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Why Oxidation Should Be Still More Feared in NABLABs: Fate of Polyphenols and Bitter Compounds

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Abstract: Nowadays, non-alcoholic (NAB) and low-alcoholic beers (LAB) still significantly suffer from staling defects when fresh, partially due to absence of ethanol as antioxidant. In the current work, the fate of flavan-3-ols (monomers, dimers, and trimers) and bitter compounds (isohumulones, humulinones, etc.) of 11 commercial NABLABs available on the Belgian market was monitored through one year of aging at 20 °C in the dark. Fresh NABLABs contained variable flavan-3-ols and bitter compounds levels (between 3.0–10.0 mg/L and 8.0–39.0 mg/L, respectively), depending on different technological processes used. Chill haze and color were also investigated as potential oxidation markers of fresh and aged beers. Surprisingly, contrary to conventional beers, the oligomers' concentration (dimer and trimer procyanidins) exhibited a strong correlation (R^2 = 0.95) with chill haze before aging, suggesting prematured oxidation of the samples. After a year of storage, significant degradation occurred as for regular dry hopped beers (process very sensitive to oxidation), only 27% remaining for flavan-3-ol dimers and an average 16% for *trans*-isohumulones. Oxidation risk appears here as the main weakness of NABLABs, which could be probably improved by spiking very efficient antioxidants.

Keywords: NABLABs; flavan-3-ols; isohumulones; oxidation; color; chill haze

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1. Introduction

A growing interest for non-alcoholic (NAB, ≤0.5% (*v*/*v*)) in most of the European Union, EU) and low-alcoholic beers (LAB, >0.5–1.2%, (*v*/*v*) in most of the EU) emerged in the last decade [1]. They meet the restrictions in terms of road safety, work rules, or even religious grounds [1–3] and likewise become alternative drinks for sportsmen, pregnant women, or people under medication [2].

Two main types of technological processes are used to produce NABLABs: physical and biological methods [1–3]. Physical methods remove the alcohol from conventional beer by thermal systems (e.g., vacuum distillation) or membrane separation (e.g., reverse osmosis). On the other hand, biological approaches limit ethanol formation during beer fermentation by adjusting process conditions [1–3] (e.g., cold contact fermentation [2,4] or use of special microorganisms [5], etc.). Unfortunately, most NABLABs already exhibit major staling defects when fresh.

NABLABs suffer from a lack of fruity fermentation aromas, and a persistent worty taste attributable to methional (low perception threshold of 0.47 µg/L) [6–9]. Recent studies also highlight that other stale odorant compounds could eventually participate in fresh NABLABs' flavor: sotolon (curry, madeira), phenylacetaldehyde (floral, honey), (E)-β-damascenone (cooked apple), and dimethyltrisulfide (onion, garlic) [9–11].

However, the main weakness of NABLABs remains the absence of ethanol. As a good radical scavenger of hydroxyl radicals (HO°) and other reactive oxygen species ROS (e.g., $O_2^{\circ -}$, HOO° issued from metal-induced Fenton and Haber-Weiss reactions [12–16]), it can prevent volatile and non-volatile compounds to be oxidized during storage in regular

beer. Polyphenols and bitter compounds are recognized to be particularly sensitive to degradation during beer storage, especially in dry hopped beers [17–21]. In NABLABs, we can suspect that they will be still less protected in the absence of radical scavengers.

Polyphenols, secondary plant metabolites, originating from both malt (70%) and hops (30%) [22], are natural and potent antioxidants [20,23,24], and particularly those of low molecular weight [12,22]. Unfortunately, they also contribute to colloidal haze, astringency, and color during beer storage [25–27]. Dissolved oxygen, agitation, light, metallic ions (iron and copper), pH, and high temperature are known to strongly accelerate aging [28]. During aging, loss of monomers ((+)-catechin and (-)-epicatechin) and dimers often occurs in the first 4–5 weeks [19], while derived «tannoids» rise later [25]. Oligomeric flavan-3-ols (dimers and trimers, monomers to a lower extent [29]) are suspected to be oxidized during the lag time (creating small A-type derivatives once oxidized) [27–29], further leading to a visible physicochemical haze as the result of non-covalent interactions with malt proteins (rich in proline) [28–31]. According to Guyot et al. [32] and Callemien et al. [25], only natural monomers participate in the color. Once oxidized to *o*-quinones or semiquinones by free radicals, they react together to form colorless "chemical" dimers (polar dehydrodi(epi)catechins) [20] further dehydrated into yellow-brown-colored dehydrodi(epi)catechins A [25,32]. To avoid the occurrence of these haze- and color-active molecules, the addition of other antioxidants, such as sulfites, ascorbic acid, gallic acid, glutathione, and other yeast-derived antioxidants, is possible [33].

Isohumulones (co-, n-, and ad-analogues of *cis*- and *trans*-isomers) rapidly degrade in the presence of ROS and generally undergo autoxidation during storage [12,15,17]. *trans*-Isohumulones, much less stable than their isomer [17], mainly deteriorate by proton-catalyzed cyclization, leading to the harsh and lingering bitter-tasting tricyclohumols, tricyclohumenes, isotricyclohumenes, tetracyclohumols, epitetracyclohumols, and scorpiohumols [18,34,35]. *cis*-Humulinones can also be degraded, leading to similar cyclized derived products [35].

The aim of the present work was to investigate the fate of polyphenols and bitter compounds of 11 commercial NABLABs through one year of storage at 20 °C in the dark. Chill haze and color were also monitored. Standard global quantitation methods (total polyphenols and total flavanoids) were first applied. Then, flavan-3-ols and bitter compounds levels were determined by Reverse-Phase High-Performance Liquid Chromatography (HPLC) with ESI(-)-MS/MS and UV detections. The fate of these attributes was further briefly compared to similar data recently published for the top dry hopped fermentation beers [18,21].

2. Materials and Methods

2.1. Chemicals

Acetone, acetonitrile, ammonia solution 28–30%, citric acid monohydrate, formic acid 99–100%, hydrochloric acid 37%, isooctane, and methanol were purchased from VWR International (Leuven, Belgium). Ammonium iron (III) citrate 16% and titriplex III were purchased from Merck (Darmstadt, Germany). Carboxymethylcellulose sodium salt and *p*-dimethylaminocinnamaldehyde were purchased from Sigma-Aldrich (St.-Louis, MO, USA). Potassium hydroxide was from Chem Lab (Zedelgem, Belgium). (±)-Catechin hydrate (purity superior to 98%) and (-)-epicatechin (purity superior to 90%) standards were also purchased from Sigma-Aldrich (Overijse, Belgium). Procyanidins B2 (purity superior to 90%), B3 (purity superior to 95%), and C1 (purity superior to 90%), as well as (+)-taxifolin (purity superior to 99.9%) standards, were from Extrasynthèse (Genay, France). Humulones and lupulones mixture standard (ICE-3), isohumulone standard (ICS-I3), and tetrahydro-isohumulone standard (ICS-T2) were purchased from Labor Veritas Co. (Zürich, Switzerland). The humulinone standard (purity superior to 95%) was produced in our laboratory following the method proposed by Cook et al. [36]. The hulupone standard (purity of 62.2%) was kindly provided by Hopsteiner (Mainburg, Germany). Milli-Q water was used (Millipore, Bedford, MA, USA).

