Evolution of Antioxidant Activity during Kilning: Role of Insoluble Bound Phenolic Acids of Barley and Malt

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Phenolic acids, bound to the cellular walls of the germinated barley, are very interesting potential antioxidants of beer. Indeed, the antioxidant activity due to these bound compounds is about 2-fold higher than the activity due to the free phenolic compounds. Seven phenolic acids were separated by HPLC after alkaline hydrolysis of the cake resulting from a methanolic extraction of barley or malt. Three main compounds, *trans*-ferulic acid, *trans*-*p*-coumaric acid, and *cis*-ferulic acid, were quantified using an external standard method. They respectively represent 177.79, 78.50, and 47.15 μg/g of dry matter of germinated barley. Responsible for 20–40% of the antioxidant activity of the hydrolyzed cake, they probably have a real influence on the evolution of this activity during kilning.

Keywords: *Antioxidant activity; barley; malt; phenolic acids*

INTRODUCTION

Germinated barley can contain up to 45 mg/g of its dry weight as lipids, with linoleic acid as the main constituent (50–60%). During malting, a significant decrease in the lipid content can be observed, indicating a rapid degradation (Doderer *et al.*, 1992). The oxidation of linoleic acid yields *trans*-nonen-2-al, which is responsible for a cardboard ("papery") flavor and has a flavor threshold of as little as 0.1 ppb (Moll and Moll, 1985).

To inhibit the oxidative deterioration of beer, natural antioxidants of barley must be protected and the production of new antioxidants *in situ* could be favored by optimizing the malting procedure.

Among antioxidants naturally present in germinated barley, free phenolic compounds have often been pointed out (Jerumanis, 1979, 1985; McMurrough, 1981; Mulkay *et al.*, 1981). However, insoluble bound phenolic compounds could play an important role. Ferulic and *p*-coumaric acids have already been identified in barley as the most important quantitatively (Nordkvist *et al.*, 1984; Ahluwalia and Fry, 1986). They are known to be linked to lignin (Salomonsson *et al.*, 1980) and to arabinoxylans (Nordkvist *et al.*, 1984), respectively. The content of *p*-coumaric acid is lowest in the center of the kernel and rapidly increases toward the outer layers, such as the lignified husk (Salomonsson *et al.*, 1980). The content of ferulic acid is highest in the cell walls of the aleurone layer, rich in arabinoxylans (McNeil *et al.*, 1975) with a main chain of β-(1–4)-linked xylopyranose unit (Ahluwalia and Fry, 1986). Both have already been quantified by gas–liquid chromatography (GLC) (Salomonsson *et al.*, 1978; Nordkvist *et al.*, 1984) and by high-performance liquid chromatography (HPLC) (Wulf and Nagel, 1976; Nordkvist *et al.*, 1984). *cis* and *trans* isomers of phenolic acids have been separated by GLC (Nordkvist *et al.*, 1984), but the HPLC methods are more rapid since no derivatization of the acids is necessary (Hartley and Buchan, 1979; Conkerton and Chapital, 1983; Romeyer *et al.*, 1983).

In this work, *cis* and *trans* isomers of *p*-coumaric and ferulic acids are quantified by HPLC. The potential role of the insoluble bound phenolic acids on the antioxidant activity of malts is studied.

MATERIALS AND METHODS

Reagents. The methanol used was of HPLC grade from Carlo Erba (Milano, Italy). The butan-1-ol and the anhydrous dodecane were from Aldrich (St. Quentin Fallavier, France). The ethyl acetate was of Pestinorm grade from Prolabo (Paris, France). Vanillic acid, *trans*-*p*-coumaric acid, and *trans*-ferulic acid were purchased from Extrasynthèse (Genay, France).

Plant Material. Malt from barley (*Hordeum vulgare*) var. Triumph harvested in 1993 was prepared at the French Institute of Brewing and Malting (Nancy, France), in a micromalthouse (2 kg) using standard malting conditions. The kilning procedure consists in five successive steps of heating of the germinated barley: 50 °C for 8 h (step 2); 64 °C for 10 h (step 4); 80 °C for 4 h (step 6); 85 °C for 4 h (step 7); and 90 °C for 2 h (step 8).

