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Impact of temperature during beer storage on beer chemical profile

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ABSTRACT

Aiming to gain insights into the impact of storage conditions on the chemical profile of beer samples, changes on the relative amount of several chemical classes of compounds was monitored. The influence of storage conditions was statistically discerned using the hierarchical cluster analysis complemented by *heatmap* date visualization. Aldehydes, furanic compounds and esters showed a clear role in beers stored at 37 ± 1 °C (contribution >1, as obtained in the *heatmap* data visualization). The reaction rate constant and temperature dependence was well described by the Arrhenius equation for these compound classes, for which the reaction rate increased with increasing temperatures. The rate of development of furanic compounds, aldehydes and esters showed to be almost 140, 90 and 20 times higher in beers stored at higher temperatures (37 ± 1 °C) when compared to beers stored at 4 ± 1 °C, respectively. These results indicate that temperature was the main parameter contributing to the major changes in beer chemical profile. Through olfactometric analysis, the major difference was found in aged beers, both naturally and forced aged, where the prevalence of sweet and papery aroma notes were noticed, linked to the development of β-damascenone and *E*-2-nonenal, respectively.

1. Introduction

Beer aging is considered one of the most serious problems currently faced by the brewing industry. Hence, changes in the beer chemical composition during storage can be considered the main quality problem faced by brewers. Flavor instability or beer staling is a highly complex process owing to the numerous different oxidative and non-oxidative reactions that take place throughout beer storage (Lehnhardt, Gastl, & Becker, 2018). Consequently, utmost attention has been given to the study on the mechanisms behind the appearance of undesirable aromas in beer (Murakami, Goldstein, Navarro, Seabrooks, & Ryder, 2003; Schieberle & Komarek, 2003, pp. 70–79).

A vast variety of flavors may arise, depending on the beer type as well as on the storage conditions (Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006). The course of aging of lager beers is well documented since it is especially susceptible to chemical changes after 3–6 months of storage at room temperature (Baert, De Clippeleer, Hughes, De Cooman, & Aerts, 2012; Guido et al., 2007; Lehnhardt et al., 2018; Saison, De Schutter, Uyttenhove, Delvaux, & Delvaux, 2009; Vanderhaegen et al., 2006). The occurrence of changes in beer chemistry and flavor during storage is mainly due to the development of aldehydes, esters, higher alcohols, and other compounds arising from several steps of the brewing process (Ferreira & Guido, 2018). Aldehydes are a group of compounds with a high contribution to beer staling. These compounds are produced mainly through the Maillard reactions, Strecker degradation and lipid oxidation (Baert et al., 2012). An increase of their concentration in aged beers can significantly enhance the appearance of unpleasant flavors, such as, nut, fat, fruit, cardboard, caramelic and bready flavors (Gonçalves et al., 2010; Wang, Li, Ji, Hu, & You, 2014). Esters of short-chain and branched-chain fatty acids, which are most aroma-active, are arguably the most important volatile compounds in beer. They have a positive impact on the overall beer flavor, especially aroma, but excessive levels of esters can lead to overly fruity, fermented off-flavors (Liu, 2015, pp. 357–374; Verstrepen et al., 2003). Esters, as well as higher alcohols, are produced by the yeast metabolism during fermentation. Besides ethanol, higher alcohols are the major alcohols that impart sensory properties to beer. Higher alcohols impart a range of organoleptic attributes such as alcoholic, fruity, pungent, solvent-like and rose-like or floral, depending on the concentration and type of alcohol (Liu, 2015, pp. 357–374). Hops (*Humulus lupulus* L.) are essential

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for the brewing process in order to confer singular sensory properties of beer. Hop-derived compounds play a crucial role in beer flavor by contributing not only to the bitter taste but also to the fruity, citrus-like and floral aromas; the latter is ascribed to terpenes in hops. The specific flavor notes derived from hops depends on the type of hopping procedure applied, during the wort boiling (Liu, 2015, pp. 357–374; Steven, 2017).

As beer undergoes a huge variety of chemical reactions leading to the development of staling compounds, and consequently staling aromas, the identification of chemical markers of beer aging, is extremely important. To achieve this, a sample preparation methodology is required in order to pre-concentrate/isolate the target analytes. Solid phase microextraction (SPME) has proven to be a suitable technique providing many advantages over conventional sample preparation techniques (ben Hammouda, Freitas, Ammar, Da Silva, & Bouaziz, 2017; Branco et al., 2020; Martins et al., 2018; Santos, da Silva, & Cabrita, 2020) for volatile organic compounds (VOCs) analysis. Frequently it is associated to gas chromatography/mass spectrometry (GC/MS) techniques (Martins et al., 2020; Ribeiro, Freitas, & da Silva, 2008) in order to characterize complex matrices, namely in beer analysis (Dennenlöhr, Thörner, Manowski, & Rettberg, 2020; Giannetti, Mariani, Torrelli, & Marini, 2019).

In recent years, intensive studies have been carried out regarding the sensory activity of the individual components of food and alcoholic beverages, like beer. The majority of accomplishments within this area can be attributed to the combination of gas chromatography with olfactometry detection (GC-O) (Plutowska & Wardencki, 2008). The determination of analyte's odor is possible thanks to the presence of a special attachment, a so-called olfactometric port, connected in parallel to conventional detectors, such as flame-ionization detector (FID) or mass spectrometer (MS) (Delahunty, Eyres, & Dufour, 2006).

The present work combines the use of HS-SPME with gas chromatography/mass spectrometry (GC/MS) and gas chromatography – olfactometry (GC-O) analysis for the assessment of the volatile profile in fresh, naturally and forced aged beers. These techniques, together, allowed the detection of chemical markers of beer aging and, at the same time, the identification of the main aromas developed through the storage time, contributing to the changes in beer flavor. The ability to distinguish beers submitted to different storage conditions, regarding their volatile and aroma fingerprints, has been evaluated by multivariate techniques, namely hierarchical cluster analysis (HCA) and heat mapping and multiple correspondence analysis (MCA).

2. Materials and methods

2.1. Sampling and aging assay

All beer samples were kindly provided by Super Bock Group (Porto, Portugal). Beers were submitted to a naturally and forced aging process. During forced aging, beers were stored in an oven (Raypa, Incuterm, Barcelona, Spain) under controlled temperature at 37 ± 1 °C, in order to reproduce warm storage conditions. The differences in the relative levels of several chemical classes were monitored during 7 and 14 days. Additionally, the study was also conducted in natural aging conditions. For this purpose, beers were stored during 3 and 6 months at 20 ± 2 °C (room temperature) and at 4 ± 1 °C (considered as control samples).

