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Release of phenolic flavour precursors during wort production: Influence of process parameters and grist composition on ferulic acid release during brewing

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ABSTRACT

In this study, the effects of mashing variables such as mashing-in temperature, time and pH, mash thickness, grist coarseness and composition, and stirring regime on the release of ferulic acid were examined. Ferulic acid is a precursor for the formation of flavour-active volatile phenols and a potent natural antioxidant in beer. Given one barley malt variety, the multitude of choice in setting various process parameters and adding brewery adjuncts during brewhouse operations can give rise to worts with widely varying ferulic acid levels. A clear difference in temperature- and pH-dependence between the release of the water-extracted and the enzymatically hydrolyzed fraction was found. The T,t-dependencies of arabinoxylan-degrading enzyme activities were correlated with ferulic acid release during mashing. Results from laboratory-scale mashing experiments were validated with those from a pilot-scale (5 h) wort production process. Enhancing the enzymatic release of phenolic flavour precursors from bound forms during mashing can greatly enhance the phenolic aroma potential of wort. Optimising this precursor release during mashing may be a means for controlling final volatile phenol levels in beer.

1. Introduction

Volatile phenols have been reported to contribute to the aroma of non-alcoholic beverages like fruit juices and coffee (Dorfner, Ferge, Kettrup, Zimmermann, & Yeretzian, 2003; Fallico, Lanza, Maccarone, Asmundo, & Rapisarda, 1996), as well as alcoholic drinks such as beer, wine, sherry and whisky (Back, Diener, & Sacher, 2000; Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993; McMurrough et al., 1996; Narziss, Miedaner, & Nitzsche, 1990; Tressl, Renner, & Apetz, 1976; van Beek & Priest, 2000; Wackerbauer, Kramer, & Siepert, 1982). An important flavour-active volatile phenol in beer is 4-vinylguaiacol (4VG). This phenolic compound is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers. Despite being historically catalogued as an off-flavour, the compound is known to be an essential flavour contributor to the characteristic aroma of Belgian white beers (made with unmalted wheat), German Weizen beers (made with malted wheat) (Back et al., 2000; Narziss et al., 1990; Wackerbauer et al., 1982) and Rauch beers (Tressl et al., 1976). However, in many other top-fermented blond and dark specialty beers, the phenolic flavour is essential for the overall flavour perception (Vanbeneden, Gils, Delvaux, & Delvaux, 2007). The volatile phenol 4-vinylsyringol has been reported to increase during the ageing of lager beers where it probably originates from the acid hydrolysis of glycosides or the thermal decarboxylation of sinapic acid (Callemien, Dasnoy, & Collin, 2006).

4VG is the decarboxylation product of ferulic acid (FA). This flavour-inactive phenolic acid, having a flavour threshold in beer as high as 600 ppm (Meilgaard, 1975), can be decarboxylated to the highly flavour-active volatile phenol 4VG by thermal impact during high temperature treatments in the beer production process, e.g. wort boiling, whirlpool holding and pasteurization, or through enzymatic decarboxylation during fermentation by phenylacrylic acid decarboxylase activity of top-fermenting yeast strains (Pad1-enzyme) (Clausen, Lamb, Megnet, & Doerner, 1994). Although phenolic acids have high threshold values and do not affect the aroma of beer, they are appreciated for their antioxidant activity (Pascoe, Ames, & Chandra, 2003), enhancing the stability of beer. Phenolic acids, and more specifically hydroxycinnamic acids (HCA), are mainly associated with polysaccharides in the plant cell wall. In cereal grain, they are mainly esterified with arabinoxylans (AX). AX are important structural carbohydrates in the husk, pericarp,

Abbreviations: 4VG, 4-vinylguaiacol; FA, ferulic acid; Pad1, phenylacrylic acid decarboxylase enzyme; HCA, hydroxycinnamic acid; AX, arabinoxylan; EBC, European Brewery Convention; rpm, rounds per minute; ECD, electrochemical detection; EU, enzyme units; 4NPF, 4-nitrophenyl ferulate; DMSO, dimethylsulfoxide; MES, 2-morpholino ethane sulfonic acid.

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aleurone and endosperm in cereal grains. AX consist of a β-(1-4)-xylan backbone in which xylose residues may be substituted with arabinose at C2 and/or C3. Feruloyl and *p*-coumaroyl groups can be esterified to the arabinofuranosyl residues at the O5. AX are high molecular weight, partly water-soluble polymers. During the brewing process, they are both water-extracted and solubilised by AX-hydrolases from the malt into the wort (Debyser, Derdelinckx, & Delcour, 1997a). AX can be degraded by several enzymes (Debyser, Delvaux, & Delcour, 1998). The β(1-4)-endoxylanases (EC 3.2.1.8) generate (un)substituted xylo-oligosaccharides. These oligomers can be further degraded by β-D-xylopyranosidase (EC 3.2.1.37), which releases β-D-xylose from the non-reducing end. The α-L-arabinofuranosidase (EC 3.2.1.55) liberates α-L-arabinose units from the main chain. Esterified HCA can be released as free acids by cinnamoyl esterase (EC 3.1.1.73) activity during mashing which makes them available for decarboxylation. Cinnamoyl esterases, also known as feruloyl esterases, *p*-coumaroyl esterases or ferulic acid esterases, have been reported natively in barley, germinated barley (Sancho, Faulds, Bartolome, & Williamson, 1999), and barley malt (Bartolome, Garcia-Conesa, & Williamson, 1996; Humberstone & Briggs, 2000; Sun, Faulds, & Bamforth, 2005).

Since volatile phenols are mainly formed by enzymatic decarboxylation during fermentation, the choice of an appropriate yeast strain is the first way to control the final volatile phenol concentration in beer (Vanbeneden et al., 2007). However, using an identical yeast strain and fermentation procedure, brewers still experience large variations in final volatile phenol levels between subsequent production batches. This illustrates the need to control precursor release during mashing. One important parameter is the choice of a suitable barley malt variety. However, given one malt variety, the multitude of choice in the setting of various process parameters during brewhouse operations can give rise to worts with varying concentrations of POF precursors and, hence, phenolic flavour potential in the final beer. The objectives of this study were to examine the release of free and ester-bound FA during brewhouse operations. The influences of various process parameters (mashing-in temperature, time and pH, mash thickness, grist coarseness and composition, stirring regime) were studied. The T,t-dependence of the activity of AX-degrading enzymes (and more specifically the activity of the cinnamoyl esterase) were correlated with free and ester-bound FA release during mashing. Finally, a pilot-scale (5 h) wort production process was conducted to validate laboratory-scale mashing experiments.

