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Beer's Bitter Compounds – A Detailed Review on Iso-α-acids: Current Knowledge of the Mechanisms for their Formation and Degradation

Iso-α-acids are quantitatively the most important hop-derived fraction in beer. In addition to CO_2 and ethanol, iso-α-acids are considered the primary beer flavour components. They impart the typical bitter taste and depending on the desired beer bitterness, their concentration varies from 10 up to 100 mg/L, according to the beer type. This article is to give a review on the knowledge about the formation of iso-α-acids and the mechanisms of their degradation in the beer matrix during storage. Iso-α-acids arise from the isomerisation of hop α-acids during wort boiling. In addition to the isomerisation reaction, the beer's bitter acids degradation has extensively been studied. Iso-α-acids are subjected to both oxidative and nonoxidative transformations. As a result, both the intensity and the quality of the beer bitterness are adversely affected. Both *trans*- and *cis*-iso-α-acids are susceptible to degradation in the presence of reactive oxygen species and light. Stored in the dark, *trans*-iso-α-acids in beer were found to be markedly more unstable than their *cis*-counterparts, and a harshy, lingering bitter taste simultaneously develops. This flavour defect occurring during beer ageing is attributed to the proton-catalysed cyclisation of *trans*-iso-α-acids into tri- and tetracyclic degradation products. These non-volatile degradation products of *trans*-iso-α-acids have only recently been identified. In contrast, only few reports deal with the formation of volatiles from iso-α-acids upon beer ageing. The relation between bitter acids degradation and aldehyde formation is cited in this review.

Descriptors: hop bitter acids, isohumulones, isomerisation, photodegradation, cyclisation, autoxidation, degradation, beer

1 Formation of iso-α-acids

1.1 Thermal isomerisation of α-acids

Iso-α-acids (Fig. 1) are formed by isomerisation of α-acids in a variety of reaction conditions. In the conventional brewing process, hops are boiled in the aqueous wort with a pH value of 5.0–5.5, in order to convert α-acids into the bitter tasting iso-α-acids, the latter being much more water soluble [1, 2]. The α-acids themselves have practically no bitter taste [3], but during wort boiling they are extracted from the hop cones and undergo thermal isomerisation to iso-α-acids via an acyloin-type ring contraction [1]. This isomerisation mechanism was revealed by *De Keukeleire* and *Verzele* and is depicted in figure 2 [4]. The ionisation of the β-triketo system in the α-acids is considered as the rate-limiting step of the isomerisation reaction [5]. After formation of the anion, the remaining enol function in the six-membered ring ketonises in a stereoselective way, so that the two 3-methyl-2-butenyl (prenyl) side chains at C4 and C6 are oriented in *trans* position. As a consequence, an acyloin entity is formed. The tertiary alcohol function of the latter functional group undergoes the known α-ketol rearrangement, which occurs with ring contraction, yielding another acyloin system and a new chiral centre [1]. As this ring contraction appears not stereoselective, two epimeric five-membered ring compounds are formed, namely *trans*- and *cis*-iso-α-acids, depending on the spatial orientation of the tertiary alcohol function at C4 and the prenyl side chain at C5. The terms *trans* and *cis* signify that these functional groups respectively point to the opposite or to the same faces of the five-membered ring [4]. Considering the three major α-acids in hops (humulone, cohumulone and adhumulone), accordingly 6 major iso-α-acids occur in beer: *trans*-/*cis*-isohumulone, *trans*-/*cis*-isocohumulone, and *trans*-/*cis*-isoadhumulone (see Fig. 1). Under normal brewing conditions, the ratio of *trans*-iso-α-acids/*cis*-iso-α-acids in beer is 32:68 [1, 2]. Since the two large vicinal side chains at C4 and C5 of the ring (the 4-methyl-3-pentenoyl side chain at C4 and the 3-methyl-2-butenyl side chain at C5) are in *trans* configuration in the *cis*-iso-α-acids, steric hindrance between these large groups is smallest, making the *cis*-iso-α-acids thermodynamically more stable compounds than the *trans*-iso-α-acids [1, 6].

Since the iso-α-acids, which are largely responsible for the characteristic fine beer bitterness, are originally not present in hops but formed by thermal isomerisation from the α-acids at the wort boiling stage of beer production, this formation reaction is com-

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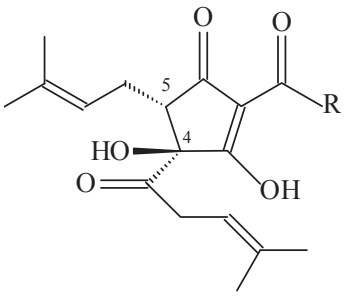
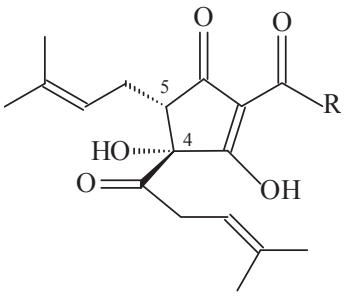
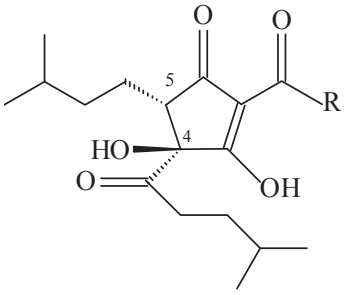


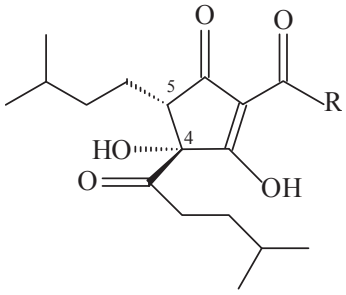
Trans-isocohumulone (**1a**)
Trans-isohumulone (**1b**)
Trans-isoadhumulone (**1c**)



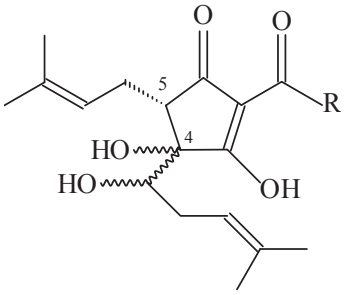
Cis-isocohumulone (**2a**)
Cis-isohumulone (**2b**)
Cis-isoadhumulone (**2c**)



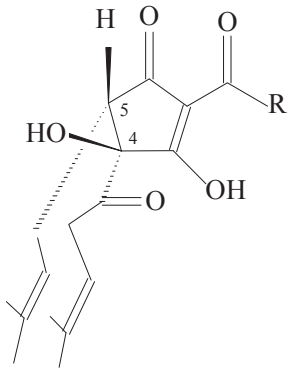
Trans-tetrahydroisocohumulone (**3a**)
Trans-tetrahydroisohumulone (**3b**)
Trans-tetrahydroisoadhumulone (**3c**)



Cis-tetrahydroisocohumulone (**4a**)
Cis-tetrahydroisohumulone (**4b**)
Cis-tetrahydroisoadhumulone (**4c**)



Dihydroiso-α-acids



Trans-iso-α-acids (**1a-c**)

$R_a = CH(CH_3)_2$
$R_b = CH_2CH(CH_3)_2$
$R_c = CH(CH_3)CH_2CH_3$

Fig. 1 Chemical structures of iso-α-acids, tetrahydroiso-α-acids, and dihydroiso-α-acids

monly regarded as the key reaction of hops with respect to the brewing process. Kettle hopping however, i.e. the isomerisation of the α-acids during the wort boiling step, remains a weak point in the brewery. Isomerisation under real wort boiling conditions is variable and incomplete (at most 50–60 %) [7, 8], which is partly attributed to the low solubility of the α-acids in the wort medium (60 mg/L at 100 °C and pH 5.0) [2, 9]. Moreover, when compared with alkaline conditions (pH 10–11), the conversion of α-acids at the wort pH (5.0–5.5) will be limited, because the α-acids are then partly protonated, while the anionic form is required for isomerisation [1, 10]. In addition, the isomerisation process in the kettle is influenced by temperature, vigour and length of boil, specific gravity, hopping rate, type of hop products used and addition points, and kettle design [2, 7–9, 10, 11]. Part of the α-acids and iso-α-acids are also further lost with the hot trub during wort clarification, during fermentation or maturation, and during final beer filtration [2, 7, 11]. Consequently, the final overall α-acid utilisation [(mass of iso-α-acids in beer/mass of α-acids in hops used) x 100] is typically only 30–40 % or even as low as 10–20 % [12]. In view of the negative impact of hot trub formation during wort boiling on the utilisation of hop α-acids, *Jaskula* et al. investigated the potential influence of mashing-off at 95 °C vs. 78 °C, aiming at more protein coagulation before wort boiling, resulting in lower α-acids and iso-α-acids losses during wort boiling and consequently potentially higher utilisations [8]. When mashing-off at 95 °C, a relative improvement in utilisation of approx. 37 % was observed with regard to the total iso-α-acids yield in the liquid fraction, which was indeed the result of less losses of α-acids and iso-α-acids during wort boiling. The enhanced α-acids utilisation appeared to be different for each iso-α-acid analogue. At the end of wort boiling, the formation of isohumulone was favoured when mashing-off at 95 °C compared with conventional mashing-off at 78 °C. These authors considered this alteration in iso-α-acids composition as positive for the final bitterness of beer, as a relatively high isocohumulone content is linked to a harsher bitterness. When boiling was preceded by mashing-off at 95 °C, increased formation of *cis*-iso-α-acids was monitored, which is reflected in lower T/C ratios [(*trans*-isocohumulone + *trans*-isohumulone)/(*cis*-isocohumulone + *cis*-isohumulone) x 100], 46–50 % vs. up to 65 % for mashing-off at 78 °C, irrespective of the boiling period [8]. From the point of view of bitterness stability, this finding is worth to mention as in particular *trans*-iso-α-acids are associated with rapid degradation upon beer ageing [13, 14, 15].

