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Electrochemical versus Spectrophotometric Assessment of Antioxidant Activity of Hop (*Humulus lupulus* L.) Products and Individual Compounds

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ABSTRACT: Antioxidant (AO) activity of extracts of hop cones (Serbian domestic varieties) and commercial hop products (Saaz, Spalter, Spalter select, and Magnum pellets) was determined by parallel application of recently developed direct current (DC) polarographic and widely used DPPH assay. Correlations between 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging and total phenolics (TPC) determined by the Folin-Ciocalteu assay (FC) (0.99), and between H_2O_2 scavenging (HPS) and humulone content (H) determined by conductometric method (0.94), total resins (TR) (0.85), and hop storage index (HIS) (−0.90), were found statistically significant at $p < 0.05$ level while complete lack of HPS correlation with TPC and DPPH was observed. To obtain an insight into differences between results of AO assays applied, activity of individual compounds, prevalent hop phenolics, and bitter acids was determined. By far superior HPS activity of humulone was followed by catechin, quercetin, xanthohumol, lupulone, and rutin. In contrast, DPPH scavenging activity of phenolics (quercetin > catechin > rutin > xantohumol) was found substantially higher than activity of bitter acids. According to ferric reducing antioxidant power (FRAP) and scavenging of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), higher AO activity was ascribed to phenolics, while almost neglecting humulone. Besides reliability, low cost, and an easy-to-handle procedure, an ability to recognize humulone as the major contributor of hop AO activity could allow DC polarographic assay to be applied in analysis of various hop-derived products.

KEYWORDS: *antioxidant activity, hop, hydrogen peroxide, humulone, lupulone, phenolics, polarography*

INTRODUCTION

Literature widely describes the content and composition of beer phenolics.[1,2] Antioxidant (AO) activity of different types of beer was extensively investigated by applying different AO assays.[3,4] Barley, malt, wort, and hop AO efficiency, as well as the effect of the malting, mashing, and brewing process on phenolics composition and AO potential of malt, wort, and beer were investigated.[4−11] Antioxidant activity determined by using various AO assays was in high correlation with total phenolics.[4,12] However, some opposed findings were reported. A negligible antioxidant effect of polyphenols in wort and beer was observed by using electron spin resonance (ESR) spin trapping.[13] Sulfite was found to be the only compound able to delay the formation of radicals. Some additional compounds different than phenolics were identified as contributors on beer AO activity as well. Evidence on lipid transfer protein, one of two major beer proteins,[14,15] DPPH scavenging, and AO activity was reported.[16]

Approximately 80% of beer phenols are derived from malt and about 20% from hop (*Humulus lupulus* L.), an indispensible beer ingredient. Commonly, AO activity of hop, a rich source of tannins, flavonol glycosides, and prenylated flavonoids, was linked to phenolic moiety.[17] Adding hop pellets to the kettle increased AO activity determined by using an assay based on the inhibition of linoleic acid oxidation in the presence of an initiator AAPH (2,20-azobis(2-amidinopropane) dihydrochloride), while addition of supercritical CO_2 hop extracts had no significant effect. The higher AO activity of low-α-acids samples versus bitter varieties and CO_2 hop extract was explained by large differences in hop flavonoids. Parameters of beer aging such as *trans*-2-nonenal were found lower in boiled wort exhibiting higher AO activity.[18] High scavenging ability of hop polyphenolics toward artificial radical species such as DPPH or physiologically relevant reactive oxygen species, peroxyl radical (ORAC), and hydroxyl radical (HORAC) was reported.[19] The predominant prenylflavonoid in hop, xanthohumol,[20] showed exceptionally high hydroxyl radical scavenging capacities, probably mediated by metal ion chelating properties. Xanthohumol was shown to scavenge hydroxyl and peroxyl radicals, and superoxide anion radicals.[21,22] Indeed, some studies reported findings opposite from widespread concept related to crucial contribution of phenolics to total AO activity of hop. Humulones (α-acid) and lupulones (β-acid), known for their wide biological and pharmacological properties,[23] were indicated as major hop antioxidants.[24] The study by Tagashira et al.[25] revealed for the first time that bitter acids had radical suppression activity against artificial DPPH radical. Later results were validated by using ESR by Tang et al.[26] and by Weietstok et al.[27] Significant DPPH radical quenching abilities of both α- and β-acids were evaluated by using ESR spectroscopy. Lower amounts of Strecker aldehydes in

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stored beer upon addition of hop to wort were also reported.[27] Assessment of AO activity of seven naturally derived components from hop extracts, such as humulones, lupulones, isohumulones, tetrahydroisohumulone, reduced isohumulone, hexahydroisohumulone, and xanthohumol, using seven different methods based on different reactive oxygen species, confirmed the DPPH scavenging activity of humulone.[28] AO properties of humulone, lupulone, and xanthohumole were examined by using a sensitive coulometric method.[29] Further research related to AO activity of various substances present in beer and hop, using various AO assay in parallel, would be a preferable avenue to obtain unequivocal evidence on major AOs present. Although a wide variety of AO assays was successfully applied in the analysis of brewing samples, including hop, requirement for a reliable, simple, cheap, and easy-to-handle assay capable of reflecting AO activity of all potentially relevant substances present still exists.

