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The Stability of Bitter Substances in Beer During the Ageing Process

The changes in hop bitter substances in beer over the course of ageing and the associated decline in sensory quality have been the subject of numerous studies, but none of these have explored if bitter substances react differently during ageing based on whether the hops are added on the hot or on the cold side. To elucidate this a lager beer was brewed with a late aroma hop addition in the brewhouse and additionally dry hopped. Both beers were stored for two years at 4 °C, 22 °C and 30 °C. Overall, ageing the beers at 4 °C resulted in minor changes only. Based on the analysis of the beers aged at 22 °C and 30 °C, the following observations can be drawn: Starting with the *cis*-iso-α-acids via the bittering units, total iso-α-acids, *trans*-iso-α-acids and on to the α-acids, the losses of these substances increase significantly. *Trans*-iso-α-acids are 5 to 8 times more sensitive to degradation than the *cis* form. The behaviour of co-hulupone was comparatively stable in this regard. A marked difference between the homologs (co-, n- and ad-) of the iso-α-acids could not be found in both beers. However, all of the bitter substances in the dry-hopped beers showed less degradation due to ageing than those in the late-hopped beers. Whether the higher stability of the bitter substances in dry-hopped beer can be attributed to the changes in its composition through the addition of dry hops cannot be clearly established. Additional polyphenols added by dry hopping could potentially provide a "more stable environment" with a higher antioxidative potential. A distinct decrease in xanthohumol was observed; however, it was largely offset by an equivalent increase in isoxanthohumol. Late and dry hopped beers showed no differences with regard to the prenylflavonoids.

Descriptors: beer ageing, hop bitter substances, iso-α-acids, humulinones, hulupones, flavour stability

1 Introduction

The changes in the hop bitter substances in beer over the course of ageing have been a topic of discussion in the literature for some time now. This includes not only the effects on the beer, which are measurable by means of laboratory analysis, but also the changes in the sensory properties. A good overview can be found in this source [1, pp. 65–69, 267–271] which also provides further references.

In early studies on the sensory effects of ageing on beer at different temperatures of 3, 15, 25 and 35 °C for up to 130 days, *Pangborn* et. al. [2] observed that beer is perceived as less bitter with increasing age. The bittering units tested in parallel trials decreased more rapidly at higher temperatures and as more time passed. Studies using HPLC to measure the effects of ageing on bitter substances were published as early as 1988 [3]. Despite insufficient separation and imprecise identification of the individual substances, some results were obtained. Beers were spiked with solutions formulated for use in the trials of both α-acids and β-acids (made from CO_2 extract), iso-α-acids (iso-extract), a β-fraction (base extract) and hulupone extract. The trial beers were subsequently aged at 25 °C for 10 to 20 weeks. The bittering units reacted slowly, revealing little about ageing behaviour. α-Acids undergo isomerization, which is intensified with exposure to light, while iso-α-acids decline significantly – more rapidly in the presence of light – and go on to form humulinic acids. If β-acids are present in beer at all, they can be converted into hulupones, even without light. They are only moderately stable in the absence of light. It was also found that the co-iso-α-acids are somewhat less stable than their n- and ad-homologs.

Walters et. al. identified the isohumulones as the compounds that, due to their strong dependency upon temperature, decrease over time in beer during storage (71 % at 40 °C for 156 days). A separation of iso-α-acids into three *cis* and three *trans* isomers was performed, leading to the observation that *trans*-isohumulones, in particular, experienced a decrease, while *cis*-isohumulones largely remained stable over the five-month period, even at 40 °C [4]. These results were confirmed shortly thereafter by *King* and *Duineveld* [5]. They also added that isocohumulone is degraded somewhat faster than the other two isomers. *De Cooman* observed the degradation of iso-α-acids in top-fermented and bottom-fermented beers at different temperatures and periods of time and also reported a specific decrease in *trans*-iso-α-acids [6]. Drawing on these results, he was able to derive a half-life of about one year for the *trans*-iso-α-acids in the top-fermented beers (12 °C). Consequently, the use of hops or hop products rich in *cis* isomers was recommended.

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In 2002, *Araki* et. al. even suggested using the ratio of *trans* to *cis*-iso-α-acids that they had monitored in beers stored at 20 °C for up to 90 days as an ageing indicator [7]; however, this has yet to be pursued any further.

Forster et. al. conducted storage trials (8 months, 22 °C, under dark conditions) with 25 commercially available beers. They reported a decrease of 13.8 % in the number of bittering units as well as losses of 18.2 % of the iso-α-acids and 55 % of the α-acids, on average [8].

A TU Munich working group has made considerable progress in the elucidation of ageing and conversion products related to α-acids and β-acids as well as how these substances are perceived. Their contributions can be found in three dissertations by *Intelmann*, *Haseleu* and *Dresel* [9, 10, 11] and several publications [12-15]. The authors explore the degradation of iso-α-acids in detail, revealing that the *trans* isomer of the iso-α-acids has a greater propensity for degradation compared to the *cis* isomer. A large number of degradation products, especially those originating from *trans*-iso-α-acids, were identified and also described sensorially, focused on character and quality of bitterness. They differentiate between oxidative and non-oxidative degradation pathways. If aged under oxygen influence *cis* and *trans*-humulinic acids, alloisohumulones and their hydoperoxides are generated. With the exclusion of oxygen rather tricyclic and tetracyclic degradation products such as tricyclohumol, tricyclohumene, isotricyclohumene, tetracyclohumol, epitetracyclohumol are formed in a *trans* specific and acid catalysed pathway. Increased formation of the co-homologs versus the n-homologs of the degradation products is an indication that the co-homolog precursors are more susceptible to degradation than others. The authors were able to observe a correlation between the period of time the beer was stored and a distinct decline in an early, pleasant form of bitterness, which was followed by an increase in an unpleasant bitterness later on in the ageing process. In addition to a decrease in most of the hop compounds in beer, Dresel reports a rise in *cis*-humulinic acids, hulupinic acids, hydroperoxy-alloisohumulones, tricyclohumols and tetracyclohumols [11, p. 112ff].