2. Materials and methods

2.1. Materials

In the laboratory-scale mashing experiments, a two-row pilsner barley malt variety, Scarlett (5 EBC), from Cargill Malt Division (Herent, Belgium) was used. Legat, Limes, Patrel, Meunier, Drifter, Alsace, Winnetou, Biscay, Pulsar and Nijinsky wheat cultivars were obtained from AVEVE (Landen, Belgium). Tremie wheat was obtained from Dingemans (Stabroek, Belgium). Flaked cereals were obtained from Brouwland (Beverlo, Belgium). Specialty malts were obtained from Weyermann Malzfabrik (Bamberg, Germany).

2.2. Laboratory-scale mashing

Unless otherwise specified, standard laboratory Congress wort was produced according to EBC-Analytica (1998) method 4.5.1. Laboratory-scale mashing experiments were carried out in an automated mashing bath (LB8 Electronic mashing bath, Funke Gerber GmbH, Berlin, Germany). Barley malt was ground in a Buhler-Miag malt mill (Buhler-Miag, Minneapolis, MN) set for fine grist coarseness (0.2 mm gap between the grinding discs). Ground malt (50.0 g) was mixed with 200 ml of water at 46 °C. A temperature of 45 °C was maintained in the mash for 30 min. The temperature was raised at 1 °C per min to 70 °C before 100 ml water (70 °C) were added. The temperature was maintained at 70 °C for 1 h before cooling to room temperature in 10–15 min. The mash was continuously stirred at 200 rpm. After adjusting the beaker content to 450 g, samples were centrifuged (3000 rpm; 5 min) and frozen at −18 °C prior to analysis.

2.3. Total alkali-extractable FA content in wort and quantification of FA and 4VG in wort

For the determination of the total alkali-extractable FA content in wort, ester-bound FA was released from AX by alkaline hydrolysis according to the method described by Nardini et al. (2002). Quantification of FA and 4VG in wort was performed by HPLC-ECD, as described by Vanbeneden, Delvaux, and Delvaux (2006).

2.4. Measurement of β(1-4)-endoxylanase, β-D-xylosidase (EC 3.2.1.37) and α-L-arabinofuranosidase (EC 3.2.1.55) activities

Endoxylanase activity assays of wort were performed according to Debyser et al. (1997a). Activities were expressed in enzyme units (EU) per gramme of dry malt. One EU is the activity expressed as ΔA_{590} per gramme of dry malt and per hour of incubation. *p*-Nitrophenyl glycosides were used as substrates to measure β-D-xylosidase and α-L-arabinofuranosidase activities according to the procedure of Cleemput, Bleukx, van Oort, Hessing, and Delcour (1995). Activities were expressed as EU per gramme of dry malt. One EU was defined as the amount of enzyme that released 1 μmol *p*-nitrophenol from the substrate per minute at 40 °C and pH 6.0.

2.5. Measurement of cinnamoyl esterase (EC 3.1.1.73) activity

Cinnamoyl esterase activity was assessed spectrophotometrically using 4-nitrophenyl ferulate (4NPF) as a substrate, based on a method adapted from Mastihuba, Kremnicky, Mastihubova, Willett, and Cote (2002). 4NPF was obtained from the Faculty of Chemical Technology, Slovak University of Technology, Bratislava, Slovakia. The substrate solution was prepared by mixing 9 vol. of 0.1 M potassium phosphate buffer solution, pH 6.0, containing 2.5% Triton X-100 with 1 vol of 10.0 mM 4NPF in DMSO, followed by immediate vortexing. The buffer-4NPF solution was prepared freshly before analysis. The DMSO solution of 4NPF was prepared within 24 h and kept at room temperature. The reaction mixture comprised 0.4 ml of substrate solution and 1.2 ml of wort. A blank sample was included, using water instead of wort to correct for the non-enzymic colour release of the substrate. Samples were incubated at 30 °C for 2 h. The release of *p*-nitrophenol from 4NPF was determined colorimetrically at 410 nm. The difference in absorbance (from the control sample), before and after incubation, was used to calculate the enzyme activity. Activities were expressed as units per gramme dry malt. One EU was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate per minute at 30 °C and pH 6.0.

For the determination of the cinnamoyl esterase activity of extracts of specialty malts and wheat samples, the spectrophotometric method could not be used, due to the interference of coloured and hazy samples, respectively. Cinnamoyl esterase activities of these samples were assessed against the methyl ester of FA (MFA) with HPLC analysis. Samples (3.00 g) of ground pilsner malt (Scarlett), wheat (Difter, Legat and Tremie) or specialty malt (Melanoidin, Caramunich and Carafa) were suspended in 50 mM MES, pH 6.0 (10.0 ml). After 15 min of vigorous shaking, the suspension was centrifuged (3000*g*, 15 min). The supernatant was filtered

through 0.45 μm PVDF syringe filters (Alltech, Deerfield, IL, USA). Cinnamoyl esterase activity, versus the MFA, was determined by incubating 3 ml of extract with 1 ml of substrate solution for 2 h at 30 °C. The substrate solution was prepared by dissolving MFA in methanol (80 mM) and diluting these solutions to 4 mM with 50 mM MES pH 6.0. After incubation, the reaction was stopped by adding 0.8 ml of glacial acetic acid. A blank sample was obtained by adding the substrate solution after the addition of acetic acid. All extracts were tested, both separately and in combination with the barley pilsner malt extract (1.5 ml barley pilsner malt extract + 1.5 ml wheat or specialty malt extract). Activities were expressed as EU per gramme of malt. One EU was defined as the amount of enzyme that released 1 nmol of HCA from the substrate per minute at 30 °C and pH 6.0.

2.6. Effect of the mashing-in temperature, time and pH on the release of FA

To study the effect of the mashing-in temperature, worts were produced according to the standard Congress wort procedure. Instead of the regular temperature profile, isothermal mashing-in temperatures were set at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. For the determination of free and total alkali-extractable FA contents, samples were taken after 60 min. Samples were immediately cooled by adding 200 ml of ice cold water and 5 ml of phosphoric acid (85%) were added to stop enzyme activity. After adjusting the beaker content to 450 g, samples were centrifuged (3000 rpm; 5 min) and frozen at −18 °C prior to analysis. To analyze the content of water-extracted FA, the same procedure was applied but, before mashing-in, the ground barley malt samples were placed in an oven at 130 °C for 5 h according to Debyser, Derdelinckx, and Delcour (1997b) to eliminate enzyme activity. For the determination of enzyme activities, samples were taken after 10, 20, 30, 40, 60, 80, 100 and 120 min but, during sampling, the addition of phosphoric acid was omitted.