Extraction of the Antioxidants from Barley and Malt. Ten grams of germinated barley (or malt) was finely ground in an analysis blender IKA A10. The sample was extracted three times (for periods of 15 min) with 50 mL of methanol. It was then filtered through Whatman No. 1 filter paper. The filtrate (150 mL) was evaporated to dryness at 35 °C under vacuum, and the residue was dissolved in 5 mL of butan-1-ol for the determination of the antioxidant activity of free phenolic compounds. The cake was used for the analysis of insoluble bound phenolic compounds.

Hydrolysis of Insoluble Bound Phenolic Compounds. One gram of the cake was hydrolyzed with 50 mL of 2 N NaOH under N_2 at ambient temperature for 4 h (Ribéreau-Gayon, 1968; Glennie, 1984). The mixture was then acidified with 2 N HCl at pH 1 and extracted three times with ethyl acetate (v/v). The ethyl acetate phase was then evaporated to dryness at 40 °C under vacuum. The residue was dissolved in 2 mL of methanol. Half of a milliliter of this extract was evaporated to dryness under N_2, and the residue was dissolved in 5 mL of butan-1-ol for the determination of the antioxidant activity of insoluble bound phenolic compounds. The last 1.5 mL was kept to be analyzed by HPLC. Two extraction replicates were performed.

The recovery of free phenolic acids submitted to control hydrolyses was 68% ± 0.5% for *p*-coumaric acid and 63% ± 1.5% for ferulic acid, including the formation of 3% ± 0.1% of *cis*-*p*-coumaric acid and of 6% ± 0.6% of *cis*-ferulic acid. Even if a small part of these acids is probably lost during the extraction by ethyl acetate, we can suppose that phenolic acids are partially destroyed during alkaline hydrolysis.

Antioxidant Activity Determination. The antioxidant activity was measured according to the method of Cuvelier *et al.* (1990) based on the accelerated autoxidation of methyl linoleate in anhydrous dodecane, under strong oxidizing conditions (110 °C, intensive oxygenation), for several hours. Gas

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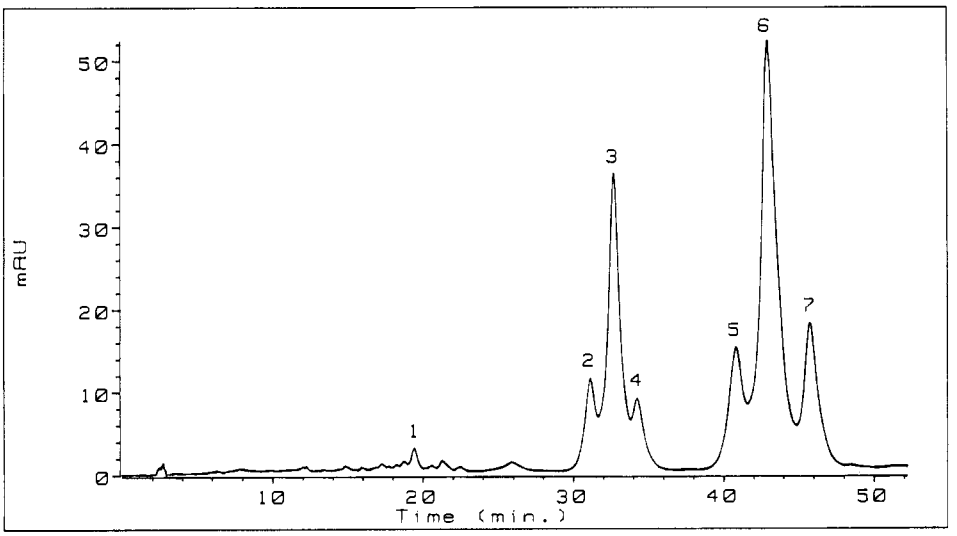

Figure 1. HPLC profile (at 308 nm) of the hydrolyzed cake of germinated barley: 1, vanillic acid; 2, *p*-coumaric derivative; 3, *trans*-*p*-coumaric acid; 4, *cis*-*p*-coumaric acid; 5, ferulic derivative; 6, *trans*-ferulic acid; 7, *cis*-ferulic acid.

