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ORIGINAL PAPER

Relationship among EPR oxidative stability and spectrophotometric parameters connected to antioxidant activity in beer samples

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Abstract

A relationship between EPR and spectrophotometric parameters related to beer staling and antioxidant activity, was identified. AUC (area under the curve), intensity at 150 min (T_{150}), radical scavenging activity (RSA), total phenolic compounds (TPC), hydroxyl radical scavenging capacity (HRSC) and one parameter linked to staling degree of beers (thiobarbituric index, TBI) were related. Temperature was modified to find the proper working conditions for EPR spin-trapping experiments and it was found that it affected the kinetic of PBN adduct evolution. For the samples reaching a maximum intensity signal, the higher the heating temperature, the shorter the time interval needed to reach it. No linear relationship was detected among parameters obtained with EPR spin trapping experiments and RSA, TPC, TPI, and HRSC when correlating one parameter with another. On the contrary, a good linear relationship was found among AUC or T_{150} and a combination of RSA, TPC, TPI, and HRSC ($R^2 = 0.9562$ and 0.9694, respectively). The goodness of fit increased to $R^2 = 1$ when a combination of AUC and T_{150} was related to a combination of RSA, TPC, and TPI, and HRSC.

Keywords Beer aging · Electron paramagnetic resonance spectroscopy · Spin trapping · Antioxidants

Introduction

Beer shelf life is influenced by raw materials, production processes [1] and storage conditions. The brewing style affects beer shelf life as raw materials (type of malt: base, caramel, crystal, chocolate, etc. [2, 3]; hop variety: aroma or bittering hop [4, 5]) with different resistance to oxidation are employed. Moreover, during storage, other factors such as heat, light and oxygen can deteriorate beer decreasing its shelf life [6, 7]. Chemical modifications of beers result in flavor deterioration, increase of the color intensity and decrease of bitterness. During storage, some volatile compounds can be degraded and some others with unpleasant flavor can be formed [8, 9]. *Trans*-2-nonenal, responsible for cardboard flavor of beers, can be formed by the oxidation of linoleic acid by an enzymatic or a chemical route [8, 10, 11]. Higher alcohols can be oxidized to the corresponding aldehydes [12].

Hydroxyl radical reacts with ethanol producing the 1-hydroxyethyl radical which degrades into acetaldehyde. Similarly, the hydroxyl radical can react with higher alcohols producing long chain aldehydes which can affect the beer flavor [8, 13]. The alpha acids from hops can also be degraded during storage of beers, leading to a decrease in bitterness [14, 15].

The Electron Paramagnetic Resonance (EPR) spectroscopy has been proposed as an analytical method to relate the resistance of beer to forced oxidation conditions with its shelf life [16]. When the beer samples are heated at 60 °C in the presence of *tert*-butylphenylnitrone (PBN), 1-hydroxyethyl radicals are trapped by PBN forming relatively stable paramagnetic adducts which can be detected by EPR spectroscopy [17]. When the EPR signal of the PBN adduct does not raise immediately, the time period it takes to increase rapidly is usually called "lag time" [18].

Despite its usefulness, EPR spectroscopy use in beer control quality is limited to international breweries. For this reason, we tried to relate the parameters of spin-trapping experiments obtained with EPR spectroscopy with those

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related to antioxidant activity and staling degree of beers obtained with a spectrophotometer.

In a previous study, we examined the influence of PBN (N-tert-Butyl-α-phenylnitrone) concentration and alcohol content on the lag-time determination of three beer samples, demonstrating that the lag time is not always present regardless of the PBN or alcohol concentrations employed [19]. This is because there is not an appreciable slope change but a continuous increase in the kinetic curve describing the intensity of the PBN adduct *vs*. time. However, the lack of a lag-time in these beer samples [19] was not related to stale beers as demonstrated by their antioxidant activity determination with the DPPH assay.

As already shown in the literature by Uchida et al. [20], increasing the temperature of spin-trapping experiments in the range 60–80 °C has the effect of decreasing the time at which there is a sudden increase of the PBN adduct signal intensity (lag time). Since the reactions producing radicals are faster at higher temperatures, it could be supposed that they become slower at lower temperatures. Therefore, at lower temperatures the lag time values should be longer, making easier their determination. With this aim we decided here to change the temperature at which the samples are heated in spin-trapping experiments in the range 40–80 °C depending on the beer sample, to check whether the lag-time parameter was present in conditions different from those usually employed (60 °C).

Moreover, as previously mentioned another objective of this work was to identify a relationship among the parameters of spin-trapping experiments obtained with EPR spectroscopy with those related to antioxidant activity and staling degree of beers.

Materials and methods

Chemicals

Gallic acid, $FeSO_4 \times 7H_2O$, N-tert-Butyl-α-phenylnitrone (PBN), absolute ethanol, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu′s phenol reagent, and 2-Thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Milan, Italy). DMPO (5,5-dimethyl-1-pyrroline N-oxide) was purchased from Enzo Life Inc. (New York, USA).

