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Occurrence of Theaspirane and its Odorant Degradation Products in Hop and Beer

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ABSTRACT: In model oxidized media, six theaspirane-derived compounds were identified by gas chromatography−high resolution mass spectrometry: 4-hydroxy-7,8-dihydro-β-ionone, 6-hydroxy-7,8-dihydro-α-ionone, dihydrodehydro-β-ionone, two monoepoxides, and a derived alcohol. Only 4-hydroxy-7,8-dihydro-β-ionone and dihydrodehydro-β-ionone have been described previously in the literature. Investigation of hop revealed five of these compounds in free form together with theaspirane (especially in the Mosaic variety), while the Citra and Amarillo hop varieties emerged as very interesting for the release of theaspirane, 4-hydroxy-7,8-dihydro-β-ionone, and dihydrodehydro-β-ionone from glucoside precursors. For the first time, theaspirane, 4-hydroxy-7,8-dihydro-β-ionone, 6-hydroxy-7,8-dihydro-α-ionone, and both monoepoxides were found in a fresh commercial top fermentation beer (only theaspirane, 4-hydroxy-7,8-dihydro-β-ionone, and dihydrodehydro-β-ionone have recently been mentioned as Gueuze constituents).

KEYWORDS: *theaspirane degradation, hop aroma, beer aging*

INTRODUCTION

Theaspirane or 2,6,10,10-tetramethyl-1-oxa-spiro[4,5]-deca-6-ene is an aromatic compound derived from C13-norisoprenoids.[1] As its chemical structure displays two chiral positions, theaspirane exists in nature as four isomers; the enantiomers (2*R*, 5*R*) and (2*S*, 5*S*), known as theaspirane A, and (2*S*, 5*R*) and (2*R*, 5*S*), known as theaspirane B. The isomers differ by their odor: camphor for both theaspirane A enantiomers; fruity, blackcurrant for the (2*R*, 5*S*) isomer; and unpleasant or naphtalene for the (2*S*, 5*R*) isomer.[2] Theaspiranes A and B have been identified in foodstuffs such as fruits, hop, fermented beverages (wine, beer, and spirits) and other cold drinks such as tea. In plants, both forms can be predominant, while in fruits, theaspirane B is always the major form.[2,3]

Daenen et al. (2008)[4] reported the presence of a high quantity of theaspirane under bound form in the Saaz hop variety (743 μg/kg theaspirane A and 854 μg/kg theaspirane B have been obtained upon release by enzymatic hydrolysis from glycoside precursors). These values make hop the second most important plant source of theaspirane, after the acerola (Barbados cherry). Recently, theaspiranes A and B were also detected in Gueuze beer at concentrations very similar to those found in "yellow wines".[4,5]

In quince, Winterhalter and Schreier (1988)[1] identified the natural preprecursor of theaspirane as a glucoside of 4-hydroxy-7,8-dihydro-β-ionol.

Under oxidative conditions, theaspirane is degraded to two interesting aromatic compounds, the dried-fruit-like dihydrodehydro-β-ionone and the grenadine-like 4-hydroxy-7,8-dihydro-β-ionone.[6] Both theaspirane-derived molecules were recently detected in yellow wines, while only dihydrodehydro-β-ionone was found in Sauternes wines.[6]

Beer aging mainly results from two chemical mechanisms: either acidic hydrolysis (e.g., the formation of cardboard-like *trans*-2-nonenal from Schiff bases,[7] of phenols from torrefied malt glycosides,[8,9] of β-damascenone from hop glycosides[10,11]) or oxidation (e.g., sotolon formation).[12] By the former process, beer could release theaspirane from hop glucosilated precursors while the latter could transform them into various derived odorants.

To better identify potential theaspirane degradation products in aged beers, harsh oxidation conditions were first applied to model media spiked with commercial theaspirane. Glucosidic forms of theaspirane analogs were further investigated in six different hop varieties, including the aromatic reference Saaz, one usual bitter cultivar (Warrior), and four dual hops (Amarillo, Citra, Hallertau Blanc, and Mosaic) whose particular aroma profiles have recently been described.[13] Finally, theaspirane degradation products were investigated in a beer, both fresh and aged for two years.