The aim of the present study was to explore the applicability of recently developed and optimized electrochemical assay, i.e., direct current (DC) polarographic measurement of the anodic current originating from hydrogen peroxide complex formation in alkaline solution, at potential of mercury dissolution[30] on hop material (extracts of cones of domestic origin, and hop pellets). Tannin extracts, commercial products containing low molecular weight hop polyphenolics, were included in the study as well. Since the first application of DC polarographic assay was reported on beer and wort samples,[4] this study represents an extension of the assay application on raw materials used in beer production. Widely accepted DPPH assay was applied in parallel. Antioxidant activity, i.e., hydrogen peroxide scavenging (HPS) activity expressed as the slope of the curve obtained by plotting the percentage of decrease of the polarographic anodic current versus volume of added hop extracts, has been compared with DPPH scavenging activity. Results of both AO assays applied were correlated with the content of total phenolics (TPC) determined by using the Folin-Ciocalteu assay, content of humulone (H) determined using standard conductometric assay, total resins (TR), and hop storage index (HSI). To identify unequivocally major contributors of hop extracts total HPS and DPPH scavenging activity phenolics and bitter acids were included in the study. Activity of prenylated chalconoid xanthohumol and the major constituents of hop tannin extract, flavan-3-ol catechin and rutin, glycoside between the flavonol quercetin, and the rutinose, was assessed, as well as quercetin itself. Two additional AO assays, ABTS and FRAP, were applied to enable more comprehensive comparison between the recently developed DC polarographic assay on one side, and the most commonly used spectrophotometric assays on the other side. Results yielded by four assays applied, especially the ability of a particular assay to recognize bitter acids as AOs, were discussed and compared enabling an insight into specific possibilities that DC polarographic assay could offer.

MATERIALS AND METHODS

Chemicals. Hydrogen peroxide was the medical grade 35% (v/v) solution, purchased from Belinka (Slovenia). Clark and Lubs (CL) buffer was made from the solution of 0.2 mol/L boric acid and potassium chloride by addition of 0.2 mol/L NaOH to the desired pH. For preparation of CL buffer pH 9.8, in 50.0 mL of the 0.2 mol/L solution of H_3BO_3 and KCl, 40.8 mL of 0.2 mol/L NaOH was added. The final concentration of KCl was 0.11 M. The substances for buffer preparations were of analytical grade quality. Phenolic compounds (catechin and rutin) were from Sigma (St. Louis, MO, USA). Working standard solutions of phenolics were prepared daily in ethanol. Concentrations of catechin and rutin were 2.0 mmol/L. The Folin-Ciocalteu reagent (phosphomolybdic/phosphotungstic acid) was from Merck (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl radical) reagent was from Aldrich (Milwaukee, WI).

Hop Samples. The water-soluble fraction from the ethanol extraction process that contains low molecular weight polyphenols, which have antioxidative properties, so-called tannin extract (dry matter 50%, total polyphenols 5.5%), as well as humulone 89%, lupulone 76%, and xanthohumol 76%, were obtained from Hopsteiner (Mainburg, Germany). The product is amended to be added into wort solution during boiling to increase AO activity (http://hopsteiner.com/pdf/20092/24_04_TanninExtract.pdf). Product specifications can be found on www.hopsteiner.com. Dried hop cones, various domestic varieties ("Bačka", "Aroma" and "Robusta"), harvest 2009, were obtained from Institute of Field and Vegetable Novi Sad (Serbia) where they were selected. Hop pellets from various producers (Saaz, Spalter, Spalter select, and Magnum pellets) were obtained from BIP (Brewing Industry of Belgrade).

Determination of Moisture. Dry matter content was determined by the method of moisture content of hops and hop products (European Brewery Convention-EBC, 7.2, 1998).[31]

Determination of Total Resins (TR). Total resins (%) were determined according to EBC 7, 1998, method 7.6.[31]

Determination of Content of Humulone (*H*). The content of α-acids in hops and hop products was determined by the widespread conductometric method based on the precipitation of bitter acids with lead salt. This method yields a conductometric value (CV) in percent (EBC 7, 7.6, 1998).[31]

Determination of Hop Storage Index (HSI). HSI was determined according to American Society of Brewing Chemists (ASBC) Method of Analysis entitled HOPS-6.