Table 1. Content of Insoluble Bound Phenolic Acids of Germinated Barley and Malt Grains during Kilning[a]

kilning step	[total phenolic acids] (μg/g of dry matter)	[*trans*-*p*-coumaric acid] (μg/g of dry matter)	[*trans*-ferulic acid] (μg/g of dry matter)	[*cis*-ferulic acid] (μg/g of dry matter)
1 (germinated barley)	303.44 ± 1.29	78.50 ± 0.16	177.79 ± 0.71	47.15 ± 0.42
2 (50 °C)	428.92 ± 9.76	99.48 ± 4.58	234.42 ± 2.81	95.02 ± 2.37
4 (64 °C)	639.37 ± 10.05	166.77 ± 1.50	360.24 ± 5.40	112.36 ± 3.15
6 (80 °C)	704.03 ± 4.09	176.05 ± 0.70	411.78 ± 1.65	116.20 ± 1.74
7 (85 °C)	625.47 ± 26.70	161.51 ± 2.58	350.31 ± 18.21	113.65 ± 5.91
8 (90 °C)	565.80 ± 5.60	152.79 ± 2.14	318.65 ± 2.23	94.36 ± 1.23

[a] Values represent means ± SD, calculated from four values.

chromatography was used to monitor the disappearance of methyl linoleate from the reaction medium (initial concentration of methyl linoleate = 40 g/L). Antioxidant activity was assessed by the percentage increase in the half-life time of a control oxidation without any antioxidant. This activity varied according to the antioxidant and its concentration. The dry residues of barley and malt were dissolved in butan-1-ol to achieve a better solubilization in the highly apolar dodecane. The butanol/dodecane ratio of the mixture was 10%. The effective quantity (EQ) required to double the half-life time of the control was determined. The lower the EQ value, the stronger was the antioxidant. To make these values easier to understand, 1/EQ, proportional to the antioxidant activity, was used. 1/EQ is expressed in milliliters of dodecane per gram of dry matter of germinated barley or malt, with a standard deviation calculated from two values.

Analysis of the Bound Phenolic Compounds by Analytical HPLC. An aliquot of 20 μL of the hydrolyzed phenolic compounds was separated using an HPLC Gilson system, a Hewlett-Packard 1040 M photodiode array detector, and an Adsorbosphere C_{18} 3U (150 mm × 4.6 mm i.d., Alltech) cartridge. The solvent system used was a gradient of A (water adjusted to pH 2.6 with orthophosphoric acid) and B (acetonitrile). The following gradient was applied: 0–10 min, 10% B linear; 10–37 min, 10 % B isocratic; 37–52 min, 15% B linear; 52–90 min, washing and re-equilibration of the column. The solvent flow rate was 0.8 mL/min. The temperature of the column was maintained at 35 °C in an oven (Violet T.55). Two HPLC analyses were realized for each replicate extract. The data were therefore the average of four results.

Quantification of the Insoluble Bound Phenolic Compounds. The three main hydroxycinnamic acids were quantified at their maximal absorbances using an external method standard: *trans*-*p*-coumaric acid and *cis*-ferulic acid at 308 nm and *trans*-ferulic acid at 322 nm.

cis isomers of *p*-coumaric and ferulic acids were produced from ultraviolet irradiation of methanolic solutions of the corresponding *trans* isomers, for 15 h (Babic, 1992).

Evaluation of the Synergistic Effect between *p*-Coumaric Acid and Ferulic Acid. The antioxidant activity of three different mixtures of *p*-coumaric and ferulic acids has been determined according to the test of Cuvelier *et al.* (1990). The ratio between the concentration of ferulic acid and the concentration of *p*-coumaric acid was 3.3 for the first mixture, 2.9 for the second one, and 2.1 for the third one.

RESULTS AND DISCUSSION

Analysis of Insoluble Bound Phenolic Compounds. A typical HPLC profile of the hydrolyzed cake of barley is shown on Figure 1. Peaks 1, 3, and 6 have been tentatively identified from their spectral characteristics and retention time, in comparison with commercial pure compounds, as vanillic acid, *trans*-*p*-coumaric acid, and *trans*-ferulic acid. *cis*-*p*-Coumaric and *cis*-ferulic acids (peaks 4 and 7) were compared to standards obtained *in vitro* by photoisomerization. The spectral analysis of peaks 2 and 5 suggested that they were *p*-coumaric and ferulic derivatives. However, their structures have not been further investigated.

The quantification of the identified phenolic acids was carried out using standard curves. However, *cis*-*p*-coumaric acid was in low amounts and not well separated from its *trans* isomer, so only the results of *trans*-*p*-coumaric acid, *cis* ferulic acid, and *trans*-ferulic acid are reported (Table 1).