MATERIALS AND METHODS

Chemicals. Theaspirane (85%), diethyl ether (>99.9%), iron chloride, theaspirane (85%), octyl-glucopyranoside (≥98%), β-glucosidase (from almonds, ≥ 6 U/mg), iron(II) chloride (98%), hydrogen peroxide solution (30 wt % in water), copper(II) sulfate (≥99.9%), and dodecane (99.9%) were purchased from Sigma-Aldrich (Bornem, Belgium). Absolute ethanol, methanol (99.9%), and acetone were obtained from Analar Normapur (Fontenay-sous-bois, France). Dichloromethane (99.9%) was obtained from Romil (Cambridge, UK) and distilled twice before use. Amberlite XAD 2 resin came from Supelco (Bellefonte, PA). Anhydrous sodium sulfate (99%) was obtained from Merck (Darmstadt, Germany) and polyvinylpolypyrrolidone from AEB Group (Brescia, Italy). Milli-Q water was used (Millipore, Bedford, MA)

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Table 1. Free Fraction (Values in Parentheses) of Theaspirane and Derived Compounds in Hop and Amounts Released by β-Glucosidase Hydrolysis (Milligrams per Kilogram, Theaspirane Equivalents for Compounds 3−7)[a]

				Amarillo	Citra	Mosaic	Hallertau Blanc	Warrior	Saaz
		substance		*alpha*-acid content (% w/w)					
				8−11	11−13	11.5−13.5	9−12	16−18	3−4.5
no.	RI (CPSil 5 CB)	name	odor (GC-O)	amount (mg/kg)					
1	1305	theaspirane A	camphor	1429.2[a] (nd)	70.7[b] (nd)	22.4[c] (7.3)	15.6[d] (nd)	nd (nd)	0.64e (nd)
2	1305	theaspirane B	blackcurrant	861.1[a] (nd)	40.7[b] (nd)	14.1[c] (6.3)	10.7[d] (nd)	nd (nd)	0.5e (nd)
3	1373	4-hydroxy-7,8-dihydro-β-ionone	grenadine	9.8[a] (nd)	0.3[b] (nd)	nd (0.1)	0.8[c] (nd)	nd (nd)	tr (nd)
4	1394	6-hydroxy-7,8-dihydro-α-ionone	–	0.3[a] (nd)	0.1[b] (nd)	nd[b] (0.1)	0.1 (nd)	nd (nd)	nd (nd)
5	1419	dihydrodehydro-β-ionone	dried fruit/ sauterne	1.8[a] (nd)	0.2[b] (nd)	nd (0.2)	4.0[c] (nd)	nd (nd)	nd (nd)
6	1560	3,4-epoxy-5,6-dehydro-7,8-dihydro-β-ionone	–	nd (nd)	nd (nd)	nd (0.1)	nd (nd)	nd (nd)	nd (nd)
7	1570	3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone	–	nd (nd)	nd (nd)	nd (0.2)	nd (nd)	nd (nd)	nd (nd)

[a]Assays in duplicate. All samples that do not share a common letter are significantly different ($p < 0.05$) according to Tukey's test. tr.: detected below the quantitation limit (0.01 μg/kg). nd: not detected.

Samples. Four hop samples were kindly supplied by Yakima Chief (Louvain-la-Neuve, Belgium): Amarillo, Citra, Mosaic, and Warrior (harvest 2013). Saaz hop (harvest 2013) and Hallertau Blanc were provided by Hopsteiner (Mainburg, Hallertau, Germany). A brown beer, bought at a local supermarket and stored for two years in a dark room at 20 °C for natural aging, was used for beer investigation.

Theaspirane Chemical Oxidation. A 0.485 g sample of commercial theaspirane (85% purity, final concentration 0.05 M), 50 mL of Milli-Q water, and 1 mL of hydrogen peroxide solution were mixed in the presence of 60 mg of iron chloride, used as catalyst. The reaction medium was heated at 100 °C for 3 h. After three diethyl ether extractions (3 × 20 mL), the extract was dried with anhydrous sodium sulfate. Dodecane was added as external standard, and the mixture was concentrated to 10 mL in a Kuderna-Danish apparatus at 39 °C (total concentration factor, 5; final EST concentration, 7 mg/L). The final extract was stored at −80 °C and analyzed by gas chromatography−mass spectrometry (GC-MS), gas chromatography−high-resolution mass spectrometry (GC-HRMS), and gas chromatography−olfactometric detection (GC-O).

Hop and Beer XAD 2 Extraction Procedure with or without Glucoside Enzymatic Release. For hop extraction, 20 g of finely ground hop and 1000 μL of internal standard (IST, 1 g/L octyl-glucopyranoside) were added to 250 mL of water/methanol (80:20 v/v) and mixed thoroughly with an Omni Mixer (Omni international, Kennesaw, GA) for ten 30 s periods interrupted by ten 30 s resting periods. The mixture was then centrifuged for 10 min at 10000 rpm; the upper phase was recovered, whereas the lower one was re-extracted in the same way. Both fractions were filtered with a Büchner funnel, and methanol was removed with a Rotavapor to a final volume of 300 mL. Polyphenols were eliminated by treatment for 1 h under agitation with 6 g of polyvinylpolypyrrolidone (AEB, Brescia, Italy). After a second Büchner filtration, the hop extract was divided into two 250 mL Schott flasks.