Extraction of Hop Cones and Pellets. To simulate hop processing in the brewing process, hop samples were extracted with hot water according to the procedure reported.[17] Determination of water content in dried cones 2010 harvest and pellets showed the values of 7.0−10.2%. For determination of antioxidant activity, 1 g of dry matrix was weighed. Before the analysis, dried cones and pellets were ground in a centrifugal mill to a particle size of 1.5 mm. After 30 min of boiling the contents of the flask was cooled and transferred quantitatively to a 200 mL volumetric flask and distilled water was added to the mark. After passage through a filtration paper and a cellulose membrane filter with a 0.45 μm pore size, the measurement of AO activity and total phenolic content was conducted.

Determination of Antioxidant Activity Using 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH) Assay. Free radical scavenging activity of hope extracts (HE) was analyzed by DPPH assay, based on determination of the remaining DPPH radical violet color after reaction with potential AOs, monitored by measurement of absorbance on 517 nm. A modified method of Blois,[32] was used: A volume of 200 μL of diluted HE was mixed with 1800 μL of a methanolic solution of DPPH (0.1 mM). After shaking and standing 30 min in dark, absorbance on 517 nm was measured. Appropriate water dilutions of HE (1:6 to 1:1) were experimentally found to satisfy the linear dependence of concentration vs absorbance. All samples were prepared in triplicate, and four dilutions of each HE were used to obtain the EC_{50} value from the graph I (%) = $f(c)$. Percentage of inhibition of DPPH radical (I (%)) of each HE was calculated according to eq 1:

$$I\ (\%) = \left[\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}}\right] \times 100 \qquad (1)$$

where A_{blank} is absorbance of DPPH with water instead of the sample and A_{sample} is absorbance of DPPH after reaction with tested hop sample. Results are expressed as the reciprocal value of I (%) multiplied by 100.

Determination of Total Phenolic Content (TPC). The total phenolic content of HE was determined by the Folin-Ciocalteu (FC) assay, according to the modified method of Singleton et al.[33] A volume of 200 μL of diluted HE was mixed with 1000 μL of FC reagent diluted with distilled water in proportion 1:10. After 6 min in the dark, 800 μL of sodium carbonate solution (7.5%) was added. After shaking and standing 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 HE were used and results were averaged. Appropriate dilutions of each extract were experimentally found (1:10 to 1:5) to give absorbance between 0.2 and 0.7 on 740 nm. Each absorbance was adjusted for the value of blank probe. The same procedure was used for four concentrations of gallic acid standards (10, 25, 50, and 100 μg/mL), and a calibration

Table 1. Hop Pellets and Cones Moisture (M), Total Resins (TR) Content, Hop Storage Index (HSI), α-Acids Content (H), Total Phenolic Content (TPC), Radical Scavenging Activity against DPPH Radical (EC_{50}^{-1}), and Hydrogen Peroxide Scavenging Activity (HPS) Determined Using DC Polarographic Assay[a]

	hop pellet				hop variety		
	Saaz	Spalter	Spalter select	Magnum	Bačka	Aroma	Robusta
M (%)	7.0 ± 0.3a	7.1 ± 0.3a	8.2 ± 0.3b	7.9 ± 0.3b	9.7 ± 0.c3	10.7 ± 0.3d	10.2 ± 0.3c
TR (%)	16.31 ± 0.01c	16.02 ± 0.01b	18.37 ± 0.01f	31.41 ± 0.01g	9.91 ± 0.01a	16.47 ± 0.01d	17.99 ± 0.01e
HSI	1.47 ± 0.65a	1.16 ± 0.43a	1.09 ± 0.29a	0.73 ± 0.26a	1.57 ± 0.73a	1.04 ± 0.53a	0.99 ± 0.28a
H (%)	3.55 ± 0.27d	2.59 ± 0.27c	5.45 ± 0.27a	14.10 ± 0.27	0.68 ± 0.27b	5.97 ± 0.28a	6.83 ± 0.27e
TPC (GAE)	182.7 ± 6.5c	137.5 ± 2.8a	190.8 ± 4.6c	127.0 ± 5.3d	139.2 ± 2.2a	117.9 ± 3.7b	139.1 ± 1.8a
EC_{50}^{-1} ($\%^{-1}$)	49.3 ± 1.4e	38.0 ± 0.9d	55.5 ± 1.7f	31.3 ± 0.9c	34.9 ± 1.3a	27.9 ± 1.0b	34.4 ± 1.1a
HPS (%/mL)	23.3 ± 1.8a	25.0 ± 1.9abc	32.6 ± 3.3cd	47.5 ± 4.2e	23.7 ± 2.1ab	32.3 ± 3.4bcd	39.9 ± 3.5de