At the optimum mashing-in temperature (40 °C), the effects of mashing-in time and pH on free and ester-bound FA release were studied. The effect of the mashing-pH was investigated by adjusting the pH of the mash to 4.3, 4.6, 4.9, 5.2, 5.5, 5.8, 6.1 and 6.4 by the addition of adequate amounts of 1 N H_2SO_4 or NaOH. The mashes were kept at 40 °C for 60 min. For the effect of the mashing-in time, the temperature was held at 40 °C for 15 min, 30 min, 1, 2, 3, 4 and 5 h.

2.7. Variation of stirring regime, grist coarseness and mash thickness

To study the effect of the stirring regime on FA release during brewing, laboratory Congress worts were prepared according to the standard procedure. The effects of continuous stirring at 100 and 200 rpm, intermittent stirring at 10 min intervals at 100 and 200 rpm, and no stirring were investigated. The effect of the grist coarseness was evaluated by adjusting the distances between the grinding discs to 0.1, 0.6, 1.2 and 1.9 mm. To study the effect of the mash thickness, the amount of finely ground grist used for mashing-in was set at 45.2, 58.5, 71.9, 85.2, 98.5, 111.9 and 125.2 g (corresponding with final wort densities of 8, 10, 12, 14, 16, 18 and 20 °P).

2.8. Addition of wheat, flaked cereal adjuncts and specialty malts

Wort was prepared according to the Congress wort standard procedure, replacing 25.0 g of pilsner barley malt with 25.0 g of finely ground wheat, cereal flakes or specialty malt. Wort samples were filtered over a folded filter (MN 614 ¼ 32 cm diameter, Macherey-Nagel GmbH).

2.9. Pilot-scale wort brewing (infusion mashing)

Pilsner malt (103 kg) was ground with a single roller malt mill and mashed-in with 3.3 h of water at 45 °C. The mashing-in pH was adjusted to 5.6 with lactic acid (PURAC FCC, VOPAK, Brussels, Belgium). After 15 min, the temperature was raised (1 °C/min) to 78.5 °C with a 10 min hold at 52.5 °C, a 40 min hold at 62 °C and a 15 min hold at 72 °C. The mash was transferred to a classic lauter tun with perforated bottom plate for wort separation. The spent grains were washed with 2 h of sparging water. The sweet wort was collected in the boiling kettle and heated to boiling temperature by steam injection. The pH was adjusted to 5.3 by the addition of lactic acid. The wort was boiled for 90 min. After 30 min, hop pellets were added to achieve a final bitterness of 20 EBU and, after 60 min of wort boiling, additional sugar (1 °P sucrose) was added into the boiling kettle. After boiling, the bitter wort was transferred to the whirlpool for clarification (20 min). Finally, 5 h wort of 14 °P were obtained. Duplicate samples were taken after 15 min at 45 °C, 10 min at 52.5 °C, 0 min at 62 °C, 40 min at 62 °C, 10 min at 72 °C, mashing-off at 78.5 °C, at the start of wort filtration, during wort filtration before the addition of sparging liquor, at the start of the wort boiling process, during the wort boiling process before the addition of hops, at the end of the wort boiling process and at the end of the whirlpool holding time.

2.10. Statistical analysis

Results are represented as means ± standard deviation and considered significantly different at the 95% confidence level (Tukey's *t*-test statistic; $p < 0.05$). The statistical package, XLSTAT 2006 Version 2006.06 (Addinsoft, Paris, France) was used for the statistical calculations.

3. Results and discussion

3.1. Effects of mashing-in temperature, time and pH on FA release during wort production

The effect of the mashing-in temperature on the release of *free* FA from malt, with and without enzyme activity, is shown in Fig. 1A. FA released from malt without enzyme activity corresponded with the amount which is water-extracted, while FA released from normal malt corresponded with FA which is either water-extracted or enzymatically solubilised by cinnamoyl esterase activity. A clear difference in temperature dependence between the release of water-extracted and enzymatically-solubilised phenolic acids was observed. The amount of water-extracted FA was independent of the mashing-in temperature within the observed temperature range. The amount of water-extracted FA corresponded with the amount of FA present in free form in barley malt, which originated from cinnamoyl esterase activity during barley germination and the first stages of the green malt kilning. If FA were to be released by additional chemical hydrolysis during mashing, the water-extraction of FA would increase with higher temperatures. Maximal release of FA from malt with enzyme activity occurred at 40 °C. This is comparable to the 43 °C reported by Narziss et al. (1990) and may explain the elevated concentrations of 4VG reported in beers with an intensified protein residue, e.g., Weizenbeers (Back et al., 2000). The parabolic curvature of the FA release is typical for an enzyme-catalyzed reaction. At 65 °C, almost no FA is released enzymatically and FA levels in wort decrease to the amount of FA already present in unbound form in malt. McMurrough et al. (1996) also showed that increasing the mashing-in temperature from 45 to 65 °C, significantly decreased the level of free FA in the wort.

