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CHANGES OF FREE FERULIC AND COUMARIC ACID CONTENTS DURING MALTING OF BARLEY GRAIN

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The aim of the presented work was to evaluate changes in free ferulic and coumaric acid concentrations during malting. In general, malting is performed at 14°C and in steeping water of pH 7.4. The influence of an elevated temperature of the malting process (22°C, "Activated Germination Malting" technique) as well as a decreased pH value of steeping water (pH 5.2) on free ferulic acid and coumaric acid contents in kilned malts was estimated. The application of "Activated Germination Malting" caused a limited increase in the concentrations of ferulic acid and coumaric acid in malt Rudzik, whereas a higher temperature during malting of Krona barley resulted in approx. 2-fold higher concentration of both free phenolic acids in kilned malt. The use of steeping water of pH 5.2 instead of 7.4 resulted in a significant increase in the contents of free ferulic and coumaric acids in malt of both tested varieties, which could lead to an increase in the contents of these phenolic acids in beer.

INTRODUCTION

Beer is the most popular alcohol beverage consumed in the world and it is likely that it will not lose its position in the future. Beer, as a popular drink, is a potential cause of some deseases and body disfunctions due to the content of alcohol. For this reason, beer should not be promoted. Nevertheless, beer will be consumed widely world-wide and research aimed at increasing the health-promoting properties of beer should be carried on. Beer is rich in phenolic compounds which are reponsible for the antioxidant potential of this beverage [Bellmer *et al.*, 1995 a, b; Montanari *et al.*, 1999; Samotyja *et al.*, 2002]. Unfortunately, most brewing technologies tend to a maximal removal of phenolic compounds from beer, since this class of compounds, together with beer polypeptides, is responsible for beer turbidity. For this reason, the total antioxidant activity of beer, measured *e.g.* in a system containing low density lipoproteins (LDL) and human plasma *ex vivo*, is low in comparison to antioxidant activities of such beverages like wine and grape juice, or green and black tea [Vinson *et al.*, 1999]. On the other hand, compared to other beverages containing high levels of phenolic compounds, *e.g.* white wines, *in vitro* antioxidant activity of beer is high, due to a high content of proanthocyanidins, epicatechin and ferulic acid [Gorinstein *et al.*, 2000]. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is the main phenolic acid in barley, malt and beer. This compound is present in barley mainly in a bound form as ester with arabinoxylan polymer, and only about 10% of the total ferulic acid in barley kernel is present in a free form [Maillard *et al.*, 1996; Maillard & Berset, 1995]. The esterificaton of ferulic acid to arabinoxylan polymers as well as the role of ferulic acid in the structure of a cell wall has been described in detail [Ahluwalia *et al.*, 1986; Renger *et al.*, 2000; Wallace *et al.*, 1995; Jacquet *et al.*, 1995; Ahluwalia *et al.*, 1986]. The interest in studies into the increase of ferulic acid or coumaric acid concentrations in beer can be explained by a higher ability of phenolic acids to be absorbed into blood plasma in comparison to other, more complex compounds, for example anthocyanins [Deprez *et al.*, 2000]. During digestion and absorbtion, relatively simple structures like phenolic acids are not subject to great changes and are likely to play an important role in increasing the total antioxidant activity *in vivo* [Murkovic *et al.*, 1999; Choudhury *et al.*, 1999; Rechner *et al.*, 2001; Azuma *et al.*, 2000; Izquierdo *et al.*, 2002]. The aim of the presented work was to determine concentrations of free ferulic acid during malting as well as to identify factors influencing the release of free ferulic acid from its bound form with arabinoxylans during malt production. Ferulic acid originating from barley and malt can be a very attractive, natural antioxidant in beer that does not draw a strong response among consumers who vigourously react against the application of some synthetic antioxidants in foods.

MATERIALS AND METHODS

Barley. Nine barley varieties were used: Orlik – ZDHAR Bąków; Atol, Rodos, Rasbet – ZDHAR Strzelce; Brenda, Krona, Madonna – Semundo Saatzucht GmbH; Rudzik, Mobek - Hodowla Roślin Szelejewo. The samples were gathered in the year 2000.

