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Effect of quality parameters on lipoxygenase activity of wheat malt

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RESEARCH ARTICLE

Abstract

Wheat malt is one of the main materials used in China's brewing industry, but in previous studies the relationship between the extent of proteolysis (Kolbach index), lipoxygenase (LOX) activity, 2-thiobarbituric acid (TBA) value, and other quality indices of the wheat malts was not sufficiently clear to guide wheat malting. In this study eight wheat malts with different Kolbach indices from the same wheat variety (*Triticum aestivum* L.) L-2, were prepared. After malting, the LOX activity was found to have decreased by 38.45 to 66.1%. When the Kolbach index was in the range of 31.4-54.2%, LOX activity of the malts increased alongside the increase in the Kolbach index until the Kolbach index was 39.5% then decreased, and there was a significant positive correlation between the Kolbach index and the TBA value: the correlation coefficient was 0.951 (P<0.01). When the Kolbach index was 37.0%, the LOX activity and the TBA value were lower and the other quality indices of the wheat malt were better. The higher TBA value of the wheat malts would relate to the linear increase in the colour of the congress wort.

Keywords: 2-thiobarbituric acid value, Kolbach index, lipoxygenase, *Triticum aestivum*, wheat, wheat malt

1. Introduction

Flavour stability has become one of the most important topics in brewing research over the past decades. Several mechanisms are reported to be involved in the formation of a stale flavour. The most reliable one is the oxidation of polyunsaturated fatty acids with a 1,4-cis-cispentadiene structure by lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) (Sovrano *et al.*, 2006). 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 (0.05-0.5µg/l). Among these, 2-trans-nonenal, which has a papery, cardboard-like flavour, is one of the major components in stale beer (Hirota *et al.*, 2006; Kuroda *et al.*, 2002). Therefore, the rate of LOX reaction should be considered as a quality factor of malt (Kaukovirta-Norja *et al.*, 1998).

The LOX activity is affected by the malting process (Kaukovirta-Norja *et al.*, 1998): steeping, germination and the subsequent drying render the state of kernels unstable with respect to the LOX reaction for at least two to three weeks. Furthermore, the malt LOX has an important influence on the quality of beer (Sovrano *et al.*, 2006). Guido *et al.* (2005) studied the change in LOX activity, the nonenal potential, the temperature and the moisture content at various stages of an industrial kilning process in the top and bottom malt layers. Significant differences occurred between the lower and upper malt bed, suggesting that the moisture content and temperature gradient play a key role in the production of E-2-nonenal during the early stages of kilning. The residual nonenal potential already present in the finished malt may account for approximately 25% of the total nonenal potential in the mash, depending on the residual LOX activity. LOX was strongly related to the nonenal potential for micro-malts. To reduce the negative effect in brewing, several procedures have been proposed to brewers, such as selection of malts with very low LOX concentrations (Jin *et al.*, 2008, 2011).

At present, wheat malt is widely used in China's brewing industry. There are many wheat varieties. The LOX activity in wholemeal flours from *Triticum monococcum*, *Triticum turgidum* and *Triticum aestivum* was studied by Hidalgo and Brandolini (2012). The highest LOX activity was observed in *T. aestivum*, followed by *T. turgidum* and *T. monococcum*. They also found that enzymatic activity was highest in the pH range 5-6; LOX activity was higher in the germ than in the bran or in the endosperm. As a result Hidalgo and Brandolini (2012) stated that selecting genotypes with a low LOX is feasible, however common wheats (*T. aestivum* L.) are most widely cultivated in China, and consequently widely used in China's brewing industry. Feng and his colleagues (Feng *et al.*, 2010) isolated the full-length nucleotide sequences of two LOX genes (*TaLOX1* and *TaLOX2*) from common wheat. They found that *TaLOX1* and *TaLOX2* were both expressed in the developing grains of two common wheat varieties, indicating that they may contribute to the total LOX activity in common wheat seeds. Though deletion of *TaLOX1* and/or *TaLOX2* is possible, no LOX-gene-deficienct common wheat has so far been reported and cultivated in China.

