Journal of the American Society of Brewing Chemists
The Science of Beer
ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ujbc20

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To cite this article: Carlos Silva Ferreira, Margaux Simon & Sonia Collin (2022): Why Catechin and Epicatechin from Early Hopping Impact the Color of Aged Dry-Hopped Beers while Flavan-3-ol Oligomers from Late and Dry Hopping Increase Colloidal Instability, Journal of the American Society of Brewing Chemists, DOI: 10.1080/03610470.2022.2062156

To link to this article: https://doi.org/10.1080/03610470.2022.2062156

JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS
https://doi.org/10.1080/03610470.2022.2062156

Why Catechin and Epicatechin from Early Hopping Impact the Color of Aged Dry-Hopped Beers while Flavan-3-ol Oligomers from Late and Dry Hopping Increase Colloidal Instability

Carlos Silva Ferreira, Margaux Simon and Sonia Collin

Unité de Brasserie et des Industries Alimentaires, Louvain Institute of Biomolecular Science and Technology (LIBST), Faculté des Bioingénieurs, Université catholique de Louvain, Louvain-la-Neuve, Belgium

ABSTRACT

Dry hopping imparts distinct aromas but also a series of non-volatile compounds suspected of causing flavor and physical instability during beer storage. In this work, color, chill haze, total polyphenols, total flavanoids, and flavan-3-ol monomers (catechin and epicatechin) and oligomers (procyanidin dimers and trimers) were monitored in five commercial pale-colored Belgian dry-hopped beers over 24 months of storage at 20 °C in the dark. Fresh dry-hopped beers contained unusually high levels of flavan-3-ol monomers (up to 6.6 mg/L) and oligomers (up to 14.1 and 10.2 mg/L dimers and trimers, respectively). The increase in color intensity during storage (up to 6.4°EBC) correlated with fresh beer monomer levels, while the oligomer content correlated with chill haze formation (up to 25.7°EBC). The evolution of these two physical attributes also correlated with the level of total polyphenols in the fresh beers. In a pilot-scale production, kettle hopping was shown to impart either monomers (early) or oligomers (late), while dry hopping promoted efficient extraction of both monomers and dimers (extraction yields of 62 and 74%, respectively). Dry hopping thus plays an important role in color and chill haze increase.

Abbreviations: RP-HPLC-ESI(-)-MS/MS: Reversed phase high pressure liquid chromatography with electrospray ionization in negative mode and mass spectrometer detector operating in MS/MS mode; DH: dry-hopped; NDH: non-dry-hopped

KEYWORDS

Beer aging; beer color; beer haze; dry-hopped beer; flavan-3-ol; procyanidin

Introduction

Color and turbidity are two important physical attributes of beer, directly related to its style and consumer acceptance.[1] While color is mainly given by malt melanoidins and polyphenols, the origin of haze might be biological (yeast and dead bacteria) or non-biological (protein-polyphenol, metal ions, lubricants, calcium oxalate, residual starch, glucans, etc.).[1–3] The polyphenols involved in these two attributes are mainly monomers, dimers, and trimers of flavan-3-ols[4–6] (Figure 1a). Most of them are imparted by barley malt, which contains around 6–95 mg/kg (+)-catechin, 9–350 mg/kg procyanidin B3, and 7–119 mg/kg procyanidin C2.[7–13] Yet hop also contributes to the flavan-3-ol content of beer, since it can contain more than 2,500, 1,400, and 800 mg/kg of (+)-catechin, procyanidin B3, and procyanidin C2, respectively, according to the variety[12,14,15] (the lower the level of α-acids, the higher the flavan-3-ol content[16,17]). In addition, hop is also a source of epicatechin and its oligomers, mostly absent from barley malt.

Flavan-3-ols are the main antioxidants in terms of activity present in beer, but their presence can be associated with positive or negative factors, depending on the quality view.[3,15,18–20] In their natural state, flavan-3-ols do not impact color or haze, but their oxidation produces, in the B ring of the molecule, *o*-quinones or semi-quinones.[3,21] In the case of monomers, the *o*-quinone might react with another flavan-3-ol monomer to form, among others, yellow-brown-colored compounds such as dehydrodicatechin A.[4] In dimers and trimers, however, the *o*-quinone triggers an intramolecular reaction leading to formation of A-type procyanidins, recognized as haze-active molecules[6,22,23] (Figure 1b). Since high temperature, agitation, dissolved oxygen, and metallic ions (iron and copper) accelerate the development of reactive oxygen species and therefore quinones formation, special attention is required to avoid their occurrence, for instance by increasing the level of other antioxidants such as sulfites, ascorbic acid (only effective if very few dissolved oxygen; otherwise, pro-oxidant activity leading to superoxide anions and hydroxyl radicals), hydroxycinnamic or benzoic acids, glutathione, and other yeast-derived antioxidants.[20]

Dry hopping, i.e., the addition of hops during or after the main fermentation, has become a popular process for

CONTACT Sonia Collin sonia.collin@uclouvain.be
Supplemental data for this article is available online at https://doi.org/10.1080/03610470.2022.2062156.