In the case of beer extraction, 150 mL of degassed beer and 500 μL of internal standard (IST, 1 g/L octyl-glucopyranoside) were poured into a Schott flask.

Six grams of Amberlite XAD 2 resin thoroughly rinsed with Milli-Q water (approximately 400 mL) were poured into each hop or beer flask. After being shaken in a dark room for 2 h, the mixtures were poured into a glass column for separation. After natural elution from the XAD 2 resin, 50 mL of water and 25 mL of diethyl ether were consecutively poured for resin washing. Glucosides were finally eluted with 25 mL of methanol. The extract was evaporated to dryness and resuspended in 25 mL of acetate buffer (pH 5). Enzymatically treated samples and controls (giving also free amounts of theaspirane analogs) were incubated at 35 °C for 2 h, with or without β-glucosidase (14 mg). Both were extracted three times with 15 mL of diethyl ether

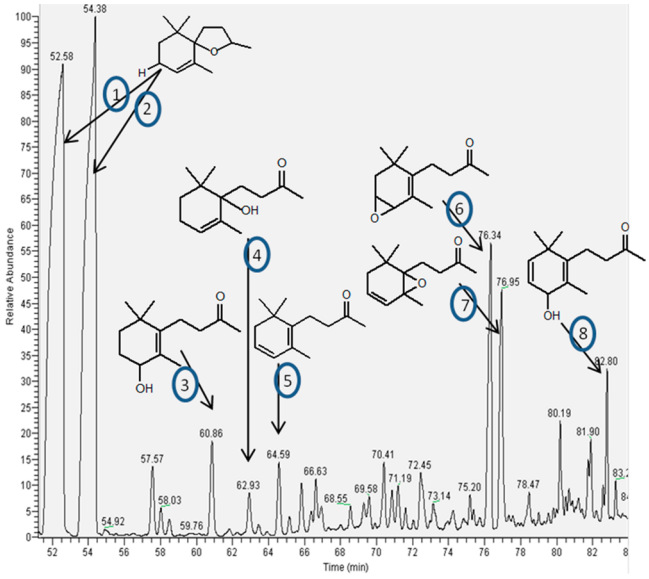

Figure 1. Total ion MS chromatogram of the theaspirane chemical oxidation media. (**1**) Theaspirane A, (**2**) theaspirane B, (**3**) 4-hydroxy-7,8-dihydro-β-ionone, (**4**) 6-hydroxy-7,8-dihydro-α-ionone, (**5**) dihydrodehydro-β-ionone, (**6**) 3,4-epoxy-5,6-dehydro-7,8-dihydro-β-ionone, (**7**) 3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone, and (**8**) 4-hydroxy-2,3-dehydro-7,8-dihydro-β-ionone.

(10 min, 1000 rpm). The combined organic phase was dried with anhydrous sodium sulfate, and 0.5 mL of EST (20 mg/L dodecane; final EST concentration, 20 mg/L) was added to the extract before concentration to 0.5 mL in a Danish-Kuderna apparatus at 39 °C. The final extracts were stored at −80 °C and analyzed by GC-MS.

Gas Chromatography−Mass Spectrometry. A 1 μL sample of hop, beer, or model extract was analyzed with a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a low-bleed MS capillary column (CP-Sil5-CB, 50 m × 0.32 mm i.d., 1.2 μm film thickness) and a splitless injector (250 °C). The split vent was opened 0.5 min postinjection. The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C at 3 °C/min. The carrier gas was helium, and the pressure was set at 100 kPa. Electronic impact (EI) mass spectra were recorded at 70 eV (full scan with a mass range from 40 to 380 m/z, or SIM mode on m/z = 70/84 for octanol; 57/71 for dodecane; 82/138 for theaspirane; 126/158 for 4-hydroxy-7,8-dihydro-β-ionone and