2.2. Analysis of beers volatile fingerprint by HS-SPME-GC/MS and HS-SPME-GC-O/TOF-MS

2.2.1. Preparation of the samples: HS-SPME extraction

The HS-SPME extraction was performed using a carboxen/divinylbenzene/polydimethylsiloxane fiber (Carb/DVB/PDMS, 1 cm, 50/30 μm film thickness (d_f)) supplied from Supelco (Bellefonte, PA, USA). Prior to use, the fiber was conditioned following the manufacturer's recommendations. Fiber blanks were run periodically to ensure the absence of contaminations and/or carryover. The samples, with 4.0 mL volume each, with 0.8 g of sodium chloride (pure, LabChem, Loures, Portugal), were introduced in a 20 mL vial and sealed with Teflon-lined rubber septum/magnetic screw cap. The vials were stored at 4 °C, overnight, to promote degasification of the samples. For the extraction, the vials were equilibrated for 10 min at 40 °C and then extracted for 30 min at the same temperature. The thermal desorption of analytes was carried out by exposing the fiber to the GC injection port at 260 °C for 3 min in a splitless mode.

2.2.2. GC/MS conditions

The analyses were performed on a GC/MS system consisting of a Bruker GC 456 with a Bruker mass selective detector Scion TQ (Bruker, Billerica, MA, USA). An automatic sampler injector was used: CTC Analysis auto sampler CombiPAL (Agilent, Santa Clara, CA, USA). Data were acquired with MSWS 8.2 Bruker and analyzed with Bruker MS Data Review 8.0. The chromatographic separation was achieved on a ZB-WAX PLUS capillary column (60 m × 0.32 mm i.d., 1.0 μm d_f) supplied by Phenomenex (Torrance, CA, USA). The oven temperature program began at 40 °C hold for 5 min, raised at 4 °C.min^{-1} up to 240 °C and hold for 15 min. An acquisition delay of 15 min was carried out in order to avoid the detection of the ethanol peak present in beer samples. Helium was used as carrier gas at a constant flow of 1.7 mL min^{-1}. The MS transfer line and source temperatures were set at 240 °C and 220 °C, respectively.

The spectra were matched by NIST MS Search Program Version 2.3 g. To determine the retention times and characteristic mass fragments, electron ionization (EI) at 70 eV mass spectra of analytes were recorded at full scan, from 40 to 450 Da. The linear retention index (LRIs) values were calculated by analyzing the commercial hydrocarbon standard solution $C_8 - C_{20}$ (Sigma-Aldrich, Darmstadt, Germany), using the same chromatographic conditions. The relative amount of individual components are expressed as area ratio (AR) relative to the peak area of 1-octanol.

2.2.3. GC-O/FID conditions

The analyses were performed on a GC-O/FID system consisting of an Agilent 6890 chromatograph equipped with a GL Science PHASER GC Olfactory Port and a flame ionization detector (FID) (Agilent, Santa Clara, CA, USA). Chromatographic separation was achieved on a DB-5ms capillary column (15 m × 0.25 mm i.d., 0.25 μm d_f) supplied by Phenomenex (Torrance, CA, USA). About 1 m of deactivated silica column, with 0.25 mm i.d., was connected through a "Y" piece, to link the column to olfactometer and 1 m of deactivated silica column, with 0.25 mm i.d., was connected to the FID detector. The oven temperature program began at 35 °C, holds for 2 min, raised at 4 °C/min up to 140 °C, and raised at 20 °C.min^{-1} up to 270 °C. Hydrogen was used as carrier gas at constant flow of 1.2 mL min^{-1}. The water vapor flow at the olfactometer was set at 24 mL/min. The transfer line temperature was set at 270 °C. LRIs values were calculated by analyzing the commercial hydrocarbon standard solution $C_8 - C_{20}$ (Sigma-Aldrich, Darmstadt, Germany), using the same chromatographic conditions.

2.2.4. GC/TOFMS conditions

The analyses were performed on a LECO Pegasus BT GC/TOFMS comprised of an Agilent 7890B GC with a split/splitless injector (Agilent, Santa Clara, CA, USA) and a LECO Time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA). Injections were performed by a CTC-Analytics L-PAL3 autosampler fitted with a 10 μL syringe (SETonic, Ilmenau, Germany). Chromatographic separation was achieved on an HP-5ms Ultra-inert column (30 m × 0.25 mm i.d., 0.25 μm d_f) supplied by Agilent, CA, USA. The oven temperature program began at 35 °C hold for 2 min, raised at 4 °C.min^{-1} up to 140 °C, and raised at 20 °C.min^{-1} up to 270 °C. Helium was used as carrier gas at constant flow of 1.2 mL min^{-1}. The MS transfer line and source temperatures were set at 300 °C and 270 °C, respectively. LRIs values were calculated by analyzing the

commercial hydrocarbon standard solution C_8 – C_{20} (Sigma-Aldrich, Darmstadt, Germany), using the same chromatographic conditions.

2.3. Statistical analysis

Peak areas of identified compounds were extracted from the chromatograms and used to calculate the area ratio (AR) relative to the peak area of 1-octanol. These values were used to build the full data matrix, consisting of 21 observations (7 beer samples, each one by three independent assays) and 42 variables (analytes). The complete list of these analytes is provided in Table 1.

The differences between the sum of the relative content, represented as the total area ratio (AR) ± SD, of each compound's family found in aged beers (naturally and forced aged beers) and those found in fresh beers (FB, considered as control samples), were compared using the *t*-test. Statistically significant differences were considered for $p < 0.05$, $p < 0.01$ and $p < 0.001$, to evaluate the strength of the observed differences.

Multivariate analysis, in particular hierarchical cluster analysis (HCA) and heat mapping and multiple correspondence analysis (MCA) were applied to the present data. HCA is an exploratory tool, being applied to characterize the data set, revealing natural groupings (or clusters) within it, through the representation of dendrogram (tree diagram) and a heatmap. Squared Euclidean distances were used, and clustering algorithm used was Ward's minimum variance. Both outputs, tree diagram and heatmap, provided a clear and rapid visualization of similarities and differences between the seven samples under study.

