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Fate of Bitter Compounds through Dry-Hopped Beer Aging. Why *cis*-Humulinones Should be as Feared as *trans*-Isohumulones?

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ABSTRACT

The evolution of isohumulones, main bitter compound of beers, through storage and its bitter impact has been studied for many years. With the uprising of highly hopped and, especially, dry-hopped beers during the last decade, other compounds such as humulones, *cis*-humulinones and hulupones have been shown to participate in both the sensory and measured bitterness of these beers. However, the fate of these compounds through aging is still unclear. The present work evaluated, by means of spectrophotometric and RP-HPLC-UV analyses, the evolution of bitterness units (BU), *cis*- and *trans*-isohumulones, humulones, *cis*-humulinones, hulupones, and tetrahydro-isohumulones in 21 Belgian dry-hopped beers after two years of storage. Besides the expected degradation of *trans*-isohumulones, it was observed an average decrease of 91% of humulones and 73% of *cis*-humulinones. Together, these results indicate a decrease of 18 – 43% of the calculated bitterness intensity of dry-hopped Belgian beers. The losses of *cis*-humulinones in dry-hopped beers bring seven new oxidation compounds, some of them very similar to those issued from *trans*-isohumulones. The bitterness impact of these compounds is yet to be determined.

Abbreviations used: DH: dry-hopped; RP-HPLC-UV: Reversed phase high pressure liquid chromatography with UV detector; RT: Retention time; HRMS: High Resolution Mass Spectrometer

KEYWORDS

Beer aging; bitterness; bottle refermentation; dry-hopped beer; humulinone

Introduction

Beer flavor is known to alter greatly during storage, depending on the beer style, packaging, and storage conditions. A challenge for brewers nowadays is to achieve stability of dry-hopped (DH) beers. This is especially true of Belgian beers, the market for which is largely export-oriented.

In addition to *trans*-2-nonenal (cardboard),[1,2] dimethyltrisulfide (onion soup),[3] methional (wort/stale),[4] β-damascenone (cooked apple),[5] furfuryl ether (solvent),[6] 4-vinylsyringol (tobacco),[7] guaiacol (burnt),[8] and sotolon (madeira),[9] some unpleasant polyfunctional thiols such as 3-methyl-2-buten-1-thiol (skunky)[10–12] and 3-sulfanyl-3-methylbutyl formate (ribes) can quickly appear through aging.[13] On the other hand, the pleasant 3-sulfanylhexanol (citrus-rhubarb) and 4-sulfanyl-4-methyl-2-pentanone (blackcurrant), often found in DH beers, are strongly degraded in the first year of storage.[14]

Beer bitterness is also impacted by aging. Isohumulones (co-, n- and ad- analogues of *cis* and *trans* isomers) undergo autoxidation during storage, resulting in a linear decrease of overall bitterness intensity.[13,14] While the *cis* isomers appear relatively stable, *trans*-isohumulones are mainly degraded by proton-catalyzed cyclization leading to formation of harsh and lingering bitter-tasting tricyclohumols, tricyclohumenes, isotricyclohumenes, tetracyclohumols, epitetracyclohumols, and scorpiohumols.[15–18] In addition, humulinic acids (*cis* and *trans*) can be produced from *cis*- and *trans*-isohumulones during beer storage.[17]

The spiking of hops during fermentation and/or lagering (dry hopping) can bring humulones (co-, n- and ad- analogues) and *cis*-humulinones (co-, n- and ad- analogues) to such beers. Although levels of humulones are low (up to 7 mg/L in Belgian DH beers[19]) because of their low solubility (14 mg/L[20]), *cis*-humulinones are more soluble and have been observed at 1 to 13 mg/L in Belgian DH beers[19] and at 3 to 24 mg/L in American DH IPAs.[21] In non-dry-hopped beers, on the other hand, *cis*-humulinones are rarely found, being degraded through wort boiling.[19] Yet, the use of aged hops[22] (lower humulones levels but higher *cis*-humulinones content) can occasionally lead to residual *cis*-humulinones in them. The *cis*- humulinones interfere with the spectrophotometric measurement of bitterness units (BU) at 275 nm[19,23] and can significantly increase sensory bitterness,[21,24] being 66% as bitter as isohumulones.[25]

During storage in a pilsner beer (where they were present at concentrations below 1 mg/L in the fresh sample), *cis*-humulinones have been shown to be degraded to an extent similar to *trans*-isohumulones.[17] In addition, a recent report describes the oxidative pathway of *cis*-humulinones (in model medium stored at 40 °C) to resemble that of *trans*-isohumulones, producing first 4′-hydroxyallohumulinones and then scorpiohumulinols A and B and dicyclohumulinols A and B.[26]

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Humulone degradation has been studied only in hops. Humulones are oxidized mainly to *cis*-humulinones[21,27] and these can be further degraded to 4′-hydroxyallohumulinones and, to a minor extent, tricyclooxyisohumulones A and B.[28] A similar degradation pathway might be expected in beer.

The aim of the present work was to investigate the bitter compounds of 21 Belgian DH beers after two years of storage at 20 °C in the dark. As previously described for the corresponding fresh beers,[19] the bitterness units and isohumulone levels obtained by spectrophotometric methods were first compared to the isohumulone, tetrahydro-isohumulone, humulone, *cis*-humulinone, and hulupone concentrations determined by Reverse Phase High Performance Liquid Chromatography with a UV detector (RP-HPLC-UV). The impact on the perceived bitterness (calculated according to two different published models[19,23]) has been also assessed. Finally, *cis*-humulinone degradation products were investigated in model media and aged beers by RP-HPLC-MS/MS and HRMS.

Experimental

Chemicals

Methanol, acetonitrile, HCl (37%), NaOH, and citric acid monohydrate were purchased from VWR International (Leuven, Belgium). Milli-Q water was used (Millipore, Bedford, U.S.A.). The humulone and lupulone mixture standard (ICE-3), isohumulone standard (ICS-I3), and tetrahydro-isohumulone standard (ICS-T2) were purchased from Labor Veritas Co. (Zürich, Switzerland). Humulinone standard (purity superior to 95%) was produced in our laboratory following the method proposed by Cook et al.[29] Hulupone standard (purity of 62.2%) was kindly provided by Hopsteiner (Mainburg, Germany).

Twenty-one Belgian DH beers were investigated: Orval (A), Bastogne Pale Ale (B), La Trouffette Blonde (C), Vedett Extra Ordinary IPA (D), IV Saison (E), V Cense (F), Leffe Royale Cascade IPA (G), Leffe Royale Mapuche (H), Leffe Royale Mount Hood (I), Houblon Chouffe (J), Taras Boulba (K), St. Feuillien Saison (L) St. Feuillien Grand Cru (M), Guldenberg (N), Houppe (O), Duvel Triple Hop 1 – Amarillo (P), Duvel Triple Hop 2 – Citra (Q), Duvel Triple Hop 3 – Sorachi Ace (R), Duvel Triple Hop 4 – Mosaic (S), Duvel Triple Hop 5 - Equinox (T), and Duvel Triple Hop 6 – HBC291 (U). All beers were stored for two years at 20 °C in the dark. For each beer sample, two bottles were analyzed.

