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Effect of Modification of the Kilning Regimen on Levels of Free Ferulic Acid and Antioxidant Activity in Malt

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ABSTRACT: Barley phenolic antioxidants change in response to the kilning regimen used to prepare malt. Green malt was kilned using four different regimens. There were no major differences among the finished malts in parameters routinely used by the malting industry, including, moisture, color, and diastatic activity. Ferulic acid esterase activity and free ferulic acid were higher in malts subjected to the coolest kilning regimen, but malt ethyl acetate extracts (containing ferulic acid) contributed only ~5% of the total malt antioxidant activity. Finished malt from the hottest kilning regimen possessed the highest antioxidant activity, attributed to higher levels of Maillard reaction products. Modifying kilning conditions leads to changes in release of bound ferulic acid and antioxidant activity with potential beneficial effects on flavor stability in malt and beer.

KEYWORDS: antioxidant activity, ferulic acid, kilning conditions, malt, Maillard reaction

INTRODUCTION

Flavor instability due to volatile aldehyde production and resulting in product deterioration is a major problem for the brewing industry. Therefore, maintaining antioxidant activity throughout brewing is important to limit off-flavor development in beer.[1] Compounds possessing antioxidant activity that are present in barley include phenolic species. These are released during malting and mashing and transferred to the final product, so preservation of naturally occurring antioxidants of barley is of great interest.[2] Reductones and melanoidins that possess reducing power are generated during kilning by the Maillard reaction,[3] and optimization of their levels in beer is also worthy of study. Our previous studies[4,5] have indicated that levels of free phenolic compounds possessing antioxidant activity increase during the kilning stage of malting. Thus, the ability to manipulate the kilning process to enhance the level of naturally occurring compounds containing antioxidant activity is of interest.

Barley phenolics have been detected in both the free and bound forms[6] and are generally located in the husk, pericarp, testa, and aleurone layers of the grain.[7] Their level and profile in barley depend upon both variety and growing conditions.[8] About 80% of the phenolics in beer originate from the malt,[6] the most common representatives being catechin, epicatechin, and ferulic, vanillic, and *p*-coumaric acids.[9]

Ferulic acid, the most abundant phenolic acid in barley and malt,[10] is an effective antioxidant.[11] In barley, it is concentrated mainly in the aleurone layer, in the husk, and in the endosperm[12] and exists in barley and malt in both free and bound forms and in both monomer and oligomer forms.[13] Ferulic acid is esterified to the C_5-hydroxyl group of α-L-arabinofuranosyl subunits of the xylan backbone. Cross-linking of ferulates can lead to the formation of dimers and oligomers, which are important structural components of cell walls because they increase mechanical and thermal stabilities.[13]

Kilning can be divided into five stages, each involving different air temperatures, leading to changes in grain moisture content.[5] The end of the grain free-drying stage, when the drying front has reached the top of the grain bed, is known as the break-point. Levels of free ferulic acid have been shown to double during kilning and then increase during mashing.[14] Hydrolytic enzymes, such as esterases, can lead to the release of bound phenolic compounds associated with lignin and arabinoxylans.[15] Kilning leads to more friable tissue and allows better extraction of the phenolic acids.[9]

The rate and extent of the release of ferulic acid, by ferulic acid esterase (FAE), are increased in the presence of xylanases and arabinoxylanases, which hydrolyze the main chain.[11] Also, the presence of FAE increases the rate of degradation of arabinoxylans by xylanases.[15] FAE appears to release only a small proportion of the bound ferulic acid, but Moore et al.[15] indicated that FAE acted as a "solubilase", releasing both β-glucan and arabinoxylan for further breakdown by β-glucanase.

FAE is released during the germination and kilning steps of malting[12,14] and the mashing step of brewing.[16] Although all three steps are key to optimizing FAE activity and, thus, ferulic acid levels in malt and beer, the current study focused on kilning. The conditions required to maximize enzymatic release of bound phenolics throughout kilning have not been reported.[2] Although there is a recent report on optimization of the enzymatic extraction of ferulic acid from wheat bran,[17] there have been no studies on the effect of modifying kilning conditions on the extractability of phenolic acids from malt. FAE is active at 45−65 °C,[18] a temperature range encountered during kilning. Therefore, the aim of this study was to determine the effect of kilning conditions on free ferulic acid concentration and antioxidant activity by analyzing malts produced by different kilning regimens.

EXPERIMENTAL PROCEDURES

Materials. HPLC grade water and methanol were from Fisher Scientific (Leicester, U.K.). High-purity water from a Purite (High Wycombe, U.K.)

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Table 1. Kilning Conditions Used for Production of SKR and MKR Malts[a]

	kilning time (h)		total kilning time (h)		air-on temperature (°C)				air-off temperature[b] (°C)			
kilning stage	SKR	MKR	SKR	MKR	SKR	MKR(45)	MKR(55)	MKR(65)	SKR	MKR(45)	MKR(55)	MKR(65)
1	8	8	8	8	60	60	60	60	20−26	20−26	20−25	20−27
2	2	2	10	10	60	60	60	60	26−28	26−27	25−25	27−27
3	2	2	12	12	65	65	65	65	28−41	27−34	25−33	27−36
4	2	2	14[c]	14[c]	70	70	70	70	41−54	34−35	33−45	36−46
5	*d*	2.5	*d*	16.5	*d*	45	55	65	*d*	45−43	45−52	46−63
6	1	1	15	17.5	80	80	80	80	54−52	43−43	52−50	63−60
7	1	1	16	18.5	85	85	85	85	52−61	43−46	50−58	60−68
8	1	1	17	19.5	90	90	90	90	61−70	46−66	58−68	68−73
9	2	2	19	21.5	95	95	95	95	90−85	66−83	68−82	73−86
10	5	5	24	26.5	105	105	105	105	85−100	83−99	82−97	86−99

[a] SKR, standard kilning regimen; MKR, modified kilning regimen; MKR(45), modified kilning regimen with a stabilization hold at 45 °C; MKR(55), modified kilning regimen with a stabilization hold at 55 °C; MKR(65), modified kilning regimen with a stabilization hold at 65 °C. [b] The range of air-off temperatures shows the difference from the beginning to the end of the kilning stage as the grain heats up throughout the stage. [c] Break-point occurred at 14 h. [d] Kilning stage 5 was omitted for SKR malt.