[a]Values with the same letter (a–f) are not statistically different at the $p < 0.05$ level (post hoc Tukey's HSD test); data represent the means of a triplicate experiment ± standard deviation.

curve $A = f(c)$ was plotted. The total phenolic content was expressed in gallic acid equivalent (GAE), as the concentration of gallic acid (μg/mL) that corresponds to the dilution of HE with the same value of absorbance at 740 nm.

Determination of Total Reducing Power Using Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was performed according to the procedure previously described by Benzie and Strain[34] with some modification. The FRAP reagent solution was made by mixing acetate buffering agent (pH 3.6), TPTZ (10 mM TPTZ solution in 40 mM HCl) and $FeCl_3 \times 6H_2O$ in volume ratio 10:1:1, respectively. All samples, standards, and reagents were preincubated at 37 °C. An aliquot of each diluted HE sample (0.1 mL) was mixed with distilled water (0.3 mL) and FRAP reagent (3 mL). After the reaction at 37 °C for 40 min, the absorbance at 593 nm was measured. The calibration curve was prepared with Trolox solution and the results were expressed as mM of Trolox equivalent per liter of sample (mM TE/L). Measurements were done in triplicate

Determination of Antiradical Activity Using 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Assay. The ABTS assay was conducted according to the procedure described by Re et al.,[35] with some modifications. A solution of 14 mM ABTS and 4.9 mM potassium persulfate was prepared in a phosphate buffer (pH 7.4), and mixed in equal volumes to produce a stable $ABTS^{\bullet+}$ stock solution. The obtained dark blue-green stock solution was left in the dark at room temperature for 12–16 h before use. A working $ABTS^{\bullet+}$ solution was prepared by diluting a $ABTS^{\bullet+}$ stock solution (approximately 1/80 dilution) to an absorbance of 0.70 ± 0.02 absorbance units (AU) at 734 nm and 30 °C, using phosphate buffer. After addition of 3.0 mL of working $ABTS^{\bullet+}$ solution to 30 μL of HE or Trolox standards (2.50, 1.25, 0.625, 0.312, and 0.156 mM Trolox solutions in phosphate buffer) the absorbance reading was taken at 30 °C, 6 min after initial mixing. Appropriate solvent blanks were run in each assay. The fractional inhibition of $ABTS^{\bullet+}$ radical was calculated and plotted as dose-dependence curves (i.e., concentration vs fractional inhibition) for Trolox and each sample. The TEAC value (Trolox equivalent antioxidant capacity) was calculated by using the following equation: TEAC (mM) (slope of the dose-dependence of sample/slope of the dose-dependence of Trolox).

Determination of Hydrogen Peroxide Scavenging (HPS) Activity by DC Polarographic Assay. A recently developed polarographic method,[30] based on atypical anodic current shape obtained on dropping mercury electrode (DME) in the presence of hydrogen peroxide in alkaline solution and its decrease in the presence of antioxidants, was used. Dependence of anodic limiting current (peak) decrease on the amount of added sample, during its addition in equal aliquots, was followed and plotted. The slope of the starting linear part of that plot was considered as a measure of hydrogen peroxide scavenging (HPS) activity of that sample. It was expressed as percentage of peak height decrease per volume of complex samples added or amount of individual compounds. Polarographic current–potential (i–E) curves were recorded on a Houston Instrument Omnigraphic 2000 X–Y recorder, using the Princeton Applied Research 174 Polarographic analyzer. The dropping time of the DME working electrode was programmed on time $\tau = 1$ s, while the low pass filter was set at 3 s. A saturated calomel electrode (SCE) was used as reference and platinum foil as auxiliary electrode. The initial potential was 0.10 V vs SCE and the potential scan rate was 10 mV/s. Clark Lubs (CL) buffer of pH 9.8 was prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl, and 40.8 mL of 0.2 M NaOH. The initial solution was prepared by addition of 0.100 mL of 1.00 M solution of H_2O_2 in 19.9 mL of CL buffer at pH 9.8. Before every i–E curve recording (of initial solution as well as after addition of every aliquot of investigated sample) solution was deaerated with pure gaseous nitrogen.