Standard beer analyses

Prior to analysis, beers were filtered through paper filters (MN 614 ¼ Macherey-Nagel, Düren, Germany) and degassed by shaking. Alcohol content, pH, and color were analyzed by means of Analytica-EBC methods 9.2.6, 9.4, and 9.6.[30] Apparent and original extracts were determined with a density meter (DM4500, Anton Paar GmbH, Graz, Austria). First, bitterness was measured and total isohumulones determined with the international standard methods (Analytica EBC Method 9.8 and ASBC-BEER23.B):[30,31] 25 mL filtered beer was mixed with 2.5 mL of 6 N HCl and extracted with 50 mL isooctane by vigorous manual shaking for 1 min. The absorbance at 275 nm (A275) of the isooctane phase was measured exactly 5 min after the shaking period. The number of BU was determined as $A275 \times 50$. Subsequently, 20 mL isooctane extract was washed with 20 mL methanol:4 N HCl (68: 32) and 5 mL of the washed extract was diluted 5 times in methanol:1.5 N NaOH (99.8: 0.2). The absorbance at 255 nm (A255) of the diluted extract was measured and the total isohumulone content was determined as $A255 \times 192.3 + 0.8$.

Extraction of bitter compounds for RP-HPLC-UV

Beer samples devoid of yeast were degassed by shaking and diluted twice in methanol. After 15 min, the mixture was filtered through a Chromafil polyester filter (0.45 µm, Macherey-Nagel, Düren, Germany).

RP-HPLC-UV

Separation was performed on two C8 columns in tandem: the Zorbax Eclipse XDB-C8 150×4.6 mm, 5 μm and the Zorbax Eclipse XDB-C8 150×4.6 mm, 3.6 μm (Agilent Technologies, Santa Clara, U.S.A.), using the binary solvent system of Analytica-EBC method 9.47[32] with A: methanol; B: 1% aqueous citric acid solution (pH 7.0):acetonitrile (70: 30). Compared to EBC method 9.47, the second Zorbax Eclipse XDB-C8 column allowed slightly improved peak resolution. Gradient elution was as follows: 15% A for 5 min, increasing A to 80% over 25 min, and 80% A for 3 min. The column temperature was kept at 35 °C, the flow rate at 1.0 mL/min, and the injection volume was 50 µL. Chromatograms were recorded throughout elution with the Empower software (Build 1154, Waters Corporation). The retention time and absorption spectrum of each compound were obtained by injection of standards. An absorbance wavelength of 270 nm was chosen for isohumulone, tetrahydro-isohumulone, and *cis*-humulinone quantitation, while 325 nm was preferred for humulones and hulupones.[19] Quantitation was performed using a single point calibration, as suggested by EBC method 9.47.[32] Calibration solutions were freshly prepared from standards before each series of analyses and stored at −20 °C in the dark. They contained 30 mg/L of isohumulones and 10 mg/L of tetrahydro-isohumulones, humulones, *cis*-humulinones and hulupones. The molar attenuation coefficient of each compound was periodically controlled in order to verify the standard stability.

Bitterness intensity models applied to dry-hopped beers

On the basis of the work of Algazzali and Shellhammer[25] on humulinone bitterness, Maye and Smith[21] proposed the following model to calculate the bitterness of DH beers: Calculated Bitterness Intensity (CBI) = mg/L isohumulones + $0.66 \times$ mg/L humulinones. We recently added to this equation the contribution of tetrahydro-isohumulones

(1.4 × mg/L).[19] Hahn et al.[23] further proposed an alternative sensorial model for bitterness intensity without the 0.66 correction factor, but taking into account the %ABV of beer: Hahn's bitterness intensity (Hahn's BI) = − 0.408 + 0.279 × (mg/L isohumulones + mg/L humulinones) − 0.001 × (mg/L isohumulones + mg/L humulinones)^2 + 0.327 × (%ABV).

Selective analysis of aging compounds by RP-HPLC − ESI(-)− MS/MS and HRMS

The *cis*-humulinone media and beer D analyses were performed on a Kinetex 150 × 2.1 mm, 3 μm, (Phenomenex, Torrance, U.S.A.). Water with 1% of acetonitrile and 0.1% formic acid was used as solvent A, and acetonitrile with 0.1% formic acid was used as solvent B. The elution gradient was linear from 97% A to 97% B in 30 min. The flow rate was set at 0.2 mL/min. A total of 10 μL sample was injected into the column at 30 °C. The HPLC system was composed of an Accela 1250 quaternary pump and an Accela autosampler 3.0 (Thermo Fischer Scientific, Eindhoven, Netherlands). Mass spectrum determinations were performed with a Q-Exactive Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Eindhoven, Netherlands) equipped with an electrospray ion source operating in negative mode (ESI-). The ESI inlet conditions were as follows: spray voltage, 5.5 kV; capillary temperature, 320 °C; sheath gas (nitrogen), 45 psi; and auxiliary gas (nitrogen) flow rate, 15 mL min^{-1}. Identification was performed by using tandem mass spectroscopy (MS/MS), collision induced dissociation spectra at a relative collision energy of 22 (arbitrary units). To confirm the molecular formula of the degradation compounds, *cis*-humulinone model media were analyzed by the same equipment in HRMS mode. The system was controlled with Xcalibur software version 4.0 (Thermo Fisher Scientific, Eindhoven, Netherlands).

Statistical analysis

Beer characteristics before and after aging were compared using the Student's t-test for average comparison in the Microsoft Excel 365, with a significance level (α) of 0.05. The repeatability error was used for spectrophotometric assays while experimental standard deviation was used for HPLC data.

Results and discussion

Humulones, isohumulones, *cis*-humulinones, hulupones, and tetrahydro-isohumulones have recently been quantitated and compared in DH and non-dry-hopped samples.[19,21,23] Because of their instability through boiling, *cis*-humulinones were pointed out as key bitter compounds specific to DH beers.[19] It was therefore of prime interest to assess to what extent they persist through natural aging and how other bitter compounds evolve in such beers.

Standard beer analyses of aged samples

After two years of aging, the 21 Belgian DH beers were first subjected to standard analyses. Alcohol content, original extract, and apparent attenuation remained constant for most beers (Table 1). Beers A, B, C, K and O displayed subtle but significant changes (t-test, α = 0.05): alcohol production and extract loss. As these beers are dry-hopped and refermented in the bottle, the residual yeast activity was probably a result of the presence of hop dextrin hydrolases.[33] The pH decreases slightly in most samples, by 0.15 on average. Beer A, which contains *Brettanomyces* yeasts, logically showed the greatest pH decrease (−0.48). These yeasts are known to metabolize dextrins slowly, producing organic acids, which reduce the beer pH. Color increased in most beers, by 3°EBC on average. This was

Table 1. Standard analyses of the 21 two-year-aged dry-hopped beers. Values in parentheses indicate differences observed with respect to the values in fresh beers in ref. [19].

