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On the Autoxidation of Bitter-Tasting Iso-α-acids in Beer

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The iso-α-acids, the major contributor to bitter beer taste, is well-known to strongly degrade during beer aging. The storage of beer in brown glass bottles revealed a strong depletion of the trans-configured isomers in a highly specific manner, whereas the corresponding *cis*-iso-α-acids seemed to be hardly affected. In comparison, storage of beer in polyethylene terephthalate bottles, which are known to be permeable to oxygen, induced a drastic degradation of both isomers independent of their *cis/trans* configuration. To investigate the chemical transformation of iso-α-acids under oxidative storage conditions, suitable model experiments were performed, and the reaction products that formed were identified as previously not reported hydroperoxy- and hydroxyl-allo-iso-α-acids by means of one-/two-dimensional NMR and liquid chromatography−mass spectrometry experiments; for example, *cis*- and *trans*-configured hydroperoxy-alloisohumulone as well as the corresponding hydroxy-alloisohumulones were generated upon oxidation of *cis*- and *trans*-isohumulone independent of their cis/trans configuration. In addition, the oxidation products formed from the various iso-α-acid congeners were quantitatively determined in a series of beer samples stored under defined conditions. For the first time, these data help to understand the molecular mechanism involved in the autoxidative degradation of iso-α-acids in beer.

KEYWORDS: Iso-α-acids; hop; beer; hydroperoxides; alloisohumulone; tricyclocohumol

INTRODUCTION

Made from water, barley malt, yeast, and hops, freshly brewed beer has been attracting consumers for thousand of years due to its refreshing character, desirable aroma, and its typical bitter taste profile. In the past, microbiological spoilage and haze formation have been addressed as the limiting factors determining the shelf life of beer. As technological improvements in beer manufacturing and a better understanding of the chemical transformations occurring during the brewing process helped to keep these problems largely under control, the instability of the attractive aroma as well as the typical bitter taste of beer have become nowadays the shelf life limiting factor of beer products. The aging of beer has been long known to induce a significant decrease of bitter intensity as well as a change of the bitter taste quality toward a long-lasting, lingering, and harsh bitterness and, in consequence, has caused severe quality problems in the brewing industry (*1*−*4*).

On the basis of the outcome of about 100 years of research on the molecules contributing to the typical flavor signature of beer, it can be concluded that the bitter taste of the beverage develops upon isomerization of the α-acids cohumulone (**1a**), humulone (**1b**), and adhumulone (**1c**) after the addition of cones, pellets, or extracts of hop (*Humulus lupulus* L.) during the wort boiling process (**Figure 1**). The α-acids **1a**−**c** are transformed into epimeric pairs of intensely bitter tasting *cis*- (**2a**−**c**) and *trans*-configured iso-α-acids (**3a**−**c**), which were found to exist in freshly brewed beer in a *trans*/*cis* ratio of about 0.4 (*5*−*9*). Because of the various side chains of the parent α-acids, the isomerized products appear as *cis*- and *trans*-configured isocohumulone (**2a** and **3a**), isohumulone (**2b** and **3b**), and isoadhumulone (**2c** and **3c**), respectively (**Figure 1**). Various studies revealed that the isomerization is vital to the taste of beer, since it yields the most potent bitter compounds (*6*, *10*), whereas the remaining α-acids (**1**) seem not to have any major impact on the taste of the final beverage (*11*).

A series of investigations demonstrated that the aging of beer induces a strong degradation of the *trans*-iso-α-acids **3a**−**c**, whereas the corresponding *cis*-configured isomers **2a**−**c** seem to be much more stable (*1*, *3*, *4*, *12*−*16*). Very recently, the molecular basis for this *trans*-specific degradation of the bitter-tasting iso-α-acids induced upon storage of bottled beer was elucidated, and the chemical structures of the reaction products generated from *trans*-iso-α-acids were successfully determined by means of sophisticated liquid chromatography−mass spectrometry (LC-MS/MS) and one-/two-dimensional NMR studies (*17*). Among these previously unreported molecules, the so-called tricyclocohumol (**4a**), tricyclohumol (**4b**), and tricycloadhumol (**4c**) (**Figure 1**) were found as the quantitatively predominant reaction products generated from **3a**−**c** by means of a *trans*-specific, proton-catalyzed cyclization reaction (*18*). This proton-catalyzed cyclization reaction was found to induce the rapid degradation of *trans*-iso-α-acids in bottled beer, but it does not at all explain the slight decrease of the amounts of *cis*-iso-α-acids by up to 20% within 8−12 months of beer storage (*1*, *12*, *15*).

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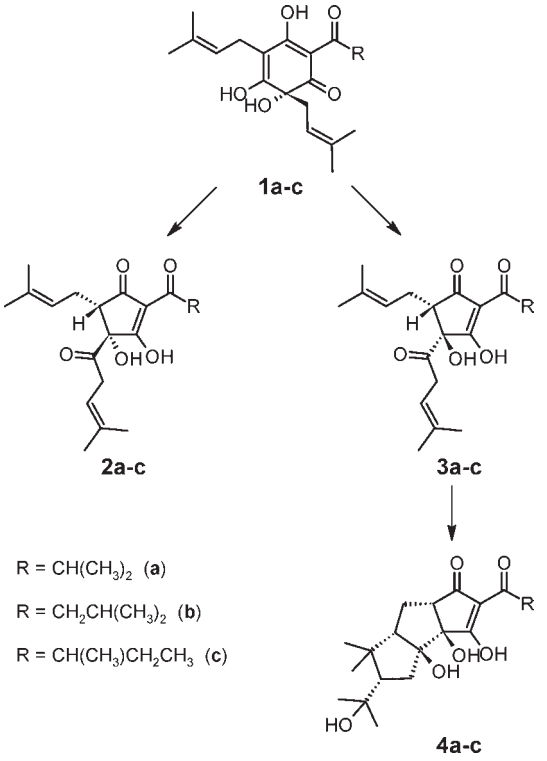

Figure 1. Thermally induced isomerization of the α-acids cohumulone (**1a**), humulone (**1b**), and adhumulone (**1c**) to give the diastereomeric *cis*- and *trans*-isocohumulone (**2a** and **3a**), isohumulone (**2b** and **3b**), and isoadhumulone (**2c** and **3c**) and proton-catalyzed degradation of the *trans* isomers **3a**−**c** to give the tricyclic products tricyclocohumol (**4a**), tricyclohumol (**4b**), and tricycloadhumol (**4c**), respectively.

Although numerous publications proposed chemical structures of putative degradation products formed from both *cis*- and *trans*-iso-α-acids such as alloisohumulones and humulinic acids (*19*−*21*), respectively, most of these compounds have been isolated from model experiments performed under rather artificial model conditions, and only a few of them have been structurally confirmed by means of NMR spectroscopy (*22*−*26*). The objective of the present study was, therefore, to answer the question as to whether oxidative reactions are involved in the depletion of iso-α-acids during beer aging, to isolate and identify the oxidation products formed from model systems, and to determine their amounts in authentic beer samples stored under defined conditions.

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: formic acid, hydrochloric acid, and sodium hydroxide (Grüssing, Filsum, Germany); acetonitrile and ethylacetate (Merck, Darmstadt, Germany); and dicyclohexylamine (Fluka, Neu-Ulm, Germany). Deuterated solvents were from Euriso-top (Saarbrücken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA). Oxygen (purity grade 4.5) and nitrogen (purity grade 5.0) were from Westfalen AG (Regensburg, Germany).