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Malting in a laboratory scale. Pale malts for lager beer were produced. Malting conditions in a laboratory scale were as those described by Nischwitz *et al.* [1999], Every time, a portion of 1 kg of barley grain was malted in a glass, vertical vessel (2 L). The total volume of water in the system was 4.5 L. The temperature was controlled with water. The time-temperature program was as follows: 0–1 h – steeping the grain with simultaneous disinfection using 0.5 mL/L of hydrogen peroxide solution; 1–24 h – steeping the grain in water at pH 7.4 or pH 5.2; 24–34 h – air rest of the grain, the process was carried out in a chamber equipped with air flow and temperature control; 36–48 h – steeping the grain in water; 48 h – germination. Germination was continued until the proper rootlets length has been achieved [Kunze, 1999]. Malt heating was performed in 4 successive steps: 10 h at 40°C; 10 h at 55°C; 4 h at 72°C; and 4 h at 83°C. In order to investigate the influence of an elevated temperature on the activities of barley grain enzymes [Baca *et al.*, 1998], the malting process was performed at 22°C, as described by Kitamura *et al.* [1990]. In order to study the influence of a decreased pH value of steeping water on barley enzyme activities, two pH values were applied: pH 7.4 (tap water) and pH 5.2 (tap water with lactic acid – 150 g/L of a solution). The samples of malts were withdrawn every 24 h during steeping and germination and after every stage of kilning. Directly after kilning, grain rootlets were removed by rubbing through a strainer. Malt was stored before analyses for 4 weeks at 4°C. Malting experiments were repeated in duplicate for each of two barley varieties tested and for each malting technology applied (temp. 14°C or 22°C and pH of steeping water 7.4 or 5.2).

Methanol extraction of phenolic acids from barley and malt. Phenolic acids were extracted from barleys and malts using methanol according to the method of Maillard *et al.* [1996] with slight modifications. Barley (2 g) was finely ground in a laboratory mill, transferred into a conical flask and extracted four times with methanol (20 mL each time). During extraction, the flask with a stopper was shaken for 20 min at ambient temperature (CERTOMAT R, B. BRAUN BIOTECH INTERNATIONAL shaker, 170/min). Four extracts were collected together, solid parts were removed by centrifugation (7000 g, 30 min) Clear supernatant was evaporated to dryness and redissolved in 10 mL of methanol (35°C, 1 atm, Büchi SB, Glassapparatenfabrik Flawil, Switzerland). Samples of grains from different stages of malting were characterised by various water contents ranging from a few per cent to 46%, thus the constant dry matter of the sample could not be weighed. In this situation, use was made of the method proposed by Sancho *et al.* [1999]. Taking into consideration the weight of 1000 barley kernels, the number of kernels equal to 2 g of barley before malting was counted and used for extraction.

Extraction of bound ferulic acid from barley using acid hydrolysis. Hydrolysis of bound ferulic acid from barley kernels was performed using sulphuric acid according to the method of Pussayanavin and Wetzel [1987] with modifications proposed by Zupfer *et al.* [1998]. A sample (1 g) was finely ground in a laboratory mill, transferred into tubes containing 15 mL of 0.4 mol/L H_2SO_4 solution and heated for 1 h in a boiling water bath. The tubes were then cooled in ice-bath to the ambient temperature and 2.2 mL of aqueous sodium acetate solution (2.5 mol/L) containing α-amylase (*Aspergillus oryzae*, EC 3.2.1.1, Type X-A, 20 g/L of solution) was added. The enzyme contained side activity of ferulic acid esterase. The samples were then incubated for 1 h at 30°C and centrifuged (7000 g, 30 min) Clear supernatants were used for HPLC analyses.