Instead of calculating relationships pair-wise, the inter-relationships among all the variables were evaluated and visualized using MCA. This is a method that allows studying the association between two or more qualitative variables. It reduces the dimensionality of data and can be thought of as analogous to Principal Component Analysis (PCA) for quantitative variables. In this work, MCA was applied to determine a relationship between the different storage conditions applied to beers samples, the aromas noticed by olfactometric analysis, and the compounds identified by mass spectrometry.

All the statistical analyses were performed using Addinsoft (v.2021.2.2.1129, 2021) XLSTAT statistical and data analysis solution, New York, USA.

Table 1
Volatile compounds identified in beer samples (fresh, naturally and forced aged beers).

Nr.	RT (min)	Compound	Most abundant ions (m/z)	$LRI_{calc.}$ [a]	$LRI_{lit.}$ [b]	Odor descriptor [c]
1	**17.760**	**Isobutyl acetate**	**43/41/56**	**1025**	**1018–1034**	**Alcoholic, Fruity**
2	**18.830**	**Ethyl butanoate**	**71/43/88/41/73/42**	**1050**	**1031–1055**	**Fruity**
3	**20.936**	**Isobutyl alcohol**	**41/43/42**	**1099**	**1090–1102**	**Alcoholic**
4	**22.481**	**Isoamyl acetate**	**43/55/70**	**1136**	**1103–1137**	**Fruity, banana**
5	**23.004**	**Ethyl pentanoate**	**43/70/85/55**	**1149**	**1138**	**Fruity**
6	**23.206**	**1-Butanol**	**41/56/43/55**	**1154**	**1142–1152**	**Alcoholic, sweet**
7	**25.758**	**Isoamyl alcohol**	**55/41/42/43/70**	**1217**	**1203–1253**	**Alcoholic, fruity**
8	**26.051**	**Limonene**	**67/93/68/94/41**	**1224**	**1193–1194**	**Citrus**
9	**27.018**	**Ethyl hexanoate**	**88/43/99/70/60**	**1249**	**1202–1251**	**Sweet, fruity**
10	**29.106**	**2-Furfuryl ethyl ether**	**81/82**	**1303**	**1297**	**Nutty, coffee**
11	**30.117**	**Unknown 1**	**69/41**	**1330**		
12	**31.001**	**Unknown 2**	**106/109/108/73/41**	**1356**		
13	**31.113**	**Unknown 3**	**79/45/93/137/77/43**	**1358**		
14	**31.324**	**1-Hexanol**	**56/41/69**	**1364**	**1338–1380**	**Green, grass**
15	**33.363**	**Nonanal**	**57/41/70/81/95**	**1421**	**1388–1403**	**Aldehydic, sweet**
16	**34.446**	**Ethyl octanoate**	**88/70/101/57/41/127**	**1452**	**1407–1440**	**Fruity, sweet**
17	**34.631**	**1-Octen-3-ol**	**57/72**	**1457**	**1456**	**Sweet, perfumy**
18	**34.979**	**1-Heptanol**	**70/41/55**	**1467**	**1458–1460**	**Green, solvent**
19	**35.914**	**Furfural**	**95**	**1494**	**1463–1497**	**Caramel, bready**
20	**36.462**	**Unknown 4**	**81/96/67/79**	**1511**	**1492–1545**	**Sweet, fat, fruity**
21	**37.463**	**2-Acetylfuran**	**95/110**	**1542**		
22	**37.877**	**Linalool**	**93/71/43/69/121**	**1554**	**1511–1523**	**Roasted, backed**
23	**38.441**	**1-Octanol**	**56/55/41/69/83**	**1571**	**1546–1554**	**Citrus, floral**
24	**38.516**	**Benzaldehyde**	**105/77/51**	**1574**	**1558–1561**	**Citrus, rose**
25	**41.103**	**Ethyl decanoate**	**88/101/70/55/41**	**1656**	**1506–1547**	**Almond**
26	**41.309**	**3-(Methylthio) propyl acetate**	**73/88/43**	**1663**	**1603–1647**	**Fruity, floral**
27	**41.687**	**Furfuryl alcohol**	**98/81/41**	**1675**	**1616–1672**	**Bready, burnt**
28	**42.174**	**Phenylacetaldehyde**	**91/92**	**1691**	**1630–1669**	**Floral**
29	**42.777**	**Ethyl 9-decenoate**	**55/88/41/69/101/110/135**	**1711**	**1674–1708**	**Fruity, fatty**
30	**43.254**	**α-Terpineol**	**93/59/81/43**	**1727**	**1709**	**Citrus, floral**
31	**43.882**	**3-(Methylthio) propanol**	**75/57/106**	**1748**		
32	**44.741**	**Unknown 5**	**105/77**	**1777**	**1768–1792**	**Citrus, floral**
33	**44.894**	**Citronellol**	**69/41/60/81/95**	**1783**	**1783–1811**	**Floral, honey**
34	**46.060**	**Ethylphenyl acetate**	**91/164**	**1823**	**1777–1848**	**Floral, sweet**
35	**47.015**	**2-Phenylethyl acetate**	**104/43/91**	**1857**	**1785–1825**	**Waxy, fruity**
36	**47.115**	**Ethyl dodecanoate**	**88/101/70/55/41**	**1861**		
37	**48.331**	**Unknown 6**	**43/71/41/73**	**1904**	**1914–1945**	**Floral, sweet**
38	**48.984**	**Ethylphenyl propanoate**	**104/91**	**1929**	**1859–1989**	**Rose, perfume**
39	**49.682**	**Phenylethyl alcohol**	**91/62/65/122**	**1955**		**Caprylic, vegetable**
40	**51.193**	**Hexanoic acid**[d]	**60/73/45/43/87**	**NC**		**Caprylic**
41	**56.058**	**Octanoic acid**[d]	**60/73/45/87/101**	**NC**		**Tallowy, caprylic**

[a] LRI_{calc} – retention index calculated from C_8 – C_{20} n-linear alkanes; NC – not calculated.

[b] LRI_{lit} – linear retention indices reported in the literature for WAX capillary column (Aubert & Bourger, 2004; Bianchi, Careri, Mangia, & Musci, 2007; Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Giannetti et al., 2019; Goodner & Technology, 2008; Kishimoto, Noba, Yako, Kobayashi, & Watanabe, 2018; Z.; Li et al., 2019; Pereira et al., 2021; Welke, Manfroi, Zanus, Lazarotto, & Zini, 2012).

