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ARTICLE

Application of a Novel Antioxidative Assay in Beer Analysis and Brewing Process Monitoring

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A novel antioxidative assay based on direct current polarography has been developed. Quantification of antioxidative (AO) activity has been based on a decrease of hydrogen peroxide anodic current in the presence of antioxidants. An efficient experimental procedure, without any special pretreatment of analyzed samples, has been applied. Antioxidative activity of different kinds of commercial beers (dark, blond, and alcohol-free), some small-scale made special beers with medicinal herbs and mushroom extracts, extracts themselves, as well as individual phenolic components present in beer has been measured. In addition, changes of AO activity during the full-scale industrial process of beer production have been monitored. A strong correlation between results obtained and total phenolics content has been observed. The assay can be recommended for application in brewing industry, either to survey a process with the aim to optimize relevant technological factors or to analyze quality of final product.

KEYWORDS: Antioxidative activity; beer; brewing process; DC polarography; hydrogen peroxide; total phenolics content

INTRODUCTION

Beer is a source of antioxidants (AOs), phenolic compounds originating from barley and hop, readily absorbed, and extensively metabolized in humans (*1*, *2*). Beer AOs have significant nutritional value, being considered as potential cancer chemopreventive agents (*3*−*5*) and promoters of cardiovascular health (*6*−*8*). Except for a physiological role, the presence of phenolics in beer has technological connotation. The implications of phenolic compounds in relation to the color, the taste, and the nonbiological stability of beer are well-known. Beer rich in AOs has higher quality, more stable sensory characteristics, such as flavor and aroma, and foam stability (*9*). Also, it is resistant to oxidation and therefore may have a longer shelf life.

Amount of phenolics in beer and wine is comparable (*10*). Recent claims suggest that the in vivo AO capacity of beer and red wine are similar despite different concentrations of total polyphenols, possibly due to superior absorption of the beer phenolics as compared with those in red wine (*2*, *10*, *11*). Although beer per drink (of equivalent alcohol content) contains more than twice as many of the AOs as white wine and only half the amount in red wine (*12*), the red wine AOs may be larger molecules that are not as readily absorbed as the smaller AOs in beer (*13*, *14*). Literature widely describes the content of phenolics in barley, malt, wort, hop, and beer (*15*−*24*). Approximately 80% of beer phenols are derived from malt and about 20% from hop. The most important phenolic compounds present in beer are phenolic acids (ferulic, cinnamic, chlorogenic, vanillic, gallic, caffeic, o- and p-coumaric, syringic), derivatives of flavan-3-ol (catechin, epicatechin, procyanidin, prodelphinidin) and flavonoglycosides.

Antioxidative activity is a significant aspect of beer quality and its evaluation is important either in relation to brewing process optimization or market sale. Over the past few years, the AO activity of beer has been investigated applying numerous assays (*25*−*27*). Methods for determination of AO activity in wort and beer have been reviewed by Karabin (*28*). Studies of AO efficiencies of barley, malt, and hop are reported as well (*29*−*34*). Effect of the malting, mashing, and brewing process on the color, flavor, and content of phenolics compounds, as well as AO potential of malt, wort, and beer has been investigated (*35*−*38*). Generally, commonly used AO assays are rather time-consuming and usually require reactive species. Therefore, a search for low cost and less time-consuming parallel methods is of large interest. Development of such a direct and efficient AO assay would be of importance for the food and beverage industry, including the brewing industry. Possibilities of electrochemical techniques within this field have not been fully explored yet.

In the present study, we took advantage of direct current (DC) polarography to develop a fast, reliable, and reproducible assay, easily applicable on beer, wort, and mash samples. Antioxidant activity of different brands of commercial beers, some small-scale made special beers with medicinal herbs and mushroom extracts, extracts themselves, as well as individual compounds present in beer, are assessed using the novel assay. In addition, the slightly modified assay has been used for fast monitoring of the full scale

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industrial process of brewing in order to determine technological phases critical for AO activity changes.

MATERIALS AND METHODS

Beer, Wort and Mash Samples. *Commercial Beers*. Different kinds of beers, commercial ale (top fermented) and lager (bottom fermented) beers, and some small-scale made special beers with medicinal herbs and mushroom extracts were studied. Commercial beers, purchased from the Serbian market, can be divided into groups: blond (pale beer with a most common alcohol content of aproximately 5% (v/v), strong (with a high content of alcohol and original wort extract), alcohol-free (blond with alcohol level less than 0.5% v/v), dark (with roasted malt, or barley), and wheat unfiltered beer.

Herb Beers. Three types of small-scale made special beers brewed with the addition of extracts of *Thymus vulgaris* (thyme), *Melissa officinalis* (lemon balm) and fungus *Ganoderma lucidum*, known for its strong pharmacodynamic effect, were investigated. *Melissa* and *Thymus* extracts were prepared using the conditions prescribed according to Pharmacopoeia (*39*). Herbal drugs were cut to pieces, mixed with 70% ethanol, and allowed to stand for 24 h in darkness. Extraction was done using a percolation method at room temperature. *Ganoderma* extract was prepared by allowing mushroom tissue cut to pieces to macerate for 21 days in 70% ethanol at room temperature in darkness. Extraction was forced by a daily mixing with a magnetic stirrer for 10 min. After extractions, the ethanolic solution was filtered and concentrated in vacuum to $^1/_5$ of the initial content. Prepared extracts were added aseptically to commercial pills beer taking into account the sensory acceptability and recommended daily doses (*39*−*42*). After injection the bottles were immediately closed and maturated at 5 °C for one day.