HPLC analysis of phenolic acids. HPLC in an isocratic system was used according to the method of Pussayanavin and Wetzel [1987] with modifications proposed by Zupfer *et al.* [1998]. The mobile phase was methanol-citric acid buffer (sodium citrate-citric acid, 0.01 mol/L, pH 5.4; $13:87_{v/v}$). The HPLC unit consisted of: a piston pump KNAUER (Germany), a UV-VIS LINEAR 200 (USA) detector and a TZ 4620 recorder (Czech Republic). Symmetry® C18 (Waters, length: 250 mm, i.d. 4.6 mm, 5 μm) RP column was used for separations. The flow of mobile phase was 0.4 mL/min. Injection volume was 20 μL; the detector was set at 320 nm. Concentrations of phenolic acids in barley and malt samples were expressed on the basis of wet matter. HPLC analyses were performed in triplicate and mean values were calculated. The repeatability of the HPLC method was determined within one day using ferulic acid (FAStd) or coumaric acid (CAStd) pure standard solutions and methanol extracts of barleys (Rudzik and Krona). Each sample was injected into the HPLC system 5 times. The reproducibility of the HPLC method (within laboratory) was calculated over an 8-week period. Ferulic acid and coumaric acid pure standard solutions (FAStd and CAStd, respectively) or barley extracts after acid hydrolysis were used. During this period, ten injections (in the case of standard solutions) or seven injections (in the case of barley extracts) were performed.

RESULTS

The repeatability and reproducibility of the HPLC method were evaluated. Coefficients of variations (% CV)

TABLE 1. Repeatability of the HPLC method at determination of ferulic and coumaric acid contents.

Sample	Ferulic acid			Coumaric acid		
	Phenolic acid content[a] (μg/10 mL)	Standard deviation	Coeficient of variation (%)	Phenolic acid content[a] (μg/10 mL)	Standard deviation	Coeficient of variation (%)
FAStd	10.8	0.7	6.48	-	-	-
CAStd	-	-	-	11.4	0.8	7.02
Rudzik methanol extract	3.4	0.2	5.88	2.7	0.2	7.40
Krona methanol extract	4.2	0.3	7.14	2.2	0.2	9.09

[a]Mean value, n=5

TABLE 2. Reproducibility of the HPLC method at determination of ferulic and coumaric acid contents.

Sample	Ferulic acid			Coumaric acid		
	Phenolic acid content[a] (μg/10 mL)	Standard deviation	Coeficient of variation (%)	Phenolic acid content[a] (μg/10 mL)	Standard deviation	Coeficient of variation (%)
FAStd	1034.3[b]	88.3	8.54	-	-	-
CAStd	-	-	-	996.5[b]	38.9	3.90
Rudzik barley hydrolysate	314.4[c]	19.1	6.07	121.4[c]	10.1	8.32
Krona barley hydrolysate	287.7[c]	28.4	9.87	78.0[c]	7.6	9.74

[a]Mean value; [b]n=10, [c]n=7

were calculated on the basis of determinations of ferulic acid and coumaric acid contents in methanol extracts (Table 1, within-day repeatability) or after acid hydrolysis (Table 2, laboratory reproducibility) using two barley varieties. Standard ferulic acid and coumaric acid solutions were also used in this experiment. Standard deviation values for the samples studied were under 10%; the repeatability and reproducibility of the HPLC method was good. All presented results corresponded to the evaluated repeatability and reproducibility of the HPLC method. Table 3 presents free and total (free plus bound) ferulic acid and coumaric acid concentrations in nine varietes of spring barley. In the case of all barley varietes, free ferulic acid comprised less than 0.6% of the total concentration of this phenolic acid in barley grain. The total ferulic acid content in ten barley varieties ranged from 494.9 μg to 597.9 μg/g of the grain. Barley Rudzik had the highest total ferulic acid content (597.9 μg/g of the grain) and the lowest free ferulic acid content (1.3 μg/g of the grain). Coumaric acid was present in relatively low concentrations in barleys in comparison to ferulic acid. The differences in free and total coumaric acid contents among barley varieties were higher than the differences of both forms of ferulic acid. The total ferulic acid concentration in each barley variety was a couple of times higher than the total coumaric acid content. The content of free ferulic acid in Mobek was lower than coumaric acid content in this barley variety, and in 8 other barley varieties free ferulic acid contents were slightly higher than the concentrations of coumaric acid. In the next part of the work, the changes in ferulic and coumaric acid contents during malting were determined in selected barley varieties: Rudzik and Krona. During malting of Rudzik barley at 14°C and pH 7.4 (Figure 1), an increase in free ferulic acid content was observed, beginning from the 72 h of the process (the steeping and air rests of the grain) up to the 116 h of the process, when the so-called "green malt" was warmed at 55°C. During next steps of kilning, a decrease in free ferulic

TABLE 3. Contents of ferulic acid and coumaric acid in barleys.