Statistical Analysis. Descriptive statistical analyses for calculating the means and the standard error of the mean were performed with use of StatSoft Statistica 10 software. All obtained results were expressed as the mean ± standard deviation (SD). The evaluation of one-way analysis of variance (ANOVA) of obtained results was performed for comparison of means, and significant differences are calculated according to posthoc Tukey's HSD test at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Antioxidant Activity of Various Hop Varieties and Commercial Hop Products. Until now, DC polarographic assay developed and optimized recently[30] has been applied on a wide variety of complex food samples, including various types of commercial and small scale beers.[4] Monitoring of the full-scale industrial process of beer production was achieved. Increase in AO activity was observed during mashing, after the addition of hop and boiling, whereas there is a decrease after wort separation, fermentation, and filtration. The effect of hopping was 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 was observed.

The objective of this study was to explore a possibility of an application to analysis of hop as an important raw material in brewing. Properties of domestic hop varieties, of hop derived products, and of hop ingredients, such as phenolics and bitter acids, were analyzed. Samples of hop cones and pellets have been studied as well for hop resins (TR), storage index (HSI), content of humulone (H), and total phenolics content (TPC) (Table 1). Reported values have been in good agreement with those reported for a variety of hops.[31] Inappropriate finishing causes low HSI of hop cones while long storage has been found responsible for HSI of Magnum pellets. Decrease of the index during the storage has been observed even including two samples with an index bellow 1.0. Hop cones and pellets extracts, prepared uniformly, have been assessed for AO activity

by using electrochemical DC polarographic and spectrophotometric DPPH assays in parallel. Previously, differences between AO activity of Czech, German, USA, and Slovenian hop varieties and various hops products were followed by DPPH radical scavenging assay, earlier considered the best for the determination of AO characteristics of hops and hop products.[17] Results of the DC polarographic assay have been based on the slope of the plot of the dependence of the percentage of hydrogen peroxide anodic current decrease on the volume of hop extracts. DPPH scavenging activity has been expressed as the reciprocal value of EC_{50} since its higher value corresponds to a higher activity. The post HOC Tukey's HSD tests have been compared for the samples within each of the experimental methods used, and statistically significant differences were found in numerous samples, $p < 0.05$ (Table 1).

Polarograms obtained by gradual addition of appropriately diluted tannin extract, rich in low molecular weight polyphenols, including catechin and rutin, into buffered solution of hydrogen peroxide were shown in Figure 1. Efficiency of tannin extract to scavenge artificial DPPH radical was measured in parallel. High HPS (HPS = (17.0 ± 1.2) × 10^4%/g) and DPPH activity (EC_{50}^{-1} = 17.4 ± 0.6 mL/mg) were ascribed to tannin extract, declared by producers as a source of power phenolics AOs. Evidence on efficient peroxyl radical scavenging by tannin extracts was reported recently.[19]

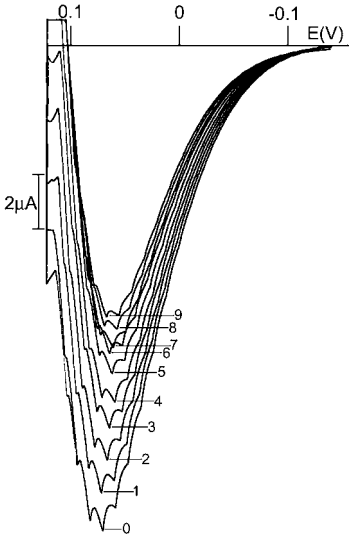

Figure 1. Polarograms (1−9) obtained by gradual addition of tannin extract in aliquots of 50 μL of solution obtained by dissolution of 11.4 mg of tannin extract in 10 mL of CL buffer at pH 9.8 in 20 mL of initial solution (0).

Correlation between Antioxidant Activity, Total Phenolics Content, Humulone Content, Total Resins, and Hop Storage Index. Antioxidant activity determined by using both DPPH and DC polarographic assay has been correlated with TPC and content of α-acids. Phenolics contribution has been confirmed to be prevalent in DPPH scavenging activity of hop but not in HPS activity. While DPPH correlates with TPC, high correlation between HPS and humulone content has been noticed (Figure 2). Strong indication that HPS activity is linked prevalently with *H* has been obtained from their unexpectedly high positive correlation. It is worth noting that the most substantial deviation from linearity has been obtained for two samples with low HSI.