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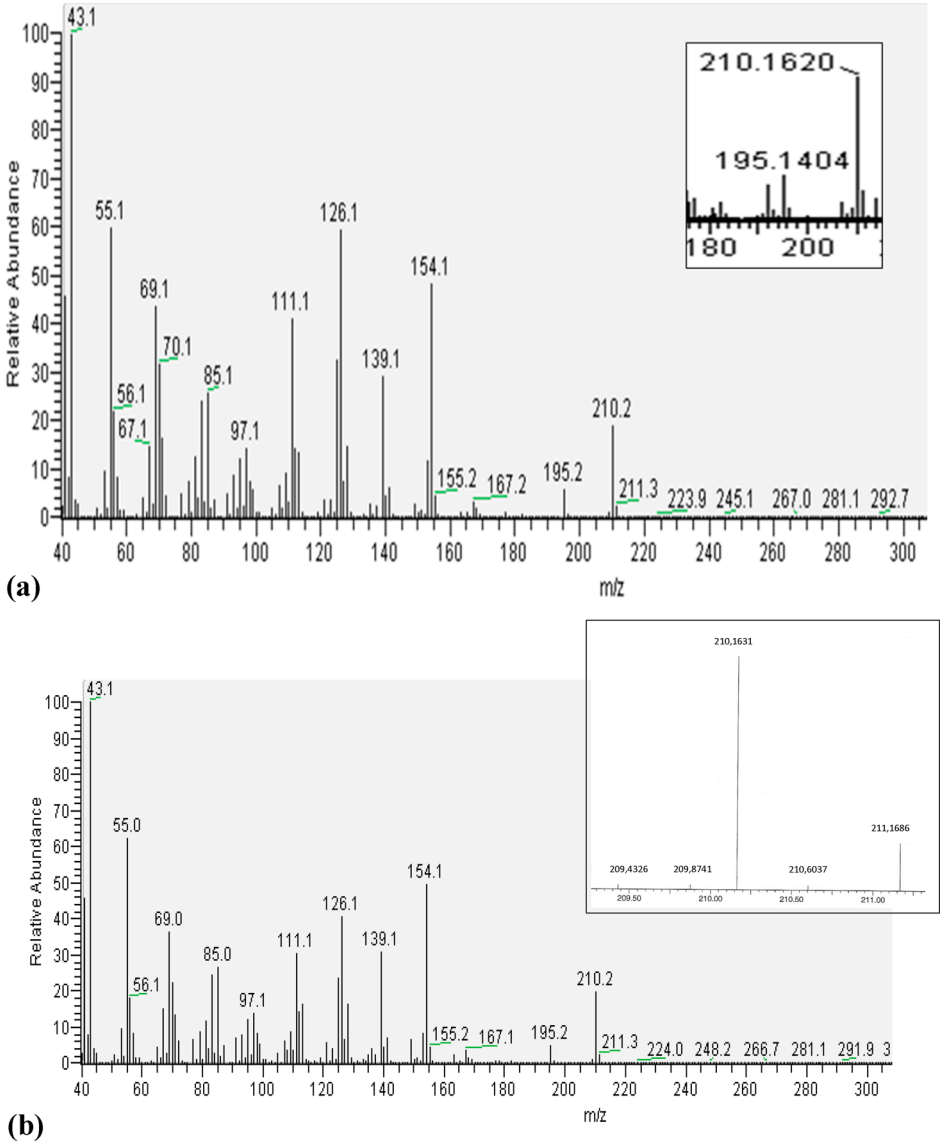

Figure 2. MS and HRMS spectra of 4-hydroxy-7,8-dihydro-β-ionone (a) and 6- hydroxy-7,8-dihydro-α-ionone (b).

6-hydroxy-7,8-dihydro-α−ionone; 119/134 for dihydrodehydro-β-ionone; 110/152 for 3,4-epoxy-5,6-dehydro-7,8-dihydro-β-ionone; 191/209 for 3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone; and 165/137 for 4-hydroxy-2,3-dehydro-7,8-dihydro-β-ionone) on a ThermoFinnigan Trace MS simple quadrupole mass spectrometer. Spectral recording was automatic throughout separation (Xcalibur software was used, NIST databank).

Gas Chromatography−High-Resolution Mass Spectrometry. The apolar column described above for GC-MS was connected to a GC-HR mass spectrometer from Waters (HRMS, GCT Premier, ToF). Perfluorotributylamine was injected on line as MS standard (the ions 69 and 131 were found in all spectra). Electron ionization (EI) mass spectra were recorded at 70 eV (trap current, 200 IA; emission current, 400 IA). Spectral recording was automatic throughout separation (Xcalibur software was used, NIST databank).

Gas Chromatography−Olfactometric Detection. A 1 μL sample of hop, beer, or model extract was analyzed with a Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250 °C; the split vent was opened 0.5 min postinjection. Compounds were analyzed with the wall-coated open tubular (WCOT) apolar CP-Sil5-CB column (50 m × 0.32 mm i.d., 1.2 μm film thickness). The carrier gas was nitrogen, and the pressure was set at 60 kPa (CP-Sil5-CB). The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C at 3 °C/min. In order to assess the olfactory potential of the extracts, the column was connected to a GC-O port (Chrompack) maintained at 250 °C. The effluent was diluted with a large volume of air (20 mL/min) prehumidified with an aqueous copper(II) sulfate solution.