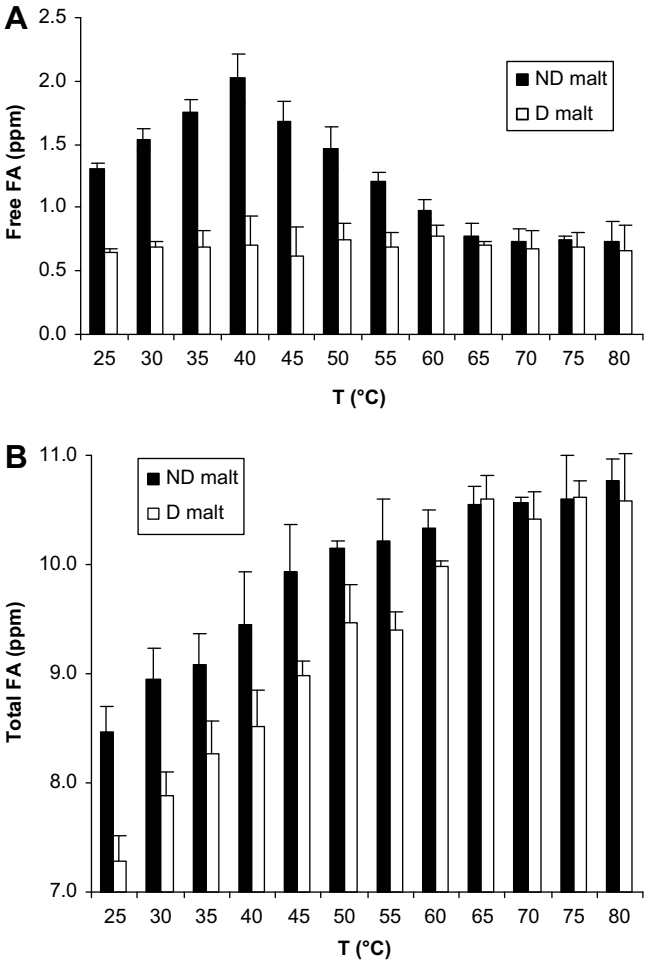

Fig. 1. Effect of mashing-in temperature on the release of FA from malt with (not denatured enzymes – ND) and without (denatured enzymes – ND) enzyme activity during wort production: (A) free FA levels; (B) total alkali-extractable FA levels.

The effect of the mashing-in temperature on the release of *total* alkali-extractable FA from malt, with and without enzyme activity, is shown in Fig. 1B. The amount of total alkali-extractable FA can be considered a measure of the AX level in wort. Hence, the amount of total alkali-extractable FA released from malt without enzyme activity is a measure of the amount of AX being water-extracted from barley malt during wort production, while the amount of total alkali-extractable FA from malt with enzyme activity is a measure of the amount of AX being either water-extracted or enzymatically solubilised by the concerted action of AX-degrading enzymes. At temperatures <65 °C, the amount of total alkali-extractable FA in wort derived from malt with enzyme activity exceeded the amount in wort derived from malt without enzyme activity, indicating an enzymatic solubilisation of insoluble AX to water-soluble forms. At temperatures ⩾65 °C, no enzymatic solubilisation of AX occurred. In contrast to the amount of water-extracted free FA, the amount of water-extracted total alkali-extractable FA did increase with temperature. Due to the polymeric structure of the AX molecules, their solubility probably increased with increasing temperature (Li, Lu, & Gu, 2005). At each temperature, most of the AX present in wort originated from the water-extractable part of the malt. Relatively little solubilisation occurred during mashing as was previously shown by Debyser et al. (1997a).

The effect of mashing-in pH on the release of free FA from malt, with and without enzyme activity, during wort production at different pH values is shown in Fig. 2. The release of water-extractable FA from malt without enzyme activity was independent of the pH. Hence, within the observed pH range, no acid hydrolysis of the ester bond between FA and AX occurred. At pH < 4.6, no significant differences could be found between the amount of FA released from malt without enzyme activity and normal malt. Hence, no significant enzymatic release of FA by cinnamoyl esterase activity did occur at low pH. This is consistent with the results of Humberstone and Briggs (2000) who found that, at pH 4, the cinnamoyl esterase enzyme in extracts from malted barley was inactive. At pH > 4.6, significant enzymatic hydrolysis of ester-bound FA did occur, with an optimal FA release at pH 5.8. Narziss et al. (1990) found a slighly higher optimal pH for FA release (6.0). The results are consistent with the results found by Back et al. (2000) who found that mash acidification leads to a decrease in FA release during wort production. Within the observed pH range, no effect was observed on the amount of total alkali-extractable FA, either from malt with, or without, enzyme activity (results not shown). Slade, Hoj, Morrice, and Fincher (1989) found that barley xylan endohydrolase maintains upto 75% of its maximal activity over a broad pH range (4.5–7.2). This relative stability of the endoxylanase can explain the independence of total alkali-extractable FA during wort production over the observed pH range (4.3–6.4).

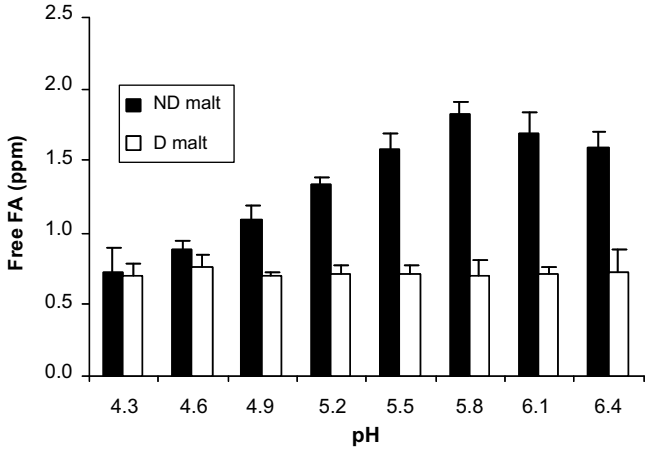

Fig. 2. Effect of mashing-in pH on the release of free FA from malt with (not denatured enzymes – ND) and without (denatured enzymes – ND) enzyme activity during wort production at 40 °C.

The influence of the mashing-in time on the release of free FA from malted barley is shown in Fig. 3. At the start of mashing-in,

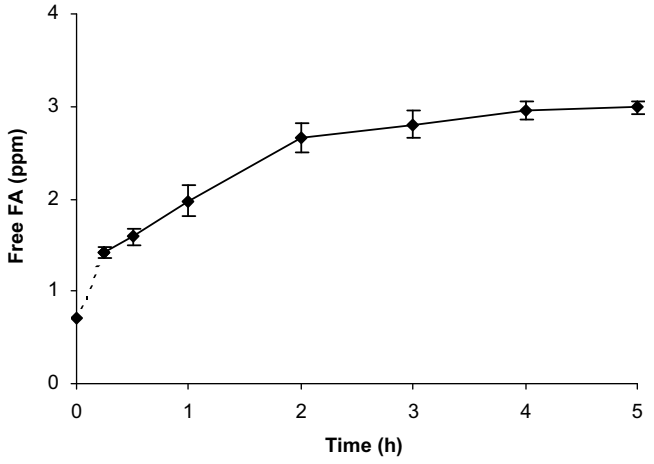

Fig. 3. Effect of mashing-in time on the release of free FA from malt with enzyme activity during wort production at 40 °C.

a rapid increase in FA concentration occurred. Already after 15 min, 1.41 ppm FA was released into the wort in free form, half of which was water-extracted (0.7 ppm), while the other half was enzymatically released. Taking into account that the flavour threshold of 4VG in blond specialty beers is 370 ppb (Vanbeneden et al., 2007), this gave rise to a wort with a 4VG potential of 3 flavour units (FU). After 1 h, free FA further increased to 2.0 ppm and further incubation led to a final free FA content of 3.0 ppm in the wort. However, taking into account the ester-bound FA content, no more than 23% of the total alkali-extractable FA in wort was hydrolyzed.