Correlation analysis applied on AO activity determined by both assays applied and parameters related to hop quality, such as TPC, TR, and *H*, yielded the correlation coefficients given in Table 2. As seen correlations between HPS and α-acids (0.941) have been found statistically significant at $p < 0.01$ level. Both content of humulone and HPS correlated positively with total resins (0.97 and 0.85) and negatively with HSI (0.88 and 0.90). As expected, total resins correlated positively with the content of α-acids (0.97), while negatively with HSI (0.84). Correlation between DPPH and TPC has been found to be very strict (0.99, $p < 0.001$). Complete lack of DPPH and TPC correlations with HPS, HSI, TR, and α-acids has been observed. Recently, similar radical inhibiting properties of commercially

Table 2. Correlations between HPS Activity, DPPH Scavenging, Total Penolic Content (TPC), Content of α-acids (*H*), Total Resin (TR) Content, and Hop Storage Index (HSI)[a]

	HIS	*H*	TPC	DPPH	HPS
TR	−0.840*	0.966*	−0.171	−0.159	0.853*
HSI		−0.881*	0.381	0.334	−0.903*
H			−0.282	−0.293	0.941*
TPC				0.986*	−0.352
DPPH					−0.362

[a]Correlations marked with an asterisk are statistically significant at $p < 0.05$ level.

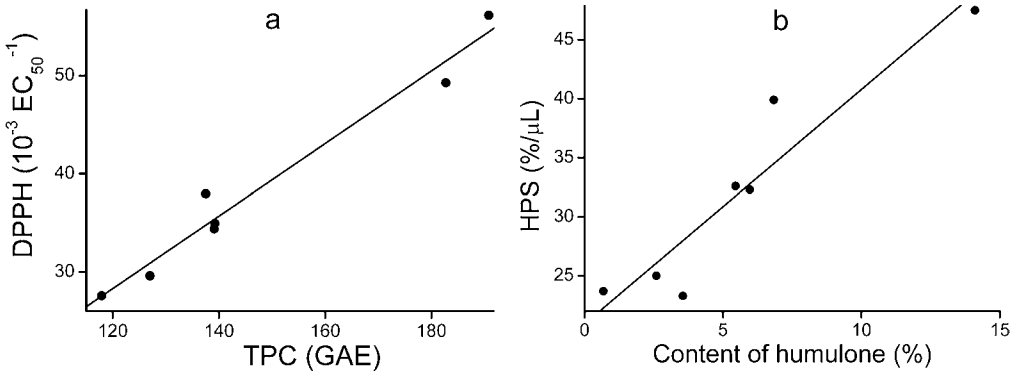

Figure 2. (a) Correlation between total phenolics content (TPC) and DPPH scavenging for HE; (b) correlation between content of humulones and HPS activity determined using DC polarographic assay.

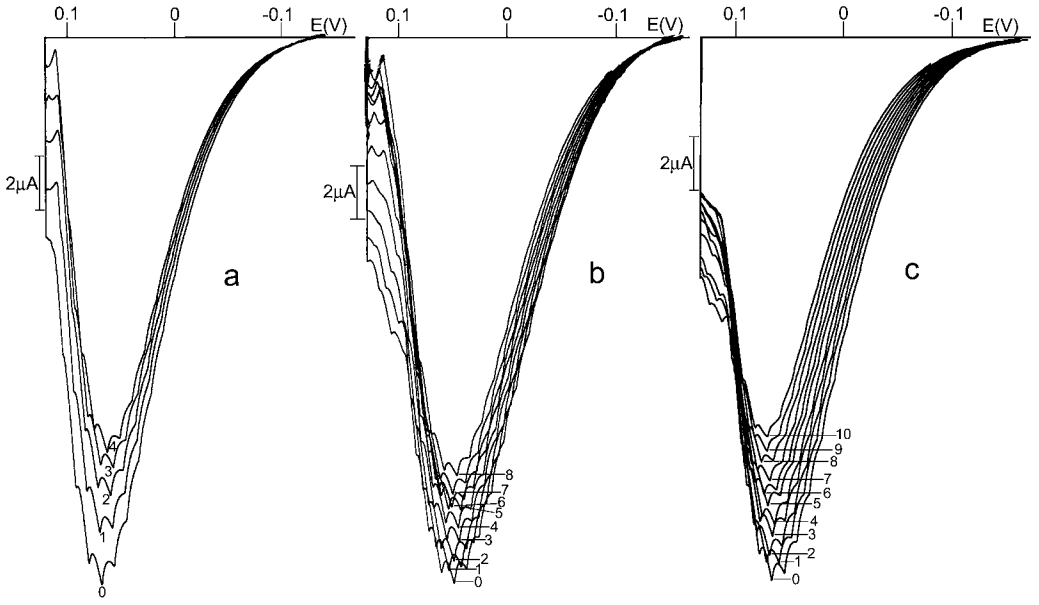

Figure 3. Polarograms (1−10) obtained by addition of aliquots of 50 μL of 2×10^{-3} M solutions of humulone (a), rutin (b), and xanthohumol (c) in 20 mL of initial solution (0).