2.2. Beer Samples

In total, 11 commercial NABLABs were investigated: Star Light (A), Energibajer (B), Pico Bello (C), Leopold 7 Road Trip (D), Palm N.A. (E), Maes 0.0% (F), Hoegaarden rosée 0.0% (G), Carlsberg 0.0% (H), Jupiler 0.0% (I), Leffe Blonde 0.0% (J), and Brugse Sport Zot alcoholvrij (K). Beers were received from brewers or bought in the market (freshly released), and the majority were in bottles except for Pico Bello and Leopold 7 Road Trip, which were in 33 cl cans. All beers, from the same batch, were stored for one year at 20 °C in the dark and analyzed in duplicate (before and after aging).

2.3. Standard Analyses

Prior to analysis, beers were degassed by shaking and filtered through paper filters (MN 614 $^1/_4$ Macherey-Nagel, Düren, Germany) except for haze, total polyphenols, and total flavanoids measurements. Chill haze (measured at 4 °C after 24 h at the same temperature) was determined according to an Analytica-EBC method 9.29 by using a Ratio2000 Turbidimeter (HACH, Loveland, CO, USA) [37]. Bitterness, total polyphenols, total flavanoids, and color were measured by means of Analytica-EBC methods 9.8, 9.11, 9.12, and 9.6 [37].

2.4. Solid-Phase Extraction of Beer Flavan-3-ols

Beer flavan-3-ols (monomers, dimers, and trimers) were extracted as described by Callemien and Collin [25]. Three grams of Sephadex LH-20 resin (Sigma-Aldrich, St.-Louis, MO, USA) packed in a 12 mL filtration tube SPE with a polyethylene frit (Supelco, Bellfonte, PA, USA) was preconditioned for 4 h with methanol: water (30:70, v/v). The flux was set at 0.5 mL/min. After loading 50 mL of degassed beer containing 2.8 mg/L of IST ((+)-taxifolin), the column was washed with 40 mL of methanol: water (30:70, v/v). Flavan-3-ols were recovered with 70 mL of acetone: water (70:30, v/v). The eluate was concentrated to dryness by vacuum rotary evaporation (35 °C) and dissolved in 2 mL of acetonitrile: water (30:70, v/v). The extracts were kept at −80 °C prior to analysis.

2.5. RP-HPLC-ESI(-)-MS/MS Analyses of Flavan-3-os

A SpectraSystem (Finnigan MAT, San Jose, CA, USA) equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump was used. A 150 × 2.1 mm, 3 µm C18 Prevail column (HICHROM, Deerfield, MA, USA) was used at a flow rate of 0.2 mL/min. Chromatographic separation was obtained using a multilinear gradient of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Gradient elution was 97–91% A, 0–5 min; 91–85% A, 5–30 min; 85–67% A, 30–60 min; 67–0% A, 60–70 min; 0–97% A, 70–75 min; and then return to the initial conditions for 15 min. Ten microliters of beer extract were injected into the column kept at 25 °C. Mass spectra were acquired with an LCQ Duo ion trap mass spectrometer 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 ions $[M\text{-}H]^{-1}$ of monomers (m/z = 289), dimers (m/z = 577), and trimers (m/z = 865). The following ESI (negative mode) inlet conditions were applied: source voltage, 4.5 kV; capillary voltage, −4 V; capillary temperature, 250 °C; and sheath gas, 50 arbitrary units. The system was controlled with Xcalibur software, version 1.2. Compound identification of monomers, B2, B3, and C1 was done by injection of commercial standards. Procyanidins B1, B4, and C2 were identified based on mass spectra and according to Callemien and Collin [26]. Quantitation was achieved using the calibration curves of (±)-catechin, (-)-epicatechin, procyanidin B3 (also used for B1, B2, and B4), and procyanidin C1 (used for all trimers) standards (at 25, 50, 75, 100 mg/L). The relative recovery between compounds and the internal standard was set at 1.

2.6. Analyses of Bitter Compounds by RP-HPLC-UV

Beer samples 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). 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, USA), using the binary solvent system of Analytica-EBC method 9.47 [37] with A: methanol; B: 1% aqueous citric acid solution (pH 7.0): acetonitrile (70:30, *v*/*v*). Compared to EBC method 9.47 [37], 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, Milford, MA, USA). 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. Quantitation was performed using a single point calibration, as suggested by EBC method 9.47 [37]. 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.

3. Results and Discussion

3.1. Chill Haze, Color Stability, Polyphenols in Fresh NABLABs, and Their Fate through Aging

Our panel of lager (A, F, H, and I), amber (E and K), white (G), abbey (J), and dry hopped (B, C, and D) fresh NABLABs explains the large distribution obtained for color (5–20 °EBC) (Figure 1a). Most fresh beers showed important chill haze (most between 0.5 (E) and 5.8 °EBC (B)), with values up to 17.1 °EBC for beer D (can refermentation) and 18.0 °EBC for beer G (non-filtered white beer) (Figure 1b) [11]. Six months of aging at 20 °C induced an increase of color and chill haze in all beers (respectively up to 8.1 °EBC in K and 4.4 °EBC in D). The next six months still slightly increased both values in most beers (respectively up to 0.5 °EBC more in K and 3.2 °EBC more in D) (Figure 1a,b).

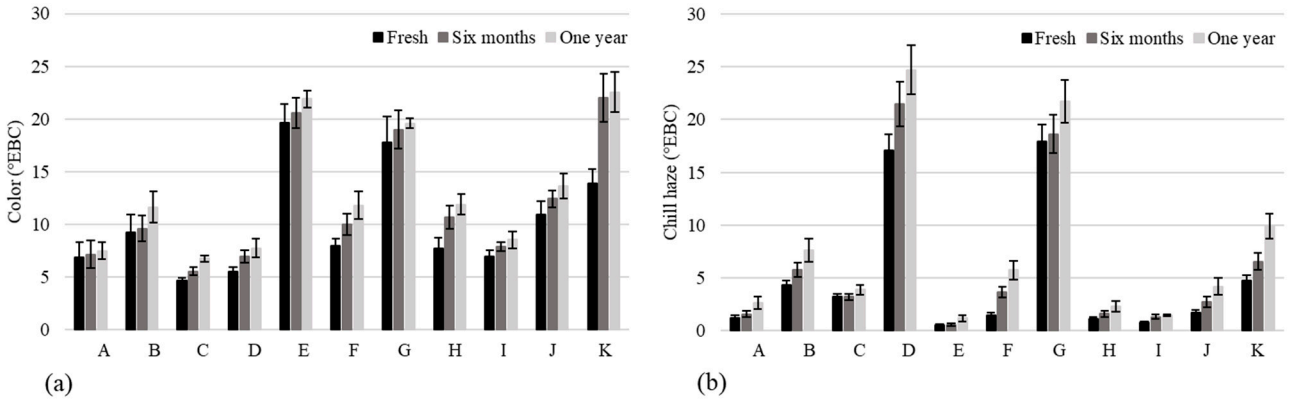


Figure 1. Evolution of color (°EBC) (**a**) and chill haze (°EBC) (**b**) of 11 commercial NABLABs through one year of storage at 20 °C in the dark.

