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Variability in the Release of Free and Bound Hydroxycinnamic Acids from Diverse Malted Barley (*Hordeum vulgare* L.) Cultivars during Wort Production

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Volatile phenols have long been recognized as important flavor contributors to the aroma of various alcoholic beverages. The two main flavor-active volatile phenols in beer are 4-vinylguaiacol and 4-vinylphenol. They are the decarboxylation products of the precursors ferulic acid and *p*-coumaric acid, respectively, which are released during the brewing process, mainly from malt. In this study, the variability in the release of free and ester-bound hydroxycinnamic acids from nine malted barley (*Hordeum vulgare* L.) varieties during wort production was investigated. A large variability between different barley malts and their corresponding worts was observed. Differences were also found between free ferulic acid levels from identical malt varieties originating from different malt houses. During mashing, free hydroxycinnamic acids in wort are both water-extracted and enzymatically released by cinnamoyl esterase activity. Esterase activities clearly differ between different barley malt varieties. Multiple linear regression analysis showed that the release of ferulic acid during mashing did not depend only on the barley malt esterase activity but also on the amount of ester-bound ferulic acid initially present in the wort and on its endoxylanase activity. The study demonstrates the importance of selecting a suitable malt variety as the first means of controlling the final volatile phenol levels in beer.

KEYWORDS: 4-Vinylguaiacol; ferulic acid; hydroxycinnamic acids; wort production; phenolic flavor; cinnamoyl esterase; barley malt; *Hordeum vulgare* L.

INTRODUCTION

Volatile phenols have long been recognized as important flavor contributors to the aroma of nonalcoholic beverages like fruit juices and coffee (*1*, *2*) as well as alcoholic drinks like beer, wine, sherry, and whisky (*3*–*6*). The two main flavor-active volatile phenols in beer are 4-vinylguaiacol (4VG) and 4-vinylphenol (4VP). The presence of these volatile phenolic compounds is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers. Hence the term "phenolic off-flavor" (POF) (*7*) is attributed to beers with a strong medicinal, clove-like aroma. Despite being historically catalogued as an off-flavor, these compounds are known to be essential flavor contributors to the characteristic aroma of Belgian white beers (made with unmalted wheat), German Weizen beers (made with malted wheat), and Rauch beers. Though, also in many other top-fermented blond and dark specialty beers the phenolic flavor is essential for the overall flavor perception.

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4VP and 4VG are the decarboxylation products of the phenolic acids *p*-coumaric acid (4-hydroxycinnamic acid) (pCA) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA), respectively. Phenolic acids (i.e., hydroxycarboxylic acids with phenolic hydroxyl groups), 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, aleurone, and endosperm in cereal grains. In barley, AX make up 25% of the cell walls of the starchy endosperm and 75–85% of the cell walls of the aleurone layer. Different levels of phenolic acids are found in different fractions of the cereal grain. The outer layers of the barley grain comprising husk, pericarp, testa, and aleurone contain the highest concentrations of total phenolic acids while their levels are considerably lower in the endosperm layers (*8*). 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

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water-extracted and solubilized by AX-hydrolases from the malt into the wort (*9*). AX can be degraded by several enzymes. The β(1–4)-endoxylanases (EC 3.2.1.8) generate (un)substituted xylooligosaccharides. These oligomers can be further degraded by β-D-xylopyranosidase (EC 3.2.1.37), which releases β-D-xylose from the nonreducing end. The α-L-arabinofuranosidase (EC 3.2.1.55) liberates α-L-arabinose units from the main chain. Esterified FA and pCA can be released as free acids by cinnamoyl esterase (EC 3.1.1.73) activity during mashing. Cinnamoyl esterases, also known as feruloyl esterases, *p*-coumaroyl esterases, or ferulic acid esterases, have been reported natively in barley and germinated barley (*10*) and barley malt (*11–13*).

Phenolic acids have high threshold values and do not affect the aroma of beer (*14*). However, they are appreciated for their antioxidant activity. Recently, bound phenolic acids also have become a topic of interest because of their potential antioxidant capacity, and methods to increase the levels of bound HCA in beer using commercial enzyme preparations have been investigated (*15*). Total FA levels in grain have been found to be highly correlated with their total antioxidant activity [as measured with the total oxyradical scavenging capacity (TOSC) assay] (*16*). Furthermore, phenolic acids from beer are absorbed and extensively metabolized in humans, and potential beneficial health effects are attributed to them (*17*). During the beer production process, the flavor-inactive phenolic acids can be decarboxylated to the highly flavor-active volatile phenols 4VP and 4VG. 4VG in beer can arise from the thermal decarboxylation of FA during high-temperature treatments during the malt and beer production process like malt kilning, wort boiling, whirlpool holding, wort transfer times, and pasteurization. Since mashing temperatures (<80 °C) are inadequate for the thermal decarboxylation of FA, no 4VG can be detected in unboiled wort. Only intensive and long boiling regimes will give rise to phenolic flavor compounds exceeding the flavor threshold (*4*). Fiddler et al. (*18*) studied the thermal decomposition of FA by thermal gravimetric analysis. The decarboxylation rate was accelerated by oxygen, indicating that the decarboxylation is probably a radical reaction. However, the major route for 4VG formation during the beer production process is the enzymatic decarboxylation of phenolic acids during fermentation by phenylacrylic acid decarboxylase activity of top-fermenting yeast strains (Pad1-enzyme) (*19*). POF has also been associated with a number of wild yeasts, the most important being *Brettanomyces/Dekkera* spp. (*20*). Also many bacteria can convert phenolic acids. Many species within the *Enterobacteriaceae*, common wort contaminants, are associated with the formation of volatile phenol off-flavors (*21*). Among the beer contaminants, many lactic acid bacteria have been found to be able to decarboxylate pCA and FA (*6*).

The objectives of this study were to investigate the variability in the release of free and ester-bound HCA during wort production, and their distribution between malt, wort, and spent grain, and to identify malt parameters that explain the observed variability in HCA concentration in worts from different barley malt varieties.