Beer samples

Six commercially available beers, three lagers, and three ales differing by production style and alcohol content, were analysed: a Strong Lager (Dortmunder Style Export) with 7.7% of alcohol, a Belgian Lager with 5.0% of alcohol, a Pilsner (Bohemian Style Pilsner) with 4.4% of alcohol, a Blonde Ale (Belgian Style Blonde) with 6.6% of alcohol, a bottle conditioned India Pale Ale (IPA) with 5.5% of alcohol, and an Irish Dry Stout with 4.2% of alcohol. All beers were purchased from a retail market and analysed at least three months before the expiring date. Beers were decarbonated by bubbling nitrogen gas followed by centrifugation (1210 g, at RT for 15 min, thrice), aliquoted and stored at − 20 °C until analysis.

EPR spin-trapping experiments

A volume of 5 µL of a PBN solution 2.5 mM in absolute ethanol was dried under a flux of nitrogen. The solid PBN was solubilized in 250 µL of decarbonated beer samples to have a final PBN concentration of 50 mM. Then 100 µL of this solution were transferred to capillary tubes and inserted in the EPR cavity heated at 40, 50, 60, 70, or 80 °C. EPR spectra were recorded for at least 150 min, acquiring spectra every 5 min. Three replicates were examined for each sample.

EPR measurements were carried out with a Bruker EMX spectrometer operating at the X-band (9.40 GHz) equipped with an HP 53150A frequency counter and with an ER 4111 VT variable temperature unit. The EPR instrument was set under the following conditions: modulation frequency 100 kHz; modulation amplitude 1.0 G; receiver gain 5×10^5; microwave power 20 mW; time constant, and conversion time 163.84 ms. According to ref. [21], the intensity of the PBN adduct after 150 min (T_{150}) of thermal treatment and the area under the curve (AUC) intensity vs. time were also considered. EPR spectra of the radicals were simulated with the software Bruker WINEPR SimFonia (version 1.26 (beta), Bruker Analytik GmbH: Berlin, Germany, 1997).

DPPH assay

A solution 1 mM of DPPH in absolute ethanol was prepared. A total of 100 µL of this solution were mixed with 150 µL of variably diluted beer samples and with 1.75 mL of absolute ethanol. The samples were kept in the dark at room temperature for 30 min and then the absorbance at 517 nm was measured with a Perkin Elmer Lambda 35 spectrophotometer.

Since the samples became turbid, they were centrifuged at 1210 g for 5 min before the measurement. The % of inhibition was calculated as follows:

$\%\ \text{of inhibition} = (ABS_{blank} - ABS_{sample})/ABS_{blank} \times 100$, where ABS_{blank} is the absorbance of a sample containing 150 µL of water instead of the diluted beer samples. A graph representing the % of inhibition as a function of the logarithm of the beer concentration in the samples was drawn and the experimental points were fitted with a straight-line model. The results of the Radical Scavenging Activity (RSA) are reported as EC_{50} values (expressed as mL beer/mg DPPH).

Total phenolic content

The total phenolic content was measured according to ref. [22]. A volume of 0.5 mL of diluted beer sample was mixed with 2.5 mL of 1:10 diluted Folin-Ciocalteu reagent and allowed to react for 5 min. Then, 2 mL of a sodium carbonate 7.5% solution was added, and the volume was brought to 10 mL with water. After 1 h at room temperature in the dark, the absorbance at 760 nm was measured and the results were expressed as mg equivalents of gallic acid (GA)/mL beer based on a calibration curve of GA in the range of concentrations 0–10 mg/L.

TBI

TBA Index (TBI) was measured according to ref. [23]. Briefly, 0.25 mL of beer samples were diluted with 2.25 mL of water and then 1.25 mL of a 0.02 M TBA solution in acetic acid 90% were added. This mixture was kept at 70 °C for 70 min and then quickly cooled at room temperature. UV–Vis spectra were measured in the range 400–600 nm and the absorption at 445 nm was corrected for the absorbance of the beer samples having the same composition except for the lack of TBA.

Color

Color (EBC values and CIEL*a*b* coordinates) was measured according to ASBC method Beer-10 [24], in quartz cells with 10 mm path length (1 mm for darker samples). Turbid samples were filtered with PES membrane syringe filters (pore size 0.45 µm).

Hydroxyl radical scavenging capacity

The hydroxyl radical scavenging activity was determined with the spin-trapping method coupled with EPR Spectroscopy. The hydroxyl radical generating systems based on the Fenton reaction used Fe(II)-Quin complex as Fe(II) sources, according to refs. [25] and [26].