Labwater R050 unit was used for all chemical analyses. Glacial acetic acid, (+)-catechin, vanillic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, sinapic acid, and ferulic acid (all >99%), 2,4,6-tripyridyl-*s*-triazine (TPTZ), ferric chloride, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were from Sigma (Poole, U.K.). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride was from Aldrich (Gillingham, U.K.). Barley, variety Optic (2002 harvest), was supplied by Campden BRI (Nutfield, U.K.).

Malting. Green malt was prepared from Pearl barley in the 50 kg pilot malting plant at Campden BRI. Barley (50 kg) was steeped by employing a three-wet protocol (water 200 L, at 16 °C per steep) divided by three air-rests. The steeping regimen was 8 h wet/14 h air-rest, 5 h wet/18 h air- rest, and 6 h wet/2 h air-rest. The temperature within the steep/germination vessel rose from 16 to 17 °C over the steeping period. The grains were allowed to germinate for 5 days, during which time the grain temperature rose from 17 to 18 °C. Germination was arrested by kilning. A separate batch of green malt was prepared under identical conditions at Campden BRI for each kilning trial, using barley from the same bulk container.

Kilning. Kilning was undertaken in the pilot plant kilns (Redler Ltd., Stroud, U.K.) at BRI, using different regimens, that is, a standard kilning regimen (SKR) and three different modified kilning regimens (MKRs) (Table 1).

Standard Kilning Regimen. The SKR used no air recirculation in the initial three stages of kilning (up to 12 h). Sixty percent recirculated air was used in the last 2 h of the prebreak period, prior to break-point (14 h). As the relative humidity (RH) of the kiln process air above the malt bed fell (air-off), the amount of air recirculation was increased from 60 to 80% by the end of kilning, and a final grain temperature of 95 °C was achieved.

Manipulated Kilning Regimens. MKRs were achieved by manipulating the percent recirculation, fan speed, and air-on temperature to allow the kiln bed to stabilize post break-point at set temperatures of 45, 55, and 65 °C, denoted MKR(45), MKR(55), and MKR(65). The grain bed was held under these conditions for 2 h (14.5−16.5 h kilning time) before the kilning regimen was resumed and completed (Table 1).

Sampling. Samples (50 g) were taken from both parallel sampling ports (front and back of the kiln), at the top, middle, and bottom of the grain bed, at monitored grain temperatures throughout kilning. Samples were taken at the beginning and after 1 and 2 h of stage 5 for the MKR (the stabilization stage at which the grain bed temperature was maintained at 45, 55, or 65 °C). Larger samples of malt (1 kg) were taken at the end of kilning from the top, middle, and bottom of the kiln. After discharge of the kiln into a bulk container, the malt was derooted, to remove rootlets produced during germination. The derooter also blended the finished malt to produce a homogeneous sample. A sample of blended grain was taken after derooting.

Routine Malt Analysis. Blended finished malts were analyzed using the following methods that are routinely applied in the malting industry,[19] unless otherwise specified. The moisture content of malt was obtained by finely grinding malt and drying at 105 °C for 3 h according to European Brewery Convention (EBC) method 4.2.[19] Material extracted by hot water from finely ground hot water extract prepared using fine-ground malt (passes through a 0.2 mm mesh) (HWE_2) and coarsely ground malt (passes through a 0.7 mm mesh) (HWE_7) malt was determined (EBC 4.6).[19] The color of the HWE_7 was measured using a Lovibond Color Comparator (measured in EBC units) (EBC 4.7.2).[19] The total nitrogen content of dry malt was measured using the Dumas method (EBC 4.3.2),[19] and total soluble nitrogen was measured using HWE_7 (EBC 4.9.3).[19] Free amino nitrogen was measured colorimetrically at 570 nm (EBC 4.10).[19] pH and viscosity were determined using HWE_7 (EBC 8.17 and EBC 4.8).[19] Friability of malt was measured using a Friabilimeter (EBC 4.15).[19] Diastatic power was determined and expressed in IOB units.[20]

Ferulic Acid Esterase Activity. The method was based on that of Sancho et al.[12] Malt samples were taken from the top, middle, and bottom of the kiln at the beginning of the stabilization period at 45, 55, and 65 °C (14.5 h). Malt extracts were prepared by grinding malt (5 g) in a coffee grinder (3 × 10 s), and the ground malt (1 g) was homogenized in 3-(*N*-morpholino)propanesulfonic acid (MOPS, 0.1 M) extraction buffer (10 mL) for 1 min. The resulting slurry was centrifuged, filtered, and retained for analysis.

For the assay, methyl ferulate (4 mM, 1 mL), extraction buffer (2 mL), and sample filtrate (1 mL) were mixed together and incubated at 30 °C for 20 min. Sample blanks contained additional extraction buffer in place of the sample filtrate. Free ferulic acid, and any residual methyl ferulate, were extracted with ethyl acetate. Extracts were centrifuged, and the organic phase was removed by aspiration. The organic phase was dried (<35 °C), redissolved (methanol/water, 1:1, v/v, 0.5 mL), filtered, and analyzed by HPLC.