Chill haze and color are known to be oxidation markers of a beer. As recently shown for dry hopped beers [21], catechin and epicatechin mainly impact color (synthesis of dehydrodi(epi)catechins A by oxidation), while flavan-3-ols oligomers (dimers and trimers, especially those including an epicatechin fragment) increase colloidal instability (synthesis of A-type oligomers by oxidation).

Spectrophotometric global methods were first applied to assess the fate of polyphenols. As depicted in Figure 2a,b, total polyphenols and total flavanoids remained relatively stable during six months of storage (87 and 84% remaining on average) except in a few samples

(e.g., drop of 125 and 20 mg/L in beer E; 172 and 23 mg/L in beer J). A similar trend was observed between 6 and 12 months of aging (45% of total polyphenols remaining in C and 43% of total flavanoids in A compared to the fresh samples). Degradation of both indicators suggested global oxidation instead of depolymerization (mean degree of polymerization (total polyphenols/total flavanoids ratio) = 4–5 on average, both for fresh and aged samples).

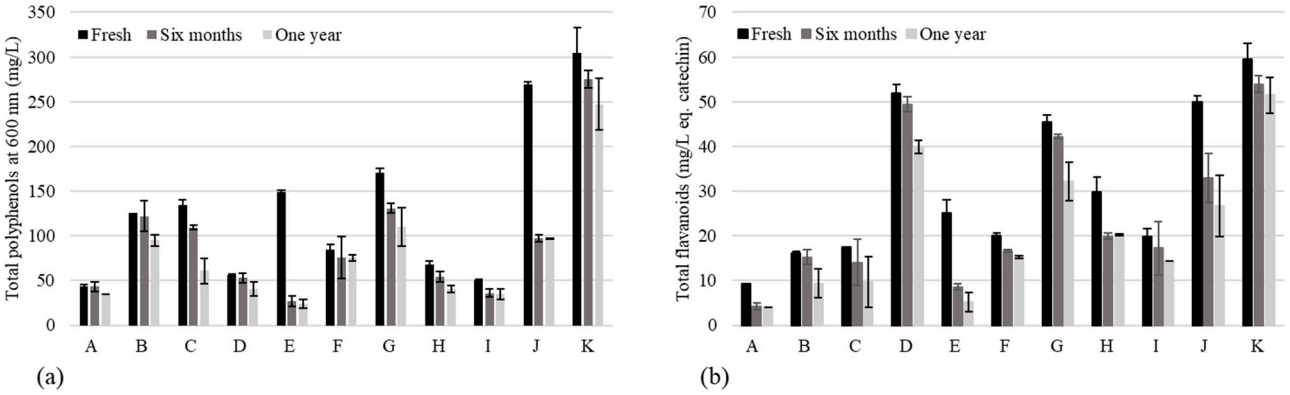


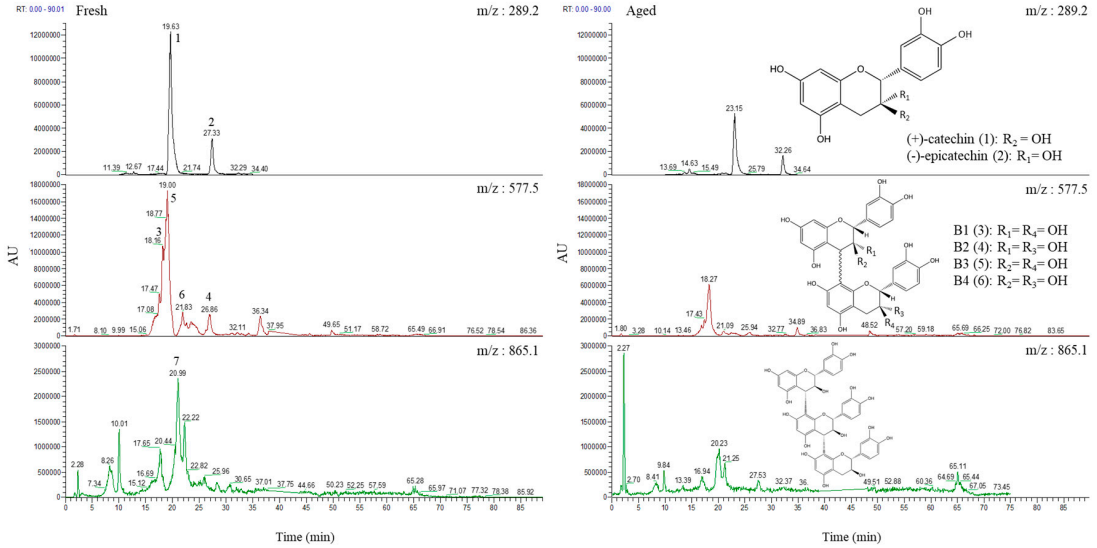
Figure 2. Evolution of total polyphenols (mg/L) (**a**) and total flavanoids (mg/L, eq. catechin) (**b**) of 11 commercial NABLABs through one year of storage at 20 °C in the dark.

In parallel, flavan-3-ol monomers ((+)-catechin and (-)-epicatechin), dimers (B1, B2, B3, and B4), and trimers (C2 and other unidentified trimers) were individually quantitated by RP-HPLC-ESI(-)-MS/MS (Table 1). Monomers were found in fresh NABLABs at amounts ranging from 1.2 (I) to 4.7 mg/L (C) (Figure 3b), with catechin/epicatechin ratios from 1.4 (E) to 6.0 (H). Procyanidin dimers and trimers ranged from 1.1 and 0.1 mg/L to 5.5 and 1.1 mg/L, respectively (Figure 3c,d). B3 and C2 emerged as the most abundant oligomers (up to 69% and more than 50% of the total, respectively), but procyanidins containing an epicatechin unit in their chemical structure (B1, B4, and B2) were also detected (less than 1 mg/L except for beer B) (Table 1). Such values are very similar to those usually reported in conventional lagers [38–40], except for beers B, C, and D, in which dry hopping brought both monomers and oligomers (values up to 10.2 mg/L in total [21]), and in beer K, where four different special malts were used.