The Fe(II)–Quin complex was prepared by solubilizing in water $FeSO_4 \times 7H_2O$ and pyridine-2,3-dicarboxylic acid (quinolinic acid, Quin) to obtain a ligand-to-metal ratio of 5/1 and a Fe(II) concentration of 0.1 mM. In the Fenton reaction, Fe(II) reacts with hydrogen peroxide to produce a hydroxyl radical and a hydroxide anion. A hydrogen peroxide solution, 9.8 mM, was prepared from an H_2O_2 concentrated solution 30% (w/w) and kept in an ice bath to avoid decomposition. The hydroxyl radicals were trapped with the nitrone spin trap DMPO. Diluted beer samples were prepared in degassed MilliQ water. In a reaction volume of 1 mL, the solutions were added in the following order (the final concentrations are reported in brackets): water, beer, DMPO (0.6 mM), H_2O_2 (0.979 mM), Fe(II)-Quin (Fe(II) 0.01 mM and Quin 0.05 mM).

A Bruker EMX spectrometer operating at the X-band (9.4 GHz) and equipped with an HP 53150A microwave frequency counter was used to detect the DMPO-OH adduct signals using a Bruker AquaX capillary cell. During the sample measurements, the Q (the quality factor of the resonator) value was kept constant, thus allowing for quantitative comparisons of the intensity of the EPR signals, in agreement with Eaton et al. [27]. The influence of other factors (filling factor, radio frequency power, etc.) was considered negligible because these were the same for all the measurements. The results are expressed as mg equivalents of gallic acid (GA)/mL beer, based on a calibration curve obtained with GA in the range of concentrations 0–30 mg/L ($R^2 = 0.988$).

Data elaboration and statistical analysis

The elaboration of kinetic curve data and the statistical analysis were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Kinetic data were fitted with a modified Boltzmann sigmoidal equation as reported by Fadda et al. [28].

The lag time was determined by fitting the initial points of the kinetic curve with a nonlinear regression available in Graph Pad "Two intersection lines –fit the crossing point", which objectively pinpoints the crossing point of the two lines. When only the experimental points before the inflection point are available, this procedure works better than the fitting with the Boltzmann sigmoidal equation. The EC_{50} values (DPPH assay) and the corresponding 95% confidence intervals (CI 95%) were calculated applying a straight-line modified model to a % inhibition as a function of the logarithm of the beer concentration graph. The lack of superimposition of the CI 95% of the EC_{50} values has been considered as a reliable criterion to distinguish statistically different values ($p < 0.05$) [29].

Results and discussion

Characteristics of the analysed beers

Table 1 reports the quality parameters of the beers tested. The alcohol concentration reported in Table 1 is the one indicated by the producers.

The six beers analysed were produced with different brewing styles that have an impact on the characteristics of each beer. The Irish Dry Stout has an EBC color value significantly larger than the other beers and is the only dark beer in this trial. Strong Lager and Belgian Lager have similar EBC color values but significantly differ for pH and the

Table 1 Alcohol content (%), apparent extract, pH and color expressed according to European Brewery Convention (EBC) and Commission Internationale de l'Eclairage (CIE) of the investigated beer samples

Sample	Alcohol (%)	Apparent extract (% w/w)	pH	EBC color	CIE		
					*L**	*a**	*b**
Strong lager	7.7	7.3 ± 0.2	4.17 ± 0.02	6.5	94.6	− 1.4	19.1
Belgian Lager	5.0	5.1 ± 0.0	4.56 ± 0.01	6.1	95.8	− 2.3	18.6
Pilsner	4.4	5.8 ± 0.1	4.87 ± 0.03	11.1	92.5	− 1.9	31.7
Blonde Ale	6.6	7.6 ± 0.0	4.43 ± 0.03	13.4	90.9	− 1.2	37.0
India Pale Ale	5.5	4.1 ± 0.1	4.44 ± 0.01	8.9	93.7	− 2.0	26.1
Dry Stout	4.2	4.3 ± 0.0	4.15 ± 0.01	106.4	34.8	31.1	66.0

The alcohol content was declared by the producer in the label

apparent extract values. The Pilsner, another lager beer, has an EBC color value remarkably higher than the other lager ones and similar to the ale beers but differs from this latter for the pH value.

Effect of temperature on the kinetic of PBN adduct

In a previous paper [19] we studied the effect of PBN and alcohol concentration on the lag-time determination of three beer samples, demonstrating that it is not always possible to obtain a lag time no matter the PBN or alcohol concentrations employed. To check whether the lag time was measurable by modifying the heating temperature, we performed the EPR experiments changing the temperature from 60 °C to lower and higher values in the range of 40–80 °C. So far very few papers deal with the effect of temperature on the evolution of PBN adduct over time and consequently on the determination of the lag time. Usually, the temperature at which the beer samples are heated in spin-trapping experiments with PBN is 60 °C; in some cases, this temperature is 55 °C [13, 30–32]. To the best of our knowledge only in one case the temperature was increased at 70 and 80 °C [20]. Here for the first time, we describe the shape of the curves intensity of the PBN adduct *vs.* time obtained at different temperatures.