HPLC analysis of ferulic acid was based on the method of Waldron et al.,[21] but adapted for an Aquasep 5 μM 100 A°, 15 cm × 2.0 mm C_{18}, reverse phase HPLC column (ES Industries, West Berlin, NJ), fitted with a Spheroclone 5 μm (ODS 2) precolumn (Phenomenex, Macclesfield, U.K.). Solvent A was 1 mM aqueous TFA/acetonitrile (10:90, v/v), and solvent B was 1 mM aqueous TFA/acetonitrile/methanol (20:40:40, v/v). A two-step linear gradient was used running from 90:10 solvent A/solvent B to 25:75 solvent A/solvent B over 15 min followed by a change to 100%

Table 2. Effect of Kilning Regimen on Moisture Content (Percent) of Malt[a]

	kiln position in											
	SKR			MKR(45)			MKR(55)			MKR(65)		
grain temperature (°C)	top	middle	bottom	top	middle	bottom	top	middle	bottom	top	middle	bottom
green malt	43.8			44.0			45.1			43.8		
45[b]				6.9	6.4	5.6						
45[c]				7.0	5.9	5.5						
45[d]				6.5	5.9	5.5						
55	4.5	4.6	4.2	5.9	5.5	4.2	6.8	5.4	5.3			
55[b]							6.5	5.6	5.1			
55[c]							6.3	5.4	4.9			
55[d]							6.2	5.3	5.0			
65	4.5	3.7	3.1	5.3	4.6	3.8	5.7	4.9	4.4	6.5	5.8	5.4
65[b]										6.4	5.6	5.3
65[c]										6.1	5.6	5.1
65[d]										6.0	5.3	5.1
75	3.5	2.9	2.2	4.4	3.7	3.1	3.6	4.2	4.9	4.4	3.8	3.4
85	3.1	3.3	1.9	3.7	3.0	2.4	3.9	3.4	2.9	3.6	3.2	2.8
95	3.1	2.2	2.4	2.7	2.1	2.5	2.6	2.0	2.4	2.7	2.3	2.3
blended		2.7			2.5			2.6			2.3	

[a] Data are the mean of three determinations. Coefficient of variation < 5%. [b] Grain temperature at the beginning of the stabilization period in MKR. [c] Grain temperature after 1 h of stabilization in MKR. [d] Grain temperature after 2 h of stabilization in MKR.

solvent B at 20 min. The flow rate was 0.2 mL/min, and the injection volume was 20 μL. Separations were monitored at 320 nm. Spectra of all peaks were recorded between 200 and 600 nm. Extracts were analyzed in duplicate. Ferulic acid was identified by matching the spectra and retention times of peaks from the sample with that of the authentic compound run under the same HPLC conditions.

Preparation of Malt Extracts. Acetate buffer extracts were prepared as previously described.[22] Briefly, ground green malt (5 g) was homogenized in acetate buffer (50 mM, pH 5.4, 40 mL) for 2 min. The slurry was centrifuged (1500*g*, 10 min) and filtered (Whatman no. 1). Samples were stored frozen prior to antioxidant analysis.

Ethyl acetate extracts were prepared from acetate buffer extracts as described by Pascoe et al.[23] In brief, the acetate buffer extract was acidified and extracted with ethyl acetate (3 × 10 mL). The organic phase was centrifuged, dried, and vacuum filtered. The ethyl acetate fractions resulting from duplicate acetate buffer extracts were combined, and the solvent was removed. The residue was then dissolved in methanol (2 mL). Duplicate extracts were prepared for each malt sample. Methanolic extracts were stored at 4 °C in sealed brown vials, prior to analysis (within 1 month).

Antioxidant Activity. The antioxidant activity of the acetate buffer and ethyl acetate extracts of malt samples was determined by two methods. The $ABTS^{\bullet+}$ method was performed exactly as previously described.[22] In summary, the chromophore solution (3.125 μL) and catechin standard or sample extract (25 μL) were mixed and then incubated at 25 °C, and the absorbance was measured at 734 nm. The ferric-reducing antioxidant potential (FRAP) assay was performed as described by Benzie and Strain,[24] but modified according to Woffenden et al.[22] Sample extract (100 μL) and FRAP reagent TPTZ (3 mL) were mixed and incubated at 25 °C. After exactly 5 min from initial mixing, the absorbance of the reaction mix was taken at 593 nm. Catechin standard solutions and blanks (using acetate buffer in place of malt extract) were run for both assays. All methanolic extracts derived from ethyl acetate extractions were diluted 1:1 in acetate buffer (pH 5.4, 50 mM) prior to analysis. Each sample was analyzed in triplicate. Calibration curves were prepared using (+)-catechin.

HPLC Analysis of Ferulic Acid in Ethyl Acetate Extracts of Malt Samples. Separations were performed using a Hewlett-Packard (HP, now Agilent Technology, Bracknell, U.K.) model 1050 series II LC with a quaternary solvent delivery system and equipped with an autoinjector and a UV−visible diode array detector. System control and data acquisition and analysis were performed using HP Chemstation software. An ACE 5Q 250 × 4.6 mm i.d., 5 μm, column (HiChrom, Theale, U.K.), fitted with a 30 × 4.6 mm guard column (HiChrom), was used with gradient elution. Solvent A was water, and solvent B was methanol. Both solvents were adjusted to pH 2.3 using glacial acetic acid. Solvent B was increased linearly from 0 to 40% over 80 min. The flow rate was 1 mL/min, and the injection volume was 20 μL. Ferulic acid was monitored at 320 nm. Calibration curves for quantification were prepared from solutions of authentic ferulic acid. All ethyl acetate extract samples and standards were passed through a 0.45 μm filter prior to analysis. Components were identified by matching their retention times and UV diode array spectra to those of the authentic standard. Each sample was analyzed in triplicate.

Statistical Analysis. Statistical analysis was performed using Minitab 13.1 for Windows (Minitab Ltd., Coventry, U.K.). One-way analysis of variance (ANOVA) was used to determine if there were any statistically significant differences between the mean values. Tukey's test was then used to determine which mean values were different. A p value of <0.05 was considered to denote a statistically significant difference.

RESULTS

Temperature and Moisture Profiles during Processing. The moisture content of the green malt used for the different trials was 44−45% (Table 2). Steeping schedules, temperature profile, and duration of germination were similar, and so it was assumed that the green malts were all in the same condition prior to kilning.

