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Identification and Quantification of the Oxidation Products Derived from α-Acids and β-Acids During Storage of Hops (*Humulus lupulus* L.)

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ABSTRACT: α-Acids and β-acids, two main components of hop resin, are known to be susceptible to oxygen and degraded during hop storage, although the oxidation products in stored hops have not been fully identified. In this study, we developed a high-performance liquid chromatography (HPLC) analysis method suitable for separation and quantification of the oxidation products. This HPLC analysis clearly proved, for the first time, that humulinones and hulupones are major products in oxidized hops. We are also the first to identify novel 4′-hydroxy-allohumulinones, suggested to be oxidative products of humulinones, by means of NMR spectroscopy and high-resolution mass spectrometry. Using the developed analytical method, changes in α- and β-acids and their oxidation products during hop storage were clearly revealed for the first time.

KEYWORDS: *hop, Humulus lupulus L., α-acids, β-acids, humulinone, hulupone, 4′-hydroxy-allohumulinone, oxidation, storage, beer*

INTRODUCTION

Hops, the female inflorescences of the hop plant (*Humulus lupulus* L.), are widely used in the brewing industry to add characteristic bitterness and aroma to beer, and possess preservative capacity. The components contributing to the bitter taste of beer mostly originate from α-acids and β-acids, which are the two main components of hop resins. The main compounds belonging to α-acids are cohumulone (**1a**), *n*-humulone (**1b**), and adhumulone (**1c**), and the main compounds belonging to β-acids are colupulone (**2a**), *n*-lupulone (**2b**), and adlupulone (**2c**). The structural differences among **1a**, **1b**, and **1c** and among **2a**, **2b**, and **2c** are the acyl side chains at C-2 (Figure 1). During the brewing of beer, α-acids isomerize into iso-α-acids via an acyloin-type ring contraction. Iso-α-acids are made up of two epimeric isomers: *cis*- (**3a**−**c**) and *trans*-iso-α-acids (**4a**−**c**), which differ in their stereochemistry at C-5 (Figure 1).[1] About 50−70% of α-acids are converted to iso-α-acids during the wort boiling process, although the amount of iso-α-acids in beer is rather low (10−40% of α-acids), due to their adsorption on the trub and loss during the postwort boiling process.[2,3] Because iso-α-acids are the major contributors to the bitter taste in beer,[4,5] beer foam stability,[6] and inhibition of Gram-positive bacterial growth,[7] brewers pay attention to the content of α-acids (precursor of iso-α-acids) in the hops they use.

It is well-known that α-acids and β-acids are oxidized rapidly during hop storage. The effects of oxidized hops on the bitterness quality of beer are controversial. There have been many reports that oxidized hops containing little or no detectable α-acids have a bittering potential, which is indistinguishable from fresh hops,[8−10] although other studies have reported that beer brewed with oxidized hops tasted mildly bitter or markedly less bitter.[5,11,12] It is considered that detailed information on the chemical composition of oxidized hops used in brewing could help to determine their influence on beer quality. However, there have been very few reports on the oxidation products of α-acids and β-acids in hops, including changes in their amount during hop storage, because the complexities of the oxidation products hamper identification of each component.

Although many studies have reported the identification of products obtained through chemical oxidation of isolated α-acids or β-acids, their occurrences in oxidized hops have remained uncertain. For example, humulinones (**5a**−**c**, Figure 1) can be readily prepared when α-acids are treated with peroxide reagent in a two-phase solvent system;[13,14] however, their occurrence in oxidized hops has not been confirmed due to a lack of suitable analytical methods.[8,15−18] The presence of certain oxidation products obtained by chemical oxidation of α-acids, such as *cis*- (**8a**−**c**) and *trans*-humulinic acids (**9a**−**c**), tricyclodehydroisohumulone (**10**), and Ashurst's compound (**11**) (Figure 1), in oxidized hops has been previously reported;[19−21] however, experimental results using low-resolution HPLC or TLC methods should be reconfirmed using high-resolution analyses.

Among the oxidation products of β-acids, hulupones (**6a**−**c**, Figure 1) were confirmed to be produced through the oxidation of hops.[22] A small amount of hulupones has also been reported to be produced during the wort boiling process and to partly contribute to the bitter taste of beer.[22,23] In addition to hulupones, Haseleu et al. recently reported the occurrence of tricyclic β-acid derivatives during the wort boiling process and their presence, as minor components, in the final beer.[23,24] Intelmann et al. also reported the degradation products and mechanisms of iso-α-acids during beer storage.[25−27] Detailed analyses of hop-derived compounds in the manufactural processes of beer and during beer storage are important in the control of beer quality.[3,28] In addition, detailed analyses of the chemical composition of the raw hop material are also important; however, oxidative changes in

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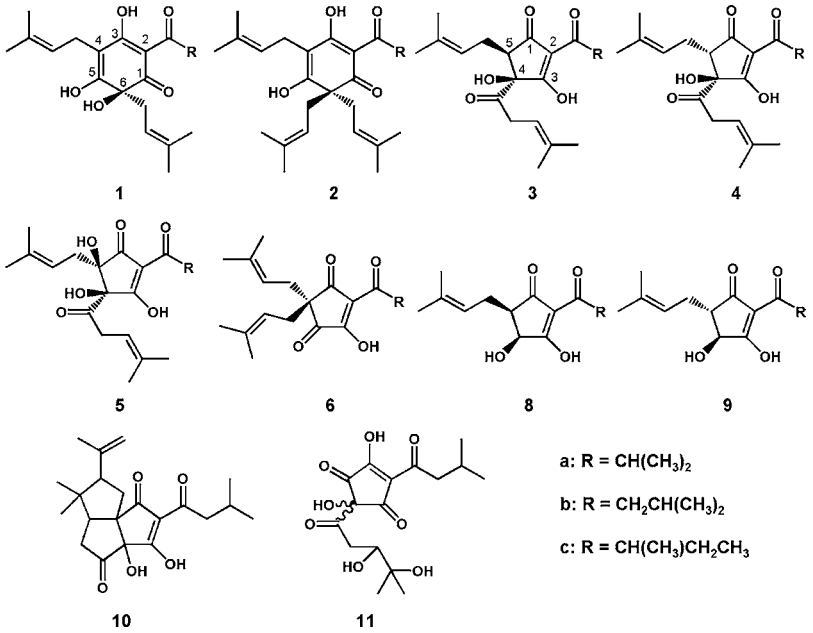

Figure 1. Structures of α-acids, cohumulone (**1a**), *n*-humulone (**1b**), adhumulone (**1c**); and β-acids, colupulone (**2a**), *n*-lupulone (**2b**), adlupulone (**2c**); and *cis*-iso-α-acids, *cis*-isocohumulone (**3a**), *cis*-iso-*n*-humulone (**3b**), *cis*-isoadhumulone (**3c**); and *trans*-iso-α-acids, *trans*-isocohumulone (**4a**), *trans*-iso-*n*-humulone (**4b**), *trans*-isoadhumulone (**4c**); and humulinones, cohumulinone (**5a**), *n*-humulinone (**5b**), adhumulinone (**5c**); and hulupones, cohulupone (**6a**), *n*-hulupone (**6b**), adhulupone (**6c**); and *cis*-humulinic acids, *cis*-cohumulinic acid (**8a**), *cis*-*n*-humulinic acid (**8b**), *cis*-adhumulinic acid (**8c**); and *trans*-humulinic acids, *trans*-cohumulinic acid (**9a**), *trans*-*n*-humulinic acid (**9b**), *trans*-adhumulinic acid (**9c**); and tricyclodehydroisohumulone (**10**); and Ashurst's compound (**11**).

