concentrations (1–6 μg/L in beers **C**, **E**, **F**, **G**, **J**). In the end, except for a few compounds, these results indicate that all the analyzed beers had a similar furan-substituted substances profile regardless of the pre/post-processing method.

While phenyl-α,β-unsaturated aldehydes and furyl-aldehydes of interest could have been expected in samples, they were not found in any, as for suspected genotoxic thiazolines, methoxyphenyl-ketones, α- and δ-damascone and 4-methyl-5-vinylthiazole.

As anticipated for products containing citrus-related ingredients (**G** and **H**, both with strong citrus aroma), the genotoxic compound *p*-mentha-1,8-dien-7-al (perillaldehyde, **35**) was found (45 and 23 μg/L in **G** and **H** respectively) (Fig. 3).

The labels of beer **G** indicated that in addition to its 5% of fruit juice (2.7% lemon, orange, lime, acerola), it also contained hop, concentrated lemon extract and an unspecified natural flavoring. Beer **H** claimed to contain coriander, orange peel, 0.6% concentrated lemon juice, 0.6% concentrated lime juice, unspecified natural flavorings, hop and apple extract. The higher concentration of perillaldehyde found in beer **G** is in line with its higher content of citrus-related ingredients. Perillaldehyde levels were in the range of its odor threshold (30–62 μg/L) (Burdock & Fenaroli, 2010). These concentrations were close to those naturally occuring in mandarin juice (71 μg/L) (Feng et al., 2018), but more data from citrus-related juices are needed to support these results. Note that, similar to other fragrances of *Citrus* spp., perillaldehyde is mainly contained in the peel, with reported concentrations ranging from 30 to 2000 mg/L in the derived essential oils (Arce & Soto, 2008).

Interestingly, perillaldehyde was not found in beer **I** which contained 1.2% fruit juices (48% raspberry juice), raspberry aroma, coriander and orange peel. Neither was it found in beer **J** containing fresh bergamot (unspecified amount), another source of perillaldehyde (Burdock & Fenaroli, 2010).

Finally, the genotoxic active methylene compound 2,4-pentanedione (**36**) was found in samples **E**, **I** and **J**, at concentrations of 6, 1 and 2 μg/L respectively, below its odor threshold of 10 μg/L (*Pubchem*, n.d.). These concentrations were below values already found in roasted chicken and mango, up to 70 and 90 μg/kg respectively (EFSA, 2004; Pino, Mesa, Muñoz, Martí, & Marbot, 2005).

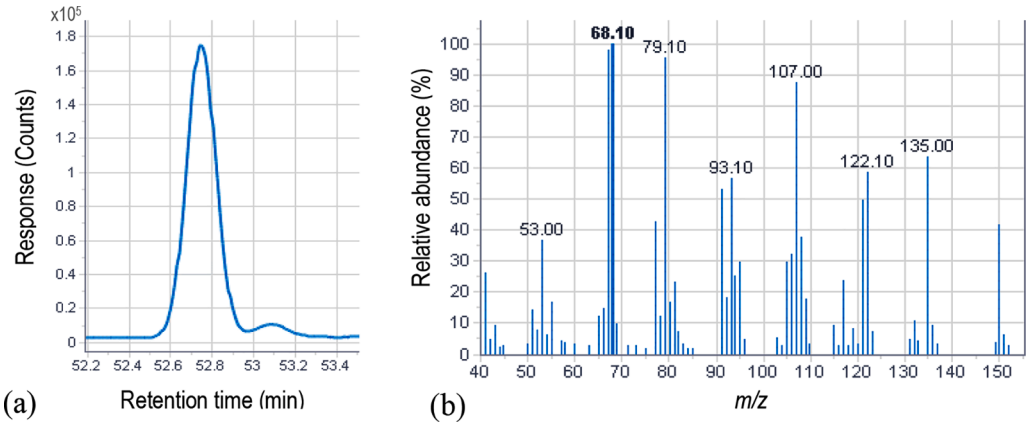


Fig. 3. (a) SIM peak of *p*-mentha-1,8-dien-7-al in beer G and (b) corresponding mass spectrum of *p*-mentha-1,8-dien-7-al.

4. Conclusions

A versatile and accurate extraction procedure using SAFE was optimized and validated for the analysis of 29 (suspected) genotoxic flavoring substances in alcohol-free beers. The validation procedure guarantees accurate results for most of the analytes, which makes this method valuable for food control laboratories. Seven flavoring substances of interest were identified and quantified in alcohol-free beers, including *p*-mentha-1,8-dien-7-al (perillaldehyde). Based on the origins of the sample ingredients and the fact that their concentrations were below or equal to their odor threshold, it can reasonably be assumed that all the identified flavoring subtances were not individually added as such. These compounds might therefore be called aromas instead of flavoring substances. Furan-substituted subtances probably originated from heat-treatment processes while perillaldehyde and 2,4-pentanedione came from plant-based extracts. No significant differences of levels of furan-substituted subtances were observed between dealcoholized beers and beers brewed with special yeasts.

The majority of the (suspected) genotoxic compounds were not identified in the analyzed samples. Analysis of upcoming new AFBs brewed with darker malt would provide relevant information on heat-formed flavoring substance levels, certainly higher than in current AFBs.

Further monitoring of flavoring substances in drinks and food is advised to broaden the occurrence assessment of these compounds and their origin; the latter not being essential for safety evaluation but still of importance to address a potential newly identified safety risk (e.g., prohibition of an ingredient). Besides alcohol-free beers, a special focus should be placed on products more likely to be consumed by sensitive people such as children or pregnant women. Perillaldehyde occurrence should be closely investigated. Additional food and drinks containing citrus-related ingredients (e.g., juices, lemonades) should be investigated to further identify the perillaldehyde content in such products.

CRediT authorship contribution statement

A. Dusart: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **B. Mertens:** Conceptualization, Methodology, Writing - review & editing, Investigation, Visualization. **E. Van Hoeck:** Conceptualization, Methodology, Validation, Formal analysis, Visualization, Supervision. **M. Simon:** Methodology, Validation, Formal analysis, Investigation. **S. Goscinny:** Conceptualization, Methodology, Validation, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision. **S. Collin:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130917.

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