





Why Humulinones are Key Bitter Constituents Only After Dry Hopping: Comparison With Other Belgian Styles

Carlos Silva Ferreira , Eloi Thibault de Chanvalon , Etienne Bodart , and Sonia Collin 

Unité de Brasserie et des Industries Alimentaires, Earth and Life Institute ELIM, Faculté des Bioingénieurs, Université catholique de Louvain, Louvain-la-Neuve, B-1348, Belgium

ABSTRACT

Although long renowned worldwide for its unique dry-hopped (DH) Trappist beer, Belgium did not develop this process for other brands until the last decade. Twenty-one commercial Belgian DH beers were investigated and compared with a few other typical Belgian beers whose production involves either late hopping or aged hop addition (Gueuze). Bitterness was determined by spectrophotometric measurements (isooctane extraction) and by reversed phase high performance liquid chromatographic with UV detector (RP-HPLC-UV) (simultaneous quantitation of humulones, *cis*-/*trans*-isohumulones, reduced isohumulones, humulinones, and hulupones). In dry-hopped Belgian beers, humulinones (found at concentrations up to 13.3 mg/L) were estimated to be responsible for up to 28% of their bitterness. As humulinones revealed to be gradually lost through boiling (22%), clarification (5%), and fermentation (14%), non-dry-hopped (NDH) beers often displayed levels below 1.7 mg/L. Even in Gueuze beers for which old, humulinone-containing hops are used, no humulinone was found. Contrary to humulones, which were detected up to 7.2 mg/L in DH beers, hulupones were found at less than 3 mg/L in all Belgian beer styles. Humulinones were not produced in the boiling wort from humulones (in contrast to hulupones, readily synthesized from lupulones) but were significantly solubilized from hop thanks to their hydrophilicity. Yet, while the co-form accounted for about 50% of the humulones, the n-form prevailed for humulinones. Some humulinone degradation products were evidenced by RP-HPLC-MS/MS, and as suggested by their retention time (RT), should be more polar than their precursors. Bottle refermentation emerged as an additional critical step of humulinone loss, explaining the low levels found even in some strongly DH beers.

KEYWORDS

Bitterness; bottle refermentation; dry-hopped beer; hop; humulinone

Introduction

Hop (*Humulus lupulus* L.) is a key ingredient of beer, improving its microbiological stability, foam, and flavors. When added at the beginning of wort boiling, humulones (mainly co-humulone, n-humulone, and ad-humulone) from hop soft resins undergo thermal isomerization yielding bitter isohumulones (mainly iso-co-humulone, iso-n-humulone, and iso-ad-humulone).^[1] The *cis*-isohumulones are the major products of isomerization, while the *trans* isomers are kinetically produced, especially when the boiling temperature is lower than 100°C.^[2] The latter isomers are known to be less stable, yielding, for instance, unpleasant tricyclohumols, tricyclohumenes, iso-tricyclohumenes, tetracyclohumols, and epi-tetracyclohumols.^[3,4] Anti-isohumulones can also be produced in the boiling kettle.^[5]

Brewers often add aromatic hop varieties at the end of the boiling step (late hopping),^[1] but few aroma compounds from this addition remain unchanged in the finished beer. Lipophilic hop aroma compounds are degraded or lost during the wort boiling^[6] and bioconversions occur during fermentation, such as the biosynthesis of geraniol through geranial reduction or geranyl acetate and geranyl isobutyrate

hydrolysis.^[7] In order to impart flavors closer to those found in hop cones (terpenols, polyfunctional thiols, and their precursors including glucosides and cystein- or glutathionyl-thiol adducts),^[8–12] hop can also be added after wort cooling (main fermentation or maturation – dry hopping (DH) process). Hop polyphenols and oxidation products of humulones and lupulones (humulinones and hulupones) are suspected of modifying the bitterness and astringency of such beers.^[13–15] Belgian craft beers are usually dry-hopped at 1–3 g of hops/L,^[6] while doses above 5 g of hops/L are often reached in the United States.^[16]

Beer bitterness is caused mainly by isohumulones. Hughes and Simpson^[17] have shown iso-n-humulone to be more bitter than iso-co-humulone, and *cis* isomers to be significantly more bitter than their *trans* counterparts.^[18] Humulinones (co-, n-, and ad-) and hulupones (co-, n-, and ad-), derived respectively from humulones and lupulones, are reported to be present at less than 0.5% w/w in hop pellets.^[13,19–21] Their bitterness intensity has been shown to be approximately 66 and 84% that of isohumulones.^[19] Maye et al.^[13] reported concentrations of humulinones from 3 to 24 mg/L in 29 DH India Pale Ales, while Oladokun et al.^[14]

detected them only in four out of 34 lagers that were exceptionally also dry-hopped.

The international standard method for determining beer bitterness is based on isooctane extraction followed by spectrophotometric measurement at 275 nm.^[22,23] The result is multiplied by 50 and expressed in Bitterness Units (BU). A similar method requiring an additional washing with acidified methanol followed by dilution of the extract with basic methanol and measuring the absorbance at 255 nm is claimed to give a value proportional to the total isohumulone content of the beer, in mg/L.^[22,24,25] Yet, such methods only provide information regarding the total amount of isohumulones and cannot distinguish *co*-, *n*-, and *ad*- analogs or *cis* from *trans* isomers. Moreover, both methods might be inaccurate for DH beers or when aged hops are used, because oxidation products of hop humulones and lupulones also contribute to the absorbance near 275 nm.^[22,26] In such cases, therefore, HPLC analysis is required to take into account each molecule contributing to beer bitterness.^[14,19,27,28] Maye and Smith^[26] recently proposed an equation for calculating the bitterness intensity of DH beers (CBI) by summing contributions of isohumulones (mg/L) and humulinones (mg/L \times 0.66). Tetrahydro-isohumulones (mg/L \times 1.4) can also be added to this equation to take into account the contribution of these reduced isohumulones.^[29]

The aim of the present work was to investigate humulones, isohumulones, reduced isohumulones, humulinones, and hulupones in Belgian DH beers, as compared with non-dry-hopped (NDH) ales and Gueuze beers. Standard global quantitation methods were first applied. Then, a reversed phase high performance liquid chromatographic with UV detector (RP-HPLC-UV) analytical method close to that recently proposed by Biendl^[30,31] enabled us to quantify all the bitter constituents individually with a single injection. To understand why high levels of humulinones were found only in DH beers, their stability was further investigated in aqueous media and throughout a pilot-scale production.

Experimental

Chemicals

Methanol, acetonitrile, 37% HCl, NaOH, and citric acid monohydrate were purchased from VWR International (Leuven, Belgium). Milli-Q water was used (Millipore, Bedford, MA, U.S.A.). Humulones and lupulones mixture standard (ICE-3), isohumulones standard (ICS-I3), and tetrahydro-isohumulones standard (ICS-T2) were purchased from Labor Veritas Co. (Zürich, Switzerland). Humulinones and hulupones were kindly provided by Hopsteiner (Mainburg, Germany).