Statistical Analyses. All analyses were carried out in duplicate. Multiple comparisons of means were performed by means of Tukey's test, with SAS software version 9.2 (SAS Institute, Inc., Cary, NC). Values in Table 1 that do not share a common letter are significantly different ($p < 0.05$).

RESULTS AND DISCUSSION

Investigation of Theaspirane Degradation Media. In order to study theaspirane oxidation products, theaspirane degradation medium was analyzed by GC-HRMS. Besides 4-hydroxy-7,8-dihydro-β-ionone ($RI_{CPSil5CB}$ = 1373, peak 3, Figure 1), having a nice grenadine-like flavor, and dihydrodehydro-β-ionone ($RI_{CPSil5CB}$ = 1419, peak 5, Figure 1), exhaling a dried fruit/Sauternes aroma, already identified by Collin et al. (2012)[6] in the same oxidation medium, four major compounds (**4** ($RI_{CPSil5CB}$ = 1493), **6** ($RI_{CPSil5CB}$ = 1657), **7** ($RI_{CPSil5CB}$ = 1667), and **8** ($RI_{CPSil5CB}$ = 1774)) were evidenced (Figure 1). As depicted

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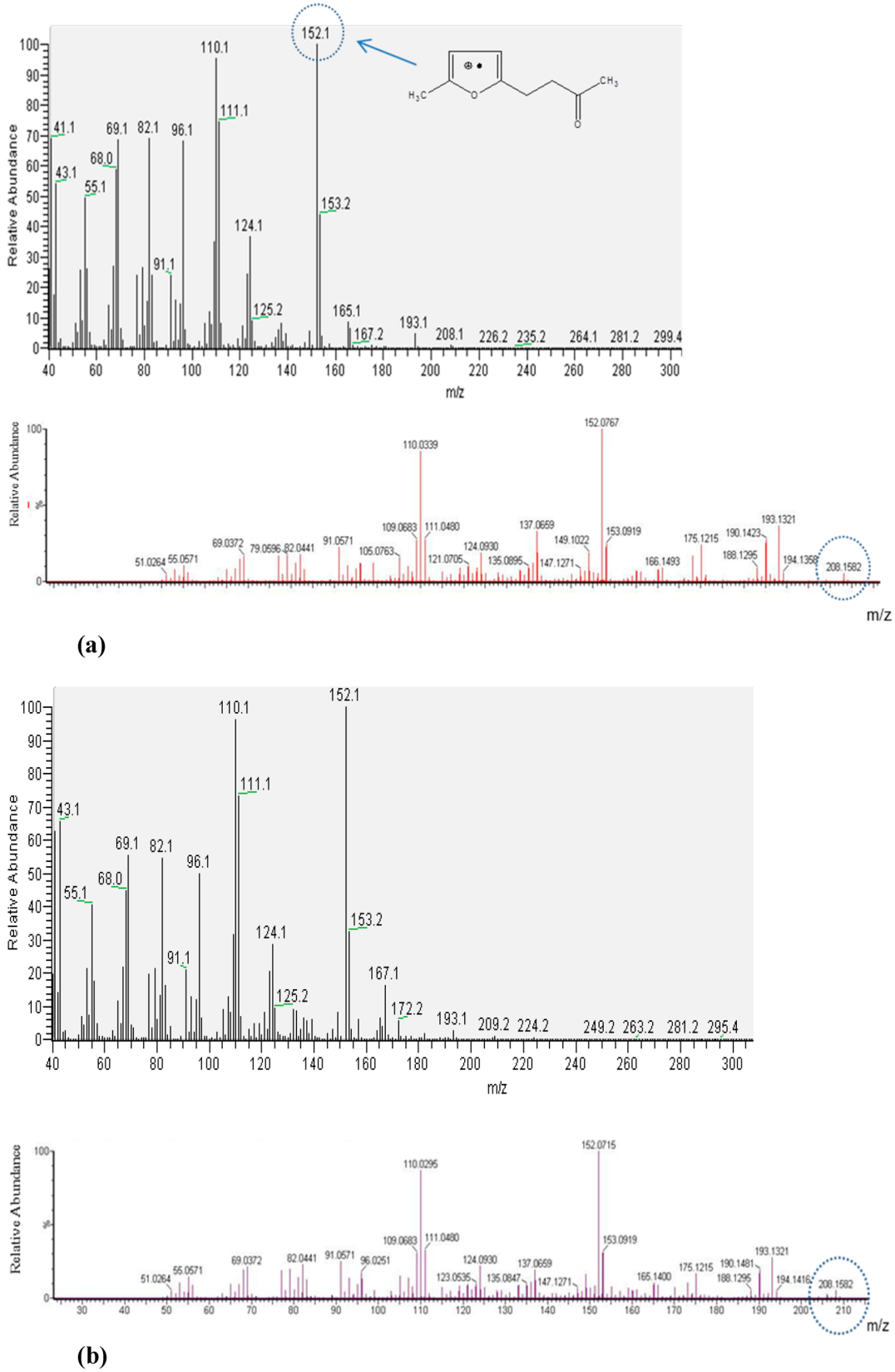