3.2. AX-degrading enzyme activities during wort production

The activity of 4 AX-hydrolyzing enzymes during isothermal mashing is represented in Fig. 4. The main-chain hydrolyzing enzyme, β(1-4)-endoxylanase, retained its optimal activity until 45 °C but the activity rapidly decreased at higher mash temperatures. At 65 and 70 °C, almost no endoxylanase activity could be detected. This explains why, at temperatures exceeding 65 °C, no additional AX were solubilised in wort made from normal malt compared to the wort made from malt with denatured enzymes (Fig. 1B). The observed temperature dependence of the endoxylanase enzyme during mashing was highly consistent with that found Li et al. (2005). Similar findings were observed by Debyser et al. (1998), although they suggested an optimal temperature of the endoxylanase enzyme at 50 °C. The α-L-arabinofuranosidase was optimally active until 50 °C. At temperatures exceeding 50 °C, activity decreased with increasing temperatures. Of the four enzymes examined, the β-D-xylosidase enzyme was the least sensitive to elevated temperatures. Only at 70 °C, was a decrease in enzyme activity observed. The same temperature dependence was observed for the α-L-arabinofuranosidase and β-D-xylosidase activity during brewing (by the infusion method) by Debyser et al. (1998). The optimal temperature of cinnamoyl esterase activity was 30 °C. After 60 min at 40 °C, the activity of the cinnamoyl esterase enzyme was only 40% of the maximal activity. This corresponded with the results obtained by Humberstone and Briggs (2000) for cinnamoyl esterase activity in an extract of barley malt with feruloyl glycerol as substrate. At higher temperatures, the cinnamoyl esterase was rapidly denatured and at 65 and 70 °C no activity could be detected. This explains why no difference was found between the FA release from malt with and without enzyme activity at temperatures exceeding 60 °C (Fig. 1). Remarkably, the temperature of maximal FA release (40 °C) does not correspond with the optimal temperature of the cinnamoyl esterase activity (30 °C). The discrepancy between the two temperatures can be explained by the synergies between the cinnamoyl esterase enzyme and other AX-degrading enzymes in the release of FA during brewing, which have been reported by Bartolome, Garcia-Conesa, and Williamson (1996) and by Sancho, Bartolome, Gomez-Cordoves, Williamson, and Faulds (2001). The concerted action of the AX-hydrolyzing enzymes not only increases the amount of feruloylated AX in wort, but also creates a depolymerising activity, decreasing the molecular weight of the AX in solution. This renders them more accessible and makes them more susceptible to cinnamoyl esterase enzymes.

3.3. Variation of stirring regime, grist coarseness and mash thickness

When the mash was stirred during brewing, significantly more FA was released into the wort than unstirred wort: 1.45 ± 0.02 ppm in the unstirred wort compared to 1.83 ± 0.02 and 2.10 ± 0.02 ppm in the continuously stirred wort at 100 and 200 rpm (corresponding to the Congress wort procedure), respectively. De-intensifying the stirring regime by stirring at 200 rpm instead of 100 rpm or intermittent stirring instead of continuous stirring (1.74 ± 0.01 and 1.93 ± 0.07 ppm at intermittent stirring at 100 and 200 rpm, respectively) decreased the amount of FA released from the malt. When the wort was continuously stirred at 200 rpm, 45% more FA was released compared to the unstirred wort. Similarly, Li et al. (2005) found that the AX content in a continuously stirred