MATERIALS AND METHODS

Barley Malt Samples and Malt Analyses. Nine commercial barley (*Hordeum vulgare* L.) pilsner malt varieties were obtained from two industrial malt houses (malt house A and malt house B). Astoria, Optic, Pasadena, Prestige, and Scarlett are two-row spring barley varieties and Esterel is a six-row winter barley variety. Malt and wort analyses (color, pH, degree of modification, degree of homogeneity, extract content, extract difference, total and soluble protein content, and moisture content) were performed according to the standard methods as defined by the European Brewery Convention (EBC Analytica, 1998). The analytical data of the malt and wort analyses are shown in **Table 1** and are generally within brewery specifications.

Table 1. EBC Analytica Malt and Wort Analyses[a]

barley malt	Md	Hg	E	E_{diff}	M	pH	C	P_t	P_s	KI
Astoria A	88.0	73.5	81.0	2.0	7.85	5.89	5.5	11.5	4.1	35.3
Scarlett A	99.5	97.0	83.3	0.5	5.30	5.89	5.5	10.2	5.0	49.0
Pasadena A	94.6	75.6	82.6	1.8	7.56	5.59	4.1	10.7	4.5	41.7
Optic A	95.4	83.6	82.5	1.1	4.60	6.09	6.1	9.6	3.4	35.0
Prestige A	91.8	70.9	81.3	0.7	5.98	5.96	3.5	10.7	4.3	40.2
Esterel A	93.9	73.6	81.8	2.2	6.37	5.98	3.7	10.2	4.2	41.6
Scarlett B	94.4	78.9	81.8	0.9	5.11	6.03	3.8	10.2	4.2	41.2
Optic B	76.5	64.7	82.5	1.8	5.00	6.13	5.3	9.7	3.6	36.9
Prestige B	93.4	81.5	81.0	0.3	5.12	5.99	3.8	10.6	4.2	39.3

[a] Md = degree of modification (%); Hg = degree of homogeneity (%); E = extract content (% dry malt); E_{diff} = extract difference (% of dry malt); M = moisture content (%); C = wort color (EBC units); P_t = total protein content (% of dry malt); P_s = soluble protein content (% of dry malt); KI = Kolbach index (P_s/P_t).

Wort Production. Standard laboratory Congress wort was produced according to EBC Analytica (1998) method 4.5.1. Barley malts were ground in a Buhler-Miag malt mill (Buhler-Miag, Minneapolis, MN) set for fine grist coarseness (0.2 mm gap between the grinding disks). Laboratory-scale mashing experiments were carried out in an automated mashing bath (LB8 electronic mashing bath; Funke Gerber GmbH, Berlin, Germany). Ground malt (50.0 g) was mixed with 200 mL of water of 46 °C. A temperature of 45 °C was maintained in the mash for 30 min. Consequently, the temperature was raised 1 °C per minute for 25 °C. When 70 °C was reached, 100 mL of water at 70 °C was 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, the mash was filtered over a folded filter (MN 614 × 32 cm diameter; Macherey-Nagel GmbH). To analyze the content of water-extracted HCA in barley malt samples, 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 et al. (*22*) to eliminate enzyme activity.

Total Alkali-Extractable HCA Content in Wort and Barley Malt. For the determination of the total alkali-extractable HCA content in wort and barley malt, ester-bound HCA were released from AX by alkaline hydrolysis. For this purpose, 5 mL of Congress wort or 100 mg of finely ground malt (0.1 mm gap between the grinding disks) was mixed with 5 mL of 2 N NaOH (Riedel-de-Haen, Seelze, Germany) in glass Pyrex tubes. The NaOH solution was supplemented with 1% ascorbic acid (Sigma-Aldrich, Bornem, Belgium) and 10 mM anhydrous EDTA (Sigma-Aldrich, Bornem, Belgium) to prevent substrate oxidation according to the method described by Nardini et al. (*23*). After the test tubes were flushed with nitrogen, the mixture was incubated on a rotary shaker for 24 h. The reaction was stopped by adding 5 mL of 4 N HCl (prepared from 37% HCl; Fisher Chemicals, Zurich, Switzerland), and 300 mg of NaCl was added. HCA were extracted three times with 10 mL of ethyl acetate (Acros Organics, Geel, Belgium). After vacuum evaporation of the combined ethyl acetate fractions to dryness at 35 °C, the HCA were resolved in 5 mL of methanol prior to HPLC analysis.

Quantification of HCA in Wort and Barley Malt Extracts. Quantification of HCA in wort and beer was performed by HPLC-ECD as described by Vanbeneden et al. (*24*). Before injection, all samples were filtered through 0.45 μm regenerated cellulose syringe filters (Alltech, Deerfield, IL) into autosampler vials and frozen at −18 °C until analysis. All samples were protected from light during operation to minimize the photoisomerization reaction to which HCA are susceptible.

Determination of Xylose Content in Wort. Since arabinose present in wort can originate both from AX and arabinogalactan and up to equal amounts of both can be present in wort, the xylose content was used as a relative measure for AX levels in wort according to Debyser et al. (*22*). For the quantification of total xylose content in wort, 2.5 mL of 4 M trifluoroacetic acid (TFA) was added to 2.5 mL of filtered

wort, and the mixture was incubated at 100 °C for 1 h. After incubation, the medium was cooled, and the TFA was evaporated by flushing the medium with nitrogen at 65 °C. The remaining residue was diluted to 50 mL. Prior to monosaccharide determination by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), samples were deionized by passing them over an anion- and cation-exchange resin. Monosaccharides were analyzed on a Dionex DX500 chromatography system (Dionex Corp., Sunnyvale, CA) consisting of a Rheodyne model 9125 automatic sample injector (10 μL Sample loop), a Dionex AS40 autosampler, a GP40 gradient pump, and an ED40 electrochemical detector with a gold working electrode. The data collection rate was set at 2 Hz. Separations were performed on a CarboPac PA1 4 × 50 mm guard column and a CarboPac PA1 4 × 250 mm analytical column. Elution was with 16 mM NaOH for 15 min followed by a 10 min column regeneration with 200 mM NaOH. Mobile phases were prepared with highly purified Milli-Q water and low carbonate NaOH (50%) (J. T. Baker, Phillipsburg, NJ). Mobile phases were degassed with helium prior to use. For detection and quantification the following pulse potentials (vs Ag/AgCl) and durations were applied to the working electrode: E_1 +0.1 V and 0.40 s, E_2 −2.0 V and 0.02 s, E_3 +0.6 V and 0.01 s, and E_4 −0.1 V and 0.06 s. Monosaccharides were identified by coelution with standard reference compounds and quantified by comparison of the peak areas with the peak areas of known concentrations of pure sugars. D(−)-Fructose, D(+)-xylose, D(−)-arabinose, and D(+)-galactose were obtained from Sigma-Aldrich (Bornem, Belgium). D(+)-Glucose was purchased at Merck Eurolab (Darmstadt, Germany). The total xylose content was compensated for free xylose present in wort by omitting the hydrolysis step and analysis of a nonhydrolyzed sample.

