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

ALEXANDRE DUSART[1], Birgit Mertens[1], Els Van Hoeck[1], Margaux Simon[2], Séverine Goscinny[1] and Sonia Collin[2]

[1] *Department of Chemical and Physical Health Risks, Sciensano, Rue Juliette Wytsman 14, 1050 Ixelles, Belgium, Alexandre.Dusart@sciensano.be*

[2] *Unité de Brasserie et des Industries Alimentaires, Louvain Institute of Biomolecular Science and Technology (LIBST), Faculté des Bioingénieurs, Université catholique de Louvain, Croix du Sud 2, Box L7.05.07, 1348 Louvain-la-Neuve, Belgium*

Abstract

The regulatory landscape of flavourings is evolving, thereby putting pressure on control laboratories to develop analytical methods for a wide range of compounds in various types of food (including drinks). In order to improve the monitoring of flavouring substances, a versatile and accurate analytical method using the solvent-assisted flavour evaporation (SAFE) technique coupled to gas chromatography-mass spectrometry in selected ion monitoring mode GC-MS (SIM) was developed and validated. Focus was put on authorised flavouring substances requiring specific attention due to a genotoxic concern based on information available in European risks assessment reports. Thirty-seven (suspected) genotoxic flavouring substances were analysed in a selection of ten alcohol-free beers. Five suspected genotoxic compounds (i.e. 1-(2-furyl)-propan-2-one, 2-acetylfuran, 2-acetyl-5-methylfuran, 2-acetyl-3,5-dimethylfuran, hex-2-eno-1,4-lactone) as well as two confirmed genotoxic flavouring substances (*p*-mentha-1,8-dien-7-al, pentan-2,4-dione) were identified and quantified among the selected samples. The relatively 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.

Keywords: Flavouring substances, Genotoxicity, SAFE, GC-MS, Alcohol-free beers

Introduction

Nowadays, thousands of chemically defined flavouring substances exist and are added to a wide variety of food and drinks to impart or modify odour and/or taste. In Europe, Regulation (EC) No 1334/2008 lays down the general provisions on the use of flavourings in food. This includes restrictions of use such as maximum levels of certain substances for certain food categories, conditions of use for some source materials, as well as substances which shall not be added as such to food. In parallel, over the past twenty years, the European Food Safety Authority (EFSA) has assessed the risks of flavouring substances. While a few compounds have already been confirmed genotoxic and are prohibited to be added as such to food, others are under evaluation due to genotoxic concern although being authorised on the market. Although different analytical methods for the analysis of flavouring substances already exist, a general lack of analytical methodology in the context of law enforcement was reported in Europe [1]. Here, the solvent-assisted flavour evaporation (SAFE) technique was used and validated for the analysis of multiple (suspected) genotoxic flavouring substances in a selection of Belgian alcohol-free beers.

Experimental

Selection of compounds

According to Regulation (EC) No 1334/2008 (consolidation of 21st of May 2019), 302 compounds were under evaluation. However, as this regulation was not consolidated each time a new EFSA opinion was available, all relevant opinions were reviewed to select only compound for which an evaluation was effectively still pending.

Alcohol-free beer samples

Ten popular commercial Belgian AFBs (A, B, C, D, E, F, G, H, I, J) were selected and analysed. 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-diene-7-al (perillaldehyde) [2]. Samples were purchased in September 2020 and stored in the dark at 20°C until analysis.

DOI:10.5281/zenodo.5518091

Characterisation of the samples

The beers were characterised by their ethanol content, colour, bitterness and pH following Analytica EBC methods 9.2.6, 9.6, 9.8, 9.35 respectively [3]. Density was also measured using a density meter (DM4500, Anton Paar GmbH, Graz, Austria).

Isolation of the 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 x 75 mL) during 20 min. After centrifugation (20 min at 2264g) of the resulting emulsion, the aqueous phase was discarded and the remaining organic phase was dried over anhydrous sodium sulphate. Non-volatile compounds were then separated by high-vacuum distillation using the SAFE system (Glasblaeserei Bahr, Manching, Germany)[4]. The conditions for the SAFE analyses were: the water bath temperature was set to 40 ºC, the pressure was kept below 10-3 Pa and the apparatus body was at 30 ºC. The distillate was recovered in a liquid nitrogen cooled flask for 15 min distillation, followed by an extraction with distilled water (3 x 25 mL) to remove any residual alcohol. The extract was dried over anhydrous sodium sulphate. To measure absolute recoveries, 25 µL of decane solution (250 mg/L) was spiked as an external standard (EST) before concentration to 500 µL in a Kuderna-Danish apparatus at 45 ºC. Extracts were stored at −80°C until analysis by gas chromatography-electron ionisation mass spectrometry.

Gas chromatography - mass spectrometry (GC-MS)

SAFE extracts were analysed 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 ionisation at 70 eV. Full-scan (FS) chromatograms (40 – 380 m/z) were also recorded on separate runs for possible qualitative retro-analysis. Data was recorded and analysed with the Agilent OpenLab software (version 2.1).