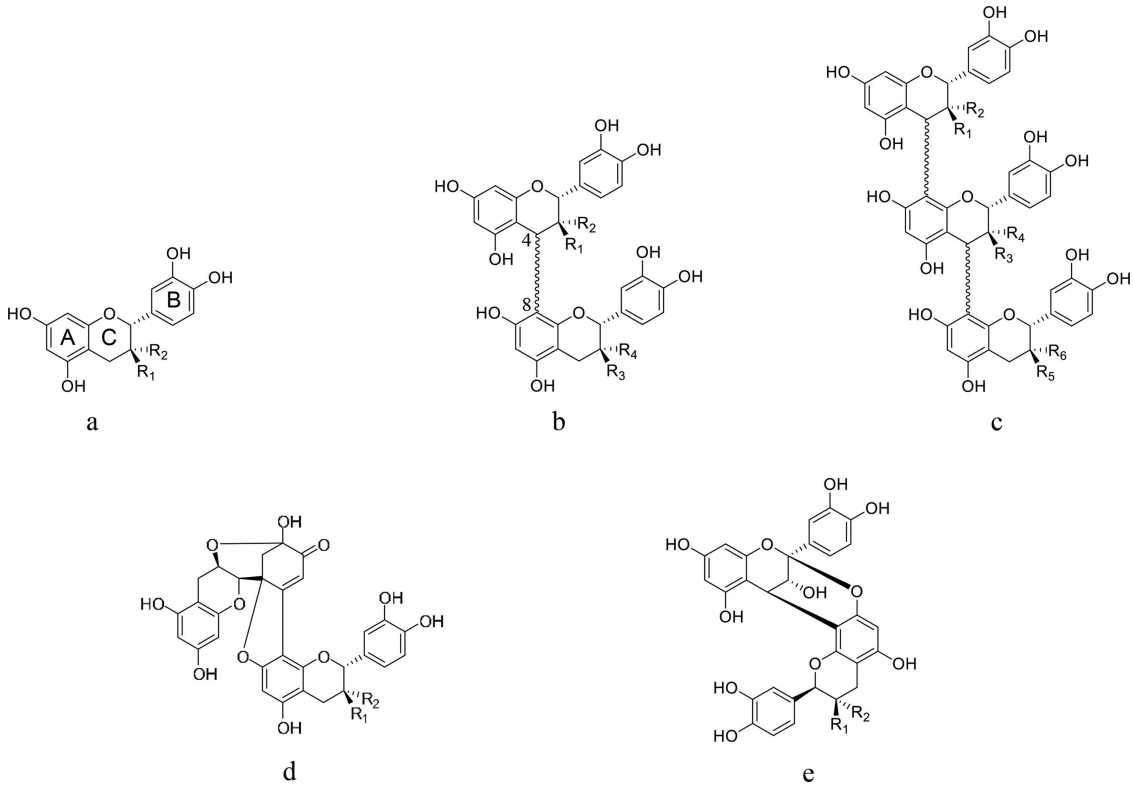

Figure 1. (a) Structure of flavan-3-ol monomers: (+)-catechin (R1 = OH, R2 = H), (−)-epicatechin (R1 = H, R2 = OH); (b) procyanidin dimers: B1 (R1 = H, R2 = OH, R3 = OH, R4 = H, 4 α→8 bond), B2 (R1 = H, R2 = OH, R3 = H, R4 = OH, 4 β→8 bond), B3 (R1 = OH, R2 = H, R3 = OH, R4 = H, 4 α→8 bond) and B4 (R1 = OH, R2 = H, R3 = H, R4 = OH, 4 β→8 bond); (c) procyanidin trimers: C1 (R1 = H, R2 = OH, R3 = H, R4 = OH, R5 = H, R6 = OH, 4 α→8 bonds) and C2 (R1 = OH, R2 = H, R3 = OH, R4 = H, R5 = OH, R6 = H, 4 β→8 bonds); (d) dehydrodicatechins A: catechin-catechin (R1 = OH, R2 = H) and catechin-epicatechin (R1 = H, R2 = OH; (e) A-type procyanidin dimers: A1 (R1 = H, R2 = OH) and A2 (R1 = OH, R2 = H).

producing innovative beers with distinct aromas.[24–26] Yet since non-volatile compounds are extracted during this process (humulinones,[27–29] starch-degrading enzymes,[30,31] hop esterases,[26] and flavor precursors[25,32,33]), hop flavan-3-ols are also expected to be imparted to the beer, although few studies have been conducted on this subject. Parkin and Shellhammer[34] report increases in beer total polyphenols (Bishop assay) after pilot-scale dry hopping under different conditions (a maximum increase of 100 mg/L for dry hopping with 16 g/L Chinook pellets for 72 h at 18 °C without agitation). They also measured total polyphenols in commercial beers before and after dry hopping but, in this case, an increase (up to 80 mg/L) was observed in only 10 out of 15 samples. Oladokun et al.[35] also report an increase in total polyphenol content after dry hopping, stating that dry hopping at 4 °C significantly reduces polyphenol extraction as compared to the same procedure at 20 °C. No studies have mentioned, however, the specific impact of dry hopping on beer flavan-3-ols monomers (source of colored dehydrodicatechins) and small oligomers (source of A derived hazing compounds).

To evaluate their initial content and their fate during aging, we have monitored the levels of catechin, epicatechin, procyanidin dimers (B1, B2, B3, and B4), and trimers (C2 and other non-identified trimers) by RP-HPLC-ESI(-)-MS/MS in five commercial pale-colored dry-hopped beers, through 24 months of storage. In these beers we have also monitored color, chill haze (measured after 24 h at 4 °C), total polyphenols, and total flavanoids (EBC methods 9.2, 9.29, 9.11, and 9.12, respectively). The relative impact of kettle and dry hopping on beer flavan-3-ols was further investigated in a pilot-scale beer production.

Experimental

Chemicals

Hydrochloric acid 37%, methanol, acetone, acetonitrile, *p*-dimethylaminocinnamaldehyde, 28–30% ammonia solution, 16% ammonium iron (III) citrate, formic acid, and ethylenediamine tetraacetic acid (EDTA) were purchased from VWR International (Leuven, Belgium); carboxymethylcellulose sodium salt, Sephadex LH-20, (+)-catechin, and (−)-epicatechin were purchased from Sigma-Aldrich (Overijse, Belgium); taxifoline, procyanidin B1, procyanidin B2, procyanidin B3, and procyanidin C1 were purchased from Extrasynthese (Rhône, France).

Beer samples

Five Belgian dry-hopped beers were investigated: St. Feuillien Grand Cru (A), St. Feuillien Saison (B), Vedett Extra

Ordinary IPA (C), Duvel Triple Hop—Citra (D) and IV Saison (E). Fresh samples (either provided by the brewers or purchased very fresh from stores) were stored for 24 months at 20 °C in the dark.

