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Food Chemistry 99 (2006) 711–717
Food Chemistry
www.elsevier.com/locate/foodchem

Rapid communication

Influence of malt browning degree on lipoxygenase activity

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Received 13 June 2005; received in revised form 15 August 2005; accepted 15 August 2005

Abstract

The development of off-flavour in pale beer is generally attributed to the oxidation of the intermediate products of lipid degradation catalysed by lipoxygenase during mashing. This paper deals with the effect of speciality malts, having different browning levels, on malt lipoxygenase. The results obtained showed that lipoxygenase activity, which was found to be present only in the pale malt, was affected by mashing temperatures above 63 °C and by the presence of Maillard reaction products in the speciality malts. In particular, the addition to pale malt of speciality malts, having high inhibitory capacity towards lipoxygenase but low colouring potential, was effective in reducing the off-flavour development in pale beer.

Keywords: Colour; Lipoxygenase; Maillard reaction products; Malt; Off-flavour; Worts

1. Introduction

Sensory degradation of packaged beer during storage is one of the most serious problems in the brewing industry. As is known, pale beer can undergo quality loss during storage due to changes in both appearance (e.g., turbidity, browning) and flavour through the formation of undesired odour compounds (Boivin, 1995; Kunze, 1999; Fix, 1999; Bamforth, 1999). Beer which undergoes off-flavour development is regarded as stale.The beer industry is generally able to prevent colour changes and turbidity by means of technologies designed to obtain colloidal and microbial stabilisation. On the other hand the control of the mechanisms responsible for stale aroma development is very difficult, due to the very low concentrations of off-flavour compounds, most of which are still unknown (Angelino et al., 1999). Besides, it must be pointed out that some of these substances are not present in freshly produced beer but can develop during storage (Evans et al., 1999).

Several mechanisms are reported to be involved in the formation of stale flavour. The most reliable one is the oxidation of polyunsaturated fatty acids with a 1,4- *cis–cis*-pentadiene structure by lipoxygenase, which is naturally present in barley. The products of the enzymatic reaction are unstable hydroxyperoxides which may undergo further reactions to form volatile carbonyl compounds, characterised by having 7–10 carbon atoms and low odour thresholds (0.05–0.5 $\mu g\, l^{-1}$) (Jamieson & Van Gheluwe, 1970; Meilgaard, 1972, 1993; Palamand & Hardwick, 1969). Among these, 2-*trans*-nonenal, which has a papery, cardboard-like flavour, is one of the major components in stale beer (Brown, 1989; Fix, 1999). It is widely accepted that lipid degradation by lipoxygenase occurs during mashing, followed by decomposition of intermediates in the brewhouse and/or in the packaged product during storage (Angelino et al., 1999; Evans et al., 1999; Hashimoto, 1972).

For these reasons, the changes in odour profile are generally considered to be the limiting factors of beer shelf-life. Therefore, retarding the development of beer stale flavour represents one of the greatest challenges for brewers (Hashimoto, 1981). Several procedures have been proposed to brewers, such as selection of malts with very low lipoxygenase concentrations, oxygen exclusion during the brewing and packaging processes, and use of exogenous

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.08.048

antioxidants (Boivin et al., 1993; Boivin et al., 1995; Boivin, 2001). In this respect, malt may contain either natural or heat-induced antioxidants, the former originating from barley (mainly phenolic compounds), the latter from the kilning and, mostly, roasting processes (mainly non-enzymatic browning products, i.e., melanoidins) (Duh, Yen, Yen, & Chang, 2001; Goupy, Hugues, Boivin, & Amiot, 1999). Although various studies have demonstrated lipoxygenase inhibition by a range of phenolic substances, it is a matter of fact that the antioxidant capacity of malt-containing phenols is unable to protect pale beer from staling (Aerts et al., 2001). On the other hand despite a very limited information on the effects of melanoidins on lipoxygenase (Boivin, 1995; Boivin et al., 1993; Richard-Forget et al., 1995), experimental evidence suggest that beers obtained by using brown speciality malts are more stable than the corresponding pale beers (Huige, 1993). In particular, the higher the browning degree of malts, the greater is the stability of beer (Boivin, 1995).