Measurement of β(1–4)-Endoxylanase (EC 3.2.1.8) Activity. Samples (3.00 g) of ground malt were suspended in 0.025 M sodium acetate, pH 4.7 (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). Endoxylanase activity assays of malted barley extracts were performed according to Debyser et al. (*9*). The extract (1.0 mL) was incubated for 5 min at 50 °C before adding an azurine-cross-linked (AZCL) wheat AX tablet (Xylazyme AX tablets, Megazyme, Australia). The incubation was then continued for 1 h at 50 °C. The reaction was terminated by adding 1% (w/v) Trizma base [tris(hydroxymethyl)aminomethane] (10.0 mL) and vigorous vortex stirring. After 5 min at room temperature, the tubes were shaken vigorously and the contents filtered through a 0.45 μm PVDF syringe filter (Alltech, Deerfield, IL). The absorbance was measured at 590 nm against a control, which was prepared by incubating the extract without the substrate tablet. A correction was made for the nonenzymic color release by the AZCL-AX tablet. Activities were expressed in ΔA_{590} per gram of malt.

Measurement of β-D-Xylosidase (EC 3.2.1.37) and α-L-Arabinofuranosidase (EC 3.2.1.55) Activity. *p*-Nitrophenyl glycosides were used as substrates to measure β-D-xylosidase and α-L-arabinofuranosidase activities according to the procedure of Cleemput et al. (*25*). Samples (3.00 g) of ground malt were suspended in 0.050 M MES [2-(*N*-morpholino)ethanesulfonic acid], 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). The activity was measured as follows: *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl β-D-xylopyranosidase (Sigma, St. Louis, MO) solutions (10 mM) were prepared in a 50 mM MES buffer, pH 6.0. Aliquots of these solutions (100 μL) were incubated with 50 μL of barley malt extract. The reaction was stopped after 30 min at 40 °C by adding 1.5 mL a 1% (w/v) Trizma base solution. In a control assay, Trizma base was added before the malt extract. The release of *p*-nitrophenol from the *p*-nitrophenyl glycoside was determined colorimetrically at 410 nm. Activities were expressed as units per gram of malt. One enzyme unit (EU) was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate per minute at 40 °C and pH 6.0.

Measurement of Cinnamoyl Esterase (EC 3.1.1.73) Activity. Cinnamoyl esterase activity in barley malt extracts was assessed spectrophotometrically using 4-nitrophenyl ferulate (4NPF) as a substrate based on a method adapted from Mastihuba et al. (*26*). 4NPF was obtained from the Faculty of Chemical Technology, Slovak Universtity of Technology, Bratislava, Slovakia. Samples (3.00 g) of ground malt were suspended in 0.050 M 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). The substrate solution was prepared by mixing 9 volumes of 0.1 M potassium phosphate buffer solution, pH 6.0, containing 2.5% Triton X-100 with 1 volume 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 barley malt extract. A blank sample was included using water instead of barley malt extract to correct for the nonenzymic color 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 (against the control sample) before and after incubation was used to calculate enzyme activity. Activities were expressed as EU per gram of malt. One enzyme unit was defined as the amount of enzyme that released 1 nmol of *p*-nitrophenol from the substrate per minute at 30 °C and pH 6.0.

Cinnamoyl esterase activity versus the methyl esters of pCA (MpCA), FA (MFA), and SA (MSA) was determined by incubating 3 mL of malt extract (prepared as described above) with 1 mL of substrate solution for 2 h at 30 °C. The substrate solution was prepared by dissolving the HCA methyl esters 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. The HCA methyl esters were tested both separately and in a mixture containing all three of them together (26.7 mM each in the stock solution). Activities were expressed as enzyme units per gram of malt. One enzyme unit 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.

Isolation of Water-Extractable AX (WEAX) from Barley Malt and Endoxylanase Treatment. WEAX was isolated from barley malt (Scarlett A) according to a procedure adapted from Cleemput et al. (*27*). The isolation was carried out at room temperature unless indicated otherwise. Barley malt (1.0 kg) was ground in a Buhler-Miag malt mill (Buhler-Miag, Minneapolis, MN) set for fine grist coarseness (0.2 mm gap between the grinding disks). The ground malt was placed in an oven at 130 °C for 5 h to eliminate enzyme activities, extracted with water [5:1 (v/w), 15 min, 30 °C], and filtered over a folded filter (MN 614 × 32 cm diameter). The supernatant was heated to 90 °C, and residual starch was hydrolyzed by addition of α-amylase solution (2.0 mL, type XII-A from *Bacillus licheniformis*, A3403; Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 90 °C for 120 min, cooled to room temperature, and filtered again over a folded filter (MN 614 × 32 cm diameter). The WEAX was precipitated by stepwise addition of ethanol (96%) to a final concentration of 65% (v/v). The mixture was stirred for 30 min, kept at 4 °C overnight, and recovered by centrifugation (10000*g*, 30 min, 4 °C). The precipitate was dissolved in water (1.0 L), and ethanol was added to a final concentration of 65% (v/v). The mixture was stirred for 30 min, kept at 4 °C overnight, and recovered by centrifugation (10000*g*, 30 min, 4 °C). The precipitate was washed with ethanol (500 mL) and with acetone (500 mL) with intermediate stirring (120 min) and centrifugation (10000*g*, 30 min, 4 °C). The final pellet was dried for 24 h at 45 °C. With this procedure, 58% of total WEAX present in the Scarlett A barley malt (0.45% w/w) was recovered. The resulting precipitate contained 7.5% protein and had a final AX content of 94% on carbohydrate basis. Contaminating sugars consisted predominantly of galactose and glucose, originating from arabinogalactan and glucan. The isolated WEAX contained 0.12% (w/w) ester-bound FA.