α-acids and β-acids occurring in the raw hop material during its storage are still not clear.

In this study, we have developed a high-resolution HPLC method suitable for simultaneous separation and quantification of oxidative products which occur during the storage of hops. In addition, we have succeeded in tracing and identifying the oxidative products derived from α- and β-acids using this method.

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: ethylenediaminetetraacetic acid (EDTA), phosphoric acid, acetonitrile, ethanol, hexane, ethyl acetate, toluene, diethyl ether, *o*-phenylenediamine (Wako Pure Chemicals, Japan); dicyclohexylamine (Aldrich, USA); cumene hydroperoxide (Tokyo Chemical Industry, Japan); and International Calibration Extract ICE2 (American Society of Brewing Chemists, USA). Deionized water for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, USA). Hop pellets, cultivar Hallertau Perle (HPE), were provided by Kirin Brewery Co. Supercritical carbon dioxide hop extracts and isomerized hop extracts were purchased from Hopsteiner (Mainburg, Germany).

Preparation of Reference Compounds. *Preparation of α-Acids (Mixtures of **1a**–**c**) and Pure n-Humulone (**1b**).* Two hundred grams of supercritical carbon dioxide hop extracts (α-acids 55.6% (w/w) and β-acids 22.6% (w/w)) was dissolved in hexane (1.5 L), and partitioned with 0.24 M disodium carbonate (2 L). In this process, α-acids were selectively extracted into the aqueous solution. The aqueous solution was acidified with 6 N HCl, and free α-acids were extracted with hexane. After being washed with saturated NaCl, the hexane layer was dried over anhydrous sodium sulfate and concentrated to dryness to give the α-acids fraction (105 g). HPLC analysis confirmed that the purity of α-acids (sum of **1a**–**c**) in this fraction was more than 90%. Pure *n*-humulone (**1b**) was prepared from the above α-acids fraction according to a previously reported method,[13] with small modifications. In brief, 50 g of the α-acids fraction was dissolved in toluene (150 mL). *o*-Phenylenediamine (11.5 g) was added to the solution and heated at 80 °C to dissolve the reagent completely. The solution was then cooled at 4 °C, and the generated precipitate (*o*-phenylenediamine/*n*-humulone complex) was collected by filtration and further purified via recrystallization using toluene (eight times) to give pure *o*-phenylenediamine/*n*-humulone complex (13.6 g). The complex was added to 2 N HCl (100 mL), and free *n*-humulone was extracted with ethyl acetate (250 mL). After being washed with saturated NaCl, the ethyl acetate layer was dried over anhydrous sodium sulfate and concentrated to dryness to give *n*-humulone (**1b**, 10.1 g). The purity of **1b** was more than 98% by HPLC analysis.

*n-Humulone (**1b**, Figure 1).* Pale yellow solid; UV (0.1 N HCl–MeOH) λ_{max} 235, 284, 323, and 355 (shoulder) nm, UV (0.1 N NaOH–MeOH) λ_{max} 226, 327, and 358 (shoulder) nm; HR-ESIMS (negative) *m*/*z* 361.2015 $[M - H]^-$ (calcd for $C_{21}H_{29}O_5$, 361.2021); ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) spectra were identical to the literature data.[29]

*Synthesis of Humulinones (Mixtures of **5a**–**c**) and Pure n-Humulinone (**5b**).* Humulinones (**5a**–**c**) and *n*-humulinone (**5b**) were prepared from the α-acids fraction described above and *n*-humulone, respectively, according to a protocol reported previously.[13] In brief, the α-acids fraction (1.0 g) and cumene hydroperoxide (0.5 mL) were dissolved in diethyl ether (5 mL). Saturated sodium bicarbonate (35 mL) was added to the diethyl ether solution, and the bilayer was kept for 4 days at room temperature in a sealed flask. Sodium salts of humulinones were deposited at the border of the bilayer under these conditions. The sodium salts were filtered and washed with cold diethyl ether and water. The washed sodium salts (600 mg) were then dissolved in MeOH (60 mL) containing 1% phosphoric acid, and 0.5 N HCl (600 mL) was added to the solution. The solution was then partitioned with hexane (600 mL × 2), and the hexane layer was concentrated to dryness to give humulinones (**5a**–**c**, 550 mg) at a purity of more than 95% (sum of **5a**–**c**) by HPLC analysis. *n*-Humulinone (**5b**, 480 mg) was prepared from *n*-humulone (1.0 g) according to the same procedure described above at a purity of more than 98% by HPLC analysis.

*n-Humulinone (**5b**, Figure 1).* White solid; UV (0.1 N HCl–MeOH) λ_{max} 226 and 282 nm, UV (0.1 N NaOH–MeOH) λ_{max} 254 and 270 (shoulder) nm; HR-ESIMS (negative) *m*/*z* 377.1964 $[M - H]^-$ (calcd for $C_{21}H_{29}O_6$, 377.1970); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) data are given in Table 1.

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Table 1. Assignment of ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) Signals of Compounds 3b, 4b, 5b, 7b, and 7a[a]