In Fig. 1, the influence of temperature is shown for four different beer samples where the final PBN concentration was kept constant at 50 mM and the alcohol content of the beer was left unchanged. Temperature affects the intensity of the PBN adduct and its growing rate over time. The shape of the curve intensity of the PBN adduct *vs.* time changes as the temperature increases. The changes in the kinetic curves are different according to the beer tested. In Belgian Lager beer (Fig. 1A), for example, the intensity of the PBN adduct is very low during the experiment. Only heating the beer at 80 °C, the PBN adduct increases up to a maximum, at 76 min, then decreases. The other beers show a similar behaviour but at lower temperatures (70 °C). The time to reach the maximum is different among beers. As an example, the Blonde Ale reaches a maximum intensity at 76 min at 70 °C, the Belgian Lager reaches it at 81 min at 80 °C, the Dry Stout at 79 min at 70 °C, and the India Pale Ale at 116 min at 70 °C.

When a maximum intensity is reached, as in Fig. 1, the shape of the curve clearly resembles those obtained in similar experiments with sunflower and extra virgin olive oils subjected to thermal treatment at 90 °C in the presence of PBN [28]. In that case, the shape of the curve was fitted by two modified Boltzmann sigmoidal equations, the first from the beginning to the maximum intensity and a reverse one from that point to the end of the experiment. The same fitting approach was applied for these adduct intensity *vs.* time curves and an example of fitting parameters is shown in Figure S1. The appearance of a maximum in the intensity of the PBN adduct *vs.* time curve can be interpreted as a consequence of the increased rate of formation and decay of the PBN adduct. This is not only formed at an increased rate at higher temperatures but also its decomposition rate is increased. Alternatively, or simultaneously, the paramagnetic PBN adduct could react with other radicals generating diamagnetic species and decreasing the intensity signal. The net result is that when the temperature is raised, the intensity increase and the subsequent decrease of the kinetic curve intensity *vs.* time takes place in shorter time intervals. Carrying out the spin-trapping experiments at the "conventional" temperature of 60 °C, the maximum intensity should appear at very long time intervals, namely hours, and for this reason, it was not previously observed. Concerning the lag time, the Belgian lager is the only beer examined in this work whose kinetic curves allow calculating this parameter. The values obtained (33.2 ± 1.8 min at 40 °C, 24.6 ± 2.4 min at 50 °C, and 20.7 ± 0.1 min at 60 °C) confirm that lag time decreases with increasing temperature, as already reported by Uchida et al. [20]. A comparison with lag time values measured in the literature for other lager beers is difficult because in our experiments PBN was not added as alcohol solution but as a solid. We have already noticed [19] that the lag time values, and the other parameters AUC and T_{150}, depend on the amount of alcohol added with the PBN solution, making difficult the comparison of the values

