Influence of pasteurization and high pressure processing on the antioxidant activity of filtered and unfiltered lager beer

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Summary
The aim of this study was to assess the impact of various final stabilization methods on the antioxidant activity of filtered and unfiltered lager beers, as well as to evaluate further changes during storage at various temperatures. Beers were processed by thermal pasteurization and by high pressure processing. The antioxidant activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and by ferric ion reducing antioxidant power (FRAP) assay. The concentration of total polyphenols was also evaluated. Changes observed in the antioxidant activity during the storage period of 8 weeks were not associated with the type of the applied stabilization method. Increased storage temperature had negative impact on the antioxidant activity. Filtered beers exhibited lower values of antioxidant activity and lower concentration of total polyphenols in comparison to unfiltered samples. The results indicate that both thermal pasteurization and high pressure processing can preserve oxidative stability of beer and are, therefore, applicable methods for its shelf-life extension.

Keywords
high pressure processing; beer; antioxidant activity; pasteurization

The worldwide increase in the number of craft breweries producing beers of great variety and distinctive styles has revolutionized the brewing market. Consequently, unfiltered beer (UB), in general, has gained popularity due to customers' preferences for natural or authentic beers and the associated craftmanship [1, 2]. In the production of UB, filtration and addition of stabilizing agents is omitted and, after the maturation phase, beer is transferred directly to its final package. Therefore, the presence of yeast cells and the related occurrence of haze is a typical feature of UB [3]. Due to microbiological instability, UB should be kept refrigerated otherwise its shelf-life will be limited. Further processing to extend the shelf-life can be applied. This comprises thermal pasteurization, a well established method in the brewing industry, or high pressure processing, also called pascalization, a novel approach adapted from fruit juice processing. Despite the beneficial impact on microbiological stability, pasteurization and high pressure processing can cause deterioration in beer flavour as a result of increased temperature or pressure, respectively [4, 5].

The further loss of pleasant flavours associated with a fresh beer taste during storage is a phenomenon described as beer staling. Beer staling is imparted by several oxidative reactions that cause degradation of various aromatic compounds. Moreover, undesirable flavours are formed de novo as oxidation products [6, 7]. On the other hand, beer contains several compounds with antioxidant properties, which, to a certain extent, can delay or prevent oxidative damage. These compounds comprise sulfites, bitter hop resins, Maillard reaction products and phenolic compounds [8, 9]. Besides their contribution to stability, polyphenols have beneficial effects on human health by preventing certain diseases related to oxidative stress and beer consumption can be associated with health-promoting effects [10, 11]. Yeasts present in UB act as a potent scavenger of reactive oxygen species that are involved in the initiation of oxidative reactions, and thereby contribute to the enhancement of flavour stability [12]. Filtered colloidally stabilized beer, from which yeast is intentionally removed and stabilizing agents are applied, is generally characterized

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by longer shelf-life due to higher microbiological and colloidal stability, in contrast to UB.

The aim of this study was to compare the antioxidant activity of filtered colloidally stabilized and unfiltered lager beer, as well as to evaluate the effect of the final stabilization method by assessing radical-scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the ferric ion reducing antioxidant power (FRAP) assay and by the determination of total polyphenol (TP) concentration immediately following the processing and after 8 weeks of storage under refrigerated or ambient conditions. After the final stabilization treatment, beers were subjected to a forced aging test and the impact on antioxidant activity was examined.

MATERIALS AND METHODS

Reagents

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, ≥ 97%), ferric(III) chloride hexahydrate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, ≥ 98%) and gallic acid (GA, ≥ 98% were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Acetic acid (≥ 99%), ammonia water (25%), sodium acetate trihydrate (≥ 98%), methanol (≥99,8%) and hydrochloric acid (≥ 35%) were obtained from Penta (Prague, Czech Republic). Carboxymethylcellulose and ammonium ferric citrate (14.5–16%) were obtained from Fluka (Buchs, Switzerland), ethylenediaminetetraacetic acid (EDTA) (≥ 98%) from Fisher Scientific (Waltham, Massachusetts, USA).