1.2 Kinetic parameters related to the hop α-acids isomerisation reaction

Jaskula et al. presented a detailed study on hop α-acid isomerisation kinetics in buffer solution, thereby determining rate constants and activation energies for the formation of the individual iso-α-acids [5]. Iso-α-acids formation was found to follow first-order kinetics and Arrhenius behaviour, with a higher rate constant for the reaction when increasing the heating temperature [5, 16]. The activation energy related to the formation of total iso-α-acids amounts to 96–97 kJ/mol. Since for the formation of isocohumulone, isohumulone and isoadhumulone, the same bonds are broken and formed during

acyloin ring contraction, identical activation energies were measured, regardless of the nature of the α-acid being converted. However, the rate constants for the formation of *cis*-isomers were always higher than the rate constants for the formation of *trans*-isomers, which is related to the noticed differences in activation energies for the formation of *trans*- and *cis*-isomers. As said by these authors, the activation energy for the formation of *trans*-iso-α-acids is approximately 9 kJ/mol lower than for *cis*-iso-α-acids. Accordingly, the lower the heating temperature, the more *trans*-isomers and the less *cis*-isomers are proportionally formed, which is reflected in higher T/C ratios. The higher activation energies involved in *cis*-isomers formation, is explained by the conformational differentiation of the keto form of the α-acids in the transition state (see Fig. 2). In this intermediary state, the intramolecular hydrogen bond between the tertiary alcohol at C6 and the carbonyl at C5, is stronger when the carbonyl is pointing downwards at a syn position (formation of *cis*-iso-α-acids) than when the carbonyl is pointing upwards (formation of *trans*-iso-α-acids). Consequently, the hydrogen of the hydroxyl group at C6 will be more readily released in the conformer that leads to *trans*-iso-α-acids. As a result, the activation energies for the formation of *trans*-isomers are lower than for *cis*-isomers. The apparent contradiction between the lower free energy of activation associated with the formation of *trans*-iso-α-acids and the lower rate constant of this reaction, in comparison with *cis*-iso-α-acids formation, is explained by the lower pre-exponential factors related to the formation of *trans*-isomers. Higher pre-exponential factors related to *cis*-iso-α-acids, explain the higher rate constants, despite of the higher activation energies required to attain their transition state, and suggest a higher probability of the *cis*-iso-α-acid transition state to become the final compound, a *cis*-iso-α-acid with a lower free energy [5].

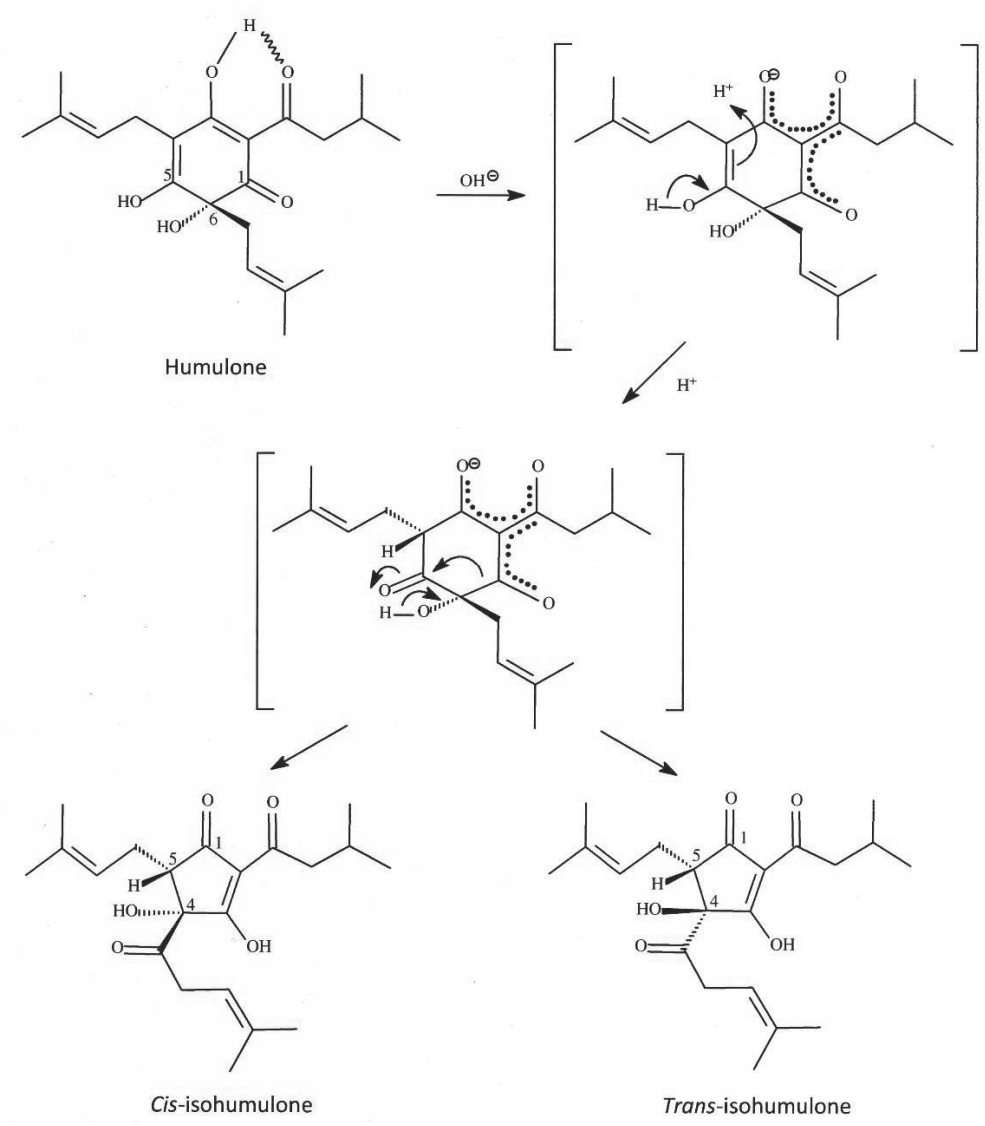


Fig. 2 The mechanism of α-acids isomerisation into iso-α-acids [1] (in cohumulone, the acyl side chain at C2 = $COCH(CH_3)_2$; in adhumulone, the acyl side chain at C2 = $COCH(CH_3)CH_2CH_3$)

1.3 Isomerisation of α-acids outside the brewhouse

The conversion of α-acids into iso-α-acids can also be performed outside the brewhouse, generally catalysed by divalent cations such as magnesium(II) and calcium(II) ions. Jaskula et al. recently showed that K^+, Na^+, Ca^{2+}, Mg^{2+}, Al^{3+}, and especially Fe^{3+} are all potential catalysts of the isomerisation reaction in wort. With Fe^{3+} even isomerisation yields of more than 80 % were obtained [10]. They explained this significant improvement of the isomerisation kinetics by the fact that Fe^{3+} will bind very efficiently to the anion formed in the initial step of the isomerisation reaction due to its high affinity towards oxygen ligands, or even induce deprotonation of the triketone moiety of the α-acids. As a result, this rate limiting step is improved. The trivalent cation Fe^{3+} should however be avoided in finished beer due to its likely negative impact on flavour stability. Besides, metal catalysis is connected to a significant increase in the ratio of *cis*-iso-α-acids/*trans*-iso-α-acids (T/C-ratio (%) is 27.2 after 60 min of boiling in wort (12 °P; pH 5.20) with 5 mg/L of Fe^{3+} added) [10]. It is suggested that metal catalysis results in lowering of the activation energies associated with the formation of *trans*- and *cis*-iso-α-acids, respectively. Besides, isomerisation of α-acids was carried out by irradiation in the wavelength region of 350–366 nm (UV-A light). Photoisomerisation of humulone happens regio- and stereoselective to *trans*-isohumulone [17, 18]. Application of bimetallic gold-based catalysts, as recently investigated by *Mertens* et al., is explored for the solvent-free production of iso-α-acids, aiming for an enhanced rate and selectivity of the reaction [19].

2 Properties of iso-α-acids in beer

Iso-α-acids are mainly present as water-soluble salts due to their low pKa values (around 3.5 in aqueous media) and the pH of lager beers, generally between 4.2 and 4.4. Their weak acid properties also explain the relatively good solubility of iso-α-acids in aqueous

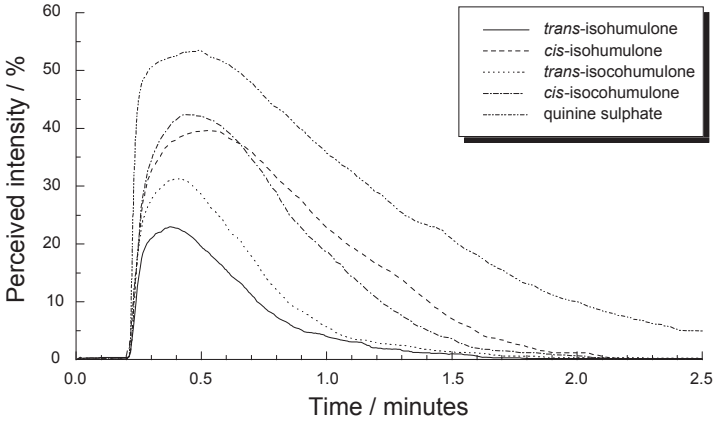


Fig. 3 Averaged time-intensity (TI) curves for four iso-α-acids (33 µM) and quinine sulphate (26 µM). Reprinted with permission from Hughes [21]. *Copyright 2000 Institute of Brewing and Distilling*

media compared with α-acids, having a pKa value of ca. 5 [20]. The hydrophobic character of the iso-α-acids is attributable to the presence of the three side chains in their molecular structures [21]. Iso-α-acids are the major bittering components of beer, with a threshold value for perception of bitterness in water of ca. 6 mg/L [22]. Bitterness elicited by buffer solutions of individual iso-α-acids has been studied by *Hughes* and *Simpson* in 1996 [22]. The *cis*- and *trans*-isomers of isohumulone and isocohumulone were therefore isolated and purified by preparative HPLC. The authors showed that these major hop bitter acids vary in the intensity of the bitter taste that they impart. *cis*-Isohumulone was found to be the most bitter hop iso-α-acid studied (ca. 1.82 times more bitter than *trans*-isohumulone), whereas *trans*-isocohumulone was perceived as the least bitter (ca. 0.74 times the bitterness of *trans*-isohumulone). Little difference in bitterness between *trans*-isohumulone and *cis*-isocohumulone was reported [22]. Despite of the masking effect of other beer components, similar differences in the bitterness intensities of hop bitter acids were demonstrated in beer [22]. Based on these results, two main traits were reported by Hughes [21]. Firstly, for a given pair of *cis*- and *trans*-isomers, the *cis*-component is thus significantly more bitter than its *trans*-counterpart. Secondly, the isohumulones are more bitter than the isocohumulones. This latter is to be expected on the basis of polarity, but the differences in the behaviour of the *cis*- and *trans*-isomers cannot be explained in terms of polarity as there is little if any difference between them [21]. According to *Rigby*, a relatively high isocohumulone content may result in a harsher, coarser bitterness with a lingering character, which was explained on the basis of the lower pKa value of isocohumulones, which will be more dissociated at the typical beer pH than isohumulones [23]. However, many authors provide little evidence for *Rigby's* finding [21, 22, 24, 25, 26, 27]. Hughes also evaluated the variation in the sensory bitterness intensity of individual iso-α-acids by characterising the time course of intensity of this persistent flavour attribute with time-intensity methodology [21]. The time-intensity curves for four iso-α-acids are depicted in figure 3. Significant differences in time-intensity behaviour of the iso-α-acids were observed, and the isocohumulones, considered by some to impart a harsher, more lingering bitterness, appear to be less potent bittering agents than their less polar counterparts [21].