cis-iso-*n*-humulone (**3b**)			*trans*-iso-*n*-humulone (**4b**)			*n*-humulinone (**5b**)			4′-hydroxy-allo-*n*-humulinone (**7b**)			4′-hydroxy-allocohumulinone (**7a**)		
position	^{1}H	^{13}C	position	^{1}H	^{13}C	position	^{1}H	^{13}C	position	^{1}H	^{13}C	position	^{1}H	^{13}C
1-CO		205.5	1-CO		203.9	1-CO		201.9	1-CO		201.7	1-CO		201.6
2		111.9	2		112.4	2		111.1	2		111.8	2		110.9
3-OH		198.1	3-OH		198.5	3-OH		198.7	3-OH		199.1	3-OH		198.9
4-OH		88.5	4-OH		90.7	4-OH		91.1	4-OH		90.0	4-OH		89.7
5	3.14 (1H, dd, *J* = 7.6, 5.7 Hz)	52.8	5	2.95 (1H, dd, *J* = 9.9, 5.7 Hz)	57.5	5-OH		81.0	5-OH		81.6	5-OH		81.4
1′-CO		210.5	1′-CO		209.9	1′-CO		210.1	1′-CO		200.2	1′-CO		200.5
2′	3.47 (2H, d, *J* = 6.8 Hz)	38.3	2′	3.47 (1H, dd, *J* = 19.7, 6.6 Hz) 3.40 (1H, dd, *J* = 19.7, 6.1 Hz)	39.7	2′	3.49 (1H, dd, *J* = 20.0, 6.5 Hz) 3.42 (1H, dd, *J* = 20.0, 6.2 Hz)	40.0	2′	6.98 (1H, d, *J* = 15.6 Hz)	121.6	2′	6.93 (1H, d, *J* = 15.6 Hz)	121.8
3′	5.22 (1H, m)	116.7	3′	5.23 (1H, m)	116.4	3′	5.21 (1H, m)	116.5	3′	6.86 (1H, d, *J* = 15.6 Hz)	155.5	3′	6.85 (1H, d, *J* = 15.6 Hz)	155.0
4′		136.5	4′		136.2	4′		136.0	4′-OH		71.4	4′-OH		71.3
5′	1.59 (3H, s)	18.1	5′	1.58 (3H, s)	18.2	5′	1.57 (3H, s)	18.2	5′	1.30 (3H, s)	29.3	5′	1.29 (3H, s)	29.3
6′	1.71 (3H, s)	25.8	6′	1.72 (3H, s)	25.9	6′	1.71 (3H, s)	25.8	6′	1.30 (3H, s)	29.4	6′	1.29 (3H, s)	29.3
1″	2.42 (1H, m) 2.36 (1H, m)	26.1	1″	2.49 (1H, m) 2.25 (1H, ddd, *J* = 15.3, 9.4, 9.4 Hz)	24.7	1″	2.45 (2H, d, *J* = 6.9 Hz)	30.8	1″	2.48 (1H, m), 2.37 (1H, dd, *J* = 15.4, 9.0 Hz)	31.3	1″	2.50 (1H, m) 2.33 (1H, dd, *J* = 15.6, 9.0 Hz)	31.7
2″	5.10 (1H, m)	121.8	2″	5.18 (1H, m)	122.3	2″	5.33 (1H, m)	119.0	2″	5.27 (1H, m)	119.3	2″	5.24 (1H, m)	119.7
3″		135.1	3″		134.7	3″		135.5	3″		135.1	3″		134.9
4″	1.59 (3H, s)	17.9	4″	1.51 (3H, s)	18.0	4″	1.46 (3H, s)	18.1	4″	1.42 (3H, s)	18.1	4″	1.42 (3H, s)	18.2
5″	1.63 (3H, s)	26.0	5″	1.67 (3H, s)	25.8	5″	1.66 (3H, s)	26.0	5″	1.62 (3H, s)	26.1	5″	1.61 (3H, s)	26.1
1‴-CO		200.5	1‴-CO		198.7	1‴-CO		199.1	1‴-CO		199.5	1‴-CO		204.3
2‴	2.71 (2H, d, *J* = 7.0 Hz)	47.0	2‴	2.76 (1H, dd, *J* = 13.8, 7.3 Hz) 2.69 (1H, dd, *J* = 13.8, 6.9 Hz)	45.9	2‴	2.77 (1H, dd, *J* = 13.8, 7.2 Hz) 2.69 (1H, dd, *J* = 13.8, 6.9 Hz)	46.3	2‴	2.74 (1H, dd, *J* = 13.7, 7.7 Hz) 2.69 (1H, dd, *J* = 13.7, 7.4 Hz)	47.9	2‴	3.64 (1H, m)	37.3
3‴	2.10 (1H, m)	27.2	3‴	2.11 (1H, m)	27.5	3‴	2.10 (1H, m)	27.6	3‴	2.11 (1H, m)	27.2	3‴	1.03 (3H, d, *J* = 6.8 Hz)	18.8
4‴	0.93 (3H, d, *J* = 6.5 Hz)	22.8	4‴	0.94 (3H, d, *J* = 6.7 Hz)	22.7	4‴	0.93 (3H, d, *J* = 6.7 Hz)	22.7	4‴	0.93 (3H, d, *J* = 7.2 Hz)	23.0	4‴	1.06 (3H, d, *J* = 6.8 Hz)	18.9
5‴	0.95 (3H, d, *J* = 6.5 Hz)	22.9	5‴	0.97 (3H, d, *J* = 6.7 Hz)	22.9	5‴	0.96 (3H, d, *J* = 6.7 Hz)	22.8	5‴	0.95 (3H, d, *J* = 7.2 Hz)	23.0			

[a]Arbitrary numbering according to structures **3b**, **4b**, **5b**, **7b**, and **7a** in Figure 4.

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*Synthesis of Hulupones (Mixtures of **6a**–**c**) and Preparation of Pure Cohulupone (**6a**).* Two hundred grams of supercritical carbon dioxide hop extracts (β-acids 41.6% (w/w) and trace amounts of α-acids) was dissolved in hexane (1 L), and partitioned with 0.6 M sodium hydroxide (2 L). β-Acids were selectively distributed to the aqueous solution. The aqueous solution was acidified with 6 N HCl, and free β-acids were extracted with hexane. After being washed with saturated NaCl, the hexane layer was dried over anhydrous sodium sulfate and concentrated to dryness to give the β-acids fraction (92 g). The purity of the β-acids (sum of **2a**–**c**) in this fraction was more than 85% by HPLC analysis. Hulupones (**6a**–**c**) were synthesized from the above β-acids fraction according to a previously reported method.[30] The purity of the prepared hulupones (sum of **6a**–**c**) was more than 95% by HPLC analysis. 360 mg of cohulupone (**6a**) was purified from the above hulupones (800 mg) by preparative HPLC (column: 150 × 10 mm i.d., 5 μm, Alltima C18 column (Systech, USA), solvent: water/phosphoric acid, 1000/10 (v/v) (solvent A) and acetonitrile (solvent B), isocratic elution at 60% B, flow rate 4.7 mL/min, detect 270 nm, temperature 40 °C, R_t 10.4 min). The purity of **6a** was more than 98% by HPLC analysis.

*Cohulupone (**6a**, Figure 1).* Yellow waxy solid; UV (0.1 N HCl–MeOH) λ_{max} 279 nm, UV (0.1 N NaOH–MeOH) λ_{max} 255 and 328 nm; HR-ESIMS (negative) m/z 317.1752 $[M - H]^-$ (calcd for $C_{19}H_{25}O_4$, 317.1759); ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) spectra were identical to the literature data.[23]

*Preparation of cis- and trans-Iso-α-acids (**3a**–**c** and **4a**–**c**).* Each *cis*- and *trans*-iso-α-acid was isolated according to previously reported methods,[29,31] with small modifications. In brief, 100 mL of isomerized hop extract (iso-α-acids about 30% (w/v)) was added to 1 N HCl (1 L) and partitioned with hexane (250 mL × 2). The hexane layer was concentrated to dryness (36 g) and redissolved in ethyl acetate (100 mL). Dicyclohexylamine (16 g) was added to the solution to precipitate trans isomers.

The precipitate (dicyclohexylamine/*trans*-iso-α-acids complex) was collected by filtration and further purified by recrystallization from EtOH, followed by preparative HPLC (column: 150 × 10 mm i.d., 5 μm, Alltima C18 column (Systech, USA), solvent: water/phosphoric acid, 1000/10 (v/v) (solvent A) and acetonitrile (solvent B), isocratic elution at 65% B, flow rate 4.7 mL/min, detect 270 nm, temperature 40 °C) as the final step. In the preparative HPLC, the *trans*-iso-α-acids were eluted separately at R_t 9.5 min (**4a**, 218 mg), 12.1 min (**4b**, 188 mg), and 13.2 min (**4c**, 45 mg).

The filtrate of the above precipitation, which contained mainly *cis*-iso-α-acids and dicyclohexylamine, was added to 1 N HCl (300 mL), and partitioned in a separating funnel to remove dicyclohexylamine. The ethyl acetate layer was concentrated to dryness to yield the mixture of *cis*-iso-α-acids. Each *cis*-iso-α-acid was finally isolated by preparative HPLC (using the same conditions applied to *trans*-iso-α-acids isolation). In the preparative HPLC, the *cis*-iso-α-acids were eluted separately at R_t 10.2 min (**3a**, 193 mg), 13.1 min (**3b**, 254 mg), and 14.4 min (**3c**, 51 mg). The purity of **3a**–**c** and **4a**–**c** was more than 95% by HPLC analysis.

*cis-Isocohumulone (**3a**, Figure 1).* Pale yellow oil; UV (0.1 N HCl–MeOH) λ_{max} 220 and 264 nm, UV (0.1 N NaOH–MeOH) λ_{max} 254 and 270 (shoulder) nm; HR-ESIMS (negative) m/z 347.1859 $[M - H]^-$ (calcd for $C_{20}H_{27}O_5$, 347.1864); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data.[29]

*cis-Iso-n-humulone (**3b**, Figure 1).* Pale yellow oil; UV (0.1 N HCl–MeOH) λ_{max} 220 and 273 nm, UV (0.1 N NaOH–MeOH) λ_{max} 254 and 270 (shoulder) nm; HR-ESIMS (negative) m/z 361.2015 $[M - H]^-$ (calcd for $C_{21}H_{29}O_5$, 361.2021); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data[29] and are given in Table 1.

