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Stability comparison of lager with dark and non-alcoholic beers using epr spin trapping technique

The spin trapping technique test effectively used in the characterisation of the flavour stability of lager beers was applied to dark and non-alcoholic beers. The induction period for the formation of free radicals as indicated by spin trapping during thermally initiated staling is determined by antioxidants naturally occurring in beer, and its length is taken as a measure of the stability of the beer (lag time). This induction period is not evident in dark and non-alcoholic beers, suggesting lower stability as compared to lager beers, although $ABTS^{\bullet+}$ tests assigned a comparable antioxidant activity to the dark beers as was found for lager beers. A possible explanation for the missing induction period in the dark and non-alcoholic beers is the presence of more effective antioxidants in lager beers, able to rapidly terminate the initially-formed reactive free radicals. Caramel used in the production of dark beers showed relatively high scavenging activity of free radicals.

Descriptors: Lager, dark and non-alcoholic beers, stability, EPR, spin trapping technique, PBN, caramel

1 Introduction

Thermally accelerated ageing, followed by EPR spin trapping technique, represents a very effective method to follow the flavour stability of lager beer [1-11]. The process leading to foul taste or odor involves free radical reactions [12, 13]. The antioxidants naturally occurring in beer terminate the free radicals formed and so prevent staling [14-20]. After antioxidants are depleted and free radicals are no longer terminated, the spin trapping agent indicates an outbreak of free radicals signaling the staling. The length of the induction period (lag time) for this process is a measure useful for predicting beer stability [1-5]. Now we also applied this technique to dark and non-alcoholic beers and compared the data obtained with those found with lager beer samples. The results obtained here point to some differences of lager to dark beers probably due to the deterioration processes of beer flavour having non-radical and non-oxidative character, like formation of (E)-2-nonenal and furfuryl ethyl ether [21, 22].

2 Experimental

2.1 Materials

Commercially available beers from Czech, German and Slovak production were investigated. Further substances used originated from the following sources: 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Fluka; a liquid substrate system for Elisa from Sigma containing 2,2´-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) salt (ABTS); 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl (TEMPOL) from Aldrich; α-phenyl-N-*tert*-butylnitrone (PBN) from Janssen Chimica served as the spin trapping agent. Ammonia caramel (E 150c, Food additives approved by the EU) was obtained from Stein Brewery (Bratislava, Slovak Republic).

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Tables see Appendix

2.2 General procedure

To 200 µl of beer sample, 10 µl of 1 M PBN in ethanol were added; the sample was bubbled with 1 ml of air and transferred to a 50 :l quartz capillary, placed in the rectangular cavity (TE_{102}) of an EMX Bruker EPR spectrometer and kept at 60 °C. Generally, the EPR spectra were monitored for 120 minutes, taking 30 spectra, each with 10 accumulations.

3 Results and discussion

Representative time courses of EPR spectra monitored during 120 minutes at 60 °C in the presence of PBN spin trapping agent for the investigation of samples of lager, dark, and non-alcoholic beers are shown in Fig. 1. Characteristic of lager beers (Fig. 1a, b) are induction periods of various lengths for individual samples; the length is a measure of the beer's stability. It is remarkable that neither dark (Fig. 1c, d) nor non-alcoholic beers (Fig. 1e, f) show such an induction period, indicating, in the terms used for the interpretation of lager beers, a very limited stability. Therefore, in further investigations we looked for an explanation for this behavior of dark and non-alcoholic beers.

Dark beers

As mentioned above, an induction period – generally well known to indicate flavour stability in the series of lager beers – was not observed (Fig. 1) in the case of dark beers. The concentration of spin adducts crosses a maximum here, then increases again in the advanced stages of thermal ageing. The question arises, do dark beers not contain naturally occurring antioxidants, which may prevent the start of radical reactions in the initial stages of the thermal ageing, or are there some other parameters responsible for the observed radical formation?

A characteristic feature in the production of dark beers is the caramelisation of the ingredients added, or the addition of caramel to the beer [23-25] as well. Consequently, we investigated the properties of caramel by means of EPR. The caramel probe was a viscous liquid, already containing free radicals, characterized by a singlet spectrum having a peak-to-peak-width of 1.1 mT and a g-value of 2.0036. Most likely it originates from carbon-centered radicals frequently formed by the thermally treated organic substances, especially sugars [23-25]. The concentration of these radicals in caramel corresponds to 10^{-4} mol dm^{-3} solutions. Under conditions analogous to those employed in the investigation of beer samples, we investigated the caramel samples. Figure 2a shows the EPR spectra of caramel radicals monitored during 120 minutes at 60 °C. The time course indicates a relatively very small decay of radicals, demonstrating their high stability. Then, an analogous experiment was carried out, but in the presence of a relatively high concentration (0.048 M) of PBN spin trapping agent. Here the decay of caramel radicals is quite evident, indicating the reaction of caramel radicals with PBN, but its spin adducts to PBN are not evident in EPR spectra.