The air-on and air-off temperatures and %RH conditions were virtually the same in each trial up to the break-point at 14 h (Table 1). The MKRs were successfully implemented by lowering the air-on temperature to the required level (45, 55, or 65 °C),

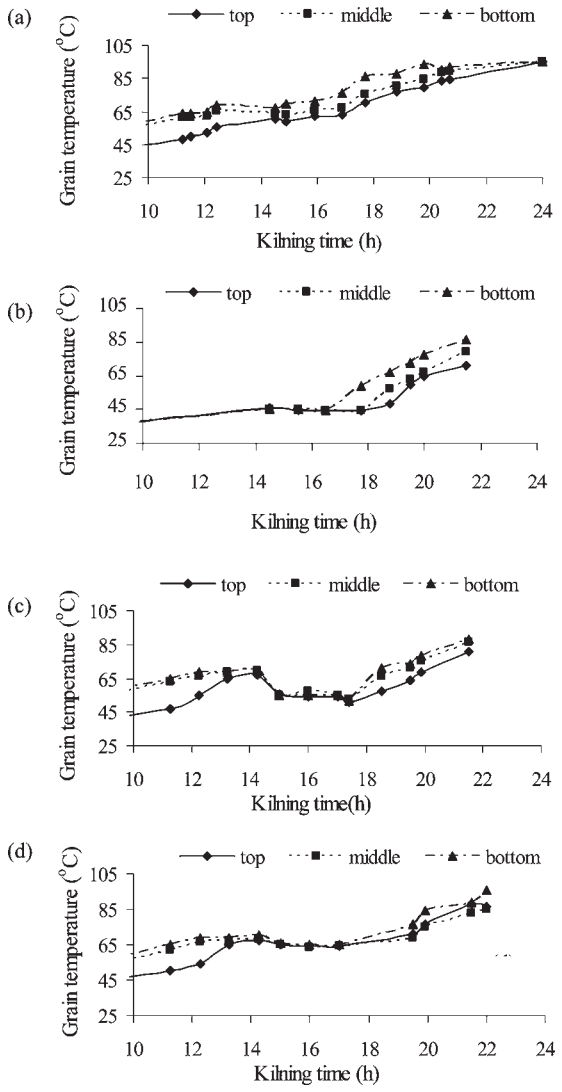

Figure 1. Grain temperatures at the top, middle, and bottom of the grain bed throughout the (a) SKR, (b) MKR(45), (c) MKR(55), and (d) MKR(65) trials. Break-point was at 14 h for all kilning regimens. No data were available in the initial 10 h of kilning for any of the trials. No data were available for the MKR(45) trial prior to the start of the stabilization hold (at 14.5 h).

introducing 100% air recirculation, and increasing the fan speed to 100%. Kiln stabilization began at the same time in all of the manipulated kilning regimens (14 h, end of stage 4) after the break-point was achieved. The grain bed temperature stabilized within 45 min and was maintained in the kiln for 2 h (14.5−16.5 h total kilning time) prior to resumption with planned increases in the air-on temperature and reductions in recirculation and fan speed.

As hot air was introduced at the base of the kiln, it absorbed moisture as it passed through the grain bed, creating a moisture gradient throughout the grain bed. In consequence, it exerted the greatest drying effect on the grains at the base of the kiln. The air-off temperature was <30 °C for the first 10 h of kilning for all kilning regimens (Table 1). In contrast, the grain temperature within the grain bed had already attained ~40 °C near the top of the kiln and ~57 °C at the bottom of the kiln by 10 h for all regimens (no data were available up to 14 h for MKR(45); Figure 1). The grain temperature throughout the kiln was >60 °C post-break-point in all

Table 3. Routine Malt Analysis of Finished Malts

analysis[a]	SKR	MKR(45)	MKR(55)	MKR(65)
moisture (%)	3.2	3.0	2.8	2.8
HWE_2 (L°/kg)	311	310	309	309
HWE_7 (L°/kg)	309	309	308	308
C/F (L°/kg)	2	1	1	1
color (°EBC)	5.2	5.0	5.2	5.2
TSN (%)	0.56	0.57	0.55	0.55
TN (%)	1.45	1.50	1.54	1.52
SNR (%)	39	38	36	36
FAN (%)	0.10	0.09	0.09	0.09
pH	5.86	5.78	5.82	5.85
viscosity (mPa s)	1.53	1.52	1.53	1.53
friability (%)	98	97	96	96
DP (°IOB)	55	56	57	58

[a] HWE_2, hot water extract prepared using fine-ground malt (passes through a 0.2 mm mesh); HWE_7, hot water extract prepared using coarse-ground malt (passes through a 0.7 mm mesh); C/F, difference between HWE_2 and HWE_7; TSN, total soluble nitrogen of HWE_7; TN, total nitrogen of malt; SNR, soluble nitrogen ratio (TSN/TN); FAN, free amino nitrogen; DP, diastatic power.

regimens and prior to kiln stabilization for MKR(45), MKR(55), and MKR(65) malts. Therefore, the grain bed was cooled to 45, 55, or 65 °C, by decreasing the air-on temperature to the desired stand temperature, by utilizing 100% fan speed and 100% recirculation to trap both air and moisture within the kiln while controlling heat input. The 100% recirculated air allowed redistribution of both moisture and temperature across the grain bed so that a homogeneous stand temperature was achieved within about 45 min. When the grain bed was held at the required temperature during the MKRs (Figure 1b−d), the temperature differential between the top and bottom of the grain bed was ≤2 °C. However, after the stabilization period, a greater temperature differential between the top and bottom of the grain bed was apparent (Figure 1b−d).

Malt Characteristics. Data for the blended finished malts obtained from each regimen using analytical tests routinely applied in the malting industry are shown in Table 3. The results indicate that manipulation of the kilning regimens did not result in major differences in any parameters routinely assessed in malts.

HPLC Analysis of Free Ferulic Acid in Ethyl Acetate Extracts. Levels of free ferulic acid were significantly higher in MKR(45) malts than those detected in samples obtained from the other kilning regimens (Figure 2). Two ferulic acid concentration maxima were detected or inferred in sample extracts from MKR(45). One was at 18.2 h, and a second was at 21.9 h. Similar maxima are also indicated by the data for the other regimens. No significant increases in free ferulic acid were observed throughout stabilization (14.5−16.5 h). In both the SKR and MKR(45) malts, the level of ferulic acid detected was significantly higher in the ethyl acetate extract of samples taken from the bottom of the kiln, compared to that determined in samples taken from the middle and top of the kiln, where the levels of ferulic acid were similar to one another. MKR(45) blended malt possessed a higher ferulic acid concentration (40 μg/mL) than the other blended samples (20−28 μg/mL).