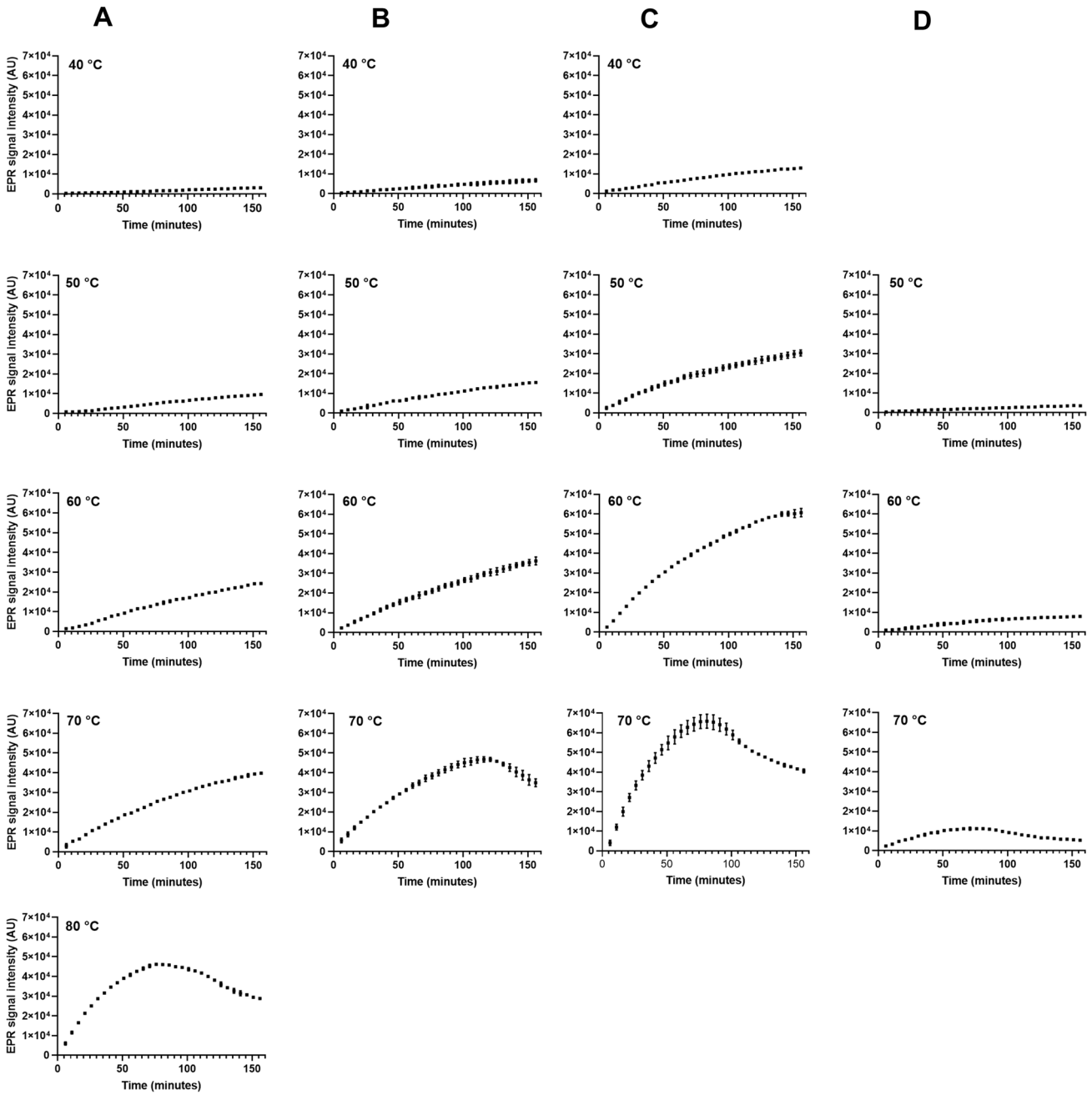

Fig. 1 Kinetic curves of the intensity of the PBN adduct as a function of time for Belgian Lager (**A**), India Pale Ale (**B**), Blonde Ale (**C**) and Dry Stout (**D**) beer samples subjected to thermal treatment at: 40, 50, 60, 70, and 80 °C. In the graphics, the means and the standard deviations of three replicates are reported

obtained for the same beer sample in different experimental conditions. Moreover, the present results demonstrate that when the lag time is not measurable, even changing the experiment temperature, there is no guarantee to obtain a measurable lag time value changing the PBN and alcohol concentrations (see ref. [19]), thus other parameters should be used for the determination of the oxidative stability of beers.

Antioxidant activity and degree of beers staling

In addition to the oxidative stability, the beers were analysed to assess the antioxidant activity and thiobarbituric acid values (Table 2). In particular, the RSA, the Total Phenolic Compounds (TPC), the Hydroxyl Radical Scavenging Capacity (HRSC), and finally the TBI, an index used to measure the degree of beer staling, were measured. Figure 2

Table 2 Parameters obtained for the beers studied in this work with spectroscopic (EPR) and spectrophotometric (UV–Vis) methods

Beer	AUC (a. u.)	T_{150} (a. u.)	EC_{50} RSA (mL beer/mg DPPH), CI 95%	TPC (mg eq GA/ml beer)	TBI	HRSC (mg eq GA/ml beer)
Strong Lager	$6.19\times10^5\pm4.11\times10^3$	$1.07\times10^4\pm1.38\times10^3$	0.851 0.818–0.883	0.340 ± 0.021	0.246 ± 0.007	70.5 ± 0.3
Belgian Lager	$1.94\times10^6\pm2.98\times10^4$	$2.42\times10^4\pm1.46\times10^2$	1.763 1.632–1.892	0.297 ± 0.021	0.219 ± 0.003	58.5 ± 1.1
Pilsner	$1.48\times10^6\pm3.96\times10^5$	$1.81\times10^4\pm4.60\times10^3$	0.909 0.842–0.974	0.339 ± 0.015	0.298 ± 0.008	46.2 ± 2.1
Blonde Ale	$5.94\times10^6\pm5.32\times10^4$	$6.07\times10^4\pm1.51\times10^3$	0.718 0.675–0.762	0.425 ± 0.020	0.576 ± 0.002	68.2 ± 0.1
India Pale Ale	$3.02\times10^6\pm1.16\times10^5$	$3.55\times10^4\pm1.10\times10^3$	0.945 0.843–1.044	0.341 ± 0.025	0.316 ± 0.004	49.8 ± 2.8
Dry Stout	$7.87\times10^5\pm4.25\times10^4$	$7.80\times10^3\pm2.52\times10^2$	1.255 1.133–1.376	0.411 ± 0.013	0.378 ± 0.006	53.1 ± 1.3

Data for AUC, T_{150} and RSA for pilsner, strong lager and blonde Ale are taken from ref. [19] and are reported here for comparison. AUC and T_{150} values are obtained from experiments performed at 60 °C. RSA stands for Radical Scavenging Activity. TPC stands for Total Phenolic Compounds. TBI stands for ThioBarbituric Index. HRSC stands for Hydroxyl Radical Scavenging Capacity

