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# Impact of Contact Time, Temperature, and Ethanol Content on Hop Creep-Related Enzymatic Activities in Beer

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## ABSTRACT

In the production of hop-forward India Pale Ales (IPA), the “hop creep” phenomenon, resulting from hop enzyme activities (combined with yeast fermentation), is a major issue in breweries worldwide. In order to improve our knowledge of the parameters that can modulate these enzymatic activities, the present work aimed to assess to what extent hop contact time, incubation temperature, and beer ethanol content can affect the enzymatic activities of hops. To this end, various hop varieties were incubated in beer over a three-week period under different conditions of temperature and ethanol content. Samples were periodically collected to monitor changes in fermentable sugar and fermentation ester concentrations over time. A longer contact time favored  $\alpha$ -glucosidase and esterase activities, leading to a higher glucose concentration and lower maltose and isoamyl acetate concentrations. An assay at 50°C allows predicting this phenomenon more quickly. As expected, dry heat pre-treatment of hop was found to delay glucose release and isoamyl acetate hydrolysis. Ethanol at 10% v/v was required to inhibit hop amylolytic enzymes in beer, but had no effect on hop esterases.

**Abbreviations:** IPA: India Pale Ales; NAB: non-alcoholic beer; LAB: low-alcoholic beer; HPLC: high performance liquid chromatography; RID: refractive index detector; IST: internal standard; HS: headspace; GC: gas chromatography; MS: mass spectrometry; WCOT: wall-coated open tubular; ANOVA: analysis of variance

## KEYWORDS

Dextrin-reducing enzymes; dry-hopping; enzymes; esterases; hop creep; hops

## Introduction

Hop (*Humulus lupulus* L.) is added during boiling to impart bitterness and aromas, protect the wort against infections, and improve foam stability of the beer. Adding dual-purpose hop varieties at the end of wort boiling (late hopping) and/or during beer fermentation and/or lagering (dry-hopping) has become widespread in order to confer specific aromas to the product, usually associated with citrus, floral, and exotic fruity notes. For the past fifteen years, the production of hop-forward India Pale Ales (IPA) has constituted a large part of the craft beer market economy, representing around 20.3% of total sales in 2017.<sup>[1]</sup>

With the increasing use of dry-hopping, unexpected variations have been observed in the finished beer, such as over-attenuation, out-of-specification alcohol content, excessive carbon dioxide production, and off-flavors.<sup>[2–6]</sup> This phenomenon has been termed “hop creep” by the brewing community and refers to the refermentation of a fully attenuated beer following dry-hopping in presence of live yeast.<sup>[7]</sup> Investigations have led researchers to identify in hops amylolytic enzymes capable of breaking down residual non-fermentable dextrins to fermentable sugars. Subsequently, yeast can metabolize these new fermentable sugars, increasing the alcohol and CO<sub>2</sub>

contents over time. Hop creep is a major issue in breweries worldwide. It is particularly difficult for brewers to anticipate all the consequences, since both hop enzymatic activities and yeast activity depend on many parameters.<sup>[5,7]</sup> For this reason, numerous studies have focused on the problem to provide new insights into its causes.

First evidence of hop amylolytic enzymes was reported in 1893 by Brown and Morris.<sup>[8]</sup> Observing that dry-hopping led to more persistent fermentation of beers, they rigorously demonstrated that hops contain a diastase capable of hydrolyzing dextrins of the beer to “a readily fermentable sugar” (in this case, maltose). Later, Janicki et al.<sup>[9]</sup> confirmed these results and demonstrated the presence of a maltase capable of generating glucose from maltose. Since 2015, two drafts of the hop genome have highlighted numerous sequences that could potentially encode glucosidases and amylases, over 250 and 120 respectively in Cascade hops.<sup>[10,11]</sup> In 2018, the presence of  $\alpha$ -amylase,  $\beta$ -amylase,  $\alpha$ -glucosidase, and limit dextrinase was confirmed by Kirkpatrick and Shellhammer.<sup>[6]</sup>