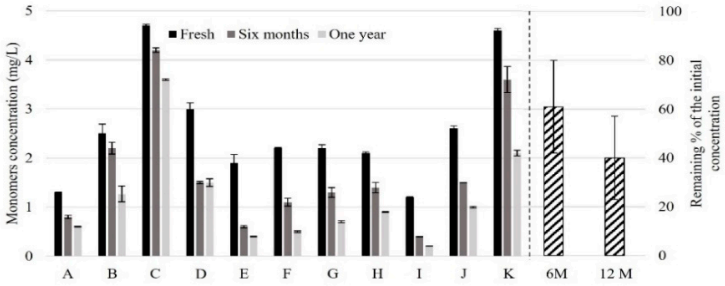
Table 1. Concentrations (mg/L) of (+)-catechin; (-)-epicatechin; dimers B1, B2, B3, and B4; total dimers (S/N > 9); trimer C2; and total trimers (S/N > 9) in the 11 fresh and aged (12M) investigated NABLABs. Values in parentheses indicate remaining percentages after one year of storage at 20 °C in the dark.

	Biological Processes										**Physical Processes**											
	Special Yeast						**Mixed Fermentation**		**Cold Contact**		**Distillation**										**Membrane Filtration**	
Compound	**A**		**B** ▲		**C** ▲		**D** *▲		**E**		**F**		**G**		**H**		**I**		**J**		**K**	
	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M
(+)-Catechin	1.0 +	0.5 (50)	1.5 +	0.8 (51)	3.5 +	2.7 (77)	2.1	1.1 (52)	1.1 +	0.3 (27)	1.4	0.4 (29)	1.6	0.6 (37)	1.8	0.7 (39)	0.9 +	0.1 (11)	1.9	0.7 (37)	3.6 +	1.8 (50)
(-)-Epicatechin	0.3 +	0.1 (33)	1.0	0.5 (51)	1.2 +	0.9 (75)	0.9 +	0.4 (44)	0.8	0.1 (12)	0.8	0.1 (12)	0.6	0.1 (17)	0.3	0.2 (67)	0.3	0.1 (33)	0.7	0.3 (43)	1.0	0.3 (30)
Total monomers	1.3 +	0.6 (46)	2.5 +	1.3 (51)	4.7 +	3.6 (77)	3.0	1.5 (50)	1.9 +	0.4 (21)	2.2 +	0.5 (23)	2.2 +	0.7 (32)	2.1 +	0.9 (43)	1.2 +	0.2 (17)	2.6 +	1.0 (38)	4.6 +	2.1 (46)
Procyanidin B1	0.3	0.1	1.8	0.4	0.6	0.3	0.2	0.1	0.2	nd	0.2	nd	0.5	0.1	0.6	0.1	0.3	nd	0.4	0.1	0.9	0.4
Procyanidin B2	0.1	nd	0.6	0.1	0.3	nd	0.3	0.1	0.1	nd	0.1	nd	0.1	nd	0.2	nd	0.1	nd	0.1	nd	0.4	nd
Procyanidin B3	1.1	0.4	2.0	0.9	1.0	0.8	1.2	0.7	0.7	0.1	0.9	0.3	0.9	0.1	1.0	0.2	0.7	0.1	1.1	0.3	2.6	1.0
Procyanidin B4	0.1	nd	1.1	0.3	0.5	0.2	0.4	0.2	0.1	nd	0.1	nd	0.2	nd	0.2	nd	0.1	nd	0.2	nd	0.7	0.1
Total dimers ♦	1.6 +	0.5 (31)	5.5 +	1.7 (31)	2.4 +	1.3 (54)	2.0+	1.1 (55)	1.1	0.1 (9)	1.3	0.3 (23)	1.7	0.2 (12)	2.0 +	0.3 (15)	1.2+	0.1 (8)	1.8 +	0.4 (22)	4.6 +	1.5 (33)
Procyanidin C2	0.2		0.5		0.3		0.2		0.2		0.4		0.1		0.1		0.1		0.5		0.5	
Total trimers ♦	0.3 +	0.1 (33)	1.1	0.3 (24)	0.4	0.1 (25)	0.3	0.1 (33)	0.4	0.1 (25)	0.7	0.2 (29)	0.2	nd (0)	0.1	0.1 (100)	0.2	0.1 (50)	0.9 +	0.2 (22)	1.0	0.3 (30)

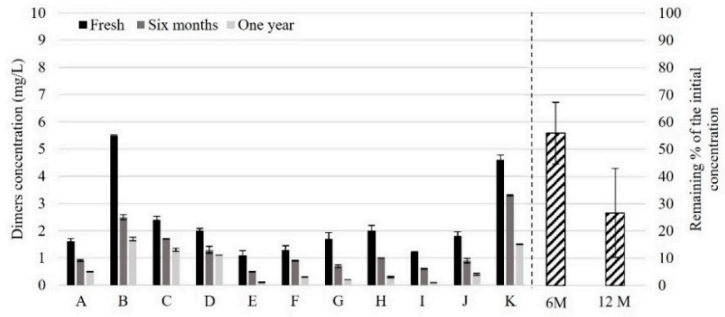
*: with bottle/can refermentation; ▲: with dry hopping; +: significant difference fresh and aged NABLABs concentrations (*t*-test, $\alpha = 0.05$). LOQ was inferior to 0.1 mg/L for all compounds; ♦: signal to noise ratio S/N > 9; nd: not detected (inferior to LOD, of 0.05 mg/L); coefficient variation <10%.



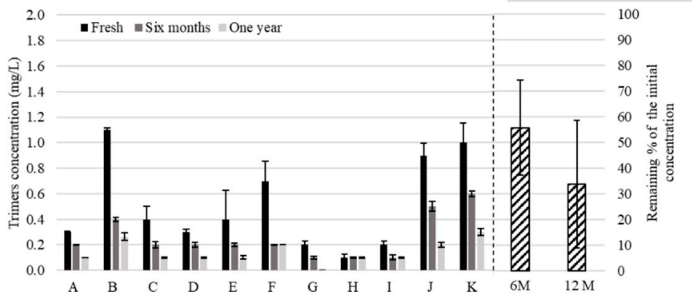
(**a**)



(**b**)



(**c**)



(**d**)

Figure 3. (**a**) Chemical structures of flavan-3-ols and RP-HPLC-ESI(-)-MS/MS chromatograms of fresh and one-year-aged beer K. 1: (+)-catechin, 2: (-)-epicatechin, 3: procyanidin B1, 4: procyanidin B2, 5: procyanidin B3, 6: procyanidin B4, and 7: procyanidin C2. (**b**) Monomers, (**c**) dimers, and (**d**) trimers concentrations (mg/L) in fresh, six-months-aged (6M), and one-year-aged (12M) NABLABs, measured by RP-HPLC-MS/MS and the respective remaining % of their initial concentration.