Beer samples

Unfiltered lager beer (12 °P, Pilsner type) was kindly donated from a local industrial-scale brewery in Krušovice, Czech Republic. Beer was transferred from a stainless steel keg into polyethylene terephthalate plastic bottles (volume 1 l) under a CO_2 protective atmosphere using a manual bottling machine (Pegas, Vilnius, Lithuania). Filtered beer was obtained by filtration of the unfiltered beer through a candle filter precoated with diatomaceous earth and subsequently stabilized by a poly(vinylpolypyrrolidone) filter aid. Afterwards, samples of unfiltered and filtered colloidally stabilized beers were divided in 4 groups and each group was subjected to one of following types of final stabilization method:

- H250 – high pressure processing (HPP) at 250 MPa for 5 min at 25 °C,
- H550 – HPP at 550 MPa for 5 min at 25 °C,
- P60 – thermal pasteurization at 60 pasteurization units (PU, 1 PU is defined as a holding time of 1 min at 60 °C) using a water bath,
- C – the last group of samples was left untreated and considered as a control.

Treatments H250 and H550 were carried out using a Hiperbaric 135 pressurizer (Hiperbaric, Burgos, Spain). To assess the impact of storage temperature on antioxidant activity during storage, all beers were stored either refrigerated at 2–4 °C or at ambient temperature (22–24 °C) for a period of 8 weeks and analysed before treatment, and then after 1, 2, 6 and 8 weeks. Prior to the measurements, beer samples were degassed on a horizontal shaker for 15 min and centrifuged at 4430 ×*g* for 10 min.

Antioxidant activity

Antioxidant activity was assessed by DPPH assay, which measures radical-scavenging potential, and by the FRAP method, which evaluates reducing power. For the DPPH assay, a modified method of GORJANOVIC et al. [13] was used. A methanolic stock solution of DPPH (1.86×10^{-4} mol·l^{-1}) was mixed with acetate buffer (0.1 mol·l^{-1}, pH 4.3) in a ratio of 2:1. From this working solution, 1.4 ml was mixed in a cuvette with 0.1 ml of beer sample and incubated in the dark for 15 min. The absorbance at wavelength 525 nm (A_{525}) was measured against distilled water using DU 730 UV-Vis spectrophotometer (Beckman Coulter, Brea, California, USA). A calibration curve was constructed from measurements of gallic acid solution and the results of DPPH radical-scavenging activity were expressed as milligrams of gallic acid equivalents per litre of sample.

For the FRAP method, the reaction mixture was prepared by mixing acetate buffer (2.3×10^{-2} mol·l^{-1}, pH 3.6), iron(III) chloride hexahydrate solution (3.3×10^{-2} mol·l^{-1}) and 1×10^{-2} mol·l^{-1} TPTZ solution in hydrochloric acid (4×10^{-2} mol·l^{-1}) in a ratio of 10:1:1. The reaction mixture was transferred to a plastic cuvette (volume 1.5 ml) and mixed with 50 μl of diluted sample and 150 μl of distilled water. The mixture was incubated in the dark at ambient temperature for 30 min. After the incubation period, absorbance at wavelength 593 nm (A_{593}) was measured against distilled water using the DU 730 spectrophotometer. A calibration curve was constructed from measurements of Trolox solution and the results of FRAP reducing power were expressed as milligrams of Trolox equivalents per litre of sample.

Total polyphenols

TP were quantified by the European Brewery Convention method 9.11 [14].

Forced aging test

Beer samples were subjected to a forced aging procedure by storing the bottles at 37 °C. After 7 days of storage, antioxidant activity (DPPH and FRAP assays) and TP were measured.

Statistical analysis

Analyses were performed in triplicate and results were expressed as mean ± standard deviation. Statistical significance of differences within means was determined by the Student's *t*-test and the influence of storage temperature and time was evaluated by two-way ANOVA followed by post-hoc Tukey's test. A *p*-value below 0.05 was considered statistically significant. Statistical analyses were performed using Statistica data analysis software, version 13 (TIBCO Software, Palo Alto, California, USA).