The aim of this investigation was to study the effect of non-enzymatic browning (NEB) products on lipoxygenase activity of barley and to establish possible relationships between the antioxidant activity of NEB products and their inhibiting effect on lipoxygenase activity. Research was also aimed to suggest, on the basis of the obtained results, technological strategies, other than those commonly adopted, for producing pale beer with improved sensorial quality and longer shelf-life.

2. Materials and methods

2.1. Materials

Experiments were performed using pale malt and three kinds of speciality malts, namely melanoidin, caramel and black, characterised by increasing browning degree. According to the supplier, the pale, melanoidin, caramel and black malts were characterised by colour values, defined by European Brewery Convention (EBC) values, of 3.5, 60, 120 and 1400, respectively.

2.2. Sample preparation

2.2.1. Malt extracts

Malt extraction was carried out according to the method of Baxter (1982). Five gramme of malt were frozen at −30 °C for 15 min and subsequently finely ground in a Bühler Universal Laboratory disc mill (type DLFU, Bühler-Miag Gmbh, Braunschweig, Germany). The gap between the grinding discs was 0.20 mm. Samples were then extracted at 4 °C with 0.1 M acetate buffer (pH 5), containing 0.1 M NaCl. After centrifugation at 12,000*g* for 30 min at 4 °C, extracts were filtered with a polypropylene filter (0.45 μm Whatman, Clifton, USA). Fresh malt extracts were prepared in duplicate. Low temperature was adopted to minimise the oxidation of malt antioxidants and loss of lipoxygenase activity.

2.2.2. Worts

Worts were obtained by mashing according to De Buck, De Rouck, Aerts, and Bonte (1998). Twenty-five gramme of ground pale malt were suspended in 50 ml of 0.1 M acetate buffer (pH 5.5), pre-warmed to 50 °C and stirred for 2 min. The obtained mixture malt/buffer (1:2 w/w) was then mashed in a water bath at 50 °C for 15 min and afterwards at 63 °C for a further 30 min. The temperature was raised by 1 °C/min. An identical test was carried out adding to the pale malt increasing aliquots of speciality malts. At pre-fixed lengths of time, aliquots of the mixtures were quickly chilled on ice and centrifuged at 800*g* for 20 min at 4 °C. The supernatant was filtered with a polypropylene filter and immediately analysed.

2.3. Analytical determinations

2.3.1. Antiradical activity

Antiradical activity was determined according to the method of Brand-Williams, Cuvelier, and Berset (1995). The bleaching rate of a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH·) (Sigma Chemical Co., St. Louis, MO, USA), at a characteristic wavelength in the presence of the sample was monitored. In its radical form, DPPH· absorbs at 515 nm, but, upon reduction by an antioxidant or a radical species, its absorption disappears. Aliquots of samples (from 20 to 150 μl) were added to a 6.0×10^{-5} M methanol DPPH· solution to a final volume of 2 ml. The bleaching of DPPH· was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 10 min.

Reaction rates were calculated using Eq. (1), proposed by Manzocco, Anese, and Nicoli (1998):

$$\frac{1}{\mathrm{Abs}^3} - \frac{1}{\mathrm{Abs}_0^3} = -3kt, \tag{1}$$

where k is the DPPH· bleaching rate, Abs_0^3 is the initial absorbance value and Abs^3 is the absorbance at increasing time t. The chain-breaking activity was expressed as the slope obtained from Eq. (1) ($-\mathrm{Abs}^{-3}\,\mathrm{min}^{-1}$) per gramme of dry matter. All the dry matter of the sample was assumed to possess antioxidant properties.