*cis-Isoadhumulone (**3c**, Figure 1).* Pale yellow oil; UV (0.1 N HCl–MeOH) λ_{max} 223 and 269 nm, UV (0.1 N NaOH–MeOH) λ_{max} 255 and 273 (shoulder) nm; HR-ESIMS (negative) m/z 361.2015 $[M - H]^-$ (calcd for $C_{21}H_{29}O_5$, 361.2021); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data.[29]

*trans-Isocohumulone (**4a**, Figure 1).* Colorless crystal; UV (0.1 N HCl–MeOH) λ_{max} 220 and 270 nm, UV (0.1 N NaOH–MeOH) λ_{max} 253 and 270 (shoulder) nm; HR-ESIMS (negative) m/z 347.1860 $[M - H]^-$ (calcd for $C_{20}H_{27}O_5$, 347.1864); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data.[29]

*trans-Iso-n-humulone (**4b**, Figure 1).* Colorless crystal; UV (0.1 N HCl–MeOH) λ_{max} 220 and 277 nm, UV (0.1 N NaOH–MeOH) λ_{max} 253 and 272 (shoulder) nm; HR-ESIMS (negative) m/z 361.2014 $[M - H]^-$ (calcd for $C_{21}H_{29}O_5$, 361.2021); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data[29] and are given in Table 1.

*trans-Isoadhumulone (**4c**, Figure 1).* Colorless crystal; UV (0.1 N HCl–MeOH) λ_{max} 220 and 275 nm, UV (0.1 N NaOH–MeOH) λ_{max} 254 and 272 (shoulder) nm; HR-ESIMS (negative) m/z 361.2015 $[M - H]^-$ (calcd for $C_{21}H_{29}O_5$, 361.2021); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) spectra were identical to the literature data.[29]

*Isolation of 4′-Hydroxy-allocohumulinone (**7a**) and 4′-Hydroxy-allo-n-humulinone (**7b**) from Oxidized Hops.* One hundred grams of hop pellets was stored at 60 °C for 120 h (in this process, α- and β-acids in hops decrease to trace amounts) and extracted with EtOH (1 L). The extract (25.5 g) was applied to Diaion HP-20 (Mitsubishi Chemical, Japan) column chromatography (3.0 cm i.d. × 25 cm), and eluted stepwisely with 10% EtOH (2 L; 3.8 g), 30% EtOH (3 L; 4.7 g), 60% EtOH (2 L; 5.6 g), 80% EtOH (2 L; 5.0 g), and 100% EtOH (2 L; 7.7 g). The 30% EtOH fraction was further chromatographed by preparative HPLC (column: 150 × 10 mm i.d., 5 μm, Alltima C18 column (Systech, USA), solvent: water/phosphoric acid, 1000/0.2 (v/v), containing EDTA (0.02% w/v) (solvent A) and acetonitrile (solvent B); a linear gradient from 15% to 28% B in 0 → 25 min, and 28% to 80% B in 25 → 30 min, then 80% B for 30 → 35 min, flow rate 8.5 mL/min, detect 270 nm, temperature 40 °C). 4′-Hydroxy-allocohumulinone (**7a**) and 4′-hydroxy-allo-*n*-humulinone (**7b**) were eluted at 10.2 and 14.4 min, respectively. To remove phosphoric acid and EDTA, each eluate was diluted with H_2O (five times) and applied to an Oasis HLB column (Waters, USA) developed with H_2O, and eluted with EtOH. The yield of **7a** and **7b** from the HP-20 30% EtOH fraction (500 mg) was 9.4 and 18.3 mg, respectively.

*4′-Hydroxy-allocohumulinone, rel-(4S,5R)-3,4,5-Trihydroxy-4-[(2E)-4-hydroxy-4-methyl-pent-2-enoyl]-2-isobutyryl-5-(3-methyl-but-2-enyl)-cyclopent-2-enone (**7a**, Figure 4).* Pale yellow oil; UV (0.1 N HCl–MeOH) λ_{max} 230 and 280 (shoulder) nm, UV (0.1 N NaOH–MeOH) λ_{max} 253 and 270 (shoulder) nm; HR-ESIMS (negative) m/z 379.1754 $[M - H]^-$ (calcd for $C_{20}H_{27}O_7$, 379.1762); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) data are given in Table 1.

*4′-Hydroxy-allo-n-humulinone, rel-(4S,5R)-3,4,5-Trihydroxy-4-[(2E)-4-hydroxy-4-methyl-pent-2-enoyl]-5-(3-methyl-but-2-enyl)-2-(3-methyl-butyryl)-cyclopent-2-enone (**7b**, Figure 4).* Pale yellow oil; UV (0.1 N HCl–MeOH) λ_{max} 230 and 282 nm, UV (0.1 N NaOH–MeOH) λ_{max} 253 and 270 (shoulder) nm; HR-ESIMS (negative) m/z 393.1912 $[M - H]^-$ (calcd for $C_{21}H_{29}O_7$, 393.1919); ^{1}H NMR (400 MHz, CD_3OD, COSY) and ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) data are given in Table 1.

High-Performance Liquid Chromatography (HPLC). A Shimadzu Prominence UFLC system, equipped with a LC-20AD pump, a SIL-20ACHT automated sample injector, a thermostatted column compartment CTO-20AC, and a SPD-M20A photodiode array detector, was used to analyze hop constituents. Data were processed with LCsolution software (Shimadzu, Japan). The used column was 150 × 2.1 mm i.d., 3 μm, Alltima C18 (systech, USA), maintained at 40 °C. The method utilized a gradient of water/phosphoric acid, 1000/0.2 (v/v), containing EDTA (0.02% w/v) (solvent A) and acetonitrile (solvent B). The elution conditions were 0.6 mL/min, a linear gradient from 10% to 52% B in 0 → 40 min, 52% B for 40 → 45 min, 52% to 75% B in 45 → 49 min, 75% to 85% B in 49 → 55 min, and 85% B for 55 → 56.5 min. The injection volume was 3.0 μL.

Qualitative and Quantitative Analysis of Hop Constituents During Hop Storage. Fresh hop pellets were ground to a powder