Pilsner type beer samples were provided by a German brewery. In detail, Pilsner type beer samples were used fresh (sample I) and after storage in a glass bottle for 8 months at 28 °C (sample II), for 4 years at 20 °C (sample III), for 10 years at 20 °C (sample IV), and for 4 years at 20 °C in a PET (polyethyleneterephthalate) bottle (sample V). Furthermore, a stream of oxygen was bubbled through a sample of fresh beer for 5 min at 22 °C, and then, the sample vial was closed and incubated for 24 h at 22 °C (sample VI). Following a literature protocol (*15*), *cis*- (**2a**−**c**) and *trans*-iso-α-acids (**3a**−**c**) were isolated from a commercially available iso-α-acid extract (Hallertauer Hopfenveredelungsgesellschaft mbH, Mainburg, Germany) in a purity of more than 98% [high-performance liquid chromatography (HPLC) and ^{1}H NMR].

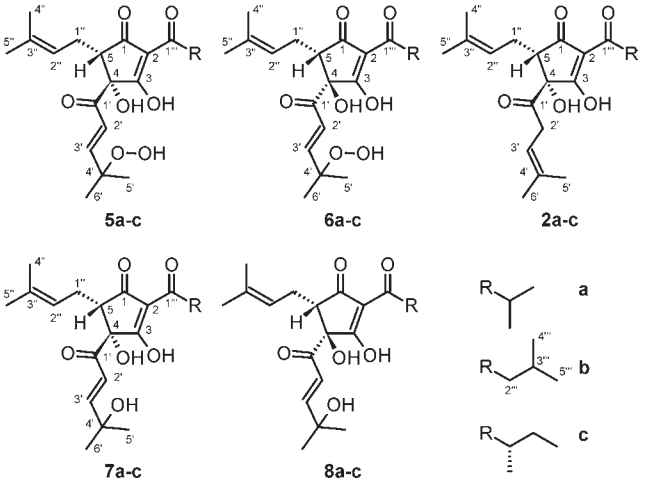

Figure 2. Chemical structures of the *cis*-isohumulone congeners (**2a**−**c**) and the oxidized iso-α-acid degradation products *cis*- and *trans*-hydroperoxy-alloisocohumulone (**5a**/**6a**), hydroperoxy-alloisohumulone (**5b**/**6b**), hydroperoxy-alloisoadhumulone (**5c**/**6c**), *cis*- and *trans*-hydroxyl-alloisocohumulone (**7a**/**8a**), hydroxyl-alloisohumulone (**7b**/**8b**), and hydroperoxy-alloisoadhumulone (**7c**/**8c**), respectively.

Oxidative Degradation of an Iso-α-acid Mixture and Purified *trans*-Isohumulone (3b). A solution of an iso-α-acid mixture (**2a**−**c** and **3a**−**c**; 10 g) in ethylacetate (10 mL) and a solution of purified *trans*-isohumulone (**3b**; 0.1 g) in ethylacetate (0.5 mL), respectively, were incubated under an atmosphere of oxygen for 2 weeks at 20 °C in the dark. Thereafter, the reaction mixtures were separated from solvent in vacuum, freeze-dried, and taken up in acetonitrile, and then, products **5a**−**c** and **6a**−**c** (from oxidation of the iso-α-acid mixture) and compound **6b** (from oxidation of purified *trans*-isohumulone; **Figure 2**) were isolated by means of preparative RP-HPLC using an UV/vis detector operating at 272 nm. Compounds **5b** and **6b** (**Figure 2**) were isolated in amounts that enabled us to perform a full structure determination by means of NMR, LC-MS/MS, and liquid chromatography−time-of-flight−mass spectrometry (LC-TOF-MS) experiments. Because of their instability, compounds **5a**,**c** and **6a**,**c** could not be obtained in high purity to allow NMR spectroscopic measurements; therefore, these isomers were tentatively identified by means of UV/vis, LC-MS/MS, and LC-TOF-MS analysis, as well as by considering their typical elution pattern on a RP-HPLC column.

Hydroperoxy-cis-alloisocohumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-(*2-methylpropanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***5a***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 238 nm. LC-TOF-MS: Found, *m*/*z* 379.1799; calculated for $[C_{20}H_{28}O_7-H^+]^-$, 379.1762. LC/MS (ESI$^-$): *m*/*z* (%) 379 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 379 (25), 347 (100), 303 (80), 249 (25), 234 (60), 181 (35).

Hydroperoxy-cis-alloisohumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-(*3-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***5b***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 234 nm. LC-TOF-MS: Found, *m*/*z* 393.1942; calculated for $[C_{21}H_{30}O_7-H^+]^-$, 393.1919. LC/MS (ESI$^-$): *m*/*z* (%) 393 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 393 (30), 361 (100), 317 (90), 263 (25), 248 (50), 195 (30). ^{1}H and ^{13}C NMR data are given in **Tables 1** and **2**.

Hydroperoxy-cis-alloisoadhumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-((*2S*)-*2-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***5c***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 241 nm. LC/MS (ESI$^-$): *m*/*z* (%) 393 (100) $[M - H^+]^-$.

Table 1. Assignment of ^{1}H NMR Signals (400 MHz, CD_3OD) of Compounds **2b**, **5b**, **6b**, and **7b**[a]

	compound no.[a]			
	δ_H[b] (m;[c] *J* in Hz)[d]			
proton at carbon	**2b**	**5b**	**6b**	**7b**
H−C(5)	3.15 (dd, 1H; 7.6, 5.7)	3.02 (dd, 1H; 7.4, 5.5)	2.72 (m, 1H)	3.02 (dd, 1H; 7.4, 5.5)
H−C(2′)	3.48 (d, 2H; 6.9)	6.80 (d, 1H; 15.9)	6.78 (d, 1H; 15.9)	6.79 (d, 1H; 15.6)
H−C(3′)	5.23 (dd, 1H; 6.9, 6.9)	7.04 (d, 1H; 15.9)	6.93 (d, 1H; 15.9)	7.02 (d, 1H; 15.6)
H−C(5′)	1.60 (s, 3H)	1.34 (s, 3H)	1.30 (s, 3H)	1.31 (s, 3H)
H−C(6′)	1.72 (s, 3H)	1.35 (s, 3H)	1.31 (s, 3H)	1.31 (s, 3H)
Hα−C(1″)	2.36 (ddd, 1H; 14.5, 6.6, 5.7)	2.38 (ddd, 1H; 14.8, 6.7, 5.5)	2.13 (m, 1H)	2.40 (m, 2H)
Hβ−C(1″)	2.45 (ddd, 1H; 14.5, 8.1, 7.6)	2.44 (ddd, 1H; 14.8, 8.0, 7.4)	2.54 (m, 1H)	
H−C(2″)	5.11 (dd, 1H; 8.1, 6.6)	5.11 (dd, 1H; 8.0, 6.7)	5.13 (m, 1H)	5.11 (dd, 1H; 8.0, 6.7)
H−C(4″)	1.60 (s, 3H)	1.60 (s, 3H)	1.45 (s, 3H)	1.60 (s, 3H)
H−C(5″)	1.64 (s, 3H)	1.62 (s, 3H)	1.61 (s, 3H)	1.62 (s, 3H)
H−C(2‴)	2.72 (d, 2H; 6.9)	2.71 (d, 2H; 7.0)	2.71 (m, 2H)	2.71 (d, 2H; 7.0)
H−C(3‴)	2.11 (m, 1H)	2.11 (m, 1H)	2.13 (m, 1H)	2.11 (m, 1H)
H−C(4‴)	0.95 (d, 3H; 6.8)	0.94 (d, 3H; 6.7)	0.93 (d, 3H; 6.8)	0.94 (d, 3H; 6.7)
H−C(5‴)	0.95 (d, 3H; 6.8)	0.95 (d, 3H; 6.7)	0.94 (d, 3H; 6.8)	0.95 (d, 3H; 6.7)

[a] Arbitrary numbering according to structures **2b**, **5b**, **6b**, and **7b** in **Figure 2**. [b] Chemical shift (ppm) of proton in relation to CD_3OD. [c] Multiplicity of signal and number of protons. [d] Coupling constants of multiplet signals are obtained by means of *J* simulation (MestreC, Mestrelab Research, La Coruña, Spain).