2.3.2. Redox potential

The redox potential was determined according to the method proposed by Manzocco et al. (1998). Measurements were made using a platinum indicating electrode and a silver/silver chloride reference electrode, connected to a digital voltmeter (Hanna Instruments, model 8417, Milan, Italy). Calibration was done against a redox standard solution ($E^\circ = 220$ mV at 25 °C). The electrode was inserted into a 50 ml three-neck flask containing 15 ml samples, previously diluted with water (the ratio sample: water was 1:3 w/w). Prior to analysis, oxygen was removed from the flask by continuous flushing with nitrogen for 10 min. The redox potential (E) was recorded for at least 20 min, until a stable reading was reached, that is, until E changed by <1 mV over 2 min.

2.3.3. Absorbance

Absorbance measurements were recorded at 350 nm with a spectrophotometer (Uvikon 860, Kontron Instruments, Milan, Italy). This absorbance represents the index of sample browning degree (Billaud, Maraschin, & Nicolas, 2004). When necessary, appropriate dilutions were made in order to obtain absorbance signals on scale.

2.3.4. Lipoxygenase activity

Lipoxygenase activity was assayed using the method of Anthon and Barret (2003) based on absorption at 234 nm of the conjugated dienes formed when linoleic acid (used as substrate) was oxidised in the presence of lipoxygenase. The substrate consisted of 10 μl of linoleic acid, 4 ml of H_2O, 1 ml of 0.1 N NaOH and 5 μl of Tween 20. The mixture was shaken and diluted to 25 mL with water. Assays were performed at 25 °C by adding 50 μl of samples (either malt extracts or worts) to 100 μl of substrate and 1.85 ml of phosphate buffer (0.1 M, pH 6.8) in a 1 cm cell. The increase in absorbance at 234 nm (A_{234}) was monitored using a Varian DMS 80 spectrophotometer (Varian Technotron Pty. Ltd., Mulgrave, Australia). In order to overcome interference due to absorbance, at this wavelength by other malts components, 1 ml of 1 N NaOH was added to the system after 5 min of reaction (Anthon & Barret, 2003). The addition of NaOH caused enzyme denaturation as well as a shift of the acid–base equilibrium of linoleic acid to its salt form, thus a clear solution was obtained. Preliminary trials showed the absence of interferences in absorbance values due to interactions between the NEB products and the substrate. The residual enzymatic activity was expressed as $A_{234}\,\mathrm{min}^{-1}$ or as the percentage ratio between the lipoxygenase activity assessed in the pale malt and that measured in the pale-speciality malt mixtures. One unit of activity was defined as the increase of 0.10 $A_{234}\,\mathrm{min}^{-1}$ in the conditions previously described.

2.3.5. Colour

Colour analyses were carried out using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before measurements. Colour was expressed in L^*, a^* and b^* Hunter scale parameters and a^* and b^* were used to compute hue angle ($\tan^{-1} b^*/a^*$) (Clydesdale, 1978).

2.3.6. Total solid content

Total solid content determinations were carried out by a gravimetric method (AOAC, Official method 985.14, 1995).

2.4. Data analysis

The results reported here are the averages of at least three measurements, and the coefficients of variation, expressed as the percentage ratio between the standard deviation (SD) and the mean values, were lower than five for total solid content and colour, 15 for enzymatic and chain breaking activity, and eight for redox potential. One-way analysis of variance was determined using the Tukey–Krammer test by using a JMP 3.2.5 package (SAS Institute Inc., Cary, NC). Differences between means were considered to be significantly different at $P < 0.05$.

Goodness-of-fit was evaluated by means of the determination coefficients (R^2) and the corresponding P values, and the sum of squares (SS) of residuals.