Ferulic Acid Esterase Activity. FAE activity was defined as the concentration of ferulic acid (μg/mL) released from methyl ferulate in the assay under the stated analytical conditions. Base levels of ferulic acid (i.e., prior to incubation of the malt sample

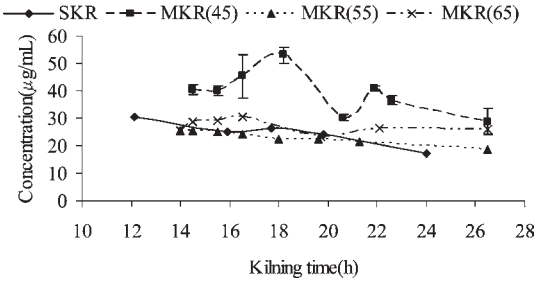

Figure 2. Changes in ferulic acid concentration in malts produced by different kilning regimens. Each data point represents the mean of duplicate analyses of each of two extracts. All samples were from the center of the kiln. Error bars represent ± one standard deviation. Note: the *x*-axis begins at 10 h to clarify the data.

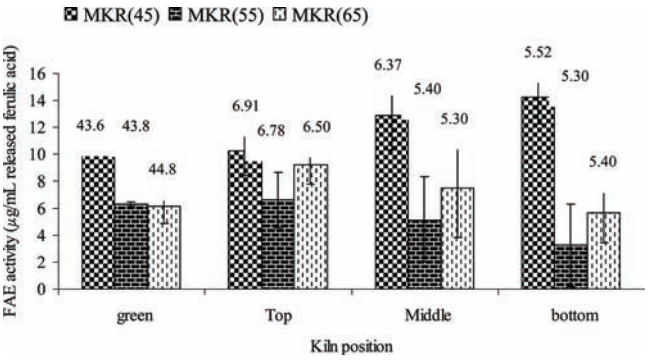

Figure 3. Activity of FAE in malt extracts at the beginning of the stabilization period. Figures represent the mean of triplicate analyses of duplicate extracts. Error bars represent ± one standard deviation. Figures above the bars represent moisture content (%).

filtrate with methyl ferulate in the FAE assay) varied in the samples taken throughout the kiln for the different MKRs, due to different temperature/time/moisture conditions (Figure 3). For MKR(45) malt, the base level of ferulic acid was highest at the top of the grain bed, where the grain had been coolest and most moist (6.9% moisture), and lowest at the bottom of the kiln, where the grain had reached temperatures of up to 57 °C. In contrast, the level of ferulic acid was highest in samples taken from the bottom of the kiln in MKR(55) and MKR(65). No SKR sample analysis was undertaken.

There were also differences in FAE activity in each of the regimens, depending upon the position of the sample in the grain bed (Figure 3). FAE activity increased in the order top < middle < bottom of the kiln for MKR(45) malt, but the order was bottom < middle < top for MKR(55) and MKR(65) malts. For example, compared to the base level (Table 4), there was a 24-fold increase to 14.22 μg/mL in ferulic acid released from methyl ferulate by the MKR(45) malt taken from the bottom of the kiln. This compares with a 10.7 μg/mL increase (5-fold) in the level of ferulic acid present in the green malt extract after incubation with methyl ferulate, indicating less FAE activity in the green malt than the malt taken from the bottom of the kiln at the beginning of the stabilization period. MKR(55) and MKR(65) malts taken from the top of the kiln were able to release more ferulic acid from methyl ferulate compared to the green malts used for their respective trials, but the increases were lower compared to the MKR(45) malt samples.

Table 4. Base Ferulic Acid Levels at the Start of the Stabilization Period[a]

	ferulic acid (μg/mL)		
malt	MKR(45)	MKR(55)	MKR(65)
green malt	2.1	0.2	1.2
top	2.8	3.4	2.3
middle	1.2	3.2	0.7
bottom	0.6	6.1	2.7

[a] Data are the mean of of triplicate analyses of duplicate extracts. Coefficient of variation < 50%.

The effect of moisture on FAE activity was different for MKR(45) malts compared to malts from the other two regimens (Figure 3). As the moisture content of MKR(45) samples decreased from top to bottom of the malt bed (Table 2), there was an increase in FAE activity. Malt from the bottom of the kiln that had received the most extreme heat treatment and which possessed the lowest moisture content (5.5%) exhibited the highest FAE activity. For MKR(55) and MKR(65) samples, as moisture content reduced down through the grain bed, due to the temperature gradient that developed during kilning, malt FAE activity decreased. However, there were no significant differences in moisture content between samples from the bottom and middle of the kiln for MKR(55) or MKR(65) and, as a consequence, no significant differences in FAE in the samples.

Antioxidant Activity. Finished malts prepared using all regimens showed an overall increase in antioxidant activity as the grain temperature increased and its moisture content decreased as a result of kilning, but the increases were not linear (Figure 4). Each regimen resulted in a unique pattern of antioxidant activity with kilning time.

There were no significant increases in antioxidant activity of the acetate buffer extracts of MKR malts taken from the center of the kiln throughout the stabilization phase (14.5−16.5 h kilning) as measured by both the $ABTS^{\bullet+}$ and FRAP assays (Figure 4a,b). The acetate buffer extract of the finished malt from MKR(65) demonstrated higher antioxidant activity than the other finished malts when measured using the FRAP assay, but no significant differences were detected using the $ABTS^{\bullet+}$ assay between the samples. For all regimens, samples of finished malt showed no difference in antioxidant activity with kiln position. Compared to the green malt used to prepare them, the blended finished malts showed the following increases in antioxidant activity using the $ABTS^{\bullet+}$ assay: SKR, 2-fold; MKR(45), 2-fold; MKR(55), 2.5-fold; and MKR(65), 1.8-fold. Increases in antioxidant activity determined by the FRAP assay were higher: SKR, 4-fold; MKR(45), 3-fold; MKR(55), 3.5-fold; and MKR(65), 3-fold.