As expected, the color increase through aging was revealed to be correlated to the monomers' (catechin and epicatechin) level in fresh beers (R^2 = 0.71, Figure 4a, very pale beer C not included). Surprisingly, contrary to conventional beers, the oligomers' concentration (sum of dimers and trimers) exhibited a strong correlation with the chill haze of fresh samples (R^2 = 0.95, Figure 4b, beer D can-refermented and G white beer not here included; R^2 = only 0.43 with chill haze after aging), suggesting intense prematured oxidation in NABLABs.

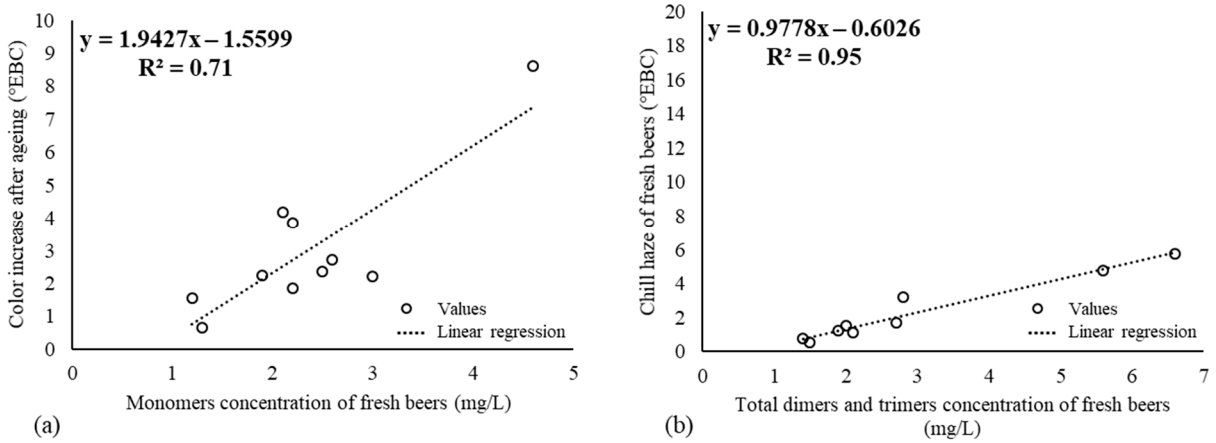


Figure 4. Correlations between (**a**) (+)-catechin and (-)-epicatechin level in fresh beers (mg/L) and color increase (°EBC) after one year at 20 °C; (**b**) Flavan-3-ol dimers and trimers concentration (mg/L) and chill haze of fresh beers (°EBC).

HPLC-MS/MS data also showed significant oxidation of all polyphenolic fractions after six months: only 61% of the initial amount of monomers, 56% of dimers, and 56% of trimers still found on average (Figure 3b–d). The losses still continued for the next six months (40, 27, and 34% remaining, respectively, on average).

3.2. Bitter Compounds in Fresh NABLABs and Their Fate through Aging

NABLABs bitterness (in BU) varied widely among styles (lager, amber, white, abbey, and dry hopped), ranging from 7 (D) to 37 (K) BU (Table 2). The bitter compounds of NABLABs were determined by an optimized RP-HPLC-UV method allowing for individual quantification of *cis*- and *trans*-isohumulones, tetrahydro-isohumulones (tetra), humulones, *cis*-humulinones, and hulupones (Table 2). RP-HPLC-UV analyses logically revealed big differences within NABLABs (differences in hopping rate, hop freshness, and variety: kettle-, late-, and/or dry hopping,…). Total isohumulones ranged from 4.5 (D) to 35.1 mg/L (K), with 86% on average of the *cis*-isomers (Figure 5b). Values of *trans*-isohumulones, below 0.5 mg/L (C, D and G), can only be obtained with a high boiling temperature or by using pre-isomerized hops [35] (Figure 5c).

Table 2. Concentrations (mg/L) of isohumulones (*cis* and *trans*), tetrahydro-isohumulones (tetra), humulones, *cis*-humulinones, and hulupones in the 11 fresh and aged (12M) investigated NABLABs. Values in parentheses indicate remaining percentages after one year of storage at 20 °C in the dark.

	Biological Processes										**Physical Processes**											
	Special Yeast						**Mixed Fermentation**		**Cold Contact**		**Distillation**										**Membrane Filtration**	
Compound	**A**		**B** ▲		**C** ▲		**D** *▲		**E**		**F**		**G**		**H**		**I**		**J**		**K**	
	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M	Fresh	12M
Bitterness units (BU)	12.8 (72)	9.2	30.7 (50)	15.4	11.0 (50)	5.5	6.8 (53)	3.6	19.2 (72)	13.7	15.2 (57)	8.7	10.2 (39)	4.0	12.6 (76)	9.6	19.9 (42)	8.4	20.6 (52)	10.8	37.3 (59)	22.1
cis-Isohumulones	5.5 (87)	4.8	16.0 + (55)	8.7	5.8 (69)	4.0	4.3 (47)	2.0	10.9 (85)	9.3	12.0 + (74)	8.9	9.4 + (23)	2.2	10.7 + (74)	7.9	15.3 + (50)	7.7	18.9 + (49)	9.3	28.9 (62)	18.0
trans-Isohumulones	1.7 + (12)	0.2	4.7 + (42)	2.0	0.3 (0)	nd	0.2 + (0)	nd	3.6 (25)	0.9	2.6 (42)	1.1	0.5 (0)	nd	1.8 (22)	0.4	1.2 + (8)	0.1	3.2 (6)	0.2	6.2 (21)	1.3
Total isohumulones	7.2 (69)	5.0	20.7 + (52)	10.7	6.1 (66)	4.0	4.5 (44)	2.0	14.5 (70)	10.2	14.6 + (68)	10.0	9.9 + (22)	2.2	12.5 + (66)	8.3	16.5 + (47)	7.8	22.1 + (43)	9.5	35.1 (55)	19.3
cis/trans	3		3		nc		nc		3		5		nc		6		nc		6		5	
Tetra	4.0 (77)	3.1	2.1 (52)	1.1	2.0 + (75)	1.5	nd (100)	nd	nd (100)	nd	nd (100)	nd	nd (100)	nd	3.2 (56)	1.8	3.2 + (62)	2.0	3.1 (52)	1.6	nd (100)	nd
Humulones	nd (100)	nd	0.5 (0)	nd	0.2 (0)	nd	nd (100)	nd	nd (100)	nd	1.5 + (0)	nd	nd (100)	nd	nd (100)	nd	nd (100)	nd	nd (100)	nd	0.9 (0)	nd
cis-Humulinones	0.3 + (33)	0.1	7.0 + (76)	5.3	2.8 + (11)	0.3	2.7 (74)	2.0	3.8 + (21)	0.8	0.4 (50)	0.2	0.4 (25)	0.1	0.4 (25)	0.1	0.2 (0)	nd	0.9 (56)	0.5	1.6 (12)	0.2
Hulupones	0.3 (67)	0.2	1.5 (69)	1.0	1.2 + (25)	0.3	0.8 (75)	0.6	1.7 (71)	1.2	nd (100)	nd	nd (100)	nd	nd (100)	nd	0.5 (40)	0.2	0.2 (50)	0.1	1.0 (40)	0.4

*: with bottle/can refermentation; ▲: with dry hopping; +: significant difference fresh and aged NABLABs 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); nc: not calculated; coefficient variation <10%.