Color, chill haze, total polyphenol, and total flavanoid measurements

Prior to analysis, the beers were degassed by shaking. Color was measured by spectrophotometry at 430 nm (EBC method 9.2), chill haze (measured at 4 °C after 24 h at the same temperature) was determined by turbidimetry at 90° (EBC method 9.29) with a Ratio2000 Turbidimeter (HACH, Loveland, U.S.A.). Total polyphenols were quantitated by the Bishop assay (EBC method 9.11) and total flavanoids by the *p*-dimethylaminocinnamaldehyde method (EBC method 9.12).[36]

Extraction of flavan-3-ol monomers, dimers, and trimers from dry-hopped beers and wort

Extractions were performed according to the procedure of Callemien et al.,[37] slightly modified. Three grams of Sephadex LH-20 was packed in a 12-mL filtration tube with polyethylene frits (Supelco, Bellfonte, U.S.A.) and preconditioned for 4 h with methanol/water (30/70, v/v). The flux was set at 0.5 mL/min and 50 mL degassed beer (or wort) containing 2.8 mg/L taxifoline (internal standard) was loaded onto the Sephadex LH-20 column. The column was further washed with 40 mL methanol/water (30/70, v/v), and flavan-3-ols were then eluted with 70 mL acetone/water (70/30, v/v). The eluate was concentrated to dryness by vacuum rotatory evaporation (35 °C), resolubilized in 2 mL acetonitrile/water (30/70, v/v) and kept at −80 °C prior to analysis.

Pilot-scale production of a dry-hopped and a non-dry-hopped beer

Beers were produced in a 60-L microbrewery (Coenco, Oostkamp, Belgium). Pilsen malt (Boortmalt 2-row spring malt, Antwerpen, Belgium) was brewed in water (14.0 kg malt in 39 L water) according to the following mashing program: 60 min at 63 °C and 15 min at 72 °C. The wort was then heated to 78 °C and filtered through a lauter tun. After sparging, 61.8 L wort with a 14°P extract was obtained. At the beginning of wort boiling, 0.65 g/L Mandarina Bavaria hop (harvest 2020) was added to the kettle. Boiling lasted for 90 min (10% evaporation), and the final extract was adjusted to 19°P by addition of sugar. Then, 0.65 g/L Mandarina Bavaria (harvest 2020), 0.65 g/hL Citra (harvest 2020), and 0.65 g/hL Amarillo (harvest 2019) were added to the wort, which was clarified by gravity for 20 min, cooled, and transferred to two 60-L cylindroconical tanks. Fermentations were carried out for 7 days at 24 °C with an ale-type yeast (Saf Ale S-33, Fermentis, Belgium) added directly to each fermenter at 60 g/hL. For production of the dry-hopped beer, 0.65 g/L Mandarina Bavaria (harvest 2020), 0.65 g/hL Citra (harvest 2020), and 0.65 g/hL Amarillo (harvest 2019) were added to one of the two tanks on the third day of fermentation (pellets were put inside a permeable bag and introduced via the top of the tank). Maturation was conducted for 14 days at 16 °C in the presence of yeast. The beers were then cooled to 4 °C for two days, filtered on cellulose filter pads (8-µm pores followed by 0.5-µm pores, BuonVino, Cambridge, Canada) under a CO_2 atmosphere and bottle fermented for 15 days at 27 °C (addition of 12 g/L saccharose and 6.9 g/hL Saf Ale S-33). Wort samples were collected before the first hop addition at the beginning of boiling (wort1), before the second hop addition at the end of the boiling step (wort2), and after wort cooling (wort3). They were filtered through glass-fiber filters (3.0-µm pores, Gelman Sciences, Ann Arbor, U.S.A.) and stored at −20 °C prior to analysis. The produced dry-hopped (DH) and non-dry-hopped (NDH) beers were stored at 4 °C in the dark prior to analysis.

RP-HPLC-ESI(-)-MS/MS analysis of flavan-3-ol extracts

A SpectraSystem (Thermo Scientific, Waltham, U.S.A.) equipped with an SCM degasser, an AS3000 autosampler, and a p4000 quaternary pump was used. Separation was performed on a C18 Prevail column (150 × 2.1 mm, 3 µm, Hichrom, Berkshire, UK) with a binary eluent system consisting of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. Gradient elution was 97–91% A, 0–5 min, 91–85% A, 5–30 min, 85–67% A, 30–60 min, 67–0% A, 60–62 min, isocratic for 8 min, then return to the initial conditions for 15 min. The column was kept at 25 °C, the flow rate was 0.2 mL/min, and the injection volume was 10 µL. Mass spectra were acquired with a Finnigan LCQ Duo ion trap mass spectrometer (Thermo Scientific, Waltham, U.S.A.) equipped with an ESI source. Collision-induced dissociation spectra were recorded at a relative collision energy of 30, 35, and 40%, respectively, for singly charged $[M\text{-}H]^{-1}$ monomers ($m/z = 289$), dimers ($m/z = 577$ and 575), and trimers ($m/z = 865$ and 863). The ESI inlet conditions were as follows: source voltage 4.5 kW; capillary voltage −4 V; capillary temperature 250 °C; sheath gas 50 arbitrary units. The Xcalibur software version 1.2 (Thermo Scientific, Waltham, U.S.A.) was used to control the system and to record the chromatograms and spectra throughout elution. Compound identification was performed by injection of commercial standards (except in the case of procyanidins B4 and C2, which were identified on the basis of mass spectra and according to Callemien et al.[37]). Quantitations were carried out according to the calibration curves, relative to IST, of (+)-catechin, (−)-epicatechin, procyanidin B3 (used for B1-B4), and procyanidin C1 (used for all procyanidin trimers), with an IST relative recovery factor of 1.

Results and discussion

Color and chill haze stabilities of commercial pale-colored Belgian dry-hopped beers

Five bottle-refermented dry-hopped beers with 6.9 (beer A) to 15.7 (beer D) °EBC of color and with chill haze (a temporary precipitate in cold beer that resolubilizes upon warming) ranging from 1.4 (beer A) to 9.0 (beer B) °EBC (Table 1a) were monitored over 24 months of storage at 20 °C in the dark. Amber and brown dry-hopped beers were not included in this work so as to avoid the impact of roasted malt melanoidins on beer color.