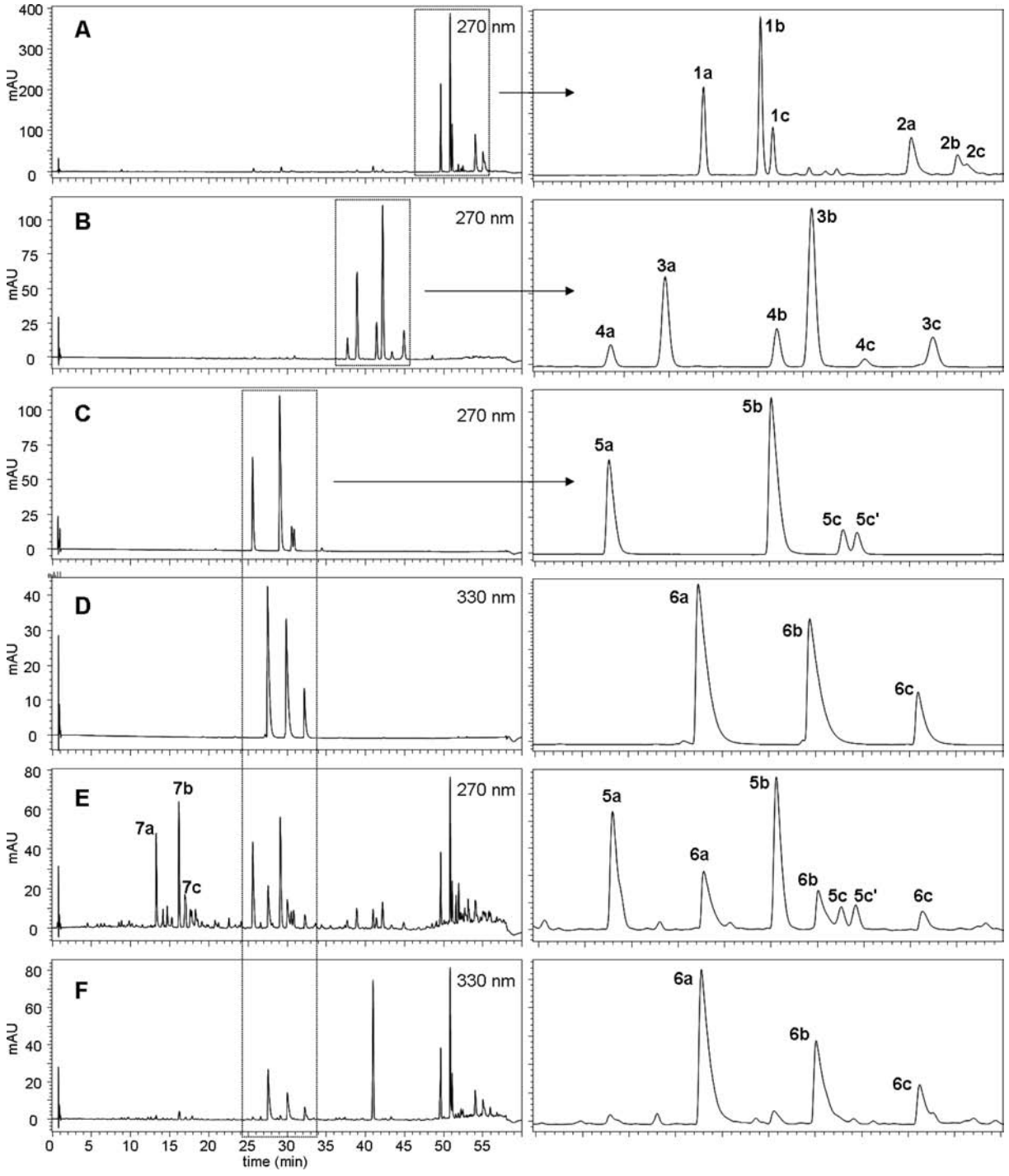

Figure 2. HPLC chromatograms of fresh hops at 270 nm (A), isomerized hop extract at 270 nm (B), reference standards of humulinones at 270 nm (C), reference standards of hulupones at 330 nm (D), and oxidized hops stored at 60 °C for 48 h at 270 nm (E) and 330 nm (F). The right side chromatograms correspond to the enlarged views of the enclosed regions. Structures of compounds are given in Figures 1, 3, and 4.

and divided into three groups. Each group (200 g) was stored at 20, 40, or 60 °C under dark conditions. The hop pellets (5.0 g) were sampled at 4, 8, and 24 h and then every other day for the first week, and then once a week for the next 7 weeks in each group. For the 20 and 40 °C groups, further sampling was undertaken continuously until 40 weeks, at once a week (7−12 weeks), once every two weeks (12−24 weeks), and once every four weeks (24−40 weeks). Each sampled hop pellet (1.0 g) was extracted with 10 mL of ethanol for 1 h at room temperature, with agitation, and then centrifuged at 600*g* for 5 min. The supernatant (100 μL) was diluted 10 times with ethanol, and the diluted solution (3 μL) was injected into the HPLC.

The International Calibration Extract ICE2 (49.39% (w/w) α-acids, 24.94% (w/w) β-acids; American Society of Brewing Chemists, USA) was used for the quantification of α-acids and β-acids. Humulinones, hulupones, and 4′-hydroxy-allohumulinones were quantified using synthesized or isolated compounds. Cohumulinone (**5a**) and adhumulinone (**5c**) were quantitated using the calibration curve of *n*-humulinone (**5b**), *n*-hulupone (**6b**) and adhulupone (**6c**) were quantitated using the calibration curve of cohulupone (**6a**), and 4′-hydroxy-alloadhumulinone (**7c**) was quantitated using the calibration curve of 4′-hydroxy-allo-*n*-humulinone (**7b**). The wavelengths for quantitative determination of HPLC analysis were 314 nm for α-acids and β-acids,

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330 nm for hulupones, and 270 nm for humulinones and 4′-hydroxy-allohumulinones.

High-Resolution Electrospray Ionization Mass Spectrometry (HR-ESIMS). HR-ESIMS of the purified compounds was measured using a Thermo Scientific LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Before the samples were measured, accurate mass calibration was performed using polytyrosine-1,3,6 calibrant (CS Bio Co., Menlo Park, CA).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, and 2D NMR spectra were measured with a Bruker AVANCE400 spectrometer (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in $CDCl_3$ or CD_3OD. Chemical shifts were referenced to tetramethylsilane. Data processing was performed using TopSpin-NMR software (version 3.0) (Bruker BioSpin, Rheinstetten, Germany).

RESULTS AND DISCUSSION

Identification of Humulinones and Hulupones in Oxidized Hops. To identify compounds derived from the bitter acids in oxidized hops, we developed a HPLC method using the Prominence UFLC system (suitable for high pressure analyses) with a gradient program. This method was established to analyze the hydrophilic oxidized compounds, together with α-, β-, and iso-α-acids.

In this method, α-acids (**1a**−**c**) and β-acids (**2a**−**c**) in the fresh hops (Figure 2A), and iso-α-acids (**3a**−**c**, **4a**−**c**) in the isomerized hop extract (Figure 2B), were eluted separately. In the fresh hops and isomerized hop extract, very few oxidized derivatives derived from α- or β-acids were detected (Figure 2A and B).

To check the separation of the oxidized compounds derived from α- or β-acids, reference standards of humulinones and hulupones prepared from α- and β-acids, respectively, were analyzed using this method. In the chromatography, humulinones (cohumulinone (**5a**), *n*-humulinone (**5b**), and adhumulinone (**5c**)) were eluted earlier than iso-α-acids (Figure 2C). In detail, **5a** and **5b**, which existed as racemic mixtures (4*S*5*R* and 4*R*5*S*, Figure 3),[15,32,33] were detected as single peaks, and **5c**, which

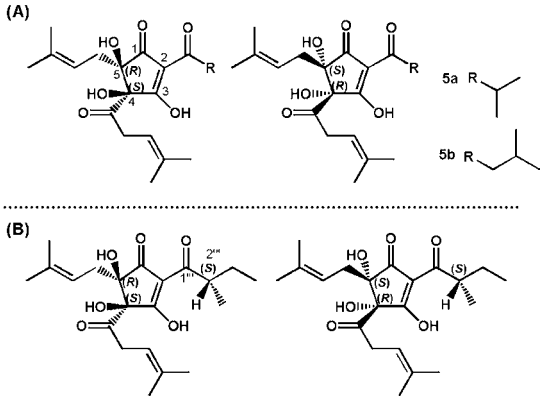

Figure 3. (A) Chemical structures of racemic cohumulinone (**5a**) and racemic *n*-humulinone (**5b**). (B) Chemical structures of two diastereomers of adhumulinone, **5c** or **5c′**.

existed as two diastereomers due to the additional fixed chiral carbon (2‴*S*) in the acyl side chain (**5c**, **5c′**, Figure 3),[25,33] was detected as two equal intensity peaks (**5c**, **5c′**) (Figure 2C). Thus, it was confirmed that this analytical method could separate all of the congeners belonging to humulinones, including the diastereomers of adhumulinone.