Recently, LOX in wheat was investigated more intensively due to its influence on the sensory quality of wheat products. Leenhardt *et al.* (2006) explored the LOX activity among cultivated diploid, tetraploid and hexaploid wheat species and bread wheat varieties. The highest LOX activity was found in bread wheat. In another research, 57 accessions, belonging to different *Triticum* species, were assessed for LOX activity by Hidalgo and Brandolini (2012). The correlation between endogenous LOX activity and 15 wheat grain quality parameters in three bread wheat populations was also analysed (Permyakova *et al.*, 2010). It was shown that enzyme activity influences the weight of the 1000 grains of wheat, dough deformation energy, dough tenacity, and mixing properties. The correlations between the enzyme activity and the basic quality parameters were negative at high activity levels and the ability of LOX to strengthen gluten was related to the lowering of dough extensibility. Cato *et al.* (2006) studied the effect of endogenous and exogenous LOX upon discolouration of white salted noodles as well as on the textural and structural attributes. It was concluded that the incorporation of the LOX preparation offers prospects for colour enhancement of white salted noodles.

As LOX activity was found to be influenced by the malting process (Kaukovirta-Norja *et al.*, 1998), recently, the preliminary characteristics of wheat LOX during malting were studied by our group (Sun *et al.*, 2012). Investigating the degree of proteolysis influence on LOX activity of wheat malt might be a good way to control the qualities of wheat malt, wort and beer. So this paper looks at the influence of the Kolbach index, proteolytic products including albumins, globulins, gliadins, glutenins and free amino nitrogen, and the TBA value on LOX activity in wheat malt.

2. Materials and methods

Materials

A wheat (*T. aestivum* L.) sample named L-2 was obtained from the Yantai Institute of Agricultural Science (Yantai, China P.R.). It is a kind of white farinaceous endosperm wheat with moisture content of 11.9%, thousand corn weight of 40.8 g on dry basis, germination rate of 96% and protein content of 13.6% dry matter. Eight malts from wheat L-2 with different Kolbach indices were acquired by controlling the different steep-out moisture and germination times. The characteristics of the wheat malts are listed in Table 1.

Main reagents

Linoleic acid (>99%; Aladdin Chemistry Co. Ltd, Shanghai, China P.R.), NaH_2PO_4, Na_2HPO_4 and acetic acid (AR, Tianjin Kaitong Chemical Reagent Co. Ltd, Tianjing, China P.R.), NaOH and NaCl (AR, Tianjin Baishi Chemical

Table 1. Characteristics of wheat malts.

Kolbach index (%)	31.4	34.9	37.0	37.6	39.5	41.0	42.7	54.2
Saccharification time (min)	10	9	5	6	6	5	6	5
Colour (EBC)	4.55	5.08	5.40	5.63	5.18	5.85	5.75	7.25
Turbidity (EBC)	2.25	2.03	2.33	2.29	2.16	2.42	2.16	2.63
Extract (%)	79.30	81.80	82.31	82.41	81.98	82.47	82.89	84.47
Free amino nitrogen (mg/100 g)	120.09	117.35	151.10	162.42	166.03	148.31	154.59	211.60
Diastatic power (WK)	384.05	385.90	456.10	449.57	496.72	439.46	426.89	477.10
Viscosity (cP)	1.54	1.66	1.59	1.66	1.61	1.63	1.66	1.54
Acidity (mg/100 ml)	0.68	0.77	0.82	0.86	0.78	0.85	0.91	1.21
pH	6.26	6.17	6.15	6.14	6.16	6.15	6.16	6.01