Schmidt et al. [16] investigated the influence of different hop products on the *cis/trans* ratio of iso-a-acids in beer and the changes in key aroma and bitter taste molecules during beer ageing. In this paper the storage behaviour of beers produced with four different hop products (showing different *cis/trans* ratios of iso-alpha-acids) was investigated not only by sophisticated HPLC-MS/MS (confirming the building of the specific degradation products as discovered by Intelmann [12] but also by GC-MS/MS. They conclude: "Beers produced with isomerized hop products showed higher ratios of *cis*- to *trans*-iso-α-acids and their concentrations of degradation products of *trans*-iso-α-acids were accordingly lower after ageing. But all of the four types of beers hopped in different ways showed very similar profiles of volatile aroma compounds even during ageing. Moreover, sensory evaluation of aged beers gave no preference for the variations higher in *cis/trans* ratios of iso-α-acids."

Other explanations have been presented regarding the degradation of iso-α-acids. As early as 1979, *Hashimoto* and *Eshima* described the degradation of iso-α-acids to 4-methylpentan-2-one and 3-penten-2-one as beer ages at 20 °C [17]. According to *Kaneda* et. al., elevated oxygen concentrations in the bottle serve to accelerate this reaction [18]. *Huvaere* et. al. demonstrated that electron acceptors other than reactive oxygen species can also be involved in an oxidation reaction [19]. *Vanderhaegen* et. al. found an increased formation of 4-methylpentan-2-one and 3-penten-2-one when monitoring the ageing of beers (12 months at 20 °C, dark) with high levels of bitterness. The concentration of these two ketones detected in fresh beer originated from the oxidative degradation of the iso-α-acids during wort boiling [20].

Another pathway for degradation was suggested by *Williams* and *Wagner* [21]. The branched carboxylic acids, 2-methylbutyric acid and 3-methylbutyric acid, may also originate from the degradation of iso-α-acids. During the ageing process, if there is a sufficient amount of ethanol present, these compounds combine with ethanol to form the corresponding esters (2-methylbutyric acid ethyl ester and 3-methylbutyric acid ethyl ester). These esters exhibit threshold values of 7–20 µg/l and 18–20 µg/l, respectively, and contribute to a vinous aroma which is indicative of ageing. In Vanderhaegen's experiments, these compounds also increased over the course of ageing and the greatest increase in these two esters was found in beers containing the highest amount of bitter substances and the highest ethanol content [20].

Hashimoto and Eshima [22] proposed a reaction pathway for the degradation of *trans*-iso-α-acids to form carbonyls known to be specifically associated with ageing such as 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. The researchers have postulated that the ageing carbonyls are formed through diacylation of specific side chains on the C2 atom. This has been questioned by *DeClippeleer* et. al. who found no correlation between the degradation of *trans*-iso-α-acids and the formation of the three aldehydes in their experiments conducted with pure substances [23]. Rather, they attribute a much greater importance to the formation of non-volatile cyclical transformation products, as described by Intelmann et. al. [13]. This is also more easily reconciled with the change in the sensory impression towards a lingering, unrefined, harsh bitterness.

Ferreira and *Collin* [24] reported on the ageing of dry-hopped beer. They compared the general properties of the beer and the content of bitter substances (isohumulones, humulones, humulinones and hulupones) of 21 dry-hopped beers when they were fresh and after two years of ageing (stored in dark conditions at 20 °C). The instability of *trans*-isohumulones was confirmed; overall, the isohumulones decreased by an average of 25 %. The humulones decreased by an average of 91 % and humulinones by 73 %. In the beer with the highest humulinone content, seven new oxidation products were found in the aged beer. This involves the degradation of 4'-hydroxyallohumulinones to scorpiohumulinolene and dicyclohumulinolene. The decrease in hulupones, widely recognized as relatively stable, is attributed to their adhesion on the cold break material which is formed during this stage of beer production.

In this research, an attempt was made to minimize the number of possible variations and to limit the change in the composition of the bitter substances (isohumulones, humulones, humulinones and hulupones as well as xanthohumol and isoxanthohumol) in beer

Table 1 **Beer analyses performed on both fresh trial beers**

	unit	late	dry
original gravity	% w/w	11.44	11.58
alcohol	% v/v	5.01	5.21
apparent extract	% w/w	1.98	1.74
real extract	% w/w	3.80	3.63
limit of attenuation	%	83.3	85.3
pH		4.51	4.57

over the course of ageing. For these trials, the effects of ageing were studied on one late-hopped beer (late) and its counterpart which received additional hops in the form of dry-hopping (dry). Both were stored at various temperatures (4 °C, 22 °C and 30 °C) for over two years.

2 Material and Methods

Two trial beers (late / dry) were produced on a 2-hl research brewery system. As the good reproducibility of the brewing equipment and the analytics have been thoroughly investigated [25] the evaluation of two single beers can be considered as justified.

The grain bill consisted of 100 % pilsner malt which was mashed using an infusion method (52/64/72/78 °C). The wort was separated in a lauter tun and hops of the 2016 crop were added in the brewhouse as follows:

- 5 g α/hl Herkules (HKS), type 90 pellets, 16.2 % alpha (EBC 7.7), added at the start of the boil
- 5 g α/hl Hallertauer Tradition (HTR), type 90 pellets, 6.6 % alpha (EBC 7.7), added at the midpoint of the boil
- 200 g/hl Hallertau Blanc (HBC), type 45 pellets, 16.8 % alpha (EBC 7.7), added to the whirlpool, which corresponds to 400 g/hl of type 90 pellets.