[c] According to the information collected from www.thegoodscentscompany.com and from (Steven, 2017).

[d] Tentative identification by NIST comparison.

3. Results and discussion

3.1. Beer chemical profile

The combination of HS-SPME with GC/MS is a powerful tool to establish volatile fingerprints with great sensitivity, namely in food samples and beverages (Alves et al., 2020). In this work, beers submitted to different storage conditions (fresh, naturally aged (3 and 6 months at 20 ± 2 °C) and forced aged beers (7 and 14 days at 37 ± 1 °C)) were analyzed. A commercial hydrocarbon mixture (C_8 – C_{20}) was used in order to calculate the linear retention indices (LRI). These LRI values were compared with those found in literature.

A total of 42 metabolites were tentatively identified based on the comparison of their mass spectra to the reference database (NIST MS Search Program Version 2.3), as well as the calculated LRI ($LRI_{calc.}$) with the values reported in the literature ($LRI_{lit.}$) for the polyethylene glycol (or equivalent) capillary column (Table 1). The total metabolites identified in this work are listed in Table 1 according to their elution order on a DB-WAX capillary column, and including their retention times (min), LRI (LRI_{calc} and LRI_{lit}), their most abundant ions and odor descriptors. The mentioned metabolites (Table 1) were identified in all beer samples (fresh, naturally and forced aged beers).

The metabolites tentatively identified by MS, were grouped into six different chemical classes. These include esters (13), aldehydes (3), alcohols (9), furanic compounds (4), fatty acids (3) and terpenes (4).

3.2. Beer chemical classes

Table 2 shows the sum of the relative content of the compounds identified in each chemical class for beers submitted to different storage conditions. The statistical *t*-test was applied to estimate the differences in the relative content of esters, alcohols, aldehydes, furanic compounds, carboxylic acids and terpenes, in comparison to the relative levels of these classes found in fresh beers.

A hierarchical cluster analysis (HCA) combined with the heatmap representation was constructed in order to evaluate if cluster analysis could be used to define chemical differences among beers exposed to different storage conditions. The heatmap (Fig. 1) shows a graphical representation of the chromatographic data achieved for the 36 detected and identified analytes subdivided into their correspondent chemical class.

The chromatic scale of the heatmap allows access to the relative amount of each chemical class (from white, minimum, to dark, maximum) and, consequently, to observe the chemical pattern for the beers under study. The dendrogram (Fig. 1) built for HCA is an exploratory tool that reveals clustering between the beers under study, gathering them according to their chemical profile's similarities.

Despite the large number of potential variables among the samples, it was possible to observe the formation of two principal clusters (Fig. 1): cluster 1 and cluster 2. Cluster 1 aggregates fresh beers (FB) and beers stored at low temperatures (4 ± 1 °C). In contrast, cluster 2 groups naturally and forcibly aged beers. Fresh beers were characterized by the lower levels of the compounds belonging to the chemical classes identified, being the alcohols those that showed higher levels in these samples. When higher temperatures were applied, the relative chemical composition of beers changes: a highlighted increase in aldehydes, esters and furanic compounds; a decrease in the relative contribution of alcohols; and a very low contribution of terpenes and fatty acids was observed, when compared to fresh beers.

Regarding the chemical profile of beers submitted to different storage conditions, it is clearly demonstrated that temperature is the principal contributor to the dissimilarities observed among beers under study. Despite naturally and forcibly aged beers are grouped in the same cluster, they belong to different sub-groups (B and A, respectively). Particularly, the higher levels of furanic compounds observed in beers exposed to higher temperature (37 ± 1 °C), are found to be the key compounds contributing to the chemical differences induced by temperature. In addition, the higher content of alcohols, terpenes and fatty acids observed in naturally aged beers, compared to forced aged beer, contributes to these dissimilarities. Regarding the fatty acids and terpenes, it is possible to observe that the main factor contributing to changes in their levels, is the time of storage, instead of temperature. Concerning the cluster 1, with the grouping of fresh beers and beers stored at low temperature (4 ± 1 °C), it is possible to observe that storing beer at low temperatures helps to protect the freshness of this beverage, conserving the original chemical profile of fresh beers, mainly by avoiding the development of aldehydes and furanic compounds.

3.3. Effect of storage temperature on the rate of development of staling compounds in beer

The effect of temperature in a chemical reaction rate may be repre-

Table 2
Sum of the relative content, represented as the total area ratio (AR) ± SD, of all the compounds identified in each defined chemical class for fresh beers (FB), natural aged beers (3 and 6 months at 20 ± 2 °C) and forced aged beers (7 and 14 days at 37 ± ± 1 °C). * represents statistical differences with $p < 0.05$; ** represents statistical differences with $p < 0.01$; and *** represents statistical differences with $p < 0.001$, in comparison to FB.

	Natural Aged Beers				Forced Aged Beers	
Fresh Beers	4 ± 1 °C		20 ± 2 °C		37 ± 1 °C	
	3m	**6m**	**3m**	**6m**	**7d**	**14d**
Esters						
100.1672 ± 10.6515	123.7924 ± 11.5267 (p-value = 0.060)	143.6694 ± 2.9959 (p-value = 0.002)**	153.5435 ± 19.1218 (p-value = 0.014)*	171.0003 ± 2.6926 (p-value = 0.000)***	137.5158 ± 1.6751 (p-value = 0.004)**	152.2136 ± 6.3479 (p-value = 0.002)**
Aldehydes						
1.1831 ± 0.0833	1.5557 ± 0.0609 (p-value = 0.003)**	1.8064 ± 0.3048 (p-value = 0.027)*	2.3645 ± 0.0519 (p-value<0.0001)***	4.0641 ± 0.2094 (p-value<0.0001)***	2.8789 ± 0.0767 (p-value<0.0001)***	4.4008 ± 0.0356 (p-value<0.0001)***
Alcohols						
146.6262 ± 13.9496	180.8338 ± 14.3349 (p-value = 0.041)*	149.6372 ± 7.1776 (p-value = 0.756)	176.1959 ± 10.6078 (p-value = 0.043)*	161.9971 ± 1.7031 (p-value = 0.131)	146.7111 ± 4.5227 (p-value = 0.992)	157.5988 ± 15.4089 (p-value = 0.412)
Furanic Compounds						
1.2027 ± 0.0409	1.1191 ± 0.0179 (p-value = 0.032)*	1.1221 ± 0.0141 (p-value = 0.032)*	1.5609 ± 0.0200 (p-value = 0.000)***	1.6781 ± 0.0143 (p-value<0.0001)***	1.9791 ± 0.1055 (p-value = 0.000)***	2.5392 ± 0.1150 (p-value<0.0001)***
Acids						
15.5308 ± 0.6679	17.3647 ± 0.1634 (p-value = 0.016)*	30.8397 ± 0.3075 (p-value<0.0001)***	19.4653 ± 1.0896 (p-value = 0.006)**	31.4073 ± 0.5203 (p-value<0.0001)***	18.5905 ± 1.3991 (p-value = 0.027)*	16.8453 ± 2.3835 (p-value = 0.410)
Terpenes						
0.5595 ± 0.0625	1.0161 ± 0.0250 (p-value = 0.000)***	1.2576 ± 0.0436 (p-value<0.0001)***	1.2059 ± 0.0422 (p-value = 0.000)***	1.2095 ± 0.0008 (p-value<0.0001)***	1.1025 ± 0.0390 (p-value = 0.000)***	0.9051 ± 0.0136 (p-value = 0.001)***