Many parameters have been shown to influence, positively or negatively, the activities of these enzymes, from hop growing to dry-hopping.<sup>[12,13]</sup> To date, it has been shown that the hop terroir, including farm management, soil,

climate, and variety, has an impact on the diastatic potential of hops, but it is still difficult to identify the key factors.<sup>[12,14]</sup> Concerning harvesting conditions, it has been evidenced how higher drying temperatures could reduce enzymatic activities.<sup>[15,16]</sup> Of course, the effect of amylolytic enzymes on beer dextrins depends also on contact time, temperature, and dose of hops, with greater release of glucose and hydrolysis of maltose at increased temperatures, contact times, and dosages.<sup>[6]</sup>

More recently it was shown that the residual enzymatic potential of hops provided by dry-hopping is more than just amylolytic activity. In the Alsace Strisselspalt and Hallertau Hersbrücker hop cultivars, Werrie<sup>[17]</sup> evidenced, for the first time, enzymes able to degrade beer isoamyl acetate and ethyl hexanoate. In 2021, the occurrence of an esterase enzyme was also confirmed, in the Mandarin Bavaria variety.<sup>[18]</sup> A total of 53 sequences predicted to encode carboxylesterases were identified in the Cascade hop genome in 2021,<sup>[11]</sup> but it had never been proven that these genes were expressed. Consequently, the definition of “hop creep” as it is known today, focusing especially on amylolytic enzymes, should be reconsidered.

The aim of this study was to provide new insights about hop enzymes by assessing how temperature and beer composition might affect their activities. To this end, various hop varieties (including dry-heat-treated hops) were incubated in beer over a three-week period under different conditions of temperature and ethanol content. Changes in levels of fermentable sugars and fermentation esters were monitored over time during the dry-hopping assays.

## Experimental

### Chemicals

Absolute ethanol (99%), acetonitrile, methanol and sodium chloride, were purchased from VWR International (Leuven, Belgium). L-cysteine hydrochloride monohydrate, ethyl hexanoate, ethyl octanoate, D-(+)-glucose, isoamyl acetate, maltotriose, 2-pentanol, and L-rhamnose were obtained from Sigma-Aldrich (Overijse, Belgium). D-(-)-fructose, maltose monohydrate and saccharose were purchased from Merck (Darmstadt, Germany). Sodium azide was provided by Acros Organics (Geel, Belgium). Milli-Q water was used (Millipore, Bedford, MA, USA). Sep-Pak C18 Plus Light Cartridges 130 mg were purchased from Waters (Antwerp, Belgium).

### Beer samples

Two freshly released commercial lager beers (Anheuser-Busch InBev, Leuven, Belgium), both selected for their consistent physicochemical parameters (see Table 1), were used as model

media. Beer A (5.4% v/v ethanol) was used for all dry-hopping assays while beer B (NAB, 0.1% v/v ethanol) was used solely for ethanol content trials.

### Hop samples

Amarillo®, Citra®, Mosaic® and Simcoe® hops (2022 harvest; T90 pellets) were kindly provided by Yakima Chief Hops (USA).

Part of the Amarillo® T90 pellets were subjected to dry heat treatment (milled hop placed in an oven at 100°C for 40 min).

### Lab-scale dry-hopping assays

All trials were carried out in duplicate according to a common protocol. Each hop sample was homogenized in a blender prior to dry-hopping assays. Hops (T90 pellets or dry-heat-treated samples) were added at 10 g/L and esters (isoamyl acetate, ethyl hexanoate, and ethyl octanoate) were incorporated at 20 mg/L into 200 mL beer A. Additionally, 0.02% (w/v) sodium azide was added to prevent microbial growth. For assessing the impact of temperature, samples were kept for three weeks at 24°C or 50°C. The influence of ethanol content was assessed with Citra hop at 24°C, by increasing the proportion of ethanol in the constant volume of ethanol-water mixture added to beer B (final ethanol concentrations: 0, 2.5, 5, 7.5 and 10% v/v).

### Basic analyses of dry-hopped samples

The Analytica EBC method 9.35<sup>[19]</sup> was used for beer pH. Real extract and alcohol content (% v/v) were determined with an Anton Paar DMA 4500 M (approved by Analytica EBC).