RESULTS AND DISCUSSION

Antioxidant activity

The presence of compounds with antioxidant activity in beer correlates with its flavour stability and, therefore, assessment of antioxidant levels can be used as a precondition to flavour deterioration during storage and as an indicator of beer aging [15]. Changes in radical-scavenging activity were evaluated by DPPH assay in filtered colloidally stabilized and unfiltered beers treated by high pressure processing at 250 MPa for 5 min (H250), 550 MPa for 5 min (H550), or by thermal pasteurization at 60 PU (P60) over a storage period of 8 weeks. The impact of temperature was assessed by storage under refrigerated conditions (2–4 °C) and at ambient temperature (22–24 °C). Results are summarized in Tab. 1. In all samples, regardless of stabilization treatment, the effect of storage temperature was significant ($p < 0.05$) and samples stored at an ambient temperature exhibited lower antioxidant activity. Any effect of the treatment method was not observed. Samples of filtered colloidally stabilized beers had overall lower radical-scavenging activity than unfiltered samples. Comparing changes in DPPH values during 8 weeks of storage, in all samples, antioxidant activity increased between the first and second week and then decreased until the end of the storage period. A similar trend was also observed in our previous experiments with different beer types (our unpublished results). The early

Tab. 1. Antioxidant activity measured by 1,1-diphenyl-2-picrylhydrazyl assay.

Storage conditions	Antioxidant activity [mg·l^{-1}]							
	Unfiltered beer				Filtered beer			
	C	H250	H550	P60	C	H250	H550	P60
Temperature 2–4 °C								
0 weeks	41.78 ± 0.11	41.26 ± 0.40	41.23 ± 0.35	41.09 ± 0.35	39.98 ± 0.34*	40.02 ± 0.29*	39.76 ± 0.36*	39.74 ± 0.47*
1 week	40.45 ± 0.08	40.21 ± 0.08*	40.15 ± 0.25	40.13 ± 0.13*	37.78 ± 0.74*	37.81 ± 0.29*	37.41 ± 0.96*	37.33 ± 0.35*
2 weeks	41.77 ± 0.18	41.89 ± 0.05	41.78 ± 0.09	41.67 ± 0.05	41.45 ± 0.59	41.69 ± 0.22	41.48 ± 0.25	41.13 ± 0.09*
6 weeks	42.50 ± 0.04	42.67 ± 0.20	42.66 ± 0.10	42.63 ± 0.17	42.67 ± 0.25	42.50 ± 0.24	42.59 ± 0.18	42.13 ± 0.29
8 weeks	38.30 ± 1.23	38.07 ± 0.27	37.35 ± 0.07	37.88 ± 0.27	34.26 ± 0.95*	30.53 ± 0.80*	33.78 ± 0.62*	31.13 ± 0.83*
Temperature 22–24 °C								
1 week	40.25 ± 0.21	40.07 ± 0.10	40.31 ± 0.10	40.00 ± 0.13	37.65 ± 0.75*	36.53 ± 0.96*	36.87 ± 0.41*	36.53 ± 0.97*
2 weeks	41.77 ± 0.09	40.07 ± 0.07	40.31 ± 0.33	40.00 ± 0.04	37.65 ± 0.15	41.72 ± 0.27	42.00 ± 0.23	41.36 ± 0.14*
6 weeks	42.19 ± 0.31	42.47 ± 0.10	42.16 ± 0.39	41.82 ± 0.38	36.99 ± 0.49*	35.85 ± 0.73*	36.89 ± 0.22*	38.53 ± 0.76*
8 weeks	37.38 ± 0.48	36.25 ± 0.48	36.67 ± 0.37	35.82 ± 0.40*	27.42 ± 0.59*	28.38 ± 0.88*	26.11 ± 0.47*	23.25 ± 0.89*

Results are expressed as milligrams of gallic acid equivalents per litre of sample and as mean ± standard deviation ($n = 3$).
C – control, H250 – high pressure treatment at 250 MPa, H550 – high pressure treatment at 550 MPa, P60 – thermal pasteurization at 60 PU, * – statistically different results within one row ($p < 0.05$).

increase could be associated with the antioxidant activity of protein thiol groups, which act as secondary radical (after exhaustion of endogenous sulfites [16].