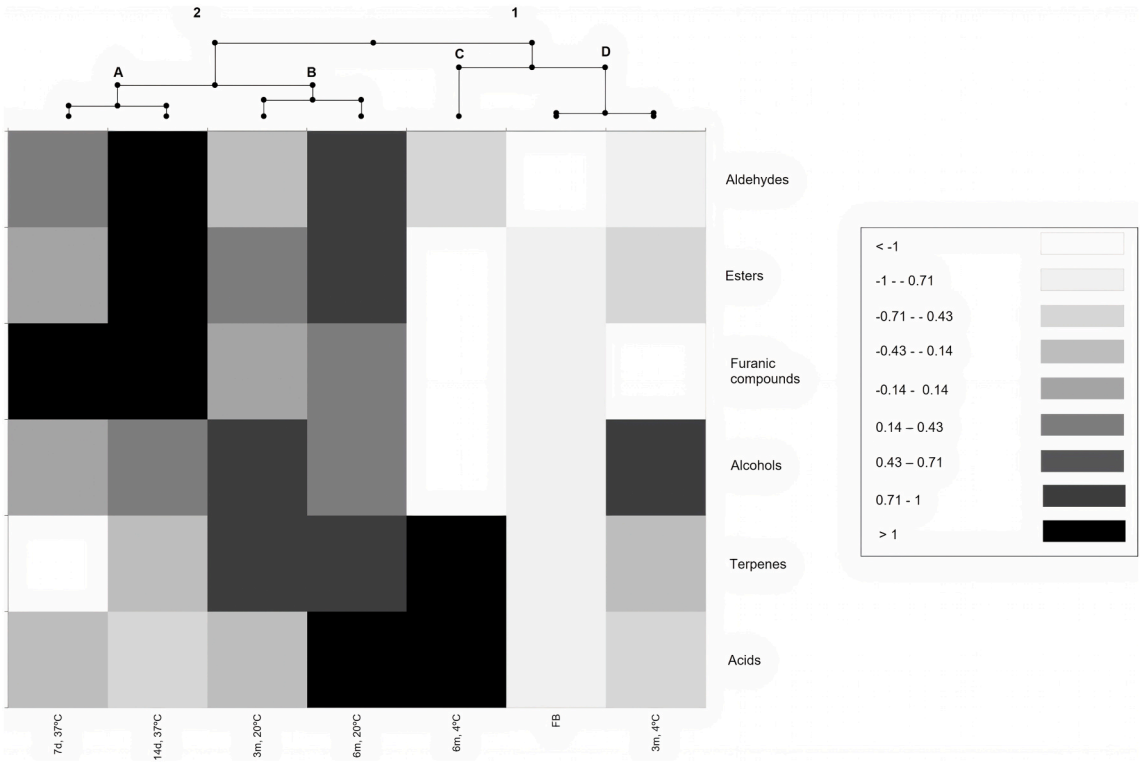

Fig. 1. Heatmap and dendrogram representation of the chemical classes identified in beers under study (fresh beers (FB), naturally aged beers (3 and 6 months at 20 ± 2 °C) and forced aged beers (7 d and 14 d at 37 ± 1 °C)). The content of each chemical class was illustrated through different colors (from white, minimum, to dark, maximum). Dendrogram for the HCA results using Ward's cluster algorithm to the data set was also included.

sented by Arrhenius equation:

$$k = A \cdot e^{-E_a/RT} \quad (1)$$

where the k is the rate constant, A is the pre-exponential factor assumed to be independent of temperature, E_a is activation energy; R is the gas constant and T is the temperature in Kelvin.

According to the rate constants of the chemical classes of compounds under study at different temperatures, together with the corresponding temperature, the rate constants for each chemical classes at different temperatures were estimated, according to Arrhenius equation (Table 3). The ratio of the rate of development of furanic compounds, aldehydes and esters in beers stored at different temperatures were calculated by dividing the rate constants at different temperatures by those at 4 ± 1 °C (Table 3).

According to several authors, temperature have a great impact on beer fermentation and maturation kinetics and ester production during fermentation (Kucharczyk & Tuszyński, 2018; Nakatani, Fukui, Nagami, & Nishigaki, 1991), on stale flavor development (H. Li, Liu, He, Cui, & Hao, 2015) and on methional and phenylacetaldehyde development during beer aging (Soares da Costa et al., 2004). In this work, the influence of different storage temperatures was studied regarding the levels of several chemical classes of compounds, such as, aldehydes, furanic compounds and esters, which showed to be the most affected by the application of higher storage temperature.

It can be seen from the results shown in Table 2 that the ratio of rate constants of aldehydes and furanic compounds at room temperature (20 ± 2 °C) is nearly 10 times that at 4 ± 1 °C. Additionally, the ratio of rate constants of furanic compounds and aldehydes in beers stored at 37 ± 1 °C is nearly 130 and 70 times that at 4 ± 1 °C, respectively, which means that the development of these chemical class of compounds in beer is temperature dependent. Regarding the ratio of rate constants of esters, they are lower when compared to the other classes of compounds. Neverthless, the ratio of rate constant of esters in beers stored at 37 ± 1 °C is nearly 15 times that at 4 ± 1 °C.