Table 2. Assignment of ^{13}C NMR Signals (125 MHz, CD_3OD) of Compounds **2b**, **5b**, **6b**, and **7b**[a]

	compound no.[a]			
	δ_C[b] (m)[c]			
carbon no.	**2b**	**5b**	**6b**	**7b**
C(1)	204.9 (C)	204.7 (C)	203.1 (C)	205.5 (C)
C(2)	111.4 (C)	112.3 (C)	113.8 (C)	112.9 (C)
C(3)	197.6 (C)	198.2 (C)	199.7 (C)	
C(4)	88.2 (C)	87.8 (C)	89.8 (C)	87.6 (C)
C(5)	52.3 (CH)	52.8 (CH)	58.8 (CH)	53.0 (CH)
C(1′)	210.1 (C)	199.5 (C)	200.4 (C)	199.9 (C)
C(2′)	37.8 (CH_2)	122.5 (CH_2)	123.8 (CH_2)	120.5 (CH_2)
C(3′)	116.3 (CH)	153.4 (CH)	152.0 (CH)	156.8 (CH)
C(4′)	135.7 (C)	82.8 (C)	82.5 (C)	71.5 (C)
C(5′)	17.5 (CH_3)	24.3 (CH_3)	24.6 (CH_3)	28.9 (CH_3)
C(6′)	25.3 (CH_3)	24.3 (CH_3)	24.7 (CH_3)	29.0 (CH_3)
C(1″)	25.6 (CH_2)	25.9 (CH_2)	25.6 (CH_2)	25.9 (CH_2)
C(2″)	121.4 (CH)	121.8 (CH)	123.5 (CH)	122.0 (CH)
C(3″)	134.6 (C)	134.8 (C)	133.7 (C)	134.4 (C)
C(4″)	17.5 (CH_3)	17.5 (CH_3)	18.2 (CH_3)	17.6 (CH_3)
C(5″)	25.6 (CH_3)	25.6 (CH_3)	26.0 (CH_3)	25.7 (CH_3)
C(1‴)	200.0 (C)	200.4 (C)	199.7 (C)	200.4 (C)
C(2‴)	46.5 (CH_2)	47.4 (CH_2)	50.0 (CH_2)	47.9 (CH_2)
C(3‴)	26.7 (CH)	27.0 (CH)	26.8 (CH)	27.0 (CH)
C(4‴)	22.3 (CH_3)	22.7 (CH_3)	23.3 (CH_3)	22.7 (CH_3)
C(5‴)	22.3 (CH_3)	22.7 (CH_3)	23.4 (CH_3)	22.7 (CH_3)

[a] Arbitrary numbering according to structures **2b**, **5b**, **6b**, and **7b** in **Figure 2**. [b] Chemical shift (ppm) of carbon in relation to CD_3OD. [c] Multiplicity of signals determined by HMQC experiments.

Hydroperoxy-trans-alloisocohumulone, (*4S,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-(*2-methylpropanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***6a***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 234 nm. LC-TOF-MS: Found, *m*/*z* 379.1768; calculated for $[C_{20}H_{28}O_7-H^+]^-$, 379.1762. LC/MS (ESI^-): *m*/*z* (%) 379 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 379 (10), 347 (100), 303 (70), 249 (35), 234 (40), 181 (30).

Hydroperoxy-trans-alloisohumulone, (*4S,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-(*3-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***6b***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 234 nm. LC-TOF-MS: Found, *m*/*z* 393.1900; calculated for $[C_{21}H_{30}O_7-H^+]^-$, 393.1919. LC/MS (ESI^-): *m*/*z* (%) 393 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 393 (30), 361 (100), 317 (85), 263 (35), 248 (50), 195 (35). ^{1}H and ^{13}C NMR data are given in **Tables 1** and **2**.

Hydroperoxy-trans-alloisoadhumulone, (*4S,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroperoxy-4-methylpent-2-enoyl*]-*2*-((*2S*)-*2-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***6c***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 237 nm. LC-TOF-MS: Found, *m*/*z* 393.1911; calculated for $[C_{21}H_{30}O_7-H^+]^-$, 393.1919. LC/MS (ESI^-): *m*/*z* (%) 393 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 393 (25), 361 (100), 317 (75), 263 (35), 248 (55), 195 (40).

Identification of the Hydroxides 7a−c and 8a−c in an Aqueous Solution of the Hydroperoxides 5a−c and 6a−c. A solution of **5b** (20 mg) in water/acetonitrile (1 mL; 50/50, v/v) was maintained for 2 weeks at room temperature and then separated by preparative RP-HPLC to yield hydroxide **7b**, the structure of which was determined by means of NMR, LC-MS/MS, and LC-TOF-MS analysis. To investigate the formation of hydroxides from the corresponding hydroperoxides, solutions of **5a**,**b** and **6a**−**c** (each 0.2−0.4 mg) in water/acetonitrile (200 μL; 50/50, v/v) were maintained for 2 weeks at room temperature and then analyzed by means of RP-HPLC coupled to an UV/vis detector and a mass spectrometer to tentatively identify the hydroxides **7a**,**c** and **8a**−**c**.

Hydroxy-cis-alloisocohumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2*-(*2-methylpropanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***7a***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 235 nm. LC-TOF-MS: Found, *m*/*z* 363.1823; calculated for $[C_{20}H_{28}O_6-H^+]^-$, 363.1813. LC/MS (ESI^-): *m*/*z* (%) 363 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 363 (100), 261 (70), 249 (65), 237 (20), 233 (25), 219 (50), 195 (30), 193 (35), 181 (70).

Hydroxy-cis-alloisohumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2*-(*3-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***7b***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 234 nm. LC-TOF-MS: Found, *m*/*z* 377.1998; calculated for $[C_{21}H_{30}O_6-H^+]^-$, 377.1970. LC/MS (ESI^-): *m*/*z* (%) 377 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 377 (100), 275 (65), 267 (50), 251 (25), 247 (35), 233 (50), 209 (35), 207 (30), 195 (60). ^{1}H and ^{13}C NMR data are given in **Tables 1** and **2**.

Hydroxy-cis-alloisoadhumulone, (*4R,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2*-((*2S*)-*2-methylbutanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***7c***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 238 nm. LC-TOF-MS: Found, *m*/*z* 377.1987; calculated for $[C_{21}H_{30}O_6-H^+]^-$, 377.1970. LC/MS (ESI^-): *m*/*z* (%) 377 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 377 (100), 275 (65), 267 (55), 251 (20), 247 (20), 233 (45), 209 (40), 207 (30), 195 (65).

Hydroxy-trans-alloisocohumulone, (*4S,5S*)-*3,4-Dihydroxy-4*-[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2*-(*2-methylpropanoyl*)-*5*-(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***8a***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 236 nm. LC-TOF-MS: Found, *m*/*z* 363.1817; calculated for $[C_{20}H_{28}O_6-H^+]^-$, 363.1813. LC/MS (ESI^-): *m*/*z* (%) 363 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 363 (100), 261 (50), 253 (50), 237 (35), 233 (40), 219 (40), 195 (25), 193 (30), 181 (60).

Hydroxy-trans-alloisohumulone, (*4S,5S*)-*3,4-Dihydroxy-4-*[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2-*(*3-methylbutanoyl*)-*5-*(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***8b***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max} = 234 nm. LC-TOF-MS: Found, *m*/*z* 377.1959; calculated for $[C_{21}H_{30}O_6-H^+]^-$, 377.1970. LC/MS (ESI^-): *m*/*z* (%) 377 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 377 (100), 275 (60), 267 (55), 251 (25), 247 (45), 233 (45), 209 (30), 207 (30), 195 (50).