Mash and Wort. Mash and wort were obtained from the industrial scale from the local brewery. Samples are withdrawn at several points of industrial process: during mashing, after wort separation, and wort boiling, before yeast pitching, after primary fermentation, at the end of beer maturation, after filtration step with Kieselguhr, and after pasteurization and bottling. Because of the significant influence of mashing on the chemical composition of wort and AO activity of the final product, four different industrial mashing processes were followed, assigned as A, B, C, and D. It was a double-mash infusion system with corn grits as adjunct. The system is based on two separate mashes: adjuncts mash boiled in a cereal cooker and malt mash heated in a mash tun. The samples of malt mash were taken at 40 °C, after mixing of the two mashes at 45 °C (B and D mashes), and 50 °C (A and C mashes), and at the end of heat treatments at 62, 68, 72, and 77 °C.

Chemicals. Hydrogen peroxide was from Merck. (+)-Catechin, caffeic, ferulic, *p*-coumaric and chlorogenic acid were from Sigma (St. Loius, MO, USA). Working standard solutions of phenolics were prepared daily by dilution in a mixture of ethanol and water (1:1). Concentration of (+)-catechin was 2 mg mL^{-1} while concentrations of phenolic acids were 4 mg mL^{-1}. Folin-Ciocalteu reagent was from Merck.

Polarographic Instrumentation and Procedure. Antioxidant activity was estimated by DC polarography, using a dropping mercury electrode (DME) as working electrode, with a programmed dropping time of 1 s. Saturated calomel electrode (SCE) served as a reference and a Pt-foil as auxiliary electrode. The current−potential ($i-E$) curves were recorded using the polarographic analyzer PAR (Princeton Applied Research Instrument), model 174A, equipped with $X-Y$ recorder (Houston Omnigraphic 2000). The volume of the experimental solution in the electrolytical cell was 20 mL. Borate buffer (pH 9.8) was prepared from 50 mL of 0.2 M H_3BO_3 and 40.8 mL of 0.5 M KOH. Starting H_2O_2 concentration of 5.7 mM was obtained by the addition of 10 μL of 35% H_2O_2 into 20 mL of borate buffer cell solution. Before each $i-E$ curve recording, the stream of the pure nitrogen was passed through the cell solution. During curve recording the inert atmosphere was kept by passing the nitrogen above the solution. The initial potentials were 0.1 or 0.15 V. The potential scan rate was 10 mV sec^{-1}. The DME current oscillation were dumped with low pass filter positioned at 3 s. Experiments were done at the room temperature. All samples were taken and measured in triplicate, and results were averaged. Beer samples were degassed by sonification. Examined samples were gradually added (in equal aliquots) into a buffered cell solution containing H_2O_2.

Determination of AO Activity. The applied polarographic assay is based on H_2O_2 oxidation on the DME and the phenomenon of decrease of oxidation anodic current in the presence of AOs. The concise explanation of the assay applied is given here while detailed discussion, providing a platform to understand electrochemistry beyond electrode reaction, is covered elsewhere.

Polarograms of H_2O_2 oxidation are recorded starting from slightly acidic to alkaline conditions. A one-step anodic wave of H_2O_2 oxidation emerges at pH 6.5. When pH value increases this single wave becomes more prominent since in the alkaline conditions dissociation of H_2O_2 is directed to the perhydroxil ion formation. The anodic reaction of H_2O_2 in alkaline solutions has been investigated by Kikuchi and Murayama (*43*), and a reaction mechanism was proposed. Anodic dissolution of electrode mercury and formation of the mercury complex $[Hg(O_2H)(OH]$ corresponding with the anodic wave was assumed (*43*). Polarographic current of H_2O_2 increases in proportion to its concentrations, in the wide concentration interval. At concentrations of H_2O_2 higher than 1.5×10^{-4} M anodic current as a peak has been developed instead of polarographic wave. Addition of gelatin or Triton X-100, suppressors of polarographic maximum, does not change the peak shape, leading us to the conclusion that its development is probably caused by superposition of polarographic current attributed to the formation of the mercury complex in the presence of OH^- ions.

Decrease of anodic limiting current (i_l) in the presence of particular AO species has been noticed. Polarograms of H_2O_2 oxidation in the presence of AOs have been recorded in solutions with pH 9.8. Effect of antioxidants on i_l declining is found to be more remarkable in slightly acidic, neutral, or slightly alkaline solutions but more alkaline values of pH (above 9) have been chosen for better reproducibility. Also, appearance of significantly marked i_l in highly alkaline solutions, for the same concentrations of H_2O_2, enable applying of standard polarographic conditions, such as sensitivity.