The shelf-life of a product, namely beer, may be defined as the time that essential characteristics are maintained under specific storage conditions. Nonetheless, it may be estimated by accelerated stability testing protocols. The determination of beer shelf-life was assessed, using an accelerated beer aging, concerning different storage conditions (different times and temperatures), regarding furanic compounds, aldehydes and esters relative contents. Based on these indicators, beer shelf-life was estimated. From the results obtained, it is not completely reliable to choose a single marker to define the beer shelf-life. According to the results obtained, furanic compounds and aldehydes proved to be good chemical markers for this purpose. Storing beer at lower

Table 3
Rate constant (k) and ratio of rate constants (in comparison to beers stored at 4 ± 1 °C) obtained for aldehydes, furanic compounds and esters, for three different temperatures (4 ± 1, 20 ± 2 and 37 ± 1 °C).

	k[a]			Ratio of rate constants		
Temperature (° C)	**Aldehydes**	**Furanic Compounds**	**Esters**	**Aldehydes**	**Furanic Compounds**	**Esters**
4 °C	0.0029	0.0005	0.1776	1.0	1.0	1.0
20 °C	0.0243	0.0058	0.7157	8.4	11.6	4.0
37 °C	0.1857	0.0632	2.6875	64.0	126.4	15.1

[a] Estimated rate constant according to the Arrhenius equation.

temperatures (4 °C) increases its shelf-life, as expected. In contrast, higher storage temperatures (37 ± 1 °C) showed to be detrimental for the beer shelf-life. Regarding furanic compounds and aldehydes, beers stored at 37 ± 1 °C for 12 days mimic the impact on beer stability of beers stored at 20 ± 2 °C during 6 months (beer *best-before date*).

3.4. Identification of volatile compounds in beer by GC-O/TOF-MS

The flavor profile of beers submitted to different storage conditions were analyzed by an untrained sensory panel composed by 8, not-trained, sensorial panel. Fresh beers, naturally aged and forced aged beers were analyzed with the purpose to understanding the impact of different storage times and temperatures on beer aromas/odors. Combining olfactometric and chromatographic (with MS detection) information, 15 compounds were olfactometric detected by all panel members, of those, 11 were recognized (Table 4) by matching mass spectra with spectra of reference compounds in NIST MS Search Program Version 2.3 g, and LRI data from literature. Several other components were identified by FID. However, no olfactory characteristics were assigned since, probably, they were below the olfactory perception threshold of the untrained panel. The HS-SPME-GC/O-FID analysis was supported by GC/TOFMS analysis with an analytical column with the same low-polarity stationary phase in order to allow LRI calculations and literature comparison. Indeed, less polar columns are known to allow much higher reproducibility in the determination of LRI, regardless of column size as well as film thickness and purchaser, as referred and studied by Mateus, Barata, Zrostlíková, da Silva, & Paiva, 2010, when compared with polar columns (Mateus et al., 2010).

To create a visual profile of "fingerprint" of product attributes, spider plots were created by plotting intensity values on each scale. Fig. 2 shows attributes (aromas/odors) identified by the olfactometric analysis in fresh, naturally (6 months at 20 ± 2 °C) and forced aged beers (14 days at 37 ± 1 °C).

These plots illustrated that sweet, sweet/floral and sweet/honey aromas were the most prominent flavor characteristics of forced aged beers (14 days at 37 ± 1 °C). In addition, the perception of several aromas, like herbal, increased in forced aged beers.

Based on these information (aromas/odors, compounds and intensities), MCA was applied to identify the odor profile of the analyzed beers. Fig. 3A shows the two-dimensional Categories plot, showing the correlation between the different identified aromas and chemical species detected in beers submitted to the different storage conditions. Fig. 3B shows the observations plot, where it is possible to see the contribution of the different beer samples analyzed for both dimensions.

The data was reduced into a two-dimensional plot, where the first dimension accounts for 10.25% of the variance and the second for 10.08%, yielding a total variance of 20.33%. Although the variability explained by MCA is 20% for the dimension 1 and 2 together, not unusual in MCA, stills relevant since qualitative data are collected, namely subjective, like the sensorial panel olfactometric perceptions. Indeed, from the obtained bidimensional plot (Fig. 3) a dataset is represented as clouds of points in a multidimensional Euclidean space as described by (Costa, Santos, Cunha, Cotter, & Sousa, 2013), showing in this case that a relationship exists and how variables are related, thus offering a valid statistical result that can be visualized and interpreted.

Analyzing the data in Fig. 3A, it is possible to observe that the fruity aromas identified in analyzed samples are due to the presence of ethyl hexanoate and isoamyl acetate in beers matrix. In addition, the detection of papery aroma is linked to the development of *E*-2-nonenal. The floral aromas perceived in analyzed beers were due to the presence of phenylacetaldehyde, ethylphenyl acetate and the unknown compounds at RT = 15.29 min and RT = 16.29 min. The herbal aromas were detected due to the presence of ethylphenyl acetate, and the sweet and honey aromas identified are linked to the detection of nonanal, β-damascenone and the unknown compound at RT = 16.49 min.

Analyzing both Figures (Fig. 3A and B), it is possible to observe that the fruity, floral and herbal aromas are correlated to all the analyzed beer samples (fresh beers and natural and forced aged beers). However, the papery and sweet aromas are highly correlated with natural and forced aging. The papery aroma was identified due to the presence of *E*-2-nonenal in aged beers, that is formed by the lipid-oxidation of fatty acids. This compound has been considered for several years the main responsible for the beer staling but, nowadays it is recognized as just a part of a more complex picture of staling (Baert et al., 2012). The sweet aromas developed in natural and forced aged beers are well correlated with the presence of β-damascenone, a compound formed by the acid-catalyzed hydrolysis of glycosides present in fresh beers (Chevance, Guyot-Declerck, Dupont, & Collin, 2002). Furthermore, nonanal was also identified as a contributor for the detection of sweet aromas in the analyzed aged beers. This compound is considered as a possible dihydro

Table 4
Identification of flavoring compounds by HS-SPME-GC/O-FID in fresh (FB), naturally (6 months at 20 ± 2 °C) and forced aged beers (14 days at 37 ± 1 °C).