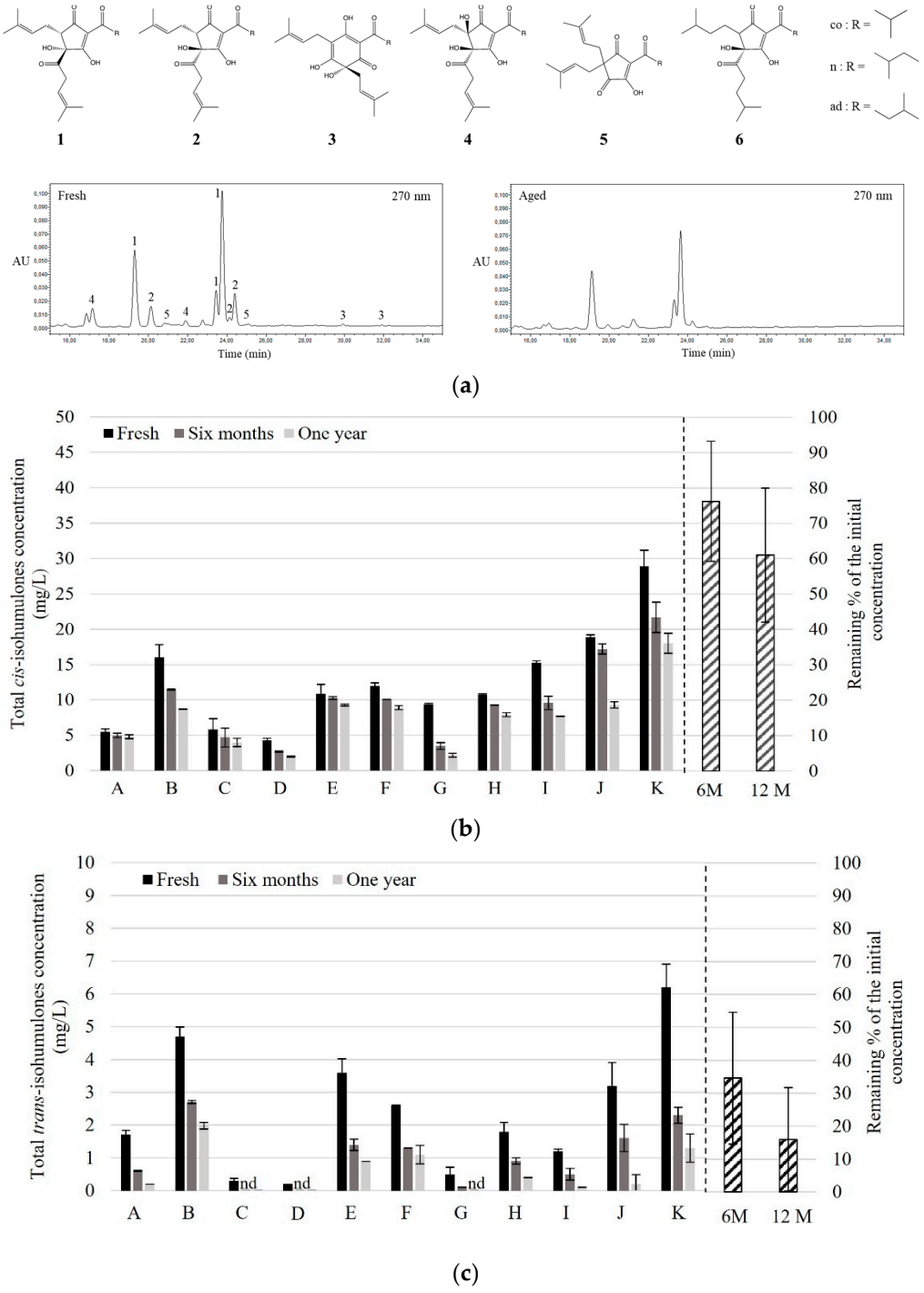
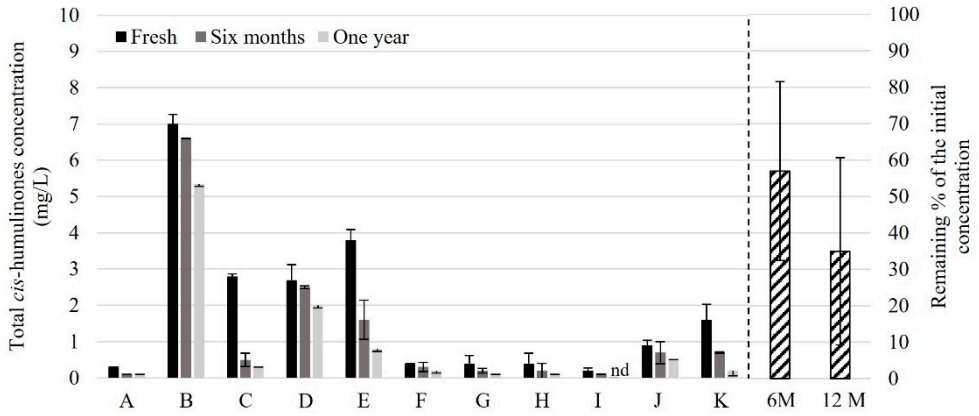


Figure 5. *Cont*.



(**d**)

Figure 5. (**a**) Chemical structures of bitter compounds and RP-HPLC-UV chromatograms of fresh and one-year-aged beer K. 1: *cis*-isohumulones, 2: *trans*-isohumulones, 3: humulones, 4: *cis*-humulinones, 5: hulupones, and 6: tetrahydro-isohumulones *. (*: not found in beer K) (**b**) Total *cis*-isohumulones, (**c**) total *trans*-isohumulones, and (**d**) total *cis*-humulinones concentrations (mg/L) in fresh, six-months-aged (6M), and one-year-aged (12M) NABLABs, measured by RP-HPLC-UV and the respective remaining % of their initial concentration.