Reagent Co. Ltd, Tianjing, China P.R.), H_2O_2 and Tween-20 (Tianjin Basifu Chemical Reagent Co. Ltd, Tianjing, China P.R.), KH_2PO_4 and $Na_2S_2O_3$ (AR, Tianjin Damao Chemical Reagent Co. Ltd, Tianjing, China P.R.), ninhydrin (AR, Shanghai Qiangshun Chemical Reagent Co. Ltd, Shanghai, China P.R.), D-fructose (BR, Sigma-Aldrich Fluka, Buchs, Switzerland), glycine (BR, Shanghai Miura Chemical Co. Ltd, Shanghai, China P.R.), $CuSO_4$ (AR, Tianjin Baishi Chemical Reagent Co. Ltd), orthoboric acid (AR, Tianjin Kaitong Chemical Reagent Co. Ltd).

Germination

Wheat was steeped at 16 °C using the following procedure: every 4 h-wet with 4 h-dry. A 0.13% H_2O_2 solution was used for the soaking water for the first 4 h steep. Compressed air was used for ventilation during the steep, and a suction method was used for oxygen during the air rest. Ventilation time was 15 min/h. The thousand corn weight of the samples was determined during the steeping process, and the moisture of the samples was calculated according to the method of Jin *et al.* (2008). Steeped for about 44-52 h, the steep-out moisture of the sample rose to 43-46%; the samples were germinated for five to seven days at 16 °C. Kilning was carried out according to the following schedule: 40 °C for 3 h, 45 °C for 3 h, 50 °C for 2 h, 55 °C for 1 h, 60 °C for 1 h, 65 °C for 2 h, 70 °C for 3 h, 75 °C for 3 h, 80 °C for 3 h.

Wheat malt analysis

After kilning, the malt samples were cleaned and the rootlets were removed, and the resulting malt was analysed according to Analytica-EBC (1998). The main quality indices of extract, diastatic power, saccharification time, Kolbach index, free amino nitrogen (α-AN), and wort viscosity were analysed.

Protein determination

Total protein content was determined by the Kjeldahl method according to Analytica-EBC (1998) and the factor used to convert nitrogen to protein was 5.70. The four protein fractions, including albumins and other soluble protein (ALSP), globulins, gliadins and glutenins, were extracted and determined according to the method of Jin *et al.* (2012).

The wheat samples were ground on a European Brewery Convention (EBC) mill (type DLFU, EBC-LF, De zhi jie Brew, Beijing, China P.R.). A sequential extraction was conducted to determine the four various proteins. The first step was to extract the ALSP. A 2.5 g aliquot of ground grain in 25 ml of distilled water was mixed with gentle shaking for 1 h and the mixture was centrifuged after extraction at 4,000×*g* for 15 min. The supernatant was collected as the ALSP extraction. The same processing was repeated two times and the distilled water amount for extraction was respectively changed to 12 ml and 8 ml; then all the supernatants were combined in a 50 ml volumetric flask and the volume was adjusted by a distilled water impregnant. The second step was to extract the globulins. The extraction process for the remainder of the ground grain was the same as that for ALSP, except that the solution was 0.5 M NaCl. The third and fourth step was a sequential extraction to determine the gliadins and the glutenins; the impregnant was 75% ethanol and 0.2% NaOH, respectively, and the extraction processes were the same as for the ALSP. The percentage of protein for each fraction was determined by the Kjeldahl method according to Analytica-EBC (1998).

Measurement of lipoxygenase activities

Preparation of crude enzyme extract

The preparation of crude enzyme extract was performed according to the method of Jin *et al.* (2011). The wheat malts samples were ground on an EBC mill. Wheat or malt powder (5.0 g) was extracted in 50 ml acetate buffer (pH 5.0, 0.1 mol/l, containing 0.1M NaCl) in an ice-bath for 15 min, then the homogenates were centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was filtered by a 0.22 μm film, collected in 100 ml volumetric flask and kept stored in ice water as the crude enzyme extract.