Beer	Alcohol content (% ABV)	Original extract (°P)	Apparent extract (°P)	pH	Bitterness units (BU)	Total isohumulones by spectrophotometry (mg/L)	Color (°EBC)
A*	7.0† (+0.4)	13.8† (−2.7)	−0.18† (−0.56)	3.8† (−0.5)	24.6 (−0.5)	16.1 (−4.5)	33.0† (+5.5)
B*	5.6† (+0.5)	13.5† (+0.3)	2.25† (−0.72)	4.3 (+0.0)	32.3† (−8.1)	25.8† (−11.9)	12.5 (−1.0)
C*	6.6† (+0.5)	15.4† (+0.3)	2.22† (−0.61)	4.4 (+0.0)	23.2† (−7.2)	14.8† (−10.7)	13.5 (+2.0)
D*	5.5 (+0.1)	13.5 (+0.0)	2.48 (−0.02)	4.1† (−0.3)	30.7 (−5.3)	22.1 (−3.1)	25.0† (+10.5)
E*	5.8† (+0.4)	12.6 (+0.2)	0.91† (−0.33)	4.5 (+0.1)	27.8† (−12.5)	19.2† (−13.3)	10.5 (−2.0)
F*	6.5† (+0.5)	13.8† (+0.4)	0.75† (−0.62)	4.5 (+0.0)	18.6† (−11.8)	10.7† (−13.7)	31.0 (−0.5)
G	7.3 (+0.1)	15.4 (−0.1)	1.12 (+0.14)	4.2† (−0.2)	26.2 (5.5)	16.6† (−15.8)	16.0† (+6.0)
H	7.0† (−0.2)	15.3† (−0.3)	1.27† (+0.18)	4.3 (−0.1)	27.8 (0.9)	19.8 (−2.0)	20.0† (+3.5)
I	6.8 (+0.0)	17.7 (+0.1)	3.86 (+0.03)	4.3 (−0.1)	21.2† (−7.3)	13.2† (−9.4)	48.0† (+4.5)
J*	8.4 (+0.1)	18.6 (+0.2)	1.85 (+0.03)	4.2 (−0.1)	40.6† (−7.1)	31.7 (+0.8)	16.0† (+5.0)
K*	4.9† (+0.5)	10.7 (+0.2)	0.93† (−0.61)	4.3 (−0.1)	41.6† (−16.4)	35.8† (−18.7)	4.5 (−0.5)
L*	6.7 (+0.1)	15.7 (+0.1)	2.26 (−0.05)	4.4 (−0.1)	21.1 (−4.5)	19.3 (−3.0)	19.5† (+5.0)
M*	9.1 (+0.0)	20.3 (−0.3)	2.17 (−0.15)	4.2† (−0.3)	26.0 (−4.75)	16.0 (−6.2)	10.0 (+1.7)
N*	7.3 (−0.1)	18.7 (−0.1)	3.94 (+0.13)	4.3 (−0.1)	32.5 (−5.6)	25.1 (−1.7)	20.5† (−8.5)
O*	7.6† (+0.2)	18.1† (+0.3)	2.78 (−0.05)	4.3† (+0.3)	32.7† (−9.6)	23.3† (−10.5)	14.5 (+0.0)
P*	9.3† (+0.2)	20.0† (+0.4)	1.30 (+0.00)	4.0 (+0.0)	29.7 (−4.8)	15.6† (−16.2)	10.5† (+5.0)
Q*	9.3 (+0.0)	19.8 (+0.1)	1.23 (+0.08)	3.9 (−0.1)	31.6 (−3.6)	21.0† (−8.4)	6.0 (+0.0)
R*	9.2 (−0.1)	19.6 (+0.0)	1.20 (+0.06)	4.0 (+0.0)	29.3† (−7.2)	22.5 (−6.1)	10.0† (+4.0)
S*	9.3 (+0.2)	19.6† (+0.4)	1.05 (+0.04)	3.9† (−0.1)	29.9 (−4.6)	10.0† (−18.2)	10.5† (+4.3)
T*	9.2 (−0.1)	19.8 (+0.0)	1.34 (+0.08)	4.0 (+0.0)	33.9 (−2.1)	22.1 (−4.2)	12.0† (+5.8)
U*	9.6 (+0.2)	20.1† (+0.4)	0.96 (+0.01)	3.9† (−0.2)	26.8 (−1.7)	18.3 (−4.0)	7.2 (+1.5)

*: with bottle refermentation

†: significant difference between fresh and aged beer concentrations (t-test, α = 0.05)

Table 2a. Concentrations (mg/L) of isohumulones and tetrahydro-isohumulones of the 21 two-year-aged dry-hopped beers. Values in parentheses indicate percentages of loss, by comparison with values given for fresh beers in ref. [19].

Compound	A*	B*	C*	D*	E*	F*	G	H	I	J*	K*	L*	M*	N*	O*	P*	Q*	R*	S*	T*	U*
cis-iso-co-Humulone	7.5†	8.1†	6.4†	5.9†	6.3	4.5	3.7†	4.2	3.7	14.0†	11.9†	5.4	5.7†	10.8†	9.4†	9.0†	8.6†	9.9†	9.3†	8.9†	7.1†
	(12)	(10)	(12)	(16)	(0)	(0)	(15)	(2)	(0)	(7)	(11)	(3)	(12)	(5)	(9)	(24)	(26)	(13)	(13)	(9)	(15)
trans-iso-co-Humulone	nd†	0.7†	0.3†	0.6†	0.5†	0.3†	0.1†	0.3†	0.2†	2.7†	0.9†	0.4†	1.1†	1.2†	0.9†	0.9†	0.8†	0.5†	0.8†	0.9†	0.9†
	(100)	(80)	(89)	(70)	(80)	(86)	(95)	(57)	(71)	(41)	(78)	(77)	(43)	(70)	(74)	(57)	(59)	(73)	(53)	(59)	(38)
cis-iso-n-Humulone	9.8†	11.0†	7.8†	7.1†	7.9†	6.1†	4.8	4.4†	4.1†	14.3†	13.4†	9.6†	9.7	10.0†	9.9†	8.7†	9.9†	9.8	9.1†	9.0†	6.8†
	(9)	(9)	(12)	(5)	(7)	(15)	(0)	(10)	(12)	(12)	(19)	(6)	(2)	(13)	(5)	(18)	(5)	(−1)	(9)	(−12)	(15)
trans-iso-n-Humulone	0.4†	1.2†	1.4†	0.8†	1.3†	0.7†	0.1†	0.2†	0.2†	2.8†	0.9†	0.9†	1.4†	1.3†	1.3†	0.8†	1.2†	0.6†	0.9†	1.0†	1.0†
	(82)	(75)	(61)	(54)	(65)	(75)	(91)	(60)	(73)	(42)	(88)	(68)	(52)	(79)	(59)	(78)	(68)	(80)	(73)	(70)	(20)
cis-iso-ad-Humulone	2.1	2.5	0.5†	1.0†	1.0†	1.2†	0.9	0.9	0.8	2.5†	3.1†	2.3	1.3†	1.5†	1.3†	2.1†	2.4†	1.6†	2.0†	1.6†	1.7
	(17)	(9)	(79)	(34)	(44)	(29)	(11)	(0)	(0)	(16)	(26)	(6)	(37)	(55)	(43)	(25)	(17)	(37)	(18)	(27)	(0)
trans-iso-ad-Humulone	nd†	nd†	nd†	nd†	nd†	nd†	nd	nd	nd	nd	nd†	nd†	nd†	nd†	nd†	nd	nd	nd	nd	nd	nd
	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Total *cis*-isohumulones	19.3	21.6	14.7	14.0	15.2	11.8	9.4	9.5	8.6	30.9	28.4	17.3	16.7	22.3	20.6	19.8	20.9	21.3	20.3	19.5	15.5
	(11)	(9)	(21)	(12)	(8)	(12)	(7)	(6)	(6)	(11)	(17)	(5)	(9)	(15)	(10)	(21)	(16)	(10)	(12)	(2)	(4)
Total-*trans*-isohumulones	0.4	1.9	1.7	1.4	1.8	1.0	0.2	0.5	0.4	6.1	1.8	1.3	2.5	2.5	2.2	1.7	2.0	1.2	1.7	1.9	1.9
	(92)	(79)	(76)	(67)	(74)	(82)	(94)	(62)	(75)	(41)	(86)	(76)	(55)	(78)	(71)	(72)	(67)	(79)	(68)	(68)	(39)
Total isohumulones	19.7†	23.6	16.4†	15.4†	17.0†	12.8†	9.6†	10.0†	8.9†	37.0†	30.2†	18.5†	19.2†	24.8†	22.8†	21.5†	22.9†	22.4†	22.0†	21.4†	17.4†
	(24)	(29)	(36)	(24)	(28)	(33)	(28)	(13)	(15)	(18)	(35)	(21)	(20)	(34)	(25)	(32)	(26)	(23)	(22)	(17)	(18)
Total Tetrahydro-isohumulones	nd	nd	nd	1.5	nd	nd	4.0†	2.2†	2.5	2.4	nd	nd	nd	nd	1.4†	1.5†	1.6†	2.1	1.6†	1.9	2.0†
	(-)	(-)	(-)	(0)	(-)	(-)	(9)	(29)	(4)	(4)	(-)	(-)	(-)	(-)	(22)	(31)	(30)	(4)	(36)	(0)	(20)