Besides bitterness, regarded as the most important hop-derived beer flavour attribute, iso-α-acids also account for beer foam stabilisation [28, 29], inhibit most Gram-positive bacteria [30, 31], protect beer against spoilage by lactic acid bacteria such as *Lactobacillus* spp. and *Pediococcus* spp. [32], and have a favourable health effect on beer consumers [33, 34]. The occurrence of iso-α-acids for beer foam stability, an important quality aspect of beer aesthetics, is vital. Iso-α-acids also create foam cling or lacing, i.e. adherence to the beer glass [21, 35]. Without doubt, foam stabilisation is largely due to the presence of foam-active polypeptides, particularly with high hydrophobicity, from barley malts. Iso-α-acids are able to form supramolecular complexes with these polypeptides [36]. Currently, it is believed that the binding forces, crosslinking the surface-adsorbed polypeptides thereby fortifying the film of the foam bubbles and stabilising the beer foam, are not ionic, but involve both hydrogen bonding, hydrophobic interactions and the formation of metal-complexes with Mn^{2+}, Al^{3+}, Ni^{2+} [21, 35, 37]. Accordingly, the less polar isohumulones and isoadhumulones stabilise beer foam to a larger extent than their isocohumulone counterparts [21, 31].The β-triketo function in iso-α-acids indeed implies that these molecules are able to chelate metal cations [21]. In view of the similar polarity of the stereoisomeric pairs of iso-α-acids, only little differences in the relative abilities of *cis*- and *trans*-isomers to stabilise beer foam are reported [21]. Conversely, *trans*-isomers partition more into beer foam compared to their *cis*-counterparts, which was put forward as one of the reasons for the enrichment of *cis*-isomers in the final beer relative to the common *cis/trans* ratios observed upstream [21]. This differential behaviour of *trans*- and *cis*-iso-α-acids is explained on the basis of their stereochemical differences. Due to the fact that the prenyl and isohexenoyl side chains are at the same side of the ring, *trans*-iso-α-acids are more rigid compounds that are in a more ordered state, resulting in a decrease in entropy. This has implications regarding the energies of hop acid-polypeptide interactions, i.e. the free energy of the interactions with polypeptides would be more negative for the *trans*-iso-α-acids [28]. In this context, Jaskula et al. studied the differential behaviour of *trans*- and *cis*-iso-α-acids in relation to the residue fraction of wort [8]. In the hot trub fraction of wort, these authors detected very high T/C ratios and defended this by the more favourable interactions between *trans*-iso-α-acids and polypeptides. Differences between *trans*- and *cis*-iso-α-acids were also shown in their adsorption onto the yeast head, leading to greater loss of *trans*-isomers during fermentation [28, 38].

3 Reduced iso-α-acids

Modification of hop extracts by chemical means to provide various reduced derivatives of iso-α-acids does not only give the advantage of convenience due to post-fermentation addition. Reduced iso-α-acids also exhibit foam enhancing properties [2, 39, 40] and different relative bitterness compared with conventional iso-α-acids [26, 41, 42]. These reduced iso-α-acids were initially developed to provide beer, bottled in green or clear glass, protection against the effects of light exposure [43, 44, 45]. In spite of the Reinheitsgebot (German beer purity law in which it is laid down that the only ingredients used in beer production are water, malt and hops), the advantages of reduced iso-α-acids are increasingly exploited by the brewing industry.

The dihydroiso-α-acids (see Fig. 1), also referred to as rho-iso-α-acids, are formed by reduction of the carbonyl group in the 4-methyl-3-pentenoyl or isohexenoyl side chain at the C4 of iso-

α-acids to a secondary alcohol using sodium borohydride ($NaBH_4$) or hydrogen gas (H_2) in the presence of a catalyst (e.g. palladium on activated carbon) [46]. A new asymmetric carbon atom occurs with this reduction, as a result of which two stereoisomers arise from each iso-α-acid. Accordingly, a mixture of rho-iso-α-acids is produced that can consist of four diastereoisomers (two *cis* and two *trans* isomers) for each of the three major analogs, rho-isocohumulone, rho-isohumulone, and rho-isoadhumulone [47]. Tetrahydroiso-α-acids (Fig. 1) can be derived from iso-α-acids by catalytic hydrogenation of the double bonds in the side chains at C4 and C5 of iso-α-acids [1]. Alternatively, tetrahydroiso-α-acids are obtained from β-acids. In this multistep production process, β-acids are initially transformed to 6-deoxytetrahydro-α-acids. Following oxidation gives rise to tetrahydro-α-acids, which are then isomerised to tetrahydroiso-α-acids [48]. Another preparation of tetrahydroiso-α-acids involves the hydrogenation of α-acids with subsequent isomerisation [47]. Simultaneous isomerisation and hydrogenation of α-acids under alkaline conditions to yield tetrahydroiso-α-acids in one step and with high purity has also been reported [49]. Hexahydroiso-α-acids are formed from iso-α-acids via a simultaneous or successive reduction of the carbonyl group to a secondary alcohol in the isohexenoyl side chain at C4 and catalytic hydrogenation of the double bonds in both the isohexenoyl side chain at C4 and the prenyl side chain at C5 [46].

The foam-stabilising characteristics of dihydroiso-α-acids are comparable to those of iso-α-acids, whereas tetrahydroiso-α-acids are frequently used for the improvement of foam stability and lacing. The role tetrahydroiso-α-acids play in the amelioration of beer foam quality is based on their enhanced hydrophobicity compared with iso-α-acids [21]. According to Hughes, increases in the molecular volume of the side-chains of hop bitter acids are positively correlated with hydrophobicity [21]. Simple double bond regions are approximately planar, while saturation gives a structure which is more bulky with the carbon atoms coordinated in an approximate tetrahedral way. The reduction of two carbon-carbon double bonds in the side-chains of tetrahydroiso-α-acids results thus in an increase in the volume of those side chains, with an increase in hydrophobicity as a result. For the same reasons, the foam-enhancing characteristics of hexahydroiso-α-acids are even further improved relative to those of tetrahydroiso-α-acids, but their application in beer production is limited due to their low solubility. On the other hand, dihydroiso-α-acids do have a larger volume than iso-α-acids, but are more polar owing to the fact that they participate in hydrogen bonds via the hydroxyl group generated by reduction of the carbonyl group of the isohexenoyl side chain at C4. Hydrophobic interactions are substantially weaker than hydrogen bonding [21].

The relative bitterness of dihydroiso-α-acids is 60–80 % of that of iso-α-acids, that of hexahydroiso-α-acids is only weakly increased with respect to iso-α-acids [50, 51], while tetrahydroiso-α-acids are the most bitter of the hop-derived bitter acids [2]. According to *Benitez* et al., the bitterness intensity of tetrahydroiso-α-acids is 1.6 to 1.8 times higher than that of the iso-α-acids [2]. Different methods have however been employed by researchers to establish the relative bitterness potentials between these hop-derived bitter compounds [2, 50, 51, 52]. In particular tetrahydroiso-α-acids were found to be less bitter than previously claimed (1.0–1.1 times the bitterness of iso-α-acids) [50, 51]. Whereas the bitter taste of dihydroiso-α-acids seems to be shorter in comparison with iso-α-acids [51], hexahydroiso-α-acids exhibit a prolonged aftertaste [53].

4 Behaviour of iso-α-acids during beer storage

Although iso-α-acids constitute the most important hop-derived fraction with regard to beer quality and are essential to distinct beer from all other beverages, control of their stability is still an urgent problem. Both *cis*- and *trans*-iso-α-acids are vulnerable to degradation by light, oxygen or free radicals, and in contrast to *cis*-isomers, *trans*-iso-α-acids also undergo a specific cyclisation reaction, all thereby adversely affecting the beer flavour. First of all, significant losses of iso-α-acids influence the perception of bitterness. Secondly, the degradation of iso-α-acids yields compounds which themselves are flavour-negative. For example, the *trans*-specific degradation of iso-α-acids results in a quality change towards a lingering, more harsh bitterness. The mechanisms of the hop bitter acids degradation are reviewed in the next section.

5 Mechanisms of iso-α-acids degradation

5.1 Photodegradation of iso-α-acids

One difficulty with the presence of iso-α-acids in beer is their limited stability in visible light. Nevertheless, this lightstruck challenge is already well-known, as there has been made a lot of efforts to understand the key aspects of the underlying reaction mechanism. Photodegradation of iso-α-acids is generally accepted to be responsible for the generation of the pungent off-odour compound 3-methyl-2-butene-1-thiol (MBT) in beer, also known as 'skunky thiol' and characterised by an extreme low flavour threshold, 7 ng/L [54]. Either direct or sensitised irradiation of iso-α-acids, resulting in photolytic cleavage of the isohexenoyl side chain, affords the precursors for MBT formation [44, 55, 56, 57, 58]. As early as 1961, *Kuroiwa* and *Hashimoto* found that a flavin (the sensitiser), iso-α-acids and a suitable sulfur source, react in the presence of light with a wavelength range of 350–500 nm to form MBT [59]. They suggested a photosensitised Norrish type I α-cleavage of iso-α-acids leading to an intermediate radical (dimethylallyl or 3-methyl-2-butenyl radical), and recombination of this radical with a thiol radical derived from a sulfur containing amino acid or (poly)peptide (see Fig. 4). This so-called Kuroiwa formalism is still valid to date [43, 44]. Light-induced carbon monoxide was already observed in beer by *Gray* et al., suggestive of a decarbonylation to yield the dimethylallyl or 3-methyl-2-butenyl radical (see Fig. 4) [60]. Very recently, the underlying formation mechanism was elucidated by applying sophisticated techniques such as laser flash photolysis spectroscopy and electron paramagnetic resonance spectroscopy. In particular, the precise role of riboflavin (RF) as the photosensitiser, and the riboflavin-mediated oxidation pathway of the iso-α-acids, including the identification of the intermediate reactive radicals derived from the iso-α-acids, have been completely unravelled [45, 57]. Also the photodecomposition of several sulfur sources and the final sulfur incorporation into the skunky thiol, was unequivocally demonstrated [61]. Consequently, the mechanism of the light-induced formation of the skunky flavour in beer is clear,