Preparation of substrate solution

The preparation of substrate solution was carried out according to the method of Sovrano (2006). A 40 μl linoleic acid was added to a 100 ml volumetric flask, then 16 ml distilled water, 20 μl Tween-20 and 4 ml 0.1 M NaOH was added. The homogenate was gently shaken in an ultrasonic ice bath until it was clear and transparent, then the volume was adjusted by distilled water.

Measurement of lipoxygenase activities

The LOX activities were measured according to the method of Sovrano *et al.* (2006). A 100 μl crude enzyme extract added 3.7 ml phosphate buffer (pH 5.5, 0.1 mol/l), followed by incubating in a 35 °C circulation water bath for 5 min, then 200 μl substrate solution was added, the reaction was kept for 5 min and was stopped by adding 2 ml 1 M NaOH. And the absorbance measured at 234 nm. 2 ml 1 M NaOH was added before substrate solution to inactive the LOX for the blanks. One unit of LOX activity was defined as 1 g of dry material that made the absorbance increase 0.1 (Jin *et al.*, 2011). LOX activity was calculated using the following formula:

$$X = \frac{A}{T} \times \frac{50{,}000}{B} \times \frac{1}{m \times (1 - \frac{M}{100})} \times \frac{1}{0.1} \quad (1)$$

Where: X = LOX activity (u); A = absorbance value of 234 nm; T = reaction of time (min); 50,000 = the total volume of the crude enzyme extract (µl); B = the volume of the crude enzyme extract (µl); m = the weight of malt (g); M = the water content of wheat malt powder (g/g).

Measurement of 2-thiobarbituric acid value

The TBA (2-thiobarbituric acid) value was detected by the method based on the improved measurement of Liu *et al.* (2008) with some modification. The wheat and wheat malts samples were ground on an EBC mill. Wheat or malt powder (5 g) was extracted in 25 ml distilled water in an oscillator (250 rpm) for 5 min, then the homogenates were centrifuged at 5,000 rpm for 5 min. The supernatant was collected. The same process was repeated. All the supernatant was collected in a 150 ml flask and filtered with double filter paper as the wort sample.

An aliquot (5 ml) of the wort sample was supplemented with 2 ml 50% acetic acid solution (including 0.33% TBA) followed by heating in a 60 °C water bath for 60 min; the reaction was stopped quickly by keeping it in an ice-water bath and the absorbance measured at 530 nm. An aliquot (5 ml) of the wort sample supplemented with 2 ml 50% acetic acid solution was used as control sample. The TBA value was calculated using the following formula:

$$TBA = A \times \frac{50}{5} \times \frac{1}{m \times (1 - \frac{M}{100})} \times 100 \quad (2)$$

Where: A = absorbance value of 530 nm; 50 = the total volume of the crude enzyme extract (ml); 5 = the volume of the crude enzyme extract (ml); m = the quality of malt (g); M = the water content of wheat malt powder (g/g).

Data analysis

Correlation and variance analysis were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and DPS 7.05 (Hangzhou Ruifeng Information Technology Co. Ltd, Hangzhou, China P.R.). The significant difference analysis between each point was analysed by SPSS. Different letters indicated a significant difference between two points, while the same indicated no significant difference (P>0.05).

3. Results and discussion

Difference analysis of TBA value, LOX activity of wheat and wheat malts

The content of carbonyl compound estimates the potential of beer staling. The TBA value is used more commonly to reflect the content of carbonyl compound of malt, wheat and beer, which can be determines by the specific reaction of carbonyl compounds in beer (Cao *et al.*, 2011; Herrmann *et al.*, 2010). Difference in TBA value, LOX activity among wheat and eight malts were analysed and shown in Table 2. It was shown that the LOX activity of the wheat was 132.00 u; after germination the LOX activity decreased. LOX activity of the malts were in the range of 50.76-87.26 u. In other words, after malting, the LOX activity decreased by 38.45% to 66.1%. Another study by our group found that the LOX was not resistant to the high temperature of the kiln (Sun *et al.*, 2012). So reducing LOX activity in malt during the kilning process could contribute to the LOX activity decrease in wheat malts.