The wort (12.0 % OG by weight) was pitched with the bottom-fermenting yeast strain W 34/70 and fermented at the low temperature of 9 °C. The wort was transferred with some residual extract remaining (apparent degree of attenuation ≈ 65 %) and the volume was split equally between two tanks. One tank was further processed without dry hopping (late) while the other tank was additionally dry hopped with 200 g/hl Hallertau Blanc (HBC) type 45 pellets (dry). Each beer was matured for 7 days at 15 °C until the diacetyl concentration reached 0.03 mg/l, well below the threshold value. This was followed by two weeks of storage at 0 – 1 °C before the beer was filtered using a diatomaceous earth filter. The filtered beer was then passed through two downstream membrane filter cartridges, pore sizes 1.2 µm 0.45 µm, respectively. The trial beers were filled into 0.5 litre Euro bottles with very low oxygen uptake. An analysis of the both fresh beers (late with only a late hop addition in the brewhouse, dry with additional dry hopping) is provided in table 1.

Original gravity and alcohol are higher in the dry hopped beer as fermentable sugars and other soluble substances from hops increase these beer characteristics during dry hopping and ongoing fermentation [26].

The beers were aged at a temperature of 4 ± 0.5 °C, at room temperature (approx. 22 °C) and at 30 ± 1 °C over a period of two years.

The two beers were tasted fresh and after 12 months of storage at 4 °C and 22 °C evaluating especially the intensity and quality of bitterness which were assessed on a five-points scale basis by 17 trained panellists.

The trial beers were analysed using a modified EBC 9.47 method for the individual determination of bitter substances using HPLC and the EBC method 9.8 [27] for measuring international bittering units (IBU). Fresh samples as well as aged samples were tested at 3, 6, 12, 18 and 24 months). Slightly hazy samples were cen-

Table 2 **Analyses of bitter substances in both beers with standard deviations (SD) for the triple determinations and corresponding confidence intervals (CI)**

	unit	late	dry	SD	CI
bitter units	IBU	33.2	34.6	0.45	0.81
iso-α-acids	mg/l	31.6	27.6	0.26	0.47
cis-iso-α-acids	mg/l	21.8	19.0	0.22	0.40
trans-iso-α-acids	mg/l	9.8	8.6	0.15	0.27
α-acids	mg/l	3.8	5.4	0.18	0.33
humulinones	mg/l	5.0	8.0	0.20	0.36
co-hulupone	mg/l	0.7	0.9	0.08	0.14
xanthohumol	mg/l	0.13	0.25	0.02	0.04
isoxanthohumol	mg/l	0.54	0.53	0.03	0.05

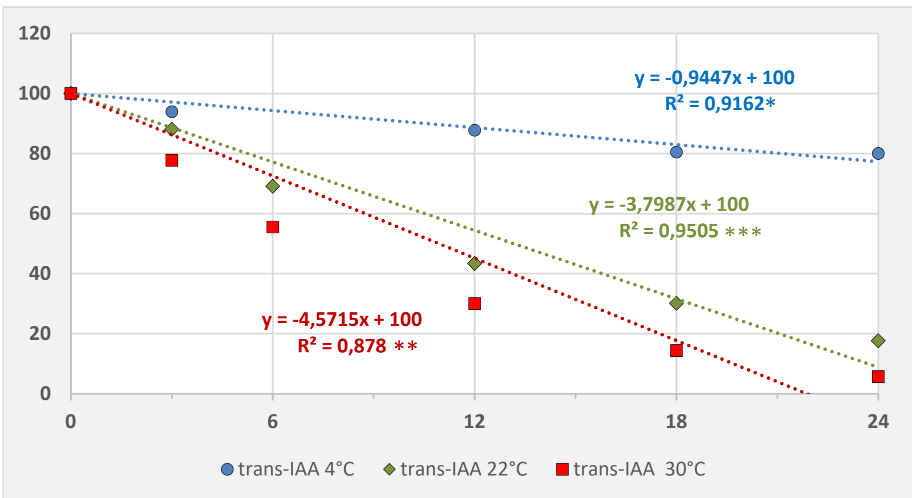

Fig. 1 **The change in *trans*-iso-α-acids (late-hopped beers) during ageing, shown in the form of a regression line over a period of 24 months**

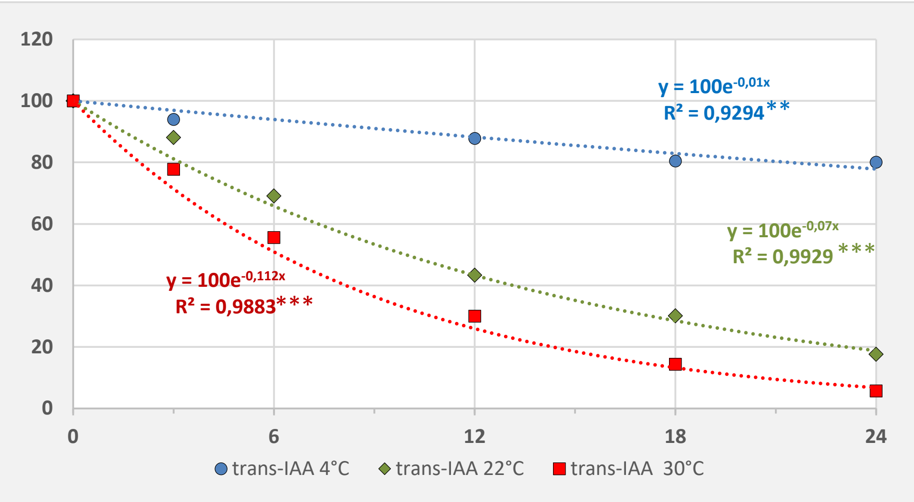

Fig. 2 The change in *trans*-iso-α-acids (late-hopped beers) during ageing shown in the form of an exponential approximation over a period of 24 months