During the first six months of storage, color and chill haze remained relatively stable, with minor increases in color (up to 1.3°EBC in beer C) and chill haze (up to 2.5°EBC in beer A, Figure 2a and 2b). Unexpectedly, the chill haze of beers D and E showed a slight drop, probably because of sedimentation of undissolved solid particles present in the fresh samples. After 24 months, however, perceptible increases in color (from 2.1°EBC in beers D and E to 6.4°EBC in beer A) and increases in chill haze (from 0.4°EBC in beer D to 25.7°EBC in beer A) were observed, and the correlation (R^2 = 0.86, Figure 2c) between the evolution of these two attributes evidenced a similar origin in beer. In general, the analyzed beers displayed fair physical stability over a short storage period (six months), but longer periods resulted in altered color and increased turbidity.

Total polyphenols and total flavanoids in fresh and aged commercial pale-colored Belgian dry-hopped beers

Compared to previous studies,[12,34,38,39] all fresh samples showed high levels of total polyphenols (EBC method 9.11), from 220 (beer E) to 358 mg/L (beer A), and of total flavanoids (EBC method 9.12), from 35 (beer E) to 65 (beer A) mg/L eq. catechin (Table 1b). Since the principles of the methods used for these two analyses are completely different (chelation of phenols with iron for total polyphenols and reaction of *p*-dimethylaminocinnamaldehyde with the A ring of either flavan-3-ol monomers or the upper extension unit of procyanidins for total flavanoids), no correlation between these two measurements was found.

Aging at 20 °C caused a decrease in total polyphenols and total flavanoids, especially between 6 and 24 months of storage (Figure 2d and 2e). Oxidative polymerization of flavanoids[4] and precipitation of procyanidins into haze[6,22,23] should logically account for most losses. Interestingly, the total polyphenol content of fresh dry-hopped beers correlated strongly with the development of chill haze (R^2 = 0.97) observed after 24 months of storage (Figure 2f) and might be used to estimate the physical stability of dry-hopped beers. Measurement of total flavanoids, however, did not provide any information about the evolution of the two studied parameters.

Flavan-3-ol monomer, dimer, and trimer stabilities in commercial pale-colored Belgian dry-hopped beers

Alongside the global EBC measurements, the stabilities of flavan-3-ol monomers (catechin and epicatechin), dimers (B1, B2, B3 and B4), and trimers (C2 and other non-identified trimers) were monitored by RP-HPLC-ESI(-)-MS/MS over the 24 months of storage (Table 1c and Figures 3 and 4). The flavan-3-ol chromatograms and mass spectra obtained for beer C (fresh and after 6 or 24 months of storage) are presented in Figure 3a–c.

Monomers ranged from 3.1 (beer D) to 6.6 mg/L (beer B) in fresh samples (Table 1c and Figure 4a), with a catechin/epicatechin ratio ranging from 2.5 (beer E) to 6.3 (beer B). Such high concentrations are not usual in non-dry-hopped beers, although sometimes observed.[40–42]

Table 1. (a) Physical attributes, (b) spectrophotometric analyses and (c) flavan-3-ol monomers, dimers, and trimers (mg/L) in fresh dry-hopped beers and after six (6 M) or twenty-four (24 M) months of storage at 20 °C in the dark.

	A			B			C			D			E		
	Fresh	6M	24M	Fresh	6M	24M	Fresh	6M	24M	Fresh	6M	24M	Fresh	6M	24M
(a) Physical attributes															
Color (°EBC)	6.9	7.0	13.3	14.6	14.5	20.6	7.0	8.3	11.0	15.7	16.5	17.8	10.7	10.2	12.8
Chill haze (°EBC)	1.4	3.9	27.1	9.0	8.8	25.4	2.3	2.9	8.5	3.2	2.1	3.6	4.9	2.5	3.8
(b) Spectrophotometric analyses (mg/L)															
Total polyphenols	358	312	288	312	319	228	278	272	239	237	249	129	220	214	173
Total flavanoids	65	57	41	60	51	30	62	55	49	60	47	26	35	36	20
(c) Flavan-3-ol monomers, dimers and trimers (mg/L)															
Catechin	5.1	6.0	3.7	5.7	4.6	2.0	3.9	3.6	2.8	2.2	2.9	2.1	3.0	3.3	2.5
Epicatechin	1.2	1.6	0.6	0.9	1.1	0.2	0.8	0.7	0.4	0.8	1.1	0.5	1.2	1.3	0.6
Procyanidin B1	1.9	1.8	0.4	1.7	0.8	0.2	2.0	1.0	0.9	1.1	0.4	0.3	0.5	0.5	0.1
Procyanidin B3	10.4	5.0	2.4	7.3	3.7	0.7	5.9	2.8	2.7	4.4	1.9	1.4	1.2	1.4	0.3
Procyanidin B4	0.9	0.6	0.2	0.3	0.3	nd	0.7	0.2	0.3	0.7	0.4	0.4	0.2	0.3	nd
Procyanidin B2	0.9	0.6	0.1	0.6	0.4	nd	0.6	0.3	0.2	0.5	0.3	0.2	0.3	0.3	nd
Procyanidin C2	4.6	1.1	0.2	2.2	0.4	nd	2.9	0.5	0.3	1.6	0.3	0.2	0.5	0.2	nd
Total monomers	6.3	7.6	4.3	6.6	5.7	2.2	4.7	4.3	3.3	3.1	4.0	2.7	4.2	4.5	3.1
Total dimers	14.1	8.0	3.1	9.9	5.2	0.9	9.2	4.3	4.1	6.7	3.0	2.3	2.2	2.5	0.4
Total trimers[a]	10.2	2.9	1.0	5.0	1.7	0.2	7.2	1.3	1.3	3.4	0.8	1.3	1.4	0.8	0.2
Total flavan-3-ols	30.6	18.5	8.4	21.5	12.6	3.3	21.1	9.9	8.7	13.2	7.8	6.5	7.8	7.8	3.7

Means of duplicates.
[a]The total trimers value is the sum of C2 and other non-identified procyanidin trimer concentrations; nd: not detected.