Quantification of the flavouring 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). The 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 slopes values were statistically compared (t-test, 95% confidence) to determine if matrix effects were similar between samples.

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 flavouring substances concentrations in the targeted matrix. Coefficient 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 $CV_{inter\text{-}r}$ [5].

Results and discussion

Selection of compounds

Recent EFSA opinions (up to June 2020), showed that only 53 out of 302 flavouring substances were still under evaluation due to a genotoxic concern. For these 53 substances, it was verified whether they could be analysed by GC-MS by collecting additional information on commercial availability of the analytical standards, retention time and mass fragmentation. This revealed that 34 substances could be analysed by GC-MS. In addition, 3 confirmed genotoxic flavouring substances that were no longer authorised to be added as such to food in Europe were also included in this study: *p*-mentha-1,8-diene-7-al (perillaldehyde), pentan-2,4-dione and 3-acetyl-2,5-dimethylthiophene. In total, 37 compounds were included in this method:

2-ethyl-5-methylfuran (1); 2-octylfuran (2); 1-(2-furyl)-propan-2-one (3); for 2-acetylfuran (4); 2-pentylfuran (5); 3-acetyl-2,5-dimethylfuran (6); 2-heptylfuran (7); 2-hexanoylfuran (8); 2-acetyl-5-methylfuran (9); 2-acetyl-3,5-dimethylfuran (10); 2-butylfuran (11); 2-butyrylfuran (12); 1-(2-furyl)butan-3-one (13); 3-methyl-2(3-methylbut-2-enyl)furan (14); 2-pentanoylfuran (15); 2-(sec-butyl)-4,5-dimethyl-3-thiazoline (16); 4,5-dimethyl-2-ethyl-3-thiazoline (17); 4,5-dimethyl-2-isobutyl-3-thiazoline (18); 4-methyl-5-vinylthiazole (19); 1-(4-methoxyphenyl)pent-1-en-3-one (20); vanillylidene acetone (21); 1-(4-methoxyphenyl)-4-methylpent-1-en-3-one (22); 2-phenylcrotonaldehyde (23), 5-methyl-2-phenylhex-2-enal (24); 4-methyl-2-phenylpent-2-enal (25); 2-phenylpent-2-enal (26); 5,6,7,7a-tetrahydro-3,6-dimethylbenzofuran-2(4H)-one (27); hex-2-eno-1,4-lactone (28); 3-(2-furyl)acrylaldehyde (29); 4-(2-Furyl)but-3-en-2-one (30); 3-(2-furyl)-2-methylprop-2-enal (31); 3-(5-methyl-2-furyl)prop-2-enal (32); *delta*-damascone (33); *alpha*-damascone (34); *p*-mentha-1,8dien-7-al (35); pentan-2,4-dione (36) and 3-acetyl-2,5-dimethylthiophene (37).

Method development

Liquid samples like alcohol-free beers could be extracted with an organic solvent before or after the SAFE [4, 6-7]. Both approaches were tested and compared. Absolute recoveries were above 80% for most of the compounds when performing the SAFE directly on beers. However, some substances were not recovered at all including 2-pentylfuran (5), 2-heptylfuran (7), 2-butylfuran (11) and thiazolines 16, 17, 18. 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, remained low.

Validation

External calibration curves at 6 concentration levels (i.e. 0.25; 2.5; 5; 7.5; 10 and 15 mg/L) were submitted a Mandel's Fitting test (R^2>0.99) and were linear. 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 5,7,11 and 16,17,18 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. These compounds could therefore only be semi-quantitatively analysed.

For each compound, the peak from the smallest standard addition spike was used to determine the limits of detection (LOD) with S/N = 3, and limits of quantification (LOQ) with S/N=10. Low LOD and LOQ were obtained, in average 0.01 µg/L and 0.05 µg/L.

Coefficients of variation on intra- and interday repeatability were respectively below 10 % and 13%. The measurement of uncertainties was comprised between 6-26%. Apparent recoveries, comprised between 101-123%, were calculated based on spiked samples of triplicate analysis performed three times on different days.

Sample analysis

Among the ten Belgian AFBs, 5 different suspected genotoxic flavouring substances were identified as well as 2 genotoxic flavouring substances (Table 1). Unsurprisingly for products containing heat-treated ingredients, malts here, furan-substituted compounds were identified. 2-Acetylfuran content did not significantly differ between dealcoholized beer and beers brewed with special yeasts (p = 0.44), and was always much below its 10 mg/L odour threshold[2]. No correlation was found between colour and 2-acetylfuran content.

Table 1: Concentrations (µg/L) of suspected and confirmed* genotoxic flavouring substances among 10 Belgian alcohol-free beers.