An amount of WEAX preparation, corresponding to 30% of ester-bound FA initially present in Scarlett A wort, was dissolved in 0.050 M NaOAc, pH 4.7 (20.0 mL), and incubated with endo-β(1–4)-xylanase (50.0 mg) from *Trichoderma viride* (Fluka, St. Louis, MO) for 60 min at 45 °C. Enzyme activity was eliminated by heating the sample for 15 min at 100 °C. The effectiveness of the endoxylanase treatment in

Table 2. Total Alkali-Extractable pCA and FA Content in Nine Malted Barley Varieties and Their Corresponding Worts (μg/g Dry Malt)[a]

	malt		wort		% wort	
barley malt	pCA (μg/g dm)	FA (μg/g dm)	pCA (μg/g dm)	FA (μg/g dm)	pCA (%)	FA (%)
Astoria A	262 ± 4 d	580 ± 28 b	10.4 ± 0.6 d,e	62.9 ± 3.9 d,e	4.0	10.8
Scarlett A	252 ± 4 c,d	532 ± 37 a,b	13.0 ± 0.7 f	63.7 ± 2.0 e	5.1	12.0
Pasadena A	263 ± 10 c	597 ± 18 b,c	6.1 ± 0.3 a	42.4 ± 1.3 a	2.3	7.1
Optic A	360 ± 12 f	634 ± 30 c,d	8.4 ± 0.6 b,c	54.7 ± 0.8 c	2.3	8.6
Prestige A	294 ± 11 e	649 ± 14 d	9.0 ± 0.4 c,d	59.0 ± 1.1 d,e	3.1	9.1
Esterel A	215 ± 8 a,b	653 ± 12 d	7.2 ± 0.8 a,b	64.6 ± 0.3 b	3.3	9.9
Scarlett B	236 ± 10 b,c	563 ± 8 b	12.5 ± 0.8 f	70.2 ± 1.6 f	5.3	12.5
Optic B	239 ± 10 b,c	481 ± 16 a	10.4 ± 0.6 d,e	57.2 ± 2.3 c,d	4.3	11.9
Prestige B	204 ± 11 a	646 ± 13 d	10.6 ± 0.6 e	55.6 ± 1.9 e	5.2	8.6

[a] Results are expressed as mean ± standard deviation. Values with no common letters differ significantly at the 95% confidence level.

reducing the molecular weight of the WEAX fragments could be evaluated by a clear visible reduction in viscosity of the solution after the procedure. A blank sample was made by adding the endoxylanase during the boiling step. HPLC analysis showed that during the treatments no release of ester-bound FA occurred in any of the two samples. Both aliquots were added at the start of Congress wort production with Scarlett A barley malt according to the procedure specified above.

Statistical Analysis. Analyses were carried out at least in triplicate (except for the standard wort and malt analyses presented in **Table 1**, which were carried out in duplicate). Results are represented as mean ± standard deviation and considered significantly different at a 95% confidence level (Tukey's *t*-test statistic; $p < 0.05$). The data obtained from the barley malt and wort analyses were analyzed using multiple linear regression (MLR). The general purpose of MLR is to study the relation between a dependent (response) variable (Y) and other independent (explanatory) variables (X_n). The general mathematical function is a first-degree equation specified as

$$Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_m X_m + \epsilon$$

where X_m represents the m experimental variables tested, α is the constant term, and ϵ is the predictive error. β_m represents the regression coefficients of the independent variables, and each represents the "weight" (correlation) of the respective independent variable. To measure the strength of the relationship between the measured variables, Pearson's correlation coefficients (r) were calculated. The statistical package, XLSTAT 2006 Version 2006.06 (Addinsoft, Paris, France), was used for the statistical calculations.

RESULTS AND DISCUSSION

Release of HCA from Malted Barley during Wort Production. The amount of total alkali-extractable pCA and FA in nine malted barley varieties from two malt houses and their corresponding worts are represented in **Table 2**. The values represent the sum of the HCA either present in free or in ester-bound form in barley malt and in wort. Mean values with no common letters differ significantly at the 95% confidence level. Total alkali-extractable FA and pCA content in malt ranged from 481 to 653 μg/g of dry malt and from 204 to 360 μg/g of dry malt, respectively, making FA the predominant phenolic acid in barley malt. These amounts are comparable with those found in unmalted barley by Hernanz et al. (*28*), which were found to be 359–624 μg/g and 79–260 μg/g for total alkali-extractable FA and pCA, respectively. The degree in variability for the pCA and FA content in malt was 18% and 10%, respectively.

Significant differences were observed between the total alkali-extractable HCA contents of the different barley varieties. Differences were also observed between subsequent harvesting years (results not shown). Possibly, smaller grains contain higher levels of HCA because of the higher surface-to-volume ratio leading to a higher percentage of outer layers. However, no correlation was found between the 1000 grain weight of different barley cultivars and their FA concentration (*29*). The HCA content in barley malt may depend not only on genotypic variations but also on environmental factors (growing environment, temperature stress, water excess, drought, solar radiation, etc.) and postharvest history. Both genotype, growing environment, and their interaction have been known to influence phenolic profiles of wheat and rye (*30*). Zupfer et al. (*29*) found phenolic acid concentrations to correlate well with agronomic factors. Variations in the steeping, germination, and kilning regime can partly explain the difference in HCA content for identical malt varieties originating from the two different malt houses. Steeping and germination time and conditions have been known to influence total FA levels in wheat grains (*31*). Fry et al. showed that the use of gibberellic acid to induce germination stimulates the synthesis of FA. They observed both an increase in soluble and macromolecule esterified FA levels in cell cultures of *Spinacia oleracea* L. (*32*). Maillard and Berset showed that the amount of insoluble bound HCA in barley malt is influenced by the kilning regime (*33*).

The release of 6.1–13.0 μg/g dry malt pCA and 42.4–70.2 μg/g dry malt FA from malted barley during Congress wort brewing corresponded to 0.70–1.53 ppm of pCA and 4.90–8.32 ppm of FA present in wort (either in free or in ester-bound form). The degree in variability in total alkali-extractable pCA and FA content in wort (22% and 13%, respectively) was higher than that in malt. During mashing, only 7.1–12.5% of total alkali-extractable FA in barley malt was released into wort (either in free or in ester-bound form), leaving the majority of esterified FA in the spent grain. Concerning the total alkali-extractable pCA in malt, only 2.3–5.3% was transferred to wort. No correlation was found between total alkali-extractable malt and total wort HCA content. This is probably due to variations in WEAX content and differences in AX-degrading enzyme activities between different barley malt cultivars. Woffenden et al. showed that, regardless of the kilning regime, also the initial moisture content of the green malt has an important effect on the level of FA in water extracts of barley malt (*34*).