Barley variety	Free phenolic acid concent (μg/g of grain)[a]		Total (free plus bound) phenolic acid content (μg/g of grain)[a]	
	Ferulic acid	Coumaric acid	Ferulic acid	Coumaric acid
Madonna	1.5	1.5	523.7	93.4
Rudzik	1.3	1.2	597.9	146.7
Rasbet	2.0	1.7	564.9	206.7
Atol	2.8	2.0	507.2	67.0
Krona	1.6	1.0	536.1	120.0
Mobek	1.4	2.0	536.1	186.7
Orlik	2.3	2.2	577.3	167.0
Rodos	1.5	1.4	585.6	86.0
Brenda	1.5	1.3	536.1	167.1

[a] values calculated for wet matter

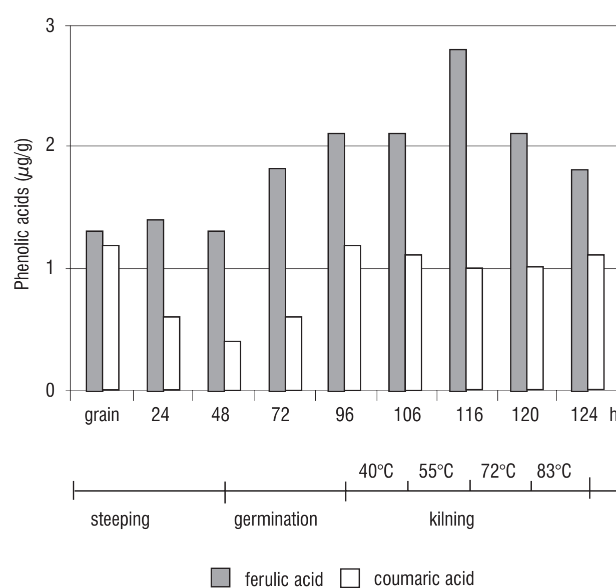

FIGURE 1. Changes of free phenolic acids contents during malting of Rudzik barley at 14°C and pH 7.4.

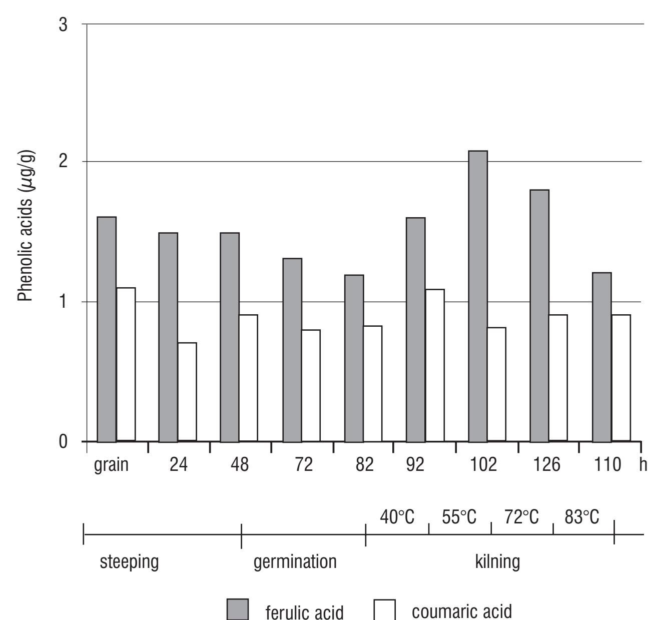

FIGURE 2. Changes of free phenolic acids contents during malting of Krona barley at 14°C and pH 7.4.