Hydroxy-trans-alloisoadhumulone, (*4S,5S*)-*3,4-Dihydroxy-4-*[(*2E*)-*4-hydroxy-4-methylpent-2-enoyl*]-*2-*((*2S*)-*2-methylbutanoyl*)-*5-*(*3-methylbut-2-en-1-yl*)-*cyclopent-2-en-1-one* (***8c***; ***Figure 2***). UV/vis (1% aqueous formic acid/acetonitrile; 30/70, v/v): λ_{max}=240 nm. LC-TOF-MS: Found, *m*/*z* 377.1981; calculated for $[C_{21}H_{30}O_6-H^+]^-$, 377.1970. LC/MS (ESI^-): *m*/*z* (%) 377 (100) $[M - H^+]^-$. MS/MS (−30 V): *m*/*z* (%) 377 (100), 275 (65), 267 (45), 251 (30), 247 (40), 233 (35), 209 (40), 207 (30), 195 (60).

Spiking of Beer with Iso-α-acids. An aliquot (20 mL) of a freshly opened, bottled beer was spiked with either ethanol (250 μL; control sample) or an ethanolic solution (250 μL) of *cis*-isohumulone (**2b**; 2.6 mmol/L) or *trans*-isohumulone (**3b**; 3.0 mmol/L), respectively, and was placed into a screwed glass bottle (25 mL) under an atmosphere or nitrogen. These samples were maintained for 2 weeks at 37 °C in the dark and then analyzed for the compounds **2a**−**c**−**8a**−**c** by means of HPLC-MS/MS in the multiple reaction monitoring (MRM) mode. In addition, an aliquot (20 mL) of a freshly opened, bottled beer was placed into a screwed glass bottle (25 mL), and the headspace was filled above the solution with oxygen gas, was shaken for 5 min, and then was kept for 2 h at 20 °C prior to HPLC-MS/MS analysis.

HPLC. The HPLC system consisted of two ProStar 210 type pumps (Varian, Middelburg, The Netherlands), a ProStar 330 type diode array detector, and a Rh 7725i injection valve with 500 μL loop (Rheodyne, Bensheim, Germany). For analytical purposes, a 250 mm × 4.6 mm, 5 μm, ODS Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type was used as the stationary phase, with aqueous formic acid (1% in water) as solvent A and acetonitrile containing 1% formic acid as solvent B. Chromatography was performed by increasing the amount of solvent B from 20 to 66% within 35 min and then to 100% within 10 min, maintaining it at 100% for 1 min, and thereafter within 2 min back to 20%. To equilibrate the HPLC system for the next run, the solvent composition was kept for a further 3 min at 20% of solvent B. Detection was performed by means of a diode array detector monitoring the effluent between 220 and 400 nm. For preparative chromatography, a 250 mm × 10 mm, 5 μm, ODS Hypersil column (ThermoHypersil) equipped with a sufficient guard column was used as the stationary phase, with aqueous formic acid (1% in water) as solvent A and acetonitrile containing 1% formic acid as solvent B. The effluent flow (4.5 mL/min) was monitored at 272 nm, and separation was achieved by increasing solvent B from 30 to 70% within 20 min and then to 100% within 5 min, thereafter maintaining it at 100% for an additional 5 min.

HPLC-MS/MS. An Agilent 1100 Series HPLC system consisting of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) or, alternatively, a PHD 4400 Hpsi type syringe pump (Harvard Apparatus, MA) was connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany), which was equipped with an electrospray ionization (ESI) source operating in the negative ion mode. After negative ionization in the ESI source, ions with distinct mass to charge ratios passed the first quadrupol (Q1) and were fragmented in Q2 by collision-induced fragmentation, using nitrogen at 4×10^{-5} torr, and finally, selected fragment ions passed the Q3 to reach the detector. The compound-specific declustering potential (DP), cell exit potential (CXP), and collision energy (CE) were optimized prior to the analysis by infusion of pure reference solutions (**Table 3**). The dwell time for each mass transition was set to 44 ms. For quantification, external calibration functions were recorded for compounds **3b**, **4b**, **6b**, and **7b**. Data acquisition and processing were performed by using the Analyst software version 1.4.2 (AB Sciex Instruments).

For quantitative analysis of iso-α-acids and their degradation products in beer samples as well as model experiments by means of HPLC-MS/MS, a 150 mm × 2 mm, 5 μm, Pursuit C18 column (Varian) was used as the stationary phase, and a gradient of acetonitrile containing 0.5% formic acid (solvent A) and aqueous formic acid (0.5% in water; solvent B) was used as the mobile phase. Using a flow rate of 250 μL/min, chromatography was performed by increasing solvent A from 5 to 10% within 15 min, then to 61% within 37 min, to 65% within 13 min, to 100% within 15 min, holding 100% for 5 min, and, finally, decreasing within 5 min to 5%. Prior to the injection of the sample (5 μL), the system was equilibrated for 5 min. An equilibration period of 5 min was necessary between the individual measurements. Each run started with the injection of an aliquot (5 μL) of the sample solution and was followed by a time-delayed injection of an internal ECHO standard following the protocol reported recently (*15*). For quantitative analysis, the mass spectrometer was operated in the MRM mode, and each sample was analyzed in triplicate.

Table 3. Specific Mass Transitions and Optimized Parameters for the LC-MS/MS Analysis of the Iso-α-acids (**2a**−**c** and **3a**−**c**), the Tricyclic Degradation Products (**4a**−**c**), and the Oxidation Products (**5a**−**c**−**8a**−**c**) using ESI in the ESI^- Mode

compound no.[a]	mass transition Q1 → Q3	mass loss (amu)	DP (V)	CE (V)	CXP (V)
2a, **3a**	*m*/*z* 347.0 → 251.0	96	−90	−22	−11
2b,**c**, **3b**,**c**	*m*/*z* 361.0 → 265.0	96	−90	−22	−11
4a	*m*/*z* 365.3 → 165.0	200	−105	−48	−9
4b,**c**	*m*/*z* 379.3 → 179.0	200	−105	−48	−9
5a, **6a**	*m*/*z* 379.0 → 347.0	32	−85	−26	−9
5b,**c**, **6b**,**c**	*m*/*z* 393.0 → 361.0	32	−85	−26	−9
7a, **8a**	*m*/*z* 363.0 → 261.0	102	−85	−28	−15
7b,**c**, **8b**,**c**	*m*/*z* 377.0 → 275.0	102	−85	−28	−15

[a] Structures of compounds are given in **Figures 1** and **2**.

LC-TOF-MS. High-resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced to sodium formate.

NMR Spectroscopy. ^1H, ^{13}C, and 2D NMR data were acquired on a Bruker DMX-400 (Bruker BioSpin, Rheinstetten, Germany). CD_3OD was used as the solvent, and chemical shifts were referenced to the solvent signal (δ_H=3.31 Hz, δ_C=49.05 Hz). For accurate NMR signal assignment, correlation spectroscopy, heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker) as well as Mestre-C (Mestrelab Research, La Coruña, Spain).