### Quantification of fermentable sugars using high performance liquid chromatography – refractive index detector (HPLC-RID)

Changes in fermentable sugar levels were monitored in dry-hopped media following representative sampling of 30 mL after 1 day, 1 week, 2 wk, and 3 wk. Fructose, glucose, saccharose, maltose, and maltotriose were quantitated using HPLC-RID. Sugars (containing L-rhamnose as internal standard IST) were recovered from dry-hopped beer samples through an SPE cartridge (Sep-Pak® C18, Waters, Milford, MA, USA). Separation was performed on a Prevail Carbohydrate ES 250×4.6 mm, 5 µm column (Grace, Columbia, MD, USA) isocratically eluted with acetonitrile–water (75:25, v/v) at a flow rate of 1.0 mL/min. The column temperature

**Table 1.** Physicochemical parameters of base beers A and B.

	alcohol (% v/v)	real extract (% w/w)	pH	glucose (g/100 mL)	maltose (g/100 mL)
Beer A	5.4±0.0	4.0±0.1	4.5±0.0	0.0±0.0	0.4±0.2
Beer B	0.1±0.0	4.6±0.0	4.4±0.0	0.6±0.1	0.4±0.1

Mean value of duplicate measurements on Anton Paar DMA 4500 M (alcohol, real extract, pH) and HPLC-RID (glucose and maltose) ± standard deviation.

was kept at 30 °C and the injection volume was 10 µL. Chromatograms were acquired with RID. Compound identification was performed using injection of commercial standards and quantitation was done with the calibration curves.

### **Quantification of residual esters by static headspace – gas chromatography – electron impact mass spectrometry (HS-GC-MS)**

The fate of the spiked esters was monitored by sampling 30 mL of the dry-hopped media after 1 day, 1 week, 2 wk and 3 wk. A total of 40 µL 2-pentanol solution (2500 mg/L; final concentration in beer: 20 mg/L), used as IST, and NaCl in excess (2 g) were added to 5 mL of sample in a headspace vial, which was immediately closed. After an incubation for 30 min at 60 °C under automatic shaking, 500 µL headspace was injected (automatic injector CTC Analytics Combipal, Hamilton 2.5-mL syringe at 70 °C). Residual esters were analyzed with the CP-Sil 5 CB wall-coated open tubular (WCOT) apolar capillary column (50 m x 0.32 mm, 1.2 µm), on an Agilent Technologies 7890 NB GC hyphenated to a single quadrupole mass spectrometer (Agilent 5977B MSD) operating in SIM mode with EI at 70 eV. The carrier gas was helium and the pressure was set at 65 kPa. The oven temperature was programmed to start at 32 °C for 5 min and then to rise from 32 to 140 °C at 8 °C/min, from 140 to 180 °C at 15 °C/min, and was finally held at 180 °C for 30 min. The following *m/z* ions were analyzed: 45 and 55 for 2-pentanol (IST), 43 and 70 for isoamyl acetate, 88 and 99 for ethyl hexanoate, 88 and 127 for ethyl octanoate. Chromatograms were recorded throughout elution. Agilent OpenLab software was used to record the resulting data. A standard addition procedure was applied for each compound. The standard addition slope *A* was used according to the following equation (IST relative recovery factor set at 1):  $X \text{ concentration (in mg/L)} = 1/A \times \text{IST concentration (in mg/L)} \times (X \text{ area}/\text{IST area})$ .

### **Statistical analyses**

All analytical measurements were carried out in duplicate. All results were analyzed with JMP Pro 17 (USA). Depending on the number of independent variables, either a two-way or three-way ANOVA was done to determine significance differences for both fermentable sugars and fermentation esters. The significance level was set at  $p < 0.05$ .

## **Results and discussion**

### **Impact of contact time**

In order to study the influence of hop contact time on the enzymatic activities of hop samples, the levels of fermentable sugars (fructose, glucose, saccharose, maltose, and maltotriose) and fermentation esters (isoamyl acetate, ethyl hexanoate, and ethyl octanoate) were measured in beer before and after 1 day, 1 week, 2 wk, and 3 wk of dry-hopping in the absence of yeast.