Beer samples

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). They were compared to four NDH ales (Saison Silly (V), Chimay Triple (W), Biolégère (X), and XX Bitter (Y)) and three Gueuze beers (Gueuze Boon (G1), Gueuze Chapeau (G2), and Gueuze Girardin (G3)). All beers were stored in the dark at 4°C.

Hop samples

Saaz (α -acids: 3–4.5%, essential oils: 0.5–1 mL/100g, harvest 2013) was purchased from Brouwland (Beringen, Belgium) and Hallertauer Mandarina Bavaria (α -acids: 7–10%, essential oils: 1.5–2.2 mL/100g, harvest 2015) was kindly provided by HVG (Wolnzach, Germany).

Standard beer analyses

Prior to analysis, beers were filtered through paper filters (MN 614 $\frac{1}{4}$ Macherey-Nagel, Düren, Germany) and degassed by shaking. Alcohol content, pH, and color were analyzed with Analytica-EBC methods 9.2.6, 9.4, and 9.6.^[23] 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)^[22, 23]: 25 mL of 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 of the isooctane phase at 275 nm (A_{275}) was measured exactly 5 min after the shaking period. The number of BU was determined as $A_{275} \times 50$. Subsequently, 20 mL of the 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 of the diluted extract was measured at 255 nm (A_{255}). The total isohumulone content was determined as $A_{255} \times 192.3 + 0.8$.^[22]

Extraction of bitter compounds for RP-HPLC-UV and Tandem Mass Spectrometry analyses

Hop humulones and lupulones were extracted prior to analysis according to the Analytica-EBC method 7.7.^[23] Humulinones and hulupones were extracted as described by Taniguchi et al.^[20] where 1 g ground pellets were mixed with 50 mL ethanol for 60 min and the supernatant was recovered.

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 μ m, Macherey-Nagel, Düren, Germany).

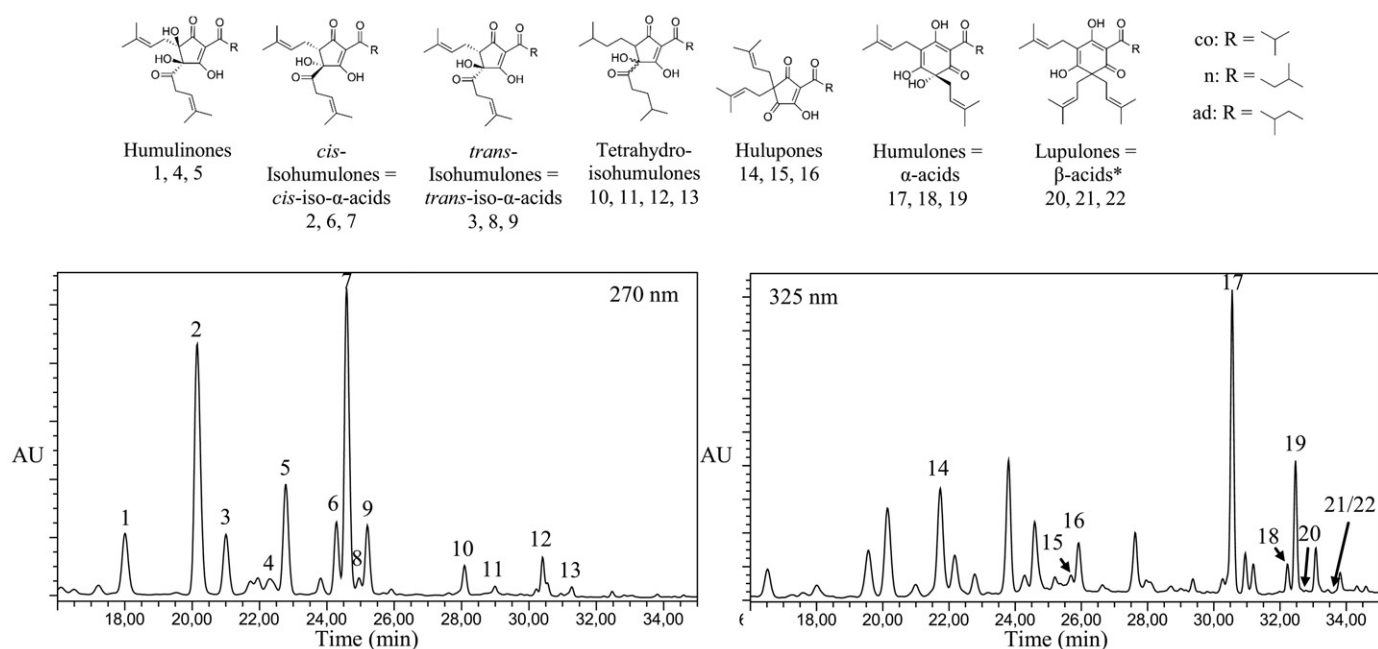


Figure 1. Chemical structures of bitter compounds and RP-HPLC-UV chromatograms of beer D extract at 270 (left) and 325 nm (right). 1: co-humulnone, 2: *cis*-iso-co-humulnone, 3: *trans*-iso-co-humulnone, 4: ad-humulnone, 5: n-humulnone, 6: *cis*-iso-ad-humulnone, 7: *cis*-iso-n-humulnone, 8: *trans*-iso-ad-humulnone, 9: *trans*-iso-n-humulnone, 10, 11, 12 and 13: tetrahydro-isohumulnone analogs, 14: co-hulupnone, 15: ad-hulupnone, 16: n-hulupnone, 17: co-humulnone, 18: ad-humulnone, 19: n-humulnone, 20: co-lupulnone*, 21: n-lupulnone* and 22: ad-lupulnone*. *, Not found in the beer.