There were increases in the antioxidant activity of the ethyl acetate extracts prepared from the MKR malts taken from the center of the kiln during the final hour of stabilization (15.5−16.5 h) detected using both antioxidant activity assays (Figure 4c,d), but increases were only significant for the MKR(55) and MKR(65) malts measured using the $ABTS^{\bullet+}$ assay. With the MKRs, there was an apparent maximum of antioxidant activity detectable in the ethyl acetate extracts as the grain temperature was increased to 55 °C in MKR(45) at 18.2 h and in MRK(55) at 16.5 h and to 65 °C for MKR(65) malt at 16.5 h. For the ethyl acetate extracts of all of these malts, the antioxidant activity then decreased as kilning progressed, followed by a further increase as the grain temperature increased to 95 °C at the end of kilning. Compared to finished malts from

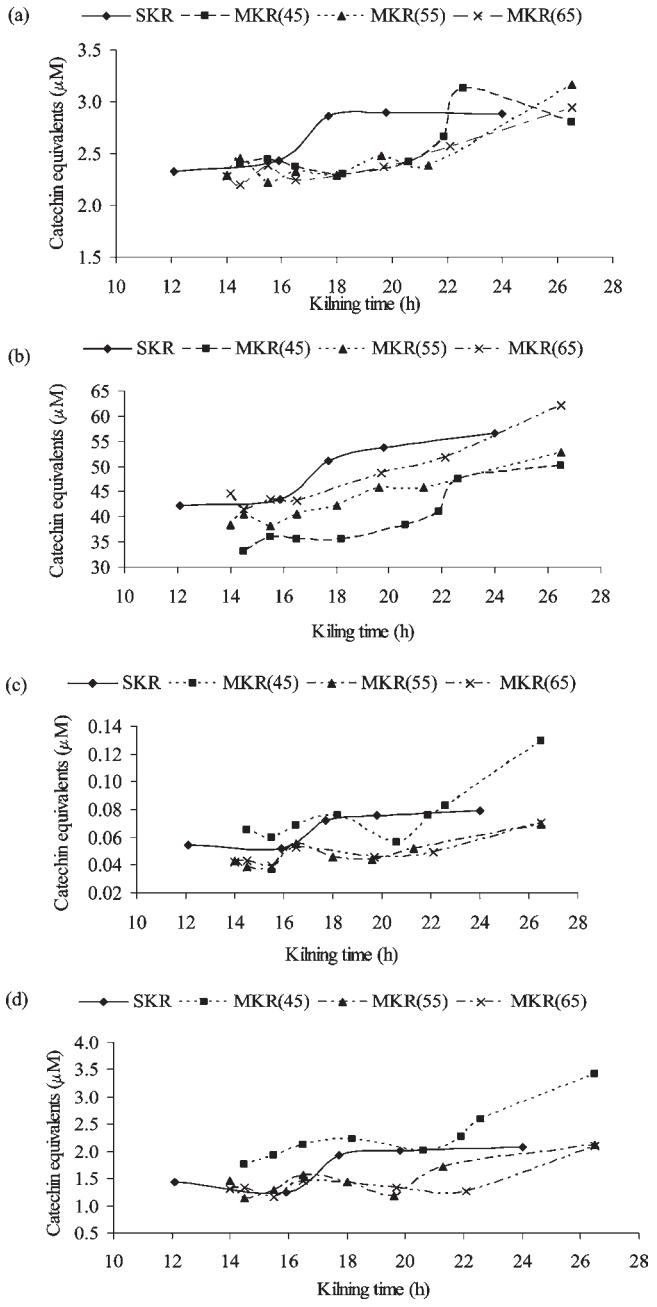

Figure 4. Comparison of the effect of kilning time on antioxidant activity: (a) acetate buffer extracts, $ABTS^{\bullet+}$ assay; (b) acetate buffer extracts, FRAP assay; (c) ethyl acetate extracts, $ABTS^{\bullet+}$ assay; (d) ethyl acetate extracts, FRAP assay. Data are the means of triplicate analyses of duplicate extracts. All samples were from the center of the kiln. The stabilization period was at 14.5−16.5 h. All results are based upon 1 g ground malt/L extract. The scale on the *x*-axis begins at 10 h and that on the *y*-axis at 1.5 μM catechin equivalents to clarify differences in data. Coefficient of variation was <10%.

SKR, MKR(55), and MKR(65), the ethyl acetate extract of the finished MKR(45) malt possessed significantly higher antioxidant activity as measured by both assays. In contrast to data obtained for the malt acetate buffer extracts, the $ABTS^{\bullet+}$ assay, but not the FRAP assay, could discriminate among ethyl acetate extracts of malt taken from different positions in the kiln (data not shown). The extract of MKR(45) malt taken from the top of the kiln possessed a significantly lower antioxidant activity (0.09 μM catechin equiv) than samples from the middle and bottom positions (0.13 μM catechin equiv). Compared to their green malts, the ethyl acetate extracts of the blended finished malts showed a 2-fold increase in antioxidant activity except for MKR(55) malt, which gave a 2.5-fold increase, when the $ABTS^{\bullet+}$ assay was applied. When the FRAP method was used, no increase was observed for SKR and MKR(65) malts, whereas the increases for MKR(45) and MKR(55) malts were 2.5- and 1.5-fold, respectively.

DISCUSSION

One of the aims of this study was to produce malts with similar characteristics, such as color, nitrogen, friability, and viscosity. In an attempt to achieve this, the stabilization period was delayed until after break-point to avoid the production of a stewed malt.

The processing conditions in all four kilning regimens were identical until the introduction of the stabilization holds in the MKRs. Recirculated air (50−60%) was introduced to the kiln in the final 2 h prior to break-point in both the standard and manipulated regimens, as it would be in a commercial operation. The effect of this was to slow the rate of moisture loss from the grain.