Tetrahydro-isohumulones (tetra, often used for foam stabilization) were detected at an average of 3.0 mg/L in half of the fresh samples (A, B, C, H, I, and J) while humulones remained under 1.5 mg/L (even undetectable in 7 NABLABs) in contrast to dry hopped beers, which displayed in a recent paper [41] concentrations between 1.1 and 7.2 mg/L. In the same way, as expected [41], *cis*-humulinones were found only as traces (0.2–0.9 mg/L) except in the three dry hopped samples (2.7–7.0 mg/L in B, C, and D) and beers E and K, for which aged hops has probably been used [42] (Figure 5d). Hulupones, which can either be produced from hop lupulones during wort boiling or solubilized from hops [41], also remained under 1.7 mg/L.

The BU values significantly dropped during storage (only 39% remaining in beer G) (Table 2). After one year at 20 °C in the dark, all bitter compounds had been partially degraded, with 70 (E) to 22 (G)% remaining for total isohumulones (up to 70 ± 14% remaining on average after six months of storage), between 75 (D) and 25 (C)% remaining for hulupones, 63 ± 11% on average for tetrahydro-isohumulones (only 52% remaining in beers B and J), 35 ± 26% on average for *cis*-humulinones (only 11% remaining for beer C) (Figure 5d), and until a complete loss for humulones (Table 2). As expected, *trans*-isohumulones were affected much more strongly, with 16 ± 16% remaining on average (versus 61 ± 19% for *cis*-isohumulones) (Figure 5b,c). These losses, a bit still higher than those previously reported for conventional dry hopped beers [18], are another strong indicator of the susceptibility of NABLABs to prematured aging. However, chemical degradation should not be the sole reason for the observed decrease (humulones, hulupones, and tetra considered very stable in absence of yeast), suggesting the removal of some of these hydrophobic compounds in oxidized colloids [18,41].

4. Conclusions

Due to the absence of ethanol, strong degradation of flavan-3-ols and bitter compounds was noticed in NABLABs (up to only 27% and 16% remaining after one year at 20 °C for flavan-3-ol dimers and *trans*-isohumulones, respectively). As expected, color increase was correlated to the pool of flavan-3-ol monomers available. More surprising was the strong correlation obtained between the level of dimers and trimers and the haze of fresh samples. Much stronger than in conventional beers, prematured oxidation needs to be avoided by developing efficient natural antioxidants (e.g., sulfites and other yeast-derived antioxidants)

able to mimic the presence of ethanol. A sensory analysis must be conducted to support these results.

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Abbreviations

NAB: non-alcoholic beers, LAB: low-alcoholic beers, EU: European Union, ROS: Reactive Oxygen Species, USA: United States of America, SPE: Solid Phase Extraction, IST: internal standard, RP-HPLC-MS/MS: Reversed-Phase High-Pressure Liquid Chromatography with mass spectrometer detector operating in MS/MS mode, ESI: Electrospray Ionization, RP-HPLC-UV: Reversed-Phase High-Pressure Liquid Chromatography with ultraviolet detector.

References

1. Brányik, T.; Silva, D.P.; Baszczyňski, M.; Lehnert, R.; Almeida, E.; Silva, J.B. A review of methods of low alcohol and alcohol-free beer production. *J. Food Eng.* **2012**, *108*, 493–506. [CrossRef]
2. Sohrabvandi, S.; Mousavi, S.M.; Razavi, S.H.; Mortazavian, A.M.; Rezaei, K. Alcohol-free Beer: Methods of Production, Sensorial Defects, and Healthful Effects. *Food Rev. Int.* **2010**, *26*, 335–352. [CrossRef]
3. Muller, C.; Neves, L.E.; Gomes, L.; Guimarães, M.; Ghesti, G. Processes for alcohol-free beer production: A review. *Food Sci. Technol.* **2019**, *40*, 273–281. [CrossRef]
4. Perpète, P.; Collin, S. State of the art in low-alcohol beer production. *Cerevisia* **1999**, *1*, 27–33.
5. Müller, M.; Bellut, K.; Tippmann, J.; Becker, T. Physical Methods for Dealcoholization of Beverages Matrices and their Impact on Quality Attributes. *ChemBioEng. Rev.* **2017**, *4*, 310–326. [CrossRef]
6. Perpète, P.; Collin, S. Contribution of 3-Methylthiopropionaldehyde to the Worty Flavor of Alcohol-Free Beers. *J. Agric. Food Chem.* **1999**, *47*, 2374–2378. [CrossRef]
7. Perpète, P.; Collin, S. Influence of beer ethanol content on the wort flavour perception. *Food Chem.* **2000**, *71*, 379–385. [CrossRef]
8. Perpète, P.; Collin, S. Evidence of Strecker Aldehyde Excretion by Yeast in Cold Contact Fermentations. *J. Agric. Food Chem.* **2000**, *48*, 2384–2386. [CrossRef]
9. Piornos, J.A.; Balagiannis, D.P.; Methven, L.; Koussissi, E.; Brouwer, E.; Parker, J.K. Elucidating the odor-active aroma compounds in alcohol-free beer and their contribution to the worty flavor. *J. Agric. Food Chem.* **2020**, *68*, 10088–10096. [CrossRef]
10. Piornos, J.A.; Delgado, A.; de La Burgade, R.C.J.; Methven, L.; Balagiannis, D.P.; Koussissi, E.; Brouwer, E.; Parker, J.K. Orthonasal and retronasal detection thresholds of 26 aroma compounds in a model alcohol-free beer: Effect of threshold calculation method. *Int. Food Res. J.* **2019**, *123*, 317–326. [CrossRef]
11. Simon, M.; Vuylsteke, G.; Collin, S. Flavor defects of fresh and aged NABLABs: New challenges against oxidation. *J. Am. Soc. Brew. Chem.* **2022**, Submitted.
12. Vanderhaegen, B.; Neven, H.; Verachtert, H.; Derdelinckx, G. The chemistry of beer aging—A critical review. *Food Chem.* **2006**, *95*, 357–381. [CrossRef]
13. Andersen, M.L.; Skibsted, L.H. Electron spin resonance spin trapping identification of radical formed during aerobic forced aging of beer. *J. Agric. Food Chem.* **1998**, *46*, 1272–1275. [CrossRef]
14. Andersen, M.L.; Outtrup, H.; Skibsted, L.H. Potential Antioxidants in Beer Assessed by ESR Spin Trapping. *J. Agric. Food Chem.* **2000**, *48*, 3106–3111. [CrossRef]
15. Kaneda, H.; Kano, Y.; Osawa, T.; Kawakishi, S.; Kamada, K. The Role of Free Radicals in Beer Oxidation. *J. Am. Soc. Brew. Chem.* **1989**, *47*, 49–53. [CrossRef]
16. Bamforth, C.W.; Muller, R.E.; Walker, M.D. Oxygen and Oxygen Radicals in Malting and Brewing: A Review. *J. Am. Soc. Brew. Chem.* **1993**, *51*, 79–88. [CrossRef]