3. Results and discussion

Table 1 shows the total solid content, absorbance at 350 nm and colour values, as well as residual lipoxygenase activities of pale, melanoidin, caramel and black malts. In agreement with the EBC colour values, the browning degree decreased as follows: black > caramel > melanoidin > pale. With reference to the enzymatic activity, only the pale malt presented lipoxygenase activity. Although some authors have reported some residual activity in dark malts (Baxter, 1982; Coghe, Vanderhaegen, Pelgrims, Basteyns, & Delvaux, 2003), it can be suggested that the kilning and roasting temperatures used to prepare the considered speciality malts led to enzyme inactivation.

Fig. 1 shows the effects of temperature and of the addition of caramel and black speciality malts on the lipoxygenase activity of pale malt during mashing. Although a maximum of activity was detected after 10 min, in agreement with Martel, Kohl, and Boivin (1991), only slight changes of enzymatic activity were observed during mashing at 50 °C. This result was expected because the optimum for lipoxygenase activity is around 40 °C (Lulai & Baker, 1976). On the other hand, lipoxygenase activity greatly decreased in mashing step carried out at 63 °C, in accordance with the literature reports (Kobayashi, Kaneda, Kano, &

Table 1
Total solids content, absorbance at 350 nm, colour and residual lipoxygenase activity[a] of pale, melanoidin, caramel and black malts

Malt type	Total solids content (%)	Absorbance at 350 nm	Colour				Lipoxygenase activity ($A_{234}\,\mathrm{min}^{-1}$)
			L^*	a^*	b^*	Hue angle	
Pale	96.9 ± 0.08	2.0	60.5 ± 2.1	4.6 ± 0.3	23.1 ± 0.4	78.8	0.086 ± 0.001
Melanoidin	97.1 ± 0.13	10.8	54.7 ± 0.9	5.8 ± 0.3	23.3 ± 0.7	75.9	n.d.
Caramel	97.0 ± 0.41	17.2	47.1 ± 1.7	8.3 ± 0.7	24.5 ± 0.7	71.3	n.d.
Black	96.0 ± 0.01	77.0	23.6 ± 0.7	7.0 ± 0.7	5.1 ± 0.8	36.0	n.d.

Hue angle ($\tan^{-1} b^*/a^*$).
n.d., not determined; i.e., value lower than the detection threshold.
[a] Data are presented as the means ± SD ($n = 3$).

Koshino, 1993; Martel et al., 1991). In this case, the enzyme was inactivated after 45 min. These results suggest that the enzymatic inactivation can be attributable only to the effect of temperature. When 10% caramel and 2% black malts were added to the pale malt a contextual temperature-speciality malt addition effect was observed on lipoxygenase activity (Fig. 1). In fact, it can be observed that these malts caused, per se, a great decrease in the enzymatic activity (more than 50%). In particular, the higher the browning degree the greater was the decrease.

Fig. 2 shows the changes of lipoxygenase activity of pale malt added with increasing concentrations of melanoidin, caramel and black malts, measured after 7 min from the beginning of the mashing process at 50 °C. The enzymatic activity decreased with the increasing of malt concentration and the browning degree. It can be observed that, compared with the melanoidin and caramel malts, very small amounts of the black malt were sufficient to cause a marked activity reduction.

In order to evaluate the influence of the speciality malts on the lipoxygenase activity, the inhibition indices (m) were calculated by linear regression analysis of the changes of enzymatic activity as a function of malt concentrations

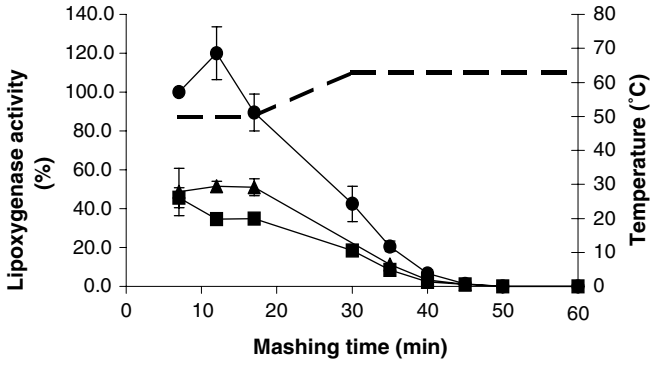

Fig. 1. Effect of addition of 10% (w/w) caramel (▲) and 2% (w/w) black (■) speciality malts on lipoxygenase activity of pale malt during mashing. The influence of mashing temperature (----) on enzymatic activity of pale malts (●) is also shown.