Free versus Ester-Bound Hydroxycinnamic Acids in Wort. The combined activity of AX-hydrolyzing enzymes leads to the release of both free and AX-esterified HCA in wort. Only free HCA are available for decarboxylation by the yeast later in the brewing process. HPLC analysis of wort before and after alkaline hydrolysis permits determination of the amount of pCA and FA present in either free form or AX-ester-bound form (**Table 3**). Significant differences in both free and ester-bound pCA and FA levels between worts from nine barley malt varieties were observed. Especially concerning free pCA and FA levels, a high degree of variability was observed (25.7% and 31.0%, respectively). Levels of free phenolic acids in wort varied from 0.95 to 3.45 ppm for FA and from 0.53 to 1.20

Table 3. Release of Free and Ester-Bound pCA and FA in Congress Wort from Nine Malted Barley Varieties (ppm)[a]

	free		ester-bound		% free	
barley malt	pCA (ppm)	FA (ppm)	pCA (ppm)	FA (ppm)	pCA (%)	FA (%)
Astoria A	0.88 ± 0.03 d	3.12 ± 0.04 e	0.31 ± 0.08 a,b	4.13 ± 0.45 a,b	74.0	43.1
Scarlett A	1.10 ± 0.03 e	2.23 ± 0.09 c,d	0.44 ± 0.09 b	5.31 ± 0.26 c	71.5	29.5
Pasadena A	0.53 ± 0.04 a	0.95 ± 0.19 a	0.17 ± 0.05 a	3.95 ± 0.25 a	75.7	19.3
Optic A	0.54 ± 0.05 a	1.81 ± 0.06 b	0.46 ± 0.09 a,b	4.71 ± 0.11 b	53.7	27.8
Prestige A	0.76 ± 0.02 c	2.46 ± 0.13 d	0.30 ± 0.06 a,b	4.48 ± 0.18 a,b	71.2	35.5
Esterel A	0.67 ± 0.01 b	1.83 ± 0.17 b	0.17 ± 0.09 a	5.73 ± 0.17 d	79.3	24.3
Scarlett B	1.20 ± 0.05 f	3.45 ± 0.26 e	0.29 ± 0.10 a,b	4.87 ± 0.32 b,c	80.8	41.5
Optic B	0.73 ± 0.03 c	2.35 ± 0.08 c	0.50 ± 0.12 a,b	4.43 ± 0.28 a,b	59.7	34.7
Prestige B	0.78 ± 0.07 c,d	2.05 ± 0.19 b,c	0.48 ± 0.10 a,b	4.55 ± 0.30 a,b,c	62.2	31.0

[a] Results are expressed as mean ± standard deviation. Values with no common letters differ significantly at the 95% confidence level.

Table 4. Water-Extracted versus Enzymatically Released Free pCA and FA in Congress Wort from Nine Malted Barley Varieties (ppm)[a]

	water-extracted		enzyme-released		% enzyme	
barley malt	pCA (ppm)	FA (ppm)	pCA (ppm)	FA (ppm)	pCA (%)	FA (%)
Astoria A	0.33 ± 0.05 a,b,c	0.73 ± 0.04 b	0.55 ± 0.06 c	2.39 ± 0.06 e	62.6	76.5
Scarlett A	0.38 ± 0.05 b	0.67 ± 0.09 a,b	0.72 ± 0.05 d	1.55 ± 0.05 c,d	65.3	69.8
Pasadena A	0.24 ± 0.08 a,b	0.51 ± 0.04 a	0.29 ± 0.09 a,b	0.43 ± 0.09 a	54.7	45.7
Optic A	0.29 ± 0.01 a	0.69 ± 0.01 b	0.25 ± 0.05 a	1.12 ± 0.05 a,b	47.0	61.9
Prestige A	0.27 ± 0.08 a,b,c	0.64 ± 0.07 a,b	0.49 ± 0.08 b,c	1.82 ± 0.08 d	64.6	73.9
Esterel A	0.47 ± 0.01 d	0.94 ± 0.03 c	0.19 ± 0.01 a	0.90 ± 0.01 a,b	28.6	48.9
Scarlett B	0.39 ± 0.02 c	0.53 ± 0.02 a	0.81 ± 0.05 d	2.92 ± 0.05 f	67.6	84.6
Optic B	0.30 ± 0.04 a,b,c	0.75 ± 0.09 b	0.43 ± 0.04 b	1.60 ± 0.04 c,d	58.7	68.1
Prestige B	0.29 ± 0.02 a	0.78 ± 0.11 b	0.49 ± 0.07 b,c	1.26 ± 0.07 b,c	62.9	61.8

[a] Results are expressed as mean ± standard deviation. Values with no common letters differ significantly at the 95% confidence level.

ppm for pCA. The majority of pCA occurred in free form (53.7–80.8% of total wort pCA) whereas FA is mainly ester-bound to AX as only 19.3–43.1% of total wort FA occurred in free form. However, because total wort FA levels were higher than total pCA concentrations, FA is the most abundant free phenolic acid in wort.

Water-Extracted versus Enzymatically Released Free HCA in Wort. The amount of free pCA and FA was determined both in normal Congress wort and in wort produced according to the Congress wort procedure but using malts without enzyme activity. HPLC analysis of both worts allows discrimination between the water-extracted and the enzymatically solubilized free HCA. The results are represented in **Table 4**. Of total free wort pCA, 28.6−67.6% was enzymatically released compared to 48.9−84.6% for FA. This corresponded to wort levels of 0.19–0.81 ppm of pCA and 0.43–2.92 ppm of FA, which were enzymatically released from AX-esterified forms by cinnamoyl esterase activity during mashing. Water-extracted free FA levels in wort ranged between 0.51 and 0.94 ppm corresponding to 4.08–7.52 μg/g of free FA in barley malt. These values were higher than the free FA levels found in water extracts of unmalted barley (2.51–3.87 μg/g) by Zhao et al. (*35*). Samaras et al. (*36*) also found higher free FA concentrations in pale malt and lager malt than in unmalted barley. Probably this is due to cinnamoyl esterase activity during the germination and the first stages of the green malt kilning.