acid concentration reached approx. 35%. As for coumaric acid, after 96 h of malting, an increase in free coumaric acid concentration was detected following stabilisation of phenolic acid content until the end of the process. During malting of Krona barley (Figure 2) at 14°C and pH 7.4, an increase in free ferulic acid content was observed beginning from the 92 h of the process (the first stage of the so-called "green malt" kilned at 40°C) up to the 102 h of the process, when malt was kilned at 55°C. During next steps, a decrease in free ferulic acid concentration reached 40%. Coumaric acid concentration was not significantly changed during the entire malting process under the applied conditions. Malting of Rudzik grain at 14°C and pH 5.2 (Figure 3) caused an increase in free ferulic acid content beginning from 48 h of malting (steeping/air rest stage of grain). Elevated free ferulic acid levels decreased by approx. 30% during the last stage of kilning at 83°C, nevertheless the content of free ferulic acid in this malt was approx. 3-fold higher than in malts produced using water of pH 7.4. A substantial increase in free coumaric acid concentration by 106 h of the process and a decrease in free coumaric acid content during the last stage of kilning at 83°C were recorded. Elevated ferulic acid and coumaric acid concentrations were observed during malting of barley Krona at 14°C and pH 5.2 (Figure 4). A considerable increase in free ferulic acid concentration was observed while kilning at 55°C and 72°C, following a slight decrease in free ferulic acid at 83°C. Similarly, as in the case of Rudzik malted using water of pH 5.2 (Figure 3), free ferulic acid content in kilned malt Krona was considerably higher than the corresponding ferulic acid content in malt produced using water of pH 7.4. Malting of Rudzik barley at 22°C and pH 7.4 (Figure 5) resulted in an increase

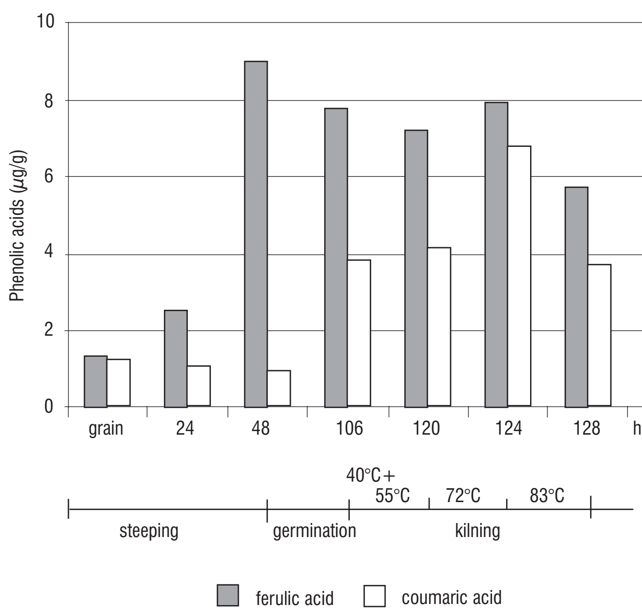

FIGURE 3. Changes of free phenolic acids contents during malting of Rudzik barley at 14°C and pH 5.2.

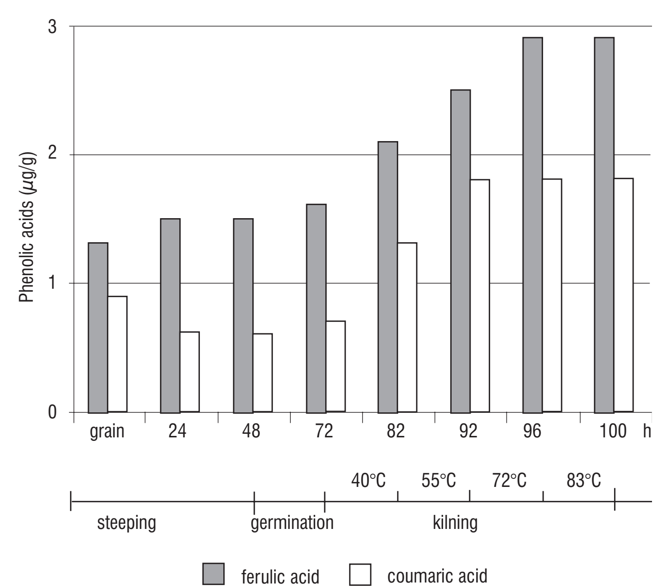

FIGURE 5. Changes of free phenolic acids contents during malting of Rudzik barley at 22°C and pH 7.4.