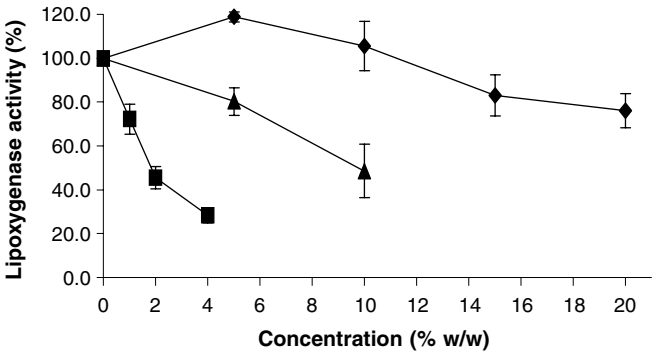

Fig. 2. Changes of lipoxygenase activity of pale malt as a function of increasing concentrations of melanoidin (◆), caramel (▲) and black (■) speciality malts.

Table 2
Inhibition indices (m), and corresponding determination coefficients (R^2), corresponding P values and sum of squares (SS) of residuals, of speciality malts on lipoxygenase activity of pale malt

Speciality malt	Inhibition index (m)	R^2	P	SS
Melanoidin	3.0	0.909	$<10^{-3}$	227
Caramel	5.1	0.918	$<10^{-2}$	249
Black	17.2	0.906	$<10^{-3}$	531

(Table 2). As expected, the capacity of speciality malts to inhibit lipoxygenase increased with the increasing of their browning degree. In particular, it can be stated that the inhibition index for the black malt was approximately 3- and 6-fold higher than those of caramel and melanoidin malts, respectively.

As already pointed out, the inhibiting capacity of malts on the lipoxygenase activity can be attributable to the antioxidant properties of the NEB products and, in particular, to the brown high molecular weight polymers, named melanoidins (Billaud, Garcia, Boivin, & Nicolas, 1997; Boivin, 1995). These compounds, which are formed during thermal treatments (e.g., kilning and roasting), may exhibit antioxidant activity through several mechanisms. In fact, they may possess reducing properties, chain-breaking activity, oxygen consuming and metal-binding capacity, which can be exhibited simultaneously (Bressa, Tesson, Dalla Rosa, Sensidoni, & Tubaro, 1996; Homma & Murata, 1995; Lingnert & Waller, 1983; Namiki, 1990; Nicoli, Anese, Manzocco, & Lerici, 1997).

The chain-breaking activity and redox potential values of the considered malts are shown in Fig. 3. It must be pointed out that these two measurements give different information on the overall antioxidant activity of a food system. The chain-breaking activity evaluates the kinetic rate of the most reactive antioxidants toward a radical species. On the other hand, the redox potential, which is a thermodynamic measure, gives indication of the oxidising/reducing efficiency of all the food antioxidants, including those which react slowly (Nicoli & Anese, 2001; Nicoli, Toniolo, & Anese, 2004). In broad terms, that means that the "slow" antioxidants could have an important role in