17. de Cooman, L.; Aerts, G.; Overmeire, H.; de Keukeleire, D. Alterations of the profiles of iso-α-acids during beer ageing, marked instability of trans-iso-α-acids and implications for beer bitterness consistency in relation to tetrahydroiso-α-acids. *J. Inst. Brew.* **2000**, *106*, 169–178. [CrossRef]
18. Silva Ferreira, C.; Collin, S. Fate of bitter compounds through dry-hopped beer aging. Why cis-humulinones should be as feared as trans-isohumulones? *J. Am. Soc. Brew. Chem.* **2020**, *78*, 103–113. [CrossRef]
19. McMurrough, I.; Madigan, D.; Kelly, R.J.; Smyth, M.R. The Role of Flavanoid Polyphenols in Beer Stability. *J. Am. Soc. Brew. Chem.* **1996**, *54*, 141–148. [CrossRef]
20. Aron, P.M.; Shellhammer, T.H. A Discussion of Polyphenols in Beer Physical and Flavour Stability. *J. Inst. Brew.* **2010**, *116*, 369–380. [CrossRef]
21. Silva Ferreira, C.; Simon, M.; Collin, S. 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. *J. Am. Soc. Brew. Chem.* **2022**, 1–10. [CrossRef]
22. Buggey, L.A. A review of polyphenolic antioxidants in hops, brewing and beer. *Brew. Int.* **2001**, *4*, 21–25.
23. Plumb, G.W.; de Pascual-Teresa, S.; Santos-Buelga, C.; Cheynier, V.; Williamson, G. Antioxidant properties of catechins and proanthocyanidins: Effect of polymerisation, galloylation and glycosylation. *Free Radic. Res.* **1998**, *29*, 351–358. [CrossRef] [PubMed]
24. Counet, C.; Collin, S. Effect of Number of Flavanol Units on the Antioxidant Activity of Procyanidin Fractions Isolated from Chocolate. *J. Agric. Food Chem.* **2003**, *51*, 6816–6822. [CrossRef] [PubMed]
25. Callemien, D.; Collin, S. Involvement of Flavanoids in Beer Color Instability during Storage. *J. Agric. Food Chem.* **2007**, *55*, 9066–9073. [CrossRef] [PubMed]
26. Callemien, D.; Collin, S. Use of RP-HPLC-ESI(–)-MS/MS to Differentiate Various Proanthocyanidin Isomers in Lager Beer Extracts. *J. Am. Soc. Brew. Chem.* **2008**, *66*, 109–115. [CrossRef]
27. Asano, K.; Ohtsu, K.; Shinagawa, K.; Hashimoto, N. Affinity of proanthocyanidins and their oxidation products for haze-forming proteins of beer and the formation of chill haze. *Agric. Biol. Chem.* **1984**, *48*, 1139–1146. [CrossRef]
28. McGuinness, J.D.; Eastmond, R.; Laws, D.R.J.; Gardner, R.J. The Use of14c-Labelled Polyphenols to Study Haze Formation in Beer. *J. Inst. Brew.* **1975**, *81*, 287–292. [CrossRef]
29. Gramshaw, W.J. Phenolic constituents of beer and brewing materials. IV. Further observations on anthocyanogens and catechins as haze precursors in beer. *J. Inst. Brew.* **1969**, *75*, 61–83. [CrossRef]
30. Poupard, P.; Sanoner, P.; Baron, A.; Renard, C.M.G.C.; Guyot, S. Characterization of procyanidin B2 oxidation products in apple juice model solution and confirmation of their presence in apple juice by high-performance liquid chromatography couplet to electrospray ion trap mass spectrometry. *J. Mass Spectrom.* **2011**, *46*, 1186–1197. [CrossRef] [PubMed]
31. Millet, M.; Poupard, P.; Guilois-Dubois, S.; Zanchi, D.; Guyot, S. Self-aggregation of oxidized procyanidins contributes to the formation of heat-reversible haze in apple-based liqueur wine. *Food Chem.* **2019**, *276*, 797–805. [CrossRef] [PubMed]
32. Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279–1288. [CrossRef]
33. Viana, A.C.; Pimentel, T.C.; do Vale, R.B.; Clementino, L.S.; Januario Ferreira, E.T.; Magnani, M.; dos Santos Lima, M. American pale Ale craft beer: Influence of brewer's yeast strains on the chemical composition and antioxidant capacity. *LWT* **2021**, *152*, 112317. [CrossRef]
34. Intelmann, D.; Hofmann, T. On the Autoxidation of Bitter-Tasting Iso-α-acids in Beer. *J. Agric. Food Chem.* **2010**, *58*, 5059–5067. [CrossRef]
35. Intelmann, D.; Haseleu, G.; Dunkel, A.; Lagemann, A.; Stephan, A.; Hofmann, T. Comprehensive Sensomics Analysis of Hop-Derived Bitter Compounds during Storage of Beer. *J. Agric. Food Chem.* **2011**, *59*, 1939–1953. [CrossRef]
36. Cook, A.H.; Howard, G.A.; Slater, C.A. Chemistry of hop constituents: VIII*. Oxidation of humulone and cohumulone. *J. Inst. Brew.* **1955**, *61*, 321–325. [CrossRef]
37. *European Brewery Convention, Analytica-EBC*; Fachverlag Hans Carls: Nürnberg, Germany, 2006.
38. McMurrough, I.; Madigan, D.; Smyth, M.R. Semipreparative Chromatographic Procedure for the Isolation of Dimeric and Trimeric Proanthocyanidins from Barley. *J. Agric. Food Chem.* **1996**, *44*, 1731–1735. [CrossRef]
39. Alonso Garcia, A.; Cancho Grande, B.; Simal Gándara, J. Development of a rapid method based on solid-phase extraction and liquid chromatography with ultraviolet absorbance detection for the determination of polyphenols in alcohol-free beers. *J. Chromatogr. A.* **2004**, *1054*, 175–180. [CrossRef]
40. Callemien, D.; Guyot, S.; Collin, S. Use of thiolysis hyphenated to RP-HPLC-ESI(-)-MS/MS for the analysis of flavanoids in fresh lager beers. *Food Chem.* **2008**, *110*, 1012–1018. [CrossRef]
41. Silva Ferreira, C.; de Chanvalon, E.T.; Bodart, E.; Collin, S. Why humulinones are key better constituents only after dry hopping: Comparison with other Belgian styles. *J. Am. Soc. Brew. Chem.* **2018**, *76*, 236–246.
42. Rehberger, A.J.; Bradee, L.H. Hop oxidative transformations and control of beer bitterness. *Tech. Q. Master Brew. Assoc. Am.* **1975**, *12*, 1–8.