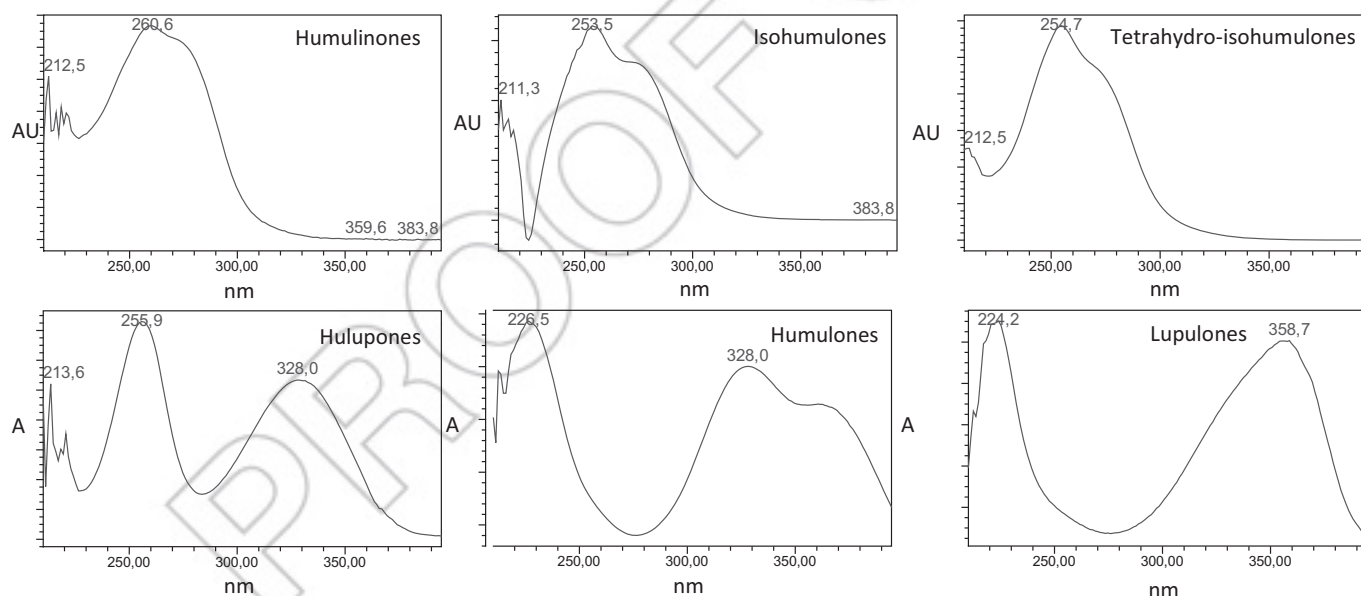


Figure 2. UV spectra of humulinones, isohumulones, tetrahydro-isohumulones, hulupones, humulones, and lupulones.

RP-HPLC-UV and MS/MS analyses

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, CA, U.S.A.), using the binary solvent system of Analytica-EBC method 9.47^[30] 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 for a

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, Miford, MA, U.S.A.).

The retention time (RT) and absorption spectrum of each compound were obtained by the injection of standards. All beer bitter compounds were clearly separated (Figure 1) except

Table 1. Standard analyses of 21 Belgian DH (A-U), 4 NDH (V-Y), and 3 Gueuze (G1-G3) beers.

Beer	Alcohol content (% ABV)	Original extract (°P)	Apparent attenuation (°P)	pH	Color (°EBC)
A ^a	6.6	16.5	97.7	4.3	27.5
B ^a	5.1	13.2	77.6	4.3	13.5
C ^a	6.1	15.1	81.2	4.4	11.5
D ^a	5.4	13.5	81.4	4.4	14.5
E ^a	5.4	12.4	90.0	4.4	12.5
F ^a	6.0	13.4	89.8	4.5	31.5
G	7.2	15.5	93.7	4.4	10.0
H	7.2	15.6	93.0	4.4	16.5
I	6.8	17.6	78.3	4.4	43.5
J ^a	8.3	18.4	90.1	4.3	11.0
K ^a	4.4	10.5	85.3	4.4	5.0
L ^a	6.6	15.6	85.2	4.5	14.5
M ^a	9.1	20.6	88.7	4.5	8.3
N ^a	7.4	18.8	79.7	4.4	29.0
O ^a	7.4	17.8	84.1	4.0	14.5
P ^a	9.1	19.6	93.4	4.0	5.5
Q ^a	9.3	19.7	94.2	4.0	6.0
R ^a	9.3	19.6	94.2	4.0	6.0
S ^a	9.1	19.2	94.8	4.0	6.2
T ^a	9.3	19.8	93.6	4.0	6.2
U ^a	9.4	19.7	95.2	4.1	5.7
V ^a	5.2	11.7	90.0	3.8	45.0
W ^a	7.8	16.2	88.3	4.2	12.4
X ^a	3.6	7.9	88.7	4.3	5.7
Y ^a	6.2	13.5	86.8	4.5	11.5
G1 ^a	7.3	14.9	91.6	3.8	16.5
G2 ^a	3.1	21.4	26.0	3.4	36.5
G3 ^a	6.2	12.8	90.5	3.4	22.5

^aWith bottle refermentation.

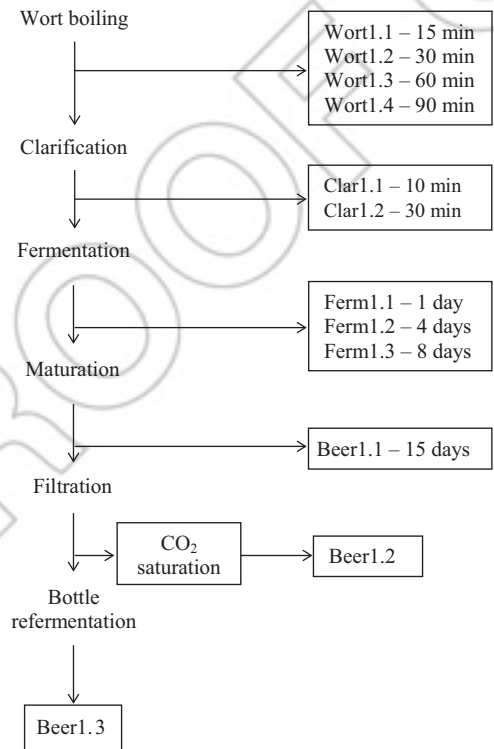


Figure 3. Scheme of the pilot scale brewing process. Samples were collected as follows: Wort1.1, Wort1.2, Wort1.3, and Wort1.4 after 15, 30, 60, and 90 min of boiling; Clar1.1 and Clar1.2 after 10 and 30 min of clarification; Ferm1.1, Ferm1.2, and Ferm1.3 after 1, 4, and 8 days of fermentation; Beer1.1 after 15 days of maturation; Beer1.2 after CO₂ saturation; and Beer1.3 after bottle refermentation.

co-humulone (n°17) and one tetrahydro-isohumulone analog (n°12). These, if present, co-eluted at 31 min. As depicted in Figure 2, the absorbance wavelengths of humulinones, isohumulones, and tetrahydro-isohumulones were similar, with two peaks around 255 and 270 nm and almost no absorbance after

320 nm, while humulones, lupulones, and hulupones showed strong absorbance above 300 nm, with a minimum around 270 nm. Therefore, 270 nm was chosen for isohumulone, reduced isohumulone, and humulinone quantitation, while 325 nm was preferred for humulones and hulupones.