The FRAP assay was used as another method for determining antioxidant activity. Treatment method had no effect on the reducing power. Nevertheless, the FRAP value of UB treated by the pressure of 250 MPa was the lowest of all UB samples during the storage period. Storage at a higher temperature caused a decrease in the reducing power after 8 weeks of storage as it was lower for samples stored at ambient temperature compared to the refrigerated ones. Generally, results of the FRAP assay on filtered colloidally stabilized beer samples were lower than on the unfiltered samples. The results of FRAP assay are summarized in Tab. 2.

Differences in antioxidant activity determined using individual methods can be explained by different sensitivity towards different standards that were used, as described by Tafulo et al. [17]. Lower levels of antioxidants in filtered beers are probably the result of the removal of phenolic compounds during Kieselguhr filtration and poly(vinylpolypyrrolidone; PVPP) stabilization [8]. Notably, in our study, we saw no significant effect of the treatment method on antioxidant activity, whereas results from previous studies were contradictory. Pascoe et al. [18] observed increased antioxidant activity in pasteurized beers in comparison to unpasteurized ones and Lund et al. [19] described a higher potential for radical formation in unpasteurized beers, indicating a lower concentration of antioxidants. Similarly, Hoff et al. [5] reported that pasteurization improves oxidative stability because faster radical formation was observed in unpasteurized beers. In contrast, a decline in endogenous antioxidants after pasteurization at 62 °C for 30 min was noted by Liu et al. [20]. Cao et al. [15] described a decrease in DPPH values in beers pasteurized at different intensities (2 PU, 8 PU and 14 PU) over a 6 month storage period, with the largest drop in DPPH values being observed during the first 2 months. According to Kaneda et al. [21], pasteurization induces radical reactions leading to beer staling and promotes development of a pasteurization off-flavour. Ambiguity of results could be explained by differences in experimental designs, treatment conditions, the storage environment or sample sizes. Variations could be also attributed to the beers tested, because different antioxidant activities were found even within the same beer type of different commercial brands [9].

The effect of HPP on antioxidant activity was not previously assessed. Our experiments did not show any influence of HPP, as the final treatment method, on the antioxidant activity of unfiltered and filtered beers, suggesting that pascalization does not induce additional radical reactions that would lead to consumption of antioxidants. This is in agreement with Yin et al. [22], who demonstrated that HPP had no effect on the sensory profile of the treated beer. However, increased Strecker aldehydes during storage after HPP were previously described, which would indicate that oxidative reactions leading to beer staling might be accelerated by high pressure treatment [23]. Therefore, further data would be needed in order to draw comprehensive conclusions on the influence of HPP on beer oxidative stability.

Total polyphenols

Polyphenols represent the main fraction of beer antioxidants. They originate in the raw materials from which they are extracted during the course of brewing. They are also constituents of important sensory features such as mouthfeel and adstringency, and are also associated with colloidal stability of the final beverage. Therefore, their concentration is considered a general quality marker [24]. Results of our study did not reveal any significant changes in polyphenol concentrations related to differences in storage temperature over the storage period of 2 months, as summarized in Tab. 3. All filtered colloidally stabilized samples had lower concentrations of polyphenols compared to UB, which is probably a result of their removal during the filtration and PVPP-stabilization steps. With regard to the effect of the final treatment method, UB processed by HHP at 550 MPa had significantly lower polyphenol levels after the first week of storage. Nevertheless, there was no clear decreasing trend in polyphenol concentration throughout the storage period in any of the treated beers. In previous studies, overall conclusions on changes in polyphenol concentrations during beer storage and its relationship with flavour stability were inconsistent. Due to oxidative degradation induced by heat load during pasteurization, the decrease in polyphenols correlated with pasteurization dose, expressed as pasteurization units (PU), and the most dramatic decrease was observed during the first two months of storage [15]. Lund et al. [19] also observed a decline in polyphenols concentration during storage of pasteurized beer. The same study also confirmed positive effects of pasteurization on oxidative stability using electromagnetic spin resonance, interpretation of which renders the role of polyphenols in antioxidant activity questionable.