*: with bottle refermentation

†: significant difference between fresh and aged beer concentrations (t-test, $\alpha = 0.05$)

LOQ inferior to 0.1 mg/L for all compounds

nd: not detected (inferior to LOD, of 0.03 mg/L)

Table 2b. Concentrations (mg/L) of humulones, *cis*-humulinones and hulupones in the 21 two-year-aged dry-hopped beers. Values in parentheses indicate percentages of loss, by comparison with values given for fresh beers in ref. [19].

Compound	A*	B*	C*	D*	E*	F*	G	H	I	J*	K*	L*	M*	N*	O*	P*	Q*	R*	S*	T*	U*
co-Humulone	0.0†	0.1†	0.1†	0.2†	0.5†	nd†	nd†	nd†	nd†	0.2†	0.1†	0.1†	0.1†	0.1†	0.3†	0.1†	0.1†	0.1†	0.1†	0.3†	0.1†
	(100)	(94)	(96)	(93)	(83)	(100)	(100)	(100)	(100)	(84)	(98)	(93)	(82)	(86)	(86)	(93)	(89)	(93)	(94)	(85)	(92)
n-Humulone	nd†	0.2†	0.1†	0.6†	0.7†	0.1†	nd†	nd†	0.1†	0.4†	0.1†	0.1†	0.2†	0.1†	0.6†	0.2†	0.2†	0.1†	0.2†	0.1†	0.2†
	(100)	(88)	(96)	(72)	(80)	(96)	(100)	(100)	(95)	(75)	(96)	(87)	(71)	(86)	(72)	(88)	(81)	(93)	(87)	(84)	(85)
ad-Humulone	nd†	nd†	nd†	0.1†	0.1†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	0.1†	nd†	nd†	nd†	nd†	nd†	nd†
	(100)	(89)	(100)	(78)	(80)	(100)	(100)	(100)	(100)	(80)	(100)	(100)	(100)	(100)	(75)	(100)	(100)	(100)	(100)	(100)	(100)
Total humulones	nd†	0.3†	0.2†	0.9†	1.3†	0.1†	nd†	nd†	0.1†	0.6†	0.2†	0.2†	0.3†	0.2†	1.0†	0.3†	0.3†	0.2†	0.3†	0.4†	0.3†
	(100)	(91)	(96)	(83)	(81)	(98)	(100)	(100)	(97)	(79)	(97)	(89)	(78)	(87)	(84)	(90)	(90)	(94)	(90)	(85)	(89)
cis-co-Humulinone	0.4	0.4†	0.3†	1.0†	0.6†	0.1†	0.3†	0.6†	0.2†	0.6†	0.7†	0.1†	0.4	0.3	0.3†	0.3†	0.2†	0.6†	0.2†	1.3†	0.5†
	(−117)	(78)	(81)	(75)	(82)	(93)	(88)	(78)	(86)	(41)	(75)	(80)	(77)	(40)	(79)	(77)	(85)	(73)	(79)	(68)	(43)
cis-n-Humulinone	0.9†	0.9†	0.4†	2.8†	0.6†	0.2†	0.4†	0.1†	nd†	1.0†	1.3†	0.3†	0.7†	0.5†	0.5†	1.0†	0.7†	0.8†	0.7†	1.6†	0.6†
	(−52)	(81)	(89)	(64)	(90)	(94)	(89)	(88)	(100)	(49)	(81)	(79)	(51)	(62)	(84)	(76)	(73)	(85)	(63)	(69)	(64)
cis-ad-Humulinone	nd	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd†	nd	nd†	nd†	nd†	nd†	nd†	nd†	nd†
	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Total *cis*-humulinones	1.3	1.3†	0.7†	3.8†	1.2†	0.3†	0.7†	0.7†	0.2†	1.6†	2.0†	0.4†	1.1†	0.8†	0.8†	1.3†	0.9†	1.4†	0.9†	2.9†	1.0†
	(−35)	(82)	(88)	(72)	(88)	(94)	(90)	(90)	(96)	(54)	(82)	(82)	(51)	(60)	(85)	(79)	(81)	(84)	(74)	(73)	(63)
co-Hulupone	nd†	1.3	0.6	0.6	0.2	0.2	0.6	0.6	0.5	0.4†	1.2†	0.9	1.3	1.0	0.6	0.7†	0.5	0.5†	0.6	0.5†	0.3
	(100)	(19)	(0)	(33)	(71)	(67)	(25)	(33)	(17)	(56)	(29)	(25)	(−44)	(9)	(14)	(36)	(38)	(50)	(33)	(62)	(50)
n-Hulupone	nd	0.7†	0.3†	0.3†	0.2	0.2	0.2	0.2	0.3	0.4	0.6†	0.3	0.6	0.6	0.2†	0.5†	0.2†	0.3†	0.2†	0.3†	0.1†
	(0)	(36)	(50)	(57)	(67)	(50)	(50)	(33)	(0)	(20)	(57)	(40)	(−20)	(0)	(60)	(38)	(67)	(50)	(67)	(67)	(80)
Total hulupones	nd†	2.0†	0.9	0.9†	0.4	0.4	0.8	0.8	0.8	0.8†	1.8†	1.2†	1.9	1.6	0.8†	1.2†	0.7†	0.8†	0.8†	0.8†	0.4†
	(100)	(26)	(25)	(44)	(69)	(60)	(33)	(33)	(11)	(43)	(42)	(29)	(−36)	(6)	(33)	(37)	(50)	(50)	(47)	(64)	(64)