The anodic current of H_2O_2 oxidation at the beginning of measurement, that is, initial i_l value (i_{l0}), obtained by recording 5.7 mM H_2O_2 solution, was always equal. Standard deviation about 2%, calculated upon 33 repetitive recording of initial H_2O_2 solution, indicated satisfactory reproducibility. The height of i_{l0} has been compared with the height of its residual values (i_{lr}) obtained upon gradual addition of tested samples. Percentage of i_l decrease was calculated upon each addition of tested sample throughout eq **1**:

$$\%\ \text{scavenged}\ [H_2O_2] = \left(\frac{i_{lr}}{i_{l0}} - 1\right) \times 100 \qquad (1)$$

Since AO activity of tested samples varied largely, different volume ranges were used. Beer, wort and mash samples were added in aliquots of 100 μL, while extracts were in 10 μL. From dose−response curves (percentage of i_l decrease vs volume of added sample) the volume of sample required for 50% decrease of i_{l0}, corresponding to 2.85 mM of H_2O_2, assigned AO_{50}, was determined. AO_{50} was expressed as μL. Reciprocal value of AO_{50}, used as criterion for AO activity, was calculated according to eq **2**:

$$AO_{50}{}^{-1} = \left(\frac{1}{AO_{50}}\right) \times 10^3 \qquad (2)$$

$AO_{50}{}^{-1}$ was expressed in mL^{-1}. Total phenolic content of commercial beers was correlated with $AO_{50}{}^{-1}$. The correlation of results was established using linear regression analysis.

Measurement of Total Phenolic Content. Total phenolic content of commercial beers was done by Folin and Ciocalteau (FC) assay (*44*). The volume of 200 μL of diluted beer was mixed with 1000 μL of FC reagent diluted with distilled water in proportion 1:10. After the mixture was allowed to stand 6 min in the dark, 800 μL of sodium carbonate solution (7.5%) was added. After the mixture was shaken and allowed to stand for an additional 2 h in the dark, absorbance at 740 nm was measured. Distilled water was used as blank. All samples were measured in triplicate. Four dilutions of each beer were used, and results were averaged. Appropriate dilutions of each beer were experimentally found (1:10−1:5) to give absorbance between 0.2−0.7 on 740 nm. Each absorbance was adjusted for the value of the blank probe. All beer samples were degassed using ultrasonic bath. The same procedure was done for four concentrations of gallic acid standards (10, 25, 50, and 100 mg mL^{-1}),

and a calibration curve was calculated. The total phenolic content was expressed in gallic acid equivalents (GAE), as concentration of gallic acid (mg mL^{-1}) that corresponds to the dilution of beer with the same value of absorbance on 740 nm.

Determination of AO Activity of Individual Phenolics. Polarograms of individual phenolics known to be present in beer, flavan-3-ol (+)-catechin, and phenolic acids (ferulic, *p*-coumaric, caffeic, and chlorogenic), are recorded in two ways. Antioxidants are gradually added in the solution of H_2O_2 and vice versa. Recording polarograms of individual AO and subsequent addition of H_2O_2 enable observation of polarographic anodic current belonging to AO itself if any. AO_{50} values determined from dose−response curves are expressed in μM. In the case when 50% decrease was not reached, concentrations required for 10% decrease were determined for comparison reasons.

Surveying of Brewing Process Using Modified Polarographic Assay. Polarographic assay was slightly modified in order to enable fast surveying of the brewing process. Instead of gradual addition, one aliquot of mash, wort, or beer samples is added into the same buffered solution of H_2O_2. Samples are taken from full scale industrial process of beer production, and the percentage of i_l maximum decrease was calculated upon the addition of 100 μL of each sample. Four industrial mashing processes were surveyed and compared, while the remaining part of the brewing process was monitored in order to identify technological phases critical for AO activity changes.

RESULTS

Antioxidative Activity of Beer. The first objective of the study has been to evaluate and compare AO activity of various types of commercial beers of habitual consumption in Serbia, either domestic or imported brands, and noncommercial herb beers, applying DC polarographic assay. Antioxidant activity of *Thymus vulgaris*, *Melissa officinalis*, and *Ganoderma lucidum* are assessed and included into the study too.

Polarograms of H_2O_2 before and after the addition of two beer samples, dark beer with high content of original wort extract and alcohol-free beer with low content of extract (**Figure 1A**), and two medicinal plant extracts, *Thymus* and *Melissa* (**Figure 1B**) are shown. As seen, the i_l decrease indicates significant AO activity of both beers and very strong activity of both extracts. The prominent difference obtained between polarograms of strong, dark beer and nonalcoholic beer brewed from less concentrated wort show assay capability to distinguish beers according to AO activity. Assay application on plant extracts verifies its competence not only for moderate but for powerful AOs also.