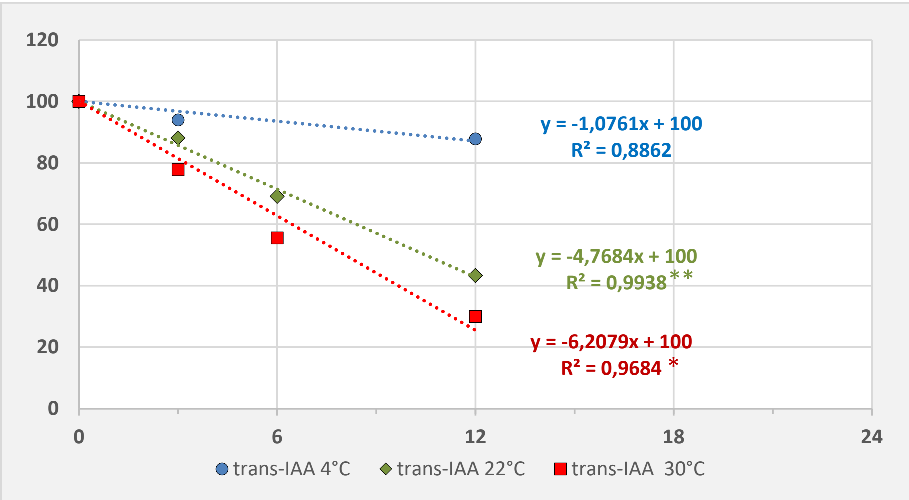

Fig. 3 The change in *trans*-iso-α-acids (late-hopped beers) during ageing, shown in the form of a regression line over a period of 12 months

trifugated for 5 min with 3000 rpm. HPLC-DAD analysis enables at different wavelength the separation of individual homologs of the α-acids (335 nm) and iso-α-acids (co-, n- and ad-homologs at 270 nm), the *cis* and *trans* isomers of the iso-α-acids, the humulinones and the hulupones (280 nm). Xanthohumol (XN) at 370 nm and isoxanthohumol (IX) at 280 nm are also detected. Calibration standards for α- and β-acids (ICE3, Labor Veritas), for iso-α-acids (ICS3, Labor Veritas), for humulones and hulupones (Hopsteiner), for XN (Orgentis) and for IX (Sigma-Aldridge) were used at the individual wavelengths.

The data recorded for the ageing processes (reduction in % relative to the initial concentration) were subjected to a linear regression in MS Excel. The coefficient of determination **R²** and the slopes **a** of the regression lines were taken from the graphs generated from these data. Its significance can be determined using a table [28] and the limits at 5 %, 1 % and 0.1 % for the correlation coefficient **r**, the square root of the coefficient of determination. The significance level is indicated as significant (*), very significant (**) and highly significant (***) and is marked accordingly in the figures and tables.

3 Results and Discussion

3.1 Evaluation scheme

Table 2 contains the initial values obtained from the analysis of bitter substances in both beers. The mean standard deviations for the triple determinations of the fresh, unaged beers and the corresponding confidence levels are also included. Of the three hulupones present, only co-hulupone could be quantified in reasonable amounts, with n-hulupone occurring in traces and ad-hulupone always below limit of detection. In the dry-hopped beer, β-acids were determined in traces below the detection limit.

The progression of ageing is not depicted in absolute values (e.g. mg/l) but instead in terms of residual content expressed as a percentage relative to the initial content (% rel.). This form of evaluation enables a direct comparison of all substances by calculating their relative residual content after ageing:

Residual content in % rel. = value after ageing ÷ initial value × 100 %

The slopes of the straight lines represent a measure for the rate of ageing. The slopes are close to linear for all substances in beers that have been aged up to 12 months, with several continuing this pattern over the course of the 24-month trial, namely bittering units, total iso-α-acids, *cis*-iso-α-acids and individual homologues of the iso-α-acids (co-, n- and ad-). *Trans*-iso-α-acids, α-acids and humulinones as well as the prenylflavonoids xanthohumol and isoxanthohumol exhibit a non-linear pattern which is best described with an exponential function (type of function: $y = 100e^{-k*t}$). A direct comparison of the exponential function with the slopes of the linear curves is not possible. Consequently, the slope from the curve plotted for the one-year period is used in these instances.

This can be illustrated by the example of *trans*-iso-α-acids (*trans*-IAA) content in late-hopped beer. Despite the fact that a linear trend is observed over 24 months (Fig. 1) resulting in significant best-fit lines (n = 6); nevertheless, the curve progression is not satisfactory.

The exponential form depicted in figure 2 comes much closer to the data points for the true progression, which is confirmed by the higher coefficients of determination (R²), especially those associated with the 22 °C and 30 °C curves.

The slopes of the first 12 months are used to facilitate a comparison of the linear progression of the other bitter substances (Fig. 3).

The coefficients of determination (R²) for the exponential regressions (22 °C and 30 °C) of the substances (*trans*-iso-α-acids,

Table 3 **Coefficients of determination and significance for both storage temperatures, 22 °C and 30 °C, for all substances with non-linear degradation behavior and mean values**

		late			dry		
bitter components	**temperature**	**linear 24 m**	**linear 12 m**	**exponential 24 m**	**linear 24 m**	**linear 12 m**	**exponential 24 m**
trans-Iso-α-acids	22 °C	0.9505 ***	0.9938 **	0.9929 ***	0.9681 ***	0.9908 **	0.9862 ***
	30 °C	0.8780 **	0.9684 *	0.9883 ***	0.9096 **	0.9874 **	0.9944 ***
α-acids	22 °C	0.8080 **	0.9532 *	0.9910 ***	0.9047 **	0.9627 *	0.9674 ***
	30 °C	0.7113 **	0.9019 *	0.9917 ***	0.7529 **	0.9056 *	0.9981 **
Humulinones	22 °C	0.9811 ***	0.9939 **	0.9813 *	0.9850 ***	0.9917 **	0.9827 ***
	30 °C	0.9847 ***	0.9999 ***	0.9407 ***	0.9282 **	0.9845 **	0.9851 ***
Xanthohumol	22 °C	0.8445 **	0.9806 **	0.9884 ***	0.8114 *	0.9687 *	0.9885 ***
	30 °C	0.4897	0.8129	0.8932 **	0.5145	0.8384	0.9507 ***
mean values		**0.831**	**0.951**	**0.971**	**0.847**	**0.954**	**0.982**

α-acids, humulinones and xanthohumol), which clearly exhibit degradation behaviour that is non-linear (“12 m exponential”), are listed in table 3. These are subsequently compared with the R^2 of the linear regressions over periods of 12 or 24 months (= “12 m linear curve” or “24 m linear curve”). Mean values of the four bitter components are calculated too.