For sugars, the results are shown only for glucose and maltose, since changes in the fructose, saccharose, and maltotriose contents were not significant. At 24 °C, as depicted in Figure 1a, a longer contact time resulted in higher saccharification of residual soluble dextrins in the beer. As soon as the hops were added, glucose release was continuous, its concentration increasing in the beer throughout incubation. As glucose is the non-hydrolysable monomer, it cannot be degraded in the absence of yeast.

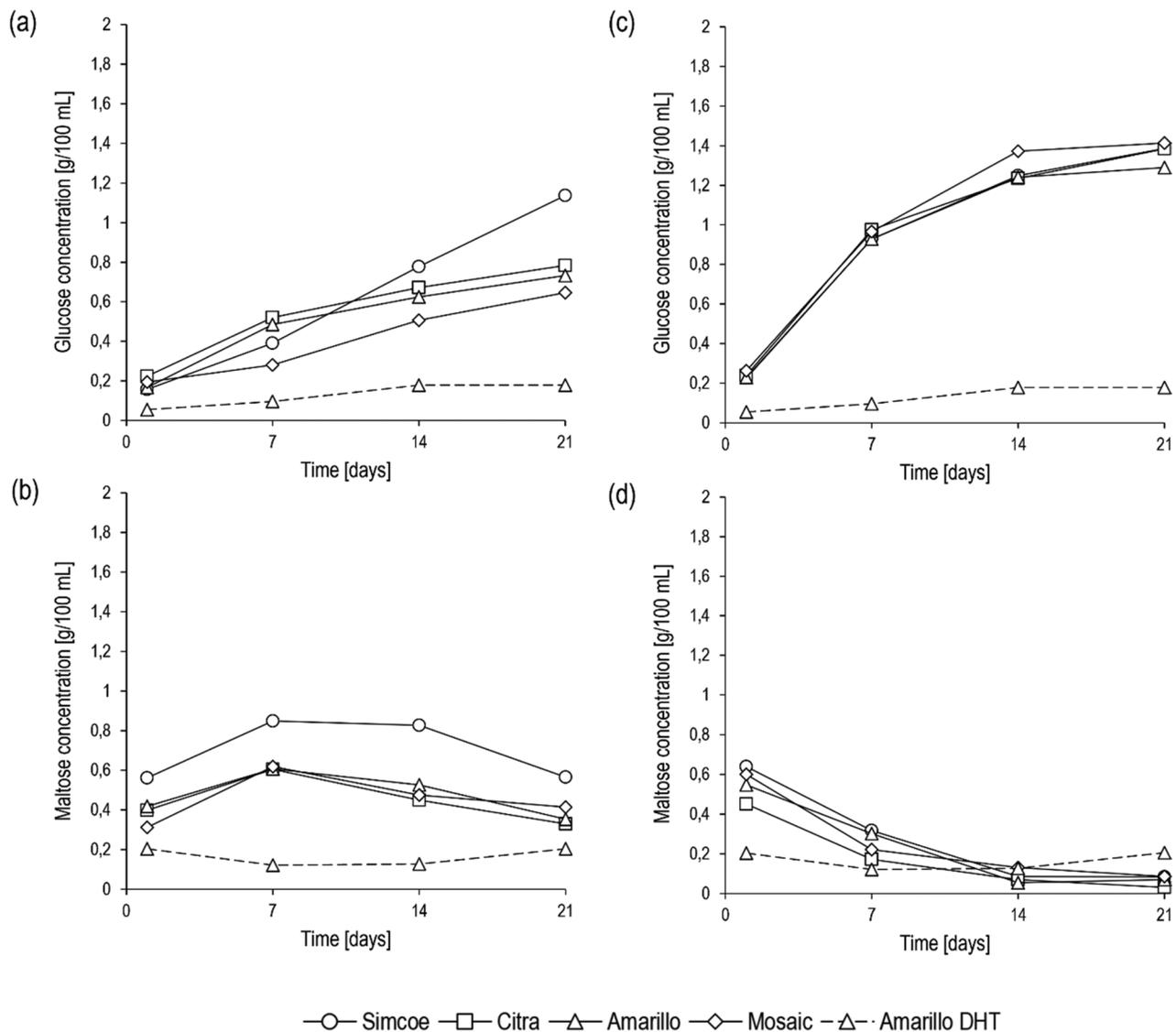
In contrast, as previously reported for Cascade hops monitored over a 2-week period (20 °C) at the same dosage (10 g/L),<sup>[6]</sup> maltose can be degraded in the absence of yeast: its hydrolysis can in some cases balance its synthesis from higher oligosaccharides. As depicted in Figure 1b, our results show an increase in maltose concentration during the first week at 24 °C, followed by a decrease over the following two weeks.