*: with bottle refermentation
†: significant difference between fresh and aged beer concentrations (t-test, $\alpha = 0.05$)
LOQ inferior to 0.1 mg/L for all compounds
nd: not detected (inferior to LOD, of 0.03 mg/L)

Table 2c. Calculated bitterness intensity (CBI) and Hahn's BI [23] of the 21 two-year-aged dry-hopped beers. Values in parentheses indicate percentages of loss, by comparison with values given for fresh beers in ref. [19].

	A*	B*	C*	D*	E*	F*	G	H	I	J*	K*	L*	M*	N*	O*	P*	Q*	R*	S*	T*	U*
CBI	20.6	24.5	16.9	20.0	17.8	13.0	15.7	13.6	12.5	41.4	31.5	18.8	19.9	25.3	25.3	24.5	25.7	26.3	24.8	26.0	20.9
	(23)	(37)	(43)	(36)	(41)	(42)	(35)	(34)	(26)	(18)	(41)	(24)	(22)	(35)	(31)	(37)	(31)	(31)	(27)	(27)	(21)
Hahn's BI	7.3	7.8	6.2	6.4	6.2	5.2	4.8	4.8	4.3	11.6	9.1	6.7	7.8	8.5	8.1	8.5	8.7	8.7	8.5	8.8	7.5
	(15)	(29)	(34)	(34)	(35)	(33)	(34)	(30)	(25)	(14)	(34)	(19)	(15)	(26)	(24)	(27)	(23)	(26)	(18)	(23)	(14)

*: with bottle refermentation

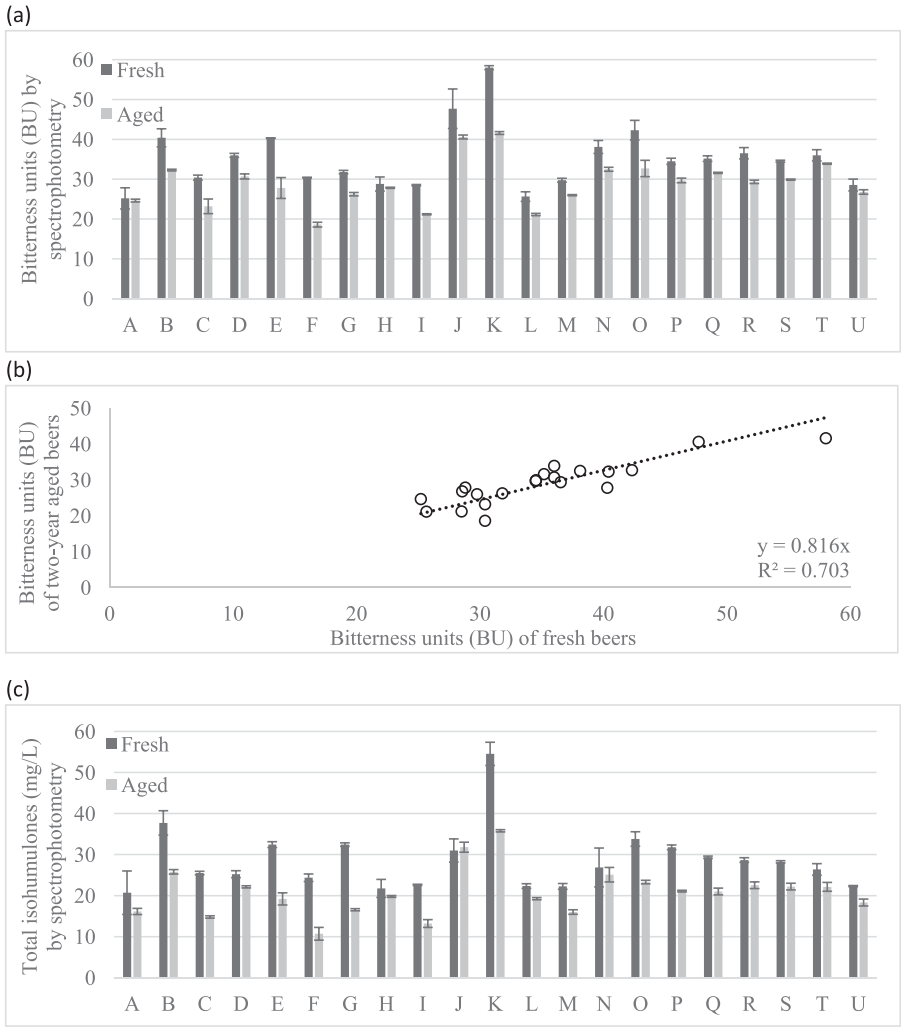


Figure 1. (a) Bitterness units (BU) in fresh (black) and two-year-aged (gray) dry-hopped beers. (b) Correlation between BU values determined before and after aging. (c) Total isohumulone concentrations (mg/L) in fresh (black) and two-year-aged (gray) dry-hopped beers. Values of fresh beers from ref.[19]

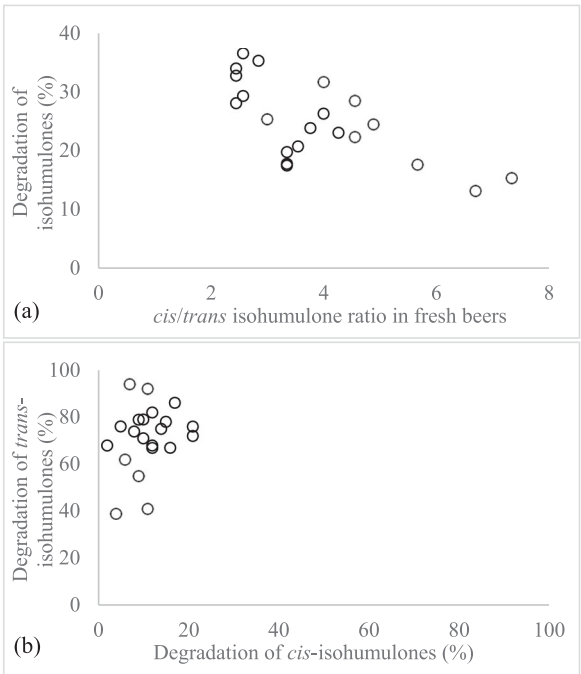


Figure 2. (a) Degradation of isohumulones observed after two years in relation to the *cis*/*trans* isohumulone ratio measured in fresh samples. (b) Comparison of *trans*- and *cis*-isohumulone degradation after two years (%).

likely due to flavanoid oxidation leading to formation of yellow dimers.[34]

Degradation of bitter compounds in Belgian beers through aging

The BU and bitter compounds of the 21 aged DH beers were determined by the global spectrophotometric method (Table 1) and an optimized RP-HPLC-UV method allowing individual quantitation of co-, n- and ad- analogs of humulones, *cis*- and *trans*-isohumulones, *cis*-humulinones, hulupones, and tetrahydro-isohumulones (Tables 2a and 2b).