The following can be concluded from these data:

- Hardly any difference exists for the coefficients of determination for the late-hopped and dry-hopped beer samples (the dry-hopped beers were slightly better). Therefore, mean values may be calculated.
- The mean value of the coefficient of determination of the 24 m exponential curve (0.977) is only slightly higher than that of the 12 m linear curve (0.953).
- The mean coefficient of determination of the 24 m linear curve (0.839) is significantly worse than that of the 12 m linear curve (0.953).

These data provide the justification for replacing the 12 m linear approximation with the 24 m exponential curve, in order to create a common basis for comparing all of the substances with the slope of the straight line.

With regard to the significance of the coefficients of determination, it should be noted that for the two-year data which are comparable with each other, the 24 m exponential curve has, on average, a higher significance level than the 24 m linear curve. Inevitably, the 12 m linear curve is not as accurate because of the fewer number of measurements (four instead of six).

3.2 Results for individual bitter substances

Table 4 shows the individual slopes of the regression lines plotted with the data for residual concentration of bitter substances in both beers after ageing at 4 °C, 22 °C and 30 °C. The stars indicate the respective significance levels.

The rates of degradation are extremely moderate at 4 °C; thus, no significance levels can be determined for the regression lines. Therefore, the discussion below is limited to the slopes of the lines plotted with the values measured for beer aged at 22 °C and 30 °C. The following observations can be made:

- The IBUs exhibit a more moderate reaction to ageing than the iso-α-acids. This can be explained by the fact that the degradation of iso-α-acids results in products that can absorb light in the non-specific spectrophotometric determination of IBU at 275 nm.
- If a quotient is calculated from the slope of the *trans*-iso-α-acids and the *cis*-iso-α-acids as a measure for the degradation intensity the following ratios are obtained: 5.8 (22 °C late), 5.0 (30 °C late), 8.3 (22 °C dry) and 7.6 (30 °C dry). The differences between the storage temperatures are not significant. This confirms the conclusions drawn by *Hofmann* [15], who reported factors ranging from 4 to 6. The comparatively higher stability of the *cis* form is derived from the *trans*-specific conversion to cyclical degradation products [9].
- The α-acids are particularly unstable. The design of this experiment does not provide clarification regarding whether they can undergo isomerization. In an older experimental model (beer simply mixed with a CO_2 hop extract), iso-α-acids could be detected; therefore, an isomerization reaction was assumed to have taken place [3].
- The humulinones exhibit a susceptibility to degradation com-

Table 4 **Slopes with significance levels for the regression lines (24 and 12 months) plotted with values for the residual concentration of all bitter substances measured in both beers, after storage at 4 °C, 22 °C and 30 °C**

	4 °C		22 °C		30 °C	
	late	**dry**	**late**	**dry**	**late**	**dry**
bitter units	– 0.02	– 0.03	– 1.24 ***	– 1.13 ***	– 1.55 ***	– 1.30 ***
iso-α-acids	– 0.58	– 0.25	– 1.75 ***	– 1.37 **	– 2.28 ***	– 1.81 **
cis-iso-α-acids	– 0.31	– 0.10	– 0.82 **	– 0.49	– 1,24 **	– 0.74 *
trans-iso-α-acids	– 1.08	– 0.72	– 4.77 **	– 4.06 **	– 6.21 *	– 5.59 **
α-acids	– 1.57	– 2.54	– 6.44 *	– 5.51 *	– 7.55 *	– 7.08 *
humulinones	– 0.96	– 0.76	– 4.12 **	– 3.79 **	– 5.60 ***	– 5.50 **
co-hulupone	– 1.33	– 1.30	– 1.64	– 0.74	– 1.41	– 0.45
xanthohumol	– 1.28	– 1.67	– 6.59 **	– 7.05 *	– 8.18	– 8.44
isoxanthohumol	+ 0.62	+ 1.14	+ 1.82 *	+ 2.31 **	+ 2.12	+ 3.35

parable to that of the *trans*-iso-α-acids.

- The aforementioned substances suffer higher losses in the late-hopped beers than in the dry-hopped beers, a phenomenon which will be discussed in greater detail below.
- The concentration of co-hulupone decreases only moderately. Its greater stability compared to that of iso-α-acids has already been established [3]. However, when evaluating these findings, it is important to consider the larger relative analytical error in terms of the generally low values measured in both beers (refer to Table 2).
- Xanthohumol and isoxanthohumol will be discussed separately.