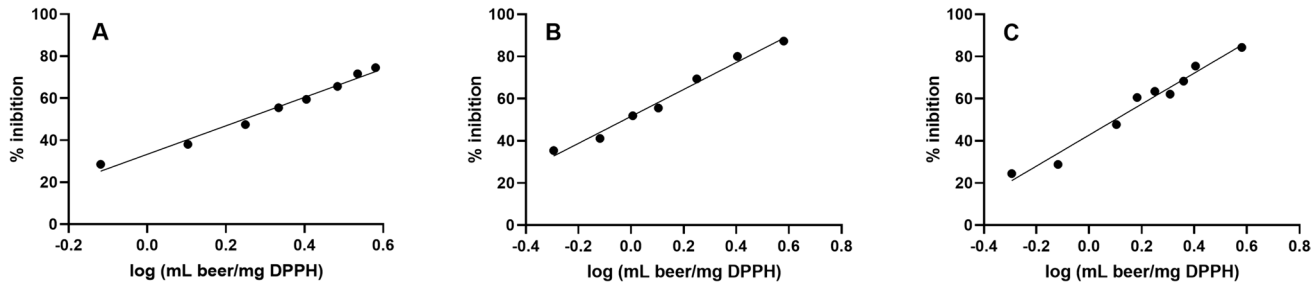

Fig. 2 Percentage of inhibition as a function of the logarithm of the concentration of: (**A**) Belgian Lager; (**B**) India Pale Ale; and (**C**) Dry Stout samples in the DPPH assay. The corresponding EC_{50} values are reported in Table 2

reports the results of the RSA of three beers (Belgian lager, India Pale Ale, and Dry stout) analysed in this paper whereas on Table 2 for comparison are also shown the values previously measured in ref. [19] for Blonde Ale, Pilsner and Strong Lager beers.

A comparison of the RSA values measured here for the first time with those already published in ref. [19] is shown in Fig. 3A. The TPC, the TBI, and the HRSC measured for the beer samples are graphically reported in Fig. 3B, C and D respectively.

Some of the antioxidant activity and radical scavenging capacity measured for the beers examined in this work could be due to hop extracts employed during the brewing process [33]. In beer 70–80% of the polyphenol fraction comes from barley malt and 20–30% from hop. Polyphenols contribute up to 60% of the antioxidant activity measured with the DPPH assay [34, 35]. However, in the literature there is no agreement about the correlation between polyphenol compound content and antioxidant activity. In fact, some authors [33, 36–38] found this correlation, while others [39, 40] found no such relationship. It is therefore not surprising that such a relationship was not found for the beers examined in this work. No relationship is found when trying to correlate the values of HRCS with those of TPC or RSA (see Figure S2), demonstrating that even if these three parameters relate to the antioxidant activity of beers, they measure different hues of the same property.

The HRSC measures the ability of beer samples to quench the hydroxyl radicals produced with the Fenton reaction and trapped by the spin trap DMPO. Therefore, it can be considered another assay measuring the antioxidant activity of beers. In this paper, DMPO was used to trap the hydroxyl radicals generated in situ by the Fenton reaction whereas in other papers [13, 18] it was used in place of PBN to trap the radicals generated during forced oxidation experiments of beer samples. The beers analysed in this paper strongly differ for the HRSC values, ranging from 46.2 to 70.5 mg eq gallic acid/mL beer.

It is expected that antioxidant activity parameters (TPC, RSA, and HRSC) are in inverse relationship with AUC, or T_{150}, since these measure the tendency of samples to scavenge the radicals formed during the thermal degradation of

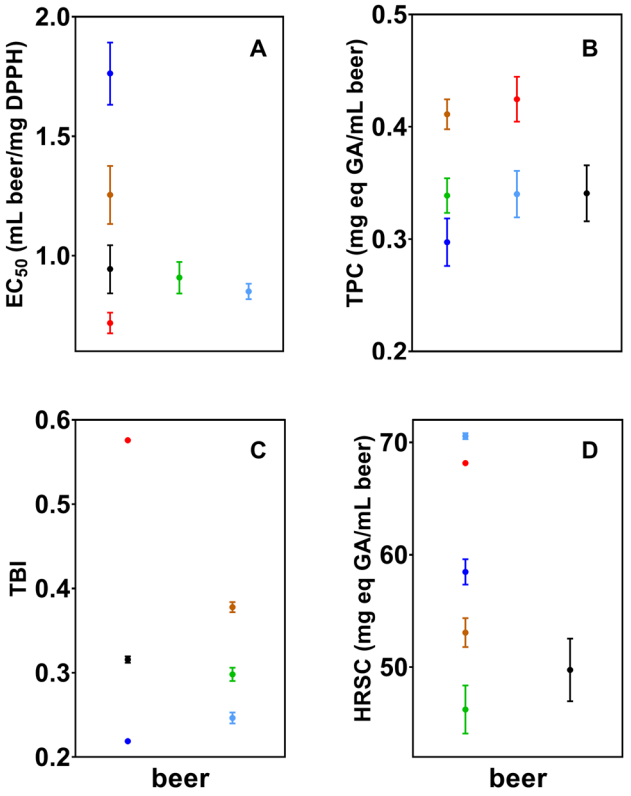

Fig. 3 Comparison of: **A** the EC_{50} values of the DPPH assay obtained for the beers examined here and in ref. [19] (for numerical values see Table 2); **B** the values of Total Phenolic Content (TPC); **C** the values of ThioBarbituric Index (TBI); and **D** the values of Hydroxyl Radical Scavenging Capacity (HRSC) measured for the beers: Pilsner (green); Strong Lager (pale blue); Blonde Ale (red); Belgian Lager (blue); India Pale Ale (black); Dry Stout (orange)