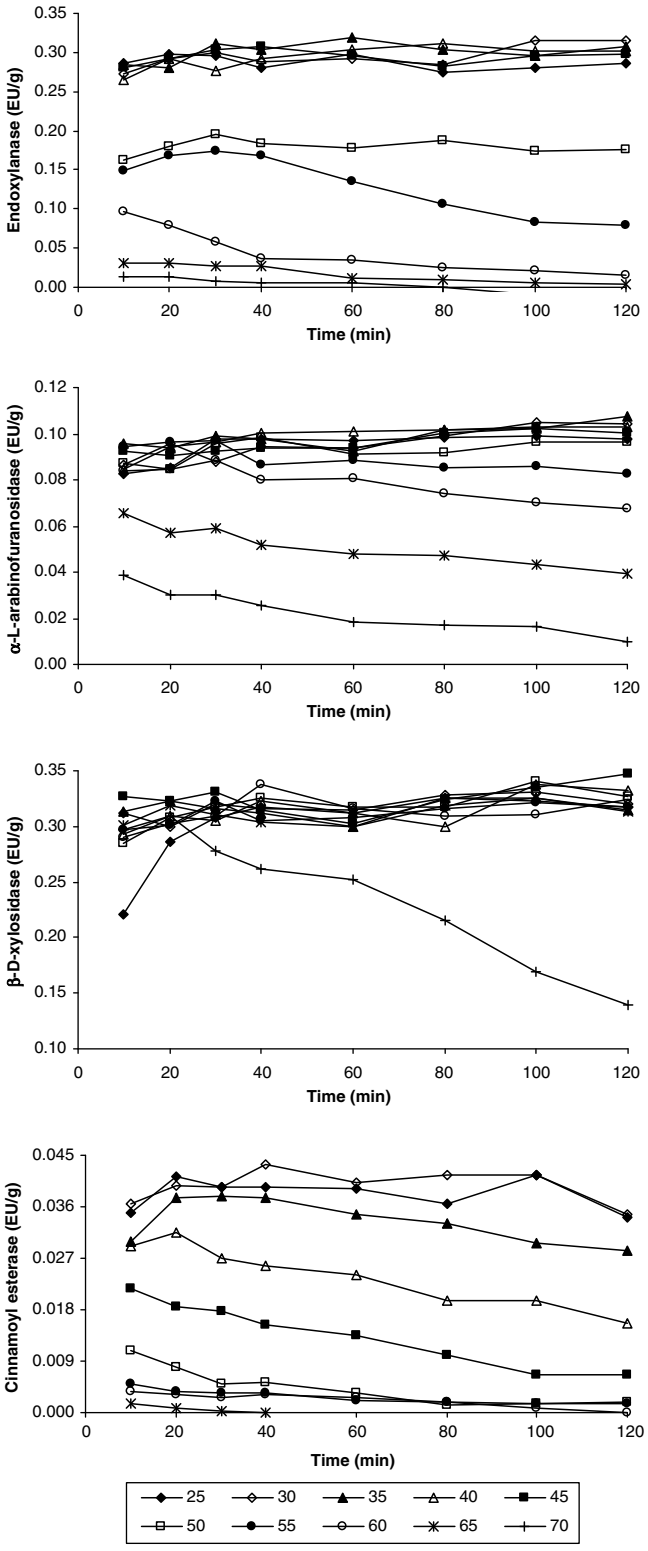

Fig. 4. AX-hydrolyzing activities (EU/g dry malt) in isothermal mashings: endoxylanase activity, α-L-arabinofuranosidase activity, β-D-xylosidase activity and cinnamoyl esterase activity (from top to bottom).

mash almost doubled compared to the AX content of an unstirred mash. Hence, less substrate is available for the cinnamoyl esterase enzyme in unstirred wort.

Free FA concentrations of, respectively, 2.04 ± 0.01, 1.82 ± 0.02, 1.75 ± 0.02 and 1.58 ± 0.04 ppm for 0.1, 0.6, 1.2 and 1.9 mm distances between the grinding discs were obtained. More FA was released from the fine grist than from coarse grist. The same effect of the grist coarseness on the solubilisation of AX during brewing was previously observed (Li et al., 2005). The coarse grist probably limited the AX solubilisation, limiting the substrate availability for the cinnamoyl esterase enzyme.

The effect of the mash thickness on the release of free FA during wort production is shown in Fig. 5. Clearly, when the mash became denser, the absolute concentration of free FA in wort increased. Increasing the amount of malt will increase both the water-extracted and the enzymatically solubilised fraction of FA present in wort. However, when each FA concentration was normalised for the amount of malt used for the wort of 12 °P, it could be seen that relatively more FA was solubilised in worts of medium density. The parabolic curvature of the effect of the mash thickness on the FA release can be explained by two counteracting mechanisms. Generally, enzymes are appreciably more stable in thicker mashes (with higher grist:water ratios) (Briggs, 1998). This may protect enzymes from thermal inactivation in more dense worts. However, in thick mashes, more water is bound by starch and the water phase becomes more concentrated. This increased viscosity may limit the mobility of the enzyme substrate in high gravity worts and the solubilisation of AX (Li et al., 2005).

3.4. Addition of wheat, flaked cereal adjuncts and specialty malts

The influence of the addition of 11 wheat varieties during brewing on the level of free FA in Congress wort is shown in Fig. 6A. Significant differences were found between worts produced with different wheat cultivars. Worts produced with 50% unmalted wheat contained 15–43% less free FA than did the corresponding wort with 100% malted barley. Cinnamoyl esterase activities of Scarlett malt and wheat (Drifter, Legat and Tremie) extracts were tested separately and in combination with the barley malt pilsner extract (v/v% 50/50). Esterase activities were 1.26 ± 0.12, 2.33 ± 0.16, 3.06 ± 0.16 and 2.30 ± 0.15 EU/g for Scarlett, Tremie, Drifter and Legat, respectively. The three wheat extracts had higher cinnamoyl esterase activities than had the Scarlett malt. Raw cereals containing more esterase activity than malted cereals can be explained by the decrease of esterase activity during germination (Sancho et al., 2001) and its heat sensitivity (Humberstone & Briggs, 2000; Sun et al., 2005) causing a further decrease during kilning. The esterase activities of the combined extracts (1.88 ± 0.23, 1.82 ± 0.34 and 2.12 ± 0.16 EU/g for Tremie, Drifter and Legat, respectively) were not significantly different from the theoretical values (½ esterase activity of barley malt + ½ esterase activity of wheat), indicating that the barley malt cinnamoyl esterase is not inhibited by wheat extracts. Hence, the lower amount of FA released in wort produced with wheat was not due to a lower esterase activity. A possible reason for the decrease in free FA is a lower release of water-extracted FA or a lower alkali-extractable FA content (i.e., cinnamoyl esterase substrate) in worts produced with wheat. However, compared to the pilsner wort (0.64 ppm water-extracted FA and 7.54 ppm alkali-extractable FA) the worts produced with wheat did not contain lower levels of water-extracted (0.49 ± 0.17 ppm) or alkali-extractable FA (7.44 ± 0.82 ppm). It seems that the limited release of FA in worts produced with unmalted wheat, is due to the lower extent of AX degradation or differences in the AX structure, rather than a direct inhibition of the esterase activity. Wort endoxylanase activity and AX molecular weight distribution may have a profound effect on the release of FA during mashing. WEAX of barley malt have a much lower molecular weight than have those from wheat: 38 kDa (Debyser, Schooneveld-Bergmans, Derdelinckx, Grobet, & Delcour, 1997) compared to 400 kDa (Cleemput et al., 1995), respectively. Hence barley malt WEAX may be better substrates for cinnamoyl esterases than wheat WEAX due to less steric hindrance. Moreover, endoxylanase activities of worts produced with unmalted wheat are lower than endoxylanase activities of worts produced with 100% barley malt (Debyser et al., 1998). This is probably due to the inhibition of the exogenous microbial xylanolytic system associated with wheat kernels by wheat endogenous endoxylanase inhibitors (Dornez, Joye, Gebruers, Delcour, & Courtin, 2006). Apart from differences in endoxylanase activity, wheat and barley malt WEAX also differ in structure. Compared to wheat AX, barley AX appear to be more structurally uniform since substituted residues are more clustered and separated by regions of unsubstituted xylosyl units. Arabinose substitution along the backbone is not random. Barley AX also have a higher proportion of arabinose substituted at the O-2 position than have wheat AX (Vietor, Angelino, & Voragen, 1992).