The compounds belonging to hulupones (cohulupone (**6a**), *n*-hulupone (**6b**), and adhulupone (**6c**)) were eluted at almost the same R_t as the corresponding congeners of humulinones (**5a**, **5b**, **5c**) (Figure 2D). Becauase hulupones show maximum UV absorption around 330 nm in the solvent used and humulinones do not show significant UV absorption at 330 nm, this wavelength is found to be suitable for the specific detection and quantification of hulupones.

Analysis of the hops stored at 60 °C for 48 h, to promote the oxidation of α-acids and β-acids, clearly revealed a decrease in α-acids and β-acids, and the production of many oxidized compounds (Figure 2E and F). Among these, humulinones and hulupones were clearly identified as major oxidized products by their retention time, UV spectra, and molecular formulas determined by the HR-ESIMS analysis. Although the presence of hulupones in oxidized hops has been previously reported,[22] the presence of humulinones remained controversial.[8,16−18] We proved for the first time that humulinones exist as one of the main oxidation products in oxidized hops. In this analysis, adhumulinone was eluted as two equal intensity peaks (**5c**, **5c′**) (Figure 2E). This pattern is the same as the reference standards (Figure 2C). Therefore, cohumulinone (**5a**) and *n*-humulinone (**5b**) in oxidized hops are suggested to exist as racemic mixtures, and the oxidation mechanism from α-acids to humulinones in hops might resemble the reaction of isolated α-acids with peroxide reagent.[13,15]

Isolation and Structural Elucidation of 4′-Hydroxy-allohumulinones. In addition to humulinones and hulupones, some unidentified hydrophilic compounds were detected in the oxidized hops. Among these compounds, two major products (**7a** and **7b**) were isolated using Diaion HP-20 column chromatography, followed by preparative ODS HPLC.

Compound **7b** was isolated as pale yellow oil. The UV spectrum of **7b** suggested that this compound possessed the same five-membered ring structure as iso-α-acid derivatives.[34] The HR-ESIMS of **7b** showed a $[M - H]^-$ peak at m/z 393.1912, indicating the molecular formula $C_{21}H_{30}O_7$ (*n*-humulinone (**5b**) + O). The ^{1}H and ^{13}C NMR spectra of **7b** were closely related to **5b**, and the ^{1}H and ^{13}C signals due to the five-membered rings (C-1−C-5) in **5b** were completely preserved in **7b**. In addition, the presence of an isovaleryl side chain at C-2 and prenyl side chain at C-5 in **7b** was confirmed by comparison with the chemical shifts in **5b** (Table 1 and Figure 4). The HMBC experiment on **7b** proved the long-range couplings from H-5′ (δ 1.30, singlet methyl) and H-6′ (δ 1.30, singlet methyl) to C-4′ (δ 71.4) and C-3′ (δ 155.5), and from H-3′ (δ 6.86, J = 15.6 Hz) and H-2′ (δ 6.98, J = 15.6 Hz) to C-1′ (δ 200.2), indicating **7b** possesses a *trans*-4-hydroxy-4-methyl-2-pentenosyl structure at C-4. This oxidative side chain is also present in hydroxy-alloisohumulones, which were recently reported to be oxidation products of iso-α-acids (Figure 5),[27] and their reported ^{1}H and ^{13}C NMR data showed good agreement with those of **7b**, except at position C-5 in the five-membered ring.

The ^{1}H chemical shift difference between H-4″ and H-5″ was Δ0.2 ppm in **7b**, indicating that the relative stereochemistry of the side chains at C-4 and C-5 in **7b** was the same as *trans*-iso-*n*-humulone (**4b**) and *n*-humulinone (**5b**), because the chemical shift difference between H-4″ and H-5″ was greater when the two large side chains at C-4 and C-5 are in the *cis*-position (>Δ0.16 ppm) as compared to the *trans*-position (<Δ0.04 ppm) in iso-α-acids and their derivatives.[27,29,35] The NOESY experiment also supported this relative configuration because the NOE was detected between H-2′ and H-1″ in **7b**, similar to *trans*-iso-*n*-humulone (**4b**) and *n*-humulinone (**5b**) (the NOE between H-2′ and H-1″ was not observed in *cis*-iso-*n*-humulone

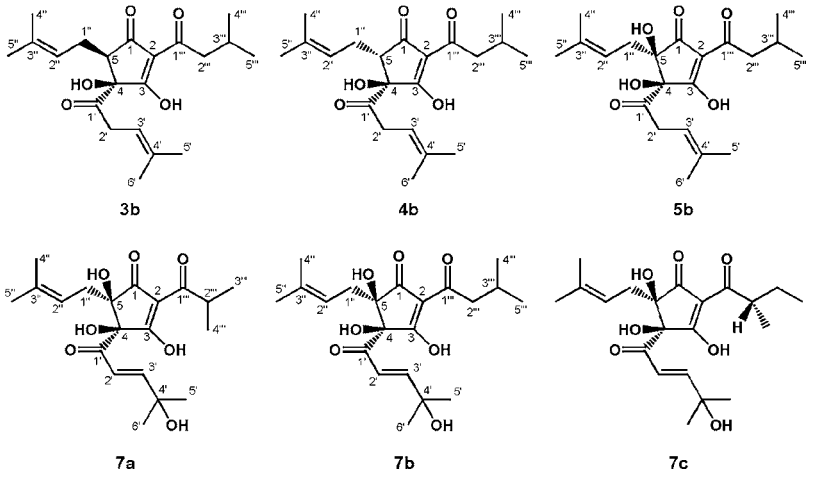

Figure 4. Chemical structures of *cis*-iso-*n*-humulone (**3b**), *trans*-iso-*n*-humulone (**4b**), *n*-humulinone (**5b**), 4′-hydroxy-allocohumulinone (**7a**), 4′-hydroxy-allo-*n*-humulinone (**7b**), and 4′-hydroxy-alloadhumulinone (**7c**).