Tab. 2. Reducing antioxidant power measured by ferric ion reducing antioxidant power assay.

Storage conditions	Reducing power [mg·l^{-1}]							
	Unfiltered beer				Filtered beer			
	C	H250	H550	P60	C	H250	H550	P60
Temperature 2–4 °C								
0 weeks	510.38 ± 12.62	482.02 ± 16.52	499.56 ± 15.71	502.49 ± 16.41	360.49 ± 3.19 *	385.22 ± 10.29 *	357.44 ± 4.30 *	343.55 ± 20.06 *
1 week	477.92 ± 20.31	457.45 ± 9.36	470.03 ± 15.60	466.52 ± 15.47	386.40 ± 13.61 *	359.50 ± 12.62 *	374.41 ± 11.91 *	423.70 ± 12.21 *
2 weeks	474.12 ± 5.69	434.65 ± 11.77 *	454.01 ± 11.03	463.89 ± 7.35	368.86 ± 6.38 *	378.27 ± 17.54 *	335.53 ± 14.96 *	334.65 ± 9.48 *
6 weeks	511.84 ± 11.83	461.84 ± 18.02 *	478.80 ± 20.44	477.05 ± 7.15 *	332.02 ± 18.73 *	364.76 ± 4.77 *	346.64 ± 9.07 *	413.60 ± 17.78 *
8 weeks	466.81 ± 16.65	500.73 ± 10.21	462.14 ± 12.08	469.15 ± 7.18	385.52 ± 16.28 *	383.19 ± 12.82 *	391.96 ± 7.45 *	413.60 ± 18.58 *
Temperature 22–24 °C								
1 week	467.40 ± 6.79	440.50 ± 7.17*	472.95 ± 11.93	452.19 ± 3.99	373.25 ± 12.95 *	352.78 ± 11.40 *	381.02 ± 3.74 *	389.33 ± 7.72 *
2 weeks	469.15 ± 6.26	440.50 ± 15.41	472.95 ± 4.47	452.19 ± 15.03	373.25 ± 15.78 *	364.47 ± 11.49 *	346.64 ± 7.52 *	358.92 ± 16.95 *
6 weeks	472.37 ± 21.09	415.06 ± 20.21	454.53 ± 10.34	456.28 ± 9.60	378.80 ± 3.23 *	386.40 ± 11.16 *	362.43 ± 14.75 *	381.14 ± 5.41 *
8 weeks	462.43 ± 11.07	416.52 ± 8.76*	458.33 ± 5.59	449.85 ± 7.52	365.35 ± 13.42 *	348.97 ± 16.40 *	329.97 ± 7.05 *	353.07 ± 17.59 *

Results are expressed as milligrams of Trolox equivalents per litre of sample and as mean ± standard deviation (n = 3).
C – control, H250 – high pressure treatment at 250 MPa, H550 – high pressure treatment at 550 MPa, P60 – thermal pasteurization at 60 PU, * – statistically different results within one row ($p < 0.05$).

Tab. 3. Concentration of total polyphenols.