The influence of the addition of specialty malts during brewing was examined by the production of Congress wort with 50% pilsner barley malt and 50% specialty malts. Three coloured malts [Vienna (6-8 EBC), Munich (15–20 EBC), Melanoidin (60–80 EBC)], two caramelized malts [Caramunich (110–130 EBC), Caraaroma (300–400 EBC)] and two roasted barley malts [Carafa (1000–1200 EBC) and Carafa special (1330–1500 EBC)] were used. The content of free FA and 4VG in Congress worts is shown in Fig. 6B. Worts produced with Vienna and Munich coloured malts did not significantly differ in FA content from the reference pilsner wort. Concerning the other specialty malts, the free FA content in Congress wort decreased with increasing wort colour. In the worts produced with the

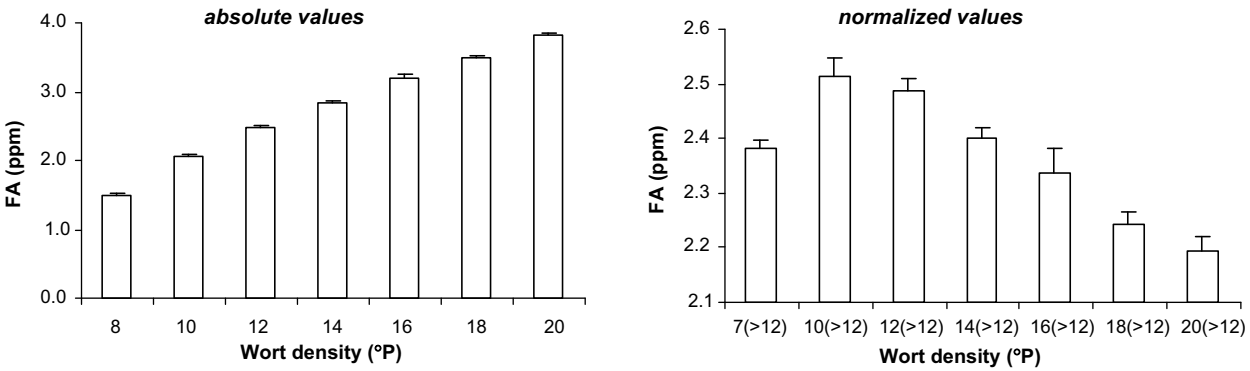

Fig. 5. Effect of the mash thickness on the release of free FA (ppm) during wort production: absolute concentration (left) and normalised concentration versus 12 °P (right).

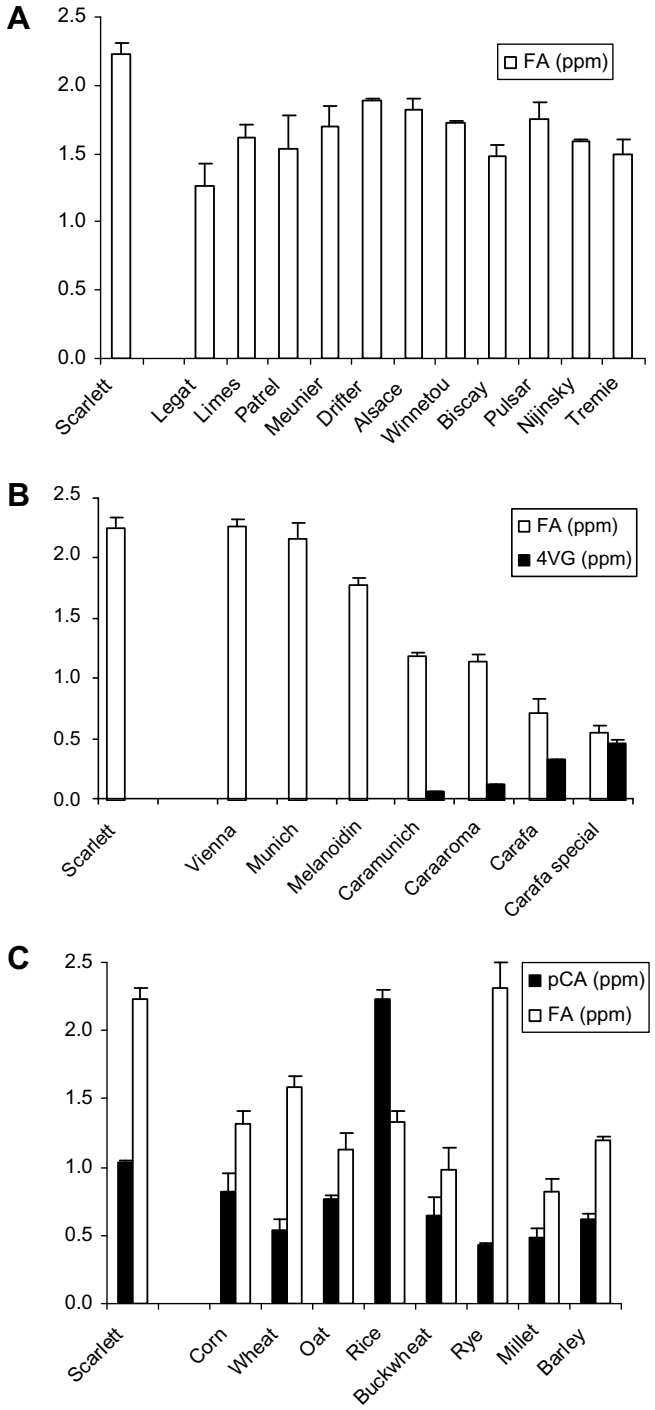

Fig. 6. Influence of the addition of adjuncts during brewing on free FA concentration (ppm) in Congress wort (50% adjuncts – 50% Scarlett pilsner malt): (A) wheat varieties; (B) specialty malts; and (C) flaked cereals.

caramelised and roasted malts, 4VG could also be detected in increasing levels with increasing malt colour. It arises from the thermal fragmentation of free FA in barley grain during the kilning and roasting of these highly coloured malts. Although the thermal decarboxylation of free FA during the malt production process can partly account for the lower levels of free FA in worts produced with specialty malts, there was still a net decrease in FA concentration. The worts produced with Carafa and Carafa special even contained less than 50% of the reference pilsner wort. Cinnamoyl esterase activities of Scarlett malt and three specialty malts (Melanoidin, Caramunich and Carafa) extracts were tested separately and in combination with the barley malt pilsner extract (v/v% 50/50). Esterase activities were 1.26 ± 0.12 and 0.45 ± 0.13 EU/g for Scarlett and Melanoidin malt, respectively. No cinnamoyl esterase activity could be detected in Caramunich and Carafa malt extracts. The esterase activities of the combined extracts (0.83 ± 0.04 and 0.65 ± 0.09 EU/g for Melanoidin and Caramunich, respectively) were not significantly different from the theoretical values (½ esterase activity of barley malt + ½ esterase activity of wheat), indicating that the barley malt cinnamoyl esterase was not inhibited by these extracts. However, the cinnamoyl esterase activity of the combined extract of Carafa (0.32 ± 0.06 EU/g) was only half the theoretical value (0.63 ± 0.06 EU/g), indicating that the extract of this roasted malt inhibited the esterase activity of the Scarlett pilsner malt. Since roasted barley or roasted malt make up only a small proportion of the grist, Congress worts were also produced with 5% specialty malt. The free FA content of these worts did not significantly differ from the reference pilsner wort. 4VG could only be detected in the worts produced with Caramunich and Carafa malt (<50 ppb). Hence, when used in small amounts, the addition of specialty malts during brewing will not significantly affect wort HCA and volatile phenol concentrations.