Figure 3. MS and HRMS spectra of (**6**) 3,4-epoxy-5,6-dehydro-7,8 dihydro-β-ionone (a) and (**7**) 3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone (b).

in Figure 2, compound **4** exhibits the same mass spectrum as 4-hydroxy-7,8-dihydro-β-ionone (compound **3**), but no odor was detected at the GC-O sniffing port in the theaspirane degradation medium. Compound **4** is here tentatively identified as 6-hydroxy-7,8-dihydro-α-ionone by GC-HRMS (Figure 2 b) (experimental m/z = 210.1631; calculated m/z = 210.1620; δ = 5.2 ppm). Upon fragmentation, peaks 6 and 7 yielded the same mass spectrum, as illustrated in Figure 3a,b. These compounds appeared to be the

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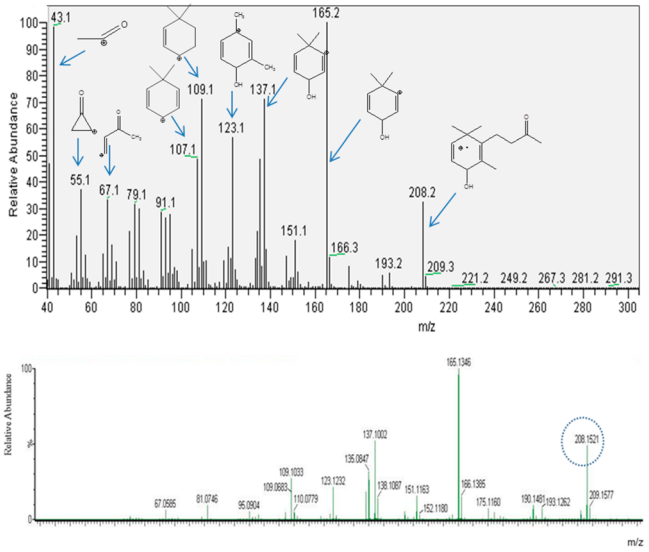

Figure 4. MS and HRMS spectra of compound **8**.

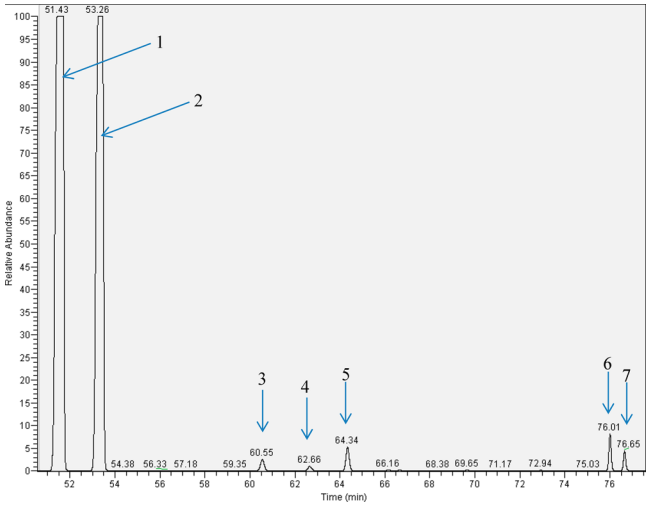

Figure 6. SIM chromatogram of Mosaic hop: (**1**) theaspirane A, (**2**) theaspirane B, (**3**) 4-hydroxy-7,8-dihydro-β-ionone, (**4**) 6-hydroxy-7,8-dihydro-α-ionone, (**5**) dihydrodehydro-β-ionone, (**6**) 3,4-epoxy-5,6-dehydro-7,8-dihydro-β-ionone, and (**7**) 3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone.

most abundant degradation products of theaspirane after harsh oxidative treatment. GC-HRMS analysis and the existence of fragment m/z = 152.0715, corresponding to isobutene loss and creation of a furan, led us to suspect that compounds **6** and **7** were epoxides (experimental m/z = 208.1582), both potentially