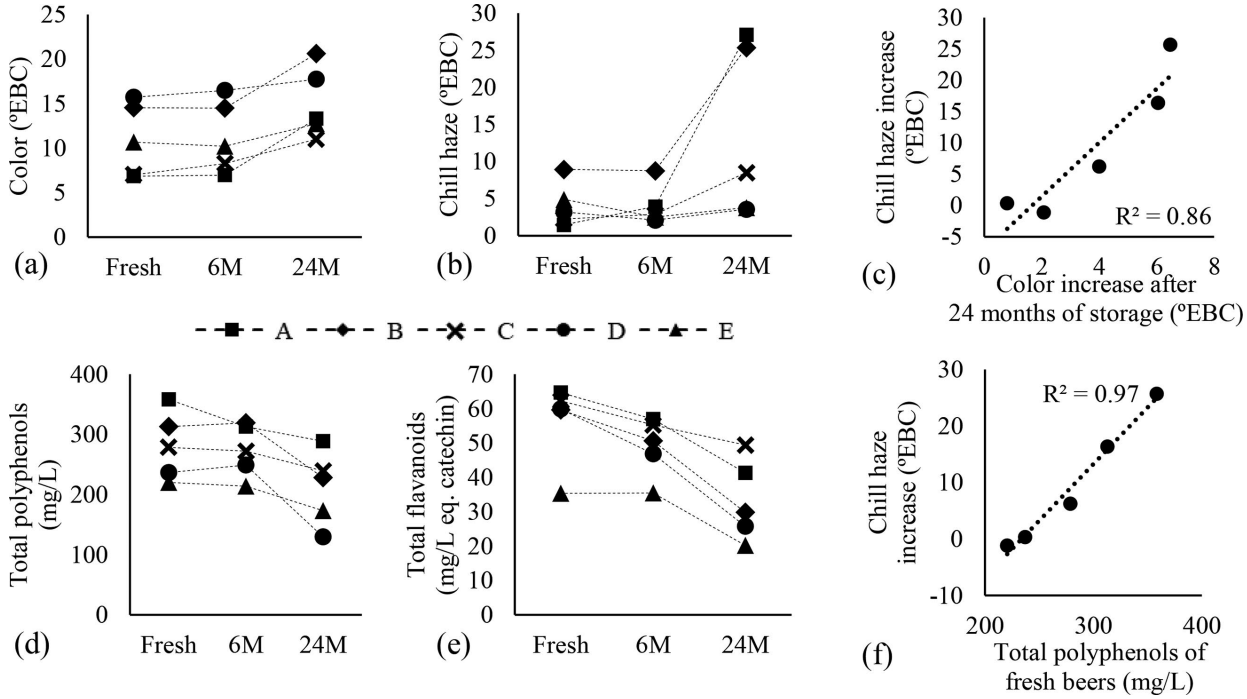

Figure 2. Evolution of (a) color and (b) chill haze in dry-hopped commercial beers over 24 months of storage at 20 °C in the dark and (c) correlation between them after 24 months of storage. Evolution of (d) total polyphenols (EBC method 9.11) and (e) total flavanoids (EBC method 9.12) in dry-hopped commercial beers over 24 months of storage at 20 °C in the dark and (f) correlation between the total polyphenol content of fresh dry-hopped beers and haze increase after 24 months of storage.