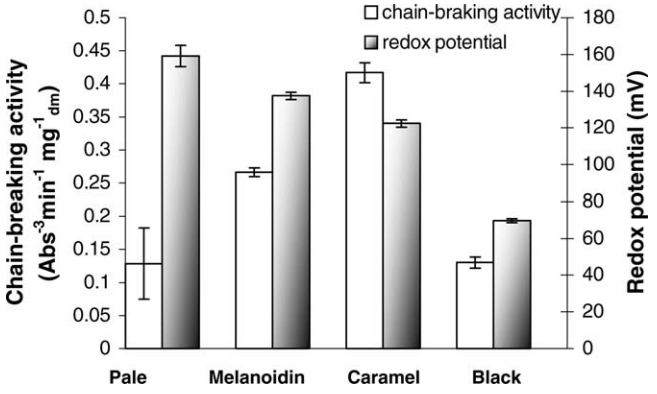

Fig. 3. Chain-breaking activity and redox potential values of pale, melanoidin, caramel and black malts.

determining and maintaining the antioxidant properties of beer during prolonged storage times. Fig. 3 shows that the redox potential decreased with the increasing of malt browning degree. Thus, in agreement with Woffenden, Ames, Chandra, Anese, and Nicoli (2002), the highest reducing capacity of the darkest malt can be related to a high content of heat-induced melanoidins (Moll & Moll, 1986). On the other hand, the chain-breaking activity did not progressively increase with the browning degree. In fact, a maximum of anti-radical activity was found for the caramel malt, having an intermediate browning degree, while the black sample presented a value similar to that of the pale malt. This result is in agreement with previous observations reported by Nicoli et al. (1997) and Woffenden, Ames, and Chandra (2001), respectively, for roasted coffee and malt. According to these authors, in fact, the intermediate products of the NEB reaction would be responsible for the high chain-breaking activity; while very intense heat treatments would prime further reactions (such as pyrolysis). These reactions would be responsible for transforming intermediate NEB products into compounds having lower chain-breaking properties. This suggests the presence, in kilned and roasted malt, of antioxidants slightly reactive toward radical species, but able to exhibit their activity throughout non-radical mechanisms. This would explain why the black malt, which had low anti-radical properties, showed the highest reducing capacity. It must also be pointed out that polyphenols, such as flavonols, flavanols and phenolic acids, as well as tocopherols and carotenoids, could contribute to the antioxidant activity of pale malts (Goupy et al., 1999; Maillard & Berset, 1995). However, it is likely that, during the heat-treatment, these natural antioxidants are progressively transformed into compounds without or with less antioxidant activity, or incorporated into melanoidin structures to form complex compounds with increased antioxidant capacity (Nicoli & Manzocco, 2005; Woffenden et al., 2001).

Table 3 shows the equations obtained from linear regression analysis of the changes of lipoxygenase inhibition indices as a function of absorbance at 350 nm, redox potential or chain-breaking activity of malts. Based on the above observations, the chain-breaking activity value of the black malt was not computed for the linear regression analysis. In all cases, a good correlation was found, suggesting that the inhibiting capacity of malts can be ascribable to both their anti-radical and their reducing properties.

In the light of these results, the study was focussed on looking for processing conditions for pale beer, able to minimise the lipoxygenase activity in order to preserve its sensorial properties. In particular, the possibility of reducing lipoxygenase activity during mashing, throughout the formulation of proper malt mixes, was investigated. Nowadays, the most used methods for minimising the activity of lipoxygenase in malts are represented by the selection of malts without or with low enzymatic activity or by the adoption of high temperatures (around 63 °C) during mashing (Back et al., 1999; Billaud & Nicolas, 2001; Boivin et al., 1995; Herrmann, 1999). The former approach is scarcely used due to the difficulty of obtaining malts with low-enzymatic activity, as well as of determining the residual activity on the industrial level. The latter approach, consisting in carrying out mashing at high temperatures, without the proteolytic step at 50–52 °C, is more commonly used. However, its application requires malts which have already been subjected to proteolysis during kilning. Besides, the absence of the proteolytic step at 50–52 °C during mashing could lead to some problems in the final product. These include physico-chemical instability, due to the formation of protein-phenol complexes and subsequent development of turbidity, as well as reduced release of free amino acids, which are indispensable for yeast metabolism and thus fermentation development.