Nr.	RT (min)	LRI*	LRI (Literature)[a]	Aroma/Odor	Compound	Odor Threshold (μg/L)[b]	Sample[c]		
							FB	6m, 20 °C	14d, 37 °C
1	2.48	826	–	Fruity/Floral	Unknown 1		1	0	0
2	6.23	875	872–875	Fruity	Isoamyl acetate	1200	1	1	1
3	6.34	1000	995–1002	Fruity	Ethyl hexanoate	210	1	1	1
4	7.44	1052	1046–1058	Floral	Phenylacetaldehyde	1600	0	1	1
5	8.59	1103	1103–1106	Sweet	Nonanal	18	0	1	1
6	10.06	1134	1115–1130	Floral	Phenylethyl alcohol	125000	2	2	2
7	11.24	1161	1162	Papery	*E*-2-nonenal	0.11	0	1	1
8	13.01	1199	1196–1205	Sweet/Floral	Ethyl octanoate	900	0	0	2
9	14.14	1234	1240–1248	Floral	Ethyl phenylacetate	160	1	1	1
10	14.40	1244	1252–1265	Herbal	2-phenylethyl acetate	3800	1	1	2
11	15.29	1272	–	Sweet/Floral	Unknown 2		0	1	2
12	16.11	1298	1297	Floral	Ethyl nonanoate	1200	0	0	2
13	16.29	1303	–	Floral	Unknown 3		1	1	1
14	16.49	1310	–	Honey/Sweet	Unknown 4		0	0	1
15	19.14	1398	1386–1393	Sweet	β-damascenone	25	0	0	1

*LRI_{calc} – retention index calculated from C_8 – C_{20} n-linear alkanes in DB-5ms capillary column.

[a] (Attchelouwa et al., 2020; Babushok, Linstrom, & Zenkevich, 2011; Cabrita, Aires de Sousa, Gomes Da Silva, Rei, & Costa Freitas, 2012; El-Sayed, Heppelthwaite, Manning, Gibb, & Suckling, 2005; Goodner & Technology, 2008; Z.; Li et al., 2019).

[b] (Miller, 2019).

[c] The different numbers displayed in the table correspond to the different intensities felt, for each compound, in the different analyzed samples: 0 – not detected; 1 – medium intensity; 2 – high intensity.

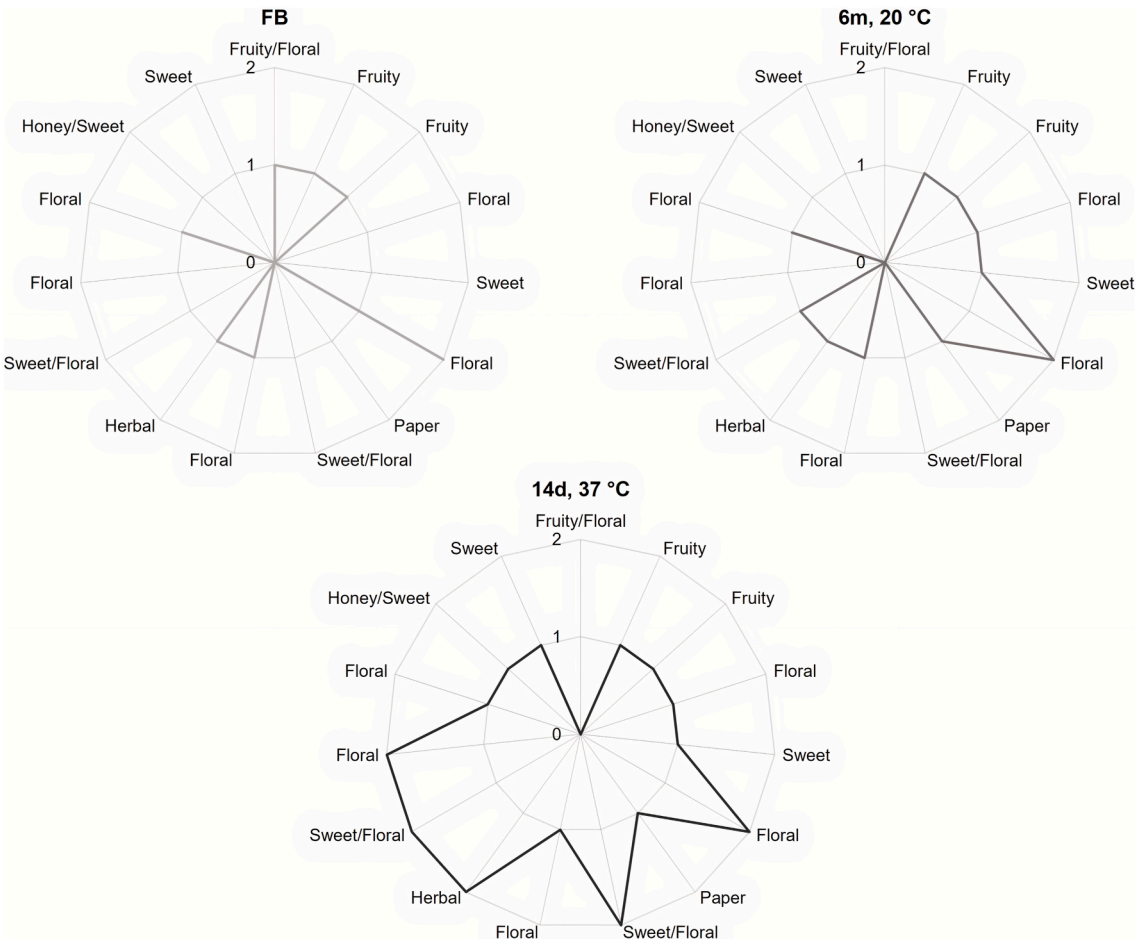

Fig. 2. Sensory profiles of beer samples (fresh beers (FB), naturally aged beers (6 months at 20 ± 2 °C) and forced aged beers (14 days at 37 ± 1 °C). Individual aromas/odors are positioned like the spokes of a wheel around a center (zero, not detected) point, with the spokes representing attribute intensity scales, with higher (more intense) values radiating outward.