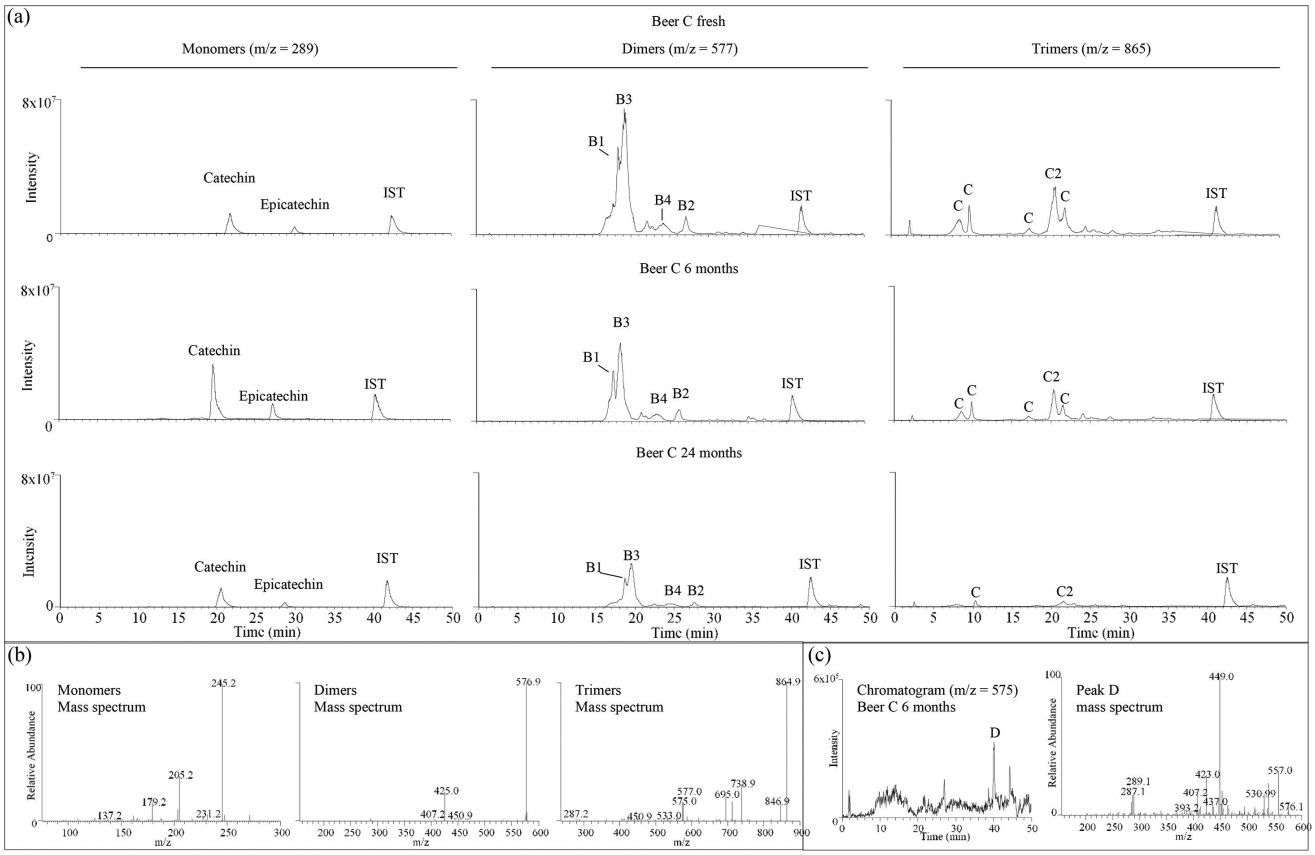

Figure 3. (a) Chromatograms of flavan-3-ol monomers (m/z = 289), procyanidin dimers (m/z = 577), and procyanidin trimers (m/z = 865) in beer C, fresh and after 6 or 24 months of storage at 20 °C in the dark; (b) mass spectra of flavan-3-ol monomers (m/z = 289), procyanidin dimers (m/z = 577), and procyanidin trimers (m/z = 865); (c) chromatogram (m/z = 575) of oxidized dimers in beer C after 6 months of storage and mass spectrum of peak D, tentatively identified as dehydrodicatechin A.

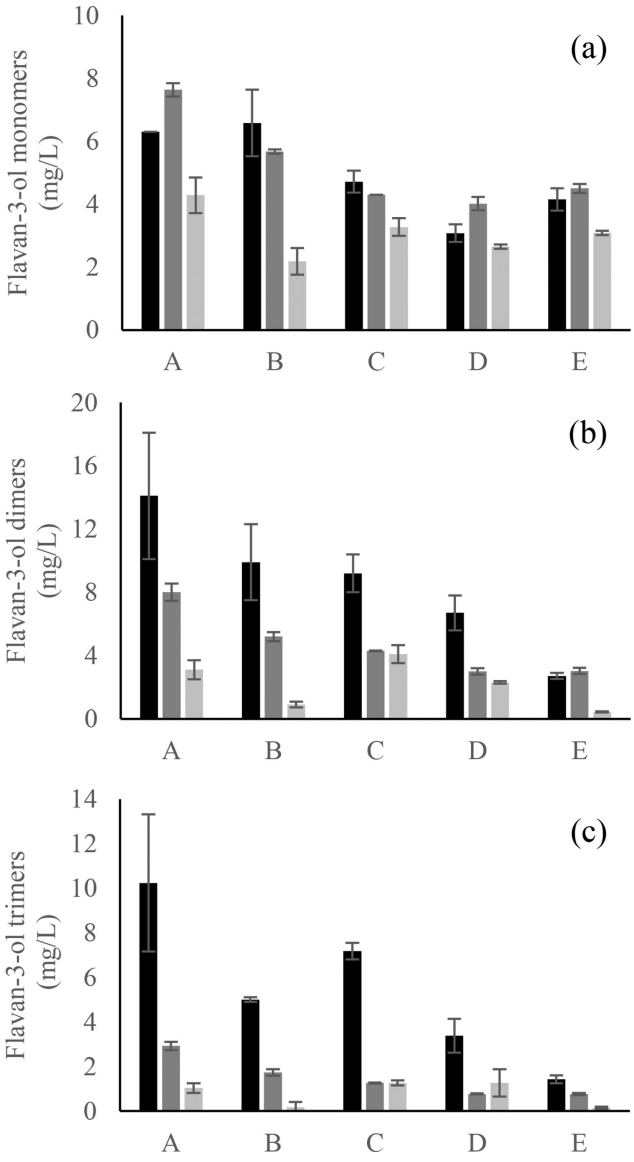

Figure 4. Concentration, in mg/L, of flavan-3-ol (a) monomers, (b) dimers, and (c) trimers in fresh dry-hopped beers (black bars) and after 6 months (dark gray bars) or 24 months (light gray bars) of storage at 20 °C in the dark. Means of duplicates.

A coefficient of determination of only 0.75 was obtained for the correlation between monomers and total polyphenol values (EBC method 9.11).

Over the first six months of storage, the total amount of monomers remained stable (except in beers A and D, where respective increases of 1.3 and 0.9 mg/L were observed, Figure 4a). This apparent stability of monomers is in accordance with previous studies[4,43] and should be due mostly to depolymerization of procyanidins to catechin and epicatechin. After 24 months, however, we observed an average 34 ± 58% decrease in the level of monomers, ranging from 13% in beer D to 67% in beer B (Figure 4a), with an increase of the catechin/epicatechin ratio in all samples. Such high variability among samples may be due to the different levels of reducing compounds, dissolved oxygen, and metallic ions (ability to protect against quinone production), and to the regeneration of monomers from oligomers according to their initial levels.

Procyanidin dimers and trimers were detected in fresh dry-hopped beers at levels ranging respectively from 2.2 and 1.4 mg/L (beer E) to 14.1 and 10.2 mg/L (beer A). B3 and C2 (up to 10.4 and 4.6 mg/L in beer A, respectively) were, logically, the most abundant oligomers, but procyanidins containing epicatechin in their structure were also present (Table 1b) and must have derived from the hop additions. The levels of procyanidin detected in the investigated dry-hopped beers seemed unusually high as compared to non-stabilized lager beers, in which McMurrough et al.[40] and Madigan et al.[44] quantified only 1.7–3.1 mg/L procyanidin B3 and Callemien et al.[42] detected 6.5 mg/L dimers and 1.3 mg/L trimers. The total polyphenol value (EBC method 9.11) measured for each dry-hopped beer was well correlated with the dimer content (R^2 = 0.88).