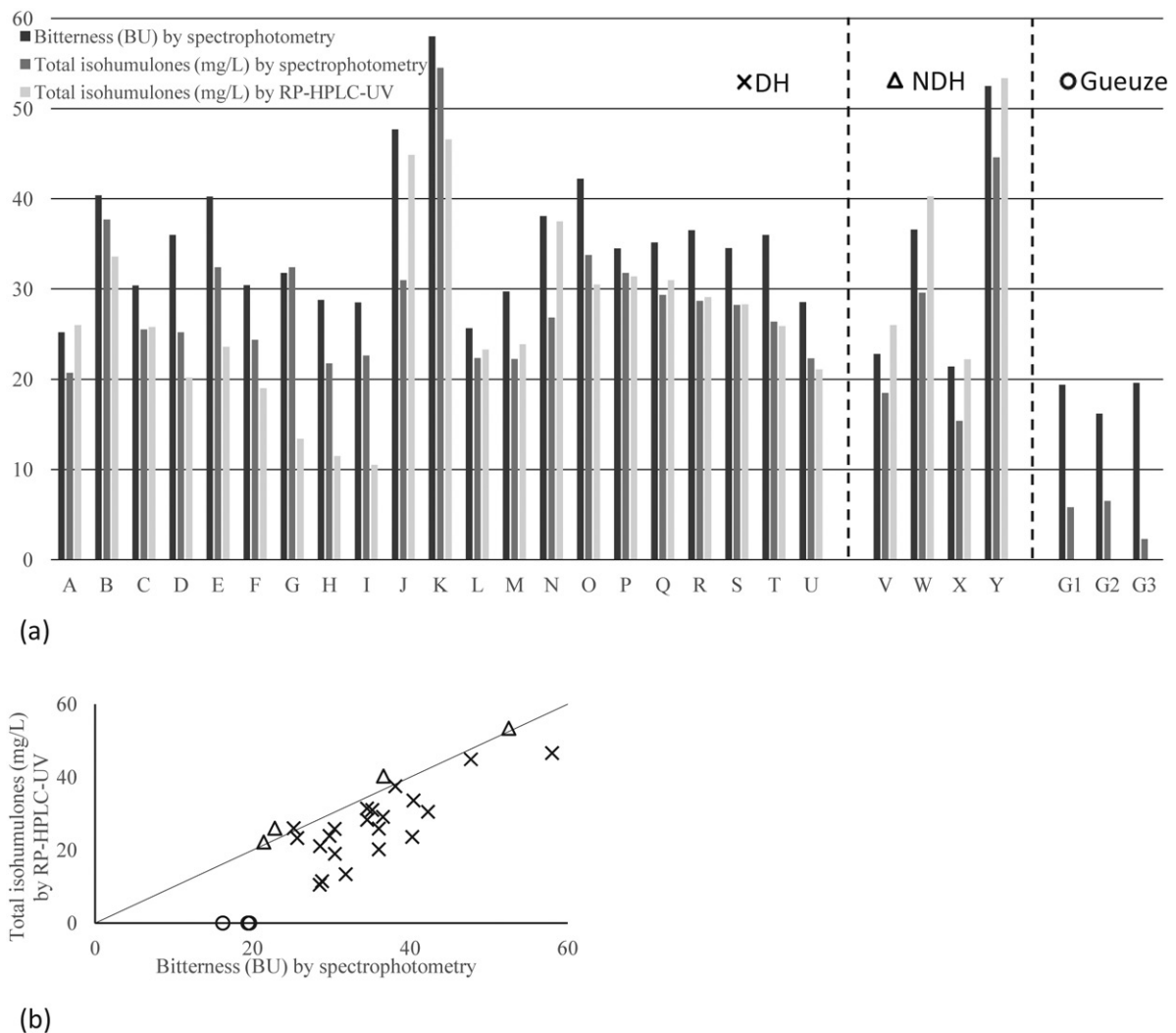


Figure 4. (a) Bitterness units (BU) and total isohumulone concentrations (mg/L) in DH (A-U), NDH (V-Y), and Gueuze (G1-G3) beers, both measured by spectrophotometry, as compared to RP-HPLC-UV quantitations (mg/L). (b) Correlation between RP-HPLC-UV total isohumulone concentrations (mg/L) and bitterness units (BU).

For analyzing humulinone degradation products in the aqueous model medium, the same columns were further connected to a Bruker Daltonics Esquire 3000 ion trap mass spectrometer equipped with an electrospray ion source operated in negative mode (ESI⁻). The ESI inlet conditions were as follows: source voltage, 3.0 kV; capillary temperature, 325°C; nebulizer, nitrogen, 40 Psi. Nitrogen was also used as drying gas, at a flow rate of 9 mL/min. For identification, collision-induced dissociation MS/MS spectra were recorded at a relative collision energy of 0.7 V.

Calculated bitterness intensity of DH beers

Based on the relation between Bitterness units (BU) and isohumulones concentration (1 mg/L of isohumulones = 1 BU) and the perceived bitterness of humulinones (66% as bitter as isohumulones), Maye and Smith^[26] proposed the following equation to calculate the bitterness of DH beers: Calculated Bitterness Intensity (CBI) = mg/L of isohumulones + (mg/L of humulinones × 0.66). We further added to this equation the contribution of tetrahydro-isohumulones (mg/L × 1.4).^[29]

Wort-boiling simulation in a model medium

A humulone and lupulone solution (47 and 2.3 mg/L, respectively, sum of co-, n-, and ad-) and a humulinone solution (70 mg/L, sum of co-, n-, and ad-) were prepared from standards in a pH 5.6 aqueous medium. Brown glass flasks were filled with 10 mL of each solution and hermetically closed. In order to simulate wort boiling, the flasks were heated in a water bath at 99°C. After 10, 25, 50, and 90 min of boiling, flasks of both solutions were removed from the bath, cooled to 20°C, and analyzed by RP-HPLC-UV and RP-HPLC-MS/MS.

Pilot-scale production of a beer enriched in humulinones

A pilot beer containing high amounts of humulinones was produced in a 60 L microbrewery (Coenco, Oostkamp, Belgium). Pilsen malt (a 2-row spring malt, from Boortmalt, Antwerpen, Belgium) was brewed in water (11.0 kg of malt in 33.9 L water) according to the following mashing program: 60 min at 60°C and 25 min at 72°C. The wort was then heated to 78°C and filtered through a lauter tun. After

Table 2. Concentrations (mg/L) of humulones, isohumulones (% of the *cis* isomer in parentheses for beers A-Y), humulinones, and hulupones in the 28 investigated beers.