After two years at 20 °C in the dark, the BU values of 9 out of 21 DH beers were significantly lower (Table 1 and Figure 1a), with up to 28% BU decrease in beer K. Interestingly, a good correlation was also found between BU values before and after aging (Figure 1b, $R^2 = 0.70$, slope = 0.8) The total isohumulone values (measured by spectrophotometry after washing the isooctane extract with acidified methanol) showed an even greater decrease (up to 49% in beer G) (Table 1 and Figure 1c), suggesting that some degradation hop compounds contributing to the absorbance at 275 nm had high polarity.

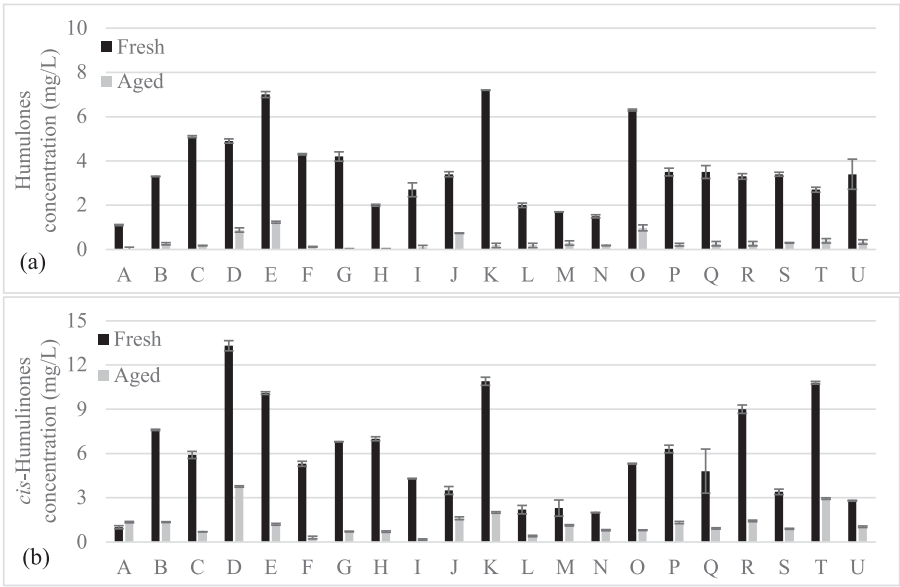


Figure 3. (a) Humulone and (b) *cis*-humulinone concentrations (mg/L) in fresh (black) and two-year-aged (gray) dry-hopped beers, measured by RP-HPLC-UV.

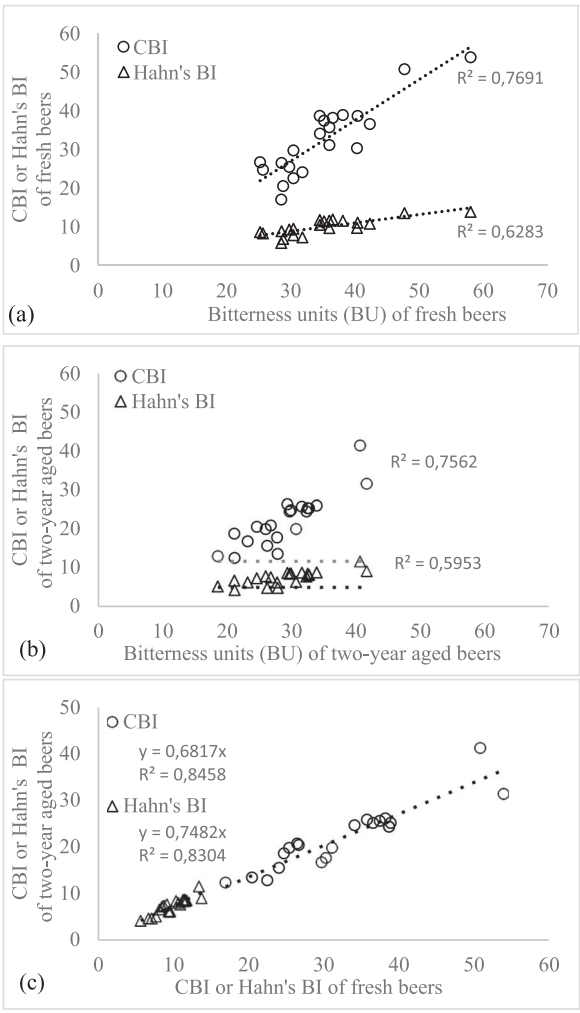


Figure 4. Correlation between calculated bitterness intensity (CBI) and bitterness units (BU) of (a) fresh DH beers (values from ref. [19]) and (b) beers aged for two years. (c) Correlation between CBI or Hahn's BI values determined before and after aging.

In all DH beers, isohumulone levels determined by HPLC were logically also lower after aging (Table 2a), by 13 (beer H) to 36% (beer C), with an average decrease of 25%. The greater stability of beer H may be partly explained by its higher *cis*/*trans* ratio[35] (possibly due to the use of pre-isomerized hops or isomerized hop extracts) (Figure 2a). As expected, *trans*-iso-humulones, known to yield tricyclo and tetracyclo derivatives, were affected much more strongly by aging than their *cis*-isomers (Figure 2b) (on the average, 71% degradation versus 11%, with a loss of up to 94% *trans*-isohumulones in beer G).

Hulupone levels, already low in all the tested fresh Belgian DH beers (up to 3.1 mg/L in beer K), showed a decrease upon aging (Table 2b). Since they are known in the literature to be stable compounds,[27] this decrease was probably caused by precipitation in the bottle during storage.

Despite the absence of double bonds in their lateral chains, tetrahydro-isohumulones also showed a decrease in half of the samples where they were present (beers H, O, P, Q, S and U), with a loss of up to 36% in beer S (Table 2a). These compounds are stable, as observed in beers D, G, I, J, T and R and mentioned by previous studies,[35,36] so degradation should not be the main cause of the observed decrease.

Humulone loss reached 91% on average (Figure 3a and Table 2b), resulting in concentrations below 1.3 mg/L in all beers. Besides degradation, this loss can also partially be caused by humulone adsorption to the bottle yeast.