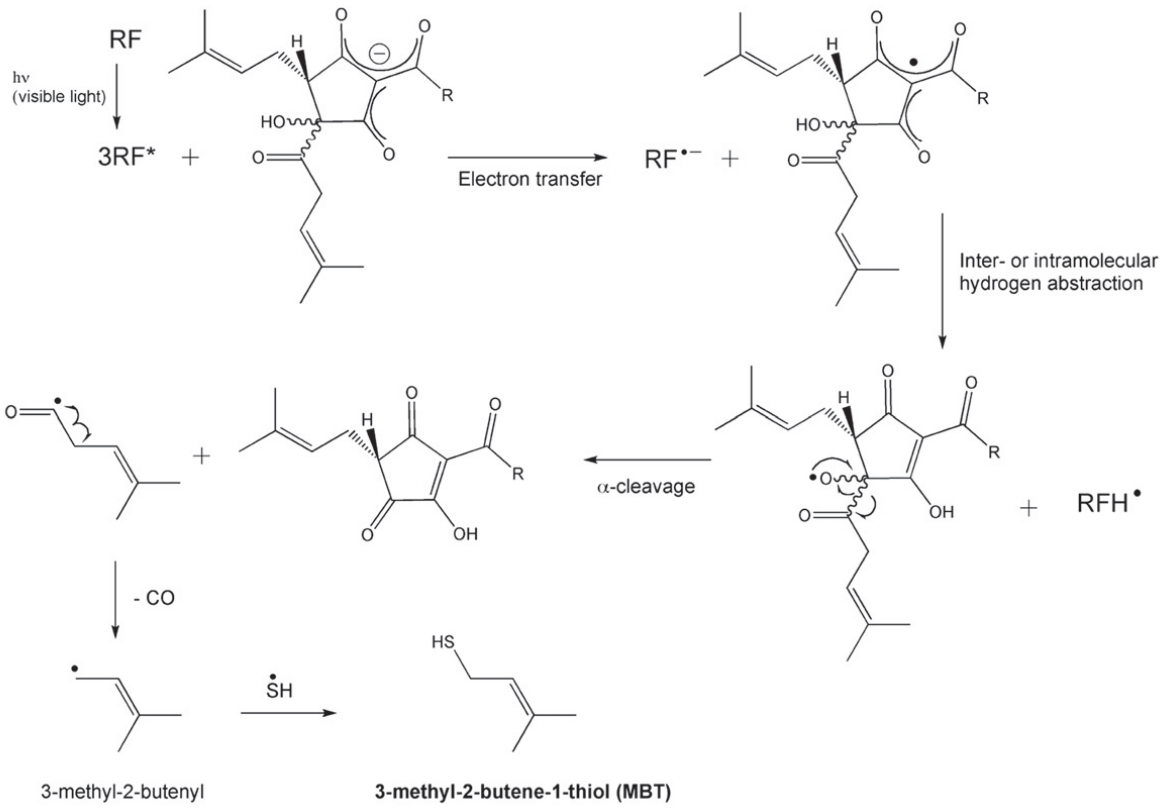


Fig. 4 Degradation products formed on visible light exposure of iso-α-acids in the presence of riboflavin (RF). Excited riboflavin (^{3}RF*) extracts an electron from iso-α-acids in their anionic form. The enolised β-tricarbonyl chromophore thereby absorbs the energy, which is then transferred to the isolated side-chain carbonyl function, eventually producing the 3-methyl-2-butenyl radical after a Norrish type I α-cleavage. Subsequent reaction with a suitable sulfur source results in the formation of MBT. Reprinted from Trends Food Sci Technol, 26, Caballero I., Blanco C. A. and Porras M., Iso-α-acids, bitterness and loss of beer quality during storage, 21–30. *Copyright (2012), with permission from Elsevier*

i.e. recombination of the 3-methyl-2-butenyl radical that is derived from photodecomposition of iso-α-acids, with the sulphydryl radical originating from photo-oxidation of cysteine, leads to MBT (Fig. 4).

When photodegradation is of concern, only storage in dark-coloured glass bottles or, alternatively, substitution of iso-α-acids for the reduced iso-α-acids, rho-iso-α-acids, hexahydroiso-α-acids and tetrahydroiso-α-acids, will prevent the formation of the typical lightstruck flavour in beer caused by the highly flavour-active MBT. Dihydroiso-α-acids lack the light-sensitive α-hydroxyketone (acyloin) group, since it is converted to a light-stable vicinal diol. For the same reason also hexahydroiso-α-acids are light-stable compounds. Conversely, photodegradation of tetrahydroiso-α-acids is likely to occur due to the fact that the α-hydroxyketone function is still present. However, MBT will not appear because the 3-methyl-2-butenyl radical is not able to be formed anymore [43, 44].

5.2 Degradation of hop bitter acids in the absence of light

As the major bittering principles in beer, iso-α-acids play an important role in the beer ageing process in terms of their instability during storage in the absence of light. Due to decomposition of in particular *trans*-iso-α-acids under these conditions, a significant decline in beer bitterness intensity and a quality change towards a lingering, more harsh bitterness are perceived. Evidence concerning the instability of the bittering principles in the beer matrix has been amassed by several investigators [13, 14, 62, 63, 64, 65, 66]. Concomitant formation of volatile carbonyl compounds associated with the deterioration of the fresh beer flavour is reported [67, 68, 69, 70, 71, 72], but mainly non-volatile structures of iso-α-acids degradation products are proposed in the literature [65, 66]. The following sections give an overview of the current knowledge on the degradation of iso-α-acids in the absence of light and describes the reported mechanisms.

5.3 Differential loss of iso-α-acids from beer during storage

Flavour instability, by far the most important quality problem of beer, may be caused by a multitude of reactions, the rapid deterioration of in particular *trans*-iso-α-acids in the absence of light is pivotal. Numerous studies have independently demonstrated that *trans*-iso-α-acids degrade much faster than their *cis*-counterparts, the latter remaining largely unaltered even after prolonged storage. This differential behaviour of the two types of stereoisomers of the bitter acids has been repeatedly demonstrated by a number of authors [13, 14, 15, 28, 63, 65, 66, 73, 74, 75, 76, 77, 78, 79]. *De Cooman* et al. concluded that the proportion of the *trans*-iso-α-acids concentration to the *cis*-iso-α-acids concentration, the so-called T/C ratio, can be regarded as a measure of the stability of beer bitter acids in aged beer [13]. Besides, the T/C ratios determined by HPLC corresponded very well with the sensory evaluation of aged beer. Therefore, iso-α-acids are considered as useful indicators to evaluate bitterness decay and the extent of flavour deterioration, and are reliable markers for beer ageing [14, 15, 63, 80, 81].

The β-tricarbonyl group of the iso-α-acids is a prime target for oxidation due to a number of ϖ and p electrons within a conjugated system incorporating three oxygen and four carbon atoms. The oxidation potential of the anions of *trans*-iso-α-acids, as measured against a normal hydrogen electrode (NHE) using E° = +630 mV versus NHE for the Fe^+/Fe couple in acetonitrile, is around 1.4 V, which results in an irreversible process and proves the high reactivity of the one-electron oxidised species produced. The undissociated *trans*-iso-α-acids have an oxidation potential of 1.8 V versus NHE, confirming the irreversibility of the process [82]. Nevertheless, the instability of the *trans*-iso-α-acids has been explained by *trans*-specific cyclisation reactions [65] and by free-radical reactions on the prenyl side chain at C5 of the five-membered ring due to the

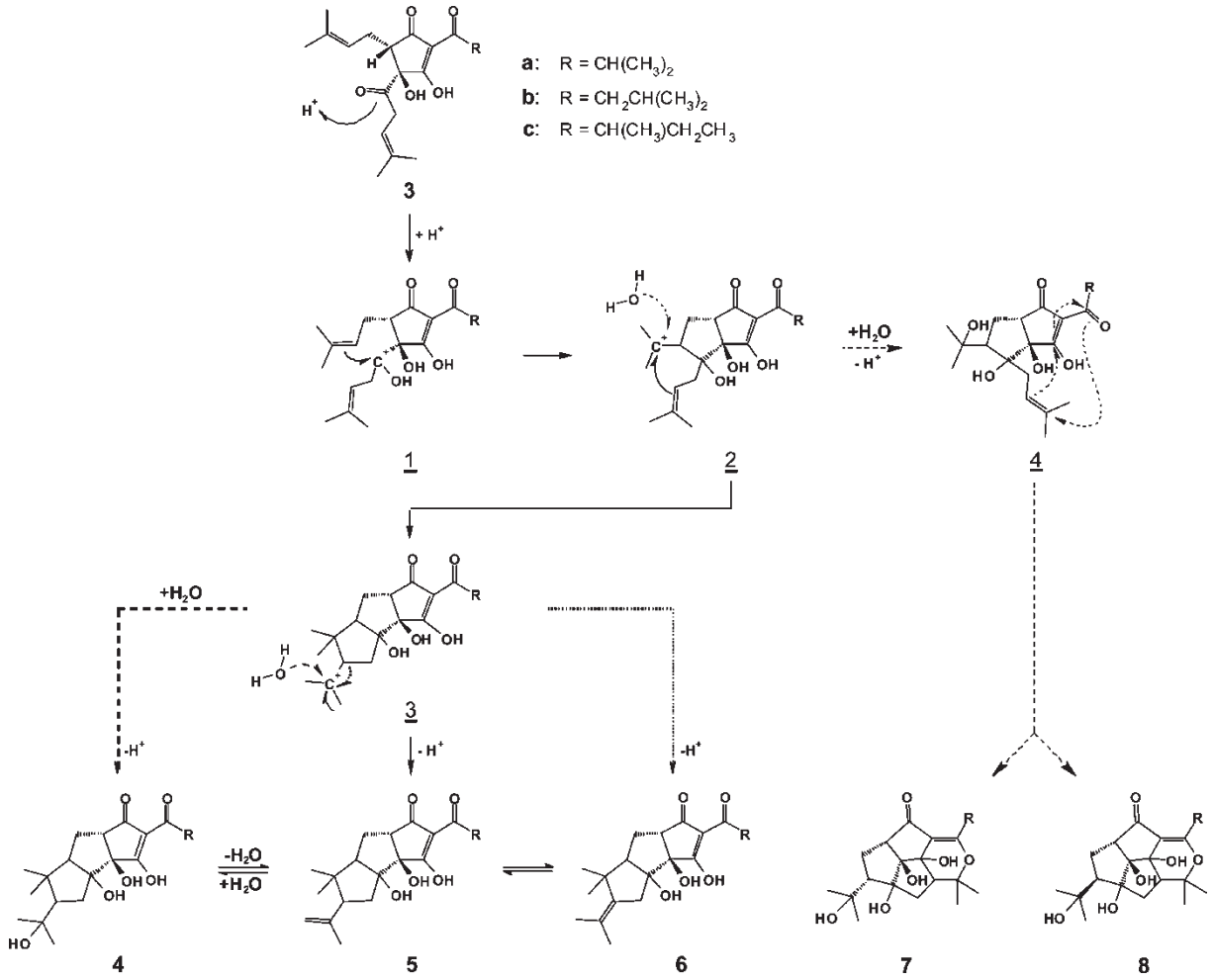


Fig. 5 Proposed reaction mechanism for the proton-catalysed, intramolecular nucleophilic cyclisation of *trans*-iso-α-acids, explaining the formation of the tricyclic (4, 5, 6) and tetracyclic degradation products (7, 8) from *trans*-iso-α-acids (3). Reprinted with permission from Intelmann et al. [65]. *Copyright 2009 American Chemical Society*

presence of active oxygen species [77]. More recently, the reactivity of the iso-α-acids towards the major radical in beer, 1-hydroxyethyl radical, has been reported [82]. The reaction mechanisms proposed by these authors will be described further on.