Whatever the variety considered, the glucose concentration, lower than that of maltose in the starting beer, far exceeded the latter after 2 and 3 wk. Noteworthy, the final glucose and maltose concentrations were significantly higher in the experiment with Simcoe (Figure 1a,b). This indicates that the enzymes involved in the saccharification of dextrins to glucose and maltose were more active in the case of this hop sample. This dependence of enzyme activity on variety has already been mentioned but remains controversial: sometimes put forward,<sup>[20]</sup> sometimes refuted.<sup>[2]</sup> At this stage it is clear that variety dependence is part of a more complex picture that also takes into account hop growing, harvesting, and post-harvesting conditions.<sup>[14,15]</sup>

Among the amyolytic enzymes already identified in hops,<sup>[6,8,9]</sup> glucose can be produced by the action of α-amylase (from linear oligosaccharides or limit dextrins) and α-glucosidase or maltase (from maltose). Maltose can be produced through the action of α-amylase and β-amylase (from linear oligosaccharides or limit dextrins) and also through that of limit dextrinase (from limit dextrins). Except for α-glucosidase, whose action is visible as it is directly related to the hydrolysis of maltose over time, it is difficult to determine precisely which enzyme acts predominantly. It can just be stated that the enzymes are still active under the test conditions (24 °C) after 3 wk of incubation and that those responsible for glucose production are more active than those responsible for maltose production.

With the aim of potentially reducing the enzymatic activities of hops, the influence of dry heat treatment (40 min at 100 °C) was assessed on milled Amarillo hop pellets. This heat treatment strongly influenced the amyolytic activity. Glucose and maltose were no longer released (Figure 1a,b). This means that both α-glucosidase and β-amylase enzymes are significantly affected.

Concerning the influence of hop contact time on fermentation ester stability at 24 °C, Figure 2 shows a significant decrease in isoamyl acetate, ethyl hexanoate, and ethyl octanoate levels over the 3-week incubation period. Quantitatively, the time course of this decrease differed considerably according to the compound studied: isoamyl acetate reached its lowest residual concentration after 3 wk (Figure 2a), ethyl hexanoate after 1 week (Figure 2b), and



**Figure 1.** Evolution of concentrations (g/100 mL) of glucose (a and c) and maltose (b and d) over a 3-week period at 24°C (left) and 50°C (right) in beer samples dry-hopped with T90 pellets (Simcoe, Citra, Amarillo, Mosaic) and dry-heat-treated T90 pellets (Amarillo DHT).