In summary, the distribution of FA between malt, wort, and spent grain and between free and bound forms is represented in **Figure 1**. Phenolic acids are covalently linked to various biopolymers (e.g., AX and lignin) in the plant cell wall by both ester and ether bonds. However, since no enzymes are known to act on this ether-bound fraction and chemical hydrolysis of these linkages requires harsh conditions, ether-bound FA present in malt will be integrally transferred to the spent grains. On the contrary, FA present in free form in malt will be water-extracted during mashing and passed on into the wort. Concerning the

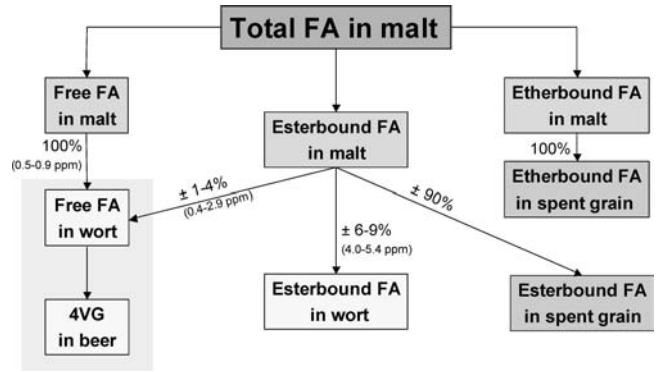

Figure 1. Fractionation of FA during mashing: distribution of free, ester-bound, and ether-bound FA in wort and spent grain.

fraction of FA, which is ester-bound to AX, almost 90% will end up into the spent grain. On average, 10% of ester-bound FA in malt will be transferred to wort: 6–9% remains ester-bound to water-extracted or solubilized AX and 1–4% is being released by malt cinnamoyl esterase activity into its free form. The combined fraction of both the water-extracted and the enzymatically released free FA can be converted to 4VG during wort boiling or fermentation.

Ester-Bound HCA and AX in Wort. In **Table 5**, wort xylose levels are presented together with the total amount of pCA and FA initially released in wort in ester-bound form. The latter can be calculated as the difference between the total wort HCA content and the water-extracted HCA content or as the sum of the enzymatically released HCA (present in free form in wort) and the final ester-bound HCA level present in wort after mashing. Good correlations can be found between wort xylose levels and these ester-bound pCA and FA levels. Pearson's correlation coefficients (r) were 0.76, 0.95, and 0.98 for pCA, FA, and their sum, respectively. Hence, a clear correlation between (either water-extracted or solubilized) wort AX and HCA being released in ester-bound form into the wort

Table 5. Xylose Content (g/L) and Initially Ester-Bound pCA and FA Content (ppm) in Congress Wort Made with Nine Malted Barley Varieties[a]

barley malt	xylose AX (g/L)	initially ester-bound pCA (ppm)	initially ester-bound FA (ppm)
Astoria A	0.629 ± 0.032	0.86 ± 0.09	6.51 ± 0.45
Scarlett A	0.766 ± 0.031	1.15 ± 0.10	6.87 ± 0.26
Pasadena A	0.452 ± 0.063	0.46 ± 0.09	4.39 ± 0.15
Optic A	0.575 ± 0.006	0.72 ± 0.07	5.83 ± 0.09
Prestige A	0.552 ± 0.008	0.79 ± 0.10	6.29 ± 0.15
Esterel A	0.585 ± 0.014	0.36 ± 0.09	6.62 ± 0.05
Scarlett B	0.846 ± 0.015	1.11 ± 0.09	7.79 ± 0.19
Optic B	0.605 ± 0.064	0.89 ± 0.12	6.04 ± 0.28
Prestige B	0.626 ± 0.076	0.97 ± 0.08	5.81 ± 0.26

[a] Results are expressed as mean ± standard deviation.

Table 6. AX Xyl:pCA and Xyl:FA Levels before and after Cinnamoyl Esterase Activity in Congress Wort from Nine Malted Barley Varieties

	start		end	
barley malt	Xyl:pCA	Xyl:FA	Xyl:pCA	Xyl:FA
Astoria A	797	125	2211	197
Scarlett A	679	135	1792	174
Pasadena A	834	104	2256	115
Optic A	878	128	1356	158
Prestige A	900	134	2341	189
Esterel A	(1760)[a]	114	(3689)[a]	132
Scarlett B	834	141	3241	225
Optic B	742	130	1334	177
Prestige B	707	139	1438	178
av ± SD	796 ± 80	128 ± 12	1996 ± 653	172 ± 33
variability (%)	10	9	33	19

[a] Outlier; result omitted from calculation.

can be found. From the amount of xylose present in the wort and either the total amount of pCA and FA being initially released into the wort in ester-bound form or the amount of ester-bound pCA and FA present in wort at the end of the mashing process, the Xyl:pCA and Xyl:FA ratios of AX before and after cinnamoyl esterase action can be calculated, respectively. The results are presented in **Table 6**. Esterel, the only six-row barley variety included in this study, had outlier values for pCA and was excluded from the calculations. Initially, one pCA and one FA molecule was bound every 796 and 128 xylose residues, respectively. Hence, the larger amount of FA being transferred to wort in ester-bound form compared to pCA is due to FA being bound in higher quantities to AX than pCA. The variabilities between the Xyl:pCA and Xyl:FA ratios of AX released from different malt varieties (10% and 9%, respectively) are quite low, indicating that, initially, AX released from different barley malt varieties were quite similar in structure. At the end of the mashing process, the Xyl:pCA and the Xyl:FA ratios were significantly higher (1996 and 172, respectively) due to the action of the cinnamoyl esterase enzyme. This corresponds with, on average, 60% of ester-bound pCA and 23.5% of ester-bound FA in wort being hydrolyzed. The increased values of the variabilities of both ratios (33% and 19%, respectively) reflect distinct variations in enzyme activities between the different malt varieties.