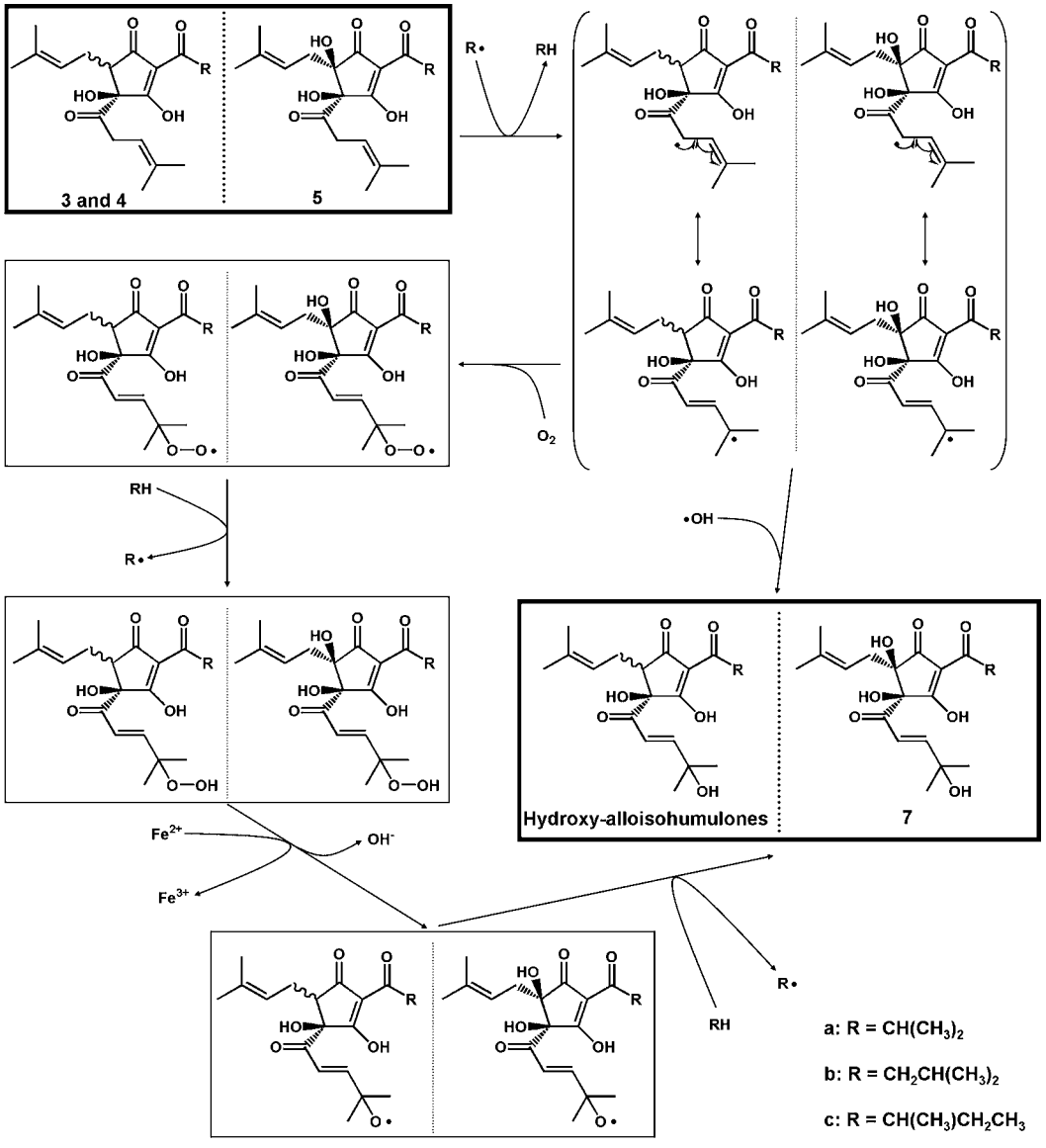

Figure 5. Proposed reaction pathway for the formation of 4′-hydroxy-allohumulinones (**7a**–**c**) from humulinones (**5a**–**c**). This pathway is the same as the reaction pathway for the formation of hydroxy-alloisohumulones from iso-α-acids (**3a**–**c** and **4a**–**c**) proposed by Intelmann et al.[27] and Almedia et al.[36]

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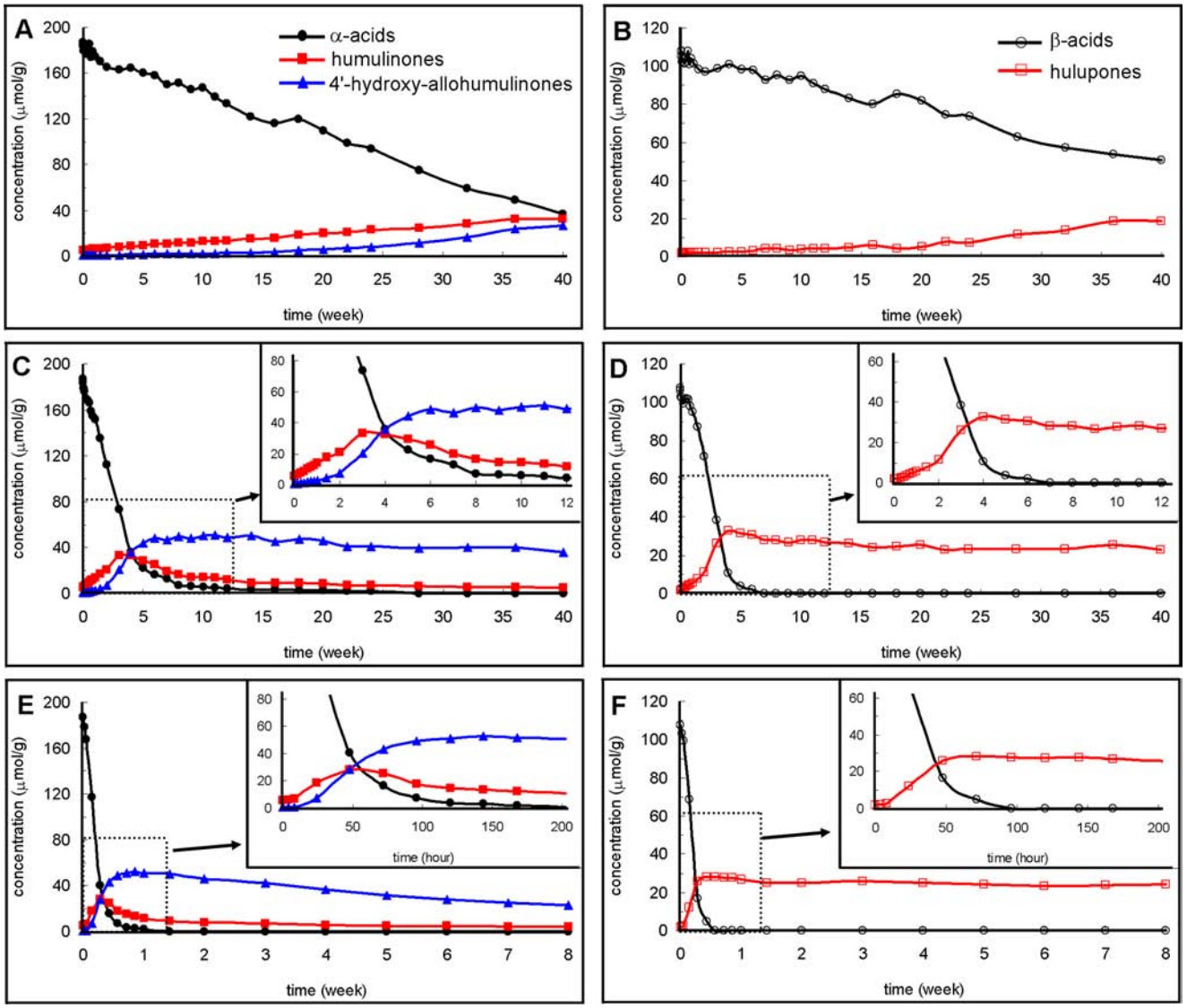

Figure 6. Concentration changes in α-acids, humulinones, 4′-hydroxy-allohumulinones, β-acids, and hulupones in hops during storage at 20 °C (A and B), 40 °C (C and D), and 60 °C (E and F).

(**3b**)). From the findings described above, compound **7b** was identified as 4′-hydroxy-allo-*n*-humulinone, which has never been reported (Figure 4).

Compound **7a** was isolated as pale yellow oil. The HR-ESIMS showed a $[M - H]^-$ peak at m/z 379.1754, indicating the molecular formula $C_{20}H_{28}O_7$ (**7b** − CH_2). The ^1H and ^{13}C NMR spectra of **7a** were almost identical to those of **7b**, except the side chain structure at C-2. The presence of isobutyryl function at C-2 in **7a** was confirmed by the long-range couplings from H-3‴ (δ 1.03) and H-4‴ (δ 1.06) to C-2‴ (δ 37.3) and C-1‴ (δ 204.3) in the HMBC experiment. Thus, compound **7a** was identified as 4′-hydroxy-allocohumulinone, which has never been reported (Figure 4).