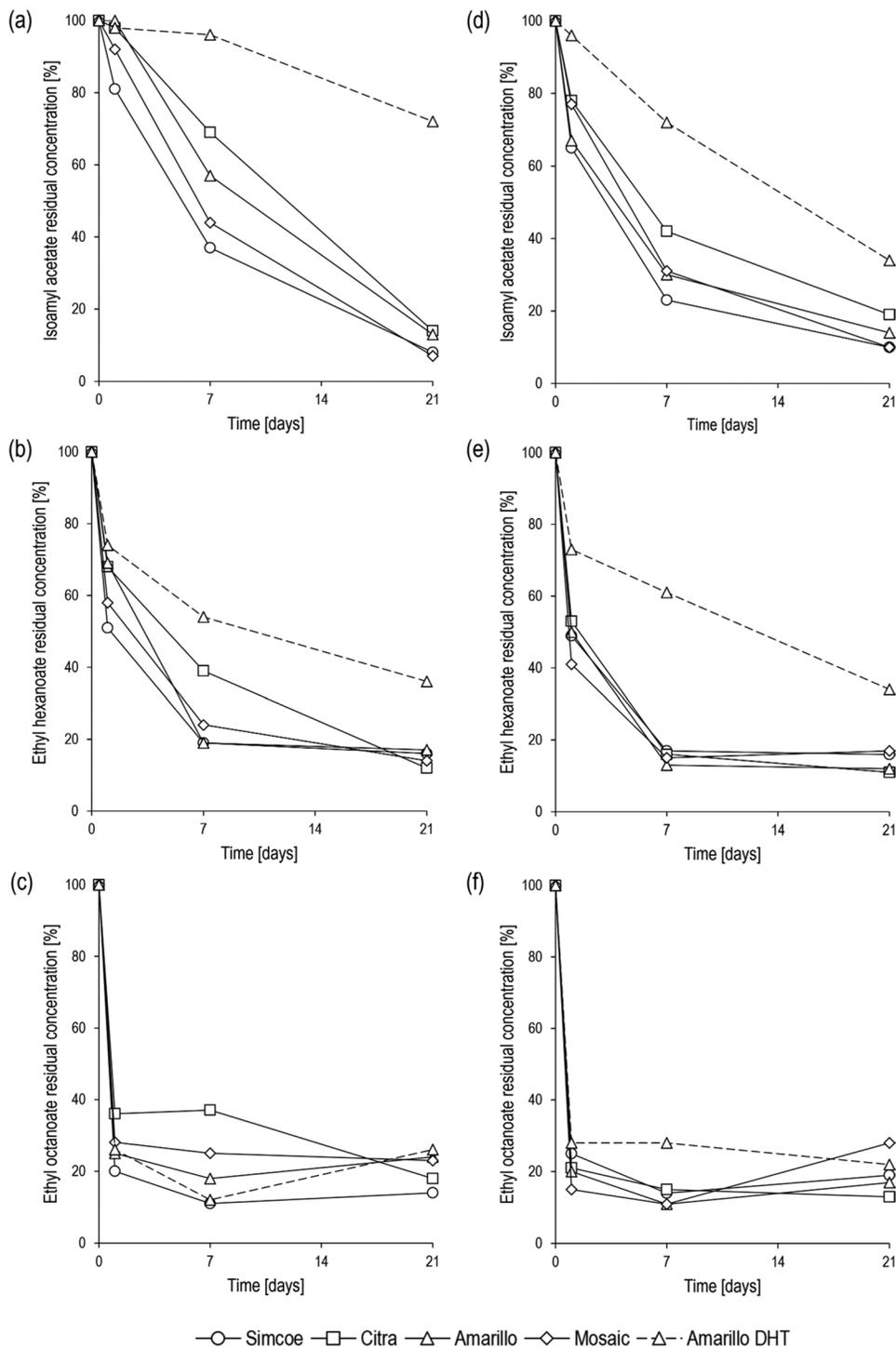
ethyl octanoate after only a day (Figure 2c). On the basis of our observations, it is clear that in the case of ethyl hexanoate and ethyl octanoate, adsorption to hops interfered with our assessment of enzymatic degradation. Such a phenomenon has been observed previously for the same compounds.<sup>[18]</sup> It is directly related to the structure of the ester concerned, the extent of adsorption being proportional to that ester's lipophilicity. We conclude that under these test conditions, enzymatic activity is observable only for isoamyl acetate, which is not adsorbed. As with amylolytic enzymes, enzymatic activity is promoted by a longer contact time and the trend is identical regardless of the variety studied.

To date, few details are available in the literature as to the nature and mode of action of the hop enzyme(s) responsible for degrading isoamyl acetate. According to Ferreira and Collin (Mandarina Bavaria, 2 g/L),<sup>[18]</sup> carboxylesterase activity can also be followed by the appearance of the carboxylic acids and alcohols corresponding to the compounds investigated.