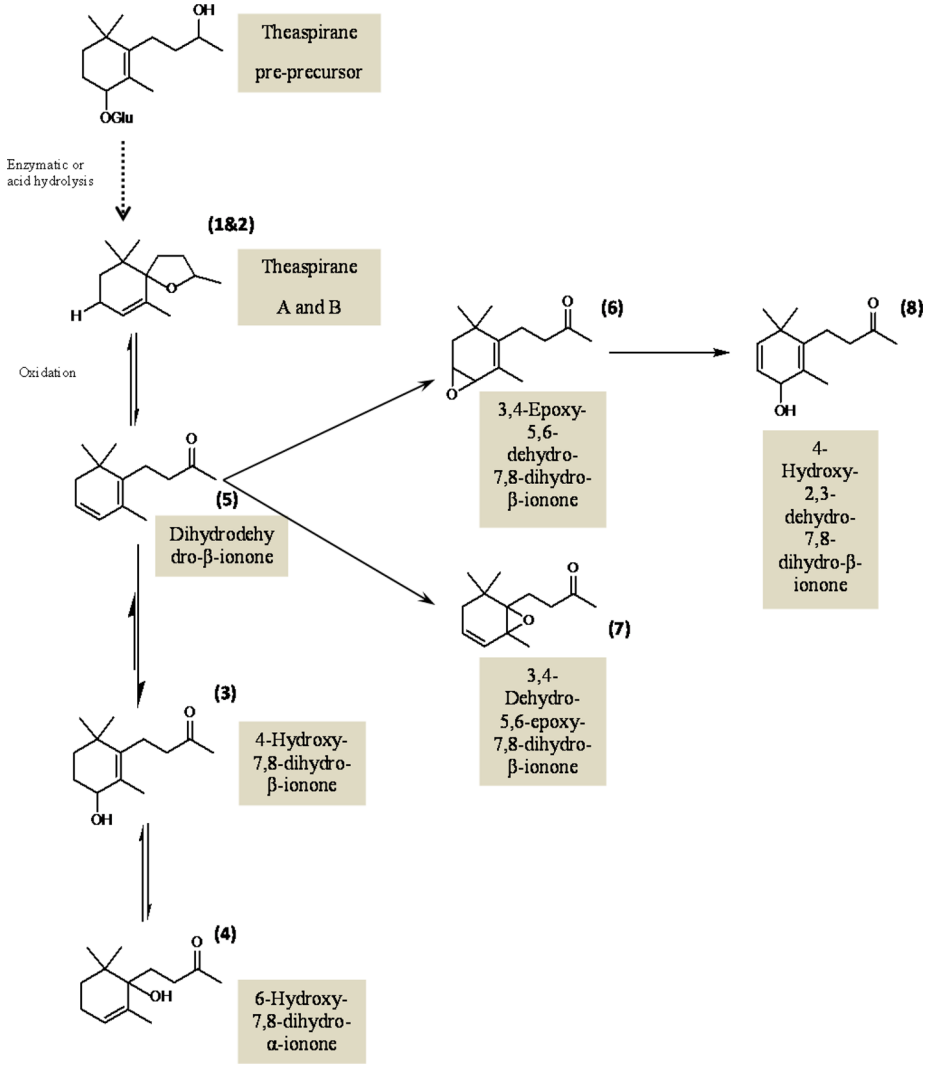

Figure 5. Hypothetical theaspirane degradation pathways.

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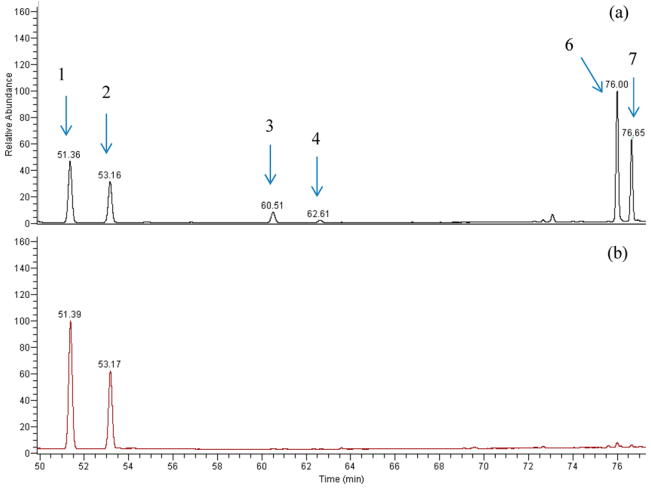