Different concentration ranges are used depending on AO efficiency of tested samples. Volume range for extracts is 1 order of magnitude lower than for beer samples. Beers, as moderate AOs, are added in aliquots of 100 μL while powerful herbal extract in aliquots of 10 μL. Percentage of i_{l0} decrease is calculated upon every addition of sample. Dose−response curves (% of scavenged H_2O_2 vs volume of sample) for herbs extract are shown in **Figure 2**. Decrease of i_{l0} is remarkable in the case of both *Thymus* and *Mellisa* extracts. Extract of *Ganoderma* shows no significant effect within the volume range applied to measure AO activity of extracts, while in concentration range applied for beer samples shows slight AO activity reaching approximately 15% of i_{l0} decrease. *Thymus* and *Mellisa* AO_{50}, determined from dose−response curves shown in **Figure 2**, are 31.95 and 64.23 μL, respectively. Dose-dependence curve for *Melissa* extract is linear within the volume range applied. In the case of *Thymus*, saturation phenomenon at volumes higher than 40 μL is noticed. As seen, at that point 50% of H_2O_2 anodic current decrease is already reached.

Dose−response curves for beers are linear within volume-range 100−500 μL. Saturation phenomenon has been noticed at volumes higher than 700 μL. Results obtained for three types of Bavaria beer, strong (super strength), standard, and nonalcoholic are shown in **Figure 3**.

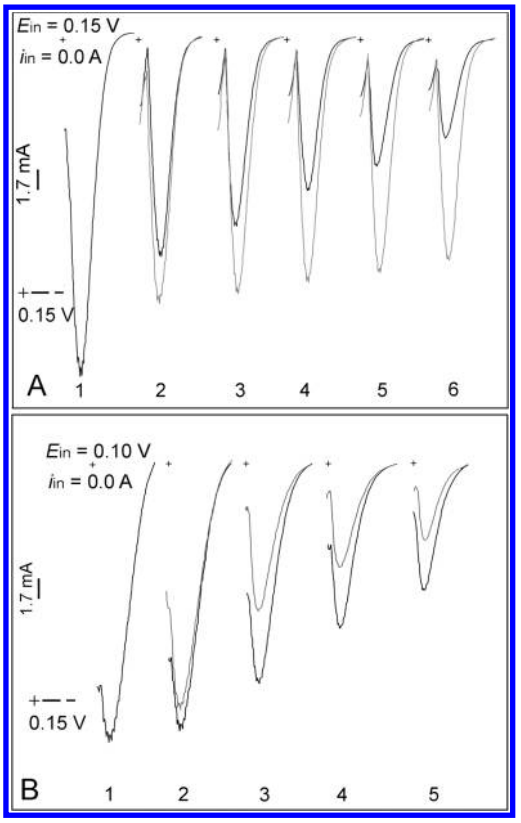

Figure 1. Anodic polarographic curves of H_2O_2 before (1) and after addition of dark beer (black line) and alcohol-free beer, with low content of extract (gray line) in aliquots of 100 (2), 200 (3), 300 (4), 400 (5), and 500 μL (6) (**A**); after addition of extracts of *Thymus vulgaris* (gray line) and *Mellisa officinalis* (black line) in aliquots of 10 (2), 30 (3), 50 (4), and 70 μL (5) (**B**).

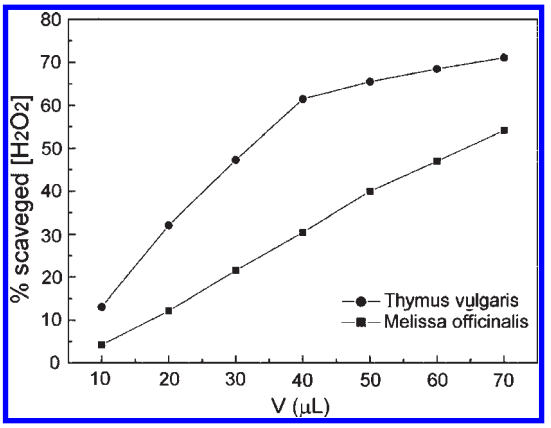

Figure 2. Dose dependence of extracts of *Thymus vulgaris* and *Mellisa officinalis* effect on H_2O_2 i_l decrease, i.e., % of scavenged H_2O_2 vs volume of extract (μL).

The AO_{50} of commercial beer and their reciprocal values AO_{50}^{-1}, accepted as criterion for total AO activity, are shown in **Table 1**. On the basis of AO_{50}^{-1}, AO activity of beers is compared and correlated with total phenolic content. According to AO_{50}^{-1}, the lowest AO activity is obtained for alcohol-free beers (Bavaria and Union) brewed from less concentrated wort, containing the lowest level of total phenolics. The highest values

of AO activity, obtained for strong and dark beers, are accompanied with high level of phenolics. Dark, strong beers have more than 2-fold higher content of phenolics and AO activity, compared to the alcohol-free beers. The relationship between results expressed as AO_{50}^{-1} and total phenolic is given in **Figure 4**. The correlation between the AO activity and total phenolics content is strong ($r^2 = 0.93$).