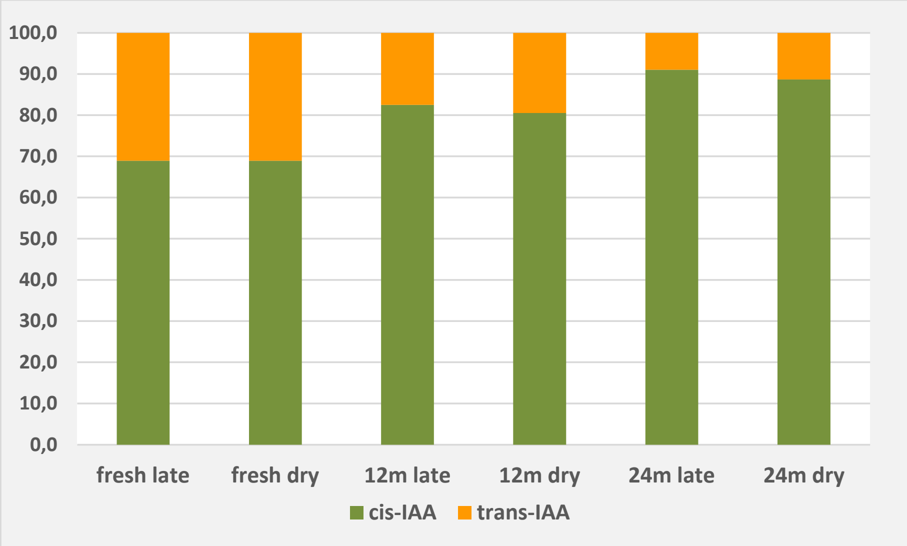

Fig. 4 **The *cis/trans* ratio [%] for iso-α-acids (in late-hopped and dry-hopped beers), measured in fresh beer and in aged beer after 12 or 24 months of storage at 22 °C**

The distinct shift in the *cis/trans* ratio is illustrated in figure 4. The ratios are shown for fresh beers and those for beers aged for 12 and 24 months at 22 °C. The *cis/trans* ratio for iso-α-acids is initially at 70/30 %. However, after 24 months of ageing at a storage temperature of 22 °C, the ratio surpasses the 90/10 % mark. For beers stored at 30 °C, the ratio climbs to above 95/5 %. This shift is less extreme in dry-hopped beers.

Ferreira et. al. [24] stored Belgian dry-hopped commercial beers at 20 °C for 2 years and measured the losses of individual bitter substances. The losses reported by them are a good approximation of the values determined in this research (Table 5).

3.3 Differences between the two beers (late-hopped and dry-hopped)

In order to ascertain the differences between the two beers, the values in table 6 were calculated from the relative losses of bitter substances over 12 months, plotted linearly and expressed as the individual as well as the mean value for each of the two storage temperatures, 22 °C and 30 °C. The table also contains a column listing how much greater the degradation rate is for late-hopped beer compared to dry-hopped beer, given as a relative percentage (= Δ). For an error analysis the absolute figures have to be considered. The differences between the absolute bitterness substance values of the late and dry hopped beers at the respective temperatures are substantially higher as the double of the standard deviation and are exceeding even mostly the double confidence intervals and so can be regarded as significant.

The conclusion can be derived from these data that the bitter substances present in dry-hopped beer are more stable than those in late-hopped beer. Furthermore, it is notable that this difference is particularly pronounced for *cis*-iso-α-acids and less so for *trans*-iso-α-acids.

The mechanism behind this more intense process of ageing, which specifically impacts the α-acids and the iso-α-acids in late-hopped beers compared to the dry-hopped beers, cannot be explained unequivocally. The answer could be due to one or both of the following:

- The residual α-acids in solution are almost completely lost after two years of storage. One explanation could be their (partial)

Table 5 **Comparison of the losses of individual bitter substances reported by Ferreira [25] stored for 2 years at 20 °C with those of this research (2 years at 22 °C)**

	Ferreira	this research
iso-α-acids	−25 %	−32 %
α-acids	−91 %	−88 %
humulinones	−73 %	−76 %

Table 6 **Losses of bitter substances [%] after 12 months including the proportion of greater degradation in late-hopped beer compared to dry-hopped beer (expressed as a percentage)**

	loss 22°C late	loss 30°C late	average loss late	loss 22°C dry	loss 30°C dry	average loss dry	Δ [% rel.]
bitter units	14.9	18.6	16.7	13.6	15.6	14.6	14.8
iso-α-acids	21.0	27.4	24.2	16.4	21.7	19.1	26.7
cis-iso-α-acids	9.8	14.9	12.4	5.9	8.9	7.4	67.5
trans-iso-α-acids	57.2	74.5	65.9	48.7	67.1	57.9	13.8
α-acids	77.3	90.6	83.9	66.1	85.0	75.5	11.1
humulinones	49.4	67.2	58.3	45.5	66.0	55.7	4.6
co-hulupone	19.7	16.9	18.3	8.9	5.4	7.1	156.3

isomerization over the course of ageing, especially at higher temperatures. Another dose of α-acids is subsequently added through dry hopping. A small portion of the newly added α-acids are soluble, leading to a "replenishment" of α-acids, thus making the amount of degradation only appear lower.

- In general, the bitterness and aroma composition of a beer are not all that changes as a result of dry hopping. Numerous other substances from the hops dissolve in the beer, e.g. polyphenols. Such an event can create a "more stable" environment overall, e.g. a higher antioxidative potential [29] or a higher pH value [30], as shown in table 1. This "more complex" beer could have a greater capacity to buffer the effects of oxygen diffusing through the crown cap, ultimately slowing the degradation of other substances, such as iso-α-acids.