N°	Compound	A	B	C	D	E	F	G	H	I	J
3	1-(2-Furyl)-propan-2-one	–	–	–	–	n.q.	–	–	–	–	n.q.
4	2-Acetylfuran	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
9	2-Acetyl-5-methylfuran	–	–	–	–	0.9±0.1	–	–	–	–	–
10	2-Acetyl-3,5-dimethylfuran	0.08±0.01[a]	–	–	–	–	–	–	–	–	–
28	Hex-2-eno-1,4-lactone	–	–	1.3 ±0.2	–	5.6 ±0.7	1.3 ±0.2	2.3 ±0.3	–	–	2.5 ±0.3
35	*p*-Mentha-1,8-dien-7-al*	–	–	–	–	–	–	45.4±6.7	23.2±3.4	–	–
36	Pentan-2,4-dione*	–	–	–	–	6.3 ±1.3	–	–	–	1.3 ±0.3	2.4 ±0.5

– = not detected (mean LOD = 0.01 µg/L); n.q. = not quantifiable (mean LOQ = 0.05 µg/L); [a]= LOQ of compound 10 is 0.02 µg/L

As anticipated for products containing citrus-related ingredients (G and H, both with strong citrus aroma), the genotoxic compound *p*-mentha-1,8-diene-7-al (perillaldehyde) was found (45 and 23 µg/L in G and H respectively). The higher concentration of perillaldehyde in beer G is in line with its higher content of citrus-related ingredients. Perillaldehyde levels were in the range of its odour threshold (30-62 µg/L) [2]. Interestingly, perillaldehyde was not found in beer I which contained raspberry juice, raspberry aroma, coriander and orange peel. Neither was it found in beer J containing fresh bergamot, another source of perillaldehyde [2].

Finally, the genotoxic active methylene compound pentan-2,4-dione was found in samples E, I and J, at concentrations of 6, 1 and 2 µg/L respectively, below its odour threshold of 10 µg/L [8]. These concentrations were below levels already found in roasted chicken and mango, up to 70 and 90 µg/kg respectively [9, 10].

Conclusion

A versatile and accurate extraction procedure using SAFE was optimised and validated for the analysis of 29 (suspected) genotoxic flavouring substances in alcohol-free beers. The SAFE technique, usually used without an appropriate validation, showed here the importance of the validation procedure to guaranty accurate results. Seven flavouring substances of interest were identified and quantified in alcohol-free beers. Based on the origins of the samples ingredients and the fact that their concentrations were below or equal to their odour threshold, it can reasonably be assumed that all the identified flavouring substances were not individually added as such. They probably originated from heat-treatment processes or from plant-based extracts. No significant differences of levels of furan-substituted compounds were observed between dealcoholized beers and beers brewed with special yeasts.

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

Further monitoring of flavouring substances in drinks and food is advised to broaden the occurrence assessment of these compounds. Besides alcohol-free beers, a special focus should be put for products more likely to be consumed by sensitive people such as children or pregnant women. Food and drinks containing citrus-related ingredients (e.g. juices, lemonades) should be assigned high priority because of the ubiquitous presence of perillaldehyde in such products.

References

1. European Court of Auditors. Chemical hazards in our food: EU food safety policy protects us but faces challenges. 2019;65.
2. Burdock GA, Fenaroli G. Fenaroli's handbook of flavor ingredients. 6th ed. Boca Raton: CRC Press/Taylor & Francis Group; 2010. 2135 p.
3. Brewers of Europe. The Analytica EBC. 2020.
4. Engel W, Bahr W, Schieberle P. Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. Eur Food Res Technol. 1999;209(3–4):237–41.
5. ISO. ISO/IEC GUIDE 98-3:2008 Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement (GUM:1995). 2008.
6. Piornos JA, Balagiannis DP, Methven L, Koussissi E, Brouwer E, Parker JK. Elucidating the Odor-Active Aroma Compounds in Alcohol-Free Beer and Their Contribution to the Worty Flavor. J Agric Food Chem. 2020;68(37):10088–96.
7. Uselmann V, Schieberle P. Decoding the Combinatorial Aroma Code of a Commercial Cognac by Application of the Sensomics Concept and First Insights into Differences from a German Brandy. J Agric Food Chem. 2015;63(7):1948–56.
8. Pubchem [Internet]. Available from: https://pubchem.ncbi.nlm.nih.gov/
9. EFSA. Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 11 (FGE.11): Aliphatic dialcohols, diketones, and hydroxyketones from chemical group 10 (Commission Regulation (EC) No 1565/2000 of 18 July 2000). EFSA J [Internet]. 2004 [cited 2020 Dec 6];(EFSA Journal). Available from: https://data.europa.eu/doi/10.2903/j.efsa.2005.166.
10. Pino JA, Mesa J, Muñoz Y, Martí MP, Marbot R. Volatile Components from Mango (Mangifera indica L.) Cultivars. J Agric Food Chem. 2005;53(6):2213–23.