Particularly illustrative are differences between AO activity of the same brand beers: standard and strong (Lav), alcohol, alcohol-free, and strong (Bavaria), alcohol and alcohol-free, (Union and Becks), dark and blond (Leffe and Nikšićko) (**Figure 5**). Alcohol-free beers are usually brewed with lower original wort extract and inhibition of alcohol formation or as normal alcoholic beers, with the alcohol removed at the last stage. Alcohol-free beers, with lower content of original wort extract (Bavaria and Union), have remarkably lower AO activity than alcoholic ones. In contrast, nonalcoholic and alcoholic Becks, with the same percentage of extracts, have close AO activity and content of total phenolics. Described differences are explained by

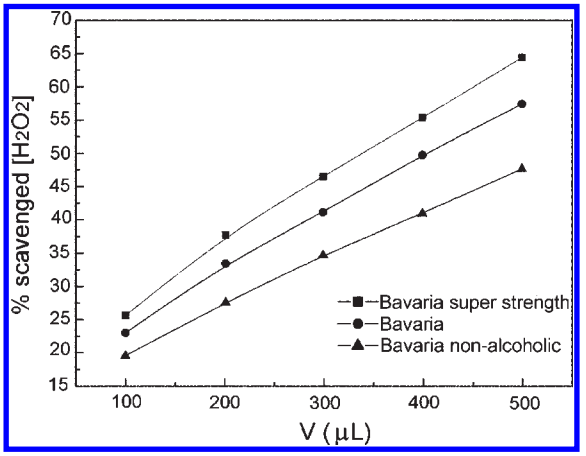

Figure 3. Dose dependence of Bavaria strong (super strength), standard, and nonalcoholic beer, with low content of original wort extract, effect on H_2O_2 i_l decrease, i.e., % of scavenged H_2O_2 vs volume of beers (μL).

various technological procedures and techniques used to inhibit alcohol formation or to remove it. Strong beers are brewed from wort with higher extract content and so are more efficient than standard ones. Lav-strong with high percentage of original extract (16.2%) exhibits considerably higher AO activity than Lav-standard (12%). The same difference has been noticed between Bavaria premium (11.4%) and Bavaria super strength (with no declared percentage of extract). Although all dark beers are not equally efficient, apparent differences between dark and blond beers of the same brand exist.

In contrast to prominent distinction between different types of commercial beers, a small difference between herb beers is noticed. Distinction has been observed only upon the addition of 400 and 500 μL of samples. The results are in accordance with extracts ratio in herb beers (0.2−0.3% (v/v)). Dose-dependence curve of herb beer with *Thymus*, in comparison to control, is shown in **Figure 6**. The AO_{50} values of *Thymus* and *Melissa* herb beers are similar to control beer. It could be assumed that at a higher ratio of extracts the difference would be more prominent.

Antioxidative Activity of Individual Phenolics Present in Beer. Beer contains a wide variety of phenolics with various structural features possessing widely different AO activity. Antioxidative activity of phenolic compounds was reported to be dependent on their molecular structures (*45*, *46*). The radical scavenging activity of the phenolic compounds depends on the numbers of hydroxyl groups, catechol moieties, and the presence of other hydrogen-donating groups. The order of AO activity of phenolic compounds ((+)-catechine, chlorogenic, caffeic, ferulic, and *p*-coumaric acid) assessed by this DC polarographic assay is in accordance with structure−activity relationship reported previously (*45*,*46*). As expected, flavan-3-ol (+)-catechin exhibits the most powerful AO activity (AO_{50} 63 μM) followed by chlorogenic acid (AO_{50} 200 μM). Chlorogenic acid dose dependence curve shows saturation phenomenon but AO_{50} is reached before saturation. Caffeic, ferulic, and *p*-coumaric acids dose response curves show saturation at lower percentage, without reaching 50% of i_{l0} decrease; values of 10% are used to make comparison with the literature data. Caffeic acid, 3,4-dihydroxycinnamic acid

Table 1. Antioxidant Activity of Commercial Beers, Expressed as AO_{50} (μL), AO_{50}^{-1} (mL^{-1}), and Total Phenolic Content (FC GAE ($\mu g\ mL^{-1}$))

brand of beer	country	type of beer	alcohol % (v/v)[a]	extract % (v/v)[a]	AO_{50} (μL)	AO_{50}^{-1} (mL^{-1})	FC GAE ($\mu g\ mL^{-1}$)
Leffe Dark	BE	top fermented, dark	6.3	15.6	252	3.97	544.3
Leffe Blond	BE	top fermented, strong	6.6		293	3.41	445.4
Guinness Extra stout	IE	top fermented, dark	4.95	11.4	308	3.25	446.6
Kilkenny	IE	top fermented, dark	4.3	9.86	362	2.76	333.6
Nikšićko Dark	ME	bottom fermented, dark	6.2	14.8	280	3.57	543.8
Nikšićko Blond	ME	bottom fermented, blond	5.0	11.4	380	2.63	278.4
Lav Strong	RS	bottom fermented, blond strong	7.2	16.2	285	3.51	408.0
Lav	RS	bottom fermented, blond	5.2	12.0	342	2.92	344.4
Bavaria super strength	NL	bottom fermented, blond	8.6		339	2.95	332.6
Bavaria Premium	NL	bottom fermented, blond	5.0	11.4	402	2.49	243.0
Bavaria Malt	NL	bottom fermented, alcohol-free	0.0	6.3	550	1.82	125.0
Beck's Pilsner	RS	bottom fermented, blond	5.0	11.2	361	2.77	268.4
Beck's, nonalcoholic	RS	bottom fermented, alcohol-free	0.5	11.3	405	2.47	244.4
Union Lager	SI	bottom fermented, blond	4.9	11.3	380	2.63	253.1
Union-UNI	SI	bottom fermented, nonalcoholic	0.5	6.5	520	1.92	183.4
Heineken	DE	bottom fermented, blond	5.0	11.4	320	3.12	292.7
Stella Artois	BE	bottom fermented, blond	5.2		353	2.83	300.0
Tuborg	DK	bottom fermented, blond	4.6	10.3	330	3.03	326.0
Budweiser	CZ	bottom fermented, blond	5.0	11.9	349	2.86	349.6
Carlsberg	DK	bottom fermented, blond	5.0	11.1	368	2.72	340.2
Holsten Pilsner	DE	bottom fermented, blond	5.22	11.6	302	3.31	387.7
Corona Extra	MX	bottom fermented, blond	4.6	11.3	430	2.33	241.5
Hoegaarden Wietbier	BE	top fermented, wheat, unfiltered	4.9	11.2	350	2.86	270.1