Our results confirm that *trans*-ferulic acid is the main bound phenolic acid of germinated barley (59%), while *trans*-*p*-coumaric acid and *cis*-ferulic acid represent 26 and 15% of total three phenolic acids, respectively. In fact, *trans* isomers of cinnamic acids are generally considered to be the native molecular forms because of their higher stability. The proportion of the *cis* isomers is yet not negligible, as it has already been reported by Nordkvist *et al.* (1984) and McMurrough and Byrne (1992). A small part of these *cis* isomers could come from an isomerization of the *trans* isomers occurring

during exposure to ultraviolet light during extraction from grains (Hermann, 1989) or during alkaline hydrolysis. However, we have shown that alkaline hydrolysis performed on pure *trans* isomers only yielded 3% *cis*-*p*-coumaric acid and 6% *cis*-ferulic acid. The alkaline hydrolysis has been preferred to an acid hydrolysis which seems to greatly reduce the yield of cinnamic derivatives (Glennie, 1984).

The proportions of the different phenolic acids are not significantly modified during kilning, but the contents of each compound are increased by about 130% until 80 °C and then decreased by 20% until 90 °C (Table 1).

To explain the increase of phenolic acids in the first steps of kilning, the hypothesis of a synthesis *de novo* must be examined. Phenylalanine ammonia lyase (PAL) is the key enzyme in phenolic biosynthesis. It has already been reported in *H. vulgare* (Koukol and Conn, 1961), but its activity at pH 6 (the pH of kilning) is not very important. Moreover, PAL is not known to be very stable at kilning temperatures. Indeed, Lourenço *et al.* (1991) have shown that PAL from cucumber (*Cucumis sativus* L.) lost 95% of its activity after a heat treatment at 55 °C for 50 min and was fully inactivated at 60 °C within 10 min. Moreover, it has been reported that when germinated barley with moisture contents over 10% is subjected to elevated temperature treatments, accelerated inactivation of the enzymes takes place (Pathirana *et al.*, 1983). Finally, the *de novo* synthesis hypothesis implies additional steps required to covalently bind phenolic acids to lignin or arabinoxylan. Since kilning involves a rapid dehydration of the grain, which denatures many enzymes and usually slows the activities of the remainders, this hypothesis seems to be quite unlikely. Therefore, the increase of the level of phenolic acids at 50 and 64 °C could rather be due to changes in extractability caused by differences in tissue moisture content: 45.5% water for germinated barley, 12.5% for kilning step 2 (50 °C), and only 5% for kilning step 4 (64 °C). This hypothesis seems to be more unlikely above 80 °C because the different kilning steps have approximately the same moisture content, between 3.5 and 5% water.

At higher temperatures, the decrease of hydroxycinnamic acids content could be explained in three ways. First of all, bound phenolic compounds could be released during kilning. The linkages between *p*-coumaric acid and lignin and between ferulic acid and arabinoxylans could be broken by the action of temperature. Second, lignin could begin to be degraded, leading to the release of derivatives of phenolic acids. Lignins are polymers of cinnamyl alcohols which derive from hydroxycinnamic acids modified by changes in substitution patterns on the aromatic nucleus or by modification of the oxidation level in the side chain (Waterman and Mole, 1994). At last, the beginning of thermal degradation could occur. Indeed, Naim *et al.* (1988) have reported the thermal sensitivity of ferulic acid in a model solution of orange juice heated at 70 °C. Moreover, experiments realized in the laboratory have shown that the recovery of pure *trans*-ferulic acid submitted to 4 h at 85 °C was 95% in an aqueous medium and only 65% in a lipophilic medium (dodecane). All of these phenomena therefore lead to a stabilization or a diminution of the phenolic content in the cake.

Evolution of the Antioxidant Activity of the Hydrolyzed Cake during Kilning. The antioxidant activity of the hydrolyzed cake doubled until kilning step 6 (80 °C) and then returned to the initial level at kilning step 8 (90 °C). If these results are compared to the evolution of insoluble bound phenolic acids content (Figure 2), we notice that the profiles of the two curves are approximately parallel. Thus, phenolic compounds probably have a real influence on the evolution of the antioxidant activity of the hydrolyzed cake, even if they are not responsible for the whole activity. Knowing the antioxidant activity of these phenolic acids, 7.9 and 13.9 L/g, respectively, for *trans*-*p*-coumaric acid and *trans*-ferulic acid (Cuvelier *et al.*, 1992), we have attempted to evaluate their participation in the antioxidant activity. It has been considered that the activities of the *cis* and *trans* isomers were the same. The following equation was therefore established to evaluate the expected antioxidant activity (expressed in milliliters per gram):

$$1/EQ_{exp} = [t\text{-pCA}](1/EQ_{pCA}) + [t\text{-FA}](1/EQ_{FA}) + [c\text{-FA}](1/EQ_{FA})$$