After six months of storage, degradation of up to 55% of dimers and 82% of trimers was already observed (Figure 4b and 4c). The lesser apparent degradation of dimers was probably due to their partial regeneration through depolymerization of higher oligomers. In beers with a very low dimer level before aging, this could even lead to a concentration increase (e.g., from 2.2 to 2.5 mg/L in beer E after 6 months of storage). Procyanidin degradation continued during storage, and after 24 months, an average decrease of 74 ± 14 for dimers and 83 ± 13% for trimers was observed. Compared to monomer degradation, dimer and trimer degradation after 24 months was more consistent among samples (the standard deviation was lower).

Relationship between flavan-3-ol levels and the development of color and chill haze in commercial pale-colored Belgian dry-hopped beers

The above-discussed investigation of color, haze, and flavan-3-ols (monomers and oligomers) in fresh and aged beers led to identifying interesting trends.

First, the color increase observed after 24 months of storage correlated strongly with the monomer content of fresh samples (Figure 5a, R^2 = 0.96, against R^2 = 0.62 with oligomers). As previously described by Callemien et al.,[4] oxidative polymerization of monomers (catechin or epicatechin) results in a yellow-brown compound known as dehydrodi(epi)catechin A, responsible for a color increase during storage. This oxidation product was tentatively identified in the beer C chromatogram after six months of storage (Figure 3c, as compared to the mass spectrum of dehydrodicatechin A from Callemien et al.[4]).

On the other hand, the formation of chill haze after 24 months of storage correlated both with the level of monomers or oligomers (sum of dimers and trimers) in fresh dry-hopped beers (Figure 5b, R^2 = 0.79 with oligomers and 0.78 with monomers) and with the oligomer degradation rate during storage (R^2 = 0.94 with oligomers against 0.37 with monomers), Figure 5c). Interestingly, the linear regression of this last correlation suggests that each mg/L of oligomer lost during storage increased the chill haze by 1.7°EBC.

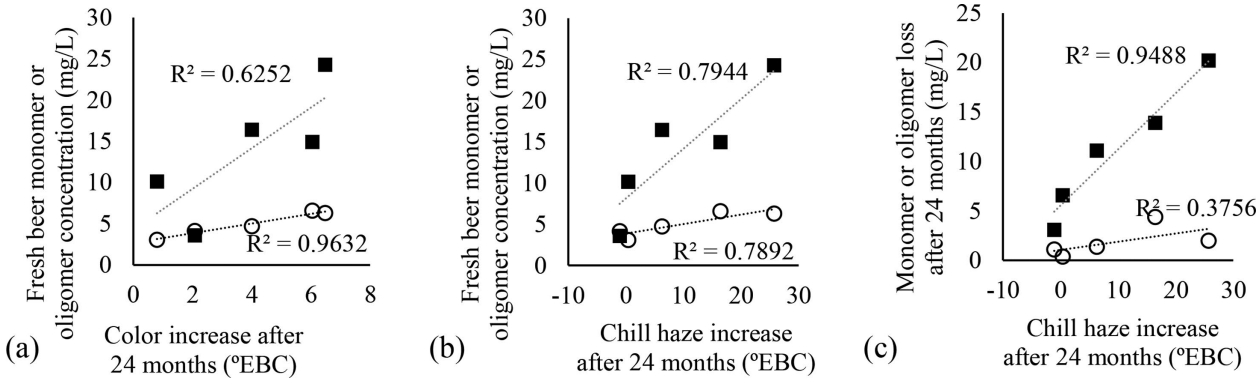

Figure 5. Correlations between (a) the flavan-3-ol monomer (o) or oligomer (■, sum of procyanidin dimers and trimers) concentration in fresh dry-hopped beers and the color increase after 24 months of storage, (b) the monomer (o) or oligomer (■) concentration in fresh dry-hopped beers and the chill haze increase after 24 months of storage, and (c) the chill haze increase and the loss of monomers (o) or oligomers (■) after 24 months of storage.

Suspected of being the main derivatives that participate in beer haze,[22,23] A-type dimers (m/z = 575) and trimers (m/z = 863) were also investigated in aged beers by RP-HPLC-ESI(-)-MS/MS. The propensity of these molecules to form haze probably hindered their detection, however, as no A-dimer peaks were found in the chromatogram of any beer.

The role of flavan-3-ols in dry-hopped beer quality is paradoxical to say the least. Their occurrence is clearly responsible for color (monomers) and colloidal (oligomers) instability of beers after two years of storage. Yet, flavan-3-ols are potent antioxidants of beer[3,15,18–20] and limit free radical propagation by scavenging reactive oxygen species. From a quality perspective, only products with short shelf-life can benefit of their antioxidant properties without the consequences for physical instability.

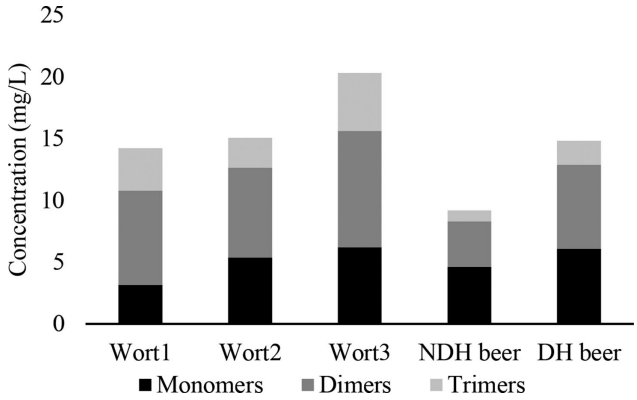

Figure 6. Concentration of flavan-3-ol monomers, dimers, and trimers throughout pilot-scale production of dry-hopped and non-dry-hopped beers.