Storage conditions	Total polyphenols [mg·l^{-1}]							
	Unfiltered beer				Filtered beer			
	C	H250	H550	P60	C	H250	H550	P60
Temperature 2–4 °C								
0 weeks	176.95 ± 5.71	161.13 ± 2.05	185.32 ± 7.38	189.83 ± 4.25	115.44 ± 3.84 *	115.62 ± 0.82 *	128.33 ± 3.69 *	115.21 ± 6.15 *
1 week	176.71 ± 4.51	165.5 ± 3.63	152.52 ± 0.82	180.40 ± 4.92	124.23 ± 6.15 *	124.64 ± 5.74 *	110.72 ± 2.08 *	124.23 ± 1.23 *
2 weeks	170.97 ± 2.05	167.28 ± 5.74	178.76 ± 3.28	166.35 ± 3.78	117.67 ± 5.33 *	113.98 ± 6.56 *	107.48 ± 4.87 *	113.98 ± 0.82 *
6 weeks	190.65 ± 8.61	167.01 ± 1.79	172.14 ± 3.79	186.64 ± 0.50	128.33 ± 9.43 *	105.42 ± 6.82 *	98.81 ± 6.15 *	121.45 ± 0.95 *
8 weeks	155.80 ± 7.38	139.81 ± 2.05	154.98 ± 4.10	168.10 ± 3.28	119.31 ± 4.51	99.61 ± 2.08 *	120.13 ± 4.51	103.32 ± 5.74 *
Temperature 22–24 °C								
1 week	183.95 ± 2.30	169.05 ± 2.80	171.79 ± 2.87	172.39 ± 4.15	114.08 ± 3.87 *	126.69 ± 6.15 *	104.55 ± 5.33 *	115.54 ± 1.68 *
2 weeks	170.56 ± 3.28	176.71 ± 4.51 *	191.47 ± 2.87	172.61 ± 2.87	111.93 ± 2.87 *	127.10 ± 6.56 *	118.48 ± 2.88 *	124.21 ± 1.74 *
6 weeks	177.77 ± 1.89	171.36 ± 2.22	176.71 ± 6.15	172.20 ± 3.28	123.00 ± 1.64 *	126.28 ± 1.64 *	114.80 ± 5.74 *	126.28 ± 3.28 *
8 weeks	185.62 ± 4.05	177.42 ± 2.15	183.68 ± 7.46	181.22 ± 3.28	113.42 ± 4.80 *	113.57 ± 4.51 *	122.90 ± 1.43 *	116.44 ± 8.20 *

Results are expressed as milligrams per litre of sampleand as mean ± standard deviation (n = 3).
C – control, H250 – high pressure treatment at 250 MPa, H550 – high pressure treatment at 550 MPa, P60 – thermal pasteurization at 60 PU, * – statistically different results within one row ($p < 0.05$).

Increased antioxidant activity and higher polyphenol concentrations in beers immediately after pasteurization were described by Pascoe et al. [18]. In agreement with our study, no further changes in the majority of polyphenolic compounds were observed during storage of pasteurized beers [18]. The effect of HPP on levels of polyphenols in the beer matrix was not previously assessed, however, in fruit juices, it was shown that processing did have an effect on the concentration of polyphenols. In particular, concentration of phenolic compounds increased, while concentration of flavanones and hydroxycinnamic acid compounds decreased over a 30 days storage period [25].

Forced aging

Forced aging is an approach used to determine flavour stability and to predict beer staling [7]. In this study, samples were maintained for 7 days at 37 °C and their antioxidant activities and TP were subsequently evaluated. Results of the DPPH assay after treatment were significantly different ($p < 0.05$) from the initial ones in UB processed by HPP at 550 MPa. Reducing power, measured by the FRAP assay, revealed significant changes in filtered pasteurized beer after the period of forced aging. Strikingly, the TP concentration before and after forced aging did not change, demonstrating the ambiguous role of polyphenols on beer antioxidant activity. Lower antioxidant activity in filtered pasteurized beer and in UB processed at 550 MPa can be explained by thermal degradation of polyphenols during pasteurization and destabilization of reducing enzymes after HPP, respectively [15, 26].

CONCLUSIONS

Our study confirmed the validity of thermal pasteurization and pascalization as final processing methods to extend microbiological and flavour stability of UB. Furthermore, a comparison of antioxidant activities and TP levels of filtered colloidally stabilized and unfiltered beers after final stabilization treatment was carried out after storage at two temperatures. During the storage period of two months, the higher storage temperature had a negative effect on the antioxidant activities of the beers. Despite some differences of the antioxidant activity between beer samples treated by the two final stabilization methods (thermal pasteurization and HPP), a clear relationship between the treatment method types was not proven. The antioxidant activity of filtered colloidally stabilized beers, as well as TP, was generally lower compared to unfiltered samples. Surprisingly, changes in polyphenol concentration were not related to the duration of storage and, in most samples, the concentration of TP did not change at all.

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