[*t*-pCA] is the concentration of *trans*-*p*-coumaric acid (g/g), [*t*-FA] is the concentration of *trans*-ferulic acid (g/g), [*c*-FA] is the concentration of *cis*-ferulic acid (g/g), $1/EQ_{pCA}$ is the antioxidant activity of *trans*-*p*-coumaric acid (mL/g), and $1/EQ_{FA}$ is the antioxidant activity of *trans*-ferulic acid (mL/g).

The results, reported in Table 2, show that *p*-coumaric and ferulic acids are together responsible for 14−22% of the antioxidant activity of the hydrolyzed cake [values of $(1/EQ_{exp})/(1/EQobs)$].

However, a synergistic effect between *p*-coumaric and ferulic acids has been pointed out. Three ratios between the concentration of ferulic acid and the concentration of *p*-coumaric acid, corresponding to the ratio observed at the different kilning steps, have been studied: a ratio

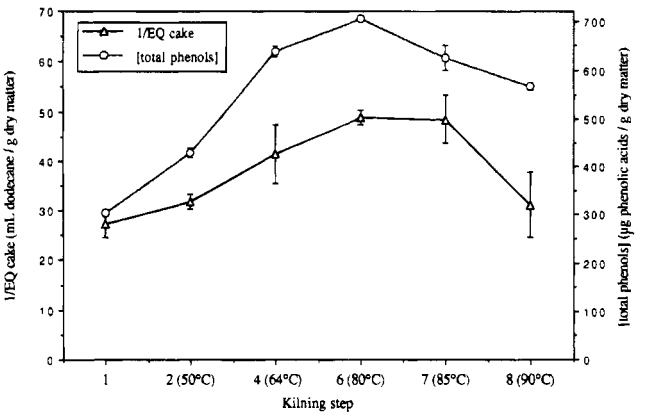

Figure 2. Comparison of the evolution of the antioxidant activity of the hydrolyzed cake and of the content of total insoluble bound phenolic compounds (bars indicate standard deviations calculated from two values for 1/EQ cake and from four values for [total phenols]).

Table 2. Determination of the Expected Antioxidant Activity, Taking into Account or Not the Synergistic Effect between *p*-Coumaric and Ferulic Acid[a]

kilning step	$1/EQ_{exp}$ (mL/g)	$(1/EQ_{exp})/(1/EQ_{obs})$ (%)	$(1/EQ_{exp+syn})/(1/EQ_{obs})$ (%)
1 (germinated barley)	3.8	14	22
2 (50 °C)	5.4	17	24
4 (64 °C)	7.9	19	30
6 (80 °C)	8.7	18	29
7 (85 °C)	7.7	16	26
8 (90 °C)	6.9	22	40

[a] $1/EQ_{exp}$ = expected value of 1/EQ, $1/EQ_{exp+syn}$ = expected value of 1/EQ taking into account the synergistic effect between *p*-coumaric and ferulic acids, $1/EQ_{obs}$, experimental value of 1/EQ.

Table 3. Antioxidant Activity of the Methanolic Extract and of the Hydrolyzed Cake of Germinated Barley and Malt Grains during Kilning[a]

kilning step	$1/EQ_{hydrolyzed\ cake}$ (mL of dodecane/ g of dry matter)	$1/EQ_{methanolic\ extract}$ (mL of dodecane/ g of dry matter)
1 (germinated barley)	27.3 ± 2.6	10.0 ± 1.0
2 (50 °C)	31.7 ± 1.5	15.5 ± 0.5
4 (64 °C)	41.4 ± 5.9	19.0 ± 0.5
6 (80 °C)	48.9 ± 1.6	22.0 ± 1.4
7 (85 °C)	48.5 ± 4.7	23.5 ± 1.8
8 (90 °C)	31.1 ± 6.5	21.0 ± 2.8

[a] Values represent means ± SD, calculated from two values).

of 3.3 for kilning step 2, a ratio of 2.9 for kilning steps 1, 4, 6, and 7, and a ratio of 2.1 for kilning step 8. The synergistic effect, determined by comparing the observed value of 1/EQ with the expected value (calculated with the above equation) is higher when the ratio is smaller: 1.4 ± 0.1 for a ratio of 3.3, 1.6 ± 0.1 for a ratio of 2.9, and 1.8 ± 0.1 for a ratio of 2.1. Such a synergistic effect had already been observed between synthetic or natural antioxidants (Kikugawa *et al.*, 1987; Coppen, 1989; Cuvelier *et al.*, 1992).