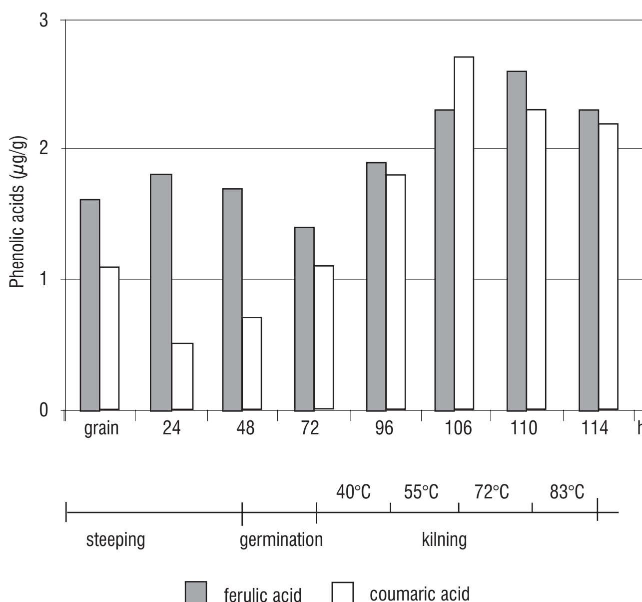

FIGURE 4. Changes of free phenolic acids contents during malting of Krona barley at 14°C and pH 5.2.

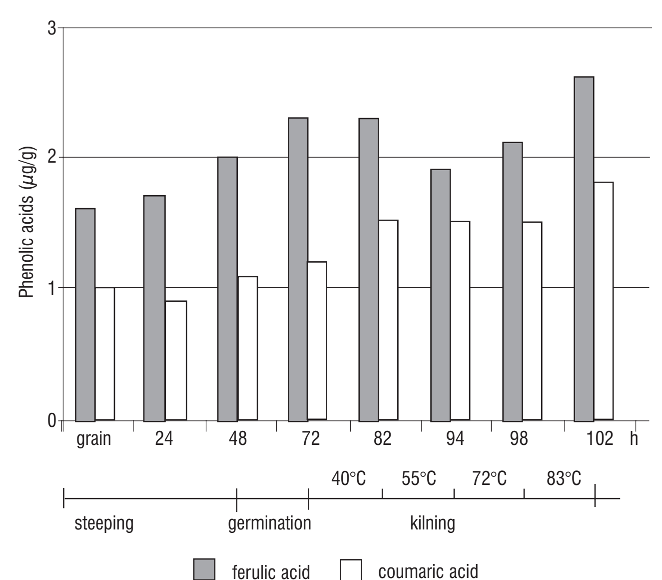

FIGURE 6. Changes of free phenolic acids contents during malting of Krona barley at 22°C and pH 7.4.

in free ferulic acid content by 8 2h of the malting process, following the stabilization of ferulic acid content during kilning at 72°C and 83°C. While malting of Krona barley at 22°C using water of pH 7.4 (Figure 6), an increase in free ferulic and coumaric acid concentrations was detected from the beginning of malting, following a slight decrease of free ferulic acid concentration after 94 h of malting and another increase in ferulic acid concentration at higher temperatures of kilning. Free ferulic and coumaric acid contents in Krona malts produced at 22°C reached a level of approx. 210% and 208% of corresponding phenolic acids contents in Krona malts produced at 14°C (Figures 2 and 6, respectively). As for barley Rudzik, the process performed at 22°C enabled a considerable increase in ferulic acid and coumaric acid contents (approx. 160% each) in comparison to phenolic acids contents in malt produced at 14°C (Figures 1 and 5). The application of steeping water with decreased pH caused over 3-fold more effective release of free ferulic and coumaric acid in kilned Rudzik malt. In the case of malt Krona produced using acidified steeping water, the release of ferulic acid and coumaric acid was similar to free acids contents in malt produced at 22°C.