Figure 7. SIM chromatograms of a fresh beer (a) and a 2-year-aged beer (b). (**1**) Theaspirane A, (**2**) theaspirane B, (**3**) 4-hydroxy-7,8-dihydro-β-ionone, (**4**) 6-hydroxy-7,8-dihydro-α-ionone, (**6**) 3,4-epoxy-5,6-dehydro-7,8-dihydro-β-ionone, and (**7**) 3,4-dehydro-5,6-epoxy-7,8-dihydro-β-ionone.

issued from degradation of compound **5**. Another compound (**8**), with an experimental m/z of 208.1521 but without any 152 fragment, was most probably issued from epoxide opening in compound **6**, leading to 4-hydroxy-2,3-dehydro-7,8-dihydro-β-ionone (fragmentation pattern proposed in Figure 4). Figure 5 gives a hypothetical succession of degradations (including oxidation, hydration, epoxidation, epoxide opening), starting from theaspirane or its preprecursor and giving rise to compounds **3**–**8**.

Investigation of Free and Bound Precursors of Theaspirane and Derived Compounds in Hop. The existence of a theaspirane glucosidic precursor has been mentioned by Daenen et al. (2008)[4] for the Saaz hop variety. Therefore, theaspirane and its degradation products were here investigated in six hop varieties before and after enzymatic treatment. The GC-MS chromatogram of Mosaic XAD-2 extract (Figure 6) revealed, for the first time, the existence of huge quantities of free theaspirane (**1** and **2**) in hop (7.3 and 6.3 mg/kg,respectively), together with 4-hydroxy-7,8-dihydro-β-ionone (**3**), 6-hydroxy-7,8-dihydro-α-ionone (**4**), dihydrodehydro-β-ionone (**5**), and both monoepoxides (**6** and **7**). Yet Mosaic emerged as the only variety among those investigated here to contain these compounds mainly in free form. Citra and Amarillo proved to be much more interesting sources of the theaspirane preprecursor (111 and 2290 mg/kg enzymatically released, respectively; Table 1). Together with theaspirane, 4-hydroxy-7,8-dihydro-β-ionone was also found in hop extracts subjected to β-glucosidase treatment, especially for the hop Amarillo (10 mg/kg). Although containing only 26 mg/kg theaspirane, the dual hop variety Hallertau Blanc emerged as the best source of the nice dried-fruit-like dihydrodehydro-β-ionone (4 mg/kg from bound form, Table 1). From the brewer's standpoint, we can suspect free compounds to be massively lost through volatilization during boiling, while glucosides should be able to release an unexpected range of flavors through enzymatic or chemical hydrolysis during fermentation or beer aging. The enzymatic treatment applied to conventional hops (the aromatic Saaz and the bitter Warrior) yielded much less interesting profiles (<2 mg/kg theaspirane and no derived compounds detected). The four dual hops investigated here have also been described, recently, to be of particular interest for their citrus-like potential, brought by very delicate polyfunctional thiols and terpenols.[13]

Investigation of Theaspirane Degradation Products in Fresh and Aged Beer. Scholtes et al. (2012)[5] have recently shown that glucosides, partially hydrolyzed and oxidized during hop or beer aging, can release theaspirane analogs in Gueuze beers. This mechanism, although less favored in usual unoxidized lager and special beers, could also be a source of interesting flavors.Theaspirane and its oxidation-derived compounds were next investigated in a Belgian top-fermentation beer, both fresh and allowed to age for two years. For the first time in a top-fermentation beer, theaspirane A (**1**), theaspirane B (**2**), 4-hydroxy-7,8-dihydro-β-ionone (**3**), 6-hydroxy-7,8-dihydro-α-ionone (**4**), and both monoepoxides (**6** and **7**) were all evidenced by GC-MS in the fresh beer samples (Figure 7).

Despite unavoidable oxidation in the bottle, analysis of the 2-year-aged beer samples revealed the presence of theaspiranes A and B in the same concentration range as found in the fresh samples (3.6 μg/L theaspirane A in fresh beer and 4.4 μg/L after 2 years; 2.4 μg/L theaspirane B in fresh beer and 2.7 μg/L after 2 years). This supports the hypothesis of a balance due to glucoside hydrolysis. Of course, complementary data are still needed to confirm the occurrence of glucosidic forms in fresh beer, and their ability to be enzymatically or chemically hydrolyzed through aging. On the other hand, 4-hydroxy-7,8-dihydro-β-ionone (**3**), 6-hydroxy-7,8-dihydro-α-ionone, and both monoepoxides (**6** and **7**), present in fresh samples (0.7, 0.2, 4.7, and 2.8 μg/L, respectively), proved to be degraded to unquantifiable levels after 2 years.

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Notes

The authors declare no competing financial interest.

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