In addition, we tentatively identified compound **7c** as 4′-hydroxy-alloadhumulinone, on the basis of HR-ESIMS analysis ($[M - H]^-$ 393.1912, indicating the molecular formula $C_{21}H_{30}O_7$) together with the typical HPLC elution patterns of the congeners of hop bitter acids.

The structures of 4′-hydroxy-allohumulinones (**7a**−**c**) suggested that they were the oxidation products from corresponding humulinones (**5a**−**c**). Recently, Intelmann et al. proposed an oxidation pathway from iso-α-acids (**3a**−**c** and **4a**−**c**) to hydroxy-alloisohumulones, which is similar to the lipid autoxidation of unsaturated fatty acids (Figure 5).[27] Furthermore, Almedia et al. proved that an oxidative reaction between iso-α-acids and 1-hydroxyethyl radicals occurs preferentially through hydrogen atom transfer at allylic positions in the side chain and not through electron transfer from the β-tricarbonyl chromophore. They also detected hydroxy-alloisohumulones as the reaction products.[36] On the basis of these reports, we speculate the reaction pathway from humulinones (**5a**−**c**) to 4′-hydroxy-allohumulinones (**7a**−**c**) is identical to that from iso-α-acids (**3a**−**c** and **4a**−**c**) to hydroxy-alloisohumulones (Figure 5).

Changes in Hop Bitter Acid Derivatives During Hop Oxidation Progression. To evaluate the oxidative changes of α-acids and β-acids during hop storage, hop pellets were stored at 20 or 40 °C for up to 40 weeks, or at 60 °C for up to 8 weeks. The stored hops were extracted with EtOH and analyzed by HPLC. Figure 6 shows the time-dependent decrease in α-acids and β-acids, and compositional changes in the oxidative α-acid derivatives (humulinones and 4′-hydroxy-allohumulinones) and oxidative β-acid derivatives (hulupones).

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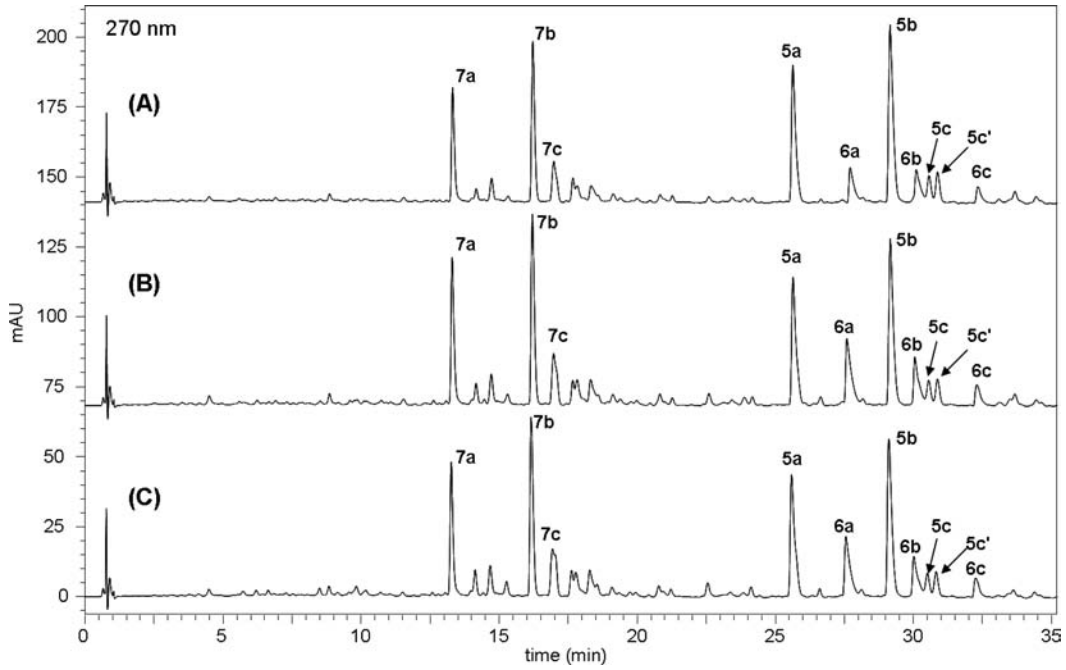

Figure 7. HPLC chromatograms of the hydrophilic oxidation products which were eluted earlier than iso-α-acids in oxidized hops. The hops were analyzed when α-acids decreased to 20% of the initial amounts due to storage at the following temperatures and periods: (A) 20 °C for 40 weeks, (B) 40 °C for 4 weeks, and (C) 60 °C for 48 h.

At 20 °C, the initial α-acids (186.9 μmol/g) decreased to 37.0 μmol/g after 40 weeks, while humulinones and 4′-hydroxy-allohumulinones reached 32.3 and 27.0 μmol/g (17% and 14% of the initial α-acids), respectively (Figure 6A). β-Acids were decreased from 107.7 to 50.9 μmol/g after 40 weeks, while hulupones increased to 18.6 μmol/g (17% of the initial β-acids) (Figure 6B).

When hops were stored at 40 °C, a rapid decrease in α-acids and β-acids was observed: their concentration decreased to less than 5% of the initial amounts after 8 or 5 weeks, respectively (Figure 6C and D). At this temperature, compositional changes in the oxidation products became clearer. Humulinones reached their maximum (33.2 μmol/g) after 3 weeks storage and then decreased rapidly (Figure 6C). In contrast, the amount of 4′-hydroxy-allohumulinones was increased rapidly for 2−5 weeks and slowly for 6−11 weeks, reaching their maximum (51.3 μmol/g, 27% of the initial α-acids) at 11 weeks, before being degraded very slowly (Figure 6C). From these observations, we confirmed 4′-hydroxy-allohumulinones to be produced by the oxidation of humulinones. Hulupones reached their maximum (32.6 μmol/g, 30% of the initial β-acids) after 4 weeks storage, and existed stably until the 40th week (Figure 6D).

At 60 °C, the observed compositional change curves resembled those at 40 °C, while the reaction speed was about 10 times faster than at 40 °C (Figure 6E and F). α-Acids and β-acids were decreased to less than 5% of the initial amounts after 96 or 72 h, respectively. Humulinones reached their maximum at 48 h before being degraded rapidly, while 4′-hydroxy-allohumulinones reached their maximum at 144 h before being degraded slowly. Hulupones reached their maximum at 72 h and were stable until the eighth week.

The results described in this section suggested that the stored temperature affected the oxidative reaction speed, but did not change the composition of generated hydrophilic oxidation products. To confirm this speculation, we analyzed the hop samples stored at 20, 40, and 60 °C, each of which contained almost the same amounts of α-acids (about 20% of the initial) (40th week for 20 °C, fourth week for 40 °C, and 48 h for 60 °C) by HPLC (Figure 7A−C). Our analyses showed that the generated hydrophilic oxidation products occurring in the stored hops at each temperature were almost identical, including minor peaks, confirming our speculation.

In conclusion, we reported suitable HPLC conditions to analyze α-acids, β-acids, and their oxidation products (humulinones, 4′-hydroxy-allohumulinones, and hulupones). Using these conditions, detailed changes in oxidation products during hop storage were clearly revealed for the first time. We hope our analytical methods enable precise prediction of the degree of hop oxidation.

Further analytical studies on the oxidation products derived from oxidized hops during the brewing process and in the final beer are in progress using this HPLC method. We expect the information will be helpful in more accurately understanding the influence of hop oxidation on beer quality.

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on March 7, 2013, with an error to Figure 5. The correct version was reposted on March 13, 2013.