DISCUSSION

The research presented in this paper focused on the changes in free ferulic acid and coumaric acid concentrations during malting, especially on the influence of such factors as malting temperature and pH of steeping water. A number of research works have pointed out that ferulic and coumaric acid are present in non-malted barley predominantly in a bound form, and only a minor part of these phenolic acids is present in free forms in barley grain. Also in this study, we attempted to evaluate ferulic acid and coumaric acid contents in free and bound forms, in nine barley varieties. The total ferulic acid content in barley varieties ranged from 494.9 μg to 597.9 μg/g of the grain, and that of coumaric acid ranged from 67.0 μg to 206.7 μg/g of grain. In all barley varieties, free ferulic acid comprised not more than 0.6% of the total concentration of this phenolic acid in barley grain. Similarly, only a minority of coumaric acid was present in the free form. The levels of ferulic acid were considerably higher than those of the second important phenolic acid in barley, namely coumaric acid, which explains a considerable interest in the role of ferulic acid in increasing the antioxidant potential of malt, wort and beer. Zupfer *et al.* [1998] determined the total (bound and free) ferulic acid contents in 18 varieties of two-row and six-row barley varieties using HPLC-UV, fluorimetry and a simple measurement of absorbance of barley extracts. The differences in the total ferulic acid contents were significant and ranged from 343.2 μg/g to 579.7 μg/g of the grain, depending on barley variety and place of growth (continental climate or not) and the sea level. In addition, the authors underlined that differences in ferulic acid concentrations depended on the morphological structure of barley grain. In similar experiments, Maillard and Berset [1995] showed that ferulic acid was present in barley grains in the bound form, and only about 10% of the total ferulic acid content was present in the free form.

The malting conditions, like temperature and pH of steeping water, are likely to influence the enzymatic and non-enzymatic changes in grains. An elevated temperature during malting (22°C) rather than the standard malting temperature of 14°C was suggested by some authors as a part of the malt production technique referred to as "Activated Germination Malting" [Kitamura *et al.*, 1990]. A temperature of 22°C was applied in order to intensify the enzymic activities and to modify grain endosperm by rapid activation of the embryo. The "Activated Germination Malting" enabled creating a malting programme in order to shorten the process without deterioration of malt quality. Nischwitz *et al.* [1999] proved that the application of an elevated temperature of 22°C during the entire malting resulted in decreased wort extract and decreased diastatic power of the malt obtained. On the other hand, the elevated malting temperature influenced more complete β-glucan degradation, thus the produced wort and beer had decreased viscosity, which in turn increased the rate of beer filtration. The increase in free ferulic acid concentration at 50°C and 64°C could be attributed to the changes of extractability of malt grains caused by the decrease of malt humidity. Maillard & Berset [1995] recorded a significant decrease in water content in malt during kilning at 64°C. Another reason of free ferulic acid and coumaric acid content increase after malting could be the degradation of grain tissues, which improved the extraction of phenolic acids. At last, during malting, there was observed an increase in the activity of ferulic acid esterase, an enzyme that plays a key role during degradation of arabinoxylan polymers present in cell walls of barley grain. Except the main desired enzyme activities induced during malting, like these of proteases, amylases, β-glucanases, a number of other accessory enzyme activities occur, including these of non-starch polysaccharides hydrolases like xylanases, acethylesterases, and esterases, which play a crucial role in arabinoxylan degradation during malting. Ferulic acid esterase activity has been detected in germinated barley but also in non-germinated barley grain. The enzyme activity dropped after one day of germination in comparison to the activity present in barley grain and then rised to a slightly higher level than in the non-germinated grain after the second day of germination. During next four days, ferulic acid esterase activity continouosly decreased and by day 6, the enzyme activity was lower than in the non-germinated barley grain [Sancho *et al.*, 1999]. The "Activated Germination Malting" enabled only a slight increase in ferulic acid concentration in Rudzik kilned malt, whereas in the case of kilned Krona malt, approx. 2-fold higher free ferulic concentration acid was gained. As for coumaric acid contents in kilned malts of both barley varieties, about 2-fold increase in comparison to coumaric acid contents in malts produced at 14°C was observed. In the presented study, the stabilization or decrease of phenolic acids contents in all final malts was recorded, except for Krona malt produced at 22°C, where a slight increase was noted. The loss of ferulic and coumaric acid in kilned malt was the subject of studies performed by Maillard and Berset [1995]. The researchers showed that in a lipophillic solution containing dodecan, the loss of *trans*-ferulic acid after 4 h of incubation at 85°C was 35%, but in a water solution, under the same conditions, the loss of *trans*-