RESULTS AND DISCUSSION

To gain a first insight into the oxidative degradation of iso-α-acids during the storage of beer, the iso-α-acids (**2a**−**c** and **3a**−**c**) as well as the recently identified, *trans*-specific degradation products **4a**−**c** (*17*) were analyzed by means of HPLC-MS/MS-MRM in a fresh sample of Pilsner beer (oxygen content < 0.2 mg/L), a sample of Pilsner beer stored in a brown glass bottle for 4 years at 20 °C, and a sample of Pilsner beer stored in a PET bottle for 4 years at 20 °C, respectively (**Figure 3**). Analysis of the fresh beer revealed a typical ratio of ∼0.4 between the corresponding *trans*- and *cis*-iso-α-acids, and only minor amounts of the compounds **4a**−**c** formed as *trans*-iso-α-acid degradation products (**Figure 3A**). After the beer was stored for 4 years in a glass bottle (**Figure 3B**), the *trans*-iso-α-acids **3a**−**c** were found to be almost completely degraded, whereas the corresponding *cis*-iso-α-acids (**3a**−**c**) seem to be hardly affected by storage. These data are well in line with findings of previous reports (*15*, *17*). In addition, high levels of the tricyclic degradation products **4a**−**c** were detected, thus confirming the acid-catalyzed transformation of **3a**−**c** into **4a**−**c** reported recently (*17*). In comparison to the sample stored in the glass bottle (**Figure 3B**), LC-MS/MS analysis of the beer sample kept for 4 years in a PET bottle revealed a quantitative degradation of both *trans*- and *cis*-iso-α-acids (**Figure 3C**). Because the *cis* isomers were recently shown to be unable to give **4a**−**c** by proton-catalyzed cyclization as found for the *trans*-iso-α-acids (*18*), there

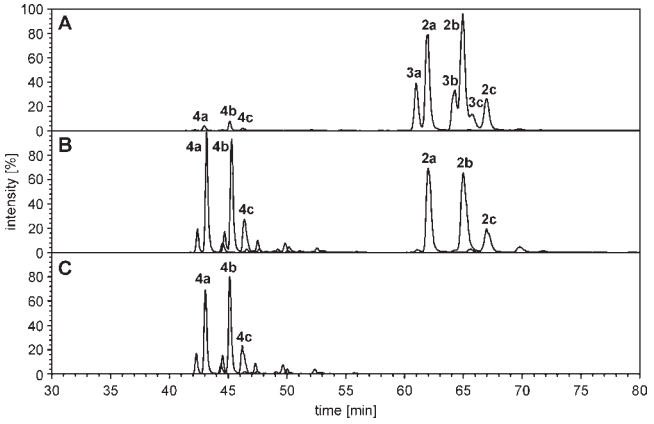

Figure 3. LC/MS/MS chromatograms of a sample of fresh beer (**A**), beer stored for 4 years in a glass bottle (**B**), and beer stored for 4 years in an oxygen-permeable PET bottle (**C**). MRM transitions (**Table 3**) are selected for the iso-α-acids **2a**−**c** and **3a**−**c** and the tricyclohumol congeners **4a**−**c**.

needs to be an alternative mechanism responsible for the degradation of *cis*-iso-α-acids in beer. As PET bottles are well-known to be not gastight and to allow the migration of air oxygen into the bottle (*27*, *28*), an oxidative degradation of iso-α-acid was assumed.

Identification of Iso-α-acid Oxidation Products. As preliminary studies did not enable a successful isolation of iso-α-acid oxidation products from aged beer, the following experiments were aimed at oxidizing iso-α-acids in model solutions under mild conditions, to identify the reaction products formed and then to confirm their occurrence in aged beer samples. To achieve this, a solution of pure iso-α-acids (**2a**−**c** and **3a**−**c**) was incubated for 2 weeks at 20 °C under an atmosphere of oxygen, and the reaction products formed were monitored by means of HPLC with UV detection (272 nm). Comparison of the chromatograms recorded before and after incubation revealed, besides the typical peak pattern of the iso-α-acids **2a**−**c** and **3a**−**c**, another group of earlier eluting peaks (**5a**−**c** and **6a**−**c**) after 2 weeks of storage (**Figure 4A**,**B**), thus suggesting the existence of one oxidation product for each parent iso-α-acid. This finding was further strengthened by the observation that each of the six purified iso-α-acids already contained a minor amount of a byproduct (**Figure 4C**−**H**), which was coeluting with one of the oxidation products detected after incubating an iso-α-acid mixture in the presence of oxygen (**Figure 4B**).

To determine their chemical structure, these oxidation products were isolated by preparative RP-HPLC, and the purity of the isolates was studied by means of analytical HPLC. Surprisingly, each of the purified compounds contained another earlier eluting compound. For example, HPLC analysis of the isolated compound **5b** revealed the earlier eluting compound **7b**, thus indicating that further degradation of **5b** occurred during the purification (**Figure 4I**). LC-TOF-MS analysis revealed the empirical formulas of compounds **5b** and **7b** to be $C_{21}H_{30}O_7$ and $C_{21}H_{30}O_6$, respectively. Comparison to the empirical formula of the parent iso-α-acid **2b** indicated the presence of two and one additional oxygen atom in **5b** and **7b**, respectively, and suggested the presence of a hydroperoxide and a hydroxide of **2b**.

In comparison to the parent *cis*-isohumulone (**2b**), the ^1H and ^{13}C NMR spectra revealed that neither the five-membered ring system with its isobutanoyl side chain at C2 (**Figure 2**) nor the prenyl chain at position C5 are involved in the oxidative modification of **2b**. Interestingly, the former isohexenoyl side chain of **2b** was found to be remarkably modified in **5b** and **7b**,

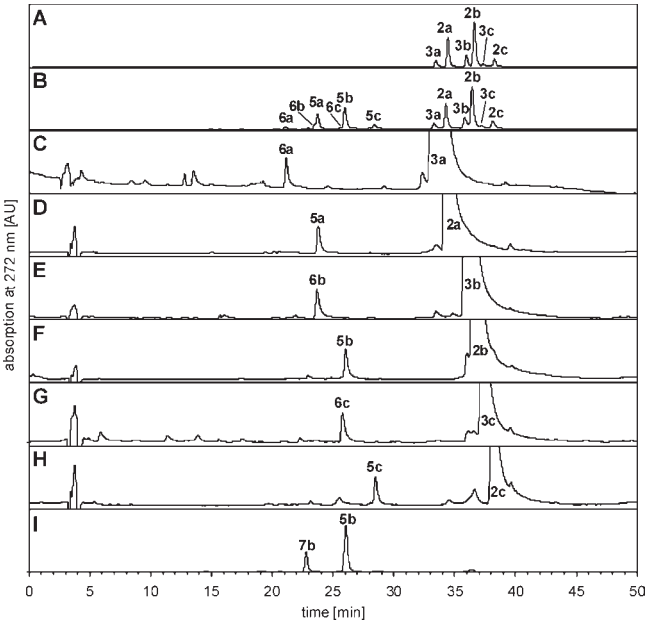

Figure 4. HPLC/UV analysis (λ = 272 nm) of a commercially available iso-α-acid mixture before (**A**) and after storage at room temperature (**B**), purified individual iso-α-acids (**C**−**H**), and rechromatography of preparatively isolated compound **5b** (**I**).

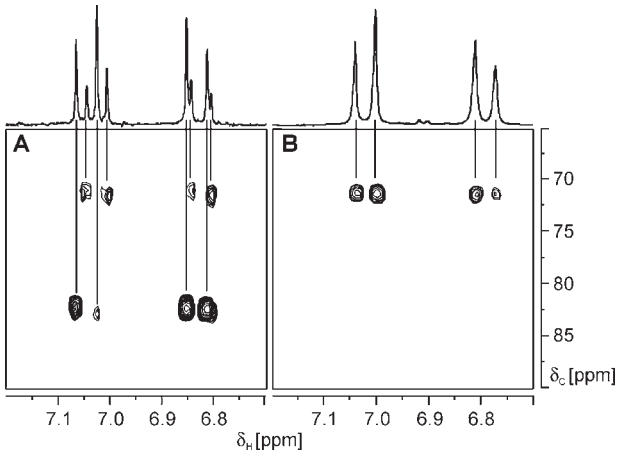

Figure 5. Excerpts of HMBC spectra of a preparatively isolated sample of **5b** before (**A**) and after (**B**) storage at 20 °C for 2 weeks.