There was a significant difference between the wheat and wheat malts LOX activity. Eight wheat malts LOX activities also exhibit significant differences, except that when the Kolbach index was 37.6 and 39.5%, 41.0 and 42.7%, 54.2 and 34.9%, 54.2 and 31.4%, the wheat malts LOX activities showed no differences.

The changes of the TBA value of wheat and wheat malt were opposite compared to the change of the LOX activity. The TBA value of wheat was 8.75, and that of the wheat malts was in the range of 21.04-37.80. There was a relatively small difference among the wheat malts TBA value.

Relationship between lipoxygenase activity and Kolbach index of wheat malt

The Kolbach index was the standard measure of malt modification in the 1970s and it remains important today. It is a measure of the extent of proteolysis that has taken place during malting and mashing. The relationship between LOX activity and the Kolbach index of wheat malt is shown in Figure 1 and it was shown that the LOX activity of the malts increased with an increase in the Kolbach index until the Kolbach index was 39.5%, then decreased (Figure 1). When the Kolbach index was 39.5% the LOX activity was 87.26 u. And, as shown in Table 2, the LOX activity of the wheat malt

Table 2. Lipoxygenase (LOX) activity and 2-thiobarbituric acid (TBA) value of wheat malts.

Kolbach index (%)	LOX activity (u)	TBA
wheat L-2	132.00±0.65^{a}	8.75±0.00^{g}
31.4	50.76±1.69^{e}	21.04±0.15^{f}
34.9	58.35±1.35^{d}	21.29±0.29^{f}
37.0	76.25±1.09^{c}	25.32±0.13^{e}
37.6	86.08±1.44^{b}	28.50±0.10^{bc}
39.5	87.26±2.75^{b}	28.09±0.10^{cd}
41.0	76.35±2.17^{c}	29.08±0.43^{b}
42.7	78.86±1.57^{c}	27.67±0.09^{d}
54.2	53.57±1.77^{de}	37.80±0.25^{a}

Values in the same column followed by the same superscript letter are not significantly different (P>0.05).

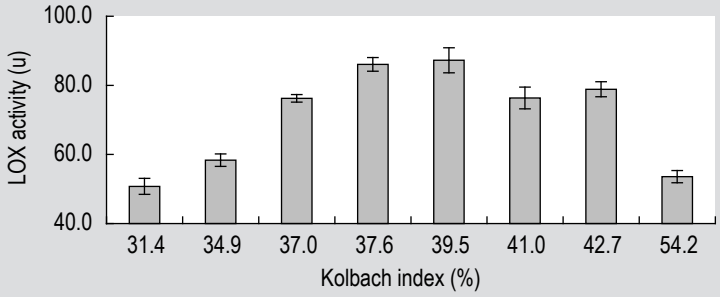

Figure 1. Relationship between lipoxygenase (LOX) activity and Kolbach index.

with Kolbach index of 39.5% was not significantly different compared to that of the wheat malt with Kolbach index of 37.6%. High LOX activity may catalyse the unsaturated fatty acids into substances that could affect the flavour of the beer. So to decrease the LOX activity of the wheat malt the most suitable Kolbach index was lower than 37.6% or higher than 39.5%.

Relationship between LOX activity and the four protein fractions of the wheat malts

The relationship between the four protein constituents (the ALSP albumins, globulins, gliadins and glutenins) and the LOX activity of the wheat malts is shown in Figure 2.

It was shown that with increasing ALSP of the wheat malts, LOX activity was not linear correlated. With the increase in globulins of the wheat malts, LOX activity increased. LOX activity had a significant positive correlation with the globulins (r=0.870**, $P<0.01$); the correlation between the LOX activity and the globulins could be interpreted as meaning that the LOX belonged to the globulins. There was no obvious correlation found between the gliadins or glutenins content and the LOX activity.