Then, in further experiments, the original caramel solution was diluted 1:100 with water (v/v) and contained 10^{-6} M of free radicals. To this diluted solution a 10^{-4} M TEMPOL or DPPH solution in ethanol was added in a volume ratio of 1:1. The EPR spectra of the individual components and the spectrum after their mixing are shown in Fig. 2c, d. The spectra of TEMPOL and DPPH, representing high concentrations of free radicals, vanished after mixing with caramel and the caramel spectrum survived with a negligible decrease of its integral intensity: 4 % in TEMPOL and 8 % in DPPH experiments. This confirms the very high stability of carbon-centered radicals occurring in caramel, and on the other hand also indicates the ability of caramel components to terminate free radicals. Similar observation was reported in [23-25].

Further, the influence of caramel added in increased concentrations to lager beer in the presence of PBN spin trap was investigated, following the time course of EPR spectra at 60 °C, similarly to that depicted in Fig. 1a, b. The results obtained are summarized in Fig. 3. For a better overview, the data obtained are shown in two different presentations: series a-d in two-dimensional and series aa-dd in three-dimensional form showing the time course of radical formation. This provides a better overview not only of the quantitative but also of the qualitative changes of radical types involved. Figs. 3a, aa presents a 12 % lager beer sample before adding caramel in an experiment similar to that shown in Fig. 1a, b. This can be considered as a standard in this series of experiments. An induction period for radical formation characteristic of lager beer is evident. Figure 3aa provides more detail on the EPR spectrum, characterized by a triplet (1:1:1) due to the interaction of an unpaired electron with a nitrogen nucleus, which is further split into a doublet, reflecting an interaction with one hydrogen nucleus (a_N = 1.564 mT, a_H = 0.335 mT; g = 2.0057). This is a characteristic EPR spectrum of free radicals added to PBN spin trapping agent [26].

In further experiments (Fig. 3b-d) caramel was added to the lager beer sample in the increased volume ratios: 3b, bb = 1 %, 3c, cc = 5 % and 3d, dd = 10 %. Only a small amount of added caramel was already significantly reflected in the EPR spectra (Fig. 3b, bb). The formation of PBN adducts characteristic of lager beers (Fig. 3a, aa) decreased considerably, and a minor indication of a singlet spectrum of caramel radical is indicated in Fig. 3bb. These trends are very pronounced with higher caramel concentrations added to the sample, as depicted in Figs. 3c, cc and 3d, dd, where finally only an EPR singlet spectrum originating from caramel is evident. These experiments show that the caramel components very intensively participate in the radical processes in the beer samples in accord with [23-25]. It is very probable that they terminate free radicals, as was also evidenced in experiments with TEMPOL and DPPH free radicals as shown in Fig. 2. So it can be assumed that with increased caramel concentrations the eventually formed radicals of PBN spin adducts are terminated with caramel components and this can well explain the behavior presented in Fig. 3, where with increasing caramel concentrations the radicals of PBN adducts vanished, and finally only caramel radical dominates. We could confirm this in an experiment generating PBN-adducts in a non-alcoholic beer sample, as shown in Fig. 1c,d. Then, caramel was added to this sample with the generated PBN-adduct and the PBN-adduct vanished. However the experiments presented so far do not explain the missing induction period in dark beer samples, possibly indicating the absence of the necessary reactive antioxidants characteristic for the samples of the lager beers.

In further experiments we investigated the antioxidant activity of caramel with $ABTS^{\bullet+}$ cation radical, which is increasingly used in antioxidant tests [17]. Figure 4 illustrates the decreasing intensity of the $ABTS^{\bullet+}$ spectrum (shown in the inset) given growing concentrations of caramel added to $ABTS^{\bullet+}$ solutions. A relatively sharp equivalent point indicates the quantitative termination of $ABTS^{\bullet+}$ radicals, thus demonstrating the effective antioxidant properties of caramel in the $ABTS^{\bullet+}$ system.