respectively; for example, the carbonyl atom C1′ of compound **5b** was found to be high field shifted by 10.6 ppm, and in addition, the carbons C(2′) and C(3′) of the olefinic double bond showed a clear downfield shift from 37.8 and 116.3 to 122.5 and 153.4 ppm, respectively (**Table 2**). Moreover, the ^1H NMR spectrum of **5b** exhibited two duplets for the olefinic protons resonating at 6.80 and 7.04 ppm and showed a coupling of 15.9 Hz each, thus indicating the presence of a *trans*-configured α−*β*-unsaturated carbonyl moiety in the molecules. Although such a *trans*-configured double bound is well in line with the structure of alloisohumulone reported more than 45 years ago (*29*), no further methine proton as present in alloisohumulone could be detected at carbon C4′ of **5b**. In addition, HMBC showed correlation signals between H−C(2′) or H−C(3′) and the carbon atom resonating at 82.8 ppm (**Figure 5A**), thus being well in line with the structure of the previously not reported hydroperoxy-*cis*-alloisohumulone (**5b**; **Figure 2**). Within this region of the HMBC spectrum (**Figure 5**), the differences between the compounds **5b**

and **7b** were particularly pronounced; for example, a heteronuclear connectivity was found between the atoms H−C(2′) or H−C(3′) and the carbon atom resonating at 71.5 ppm. The ^{1}H NMR spectrum showed a double signal set for these olefinic protons, the integral of which showed the same ratio as the peaks of the HPLC chromatogram (**Figure 4I**). Interestingly, after incubation of an aqueous solution containing **5b** and minor amounts of **7b** for 2 weeks at room temperature, the amounts of **7b** increased, whereas **5b** was not detectable anymore as displayed in the excerpt of the HMBC spectrum in **Figure 5B**. LC-MS/MS analysis of this stored aqueous solution revealed the sole presence of **7b** showing the pseudomolecular ion *m*/*z* 377, thus confirming the presence of an additional hydroxy function in the molecules. Taking all these data into account, the compound **7b** was identified as the previously not reported hydroxyl-*cis*-alloisohumulone (**Figure 2**).

To further strengthen the assumption that air oxidation of the other iso-α-acids leads to the generation of corresponding hydroperoxides, compounds **5a**,**c** and **6a**−**c** were isolated from a solution of an iso-α-acid mixture kept in the presence of air oxygen as shown above for the preparation of **5b**. By means of LC-TOF-MS, the empirical formula of **6b** could be verified to be $C_{21}H_{30}O_7$, and also, the MS fragmentation pattern was found to be almost identical to the corresponding cis isomer **5b**. The direct comparison of the ^{1}H and ^{13}C NMR data of **5b** and **6b** (**Tables 1** and **2**) led to the unequivocal structure determination of **6b** as hydroperoxy-*trans*-alloisohumulone (**Figure 2**), which, to the best of our knowledge, has not previously been reported in the literature. In contrast, the amounts of **5a**,**c** and **6a**,**c** were too low to allow a full NMR spectroscopic structure determination. Therefore, hydroperoxy-*cis*-alloisocohumulone (**5a**), hydroperoxy-*cis*-alloisoadhumulone (**5c**), hydroperoxy-*trans*-alloisocohumulone (**6a**), and hydroperoxy-*trans*-alloisoadhumulone (**6c**) were tentatively identified by means of UV/vis, LC-MS/MS, and LC-TOF-MS analysis, as well as by considering their typical elution pattern on a RP-HPLC column.

To confirm the formation of the corresponding hydroxides upon storage of these hydroperoxides, aqueous solutions of **5a**,**c** and **6a**−**c** were incubated for 2 weeks at 20 °C, and the reaction products formed were analyzed by means of HPLC-UV/vis and HPLC-MS/MS. On the basis of these spectroscopic and chromatographic data, hydroxy-*cis*-alloisocohumulone (**7a**), hydroxy-*cis*-alloisoadhumulone (**7c**), hydroxy-*trans*-alloisocohumulone (**8a**), hydroxy-*trans*-alloisohumulone (**8b**), and hydroxy-*trans*-alloisoadhumulone (**8c**) were identified to be formed from the parent hydroperoxides **5a**,**c** and **6a**−**c**.

Spiking of Beer with Iso-α-acids. To verify the oxidation of iso-α-acids in the beer matrix, samples of a freshly opened, bottled beer were spiked with either *cis*-isohumulone (**2b**), *trans*-isohumulone (**3b**), or without any additive, respectively, and were maintained for 2 weeks at 37 °C under an atmosphere of nitrogen in the dark. In addition, a sample of a freshly opened, bottled beer was incubated in the presence of oxygen for 2 h at room temperature. Thereafter, each beer sample was analyzed for the compounds **2a**−**c**−**8a**−**c** by means of HPLC-MS/MS in the MRM mode. As an example, the mass transitions tuned for the selected parent iso-α-acids (**2b**,**c** and **3b**,**c**), their corresponding hydroperoxides (**5b**,**c** and **6b**,**c**) selected as marker molecules for the oxidative iso-α-acid degradation, and the tricyclohumol congeners (**4b**,**c**) selected as marker molecules for the proton-catalyzed *trans*-iso-α-acid degradation are displayed in **Figure 6**. The chromatogram obtained after incubating the authentic beer for 2 weeks at 37 °C clearly indicated the presence of all of the target molecules including the hydroperoxides **5b**,**c** and **6b**,**c** as well as the tricyclohumol congeners (**4b**,**c**) (**Figure 6A**).

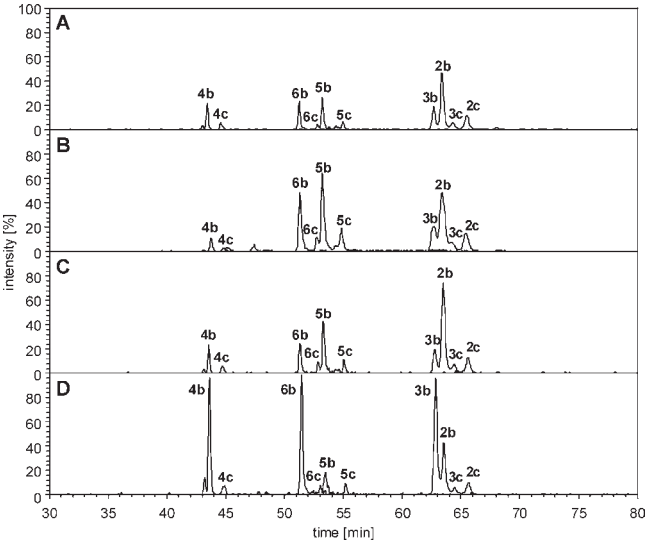

Figure 6. HPLC-MS/MS chromatograms of a sample of beer stored for 2 weeks at 37 °C in the dark (**A**), beer maintained for 2 h at room temperature under an atmosphere of oxygen (**B**), and beer sample spiked with purified *cis*- (32.5 μmol/L; **C**) and *trans*-isohumulone (37.5 μmol/L; **D**) and stored for 2 weeks at 37 °C in the dark. MRM transitions (**Table 3**) are selected for the iso-α-acids **2b**,**c** and **3b**,**c**, the tricyclic degradation products tricyclohumol (**4b**) and tricycloadhumol (**4c**), and the oxidation products *cis*- and *trans*- hydroperoxy-alloisohumulone (**5b** and **6b**) and hydroperoxy-alloisoadhumulone (**5c** and **6c**).