Fig. 4 shows the changes in hue angle of worts obtained by adding increasing concentrations of melanoidin, caramel and black malts to pale malt. The same hue angle value of 86 was obtained by the addition of 15% melanoidin, 10% caramel and 1% black malts. The samples with a hue angle of 86 were amber-coloured. As the wort colour is indicative of that of the final product, this value can be satisfactory for the sensorial quality of pale beer. Comparing Fig. 4

Table 3
Equations, and corresponding determination coefficients (R^2), corresponding p values and sum of squares (SS) of residuals, obtained from linear regression analysis of the changes of enzymatic activity inhibition index as a function of absorbance at 350 nm, redox potential or chain-breaking of malts

Equation	R^2	p	SS
$m = 0.223{*}\mathrm{Abs}_{350\,nm} + 0.583$	0.995	$<5\times10^{-3}$	3.06
$m = -0.201{*}E + 31.0$	0.983	$<10^{-2}$	0.88
$m = 17.78{*}\mathrm{CB} - 2.09$[a]	0.984	$<5\times10^{-2}$	0.18

m, inhibition index of lipoxygenase activity.
Abs 350 nm, absorbance of malts measured at 350 nm.
E, redox potential (mV).
CB: chain-breaking activity ($\mathrm{Abs}^{-3}\ \mathrm{min}^{-1}\mathrm{mg}_{dm}^{-1}$).
[a] Chain-breaking activity value of the black malt was not computed for linear regression analysis.

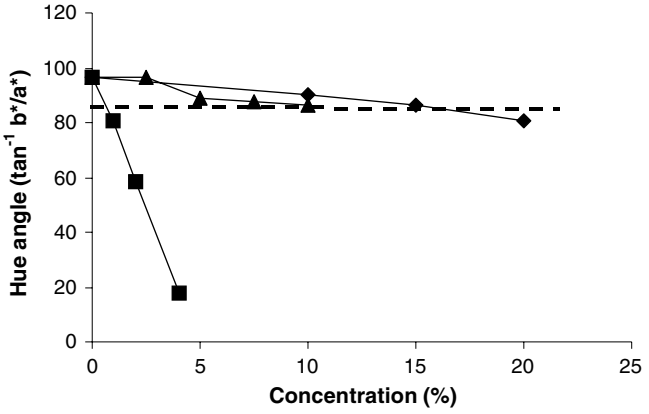

Fig. 4. Changes in hue angle of worts obtained by adding increasing concentrations of melanoidin, caramel and black malts to pale malt.

Table 4
Chain-breaking activity and redox potential of mixtures of pale malt and 15% melanoidin, 10% caramel or 1% black speciality malts, having a hue angle value of 86

Speciality malt	Chain-breaking activity (Abs^{-3} $min^{-1}mg_{dm}^{-1}$)	Redox potential (mV)
15% melanoidin	0.038	21.0
10% caramel	0.040	9.6
1% black	0.001	0.7

with Fig. 2, it is possible to observe that the addition of 10% caramel malt led to the greatest enzymatic inhibition.

Table 4 shows the chain-breaking activity and the redox potential values of the above considered worts with a hue angle value of 86. The mixture containing 10% caramel malt had a chain-breaking activity value comparable to that of the 15% melanoidin malt and higher than that of the 1% black one, while its redox potential assumed intermediate values. These results confirm that both the antiradical activity and the reducing properties contributed to the overall antioxidant capacity of caramel malt and thus to its inhibitory activity.

Although further investigation is needed, these results suggest that the addition to pale malt of speciality malts, having high inhibitory capacity towards lipoxygenase but low colouring potential, could represent a technological intervention in order to reduce the off-flavour development in pale beer. This approach, as based on a simple and fast colorimetric determination, could also be easily managed in the brew-houses.

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