available hop products with varying polyphenol content were observed. Hop products have significant DPPH$^{\bullet}$ radical quenching abilities, evaluated by using ESR spectroscopy, as a function of hop acid content.[19]

Until now, a broad variety of complex samples have been investigated by using DC polarographic assay and high correlation with TPC, as well as AO activity determined by various assays being commonly found. This is a first example of a complete lack of correlation between HPS activity of complex samples and both TPC and DPPH scavenge. High correlation between HPS and TPC was found for various beer samples (0.93)[4] while high correlation with both TPC and DPPH for various red and white wines (0.99),[36] strong alcoholic beverages (0.97 and 0.92),[37] true teas (0.80 and 0.98) and herbal infusions (0.93 and 0.91),[38] honey of various floral origin (0.90 and 0.84),[39] propolis (0.99),[40] etc. Finding that correlation between HPS and TPC for some of the analyzed samples was even higher than correlation between DPPH and TPC, indicating that HPS reflected the activity of a wider range of AOs than DPPH. In addition, high correlations were observed between HPS activity of samples, such as teas, herbal infusion and honey samples, and ABTS, FRAP, ORAC, etc.

Antioxidant Activity of Individual Hop Compounds. Correlation of hop cones and pellets extracts HPS and DPPH scavenging activity with content of total phenolics and humulones gave guidance on the main contributors of total AO activity. To confirm the assumption on prevalent contribution of humulone to HPS activity this study was extended. Assessment of DPPH and H_2O_2 scavenging of major individual compounds known to be present in hop was included. Besides bitter acids, humulone and lupulone, and predominant prenylflavonoid xanthohumol, individual phenolics present in tannin extracts, catechin and rutin, as well as quercetin, the phenolic part of rutin, have been considered. Addition of humulone into electrolytic solution showed a more significant effect than addition of chosen phenolics, as seen on polarograms presented in Figure 3.

Results of the DC polarographic assay have been compared with common spectrophotometric AO assays (Figure 4).

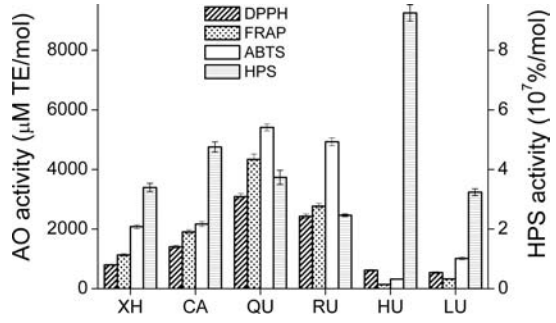

Figure 4. Antioxidant activity of xanthohumol (XH), catechin (CA), quercetin (QU), rutin (RU), humulone (HU), and lupulone (LU) deterimined using DPPH, ABTS, and FRAP assay (μM TE/mol) and HPS activity using DC polarographic assay (%/mol). Data represent the means of a triplicate experiment ± standard deviation.

Obviously, HPS and DPPH scavenging activity of individual compounds can explain correlations obtained unequivocally. Two additional assays, ABTS and FRAP, have been applied to obtain more comprehensive insight into differences between the recently developed DC polarographic assay and the most commonly applied spectrophotometric ones. Also, the diverse ability of spectrophotometric assays to recognize bitter acids as antioxidant has been enlightened, as seen on histograms shown in Figure 4.

The following rank of order of HPS activity HU > CA > QU > XH > LU > RU has been obtained. Humulone HPS activity has been found by far superior among all analyzed samples. Twofold higher activity has been ascribed to humulone than the most active phenolic catechin, and about fourfold higher activity than rutin, with the lowest activity among analyzed substances. HPS activity of lupulone has been found almost 3 times lower than humulone. Still, lupulone HPS activity has been shown to be 1.3 times higher than rutin, almost equal to xanthohumol and only 1.5 times lower than catechin. Quercetin was used for comparative purposes in the recent study of radical scavenging activity of hop-derived products. Superior peroxyl

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and hydroxyl scavenging activity of xanthohumol than quercetin was found.[19]

Lack of correlation between HPS activity and TPC of HE, and unexpectedly high correlation with humulone, has been explained by the dominance of humulone in terms of HPS activity. Results presented confirmed that pure hop compound having a six-member ring configuration (semiquinone or quinoid), such as humulone, has stronger HPS activity than phenolics. Based on corroboration between HPS activity and inhibition activity against lipid peroxidation, reported previously by Tagashira et al.,[25] the physiological relevancy of data obtained by DC polarographic assay could be assumed. Involvement of $\beta\beta$-triketone moiety in lipid peroxidation inhibition activity was assumed by Tagashira et al.[25] However, the physiological relevancy of results obtained by using DC polarographic assay needs to be further examined. Also, the structure−activity relationship remains to be established more comprehensively.