Impact of early, late, and dry-hop additions on beer flavan-3-ol monomers, dimers, and trimers

Pilot-scale beers were produced in order to better understand how each hop addition imparts flavan-3-ol monomers and oligomers to beer (Figure 6). In keeping with the nature of malt flavan-3-ols, catechin, procyanidin B3, and procyanidin C2 accounted for 2.8 of the 3.2 mg/L monomers, 6.9 of the 7.6 mg/L dimers, and 2.7 of the 3.4 mg/L trimers in the wort before boiling (wort1). The small amounts of epicatechin and its oligomers detected at this point likely resulted from epimerization of (+)-catechin to (+)-epicatechin (its unnatural epimer).[45]

Following the addition of 0.65 g/hL of Mandarina Bavaria (wort2) followed by 90 min of boiling, it was observed that monomers increased by 2.2 mg/L. Dimer and trimer concentrations were shown to decrease by 0.3 and 1.0 mg/L, respectively. Procyanidin losses through oxidation and linkage to coagulated proteins are expected to happen during boiling. Yet depolymerization to monomers[10,12,46,47] also occurs, explaining the greater increase in monomers than expected from the amounts of catechin and epicatechin available in Mandarina Bavaria hop (2.75 times its total content, Table 2).

The late hop addition (wort3), on the other hand, mainly imparted dimers (2.1 mg/L) and trimers (2.3 mg/L) to the wort, with extraction yields of 53 and 66%, respectively. Monomers were less extracted (extraction yield: 35%; level increased by only 0.8 mg/L) and oligomer depolymerization was probably unsignificant during these last 20 min at 80–85 °C. In other words, while early hop addition increases the levels of monomers responsible for color development during storage, late hopping imparts mainly oligomers, related to colloidal instability.

Cold maturation and filtration over cellulose plates caused, as expected, a major decrease in flavan-3-ols, as observed in the non-dry-hopped beer (NDH in Figure 6 and Table 2). This loss increased with the degree of polymerization from monomers (26%) to dimers (60%) and trimers (80%). In addition to cellulose adsorption, precipitation to chill haze probably also accounted for losses and explains why monomers were less impacted than dimers and trimers.

After the third day of fermentation, one of the beer tanks was dry hopped with 0.65 g/L Mandarina Bavaria, 0.65 g/hL Citra, and 0.65 g/hL Amarillo hops (DH beer in Table 2). This hop addition promoted a 61% increase in the total level of flavan-3-ols (an extra 1.5 mg/L monomers, 3.1 mg/L

Table 2. Concentrations (mg/L) of flavan-3-ol monomers, dimers and trimers throughout the pilot beer production and their availability from hops during early or late and dry hopping (see supporting information 2); means of duplicates.

Compound	Wort 1	Wort 2	Wort 3	NDH beer	DH beer	**Availability during early hopping**[a]	**Availability during late/ dry hopping**[b]
Catechin	2.8	3.8	4.2	3.3	4.2		
Epicatechin	0.4	1.6	2.0	1.3	1.9		
Procyanidin B1	0.2	1.4	1.4	0.7	1.2		
Procyanidin B3	6.9	5.4	4.9	1.8	3.5		
Procyanidin B4	nd	nd	0.8	0.3	0.6		
Procyanidin B2	0.5	0.5	0.7	0.3	0.4		
Procyanidin C2	2.7	1.2	1.6	0.4	0.7		
Total monomers	3.2	5.4	6.2	4.6	6.1	0.8	2.4
Total dimers	7.6	7.3	9.4	3.7	6.8	1.6	4.1
Total trimers[c]	3.4	2.4	4.7	0.9	1.9	1.4	3.4
Total flavan-3-ols	**14.2**	**15.1**	**20.3**	**9.2**	**14.8**	**3.8**	**9.9**

Wort results were normalized to the volume of the wort1.
[a]Calculated for 65 g/hL Mandarina Bavaria.
[b]Calculated for 65 g/hL Amarillo, 65 g/hL Citra, and 65 g/hL Mandarina Bavaria.
[c]The total trimers value is the sum of C2 and other non-identified procyanidin trimers concentrations; nd: not detected.

dimers, and 1.0 mg/L trimers) compared to the NDH beer. During dry hopping, monomers and dimers were easily extracted (respective yields: 62 and 76%), which explains the high levels detected in commercial pale-colored dry-hopped beers. Trimers, on the other hand, are less polar and thus showed a much lower extraction yield (29%).

Conclusion

Pale-colored Belgian dry-hopped beers contain comparatively high levels of flavan-3-ol monomers, dimers, and trimers (up to 6.3, 14.1, and 10.2 mg/L, respectively, in the commercial samples investigated here), as these compounds are efficiently extracted during dry hopping (extraction yields of 62, 76, and 29% for monomers, dimers, and trimers, respectively, if performed during primary fermentation as is usual in Belgian breweries). When added into the kettle, hop also imparts flavan-3-ols to the wort, mainly monomers upon early hopping and dimers and trimers upon late hopping.

Amounts of polyphenols in fresh dry-hopped beers emerged as very good markers of physical instability during storage. Monomers induced a color increase and procyanidin dimers and trimers promoted chill haze formation. Less expensive than RP-HPLC-ESI(-)-MS/MS quantitations, the colorimetric measurement of total polyphenols (Bishop assay) in fresh dry-hopped beers remains a good indicator of the evolution of these two attributes. Note that despite these two negative attributes, polyphenols remain among the best natural antioxidants. Their impact is very positive through mashing and boiling where they protect the wort against lipid oxidation (source of cardboard off-flavor).[16,48] Unfortunately, in the final beer, their oxidized forms will badly modify color, haze and also, probably, astringency.

In order to minimize the flavan-3-ol level in dry-hopped beers, brewers might use CO_2 extracts in the kettle and choose hop varieties with a low flavanoid content for dry hopping. Yet considering that other extractible non-volatiles from hop also constitute a threat for beer stability (humulinones,[27–29,49] starch-degrading enzymes,[30,31] and hop esterases[26]), we conclude that in order to produce stable dry-hopped beers, innovative hop extracts free of these harmful non-volatiles should be developed for use in dry hopping.

Acknowledgments

We are indebted to the Brasserie de Jandrain-Jandrenouille and the Brasserie St-Feuillien for kindly providing fresh beer samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Carlos Silva Ferreira http://orcid.org/0000-0003-3760-517X

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