[a] Values of alcohol and original wort extract percentage declared on beer bottle.

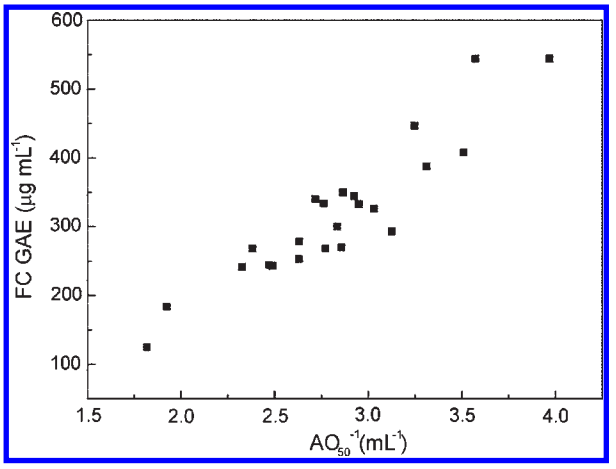

Figure 4. The relationship between AO activity of commercial beers, expressed as AO_{50}^{-1}, and total phenolics content (correlation coefficient $r^2 = 0.93$).

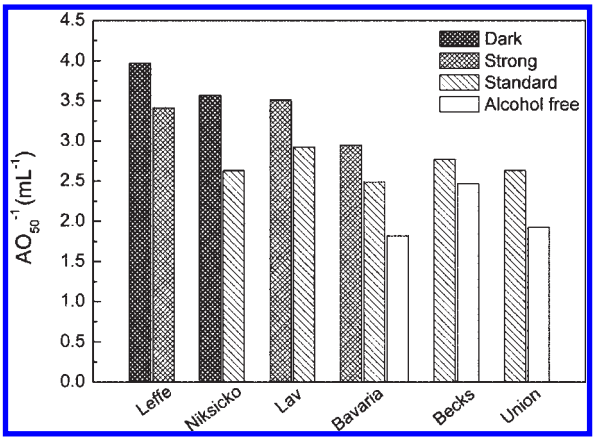

Figure 5. Antioxidative activity of dark, strong, standard, and nonalcoholic beers of different brands.

with two hydroxyl groups bonded to aromatic ring in the ortho position, has the stronger AO activity than cinnamic acids with one hydroxyl group, ferulic, and *p*-coumaric acids, the last one showing the lowest activity. Results are in accordance with a recent demonstration that caffeic acid was the strongest AO among cinnamate derivatives, as opposed to *p*-coumaric acid, which was found to be a poor H_2O_2 scavenger (*47*).

Monitoring of Brewing Process. Another objective of this study was to evaluate effects of different technological phases of brewing on AO activity changes. Modification of the assay enabled the fastest possible monitoring of the full scale process of beer production. On the basis of comparison of percentage of limiting H_2O_2 anodic current decrease upon addition of an aliquot of samples, withdrawn along the whole process of brewing, critical technological phases in relation to AO activity changes are revealed. Mashing as the crucial phase is studied more thoroughly than the rest of the process. Separate mashing processes are studied and compared in order to show differences among them depending on variations in malt quality, the ratio of corn grits added, and temperature regime (**Figure 7**). The remaining process has been assessed with the aim to reveal technological phases when AO activity changes occur (**Figure 8**).

Decrease at the beginning of mashing process, between 40 and 50 °C, explained by addition of maize adjunct, has been followed by continuous increase in AO activity. The same phenomenon was observed in all four cases, despite the differences in working

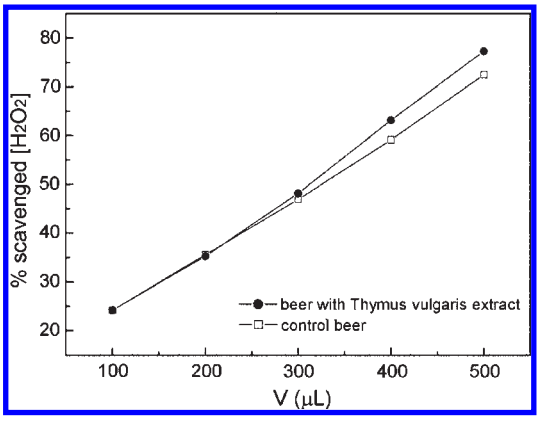

Figure 6. Dose dependence of beer with *Thymus vulgaris* extract effect on H_2O_2 i decrease in comparison to control beer, i.e., % of scavenged H_2O_2 vs volume of beers (μL).