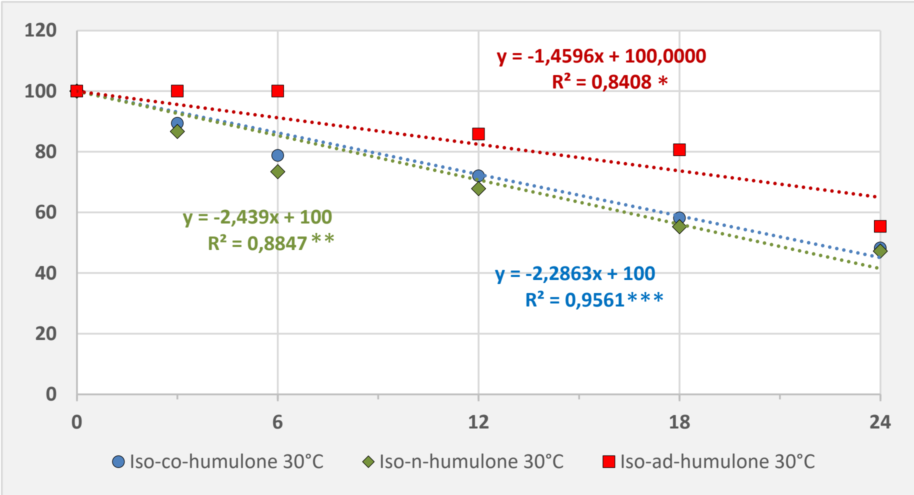

Fig. 5 **The change in the homologues of the iso-α-acids (late-hopped beers) during ageing, shown with a regression line over a period of 24 months**

3.4 Behaviour of the iso-α-acid homologs

Figure 5 illustrates the relative losses of the three homologs of the iso-a-acids in the late hopped beer during a storage time of 24 months at 30 °C. The degradation of co- and n-isohumulones proceeds very similar. Ad-iso-humulone appears to be somewhat more stable. This only partially correlates to the findings of King and Duineveld [5].

3.5 HPLC chromatograms

Figure 6 shows the HPLC chromatograms for the dry-hopped beers aged at 4 °C and 30 °C. The chromatogram for the fresh, unaged beer was less relevant because the time interval of two years makes any comparison difficult. However, since the effects of ageing at 4 °C are small when compared to the results for ageing at higher temperatures, a comparison of analyses performed on samples aged for similar periods is more reliable and provides a sufficient amount of information.

There is considerable variation in the losses of certain substances at different temperatures. Moreover, the formation of other substances is striking. Based only on the diode array detector (DAD) results, several peaks show similar spectra to those of humulinic acids. A mass spectrometer for a definite structural identification was not available.

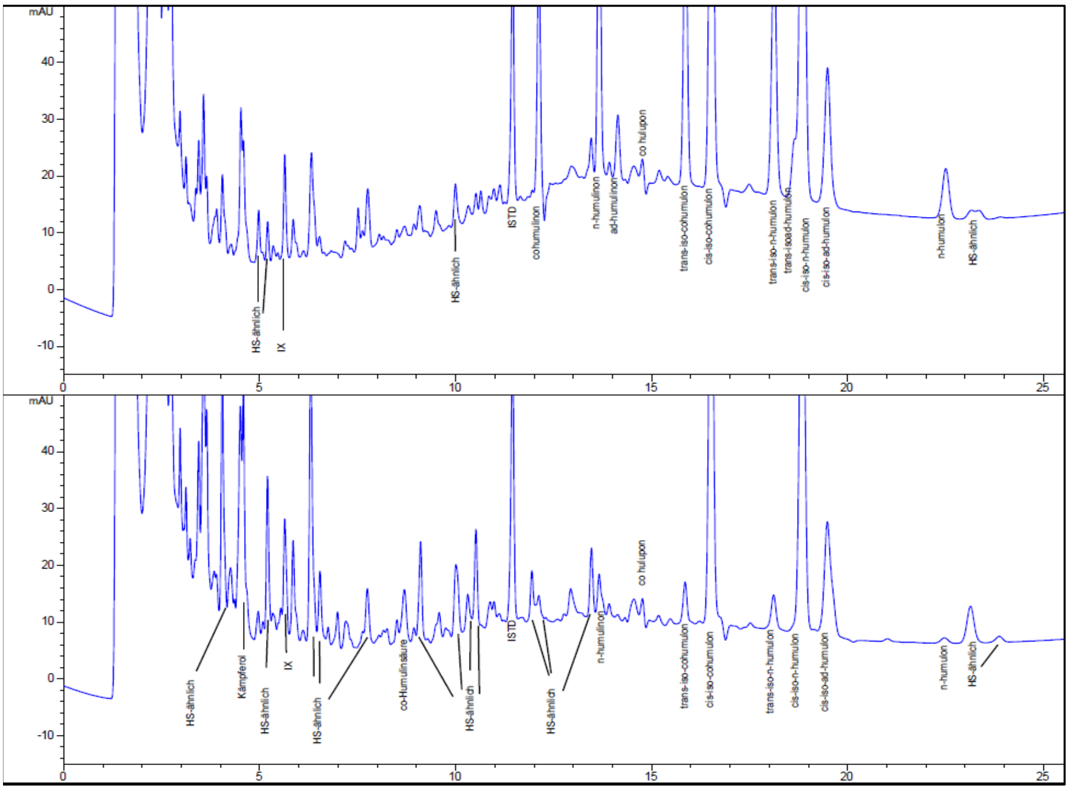

Fig. 6 **Comparison of HPLC chromatograms from the dry-hopped beer after storage for 24 months at 4 °C and 30 °C (HS-ähnlich = similar to humulinic acids)**

3.6 Behaviour of xanthohumol (XN) and isoxanthohumol (IX)

As prenylflavonoids these two substances are not grouped with the bitter substances, they belong to the polyphenols. They are less polar than all other polyphenols, which are found in the bracts and bracteoles of the hop cones. XN and IX are stored in the lupulin glands along with the aroma and bitter substances.