Relationship between TBA value and Kolbach index of wheat malt

During malting, the aldehydes of malt could be produced by the Maillard reaction between the degradation matter of the starch and the protein, such as the glucose, maltose, free amino acid and Low-molecular-mass polypeptide. The relationship between TBA value and Kolbach index of wheat malt was shown in Figure 3. It was found that when the TBA value was in the range of 31.4-54.2%, the higher the Kolbach index the higher the TBA value; there was also a significant positive correlation between the Kolbach index and the TBA value, the correlation coefficient being 0.951 ($P<0.01$). TBA value mainly reflected the aldehyde content of malt, wheat and beer; one of the main aldehydes was 5-methyl furfural. Because the TBA value represents the aging degree of beer, it was expected to be much lower, as

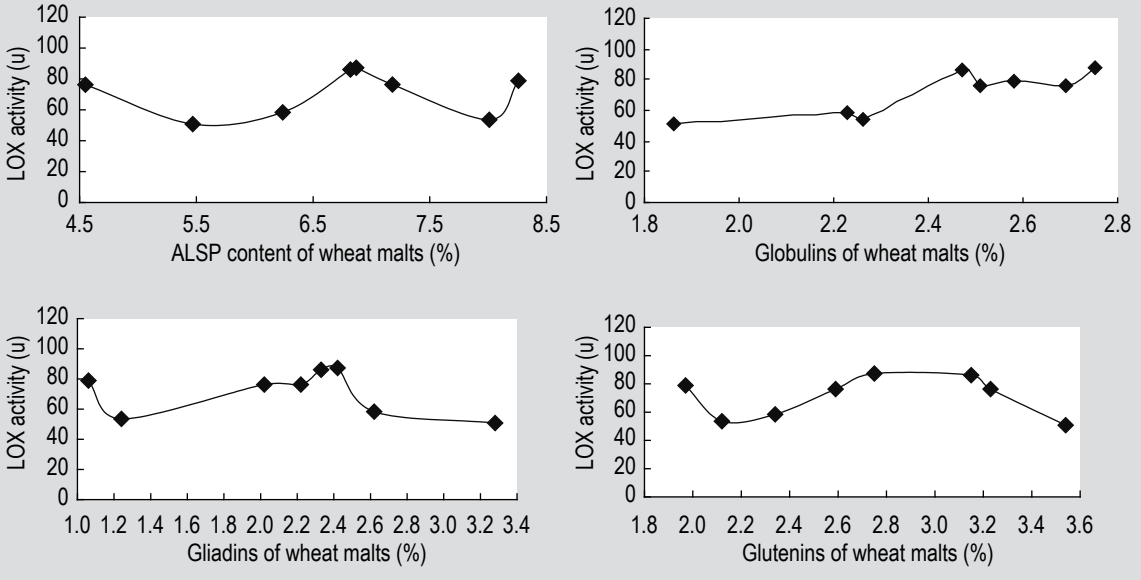

Figure 2. Relationship between four protein fractions and lipoxygenase (LOX) activity of wheat malts.