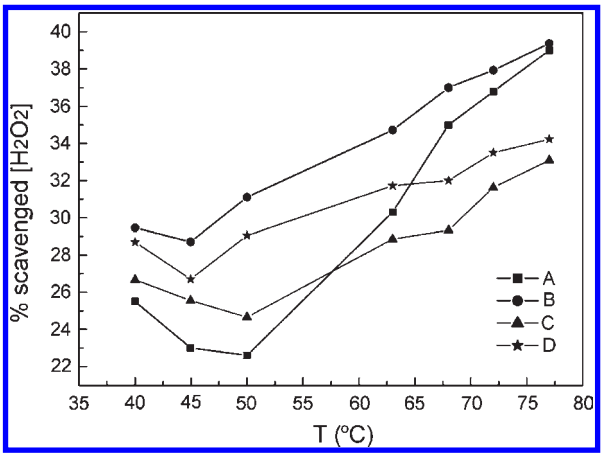

Figure 7. Changes of AO activity, expressed as percentage of scavenged H_2O_2, during the mashing process.

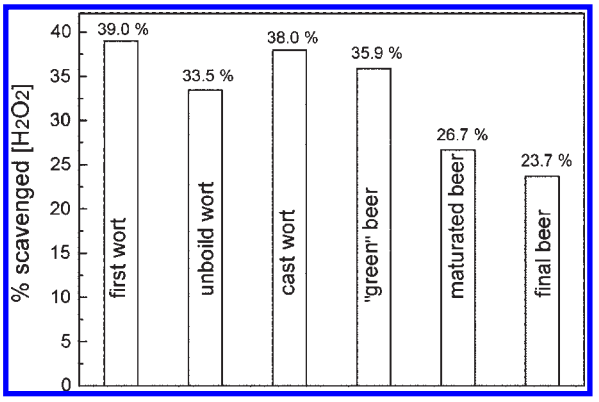

Figure 8. Changes of the AO activity, expressed as percentage of scavenged H_2O_2, from first wort to final beer.

parameters. Poorly modified malt and higher corn grist intake have direct influence on extract conversion and reduction of AO activity. In such conditions, prolonged temperatures rests are needed to achieve adequate extract yield, while prolonged hydrolysis is followed with an increase of AO activity.

Observed increase of AO during mashing is in accordance with previously published data (*37*). During mashing antioxidants are released from malt and dissolved. In addition, bound phenolics are released by enzyme hydrolysis. The release of the free and bound hydroxycinnamic acids during mashing (*48*), as well as

release of ferulic acid during malting and brewing (*49*), has been reported. Protein unfolding might contribute as well, since it leads to exposure of amino acids able to participate in the scavenging process. Antioxidants prevent excessive oxidative damage of polysaturated fatty acids during the mashing process (*37*).

Only the mashing process assigned as B, with first wort with highest AO activity, was followed. After wort separation, during the washing of the spent grains, a decrease in wort AO activity was observed due to dilution of the wort with sparging liquor (unboiled wort) (**Figure 8**). The effect of hopping is evaluated by comparing samples drawn after wort separation with those drawn after wort boiling and clarification. As hop displays much higher AO activity than pale malt, the difference between unboiled and cast wort, that is, increase in AO upon addition of hops, is observed (cast wort).

Continuous decrease of AO activity was noticed during subsequent stages of beer processing. The reason for this decay is the loss of tannins and nontannic flavonoids. As assessed by this assay, the most critical stages during the beer production process for changes in AO activity are filtration and clarification, as well as boiling, fermentation, and maturation. Obtained results are in accordance with previous studies (*35*, *36*). The decrease of AO activity from wort to beer, estimated using inhibition of oxidative modification of human LDL, was found to be 29% while the phenolic amount decay was 28% (*36*). Decrease from first wort to beer estimated using DC polarographic assay was 37.6%.

DISCUSSION

Antioxidants have attracted global attention for their free radicals scavenging ability and role in health and disease control. Various AO activity assays were developed and applied to food and beverage analysis, (*50*) and the chemistry behind them was discussed (*51*, *52*). Commonly used AO activity assays in food technology are based on the generation of free radicals easily detectable using flourometric or photometric techniques. The results in the measurement of AO activity are dependent on the method used. A single method cannot give a comprehensive prediction of AO efficiency of the different compounds. Numerous comparative studies of AO activity assays have been published. Differences between AO activity assays in terms of substrates, probes, reaction conditions, and quantification methods, as well as complex composition of food and beverages make comparison and correlation of the results challenging. The research area is very active; there is a requirement for a simple, rapid, and reliable assay, and new assays claiming to measure AO activity continue to be reported.