Release of HCA from Their Methyl Esters by Barley Malt Extracts. Cinnamoyl esterase activity of three barley malt (Astoria A, Scarlett A, and Optic B) extracts was assessed both on MpCA, MFA, and MSA separately and on the three methyl esters together. Results are represented in **Table 7**. When the enzyme activity was measured against each of the three methyl esters separately, the activity was the highest on MFA, followed by MpCA, and then MSA for all three malt varieties corresponding with the results obtained by Bartolome et al. (*11*). However, when the three methyl esters were administered in the same assay, more pCA was being released followed by FA and SA. Apparently, once bound to the active site of the cinnamoyl esterase enzyme, the turnover of ester-bound FA was faster than that of ester-bound pCA and SA. However, it seems that pCA was the substrate being preferentially bound by the active site of the enzyme compared to FA when both substrates are present together. This can explain why pCA is the HCA being preferentially released from its bound forms during mashing (60% compared to 23.5% for FA).

Table 7. Release of HCA (EU/g of Malt) by Barley Malt Extracts: (A) MpCA, MFA, and MSA Administered Separately (80 mM in Stock Solution) and (B) MpCA, MFA, and MSA Administered Together (3 × 26.7 mM in Stock Solution)[a]

barley malt	pCA	FA	SA
	Part A		
Astoria A	1.41 ± 0.11	1.76 ± 0.06	0.74 ± 0.03
Scarlett A	0.65 ± 0.02	1.04 ± 0.05	0.51 ± 0.02
Optic B	1.55 ± 0.03	2.18 ± 0.05	0.94 ± 0.02
	Part B		
Astoria A	0.52 ± 0.03	0.36 ± 0.04	0.14 ± 0.01
Scarlett A	0.37 ± 0.01	0.26 ± 0.01	0.16 ± 0.01
Optic B	0.98 ± 0.06	0.58 ± 0.03	0.30 ± 0.01

[a] Results are expressed as mean ± standard deviation.

Activity of AX-Degrading Enzymes in Barley Malt Extracts. The activity of four AX degrading enzymes [endo-β(1–4)-xylanase, β-D-xylosidase, α-L-arabinofuranosidase, and cinnamoyl esterase] in barley malt extracts is represented in **Table 8**. Apart from the clear differences in enzyme activity between the nine malted barley varieties, no correlation could be found between any pair of the four enzyme activities ($r_{xy} < 0.63$ for each combination). This is probably due to the fact that the malting process has a different impact on the level of each enzyme. While malt is considerably more active in endoxylanase activity than the corresponding barley cultivar, it is as active in arabinofuranosidase and less active in xylosidase activity (*37*). Barley cinnamoyl esterase activity is known to decrease during germination (*10*), and since it is relatively heat labile (*12*, *13*), it will probably decrease during kilning too.

Factors Influencing the Release of FA in Wort: Multivariate Data Analysis. Pearson's correlation coefficients were used to evaluate the relative significance of the malt parameters on the release of free FA in wort. Both enzymatically released FA and water-extracted FA were taken into consideration (**Table 4**). As explanatory variables, the activities of the four AX-degrading enzymes (**Table 8**), degrees of modification and extract content (**Table 1**), total malt FA (table **2**), and total ester-bound FA content being released into wort (**Table 5**) were included in this study. Results are shown in **Table 9**. There were no significant correlations between the amount of water-extracted FA and any of the malt parameters under investigation ($p > 0.05$). Probably the water-extracted FA levels depend on other characteristics such as environmental conditions, barley growing regime, harvesting conditions, and specific processes during barley germination and kilning. On the contrary, better relationships were obtained when the enzymatically released FA was considered. The endoxylanase activity, esterase activity, and total ester-bound FA levels significantly correlated to the amount of enzymatically released FA in wort at a 95% confidence level. No significant correlation between the release of FA and the extract content, the degree of modification, and the xylosidase and arabinofuranosidase activities of the different barley malt varieties was observed.

Table 8. AX Degrading Activities in Barley Malt Extracts[a]

barley malt	endo-β(1–4)-xylanase (ΔA/g of malt)	β-D-xylosidase (EU/g of malt)	α-L-arabinofuranosidase (EU/g of malt)	cinnamoyl esterase (EU/g of malt)
Astoria A	0.430 ± 0.005	0.213 ± 0.008	0.087 ± 0.003	2.72 ± 0.10
Scarlett A	0.310 ± 0.004	0.230 ± 0.004	0.047 ± 0.009	2.17 ± 0.15
Pasadena A	0.313 ± 0.001	0.162 ± 0.012	0.046 ± 0.004	1.99 ± 0.28
Optic A	0.320 ± 0.023	0.211 ± 0.002	0.086 ± 0.001	2.57 ± 0.21
Prestige A	0.394 ± 0.008	0.192 ± 0.004	0.082 ± 0.004	2.61 ± 0.09
Esterel A	0.283 ± 0.006	0.217 ± 0.004	0.078 ± 0.005	1.74 ± 0.14
Scarlett B	0.468 ± 0.004	0.265 ± 0.012	0.096 ± 0.005	3.34 ± 0.17
Optic B	0.292 ± 0.007	0.232 ± 0.008	0.067 ± 0.002	3.12 ± 0.16
Prestige B	0.388 ± 0.002	0.247 ± 0.007	0.073 ± 0.017	2.46 ± 0.10

[a] Results are expressed as mean ± standard deviation.

Table 9. Pearson's Correlation Coefficients (*r*) between the Amount of Enzymatically Released and the Amount of Water-Extracted FA and Several Malt Parameters

malt parameters	water-extracted FA	enzyme-released FA[a]
endo-β(1–4)-xylanase	−0.392	0.808*
cinnamoyl esterase	−0.356	0.787*
α-L-arabinofuranosidase	0.153	0.625
β-D-xylosidase	0.208	0.615
deg of modification	−0.219	−0.174
deg of extract	−0.253	−0.345
total malt FA	0.244	−0.328
total ester-bound FA	0.126	0.798*

[a] Values with an asterisk (*) are significantly different from 0 with a significance level of $\alpha = 0.05$.