Reduced iso-α-acids (Fig. 1) are more resistant towards oxidative decomposition in comparison with conventional iso-α-acids [14, 62, 78, 83]. It was found that a certain type of reduced iso-α-acids, namely the tetrahydroiso-α-acids, are perfectly stable during beer ageing [13, 14, 63]. The reduction of the double bonds in the side chains at C4 and C5 is accounted for their exceptional stability, resulting in a reduced susceptibility for oxidative degradation [67, 82]. Furthermore, beers fully bittered with stable tetrahydroiso-α-acids, appeared to show a significantly improved flavour stability compared to beers that were bittered with 100 % hopextracts of non-reduced iso-α-acids or compared to beers that were conventionally hopped in the boiling kettle (e.g. with hop pellets or non-isomerised hop extract). This was unanimously determined by trained taste panels, based on three independent series of pilot brews [14, 40, 63]. The light-proof dihydroiso-α-acids were not stable on storage [14]. The exceptional stability of tetrahydroiso-α-acids is obviously indicating that hopping, especially the use of *trans*-iso-α-acids that are very susceptible to degradation, is of decisive meaning with respect to the flavour (in)stability of beer. On the other hand, full bittering with tetrahydroiso-α-acids results in the appearance of an unattractive foam. Therefore, using well-balanced mixtures of dihydroiso-α-acids and tetrahydroiso-α-acids, a suitable combination of desired bitterness with enhanced stability and foam stability can be aimed for [14, 40, 63].

5.4 Trans-specific cyclisation

Even though a number of investigators demonstrated the rapid degradation of *trans*-iso-α-acids upon beer storage in contrast with the enhanced stability of *cis*-iso-α-acids, and postulated underlying degradation mechanisms, the key transformation products formed exclusively from *trans*-isomers in beer were not yet elucidated. In 2009, *Intelmann* et al. presented different studies in which they explained the storage-induced changes of the beer's bitter taste by the formation of formerly unknown *trans*-specific iso-α-acid transformation products [65, 66, 76]. By simulating the degradation of iso-α-acids in quantitative model experiments and application of sophisticated NMR spectroscopic and LC-MS/MS techniques, a series of non-volatile cyclic *trans*-specific transformation products were unambiguously identified, exhibiting the aforementioned harsh, lingering bitter taste [66]. Initially, the structure of the major transformation product from *trans*-isocohumulone was unequivocally determined on the basis of ^{1}H NMR spectroscopic data as the previously unreported tricyclic compound tricyclocohumol (**4**) (see Fig. 5). Besides tricyclocohumol, the four compounds tricyclocohumene (**5**), isotricyclocohumene (**6**), tetracyclocohumol (**7**), and epitetracyclocohumol (**8**) were detected as additional major

transformation products of *trans*-isocohumulone under hydroalcoholic conditions (Fig. 5). As all these transformation products show the isopropyl moiety (the R group in the side chain at C2 for co-isomers) in their chemical structure, their generation from *trans*-isocohumulone seems independent of the variable alkanoyl side chain of the *trans*-iso-α-acid [66]. Accordingly, the corresponding tricyclic and tetracyclic homologues of the n- and ad-isomers of *trans*-iso-α-acids were also detected by these authors, which is conform with the observations that all the *trans*-iso-α-acid congeners similarly degrade during beer storage [65, 66].

Tricyclocohumene and isotricyclocohumene exhibited a rather lingering, harsh bitterness and the lowest bitter threshold concentrations of respectively 5 and 10 µmol/L, somewhat lower compared to the 20 µmol/L measured for their precursor *trans*-isocohumulone. On the contrary, tricyclocohumol and the tetracyclocohumols have higher bitter taste threshold concentrations of 30 and 70 µmol/L, respectively. When screening for the transformation products of *trans*-isocohumulone in fresh and aged beer samples, they were found to be present in fresh beer only at trace levels, originating from the beer production process (e.g. wort boiling), or were not detectable at all (epitetracyclocohumol). Under authentic ageing conditions, tricyclocohumene and isotricyclocohumene were formed from their precursor in rather low yields, whereas the less bitter tricyclocohumol and tetracyclocohumol were detected in relatively high yields, which is in agreement with the decrease in the bitterness intensity and the alteration in quality towards a lingering and harsh bitterness observed during beer storage [66, 77]. Tricyclocohumol was the most predominant and epitetracyclocohumol the minor reaction product. These tri- and tetracyclic degradation products explain for about 91 % of the *trans*-iso-α-acid degradation on a molecular level [65].

By application of ^{18}O stable isotope labeling in addition to quantitative model studies combined with LC-MS/MS experiments, Intelmann et al. also revealed the mechanism behind this stereospecific transformation of *trans*-iso-α-acids into the tri- and tetracyclic degradation products [65]. The proposed reaction mechanism as represented in figure 5 is based on a proton-catalysed carbon/carbon bond formation between the carbonyl atom C1' of the isohexenoyl moiety and the alkene carbon C2'' of the isoprenyl moiety of the *trans*-iso-α-acids. At first, protonation of the carbonyl group of the isohexenoyl side chain in the *trans*-iso-α-acid (**3**) gives rise to the intermediary carbocation 1 that undergoes cyclisation by an intramolecular attack of the ϖ-electrons of the methine carbon of the prenyl chain, resulting in the carbocation 2 (Fig. 5). This latter intermediate might undergo a second intramolecular attack of the olefinic carbon of the isoprenyl side chain to yield the carbocation 3 (Fig. 5). This intermediate can further react to stable end products via three alternative routes. Compound **4** (e.g. tricyclocohumol) is formed by nucleophilic addition of a water molecule (Fig. 5). Elimination of a proton leads to the unsaturated compounds **5** (e.g. tricyclocohumene) and **6** (isotricyclocohumene), respectively (Fig. 5). These tricyclic compounds **4**, **5** and **6** were found to be converted into each other, reaching a molar equilibrium ratio of 6:1:30. Alternatively, carbocation 2 might add a water molecule resulting in the intermediary alcohol 4, which, when being protonated at the carbonyl function of the alkanoyl side chain, induces an intramolecular cyclisation involving the double bond of the isoprenyl side chain as well as the vinylogous carbonyl moiety in the tricarbonyl system. Either the tetracycle **7** (e.g. tetracyclocohumol) or **8** (e.g. epitetracyclocohumol) is formed (Fig. 5). Stereochemical considerations using molecular dynamics simulations further strengthened the mechanism in which exclusively *trans*-iso-α-acids and not *cis*-iso-α-acids are able to generate these cyclic degradation compounds [65].

Verzele and De Keukeleire already reported that the length of the side chains in the structures of iso-α-acids, together with the double bonds and hydroxyl groups, easily give rise to oxidative cyclisations [1]. Intelmann et al. demonstrated that the cyclic degradation products were only formed in aqueous or water/alcoholic solution and not in the absence of water [65]. The reaction was found to be strongly pH dependent and oxidative and/or photo-oxidative reaction mechanisms are believed not to play any major role. The most rapid degradation was observed at pH values as low as 1.0, whereas none of the reaction products were formed at pH 6.0 [65]. Also *Kaneda* et al. already reported the accelerated degradation of iso-α-acids with decreasing pH [84]. After total consumption of the precursor *trans*-isocohumulone, isotricyclocohumene was still formed with increasing reaction time by transformation of the most rapidly formed major reaction products tricyclocohumol and tricyclocohumene [65].

5.5 Autoxidation of iso-α-acids

The proton-catalysed cyclisation reaction that was found to induce the rapid degradation of *trans*-iso-α-acids, does not explain the slight decrease of *cis*-iso-α-acids that is also observed during storage of glass bottled beer in the absence of light. A decline of about 20 % of the initial *cis*-iso-α-acids concentration was reported after 8 to 12 months of beer storage [13, 75, 76]. According to earlier work, many authors claim free-radical reactions as the cause for the decomposition of iso-α-acids [13, 14, 62, 63, 69, 84, 85, 86, 87]. Hashimoto and *Eshima* reported the degradation of iso-α-acids in the presence of oxygen [67]. However, both by photodegradation and radical-assisted oxidation, iso-α-acids undergo oxidative decomposition by electron loss in the presence of suitable electron acceptors, which does not necessarily requires oxygen species [43, 44, 57, 58, 88]. Still, the degradation of *trans*-iso-α-acids in the absence of light is often considered to be an oxidative process according to the literature [13, 15, 84], even though it takes place in quasi oxygen-free beer.

De Cooman et al. explained the instability of *trans*-iso-α-acids by the specific stereochemical arrangement of the tertiary hydroxyl group at C4 and the prenyl side chain at C5 (see Fig. 1), as a result of which *trans*-isomers are much more prone to oxidation reactions than are *cis*-iso-α-acids [13]. The proximity of the unsaturated sites in *trans*-iso-α-acids provides a pool of high electron density, which may initiate autoxidation, either by direct reaction involving the double bonds or by allylic hydrogen abstraction from the doubly activated α-carbon of the isohexenoyl side chain at C4. Radical chemistry rapidly ensues, leading to a multitude of oxidation products [13, 82, 84].