Although AO activity assays based on electrochemical detection are easily performed, they still receive only limited attention. Due to the high sensitivity, quickness, and possibility of direct determination, various electrochemical techniques can be easily applied for evaluating the AO status. Determination of total AO activity of pure substances, different extracts, or products by cyclic voltammetry (CV), rarely by DC or differential pulse polarography (DPP), has been reported. Phenolic acids and flavonoids were characterized by CV (*53*−*55*). Easily oxidizable polyphenols in beverages are efficiently measured by CV (*56*). Phenolic antioxidants in beverages were successfully ranked by reducing strength and characterized for reversibility using CV at a glassy carbon electrode (*57*). There are a few reports on voltammetry application in beer. Cyclic voltammograms of beer reflected its high AO effect (*54*). In addition, automated voltammetric determination of reducing compounds in beer was developed (*58*). Feasibility of DC polarography for studying AO properties was demonstrated (*59*, *60*); Hamilton and Tappel developed a rapid polarographic method for lipid peroxidation measurement based on the decrease of dissolved oxygen (*59*), while Bumber et al. reported polarographic study of AO activity of proteins and amino acids, based on the shift of the first oxygen reduction potential (*60*). A water-soluble vitamin E derivative-tocopherol monoglucoside AO activity was estimated by DPP (*61*).

Electrochemical assay, based on DC polarography, reported here is recommended for its advantages such as simplicity and low cost. The assay is rapidly performed, diversely applicable and highly reproducible. Turbidity of samples does not have any influence, as well as presence of other substances. The extraction of AOs from tested samples is not needed because the other compounds presented, such as alcohol or sugars, have no influence on polarographic response. No pretreatment was needed regardless of complex composition of examined samples. Moreover, the polarographic approach evaluates the overall AO activity of samples directly, without the use of reactive species. The possibility to use peroxide as uniform equivalent is one of the main advantages of polarographic assay. An equivalent based on decrease of H_2O_2 anodic limiting current can serve as criterion of AO activity.

Polarographic assay efficiency is demonstrated by applying it on samples related to the brewing industry and individual phenolics present in beer. Commercial beers, domestic, and imported, brands are included in the study, as well as new, herb beers. Comparison between AO activity of different types of beers, blond strong, standard, nonalcoholic, and dark, demonstrates assay ability to distinguish beer according to AO activity, expressed either as AO_{50} or AO_{50}^{-1}. Differences obtained for different types of the same brand beer are particularly illustrative. As expected, nonalcoholic beers with the lower content of original wort extract exhibit the lower AO activity than standard beers, while dark and strong beers have the highest AO_{50}^{-1}. Dark beers have a high content of melanoidins, as dark malt is produced by a high-kilning process. Melanoidins may act as AOs at different levels in the oxidative sequence, in a similar way to polyphenols. Peroxyl radical scavenging activity of melanoidins isolated from beer were investigated (*62*). A linear relationship between the peroxyl radical scavenging activity and the chromophore residues in the melanoidin skeleton responsible for browning was established (*62*). Strong correlation between AO activity and phenolic content and a weak correlation between AO activity and melanoidin content in Spain beers existed (*22*). The total phenolic content is significantly different among different types of commercial beers and strongly correlated with AO activity, expressed as AO_{50}^{-1}. Considering a special types of beers, herb beers, only a slight difference between control beer and beers with *Thymus* and *Mellisa* extracts is obtained, as expected on the basis of herbal extract content and their AO activity. The lack of significant difference may be related to insufficient dose of extract added in beer while results obtained for extracts themselves are consistent with known AO properties of *Mellisa*, *Thymus, and Ganoderma*.

The efficiency of DC polarographic assay is additionally approved determining AO activity of individual phenolic compounds present in beer. AO_{50} values for (+)-catechin and chlorogenic acid are in accordance with their AO activity accessed by DPPH and ABTS assays (*46*), while the rank of AO activity of beer phenolics obtained using DC polarography is in accordance with relationship structure−activity published previously (*45*, *46*).

The applicability of DC polarographic assay for brewing process surveying was shown as well. AO activity of samples taken during a full-scale industrial process of beer production is

measured using modification of the DC polarographic assay. Results are in line with previous studies. Increase in AO activity is observed during mashing, after the addition of hop and boiling, whereas there is a decrease after wort separation, fermentation, and filtration. Fast monitoring of the brewing process could help to optimize the process in order to obtain a final product with improved AO activity. Differences between separate processes could be correlated with technological parameters and variations in raw materials, and this will be the topic of the next publication.

In conclusion, the DC polarographic assay we developed is rapid, low cost, and simple enough to be widely applied either to survey the brewing process or to analyze the final product, beer. The AO activity of beer should be declared as important quality parameter . There is a great demand for healthy drinks and an insight into beer AO activity might affect moderate consumers' choice of preferable beer brand. Measurement of AO changes during beer production would enable optimization of brewing technology in respect to level of antioxidants extracted from malt and hops. Such optimization could have a significant impact on AO activity of beer.

ABBREVIATIONS USED

AO activity, antioxidative activity; AO_{50}, volume of sample required for 50% decrease of H_2O_2 limiting current; AO_{50}^{-1}, reciprocal value of AO_{50}; SCE, saturated calomel electrode; DC, direct current; DME, dropping mercury electrode; DPP, differential pulse polarography; i_l, limiting current.

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