Thus, taking into account this synergistic effect, we can suppose that the participation of the studied phenolic acids in the antioxidant activity should vary from 20 to 40% (Table 2). Only a part of the antioxidant activity of the hydrolyzed cake has therefore been explained by the presence of *trans*-*p*-coumaric acid and *cis*- and *trans*-ferulic acids. This result may lead to several observations. First of all, phenolic compounds other than the quantified ones have been found in the hydrolyzed cake (peaks 2 and 5, Figure 1) and could have antioxidant power. Second, it has been supposed that *cis* and *trans* isomers had the same antioxidant activity. Finally, some compounds have perhaps not been detected in our working conditions.

Evolution of the Antioxidant Activity of the Methanolic Extract during Kilning. The antioxidant activity of the methanolic extract increased until step 6 (80 °C). Then it is quite stable (Table 3). However, at each step, the values of 1/EQ are about 2-fold less important for the methanolic extract than for the hydrolyzed cake.

Preliminary studies, realized by HPLC combined with mass spectrometry, have shown the presence of *trans*-*p*-coumaric and *trans*-ferulic acids in the methanolic extract, but mainly flavan-3-ols, including (+)-catechin and dimeric flavanols, are present (Maillard *et al.*, 1995). These compounds have already been reported by several authors who have identified (+)-catechin, (−)-epicatechin, procyanidins B_3 and C_2, propelargonidin, prodelphinidin B_3, and trimers of prodelphinidin in barley (Jerumanis, 1979, 1985; McMurrough, 1981; Mulkay *et al.*, 1981).

The values of 1/EQ, determined by the test of Cuvelier *et al.* (1990), are, respectively, 58.8 and 15.4 L/g for (+)-catechin and (−)-epicatechin. These values are higher than those of the studied phenolic acids. According to Ariga *et al.* (1988), procyanidins B_1 and B_3 are much stronger than (+)-catechin in their antioxidant activity, evaluated in an aqueous linoleic acid−β-carotene model system. These results tend to show that the antioxidant activity of the methanolic extract should be higher than the activity of the hydrolyzed cake.

However, a preliminary quantification (unpublished results) of *trans*-*p*-coumaric, *trans*-ferulic acids, and flavan-3-ols in the methanolic extracts would explain this result. The quantity of free phenolic acids would be 100-fold lower than the quantity of bound phenolic acids. Even if this level seems to increase 2-fold during kilning, confirming the result of McMurrough and Byrne (1992), the participation of the phenolic acids in the antioxidant activity of the methanolic extract is still negligible. This activity would mainly be due to flavan-3-ols, the content of which is yet lower than the content of bound phenolic acids. These results could therefore explain the low antioxidant activity of the methanolic extract compared to the hydrolyzed cake.

Once more, the increase of the 1/EQ value up to kilning step 6 (80 °C) could partially be explained by the release of bound phenolic compounds from the outer layers of the grain under the effect of temperature but more probably by a better extraction of the dry tissues.

It is striking to note that the decrease of the 1/EQ value at 90 °C is very slight in the methanolic extract compared to the hydrolyzed cake, perhaps because the main antioxidant compounds of these extracts are more heat stable.

Conclusion. Insoluble phenolic acids, particularly *p*-coumaric and ferulic acids bound to the cellular walls of the germinated barley grains, are very interesting potential antioxidants of the beer. To limit lipid oxidation during beer production, it may be possible to release them as early as the first step of kilning, thus increasing the level of free phenolic compounds. Two successive processes can be proposed: after an alkali treatment that renders polymers more susceptible to enzymic hydrolysis (Etok Akpan, 1993), the cross-bridges between ferulic acid and carbohydrate complexes and the linkages between *p*-coumaric acid and lignin could be broken by commercial enzymes.

However, phenolic acids should be eliminated before the end of the process because their degradation products, such as *p*-vinylguaiacol formed from decarboxylation of ferulic acid, can be responsible for off-flavors (Naim *et al.*, 1988).

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