Dry heat treatment of hop was relatively effective at inhibiting hop esterases (Figure 2a). After one week, no loss of isoamyl acetate was observed. Yet this inhibition was not complete, as the concentration of the ester started to decrease after 2 wk (72% residual concentration at 3 wk). This experiment provides further evidence of lipophilic ester adsorption to hops (Figure 2c), since the residual ethyl octanoate concentrations measured over time were the same whether dry heat treatment was applied or not, in contrast to the effect observed with isoamyl acetate.

#### Assays at 50°C for potential prediction of hop creep

The amylolytic activity of hop (10 g/L) was also investigated at 50°C. As shown in Figure 1c and Figure 1d, the results obtained for sugars at 50°C were similar to those obtained at 24°C, with glucose levels increasing over time so as even to exceed, finally, those of maltose. It is clear, however, that the glucose-releasing enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase)



**Figure 2.** Residual concentrations (% after spiking of 20 mg/L) of isoamyl acetate (a and d), ethyl hexanoate (b and e) and ethyl octanoate (c and f) over a 3-week period at 24°C (left) and 50°C (right) in beer samples dry-hopped with T90 pellets (Simcoe, Citra, Amarillo, Mosaic) and dry-heat-treated T90 pellets (Amarillo DHT).

are more active at 50°C than at 24°C. Obviously, 50°C will not be applied by brewers to industrial productions but this temperature could be used in the laboratory to assess future hop creep (maximum glucose reached already after 2 wk of incubation). Another approach for measuring the enzymatic potential of hops under forced conditions has been proposed by Bruner et al.<sup>[2]</sup> in the presence of yeasts (assessment of the degree of refermentation). Regarding the enzymes responsible for maltose production ( $\alpha$ -amylase,  $\beta$ -amylase, and limit

dextrinase), no significant differences were measured between 24°C and 50°C. At both temperatures, the release of glucose exceeded that of maltose after only one day of incubation, making it difficult to draw any conclusions about the enzymes responsible for maltose production.

Temperature emerged also as a key parameter for esterase activity (Figure 2d-f), with much less hydrolysis after one week at 24°C than at 50°C (e.g. 38% residual content with Simcoe at 24°C vs. 20% at 50°C). Yet after three weeks,

the residual levels were identical in both cases (10% in both cases). A blank test (with no hop addition) showed no significant loss of isoamyl acetate after 3 wk at 50°C.

### Influence of the ethanol content

To date, no study has investigated the influence of ethanol on the enzymatic activity of hops in beer. This question is

of major interest to brewers, given the current market for dry-hopped beers with a wide range of alcohol levels, including NABLABs. To study this influence, Citra hops were incubated for 3 wk at 24°C in the same alcohol-free beer B, to which increasing amounts of ethanol were added.

As depicted in Figure 3a, the lower the ethanol content, the higher the glucose release ( $R^2 = 0.96$  at 3 wk). At 10% v/v ethanol, glucose release became insignificant, while up to 0.4 g/100 mL glucose was released in the unspiked NAB. For maltose (Figure 3b), the influence of ethanol content was minor, yet there was no release at all at 10% v/v ethanol. At all the other ethanol levels tested, the increase in maltose of 0.2 g/100 mL, observed during the first week, was balanced by continuous hydrolysis over the next two weeks. These results are in line with observations on beer A with 5.4% v/v alcohol (see Figure 1a,b). Our results evidence, for the first time, that hop  $\alpha$ -glucosidase can be highly inhibited by ethanol in some strong beers. Literature just mentioned similar inhibition by ethanol of an  $\alpha$ -amylase issued from *Schwanniomyces castellii*.<sup>[21]</sup>

Ethanol proved much less effective as an inhibitor of hop esterases. Although the trend depicted in Figure 3c suggests that a higher ethanol content led to lower enzymatic activity, the degradation of isoamyl acetate over time was statistically the same for all ethanol levels investigated ( $p > 0.05$ ), with a final isoamyl acetate concentration in the same range as obtained previously in beer A with 5.4% v/v alcohol (see Figure 2a,d).