This study resulted in the successful production of malts with similar malting characteristics. Analysis of the blended malts from the different kilning regimens did not demonstrate any significant differences. The malt color varied only between 5.0 and 5.2 °EBC units, indicating that the same colored compounds were present in the malts; that is, the chemistry leading to their formation in all cases was similar. Also, the modification of the kilning regimens did not alter the diastase activity in the final malts.

Modification of the kilning regimen, prior to break-point, has been undertaken in the past for the production of crystal malt with the aim of stimulating amylolytic and proteolytic enzymes.[25] No modification of kilning by introducing post-break-point stabilization holds to increase the level of FAE activity has been reported in the literature.

Effect of Processing on Free Ferulic Acid. Changes were detected in the level of free ferulic acid in the malts as a result of kilning, in agreement with previous studies.[2,4,9,10] In the current study, no significant increase was detected in the level of free ferulic acid during the stabilization hold at 45, 55, or 65 °C, which suggests that the conditions within the kiln were not optimum for the enzymatic release of ferulic acid. However, there was no decrease in ferulic acid concentration during the stabilization period, which indicates that there was no thermal degradation of the compound, nor was it involved in chemical reactions.

Effect of Processing on Ferulic Acid Esterase Activity. At the start of the stabilization period, MKR(45) malts possessed the highest FAE activity throughout the kiln and the highest ferulic acid concentration in samples taken from the center of the kiln (the sole sampling point). This indicates that FAE in MKR(45) malt at the point of sampling was, or had been, more active in the release of ferulic acid than in the other MKR malts. No FAE determinations were made after the beginning of kiln stabilization, because the primary objective was to understand free ferulic acid concentration and antioxidant activity.

Low molecular weight phenolics, such as ferulic acid, are esterified with the cell-wall polysaccharides, and an increase in the free phenolic acid may be due to their enzymatic release[2] or thermal liberation.[26] Maillard and Berset[2] suggest that changes in levels of phenolic acids during kilining between 50 and 64 °C are due to changes in extractability. Also, phenolics may be

enzymatically released throughout kilning. As the grain temperature is further increased, linkages between the ferulic acid and arabinoxylans may break. Lignin degradation could also lead to the release of phenolic acid derivatives.[2]

One hypothesis that explains the observed release of ferulic acid in the current study is as follows. The initial increase of ferulic acid at grain temperatures up to 55 °C is due to the release of bound phenolics by the action of FAE and associated enzymes. As the temperature increases further, the FAE becomes less active and release is slowed. Thermal degradation of free phenolics, due to the increasing grain temperatures, causes an overall decrease in the level of ferulic acid. As the temperature is increased yet further, there is an increase in ferulic acid due to increased ease of extractability, which, until 75 °C, is greater than its thermal degradation. Beyond 75 °C, any increase in ferulic acid due to release is overtaken by its thermal breakdown.

Enzymes need to be hydrated to become active, and FAE, being a hydrolase, requires water to hydrolyze the ester bonds.[18] Thus, as the moisture content is decreased, lowered enzyme activity results. In the current study, the variation in FAE activity at the start of the stabilization period was greater for MKR(45) malts than for MKR(55) and MKR(65) samples, with the drier (5.5% moisture) MKR(45) malts at the bottom of the kiln possessing the highest activity. However, all MKR(45) samples possessed higher or nonsignifcantly different FAE compared to MKR(55) and MKR(65) malts. The higher moisture content and lower temperature achieved in the MKR(45) malts during stabilization may have enhanced ferulic acid release. However, throughout kilning, unlike antioxidant activity, there is no increase in the level of ferulic acid detected in the sample extracts as the moisture content of the samples decreased. Indeed, as the grain moisture decreased below 5%, the level of ferulic acid in the samples decreased, possibly due to thermal degradation with increasing kilning temperature. At the end of kilning (95 °C, 26.5 h), ethyl acetate extracts of MKR(45) malts demonstrated higher antioxidant activity than other malts by both $ABTS^{\bullet+}$ and FRAP assays.

Detecting the optimum conditions for the action of enzymes within a grain is difficult, and optimum operating conditions of any enzyme are dependent upon many parameters, such as pH, time of incubation, and substrate and enzyme concentrations.[27] The grain in the kiln will have enzymes in different stages of activity, due to the different operating temperatures and moisture contents. Also, there will be a complex set of enzymes present in the malt, each with specific activities. The complete breakdown of arabinoxylan to arabinose, xylose, and ferulic acid requires five separate enzymes.[18] Therefore, the conditions throughout the kiln as well as processing time will have a direct impact upon FAE activity and, thus, the phenolic acid profile and, ultimately, the antioxidant activity of the malt.

Effect of Processing on Antioxidant Activity. Changes in antioxidant activity in the malts resulting from the different kilning regimens were not identical. Also, the results from the same kilning regimen as measured by the two different assays were not the same.

The acetate buffer extract is representative of the total extractable antioxidant activity of the malt sample and includes contributions from both the free phenolic acids and Maillard reaction products (MRPs). The ethyl acetate extract contains contains ferulic acid and other phenolic acids as well as all other ethyl acetate-extractable components, including some MRPs. The acetate buffer extracts increased in color with extent of kilning, probably due mainly to the development of MRPs. The majority of MRPs will not be extracted into ethyl acetate, and therefore differences in antioxidant activity between the acetate buffer and ethyl acetate extracts may be attributed in part to MRPs possessing antioxidant activity, for example, melanoidins and reductones, which will remain largely in the aqueous phase, whereas phenolic acids, including ferulic acid, partition into ethyl acetate. Antioxidant activity increased as a result of kilning as determined by both the $ABTS^{\bullet+}$ and FRAP assays, for all kilning regimens. The pattern of antioxidant activity development was not identical for either the different kilning regimens or the assays, reflecting the different chemistries of the assays, as well as changes in the chemistry taking place within the malt. Both FRAP and $ABTS^{\bullet+}$ assays are kinetic measurements. Any slower-acting antioxidants present in the sample extracts are not measured by these assays.[28,29] The FRAP assay was more sensitive in detecting changes in antioxidant activity of the acetate buffer extracts. This assay determines the total reducing power of a sample, and components able to respond in the assay include MRPs that may act as antioxidants by various mechanisms, including scavenging of free oxygen and free radicals, reduction, and metal chelation.[30,31] In contrast, the $ABTS^{\bullet+}$ assay was better at discriminating among the ethyl acetate extracts of the malts. This was expected, because the $ABTS^{\bullet+}$ assay measures free radical scavenging activity, and phenolic compounds, including ferulic acid, are recognized for their ability to scavenge free radicals.[32]