The following rank of order of DPPH scavenging activity QU > RU > CA > XH > HU > LU can provide explanation for both high DPPH activity of tannin extracts and positive correlation between DPPH scavenge and TPC. The highest values of DPPH radical scavenging activity of quercetin were followed by rutin, catechin, and xanthohumol. Although less efficient scavengers of DPPH radical than phenolics, bitter acids have shown significant activity. Xanthohumol, catechin, rutin, and quercetin activity have been found 1.3, 2.3, 3.9, and 5 times higher than humulone and 1.5, 2.6, 4.5, and 5.7 higher than lupulone suggesting that radical scavenging activity of both humulone and lupulone should not be neglected. Results presented are in corroboration with previously published ones.[25−28] Potent DPPH scavenging activity of bitter acids revealed by Tagashira et al.[25] was supported by using electron spin resonance with a spin trap of *tert*-butylphenylnitrone.[26] Significant DPPH radical quenching abilities of hop α- and β-acids and the negligible effect of iso-α-acids and a hop polyphenol extract were evaluated using ESR spectroscopy by Weietstok et al.[27] In addition, humulone showed the highest quenching activity against the nitrite radical and DPPH radical while xanthohumol against peroxyl radical (ORAC), hydroxyl radical (HORAC), superoxide (SOD), and ferric ion (FRAP).[28]

Activities determined by ABTS and FRAP have followed DPPH scavenging activity, rather than HPS activity. The rank of order of phenolics has been found to be the same as that of DPPH scavenging. However, the opposite rank of order of bitter acids has been noticed. According to ABTS and FRAP assays, lupulone possesses about 2 and 3 times higher activity than humulone, respectively. In comparison to DPPH scavenging activity, the ratio between ABTS scavenging activity and ferric reducing activity of phenolics and bitter acids has been found lower. Obviously, the DPPH assay distinguished bitter acids as more efficient AOs than the other two spectrophotometric assays applied. Considering the ratio of phenolics and humulones activity, the difference between HPS and FRAP, especially ABTS, has been found more prominent than the deference between HPS and DPPH. The ratio between humulone and quercetin HPS activity has been found to be almost 2.5, while the ratio between DPPH, FRAP, and ABTS of quercetin, the most active phenolic according to all spectrophotometric assays applied, and humulone has been found to be about 5, 17, and 31, respectively. Xanthohumol activity determined by DPPH, ABTS, and FRAP has been found to be about 1.3, 6.5, and 8.1 times higher than humulone, while its HPS activity is 2.7 lower than humulone.

Different chemistry behind applied assays yields variations in results obtained, confirming that more than one analytical method should be applied to reliably elucidate AO capacity of complex samples.[41] Despite the recent efforts of the scientific community a widely accepted standardized method has not yet been established.[42] The number of novel AO assays and modifications introduced increased considerably. A simple, direct, and high-throughput assay such the DC polarographic one applied in this study could find wide applications in research, food industry, and drug discovery. Advantages of this assay, such as an easy-to-handle procedure, low cost, possibility to work with colored and turbid samples, and no requirement for reactive artificial radical species, were mentioned in previously published applications. An ability to recognize humulone as major AOs in hop could allow the assay to be applied in analysis of hop-derived products, both those used in brewing or traditional and novel health applications. In the future, novel DC polarographic assays developed in our laboratory will be applied on various hop samples to demonstrate additional advantages of this approach to AO activity determination.

In conclusion, in contrast to the most commonly applied spectrophotometric AO assays DPPH, FRAP, and ABTS, the recently developed DC polarographic assay recognizes humulone as a superior antioxidant in comparison with various hop phenolics. Data reported could serve as additional evidence on bitter acids efficiency as antioxidants, indicated in recent literature, and suggests that their contribution to total AO activity of complex hop samples should not be neglected. Hop cones and pellets extracts HPS activity positively correlated with the content of humulone and total resins, and negatively with hop storage index, enabling potentially important insight into the quality of hop material. Based on presented results, and having in mind previous ones, the DC polarographic assay can be recommended for wide application in the brewing industry, to analyze raw materials, to survey the brewing process with the aim to optimize relevant technological factors, and to analyze quality of final product.

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