### Conclusion

The aim of this study was to provide additional information on the behavior of hop amylolytic and esterase enzymes in different beers. As expected, a longer contact time between hop and beer dextrins gives amylolytic enzymes the opportunity to produce more glucose and maltose. The same applies to isoamyl acetate hydrolysis by esterases. Therefore, to avoid hop creep in the bottle, we would advise brewers to add their hops sufficiently early through primary fermentation (yet without interfering with yeast growth). Brewers producing mostly triple IPAs or other hop-forward beers with a high alcohol content may be less concerned, as  $\alpha$ -glucosidase tends to be inhibited in this environment. In contrast, hop samples with low enzymatic activity (e.g. heat-treated) should be preferred in dry-hopped NABLABs. For laboratory predictions, incubating the dry-hopped beer for 2 wk at 50°C could allow better prediction of the extent of hop creep.

### Acknowledgements

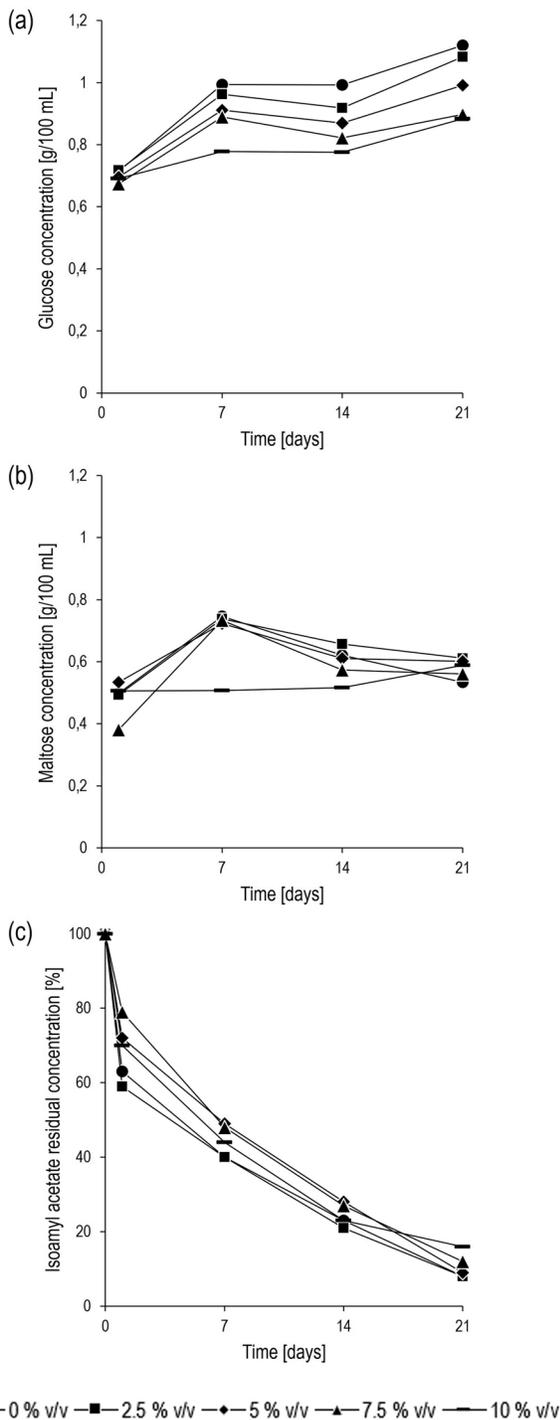
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### Disclosure statement

No potential conflict of interest was reported by the author(s).

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**Figure 3.** Evolution of concentrations of (a) glucose (g/100 mL) and (b) maltose (g/100 mL), and (c) residual concentrations of isoamyl acetate (%) over a 3-week period at 24°C in beer samples dry-hopped with Citra hop T90 pellets, in presence of increasing ethanol content (0, 2.5, 5, 7.5 and 10% v/v).

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