The antioxidant activity of the finished malts was higher than those of the green malts, in line with previous studies,[2,4] and is attributed to the antioxidant activity of the thermally generated compounds, for example, melanoidins and reductones, being greater than the antioxidant activity of ferulic acid that degraded during processing.

The modified kilning regimens of Chandra et al.,[25] used for the production of crystal malt (with the addition of prebreak holds in the kilning regimens), were demonstrated to have no significant effect upon the antioxidant activity of the finished malts, determined by both $ABTS^{\bullet+}$ and HPLC chemiluminesence quenching techniques. This observation is supported by the data obtained in the current study, which also showed no significant difference in antioxidant activity of the acetate buffer extracts of the finished malts, obtained at the center of the kiln for the different regimens, as determined by the $ABTS^{\bullet+}$ and FRAP assays.

For the MKR(45) malt ethyl acetate extracts, the observed apparent maximum in antioxidant activity at 18.2 h (55 °C) corresponded with the highest ferulic acid concentration. This suggests that the enzymes responsible for the release of ferulic acid, which is responsible for the radical scavenging ability demonstrated by the $ABTS^{\bullet+}$ assay, may have been active for longer than in the MKR(55) and MKR(65) trials.

The contribution of the ethyl acetate fraction to the total antioxidant activity of the malts was relatively small (~5%). It varied with kilning conditions and antioxidant assay. The ethyl acetate fraction always made a larger contribution to the antioxidant activity of the MKR(45) malts at 4.6 and 7.02% for blended finished malts determined by the $ABTS^{\bullet+}$ and FRAP assays, respectively, compared to ≤3.2 and ≤5.5%, respectively, for the other regimens.

The antioxidant activity of the malt extracts increased as the grain moisture content decreased below 4%, in line with earlier studies.[1,4] The effect of malt moisture content is more apparent on the antioxidant activity of the acetate buffer extracts than on the ethyl acetate extracts. The increase in antioxidant activity may

have been due to the increase in MRPs that possess antioxidant activity and which in model systems have demonstrated increased antioxidant activity at lower a_w.[33]

Relationship between Ferulic Acid Concentration and Antioxidant Activity. The antioxidant activity of malt is derived both from the naturally occurring components of the malt possessing antioxidant activity and from components generated throughout heating.[2,9] The level of phenolics varies throughout kilning as a response to the processing conditions. Components possess different antioxidant activities, and this can vary depending upon several factors including their concentration, the system concentration, and the assay. Not all phenolic compounds at the same concentration exhibit the same antioxidant properties, and antioxidant activity can also vary depending upon the assay used to quantify the antioxidant activity. Therefore, although it is possible to correlate the antioxidant activity of one phenolic compound with concentration for one assay, it is not always possible to correlate total phenolics and antioxidant activity. Mixtures of phenolics can exhibit enhanced antioxidant activity; that is, they work together synergistically.[9] Monomers, dimers, and oligomers of the same compound, for example, ferulic acid, have different antioxidant activities, and even different dimers of the same compound have been demonstrated to have different antioxidant activities.[34] Antioxidants within various categories (for example, MRPs, phenolics, and carotenoids) may exhibit different antioxidant properties in relation to their concentration, chemical structure, and oxidation state.[35]

In line with our previous studies,[5] ferulic acid does not increase throughout kilning in a linear manner, but increases and decreases according to the grain temperature and kilning conditions. Antioxidant activity has also been demonstrated to increase throughout kilning in a nonlinear manner.

In each malting regimen, the initial increase in ferulic acid (up to a grain temperature of 55 or 65 °C, depending upon the regimen) corresponded to an increase in antioxidant activity. At grain temperatures above 75 °C, the relationship between the ferulic acid concentration and antioxidant activity broke down. At these temperatures, the contribution of the ferulic acid to total antioxidant activity became less important and the influence of MRPs possessing antioxidant activity increased.

The following conclusions may be drawn. The concentration of free ferulic acid in finished malt is affected by the kilning regimen, which may be due in part to both the different FAE activities throughout kilning and the time scale over which the enzyme is active. FAE and antioxidant activity of the malt vary throughout the grain bed. This results in differences in ferulic acid concentration at different grain bed positions. Although this study involved an exploration of the effect of kilning conditions, rather than their optimization, on free ferulic acid concentration and antioxidant activity, the data indicate that the inclusion of a stabilization hold in the kiln at 45 °C for 2 h post-break-point enhances the level of ferulic acid in the finished malt, and this is attributed to enhanced FAE activity. Further trials, including some at a commercial scale, should be undertaken to confirm this finding and understand the kilning conditions leading to optimized levels of ferulic acid and antioxidant activity in finished malt for the conservation of flavor in beer.

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ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); EBC, European Brewery Convention; FAE, ferulic acid esterase; FRAP, ferric-reducing antioxidant potential; HWE_2, hot water extract prepared using fine-ground malt (passes through a 0.2 mm mesh); HWE_7, hot water extract prepared using coarse-ground malt (passes through a 0.7 mm mesh); MKR, modified kilning regimen; MKR(45), modified kilning regimen with a stabilization hold at 45 °C; MKR(55), modified kilning regimen with a stabilization hold at 55 °C; MKR(65), modified kilning regimen with a stabilization hold at 65 °C; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MRPs, Maillard reaction products; RH, relative humidity; SKR, standard kilning regimen.

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