Intelmann and *Hofmann* very recently monitored the quantitative degradation of both *cis*- and *trans*-iso-α-acids in a PET bottle,

which is not gastight and allows the migration of air oxygen into the bottle [77]. Consequently, these authors investigated whether oxygen is involved in the depletion of iso-α-acids during beer ageing. They examined the chemical transformation of iso-α-acids in model experiments under an atmosphere of oxygen, and identified hy-droperoxy- and hydroxy-allo-iso-α-acids as the primary autoxidation products of both *cis*- and *trans*-iso-α-acids in beer in the presence of oxygen. The reaction pathway leading to hydroperoxy-allo-iso-α-acids and hydroxy-allo-iso-α-acids proposed by Intelmann and Hofmann is presented in figure 6 [77]. The air oxidation of the iso-α-acids (**2**, **3**) leads primarily to the generation of corresponding hydroperoxides (**5**, **6**), which were found to be unstable compounds as the corresponding hydroxides (**7**, **8**) are formed thereof upon storage (Fig. 6). Initially, a hydrogen atom is abstracted from an iso-α-acid (**2**, **3**) by a starter radical, in analogy to the autoxidation of unsaturated fatty acids, leading to a resonance-stabilised radical in the isohexenoyl side chain of the iso-α-acid. In the presence of triplet oxygen, a peroxy radical is formed, which can abstract a hydrogen atom from another donor molecule such as an iso-α-acid. That is the start of another reaction cascade and the formation of the hydroxyperoxy-allo-iso-α-acids (**5**, **6**), respectively (Fig. 6). By means of transition metal ions like iron(II) ions or by light, the hydroperoxy function is cleaved to give an alkoxy radical. Abstraction of a hydrogen atom from another donor molecule gives then rise to the generation of the hydroxy-allo-iso-α-acids (**7**, **8**) (Fig. 6). Both hydroperoxy-allo-iso-α-acids and hydroxy-allo-iso-α-acids were shown to be already present in fresh beer, but only in rather low concentrations (a total amount of 0.39 µmol/L). Hydroperoxy-*cis*-allo-iso-α-acids and hydroxy-*cis*-allo-iso-α-acids were the most abundant oxidised isomers (respectively 0.21 and 0.11 µmol/L). Upon ageing, the hydroperoxide levels remained rather constant and the concentrations of the hydroxides increased only to some extent, while a clear relative increase in these oxidation products was revealed, implying that they are not stable oxidation products. In contrast to the *cis*-configured hydroxides, the *trans*-configured hydroxides are less stable and more rapidly further degraded via unknown pathways. The quantitative data of these quickly formed autoxidation products can be used to measure the oxidative status of beer, but are due to their instability not very suitable as oxidation indicators [77, 89].

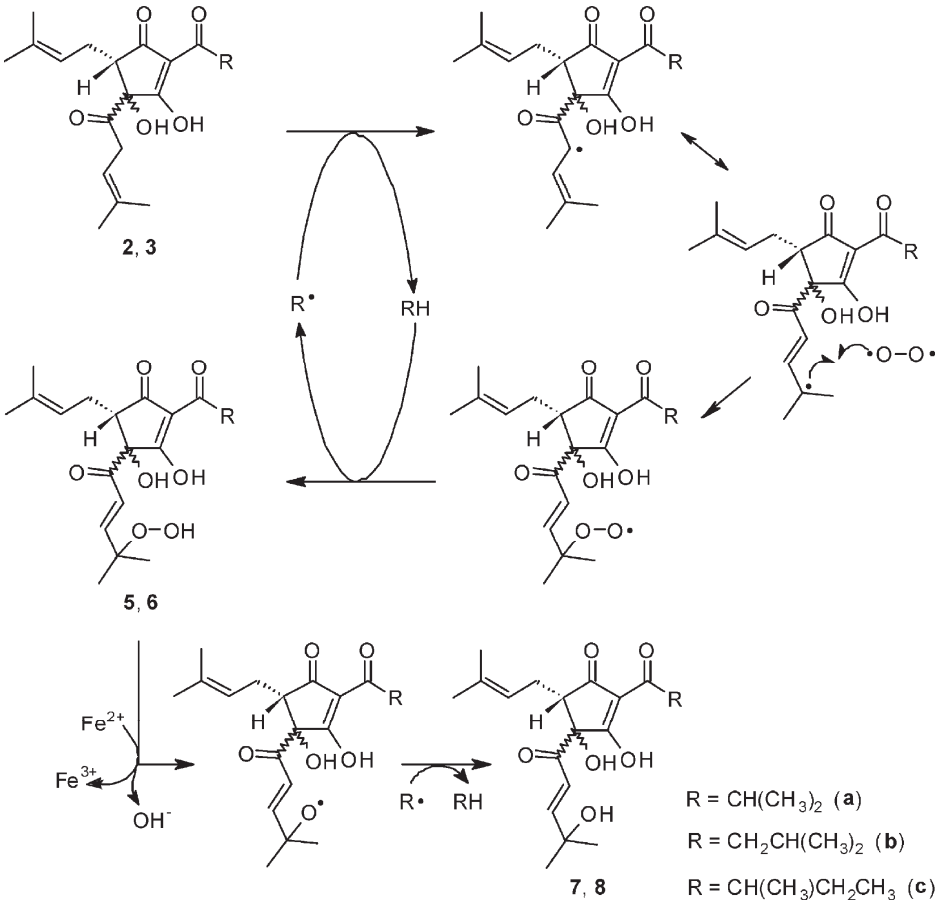


Fig. 6 **Proposed reaction pathway for the formation of *cis*- and *trans*-hydroperoxy-alloisocohumulone (5a and 6a), hydroperoxy-alloisohumulone (5b and 6b), hydroperoxy-alloisoadhumulone (5c and 6c), and *cis*- and *trans*-hydroxyl-alloisocohumulone (7a and 8a), hydroxyl-alloisohumulone (7b and 8b), and hydroxyl-alloisoadhumulone (7c and 8c), respectively. Compounds 2, 5, and 7 are *R*-configured at position C4 of the five-membered ring, whereas the epimeric structures 3, 6, and 8 exhibit a *S*-configured stereocenter. Reprinted with permission from Intelmann and Hofmann [77].** *Copyright 2010 American Chemical Society*

5.6 Reactivity of hop bitter acids towards the 1-hydroxyethyl radical

Recently, *de Almeida* et al. studied the reactivity of hop bitter acids towards the major radical identified in beer, the 1-hydroxyethyl radical [82, 90, 91]. The authors determined the apparent second-order rate constants for the reactions between this radical and the bitter acid compounds by electron paramagnetic resonance (EPR) spectroscopy and electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) [82]. Through this competitive kinetic approach, they observed that both iso-α-acids and dihydroiso-α-acids were decomposed in the presence of the 1-hydroxyethyl radical with comparable reactivities (apparent second-order rate constants 1.3 x 10^9 and 1.5 x 10^9 $L \cdot mol^{-1} \cdot s^{-1}$, respectively). Tetrahydroiso-α-acids did not react with the radical. Isolation of *trans*- and *cis*-iso-α-acids from the mixture of iso-α-acids (ratio *cis*-/*trans*-iso-α-acids 7:3) revealed that the *trans*-iso-α-acids (average apparent second-order rate constant 9.2 x 10^9 $L \cdot mol^{-1} \cdot s^{-1}$) showed higher reactivity than the *cis*-iso-α-acids (average apparent second-order rate constant 1.8 x 10^8 $L \cdot mol^{-1} \cdot s^{-1}$) towards the 1-hydroxyethyl radical. As said by de Almeida et al., the reaction between the 1-hydroxyethyl radical and the β-tricarbonyl chromophore, typical for the beer bitter acids studied, is not thermodynamically favourable as the reduction potential of the radical (0.98 V versus NHE) is significantly lower than the oxidation potential of the compounds involved [82]. No difference in reactivity of the dissociated and undissociated *cis*- and *trans*-iso-α-acids towards the 1-hydroxyethyl radical was observed. Therefore, the operating mechanism for the reactions between the bitter acids and the 1-hydroxyethyl radical was suggested to be hydrogen transfer from olefinic groups rather than electron transfer from the β-tricarbonyl system to the radical (see Fig. 7). The bond dissociation enthalpies of relevant allylic hydrogen atoms in the isohexenoyl and prenyl side chains at C4 and C5 of the five-membered ring point