Compound	DH												NDH					Gueuze											
	A ^a	B ^a	C ^a	D ^a	E ^a	F ^a	G	H	I	J ^a	K ^a	L ^a	M ^a	N ^a	O ^a	P ^a	Q ^a	R ^a	S ^a	T ^a	U ^a	V ^a	W ^a	X ^a	Y ^a	G1 ^a	G2 ^a	G3 ^a	
co-Humulone	0.4	1.6	2.4	2.4	3.0	1.6	2.3	1.1	1.2	1.5	3.4	0.9	0.8	0.7	2.7	1.5	1.6	1.5	1.7	1.8	1.5	1.5	0.0	1.4	1.2	0.9	0.0	0.0	0.0
n-Humulone	0.6	1.5	2.3	2.2	3.5	2.4	1.7	0.8	1.3	1.7	3.3	1.0	0.8	0.7	3.2	1.8	1.7	1.5	1.5	0.8	1.7	0.4	0.8	2.0	0.4	0.0	0.0	0.0	0.0
ad-Humulone	0.1	0.2	0.4	0.3	0.5	0.3	0.2	0.1	0.2	0.2	0.5	0.1	0.1	0.1	0.4	0.2	0.2	0.3	0.2	0.1	0.2	0.0	0.1	0.3	0.1	0.0	0.0	0.0	0.0
Total	1.1	3.3	5.1	4.9	7.0	4.3	4.2	2.0	2.7	3.4	7.2	2.0	1.7	1.5	6.3	3.5	3.5	3.3	3.4	2.7	3.4	0.4	2.3	3.5	1.4	0.0	0.0	0.0	0.0
iso-co-Humulone	10.2	12.5	10.0	9.0	8.8	6.6	6.3	5.0	4.3	19.7	17.6	7.3	8.4	15.4	13.8	13.9	13.6	13.3	12.4	12.0	9.8	9.9	21.0	5.6	34.5	0.0	0.0	0.0	0.0
	(83)	(72)	(72)	(78)	(72)	(69)	(83)	(86)	(88)	(77)	(76)	(76)	(77)	(74)	(75)	(85)	(85)	(86)	(86)	(82)	(85)	(73)	(72)	(70)	(70)				
iso-n-Humulone	12.7	17.0	12.5	9.2	12.2	10.0	5.9	5.5	5.3	21.2	23.7	12.9	12.8	17.7	13.6	14.2	14.1	12.9	13.1	11.4	9.2	12.8	15.9	13.4	12.7	0.0	0.0	0.0	0.0
	(84)	(71)	(71)	(81)	(70)	(72)	(81)	(89)	(88)	(77)	(70)	(79)	(77)	(65)	(77)	(74)	(74)	(75)	(76)	(70)	(87)	(77)	(74)	(72)	(71)				
iso-ad-Humulone	3.1	3.8	3.3	2.0	2.6	2.4	1.2	1.0	0.9	4.0	5.3	3.1	2.7	4.4	3.1	3.3	3.3	2.9	2.8	2.5	2.1	3.3	3.4	3.2	6.2	0.0	0.0	0.0	0.0
	(81)	(73)	(73)	(76)	(70)	(71)	(84)	(87)	(88)	(75)	(78)	(78)	(76)	(76)	(74)	(86)	(86)	(87)	(86)	(85)	(82)	(75)	(74)	(73)	(61)				
Total	26.0	33.3	25.8	20.2	23.6	19.0	13.4	11.5	10.5	44.9	46.6	23.3	23.9	37.5	30.5	31.4	31.0	29.1	28.3	25.9	21.1	26.0	40.3	22.2	53.4	0.0	0.0	0.0	0.0
	(83)	(72)	(72)	(79)	(71)	(71)	(82)	(87)	(88)	(77)	(74)	(78)	(77)	(71)	(75)	(80)	(80)	(81)	(82)	(77)	(85)	(75)	(73)	(72)	(69)				
co-Humulonone	0.2	2.0	1.6	3.9	3.3	1.4	2.4	2.9	1.2	1.0	2.8	0.5	0.6	0.5	1.4	1.5	1.3	2.3	0.9	4.1	0.8	0.4	0.9	0.4	0.9	0.0	0.0	0.0	0.0
n-Humulonone	0.6	4.7	3.6	7.8	5.8	3.2	3.7	3.4	2.7	2.0	6.8	1.4	1.4	1.3	3.2	4.1	2.7	5.3	1.9	5.3	1.6	0.6	0.8	0.7	0.5	0.0	0.0	0.0	0.0
ad-Humulonone	0.2	0.9	0.7	1.6	1.0	0.7	0.7	0.7	0.4	0.5	1.3	0.3	0.3	0.2	0.7	0.7	0.8	1.4	0.6	1.4	0.4	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Total	1.0	7.6	5.9	13.3	10.1	5.3	6.8	7.0	4.3	3.5	10.9	2.2	2.3	2.0	5.3	6.3	4.8	9.0	3.4	10.8	2.8	1.0	1.7	1.1	1.7	0.0	0.0	0.0	0.0
co-Hulupone	0.4	1.6	0.6	0.9	0.7	0.6	0.8	0.9	0.6	0.9	1.7	1.2	0.9	1.1	0.7	1.1	0.8	1.0	0.9	1.3	0.6	0.9	0.2	0.8	1.1	1.0	0.0	1.5	0.6
n-Hulupone	0.0	1.1	0.6	0.7	0.6	0.4	0.4	0.3	0.3	0.5	1.4	0.5	0.5	0.6	0.5	0.8	0.6	0.6	0.6	0.9	0.5	0.4	0.1	0.4	0.0	0.3	0.0	0.6	0.6
Total	0.4	2.7	1.2	1.6	1.3	1.0	1.2	1.2	0.9	1.4	3.1	1.7	1.4	1.7	1.2	1.9	1.4	1.6	1.5	2.2	1.1	1.3	0.3	1.2	1.1	1.3	0.0	2.1	2.1

^aWith bottle refermentation.

sparging, 62.8 L of wort with an extract of 11°P was obtained. The wort was then boiled with 3.6 g/L of aged Saaz (used here as the humulinone contributor) for 90 min (10% evaporation) and the final extract was adjusted to 11°P by addition of water. Two g/L of Hallertau Mandarin Bavaria (HMB) hop was added to the wort at the end of the boiling. Fermentation was carried out at 22°C for 8 days in a 60 L cylindroconical fermenter with an ale-type yeast (INBR-Bras212, propagated in a glucose/maltose/yeast extract/peptone medium). This strain was pitched at 7.5×10^6 cells/mL. Maturation was conducted for 14 days at 16°C in the presence of yeast. The beer was filtered on cellulose filter pads (8- μ m pores followed by 0.5- μ m pores, BuonVino, Cambridge, Canada) under a CO₂ atmosphere. Saturation at 5 g/L CO₂ was obtained either by injection of CO₂ (Air Liquide, Herenhout, Belgium) or by bottle fermentation for 15 days at 27°C with a new batch of INBR-Bras212, pitched at 5×10^5 cells/mL. The beers were then stored in the dark at 4°C. Wort/beer samples were collected throughout the brewing process (Figure 3), filtered through glass-fiber filters (3.0- μ m pores, Gelman Sciences, USA), and stored at -20°C until analysis.