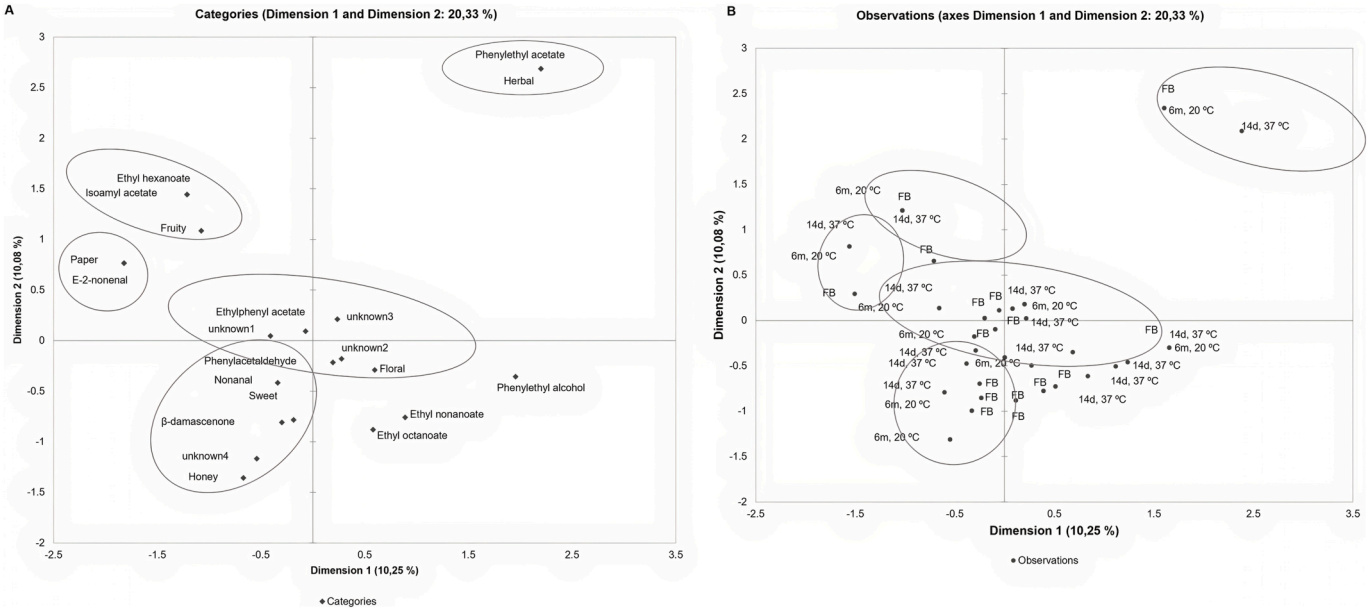

Fig. 3. Multiple correspondence analysis (MCA) of the analytical and olfactometry data obtained by the analysis of beer samples submitted to different storage conditions (fresh beer (FB), naturally aged (6 months at 20 ± 2 °C) and forced aged beers (14 days at 37 ± 1 °C) showing (A) the two dimensional categories plot (A) and (B) the two-dimensional observations plot.

derivative of the initial unsaturated aldehyde, *E*-2-nonenal (Baert et al., 2012).

4. Conclusion

This work deals with the analysis of the chemical and sensorial profiles of beer samples submitted to different storage conditions (fresh beers, naturally aged beers (3 and 6 months at 20 ± 2 °C) and forced aged beers (7 and 14 days at 37 ± 1 °C)). The chemical profile was assessed by the extraction of volatile compounds by HS-SMPE, followed by GC/MS detection. The sensorial profile was assessed by the extraction of volatile compounds using HS-SPME and the sensorial analysis was conducted by an untrained sensory panel using GC-O/TOFMS technique.

Different chemical classes of compounds were identified and some of them are of utmost importance during beer staling. Beer aging comprises a large number of chemical reactions, which may occur at different rates depending on the storage conditions applied to beers. At the storage conditions studied in this work, significant differences were found, especially for the relative contents of aldehydes, esters and furanic compounds in aged beers. Based on the HCA analysis, temperature of storage is the principal factor that allow to distinguish among beers due to its high dissimilarity concerning the overall chemical composition. Usually, the industrial beers shelf-life is about 1 year. However, even the rate of chemical reactions being low in beers stored at room temperature (20 ± 2 °C), this study shows that the chemical composition after 6 months of storage is significantly different compared to fresh beers. In addition, HCA analysis has allowed us to conclude that the storage of beer at low temperatures is the better way to preserve the freshness and the organoleptic characteristics of fresh beers.

Temperature was shown to be highly injurious with respect to the development of staling compounds, for beers stored at 37 °C. The data obtained suggest a clear role of temperature on the development of staling compounds, as confirmed by the increase on the amounts of aldehydes, furanic compounds and esters in analyzed beers. The dependence between the reaction rate constant and temperature was well described by the Arrhenius equation for these three chemical classes of compounds, for which the reaction rate increased with increasing temperatures. The rate of development of furanic compounds showed to be almost 130 times higher in beers stored at higher temperatures (37 ± 1 °C) when compared to beers stored at 4 ± 1 °C. The comparison between beers stored at 37 ± 1 °C and 4 ± 1 °C allowed us to verify that, in the first ones, the formation of aldehydes and esters was 70 and 15 times higher, respectively. The estimation of beer shelf-life allowed us to verify that higher storage temperatures are detrimental for beer shelf-life and, in contrast, lower storage temperatures helps to extend beer shelf-life. According to the obtained results, furanic compounds and aldehydes showed to be good chemical markers of beer instability. Regarding these two indicators, beers stored at 37 ± 1 °C for 12 days mimic the impact on beer stability of beers stored at 20 ± 2 °C during 6 months (beer *best-before date*).

Concerning the olfactometric analysis, different aromas were detected in the analyzed beers, such as fruity, floral, sweet, honey and paper-like aromas. The fruity and herbal aromas detected are related to the presence of esters and the floral-like aromas are related to the presence of esters and aldehydes. The major difference was found in aged beers, both naturally and forced aged, where the prevalence of sweet and papery aroma notes were noticed. The sweet aromas were linked to the presence of β-damascenone in aged beers and. The papery aroma notes were linked to the presence of *E*-2-nonenal. These two compounds, both detected only in aged beers, have been identified, by several authors, as good chemical markers of beer aging.

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Author statement

Inês M. Ferreira: Experimental part – sample preparation and chromatographic analysis, data treatment and analysis, data curation, Writing-Original draft preparation.

Flávia Freitas: GC/MS and GC/TOFMS analysis and systems maintenance.

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Maria Filipa Mourão: Statistical work design.

Luís Ferreira Guido: Conceptualization, Funding acquisition, Methodology, Supervision, Reviewing and Editing.

Marco Gomes da Silva: Conceptualization, Project administration; Resources, Analytical Methodology, Supervision, Reviewing and Editing.

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