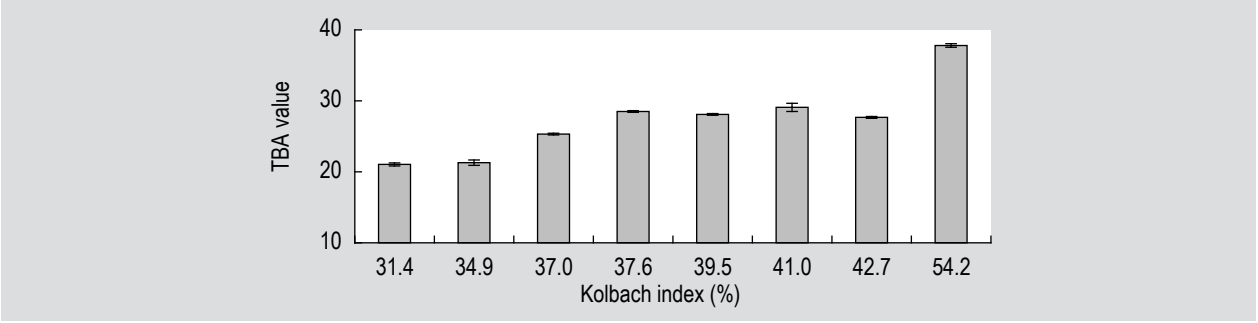

Figure 3. Relationship between 2-thiobarbituric acid (TBA) value and Kolbach index.

well as the LOX activity. To acquire a lower TBA value in wheat malts, the Kolbach index of the wheat malts must be lower.

Based on Figure 1 and 3, to lower the LOX activity and the TBA value, the Kolbach index should be lower than 37.6%. Taking into consideration the other quality indices, the suitable Kolbach index is 37.0%.

Relationship between LOX activity and TBA value and qualities of the wheat malts

The relationship between quality indices and the LOX activity of the wheat malts was shown in Table 3. There was no correlation between the LOX activity and the other quality indices of the wheat malts, i.e. the LOX activity of the wheat malts has no direct influence on the qualities of the wheat malt.

The correlation between the characteristics of the malts and TBA value was analysed and the results are shown in Table 3. The relationships were described one by one as follows:

Table 3. Correlation between lipoxygenase (LOX) activity and 2-thiobarbituric acid (TBA) value and the other quality parameters in wheat malt.

	LOX activity	TBA
Colour	-0.072	0.944**
Saccharifying time	-0.569	-0.753*
Turbidity	-0.217	0.793*
Extract	0.214	0.850**
α-AN	0.142	0.968**
Diastatic power	0.544	0.735*
Acidity	-0.155	0.922**
pH	0.007	-0.907**

** Correlation is significant at 0.01 level (2-tailed); * correlation is significant at 0.05 level (2-tailed).
α-AN = free amino nitrogen.

- TBA value had a significant positive correlation with the colour (r=0.944**). Because the TBA value represents the content of aldehydes of malt, i.e. which could reflect the degree of the Maillard reaction, higher a TBA value would result in higher colour.
- TBA had a significant positive correlation with acidity (r=0.922**) and the extract (r=0.850**), and a significant negative correlation with pH value (r=-0.907*). Because there was a significant positive correlation between the Kolbach index and the TBA value, to a certain extent the TBA value reflects the degradation degree of wheat malt. So the TBA value was correlated with the wheat malts quality indices.
- TBA had a significant positive correlation with the α-AN (r=0.968**), and had a positive correlation with diastatic power (r=0.735*); it had a negative correlation with saccharification time (r=-0.753**). The TBA value was higher because the higher α-AN content could enhance the Maillard reaction. On the other hand higher diastatic power could improve the conversion of the starch to sugars, which could to some degree increase the TBA value.

4. Conclusion

After malting, the LOX activity decreased by 33.89-61.54%. And there were significant differences between the LOX activities of the wheat and wheat malts. When the Kolbach index was in the range of 31.4-54.2%, LOX activity of the malts increased with the increase of the Kolbach index until the Kolbach index was 39.5% then decreased, and there was a significant positive correlation between the Kolbach index and the TBA value, with the correlation coefficient being 0.951 (P<0.01). Taking the positive effect of the LOX and the TBA into account, the Kolbach index of the wheat malts should be lower. In this study when the Kolbach index was 37.0%, the LOX activity and the TBA value were lower and the other quality indices were good. LOX activity had a significant positive correlation with the globulins (r=0.870**, P<0.01). So it was concluded that the LOX might belong to the globulins. The higher TBA value of the wheat malts would result in the linear increase in the colour of the congress wort.

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