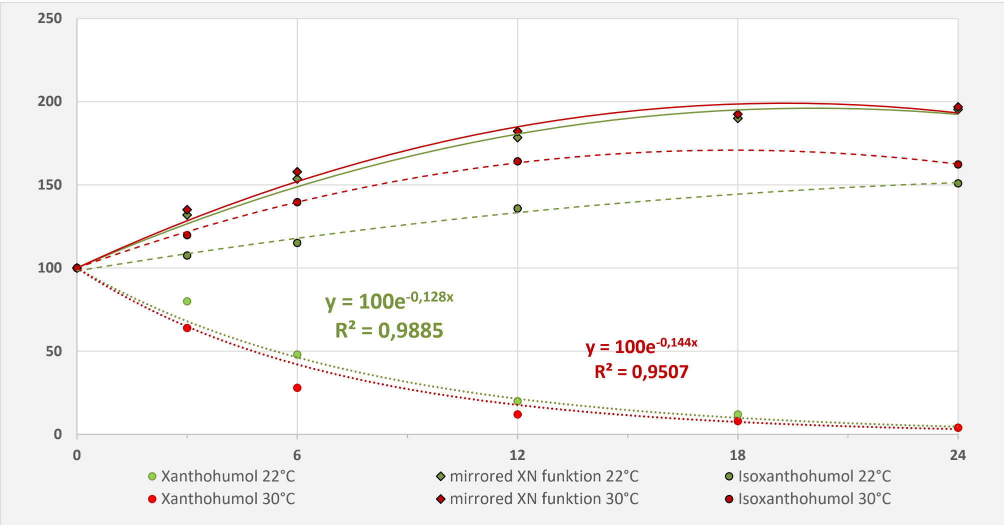

Fig. 7 **The change in xanthohumol and isoxanthohumol (dry-hopped beers) during ageing at storage temperatures of 22 °C and 30 °C, expressed in % rel.**

Dresel, in particular, has made an outstanding effort to elucidate prenylflavonoids [11].

Figure 7 shows the exponential decrease of XN (····), the parallel increase of IX (- - -) as well as a XN curve (——) that has been mirrored by the 100 % line for both 22 and 30 °C. This is imaging a theoretical course of the IX if the whole amount of XN would have been isomerized to IX. The following conclusions can be driven:

- Doubtlessly an isomerization of XN is occurring, that is not leading to an equivalent amount of IX. At 30 °C the reaction proceeds faster than at 22 °C.
- IX itself is not absolutely stable and is subject to a marginal degradation. This is hidden as long as there is XN available for an isomerization. The observation period of 24 months was too brief to confirm any degradation of IX. This could be established by experiment with a beer that only contains IX.
- In contrast to the bitter substances a difference in the degradation behaviour between the late- and dry-beers could not be observed.

A relative loss of XN of 79 % has been calculated after one year of storage at 22 °C. This corresponds fairly closely to the figure for degradation of 48 % measured over eight months described in [8], likewise conducted at 22 °C. However, no increase in isoxanthohumol could be found in this work, simply a significantly lower figure for degradation, i.e. 6 %. This may also be attributable to the low XN concentrations (0.026 mg/l) in the beers and therewith to the lacking base substance for an isomerization.

Table 7 **Point decreases in the ratings for the intensity and quality of the bitterness (late-hopped and dry-hopped beers) after storage at 22 °C; the asterisk indicating a significant difference**

	Intensity 4 °C	Intensity 22 °C	Quality 4 °C	Quality 22 °C
late	0	−0.35	−0.32	−0.58 *
dry	−0.20	−0.30	−0.32	−0.69 *

3.7 Sensory evaluation

The late-hopped and dry-hopped beers were tasted after 12 months of ageing at 4 °C and 22 °C by a trained panel consisting of 17 members. The intensity and quality of the bitterness of the beers stored at 4 °C and 22 °C were assessed on a five-points scale basis. The point decreases are provided in table 7.

In particular, there was a significant decline in the quality of the bitterness in both beers late and dry stored at 22 °C when calculating the confidence intervals. A decrease in the intensity of the bitterness with a parallel loss in the quality of the bitterness has also been observed and reported [9].

4 Summary

The changes in hop bitter substances in beer over the course of ageing and the associated decline in sensory quality have been the subject of numerous studies to date. The much higher instability of *trans*-iso-α-acids and their reaction pathways compared to those of *cis*-iso-α-acids have been clearly established. However, none of these studies have explored whether bitter substances react differently during ageing based on how the hops are added during production, that is, on the hot side (hot wort) or the cold side (dry hopping). This aspect has been examined in the research described here.

A heavily hopped lager beer was brewed with a late hop addition in the brewhouse (equivalent to 400 g/hl of Hallertau Blanc hops). This beer served as one of the two in this trial. The second beer was the same beer, dry-hopped at a rate of 400 g/hl. Both beers were stored for more than two years at 4 °C, 22 °C and 30 °C. Overall, ageing the beers at 4 °C only resulted in minor changes. However, based on the analysis of the beers aged at 22 °C and 30 °C, the following conclusions can be drawn:

- Starting sequentially with the *cis*-iso-α-acids and moving on to the bittering units, total iso-α-acids, humulinones, *trans*-iso-α-acids and on to the α-acids, the losses of these substances increase significantly, e.g. the α-acids are about 10 times more sensitive to degradation compared to the *cis*-iso-α-acids.
- *Trans*-iso-α-acids were found to be 6 to 8 times more sensitive to degradation than were those in the *cis* form.
- The behaviour of co-hulupone was comparatively stable in this regard.
- A marked difference between the homologs (co-, n- and ad-) of the iso-α-acids could neither be found in the late-hopped nor in the dry-hopped beers.
- The DAD spectra of the HPLC analyses of numerous degradation products are largely similar to those of humulinic acids.

It is notable, however, that almost all of the bitter substances in the dry-hopped beers showed less degradation due to ageing than those in the late-hopped beers. Only the difference in humulinones was within the comparative confidence limits of the analysis. The difference was particularly marked with the *cis*-iso-α-acids. Whether the higher stability of the bitter substances in dry-hopped beer can be attributed to the changes in its composition through the addition of dry hops cannot be clearly established. After all, the addition of more polyphenols through dry hopping could potentially provide a “more stable environment” with a higher antioxidative potential.

The behaviour of the prenylflavonoids were also investigated. A distinct decrease in xanthohumol was observed; however, it was largely offset by an increase in isoxanthohumol.

There was a significant decline in the quality of the bitterness in both beers late and dry stored at 22 °C.

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