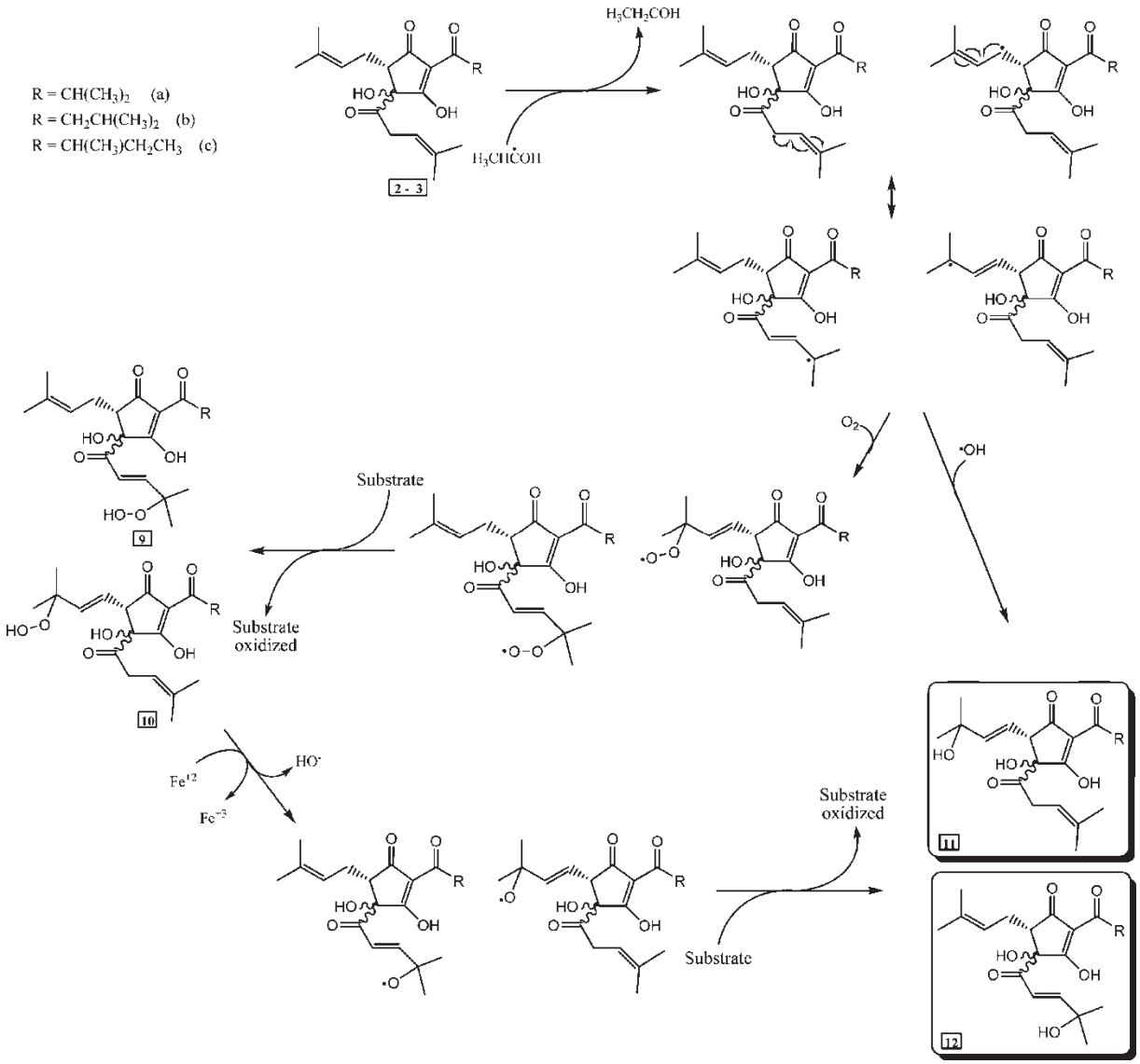


Fig. 7 Proposed reaction pathway for the formation of hydroxyl-*cis*- and hydroxyl-*trans*-alloisocohumulones (12a), hydroxyl-*cis*- and hydroxyl-*trans*-alloisohumulones (12b), and hydroxyl-*cis*- and hydroxyl-*trans*-alloisoadhumulones (12c). Compounds 11a-11c refer to hydroxylated isohumulones. Compounds 2 and 3 refer to *trans*- and *cis*-isohumulones, respectively, whereas compounds 9 and 10 refer to hydroperoxy-alloisohumulones. Reprinted with permission from de Almeida et al. [82]. *Copyright 2011 American Chemical Society*

out that hydrogen atom abstraction by the 1-hydroxyethyl radical from both groups is possible, because both possibilities deliver resonance-stabilised radicals. But as these data are comparable for *cis*- and *trans*-iso-α-acids (C-H_{prenyl} 314 and 316 kJ·mol^{-1}, C-$H_{isohexenoyl}$ 325 and 316 kJ·mol^{-1} for *cis*- and *trans*-iso-α-acids, respectively), they do not explain the different reactivity of both isomers towards the 1-hydroxyethyl radical. Consequently, the higher reactivity of *trans*-iso-α-acids can be accounted for an entropic compensation and is explained by the different stereochemical arrangement of the side chains at C4 and C5 [82]. The spatial proximity of these two chains in the *trans* form (see Fig. 1) indeed suggests a higher density of electrons, as previously reported by *De Cooman* et al. [13]. This particular stereochemistry results in different reaction rates for *cis*- and *trans*-iso-α-acids. In this context, it was also found that the *cis* configuration is more stable than the *trans* configuration, with an energy difference of 15.9 kJ·mol^{-1} [82]. Accordingly, tetrahydroiso-α-acids are explained not to be reactive towards the 1-hydroxyethyl radical because of the lack of hydrogens in allylic positions with respect to a side-chain olefinic bond, in contrast with both iso-α-acids and dihydroiso-α-acids.

By liquid chromatography-electrospray ionisation-ion trap-tandem mass spectrometry (LC-ESI-IT-MS/MS), *De Almeida* et al. identified the oxidation products resulting from the reaction between the 1-hydroxyethyl radical and *trans*-iso-α-acids [82]. 1-Hydroxyethyl radicals were derived from the presence of hydrogen peroxide and Fe^{II} ions in the reaction mixture, i.e. a solution of *trans*-iso-α-acids in ethanol. For both *cis*- and *trans*-iso-α-acids, the authors proposed the reaction pathway in figure 7, suggesting the formation of hydroxy-containing reaction products. The produced hydroxyl-*cis*- and hydroxyl-*trans*-allo-iso-α-acids (**12**) contain a hydroxyl group in the isohexenoyl side chain at C4 of the iso-α-acids (Fig. 7). These oxidation compounds have earlier been reported as the primary autoxidation products of both *cis*- and *trans*-iso-α-acids [77, 92]. In addition, de Almeida et al. proposed a mixture of positional isomers of hydroxylated iso-α-acids in which the olefinic double bond is shifted to an allylic position in the prenyl side chain at C5 (**11**) (Fig. 7) [82]. These oxidation products have not been previously reported, and are regarded as novel derivatives of the *trans*-iso-α-acids. Furthermore, residual oxygen might react with the resonance-stabilised radical derived from iso-α-acids, yielding

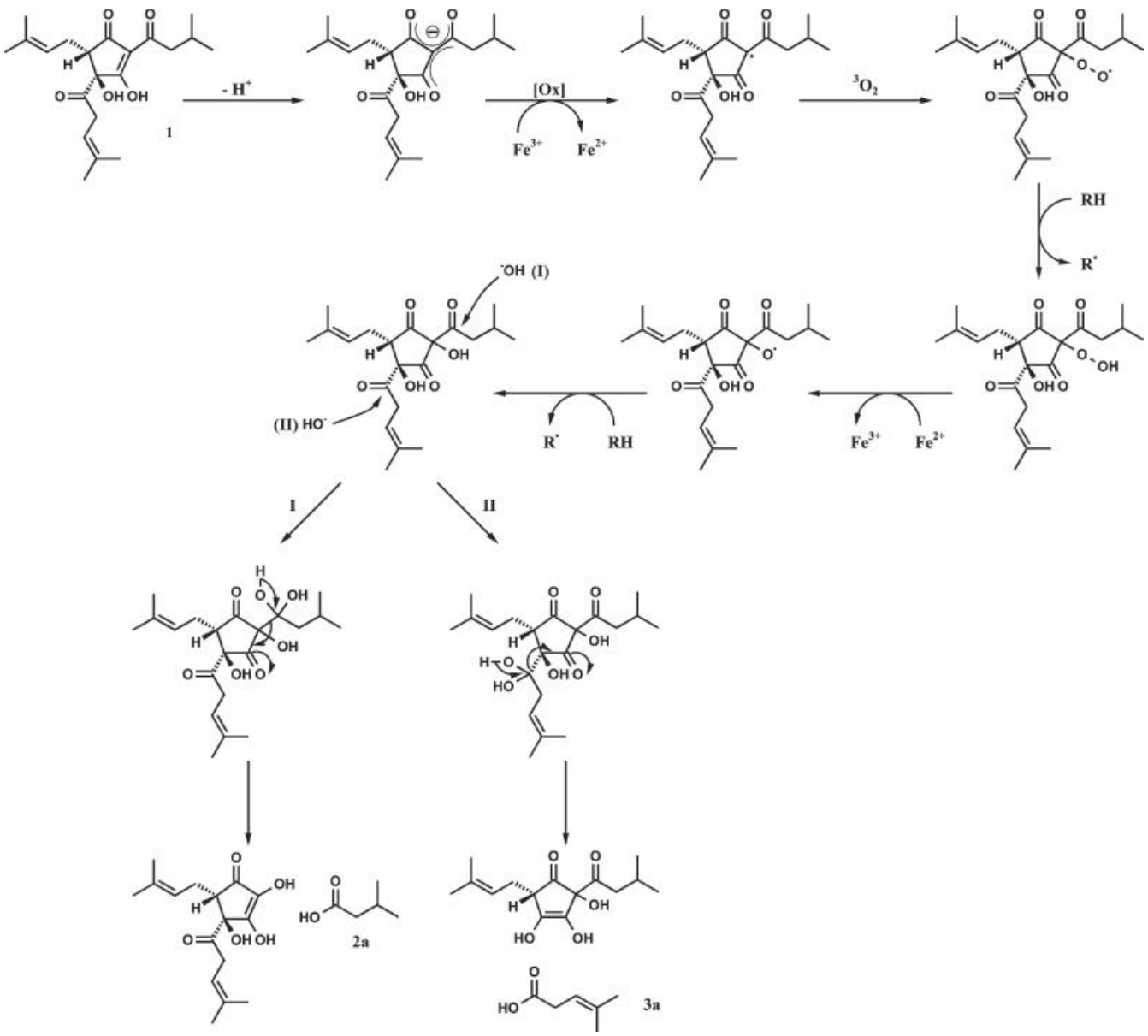


Fig. 8 Mechanism leading to the formation of 3-methylbutyric acid (2a) and 4-methyl-3-pentenoic acid (3a) from *trans*-isohumulone (1) by hydrolytic β-dicarbonyl cleavage after oxidation of the isohumulone ring structure. Reprinted with permission from Rakete et al. [93]. *Copyright 2014 American Chemical Society*

the corresponding peroxyl radical, and after hydrogen abstraction from oxidisable substrates, such as ethanol or iso-α-acids, hydroperoxides are formed thereof (**9**–**10**) (see Fig. 7). Cleavage of the hydroperoxides in the presence of high amounts of Fe^{II} generates alkoxyl radicals, which again leads to the compounds **11** and **12** after hydrogen atom abstraction from oxidisable substrates (see Fig. 7) [82].

5.7 Oxidation of iso-α-acids induces the formation of carboxylic acids by hydrolytic cleavage

Most recently, *Rakete* et al. [93] studied the formation of carboxylic acids and their corresponding amides from the reaction of *trans*-isohumulone with amines, in particular L-proline, representing approximately 50 % of the total free amino acids in beer. *Trans*-isohumulone (5 mM) was incubated at both pH 5 and pH 7 with 25 mM L-proline for up to 21 days at 50 °C in 0.1 M ethanolic phosphate buffer (10 % vol. ethanol) under aerated and deaerated conditions. At pH 5, degradation of *trans*-isohumulone was visible up to 97 %, although slightly less at deaerated conditions (95 %), whereas the amounts of unaltered *trans*-isohumulone remained relatively high at pH 7 (72 % of degradation under dearation and 85 % under oxidative conditions). The pH dependency of the *trans*-isohumulone degradation reported by Rakete et al. [93] is conform with the literature [65, 84]. Along with the degradation of *trans*-isohumulone, the levels of 3-methylbutyric acid and 4-methyl-3-pentenoic acid increased over the incubation time, preferably at neutral and aerated conditions. In parallel to the carboxylic acids, the analogous L-proline amides were also formed in the incubations, but about 40–50 times less compared to the corresponding carboxylic acids. *Trans*-humulinic acid and hydroxy-*trans*-alloisohumulone were also quantitated in the incubations. Both *trans*-humulinic acid and hydroxy-*trans*-alloisohumulone were preferentially formed at pH 7 under deaerated conditions. In particular the levels of hydroxy-*trans*-alloisohumulone were considerably higher at pH 7 when compared with pH 5, but in both cases there was a maximum after 3 days after which the levels declined.