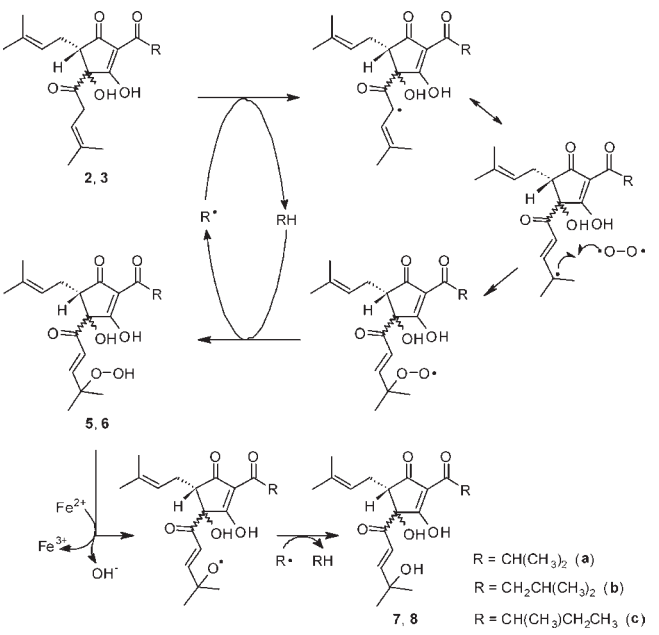

Figure 7. Proposed reaction pathway for the formation of *cis*- and *trans*-hydroperoxy-alloisocohumulone (**5a** and **6a**), hydroperoxy-alloisohumulone (**5b** and **6b**), hydroperoxy-alloisoadhumulone (**5c** and **6c**), and *cis*- and *trans*-hydroxyl-alloisocohumulone (**7a** and **8a**), hydroxyl-alloisohumulone (**7b** and **8b**), and hydroperoxy-alloisoadhumulone (**7c** and **8c**), respectively. Compounds **2**, **5**, and **7** are *R*-configured at position C4 of the ring system, whereas the epimeric structures **3**, **6**, and **8** exhibit a *S*-configured stereocenter.

After treatment of the beer in the presence of oxygen for only 2 h at room temperature somewhat lower amounts of the tricyclohumol congeners (**4b**,**c**) but significantly higher amounts of hydroperoxides and, in particular of compounds **5b** and **6b**,

were found (**Figure 6B**), thus demonstrating that both the *cis*- and the *trans*-iso-α-acids do undergo oxidation in the presence of air oxygen. This was further confirmed by the analysis of the beer samples spiked with either *cis*- (**2b**) or *trans*-isohumulone (**3b**). The increased amounts of **2b** favored the oxidative generation of **5b** (**Figure 6C**), whereas the addition of **3b** accelerated the oxidative production of **6b** besides the acid-catalyzed formation of **4b** (**Figure 6D**).

On the basis of the structures of the identified oxidation products, a reaction cascade leading to the hydroperoxides **5a**−**c** and **6a**−**d** as well as the hydroxides **7a**−**c** and **8a**−**c** is proposed in **Figure 7**. In analogy to the lipid autoxidation of unsaturated fatty acids, first a hydrogen atom is abstracted by a starter radical leading to a resonance-stabilized radical in the isohexenoyl side chain of the iso-α-acid (**2** and **3**). After addition of triplet oxygen, a peroxy radical is formed, which can abstract a hydrogen atom from another donor molecule such as the iso-α-acid to induce the start of another reaction cascade and the formation of the hydroperoxides **5** and **6**, respectively. Cleavage of the hydroperoxy function by means of transition metal ions such as iron(II) ions or by light generates an alkoxy radical, which upon abstraction of a hydrogen atom from another donor molecule gives rise to the generation of the hydroxides **7** and **8**, respectively.

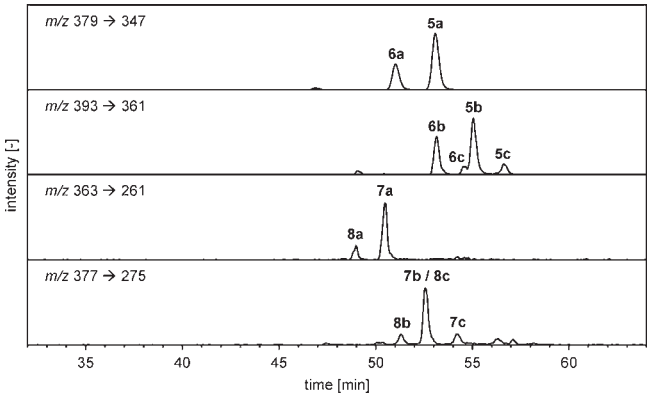

Figure 8. Excerpt of an LC-MS/MS chromatogram of a fresh beer sample, showing mass transitions (**Table 3**) selected for the iso-α-acid oxidation products **5a**−**c**−**8a**−**c**.

Quantitative Analysis of Iso-α-acid Oxidation Products in Beer Samples. To quantitatively analyze the oxidation products identified above in beer samples by means of a highly sensitive and selective HPLC-MS/MS-MRM method, the ionization and fragment parameters of each target compound were optimized (**Table 3**). Using these parameters, MRM chromatograms were recorded for the selected target analytes **5a**−**c**, **6a**−**c**, **7a**−**c**, and **8a**−**c** in beer samples. As depicted in **Figure 8**, with the exception of hydroxyl-*trans*-alloisoadhumulone (**8c**) and hydroxyl-*cis*-alloisohumulonHe (**7b**), all other target compounds were properly separated and detectable in an authentic Pilsner type beer. As chromatographic analysis optimized for the separation of **7b** and **8c** (chromatograms not shown) revealed that independent of the beer sample, the amounts of **7b** exceed the amounts of **8c** by more than a factor of 5, **8c** was not quantitatively determined. Besides these oxidation products, iso-α-acids (**2a**−**c** and **3a**−**c**) as well as tricyclocohumol (**4a**) and tricyclohumol (**4b**) were determined by means of HPLC-MS/MS.

Table 4. Concentrations of Iso-α-acids (**2a**−**c** and **3a**−**c**), the Tricyclic Degradation Products (**4a**−**c**), and the Oxidation Products (**5a**−**c**−**8a**−**c**) in Fresh and Aged Beer Samples, Respectively

	concentration[a] (μmol/L) in beer sample[b]					
target compound[c]	I	II	III	IV	V	VI
trans-isocohumulone (**3a**)	11.5 ± 0.9	3.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	9.1
cis-isocohumulone (**2a**)	29.6 ± 0.5	26.2 ± 1.2	26.2 ± 1.7	25.2 ± 1.8	0.2 ± 0.1	20.3
trans-isohumulone (**3b**)	12.6 ± 0.3	3.8 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	12.7
cis-isohumulone (**2b**)	35.7 ± 1.4	27.9 ± 2.3	21.8 ± 1.7	15.6 ± 1.0	0.2 ± 0.1	30.3
trans-isoadhumulone (**3c**)	2.9 ± 0.1	1.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	2.6
cis-isoadhumulone (**2c**)	10.0 ± 0.4	7.6 ± 0.4	6.8 ± 0.4	6.8 ± 0.3	0.1 ± 0.1	8.8
sum of iso-α-acids (**2a**−**c** and **3a**−**c**)	102.4	70.0	56.5	48.2	0.8	83.8
trans/*cis* ratio[d] of **2** and **3**	0.37	0.15	0.04	0.02	0.56	0.41
tricyclocohumol (**4a**)	1.0 ± 0.1	4.5 ± 0.1	7.2 ± 0.5	9.3 ± 0.2	6.1 ± 0.8	1.2
tricyclohumol (**4b**)	1.6 ± 0.1	7.6 ± 0.5	9.2 ± 0.5	7.4 ± 0.6	8.3 ± 0.9	1.9
tricycloadhumol (**4c**)	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
sum of **4a** and **4b**	2.6	12.1	16.5	16.7	14.4	3.1
trans-alloisocohumulone-hydroperoxide (**6a**)	0.04 ± 0.01	0.03 ± 0.01	<0.01	<0.01	<0.01	0.07
cis-alloisocohumulone-hydroperoxide (**5a**)	0.14 ± 0.01	0.17 ± 0.01	0.21 ± 0.06	0.16 ± 0.03	0.01 ± 0.01	0.23
trans-alloisohumulone-hydroperoxide (**6b**)	0.09 ± 0.02	0.05 ± 0.01	0.01 ± 0.01	<0.01	<0.01	0.23
cis-alloisohumulone-hydroperoxide (**5b**)	0.21 ± 0.02	0.22 ± 0.04	0.22 ± 0.05	0.12 ± 0.02	0.01 ± 0.01	0.39
trans-alloisoadhumulone-hydroperoxide (**6c**)	<0.01	<0.01	<0.01	<0.01	<0.01	0.03
cis-alloisoadhumulone-hydroperoxide (**5c**)	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	<0.01	0.11
sum of **5a**−**c** and **6a**−**c**	0.52	0.51	0.51	0.32	0.02	1.09
trans-alloisocohumulone-hydroxide (**8a**)	0.02 ± 0.01	0.04 ± 0.01	<0.01	<0.01	<0.01	0.16
cis-alloisocohumulone-hydroxide (**7a**)	0.11 ± 0.01	0.43 ± 0.03	0.54 ± 0.02	1.00 ± 0.07	0.02 ± 0.01	1.11
trans-alloisohumulone-hydroxide (**8b**)	0.04 ± 0.01	0.06 ± 0.05	0.02 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.41
cis-alloisohumulone-hydroxide (**7b**)	0.18 ± 0.02	0.64 ± 0.04	0.61 ± 0.02	0.83 ± 0.05	0.02 ± 0.01	2.19
trans-alloisoadhumulone-hydroxide (**8c**)	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
cis-alloisoadhumulone-hydroxide (**7c**)	0.04 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.26 ± 0.02	0.01 ± 0.01	0.46
sum of **7a**−**c** and **8a**,**b**	0.39	1.29	1.33	2.11	0.08	4.33