beer samples. On the contrary, a relationship between TBI and AUC, or T_{150}, values is not observed (see Figure S3). An almost linear relationship can be obtained when plotting the AUC *vs*. T_{150}, see Fig. 4, indicating that the two parameters are measuring for these beer samples the same thing, that is the amount of PBN–1-hydroxyethyl radical adduct formed during the first 150 min of reaction at 60 °C. As can be seen from Table 1 and Fig. 1, the dry stout beer shows the lowest value of T_{150} and the second to last value of AUC. If this beer is not considered in the plot in Fig. 4, the R^2 value of the goodness of fit increases from 0.9885 to 0.9967. The low intensity of the EPR signals of the dry stout beer can be put in relationship with the melanoidin formed during the roasting of malt [41]. Melanoidins show antioxidant activity [42, 43], which can explain the observed low intensity signals.

All the other possible combinations of measured parameters are not in linear relationship. In particular, there is no linear inverse relationship between T_{150}, or AUC, and TPC, RSA or HRSA. A linear relationship is not observed even when plotting two of the three antioxidant activity parameters (TPC, RSA and HRSA) indicating that these

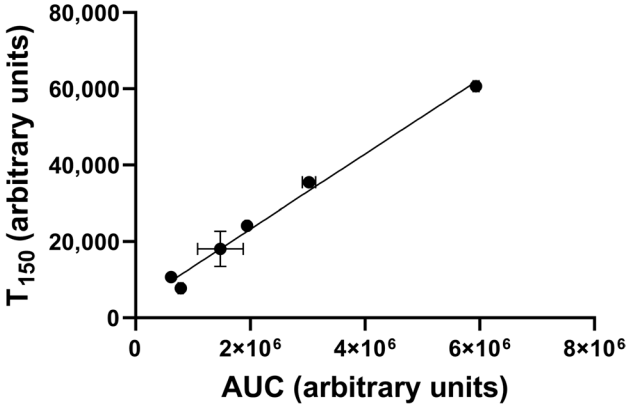

Fig. 4 Plot of the intensity of the PBN adduct measured after 150 min (T_{150}) vs. area under the curve (AUC) after the same time interval for the six beer samples examined in this work. $R.^2 = 0.988$

measure different shades of the same feature. Figures S2 and S3 summarize some plots of all possible combinations of parameters.

A linear relationship can be found when relating T_{150}, or AUC, with a combination of the values of RSA, TPC, TBI and HRSC; the R^2 values are 0.9562 and 0.9694, respectively. When the values of HRSC are not considered in this comparison the R^2 values slightly decrease to 0.9524 e 0.9668, respectively.

The goodness of fit considerably grows when a combination of T_{150} and AUC is considered in the comparison, and the R^2 value increases to 1. In other words, a combination of the values of AUC and T_{150} is compared with a combination of the experimental values of RSA, TPC, TBI, and HRSC values.

The result of this comparison is rather interesting, and the resulting plot, obtained with the following relationship, is shown in Fig. 5:

$$AUC + \beta_0 + \beta_1 \times T_{150} = \beta_2 \times TBI + \beta_3 \times RSA + \beta_4 \times TPC + \beta_5 \times HRSC$$

$$\left(Where\ \beta_0 = 447065; \beta_1 = -84.55; \beta_2 = 4151733; \beta_3 = 186796; \beta_4 = -2964661; \beta_5 = -173.2\right)$$

In this way it is possible to calculate the expected value of a combination of AUC and T_{150} after knowing HRSC, TPC, RSA, and TBI. Considering that the beer styles examined here are very different from each other, being blonde or dark, lager or ale, and differing for the alcohol content, we think we examined here a good variability among those available on the market.