As shown in figure 8, the authors postulated hydrolytic β-dicarbonyl cleavage after oxidation of the isohumulone ring structure as the mechanism of formation for the generated carboxylic acids [93]. After deprotonation, the isohumulate ion is oxidised via a one-electron oxidation, giving a carbon-centered *trans*-isohumulone radical [88]. Reaction with molecular oxygen leads to the formation of the corresponding hydroperoxy radical. After abstraction of hydrogen from a donor molecule, the hydroperoxide undergoes a Fenton-like redox reaction to form the alkoxy radical and subsequently the alcohol after a second abstraction of hydrogen from another donor molecule. The now oxidised isohumulone has lost its ability to enolize, which comes along with a significantly increased carbonyl activity. Nucleophilic attack of water or L-proline at the carbonyl function at the alkanoic or alkenoic side chain then gives the free carboxylic acid or the carboxylic acid amide based on β-dicarbonyl cleavage, respectively [93]. These carboxylic acid proline amides were also verified in beer. Although the spectrum of 3-methylbutyric acid proline amide in beer was less intense compared to the authentic standard, presence in beer was confirmed. Only traces of the 4-methyl-3-pentenoic acid proline amide were found in beer [93].

5.8 Involvement of the iso-α-acids degradation in the formation of volatile aldehydes

Taking into account the current research into flavor instability of beer, the formation of volatile flavour-active aldehydes from iso-α-acids during beer storage in the absence of light is of major concern. Nevertheless, research exploring the possible connections between the formation of these well-known aged beer off-flavours and the decline of the hop bitter acids have only scarcely been carried out. In particular radical-assisted oxidation of iso-α-acids is generally accepted to be responsible for the generation of pungent volatile degradation products, but the available information on the mechanisms of their formation from iso-α-acids is rather fragmentary [67, 69, 94, 95]. According to *Kaneda* et al., free radicals, such as hydroxyl radicals and the superoxide radical anion, oxidise iso-α-acids directly into aldehydes and ketones [69]. Besides, they proposed hydroxyl radicals to initiate a series of radical reactions which, in turn, also oxidise iso-α-acids into undesirable aldehydes. As said by Hashimoto and Eshima, the portion of isohumulone in bottled beer that is oxidatively degraded, is the carbonyl group or the unsaturated bond of the isohexenoyl side chain at C4 of the molecule, leading to the formation of minute amounts of aldehydes [67]. Hashimoto and Eshima showed the formation of carbonyl compounds with various side chain lengths, such as C3–C11 2-alkanones, C2–C10 alkanals, C4–C7 2-alkenals and C6–C7 2,4-alkadienals, in beer model systems [67, 95]. Additionally, acetone, 2-methylpropanal, 3-methyl-butan-2-one, methyl isobutyl ketone or 4-methyl-pentan-2-one, 2-methyl-3-buten-2-ol [67, 95], and several acids, namely 2-methylpropionate [96], 2-methylbutyrate [96] and 3-methylbutyrate [93, 96], were also identified as bitter acids degradation products. Because aldehydes might be involved in subsequent reactions such as aldol condensation, unsaturated alkenals and alkadienals with chain lengths of 6 to 10 carbon atoms can then be generated, causing the aged flavour of beer [94]. Accordingly, Hashimoto and Eshima assert that beer brewed without hops hardly develops any typical aged flavour even after prolonged storage [67].

Similarly, the use of dihydroiso-α-acids but especially the perfectly stable tetrahydroiso-α-acids, would prevent the development of an aged flavour according to *Zufall* et al. [97]. However, the relation between the bitter acids decline and the increase in volatile ageing aldehydes was subject of further investigation. *De Clippeleer* et al. investigated the formation of aldehydes corresponding to the *trans*-specific conversion of iso-α-acids [98]. In particular, they studied the generation of aldehydes in beers exclusively bittered with *trans*-, respectively *cis*-iso-α-acids during forced ageing (T/C ratio 790 % for *trans*-beers and 3 % for *cis*-beers). Consequently, pure *trans*- respectively *cis*-isomers were required in large amounts for post-fermentation addition to finished beer. Separation of *trans*- from *cis*-iso-α-acids in a commercial isomerised hop extract was accomplished on pilot scale by the formation of *trans*-iso-α-acids β-cyclodextrin inclusion complexes, permitting the quantitative dosage to beer in mg/L amounts [98]. The authors assumed that if the bitter acids degradation contributes to the flavour instability of beer by formation of aldehydes such as 2-methylpropanal, 2-methylbutanal and 3-methylbutanal, the development of these off-flavour compounds should be different in authentic beer samples exclusively bittered with *cis*- respectively *trans*-iso-α-acids as observed with advanced analysis techniques [98]. The distinct decline in *trans*-iso-α-acids, demonstrated in the beers exclusively bittered with *trans*-iso-α-acids, could however not be linked to an enhanced formation of ageing aldehydes when compared with the unhopped treatment and the beers exclusively bittered with *cis*-iso-α-acids, which showed enhanced bitterness stability. Formation of aldehydes by the *trans*-specific iso-α-acid degradation seems to play only a minor role in the beer matrix upon ageing [98]. The explanation of aldehyde formation upon beer ageing was found in the malt used for brewing, irrespective of the mode of bittering [98]. This means that a beer exclusively bittered with *cis*-iso-α-acids, exhibiting superior stability towards degradation compared with their *trans*-counterparts, is not necessarily more flavour stable from an aldehyde-point of view. Additionally, concomitant with the degradation of *trans*-iso-α-acids, a remarkable increase of the relative concentration of *cis*-iso-α-acids up to 150–200 % was observed during forced ageing of the beers exclusively bittered with *trans*-iso-α-acids [98]. This phenomenon was demonstrated for the first time in a beer matrix and according to the authors the only apparent reason for it, were the very low concentrations of *cis*-isomers and the very high proportion of their *trans*-counterparts in the *trans*-beer matrix [98]. A possible explanation of the considerable increase of *cis*-iso-α-acids detected in the *trans*-beers, was found in the fact that *cis*-iso-α-acids are thermodynamically more stable than their *trans*-counterparts [5], the reason for which the *cis*/*trans*-iso-α-acids ratio is typically 7:3 in commercial isomerised hop extracts [1, 38]. The observations in the *trans*-beers were ascribed to partial conversion, i.e. reverse isomerisation, of *trans*-iso-α-acids via α-acids into *cis*-iso-α-acids [98].

Starting from pure *trans*-iso-α-acids, the free energy of activation required to attain the transition state in the formation of α-acids, is lower than starting from pure *cis*-iso-α-acids (see Fig. 9) [5]. Despite the higher activation energies required for *cis*-isomer formation from α-acids, this configuration is energetically favoured, since the finished product, a *cis*-iso-α-acid, has indeed a lower free energy, i.e. a higher stability from the thermodynamic point

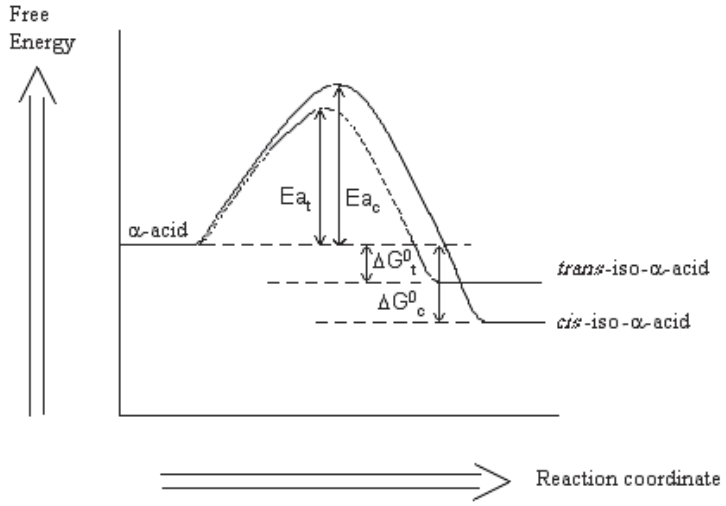


Fig. 9 Free energy diagram for the formation of *trans*- and *cis*-iso-α-acids from α-acids proposed by Jaskula et al. [5]. $\Delta G°_t$: overall free energy change related to the formation of *trans*-iso-α-acids; $\Delta G°_c$: overall free energy change related to the formation of *cis*-iso-α-acids; $E_{a\,t}$: free energy of activation related to the formation of *trans*-iso-α-acids; $E_{a\,c}$: free energy of activation related to the formation of *cis*-iso-α-acids

of view, compared to its *trans*-counterpart (Fig. 9) [5]. In *cis*-iso-α-acids, the bulky side chains at C(4) and C(5) are actually in the *trans*-configuration, indeed leading to more low energy conformations with a resultant increase in entropy, compared to that of *trans*-iso-α-acids [28]. *Trans-cis* interconversion was previously reported by Verzele and De Keukeleire [1] and demonstrated both in heated buffer systems and boiling wort, starting from pure *trans*-iso-α-acids [5].

6 Conclusion

In this article, we aimed at a comprehensive review on the current knowledge of the complex chemistry behind the formation of beer's bitter compounds, i.e. the iso-α-acids, and the mechanisms involved in their chemical degradation in the beer matrix during storage. Iso-α-acids are five-membered ring structures that arise from the isomerisation of α-acids during wort boiling. The hop α-acids isomerisation reaction has extensively been studied in the literature, and kinetic parameters related to it have been determined. During storage, beer's bitter compounds are unstable. Chemical transformation of both cis- and trans-iso-α-acids in the presence of light, reactive oxygen species and free radicals has been described. Various researchers reported that during dark storage trans-iso-α-acids in beer are much more susceptible to chemical transformations than their cis-counterparts. Next to the significant decline in beer bitterness, the decomposition of trans-iso-α-acids into a series of non-volatile cyclic compounds contributes to a harsh, lingering bitterness upon ageing. The flavour shelf life of beers brewed with a strongly increased level of cis-isomers could therefore be extended with regard to the bitterness intensity and quality. Nevertheless, the aldehyde increase in aged beers exclusively bittered with cis-iso-α-acids is equally high, since researchers demonstrated that the development of aldehydes from trans-iso-α-acids degradation is of minor importance in the beer matrix.

7 References

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