ferulic acid reached only 5%. The kilning at higher temperatures, taking under consideration the presence of fatty acids in barley grains, can lead to a decrease in the contents of phenolic acids. Free *trans*-ferulic acid and *trans*-coumaric acid contents were about 100-fold lower than the corresponding contents of the bound forms of both phenolic acids. It was also shown that during malting, the proportions of main free phenolic acids (ferulic and coumaric acid) did not change, but the concentrations of both phenolic acids initially increased by *ca.* 130% until the kilning at 80°C, and then decreased by *ca*. 20% after heating at 90°C [Maillard & Berset, 1995]. Although the final kilning temperature applied in the presented study was 83°C and not 90°C, the same initial increase followed by either a decrease or stabilization of phenolic acids contents was observed. Maillard & Berset [1995] evaluated that in kilned malt, *trans*-ferulic acid was in majority (ca. 59% of the sum of ferulic and coumaric acid content) followed by *cis*-ferulic acid (15%) and trans *p*-coumaric acid (26%).

During experiments concerning the total antioxidant activity of malt hydrolysate, in an oxidation system containing methyl ester of linoleic acid in dodekan, in the presence of oxygen and at high temperature (110°C), the parallelism between the total antioxidant activity changes and total phenolics level was recorded [Maillard & Berset, 1995]. Phenolic compounds, including main malt phenolic acids – ferulic and coumaric acid, can exert a considerable influence upon the antioxidant activity level of kilned malt, wort and beer. Maillard *et al.* [1996], using pure standards, evaluated that *cis-* and *trans-* ferulic acid and *trans- p*-coumaric acid were responsible for 14–22% of the total antioxidant activity of alkaline malt hydrolysate. The authors also detected the synergistic effect between two phenolic acids.

CONCLUSIONS

The application of "Activated Germination Malting" technology, but especially the use of steeping water of pH 5.2 instead of 7.4, might contribute to a significant increase in free ferulic and coumaric acid contents in kilned pale barleys malts. This, in consequence, could lead to the increased concentrations of phenolic acids in beer. The future experiments should be planned to identify these particular factors that influence the release of free phenolic acids from barley grain during malting. Ferulic acid, as the most abundant phenolic acid in barley and malt, but also coumaric acid present at considerable levels in malt, are very attractive, natural antioxidants that could gain applause among consumers displaying negative attitudes towards some artificial antioxidants added to foods.

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ZMIANY ZAWARTOŚCI WOLNEGO KWASU FERULOWEGO I KUMAROWEGO W CZASIE SŁODOWANIA JĘCZMIENIA

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Celem prezentowanej pracy było określenie zmian zawartości dwóch głównych kwasów fenolowych: ferulowego i kumarowego, w ziarnie jęczmienia poddanego słodowaniu. Zastosowano standardową temperaturę słodowania 14°C; ponadto, zgodnie z zaproponowaną przez innych badaczy techniką "Activated Germination Malting", prowadzono proces słodowania w podwyższonej temperaturze 22°C. Określono wpływ obniżenia pH wody użytej do zamaczania jęczmienia (z 7,4 do 5,2) na zmiany zawartości wolnych form obu kwasów fenolowych w czasie produkcji słodu. Wykazano, że słodowanie odmiany Rudzik w temperaturze 22°C wywołało niewielki wzrost stężenia wolnego kwasu ferulowego i kumarowego w gotowym słodzie, w porównaniu z procesem prowadzonym w temperaturze 14°C, podczas gdy słodowanie odmiany Krona wg. techniki "Activated Germination Malting" wywołało ok. dwukrotne podwyższenie stężenia wolnego kwasu ferulowego i kumarowego w gotowym słodzie (rys. 1, 2, 5 i 6). Po zastosowaniu wody o pH 5,2 do namaczania ziarna jęczmienia uzyskano podwyższone stężenia wolnego kwasu ferulowego i kumarowego w gotowych słodach obu odmian jęczmienia (rys. 1–4), co może przyczynić się do podwyższenia stężenia obu kwasów fenolowych w piwie.