The *cis*-humulinones, the oxidized derivatives of humulones, were also severely degraded (by 73% on the average, even more than *trans*-isohumulones) in all beers except beer A, which contains *Brettanomyces* yeast (Figure 3b and Table 2b). The highest concentration of *cis*-humulinones after aging was found in beer D (3.8 mg/L), which also contained the highest amount before aging (13.3 mg/L[19]). These results are in accordance with

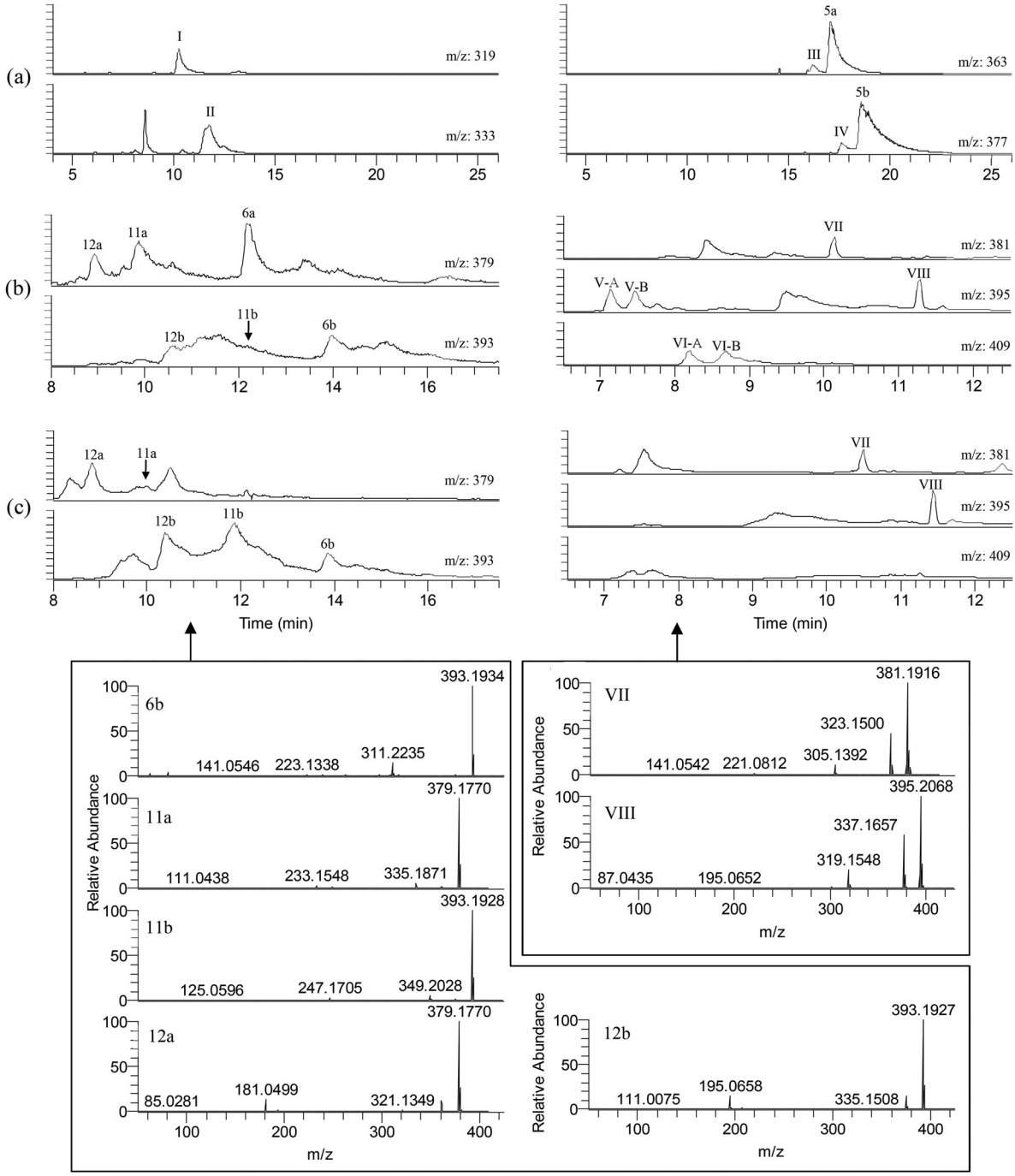


Figure 5. HPLC-ESI(-)-MS/MS chromatograms *cis*-humulinones degradation products produced in an aqueous model medium (initial concentration = 100 mg/L), subjected either to 90 min at 99 °C (a) or to 21 days at 40 °C (b), and evidence (MS/MS and HRMS) of compounds 6b, 11a- b, 12a-b, VII and VIII in beer D after two-years of storage (c).

those of Taniguchi et al. (for model medium) and Intelmann et al. (for pilsner beer), showing that *cis*-humulinones can be degraded during storage,[17] first to 4′-hydroxyallohumulinones and then to scorpiohumulinols and dicyclohumulinols.[26]

Fate through aging of the sensory bitterness models applied to Belgian dry-hopped beers

As depicted in Figures 4a and b and Table 2c, since the bitter compounds *cis*-humulinones and tetrahydro-isohumulones also absorb at 275 nm, the BU values showed a fair assessment to the calculated bitterness intensity (CBI[19] in Table 2c) both before (R^2 = 0.76) and after aging (R^2 = 0.75). These BU values revealed, to a lesser extent, also to be correlated to the Hahn's BI values, recently proposed as an alternative bitterness model for DH beers[23] (IIc, 212 R^2 = 0.62 and 0.59).

As suggested by the slope depicted in Figure 4c, the CBI and Hahn's BI of the two-year -aged beers showed a similar average decrease (31 and 26%, respectively), resulting from loss of isohumulones, *cis*-humulinones and tetrahydro-isohumulones (only for CBI). Yet, we suspect that neither

Table 3. Retention time, experimental accurate [M-H]- and four major MS/MS fragments of *cis*-humulinones and their degradation compounds in model media.

Compound	Retention time (min)	[M-H]- (HRMS)	Four major MS/MS ions m/z
5a	17.1	363.1819	363.18, 209.12, 249.11, 141.05
5b	18.6	377.1975	377.20, 223.13, 263.13, 141.05
6a	12.2	379.1770	379.18, 224.14, 264.13, 111.04
6b	13.9	393.1927	393.19, 223.13, 263.13, 125.06
11a	9.8	379.1768	379.18, 361.17, 223.15, 111.04
11b	12.1	393.1927	393.19, 375.18, 223.13, 125.06
12a	8.9	379.1768	379.18, 181.05, 361.17, 111.01
12b	10.6	393.1927	393.19, 195.07, 375.18, 125.06
I	10.2	319.1190	319.12, 163.08, 111.04, 275.13
II	11.7	333.1348	333.14, 163.08, 125.06, 289.15
III	16.1	363.1819	363.18, 249.11, 209.12, 267.12
IV	17.6	377.1971	377.20, 261.13, 223.13, 281.14
V-A	7.1	395.1718	395.17, 307.12, 283.12, 111.04
V-B	7.5	395.1718	395.17, 307.12, 283.12, 111.04
VI-A	8.2	409.1875	409.19, 321.13, 283.12, 125.06
VI-B	8.7	409.1875	409.19, 321.13, 283.12, 125.06
VII	10.1	381.1927	381.19, 363.18, 305.14, 181.09
VIII	11.3	395.2081	395.21, 377.20, 319.15, 195.07