The influence of the addition of flaked cereals during brewing was examined by the production of Congress wort with 50% pilsner barley malt and 50% flaked cereals. While, in the previous experiments, the release of pCA and FA followed parallel trends, the addition of flaked cereals had different effects on the release of FA and pCA. The contents of free FA and pCA in Congress worts with 8 different flaked cereals are shown in Fig. 6C. Except for the pCA content in wort produced with rice flakes and the FA content in wort produced with rye, the worts produced with cereal adjuncts had significantly lower levels of free pCA and FA. During the production process of the flakes, all enzymes are denatured. The lack of cinnamoyl esterase activity and endoxylanase activity of these adjuncts will generally lead to reduced levels of HCA in wort. The FA content in wort produced with rye flakes is comparable to the FA content in pilsner wort. This may be due to the high levels of AX reported in rye (Henry, 1987).

3.5. Pilot-scale wort production by the infusion mashing method: a case study

The release and the evolution of free FA during wort production (infusion mashing) in a pilot-scale brewery (5 h) were investigated by multiple sampling during the mashing process, filtration, wort

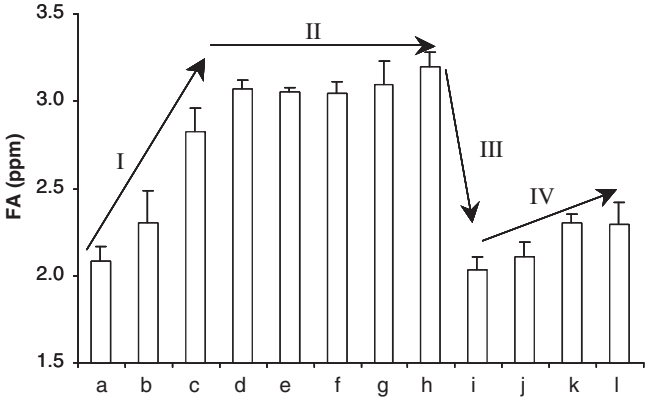

Fig. 7. Release and evolution of free FA (ppm) during wort production: a case study (a) 45 °C 15 min; (b) 52.5 °C 10 min; (c) 62 °C 0 min; (d) 62 °C 40 min; (e) 72 °C 10 min; (f) 78.5 °C mashing-off; (g) start filtration; (h) during filtration before sparging; (i) end of filtration – start wort boiling; (j) during wort boiling – before hopping; (k) end wort boiling; and (l) whirlpool end.

boiling and whirlpool holding. The results are represented in Fig. 7. Four distinct parts can be differentiated during the course of the wort production process. From the start of mashing-in until the end of the holding time at 62 °C, the FA concentration increased continuously due to the extraction of free malt FA and the enzymatic solubilisation of ester-bound FA at temperatures <65 °C. The highest amount of FA was released during the first 15 min of mashing. After the initial increase, the wort FA content levelled-off and remained constant during the mashing-off phase, the transfer to the lauter tun, the mash settling and the start of the wort filtration until the sparging of the spent grains. When the temperature exceeded 62 °C, the cinnamoyl esterase enzyme was denatured. Since no chemical hydrolysis of FA occurred at higher temperatures (72 and 78.5 °C), no additional FA was released into the wort. During the washing of the spent grains, a decrease in wort FA content was seen, due to the dilution of the wort with the sparging liquor. The drop in the FA concentration corresponded with the wort dilution factor. Hence, no free FA remained in the filter bed after washing. During wort boiling, the free wort FA concentration increased by 10%. This net increase was the result of several factors. During wort boiling, thermal decarboxylation of FA will lead to the formation of 4VG. At the end of the boiling process, 0.14 ppm 4VG was found in the wort. This thermal decarboxylation caused the wort FA concentration to diminish by 9%. However, during wort boiling, the wort volume will decrease by 7–8% due to evaporation. This will cause an apparent increase in FA content. Finally, the addition of hop pellets will cause a real increase in wort FA content by 7–11% (based on results obtained in laboratory hop addition experiments). Taking into account these three factors, a net increase of the wort FA content during wort boiling will occur. The reassociation or coprecipitation of free FA with AX, polyphenols or proteins was negligible. Otherwise, no net increase in free FA content would occur during pilot-scale wort boiling. This was confirmed during laboratory-scale wort boiling experiments under reflux (no evaporation) without hop addition. During these experiments, the increase in 4VG corresponded with the decrease in FA.

4. Conclusions

During mashing, FA is both water-extracted and enzymatically solubilised by cinnamoyl esterases. A clear difference in temperature- and pH-dependence between the release of the water-extracted and enzymatically hydrolyzed fraction was found. In contrast to the water-extracted fraction, the hydrolysis of ester-bound FA is subject to close technological control. An optimal temperature of 40 °C and an optimal pH of 5.8 were found. Also, the mash thickness, the grist coarseness and composition, and the stirring regime had a pronounced effect on the release of FA during mashing. The T,t-dependencies of AX-degrading enzymes were correlated with free and ester-bound FA release during mashing. Finally, a pilot-scale (5 h) wort production process was conducted to validate the results of the laboratory-scale mashing experiments. In previous studies, it was suggested that the selection of a suitable brewing yeast strain is probably the most important means of creating a phenolic taste profile in beer. However, the brewers' choice of a yeast strain is mostly dominated by other factors, such as fermentation and flocculation behaviour, overall flavour generation and tradition. Hence, other means for controlling volatile phenol levels in beer might be necessary. Enhancing the enzymatic release of the phenolic flavour precursors from bound forms during mashing can greatly enhance the phenolic aroma potential of wort. Optimising this precursor release during mashing is therefore another important means for controlling final volatile phenol levels in beer. This work presents a practical aid for brewers to help them in optimising this release during brewhouse operations.

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