Results and discussion

Bitterness of commercial Belgian DH beers compared with NDH ales and Gueuze beers

Together with other basic beer properties, the bitterness (in BU) of 21 DH beers (A-U), four NDH ales (V-Y), and three Gueuze beers (G1-G3) was first determined by standard global methods. As shown in Table 1, the alcohol content (% ABV) of the Belgian DH beers ranged from 4.4 (K) to 9.4 (U) (original extracts from 10.5 (K) to 20.6 (M)). Apparent attenuation exceeded 80% for most of them. Color ranged from 5 to 14.5°EBC for 16 beers, including blondes (B, C, K, M, and O), triples (P, Q, R, S, T, and U), India pale ales (D, G, and J), and saisons (E and L). A few ambers (A, F, H, and N) and one brown DH beer (I) were found on the market. In contrast to the Gueuze beers, whose pH was below 3.8, the DH beers exhibited pH values between 4.0 (O-T) and 4.5 (F, L, and M). The highest pH values measured here were recently reported as a possible consequence of the dry hopping process.^[13]

As depicted in Figure 4a, the bitterness (in BU) of Belgian DH beers ranged from 25.2 (A) to 58.0 (K). In the case of most beers, the spectrophotometric method used to determine total isohumulones produced a lower value than the BU determination, because of the unexpected loss of about 20% of isohumulones during washing with acidified methanol (checked by RP-HPLC-UV). Only beer G displayed 32 mg/L total isohumulones for a similar bitterness, probably because of the presence of more tetrahydro-isohumulones (4.4 mg/L, see hereunder).

Isohumulone, humulone, lupulone, reduced isohumulone, humulinone, and hulupone concentrations were further determined by RP-HPLC-UV (Table 2). Lupulones were undetectable in all samples. The only reduced isohumulones found were tetrahydro-isohumulones. In the case of most

DH beers, the total isohumulone content determined by the standard global spectrophotometric method did not tally with that determined by RP-HPLC-UV (Figure 4b). This supports the findings of previous studies^[14,15,26] showing that global methods are not sufficiently accurate for measuring DH beer bitterness.

RP-HPLC-UV analysis of Gueuze beers revealed the absence of humulones, isohumulones, reduced isohumulones, and humulinones (Table 2). Yet, these beers exhibited bitterness values above 16.2 BU and spectrophotometric total isohumulone values between 2.3 and 6.5 mg/L (Figure 4). These results indicate the presence of compounds, probably issued from aged hops, liable to interfere in global spectrophotometric measurements, even after washing with acidified methanol.

Humulones were found in all DH beers (first bar in Figure 5) at concentrations ranging from 1.1 (beer A) to 7.2 mg/L (beer K). In contrast, all NDH beers contained less than 3.5 mg/L and all three humulone peaks were even undetectable in Gueuze beers. In the course of a traditional brewing process with late hopping, humulones are massively lost during wort clarification and further removed by yeast during fermentation, and this led to very low amounts in the final beers. On the other hand, humulones are better solubilized in ethanolic media, as is the case during dry hopping. Most DH beers displayed similar concentrations of co-humulone and n-humulone (Table 2). This result was unexpected at first, as the n-analog constitutes the major fraction of humulones in hops. Yet, the structure of its substituent makes the co-humulone more soluble.

Likewise, humulinone concentrations (second bar in Figure 5) ranged from 1.0 (beer A) to 13.3 mg/L (beer D) in DH beers, while values below 1.7 mg/L were found in NDH beers and no humulinones were detected in Gueuze beers. Concentrations over 8 mg/L have been reported to be sufficient to impact beer bitterness.^[19] No correlation between humulone and humulinone concentrations ($R^2 = 0.35$) was observed in DH beers, and while co-humulone accounted for some 50% of humulones (as stated previously), the n-form appeared clearly as the most abundant humulinone.

Hulupone concentrations were below 3 mg/L in all beers, with no significant difference ($\alpha = 95\%$) between DH, NDH, and Gueuze beers (third bar in Figure 5). At this level, these molecules have no bitter impact.^[19] As in the case of the humulones, the least hydrophobic hulupone, co-hulupone, emerged as the major form (Table 2). Hulupones can either be solubilized from hops or be produced from hop lupulones during wort boiling.

Application of the Calculated Bitterness Intensity (CBI) equation adapted from Maye and Smith^[26] yielded values ranging from 17.0 (I) to 53.8 (K) for DH beers (Figure 6). Isohumulones remained the main source of bitterness in all samples. The contribution of humulinones to the CBI was above 10% for most DH samples, with beer D showing the greatest contribution (28%).

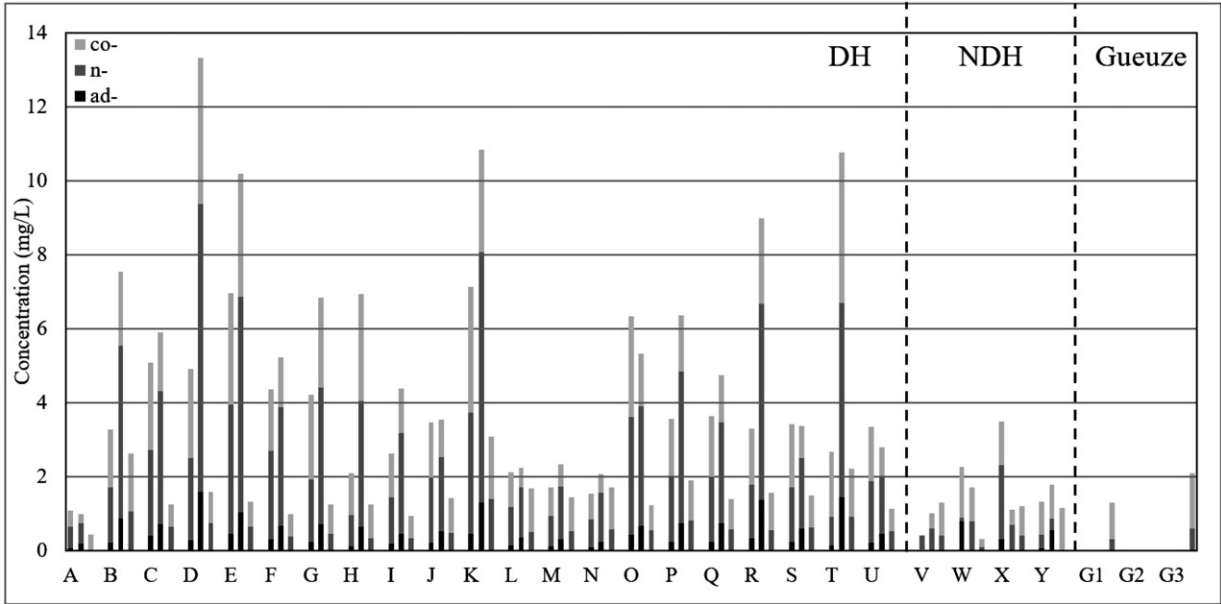


Figure 5. Concentrations (mg/L) of humulones (first bar), humulinones (second bar), and hulupones (third bar) in Belgian DH (A-U), NDH (V-Y), and Gueuze (G1-G3) beers. Analogs (co-, n-, and ad-) are shown for all compounds with different color tones in the same bar.