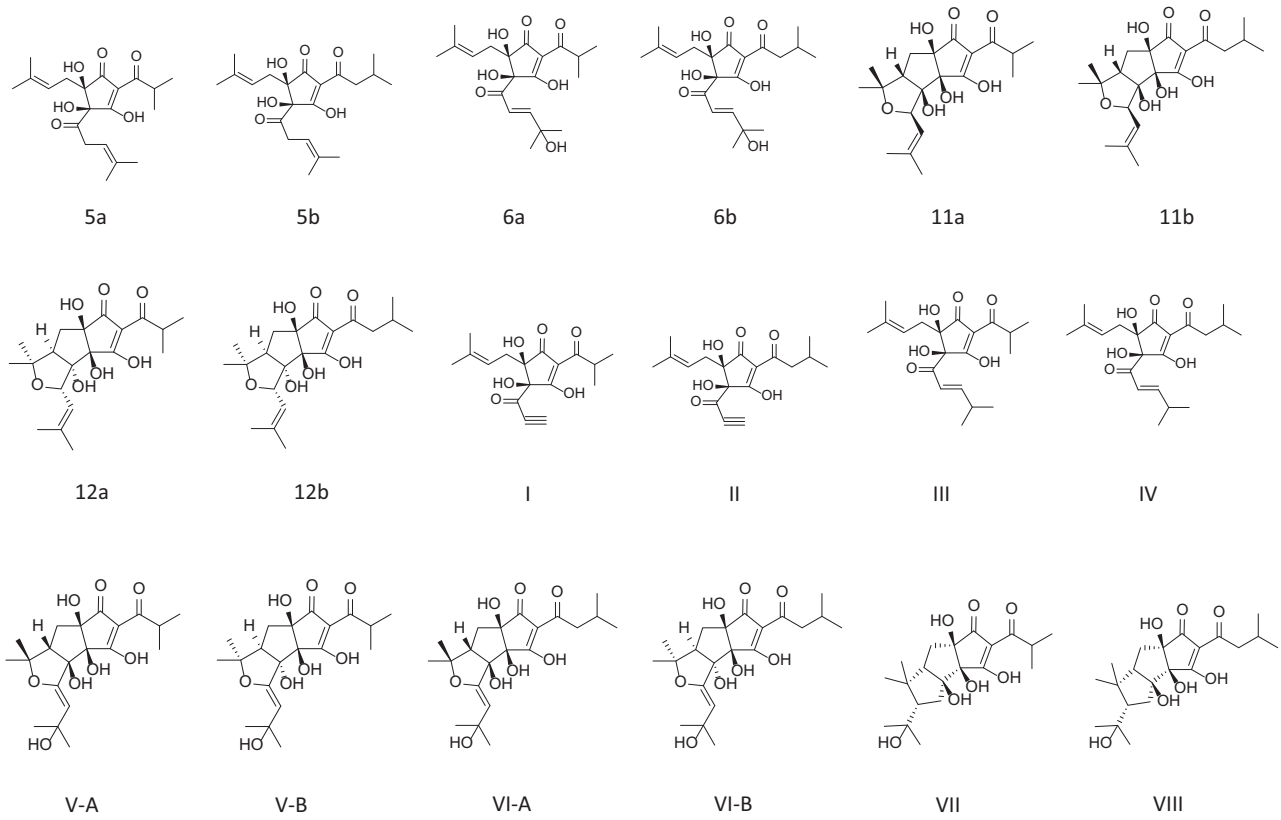


Figure 6. Structures of *cis*-cohumulinone (5a), *cis*-n-humulinone (5b), 4′- hydroxyallocohumulinone (6a), 4′-allohydroxyhumulinone (6b), scorpiocohumulinol A (11a), scorpiohumulinol A (11b), scorpiocohumulinol B (12a) and scorpiohumulinol B (12b), and proposed structures of compounds I, II, III and IV, V-A, V-B, VI-A, VI-B, VII and VIII.

CBI nor Hahn's BI could not be any longer representative after aging due to the potential bitterness of some degradation products of bitter compounds.

Evidence of cis-humulinone-derived compounds after aging

In a prelude to the present study,[19] we used ESI(-)-RP-HPLC-MS/MS to investigate a *cis*-humulinone model medium subjected to 90 min of boiling. Four polar degradation products were evidenced, I and III being issued from *cis*-cohumulinone (5a) and II and IV from *cis*-n-humulinone (5 b). Their MS/MS and HRMS spectra are depicted in Figure 5a and Table 3. On the basis of these new data, we propose co- and n-allohumulinone, respectively, as molecules III and IV (Figure 6, $[M-H]^-$ in HRMS = 363.1820 and 377.1975, $\delta = 4.4 - 4.6$, inferior to 5.0, the identification limit of the HRMS). We further propose that products I and II ($[M-H]^-$ = 319.1190 and 333.1348, $\delta = 4.4 - 4.8$) might result from cleavage of II and IV at position 3' (Figure 6).

In the present work, the *cis*-humulinone model medium (initial concentration of 100 mg/L, pH 4.0), was aged for

21 days at 40 °C (Figure 5b and Table 3). As suggested by Taniguchi et al.,[26] compounds 6a-b (4′-hydroxyallohumulinone), 11a-b (scorpiohumulinols A) and 12a-b (scorpiohumulinols B) were found at retention times from 8 to 14 min (Figure 5b and Table 3, same numeration as Taniguchi et al.[26]) Yet, we also evidenced six additional polar derivatives (RT from 7 to 12 min), suspected to be structures V to VIII (Figure 6). Our proposals are reinforced by HRMS accurate $[M\text{-}H]^-$ ($\delta = 4.2$–4.9).

As depicted in Figure 5c and Table 3, RP-HPLC-MS/MS and HRMS of beer D aged two years at 20 °C allowed detection of compounds 6 b, 11a-b and 12a-b between 8 and 14 min. Both the retention times and the MS/MS fragmentations were similar to those found in our model medium (Table 3). Interestingly, the presence (before 12 min) of two of the polar molecules here-above described were also found and verified: VII and VIII ($[M\text{-}H]^- = 381.1916$ and 395.2068), both evidenced with one less hydroxyl group by Intelmann et al.[17] when starting from *trans*-isohumulones instead of *cis*-humulinones (Figure 6).

Conclusions

In the present work, we have examined the effects of two years of storage on 21 Belgian DH beers. While standard parameters such as alcohol content, extracts, and pH remained relatively stable through aging, bitterness units (BU) suffered a major decrease, along with the CBI (decreased by 14 to 41%) and the Hahn's BI (decreased by 14 to 35%) values. As expected, beers with the highest *cis*/*trans* isohumulone ratio (use of pre-isomerized hops or isomerized hop extracts) exhibited better stability. Besides *trans*-isohumulones, the most strongly degraded fractions were humulones and *cis*-humulinones, whose residual concentrations after two years were below 1.3 and 3.8 mg/L, respectively. Seven *cis*-humulinone degradation products have been evidenced in beer D after two years of aging. Further research is needed to evaluate the extent to which these products impact the bitterness intensity and quality of DH beers.

Acknowledgments

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