A stepwise MLR analysis was applied to develop a model explaining which factors influence the amount of enzymatically released FA. Corresponding with the previous calculations, three parameters have a regression coefficient in the model that is significantly different from zero (endoxylanase activity $p = 0.036$; esterase activity $p = 0.038$; total ester-bound FA $p = 0.010$). The following function was obtained:

$$[\text{enzymatically released FA}] = -3.65 + 4.22[\text{endoxylanase}] + 0.53[\text{cinnamoyl esterase}] + 0.38[\text{total ester-bound FA}]$$

The MLR is statistically significant with a degree of significance $p = 0.001$ explaining 92% of the variation of the amount of FA released (adjusted R^2 taking into account the number of independent variables included in the model) and has a standard error of estimate (root mean squared error) of 0.21. The correlation between the observed and the calculated values of the amount of enzymatically released FA is depicted in **Figure 2**. Three new, randomly selected commercial malts were used to validate the model. Endoxylanase, esterase, and total ester-bound FA levels were determined, and the theoretically calculated FA content was compared with the measured value. All three had observed values within the 95% confidence interval of the model (**Figure 2**). The model suggests that it is not only the activity of the cinnamoyl esterase itself that affects the amount of FA being released into the wort from its ester-bound forms. Also, the amount of ester-bound FA present in the wort and the endoxylanase activity turn out to be important factors. The former represents the substrate of the cinnamoyl esterase enzyme. The latter can affect both the amount of esterase substrate (xylanase-solubilizing activity) and the molecular weight of the AX polymers (xylanase-depolymerizing activity). Both effects were mimicked by addition of isolated barley malt WEAX (untreated and pretreated with *T. viride* xylanase) during wort production. Endoxylanase activity enhancing FA release was already shown by the addition of exogenous microbial AX-degrading enzymes to barley spent grain (*11*) and wort (*15*). It has also already been shown that native barley endoxylanase activity enhances the release of FA from brewers' spent grain, combining extracts from barley and germinated barley (*38*).

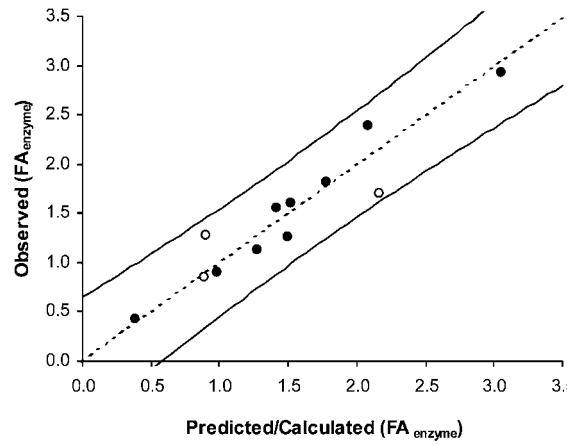

Figure 2. Comparison between observed and calculated enzymatically released FA levels in wort (●) and between observed and predicted enzymatically released FA levels in wort (○) (ppm).

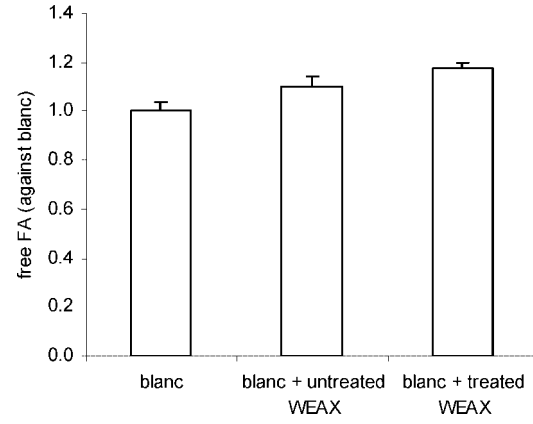

Figure 3. Release of FA (relative concentration versus blank) during wort production: influence of the addition of isolated barley malt WEAX and pretreatment with endoxylanase from *T. viride*.

Addition of Isolated Barley Malt WEAX during Wort Production. To increase the amount of ester-bound FA during wort production, isolated WEAX (corresponding with an increase of 30% of ester-bound FA initially present in wort) were added at the start of the mashing-in process. Increasing the amount of ester-bound FA leads to an additional release of FA of 10.2% (**Figure 3**). This demonstrates the dose–response relationship between the amount of FA released by cinnamoyl esterase activity and the amount of ester-bound FA present in wort, i.e., the substrate of the cinnamoyl esterase enzyme. To evaluate the effect of endoxylanase activity on the enzymatic

release of FA during mashing, an aliquot of the WEAX preparation pretreated with endoxylanase was added during mashing. When the WEAX added to the wort were pretreated with endoxylanase from *T. viride* to lower the molecular weight of the AX polymers, an increase in FA release of 17.7% versus the blank sample was seen (**Figure 3**). Reducing the molecular weight of the AX polymers generates more suitable esterase substrates by reducing the steric hindrance and enhancing the access of the cinnamoyl esterase to ester-bound HCA. Both experiments show the positive correlation between the amount and the molecular weight of the esterase substrate and amount of FA released during brewing.

In conclusion, a large variability in HCA content between different barley malt varieties and their corresponding worts was observed. Differences were also found between free FA levels from identical malt varieties originating from different malt houses. This demonstrates the importance of selecting a suitable malt variety as the first means of controlling the final volatile phenol levels in beer. It was shown that only a small part of the HCA in malt is transferred to wort during mashing, the lion share remaining in the spent grains. Free HCA in wort are both water-extracted and enzymatically released by cinnamoyl esterase activity. This esterase activity is reflected in the decrease of the Xyl:FA and Xyl:pCA ratios during mashing. Esterase activities clearly differ between different barley malt varieties, as do other AX-degrading enzyme activities. The substrate specificity of the esterase versus HCA methyl esters reflects the release of HCA during mashing. MLR was used to define the analytical data, which mostly discriminated the release of FA during mashing, and to reduce the number of variables under consideration. MLR analysis suggested that the enzymatic release of FA during mashing depends not only on the esterase activity but also on the amount of AX ester-bound FA initially present in the wort and on the endoxylanase activity of the barley malt. This was confirmed by the addition of isolated WEAX pretreated with *T. viride* xylanase.

ABBREVIATIONS USED

4VG, 4-vinylguaiacol; 4VP, 4-vinylphenol; POF, phenolic off-flavor; pCA, *p*-coumaric acid; FA, ferulic acid; HCA, hydroxycinnamic acid; AX, arabinoxylan; WEAX, water-extractable arabinoxylan; MLR, multiple linear regression.

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