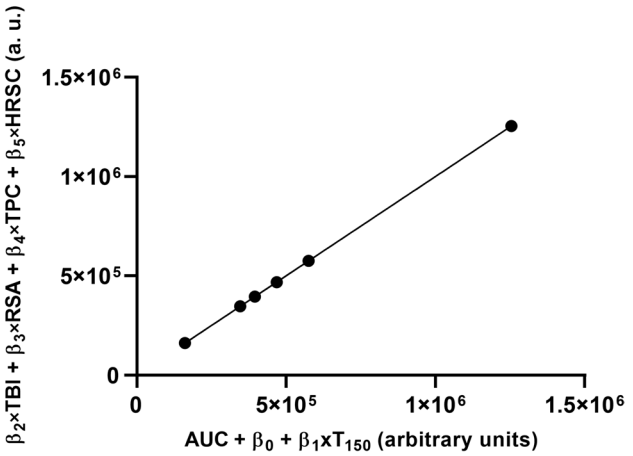

Fig. 5 Comparison between a combination of experimental EPR parameters (AUC and T_{150}) and their calculated values with a combination of other parameters (RSA, HRSC, TPC and TBI). The fitting has $R^2 = 1$

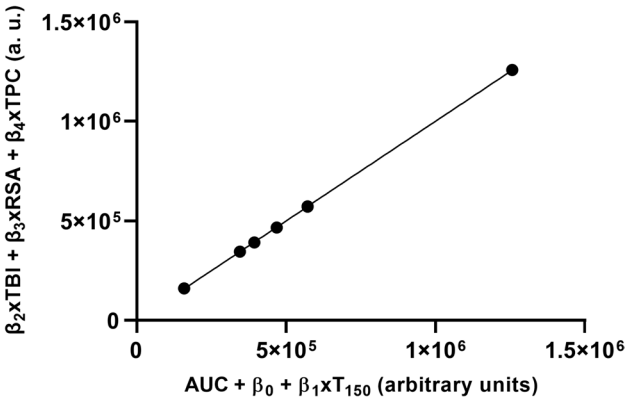

Fig. 6 Comparison between a combination of experimental EPR parameters (AUC and T150) and their calculated values with a combination of spectrophotometric parameters (RSA, TPC and TBI). The fitting has $R.^2 = 1$

Someone could raise an objection since also the HRSC parameter is obtained with a spin-trapping experiment, that is with an EPR spectrometer. However, in this case the hydroxyl radical trapped by DMPO is generated in situ by the Fenton reaction and is not produced by the thermal degradation of radical precursors already present in the beer. We tried to not consider the HRSC parameter, and another good relationship can be obtained between AUC and T_{150} with a combination of TBI, RSA, and TPC. In this way, it is possible to put in relationship the parameters obtainable with EPR spectroscopy and spin-trapping experiments (AUC and T_{150}) and those attainable with an UV–Vis spectrophotometer (TPC, DPPH and TBI), see Fig. 6.

We are aware that we examined here only six beer samples and without other experimental measurements it is impossible to affirm that this relationship has a general application. Since a parameter extracted from EPR experiments, that is AUC, has already been related to sensory staleness scores [21], this proposal aims to relate EPR parameters with antioxidant activity ones, to try to determine the staling degree of beers with different spectroscopic methods.

$$\mathrm{AUC} + \beta_0 + \beta_1 \times \mathrm{T}_{150} = \beta_2 \times \mathrm{TBI} + \beta_3 \times \mathrm{RSA} + \beta_4 \times \mathrm{TPC}$$

Where $\beta_0 = 443236$; $\beta_1 = -84.44$; $\beta_2 = 4181025$; $\beta_3 = 187159$; $\beta_4 = -3023979$

Conclusions

Of the six beers examined in this work, four have been subjected to thermal treatment in the range 40–80 °C in the presence of PBN to verify the effect of the temperature in EPR spin-trapping experiments. The results show that the shape of the curve intensity of the PBN–1-hydroxyethyl radical adduct *vs.* time changes, with the appearance of a maximum intensity shifted towards shorter time intervals as the temperature increases. To the best of our knowledge this is the first time that this behaviour is described in detail. The shape of this curve can be fitted by separating it in two parts: one from the beginning of the experiment up to the intensity maximum, and another from the maximum to the end of the experiment. A Boltzmann modified sigmoidal curve, originally proposed by Fadda et al. [28], has been used for the fitting. Among the beers examined here, a lag time can be observed only for the Belgian Lager (in the range 40–60 °C) and, as previously reported by Porcu et al. [19] for the Strong Lager at 60 °C.

For the six beers several other parameters related to their antioxidant activity (RSA), polyphenolic content (TPC), hydroxyl radical scavenging capacity (HRSC) and staling degree (TBI) were measured. The existence of relationships among these parameters and/or with those obtainable from spin-trapping experiments, i.e., the area under the curve (AUC) and the intensity at 150 min (T_{150}) were verified. There is no relationship between Radical Scavenging Activity and Total Phenolic Content of the beers. Good relationships can be found between T_{150}, or AUC, and a combination of RSA, TPC, TBI, and HRSC with $R^2 = 0.9562$ and 0.9694, respectively.

The goodness of fit considerably increases when a combination of AUC and T_{150} is considered instead of considering only one of the two. The R^2 values of the fitting do not worsen if the HRSC is not considered in this comparison. Finally, we demonstrated that the spin-trapping experiments parameters (AUC and T_{150}) can be put in relationship with

other parameters obtainable with a spectrophotometer (RSA, TPC, and TBI).

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Availability of data and materials All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Compliance with ethics requirements This research does not contain any studies with human or animal subjects.

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