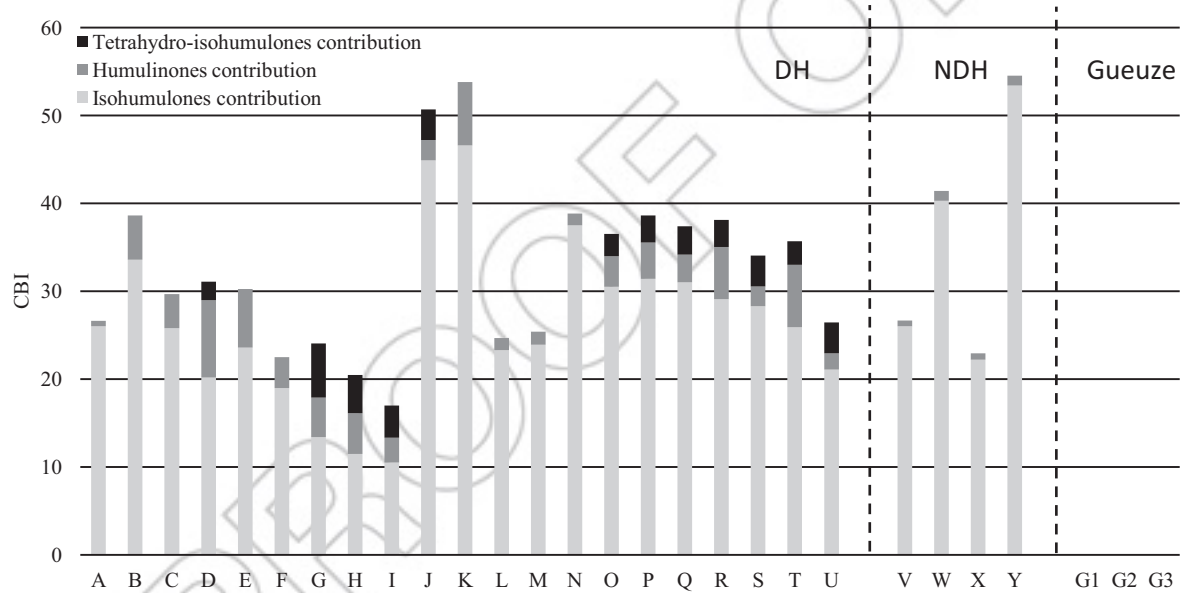


Figure 6. Calculated Bitterness Intensity (CBI) of beers. The contributions of isohumulones, tetrahydro-isohumulones, and humulinones to the CBI are expressed with different color tones in the same bar.

Thermal stability of humulones, lupulones, and humulinones in model media

The thermal stability of humulones, lupulones, and humulinones during wort boiling was investigated by heating aqueous model media (pH 5.6) at 99°C. In the first model medium, which contained humulones and lupulones (Figure 7a), 55% of the initial amount of humulones was recovered after 90 min of boiling, while 38% appeared isomerized to isohumulones (7% not recovered). Surprisingly, no humulinones were found after heat treatment. In contrast, all the lupulones already appeared to have been transformed to hulupones within 50 min.

In the second model medium, which contained only humulinones (Figure 7b), 24% of the initial amount was recovered at the end of the treatment, confirming their heat instability. As the three humulinone analogs (co-, n-, and ad-) were degraded at the same rate, it appears that the nature of the lateral chain had no significant kinetic impact.

This model medium was further investigated by ESI(-)-RP-HPLC-MS/MS. Four polar peaks (I-IV) in addition to those of humulinones were evidenced. As suggested by their MS/MS fragmentation shifted by 14 units (Figure 7c), I (RT = 4.1 min) and III (RT = 11.0 min) were probably issued from co-humulone, while II (RT = 4.1 min) and