[a] Values are arithmetic means of triplicates ± standard deviations. [b] Fresh beer (I), beer stored for 8 months at 28 °C (II), beer stored for 4 years at room temperature (III), beer stored for 10 years at room temperature (IV), beer stored for 4 years at room temperature in a PET bottle (V), and fresh beer incubated for 24 h at 22 °C with oxygen (VI). [c] Structures are given in **Figures 1** and **2**. [d] The *trans*/*cis* ratio is calculated by summing congeners **a** and **b**, respectively. [e] Could not be determined, due to coelution with other compounds.

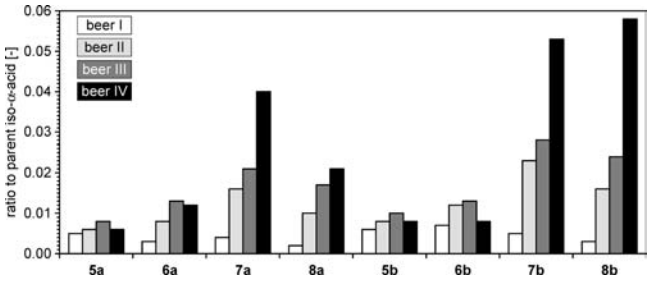

Figure 9. Ratio of the oxidation products **5a**,**b**−**8a**,**b** to their corresponding parent iso-α-acid in fresh beer (I), beer stored for 8 months at 28 °C (II), beer stored for 4 years at 20 °C (III), and beer stored for 10 years at 20 °C (IV).

The fresh Pilsner beer (sample I) was found to contain a total of 102.4 μmol/L iso-α-acids (**2a**−**c** and **3a**−**c**) with a mean *trans*/*cis* ratio of 0.37 (**Table 4**), which is well in line with literature data (*9*, *15*). Interestingly, noticeable amounts of the *trans*-specific iso-α-acid degradation products, formed upon acid-catalyzed cyclization from **3a**−**c** (*18*), were already detectable in this beer, for example, 1.0 μmol/L of tricyclocohumol (**4a**) and 1.6 μmol/L of tricyclohumol (**4b**) were found in the fresh beer sample I. In comparison, a rather low amount of 0.52 μmol/L was found for the sum of the hydroperoxy-alloisohumulones (**5a**−**c** and **6a**−**c**) formed upon iso-α-acid oxidation. Also, the hydroxyl-alloisohumulones (**7a**−**c** and **8a**,**b**) were present in this fresh beer in only rather low concentrations; for example, a total amount of 0.39 μmol/L was determined (**Table 4**). Among all of these oxidation products, the hydroperoxy-*cis*-alloisohumulone (**5b**) as well as the hydroxy-*cis*-alloisohumulone (**7b**) were found as the most abundant isomers with concentrations of 0.21 and 0.11 μmol/L.

A beer sample aged for 8 months at 28 °C (sample II) showed strongly reduced levels of *trans*-iso-α-acids (**3a**−**c**), confirming data published earlier (*9*), and about 4-fold increased concentrations of their tricyclic degradation products **4a**,**b**, respectively (**Table 4**). However, the levels of hydroperoxides (**5a**−**c** and **6a**−**c**) seem to be rather constant, and the concentrations of the hydroxides (**7a**−**c** and **8a**,**b**) increased only to some extend when compared to the fresh beer (sample I). However, calculating the ratio of these oxidation products to their parent iso-α-acids clearly demonstrated a relative increase of these hydroperoxides and hydroxides upon beer aging (**Figure 9**). These data clearly imply that, once generated, the hydroperoxy-alloisohumulones are not stable end products of oxidation but react further on during beer aging.

These findings were further confirmed by the analytical data obtained from a beer sample III, which was stored for 4 years at 20 °C, and a beer sample IV, which was stored for 10 years at 20 °C. In sample III, the absolute concentrations of hydroperoxides **6a**,**b** are further decreased, while their relative amounts are increasing (**Figure 9**). The total amount of the hydroxyl-alloisohumulones increased slightly from 1.29 (beer II) over 1.33 (beer III) to 2.11 μmol/L (beer IV), and the relative amount increased strongly (**Table 4** and **Figure 9**). In addition, the HPLC-MS/MS analysis of the long-term aged beers III and IV clearly demonstrated that, in contrast to the increase of the *cis*-configured hydroxides (**7a**,**b**), the amounts formed of the *trans*-configured hydroxides (**8a**,**b**) seem to be rather constant at a low level, thus implying the latter compounds to be less stable and to be more rapidly further degraded via unknown pathways (**Table 4**).

In the beer sample V, stored for 4 years in the gas permeable PET bottle, only the tricyclic derivatives **4a**,**b** could be found in higher amounts, whereas neither the parent iso-α-acids (**2** and **3**) nor their oxidation products (**5**−**8**) were left in higher concentrations (**Table 4**). To quantify the target analytes in a beer sample kept in the presence of oxygen, another beer sample was incubated for 24 h at 20 °C under an atmosphere of oxygen (sample VI). Whereas iso-α-acids (**2** and **3**) were found to be slightly decreased and the tricyclic degradation products (**4**) were hardly affected, the hydroperoxides (**5** and **6**) and, in particular, the hydroxides (**7** and **8**) were detectable in 2- and 11-fold elevated amounts of 1.09 and 4.33 μmol/L when compared to the fresh beer (sample I) (**Table 4**).

In conclusion, hydroperoxy- and hydroxyl-allo-iso-α-acids were identified as primary autoxidation products of both *cis*- and *trans*-configured iso-α-acids in beer samples, in particular, when maintained in oxygen-permeable PET bottles. Because these autoxidation products are formed rather quickly, their quantitative data might provide a measure for the current oxidative state of a beer sample. However, because of their pronounced instability, the compounds **5**−**8** cannot be recommended as suitable indicator molecules of the oxidation degree of beer. Hence, further research is needed to identify stable end products as appropriate marker molecules for oxidative deterioration of the beer taste.

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