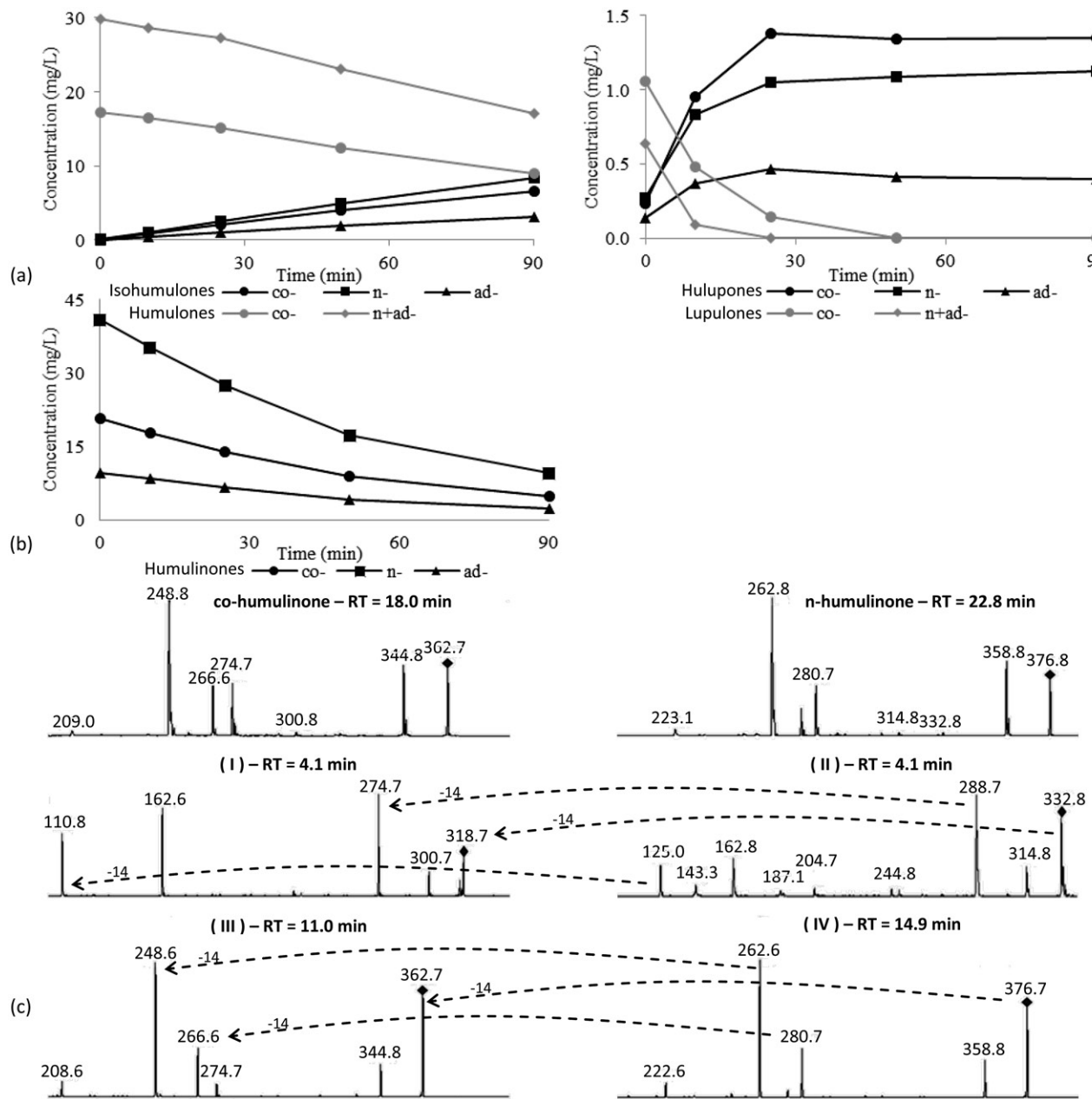


Figure 7. Degradation at 99°C of (a) humulones and lupulones together, or (b) humulones, in model media at pH 5.6. (c) ESI(-)MS/MS spectrum of co-humulone, n-humulone, and four polar degradation products (I, II, III, and IV) evidenced in model medium (b) after 90 min.

Table 3. Concentrations (% w/w) of humulones, isohumulones, humulinones, and hulupones (ratio n-/co- in parentheses) in both hops used for pilot beer production and recovery (% eq. 11°C) through the pilot scale brewing process.

	Hops		Wort and beer											
Compound (%)	Aged Saaz	HMB	Wort 1.1	Wort 1.2	Wort 1.3	Wort 1.4	Clar 1.1	Clar 1.2	Ferm 1.1	Ferm 1.2	Ferm 1.3	Beer 1.1	Beer 1.2	Beer 1.3
Humulones	1.3 (2.6)	7.8 (1.6)	100 ^a (1.8)	85 (1.8)	55 (1.8)	43 (1.8)	170 (0.8)	229 (0.9)	139 (0.7)	28 (0.5)	24 (0.5)	22 (0.5)	12 (0.3)	2 (0.5)
Isohumulones	0.0	0.0	16 (2.9)	29 (2.8)	50 (2.4)	67 (2.1)	84 (1.5)	100 ^b (1.5)	96 (1.4)	82 (1.3)	67 (1.2)	72 (1.3)	71 (1.3)	50 (1.1)
Humulinones	0.7 (4.2)	0.3 (1.9)	100 ^a (2.8)	96 (3.0)	83 (2.9)	78 (3.1)	104 (2.2)	99 (2.2)	100 (2.2)	95 (2.1)	86 (2.0)	87 (2.1)	87 (2.0)	55 (2.1)
Hulupones	0.3 (1.2)	0.1 (0.6)	93 (0.7)	97 (0.7)	100 ^c (0.6)	97 (0.6)	82 (0.3)	84 (0.5)	87 (0.5)	80 (0.5)	69 (0.5)	70 (0.5)	70 (0.5)	60 (0.4)

Recovery compared to ^aWort1.1 for humulones (= 15.0 mg/L) and humulinones (= 25.0 mg/L), to ^bClar1.2 for isohumulones (= 26.4 mg/L), and to ^cWort1.3 for hulupones (= 8.9 mg/L).

IV (RT = 14.9 min) likely came from n-humulone. Also worth stressing is the similarity of the MS/MS spectra of III and IV to those of the corresponding humulinone precursors. Complementary investigations will be needed to identify these compounds.

Humulinone and hulupone stability during beer production

To understand why high levels of humulinones were found only in DH beers, humulinone stability was also investigated throughout a pilot-scale brewing process (Figure 3 and

Table 3) in which spiking with an unusually high amount (3.6 g/L) of an aged Saaz hop sample (harvest 2013) was performed at the beginning of boiling. Hallertauer Mandarina Bavaria, harvest 2015 (HMB) was used for late hopping at 2 g/L. The concentrations of humulones, lupulones, humulinones, and hulupones in both hops are given in Table 3.

After the first 15 min of wort boiling (Wort1.1), all the humulinones present in the aged Saaz hop appeared solubilized into the wort, leading to 25 mg/L (set to 100% in Table 3). At the end of the boiling (Wort1.4), 78% of this amount was still found in the wort (concentrations normalized to 11°P initial wort extract to take into account evaporation). Losses were thus less than in the aqueous model medium previously described.

Not surprisingly, adding HMB hop at the end of boiling (Clar1.1) led to an increased humulinone concentration (from 78 to 104% = 6.4 mg/L). Given the composition of HMB hop, this means that 96% of its humulinones had been dissolved, of which only 5% appeared degraded after 30 min of wort clarification.

During fermentation, the humulinone concentration decreased by an additional 14% (Table 3), because of adsorption to yeast and vessel walls. Here again, the effect was much weaker for these polar compounds than for isohumulones.^[32]

More unexpectedly, another 32% of the humulinones was removed by yeast during bottle refermentation (this very traditional Belgian process was applied to the NDH samples but also to 18 of our 21 DH beers, as specified in Table 1). In conclusion, as humulinones are not produced from humulones during boiling (as previously mentioned) and as they are continuously degraded throughout the process (albeit to a lesser extent than in water), quite logically the NDH beers displayed concentrations under 1.7 mg/L.

The hulupone concentration was highest in Wort1.3, representing about half of the hulupones present in the Saaz hop. On the other hand, addition of the second hop (HMB) at the end of boiling did not impact the hulupone level. Significant losses occurred through fermentation (18%) and bottle refermentation (10%) (Table 3), with, as previously mentioned for other compounds, a stronger impact for the most hydrophobic n-analog.

Conclusion

This work has enabled us to highlight both the diversity and the specificity of Belgian DH beers. In these beers, no correlation was found between levels of the hydrophobic humulones and those of the more hydrophilic humulinones: the co-form accounted for about 50% of the former while the n-form of the latter prevailed.

Degraded during boiling and adsorbed onto yeast and the vessel during fermentation, humulinones emerge here as good markers of DH beers, having an estimated contribution of up to 28% of their bitterness. This makes international spectrophotometric quantitations inappropriate. On the other hand, because of their higher

hydrophobicity, hulupones appeared not to impact Belgian DH beer bitterness.

The bottle refermentation process, used by most Belgian brewers, had a strong impact, partly explaining the lower levels of humulinones found in our DH beers than in American beers.^[26] Further investigation is now needed to assess the stability of humulinones through DH beer aging and to determine the organoleptic properties of the most hydrophilic compounds issued from humulinone degradation. Their identification could also help explain the misleading bitterness values (as measured in BU) usually obtained for Gueuze beers, devoid of humulones, isohumulones, and humulinones.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Carlos Silva Ferreira  <http://orcid.org/0000-0003-3760-517X>
Eloi Thibault de Chanvalon  <http://orcid.org/0000-0001-6668-4028>
Etienne Bodart  <http://orcid.org/0000-0002-4280-3325>
Sonia Collin  <http://orcid.org/0000-0002-1929-098X>

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