There are various reasons, why the flavour stability of dark beers may differ from lager beers. The extreme temperature conditions during production of dark malts create a lower lipoxygenases activity, this may produce less staling aldehydes during storage of dark beers [27]. Dark beers are generally characterised by burnt and caramel-like flavours significantly influencing "sensory background" [28]. Further, dark beers would have a higher endogenous reducing power due the presence of antioxidants originating from the Maillard reaction [29]. A higher reduction power of dark beers was also confirmed in our $ABTS^{\bullet+}$ tests (Fig. 4).

Non-alcoholic beers

The basic behavior of non-alcoholic beers and their differences with lager beers in spin trapping experiments was evident from Fig. 1, where the lager beer showed a typical induction period (Fig. 1a, b) characterizing their stability, whereas non-alcoholic beers without an induction period (Fig. 1e, f) indicated very limited stability. In terms of the concept of such tests with lager beers, this finding for non-alcoholic beers should indicate a very limited content of antioxidants, which are actually responsible for the EPR silent induction period terminating the free radicals formed. The observed difference may originate from different production technologies as between non-alcoholic and lager beers, where for the non-alcoholic beers the antioxidants are already largely absent in the input components, or they are not formed, or may get lost in the production process, e.g., during filtration. The polyphenols in beer are from barley and hop. Non-alcoholic beers are brewed with lower original wort extract and boiled with a reduced hop addition, therefore they contain lower amounts of antioxidants [15, 17-19, 30]. The missing alcohol content can not be considered as a possible explanation for the missing induction period, because an increased ethanol concentration added to non-alcoholic beers (up to 5 %) did not induce significant changes to the spectra shown in Fig. 1e, f. One of the possible explanations is that during the limited length of the fermentation, not enough sulphites are formed, which do represent a relative powerful antioxidant [9, 11, 16]. We prove this alternative, adding increasing sulphite concentrations to the non-alcoholic beer samples in the standard spin trapping tests. Figure 5 shows the results obtained. The increasing sodium sulphite concentrations in the non-alcoholic beer samples induced an induction period such as that characteristic for the lager beers. Consequently, it can be assumed that the absence of sulphites or also some other components in their function as antioxidants [9, 11, 16, 31], may be one of the reasons for the missing radical EPR silent induction period of non-alcoholic beers. A lower content of antioxidants in non-alcoholic as compared to lager beers was also confirmed in the antioxidant tests described below.

To compare antioxidant content in non-alcoholic, lager, and dark beers, antioxidant tests using $ABTS^{\bullet+}$ cation radical solution were carried out. $ABTS^{\bullet+}$ solution was prepared according to ref. 32. The experiments started with the reference sample. To 80 µl of $ABTS^{\bullet+}$ solution were added 20 µl of 5 % aqueous ethanol, and the EPR spectrum of such a reference sample was measured. Then, under analogous conditions, the 5 % ethanol solution was replaced with an adequate amount of beer (diluted with 5 % ethanol 1:10), and the EPR spectrum was measured again. The $ABTS^{\bullet+}$ cation radical concentration in the beer samples was lower than in the reference, as the antioxidants from the beer terminated part of the $ABTS^{\bullet+}$ radicals. The higher the difference, the more active are the antioxidants in the beer sample. The differences thus evaluated are quoted in Fig. 6. The relatively highest ratio of terminated radicals, and therewith the dark beers, closely followed by the lager beers, showed the highest antioxidant activity, and the lowest activity was found in the non-alcoholic beers. The lowest antioxidant content, which was shown in $ABTS^{\bullet+}$ tests with non-alcoholic beers, could well explain the missing induction period in radical formation in the thermally-initiated stability tests by PBN spin trapping. The question of why the relatively higher antioxidant activity indicated in the $ABTS^{\bullet+}$ tests for the dark beers does not induce an induction period terminating the initially-formed free radicals as evidenced by PBN spin trapping technique found in the lager beers, thus remains open.

4 Conclusions

A possible explanation for the missing induction period in the spin trapping tests in the dark beers may originate in the different antioxidant efficiency of radicals occurring in lager and dark beers. The $ABTS^{\bullet+}$ antioxidant test indicated a comparable integral antioxidant capacity in dark and lager beers. But in PBN spin trapping tests a relatively very effective radical trapping agent – namely PBN – acts, and it competes with the beer antioxidants in the termination of free radicals. Thus only very effective antioxidants can eliminate the action of PBN in the formation of its spin adducts and thus induce an induction period. Probably these antioxidants with a rapid termination activity are mostly present in lager beers but absent in the non-alcoholic and dark beers, and so they do not terminate the initially-formed radicals in PBN tests.

5 Acknowledgements

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Appendix

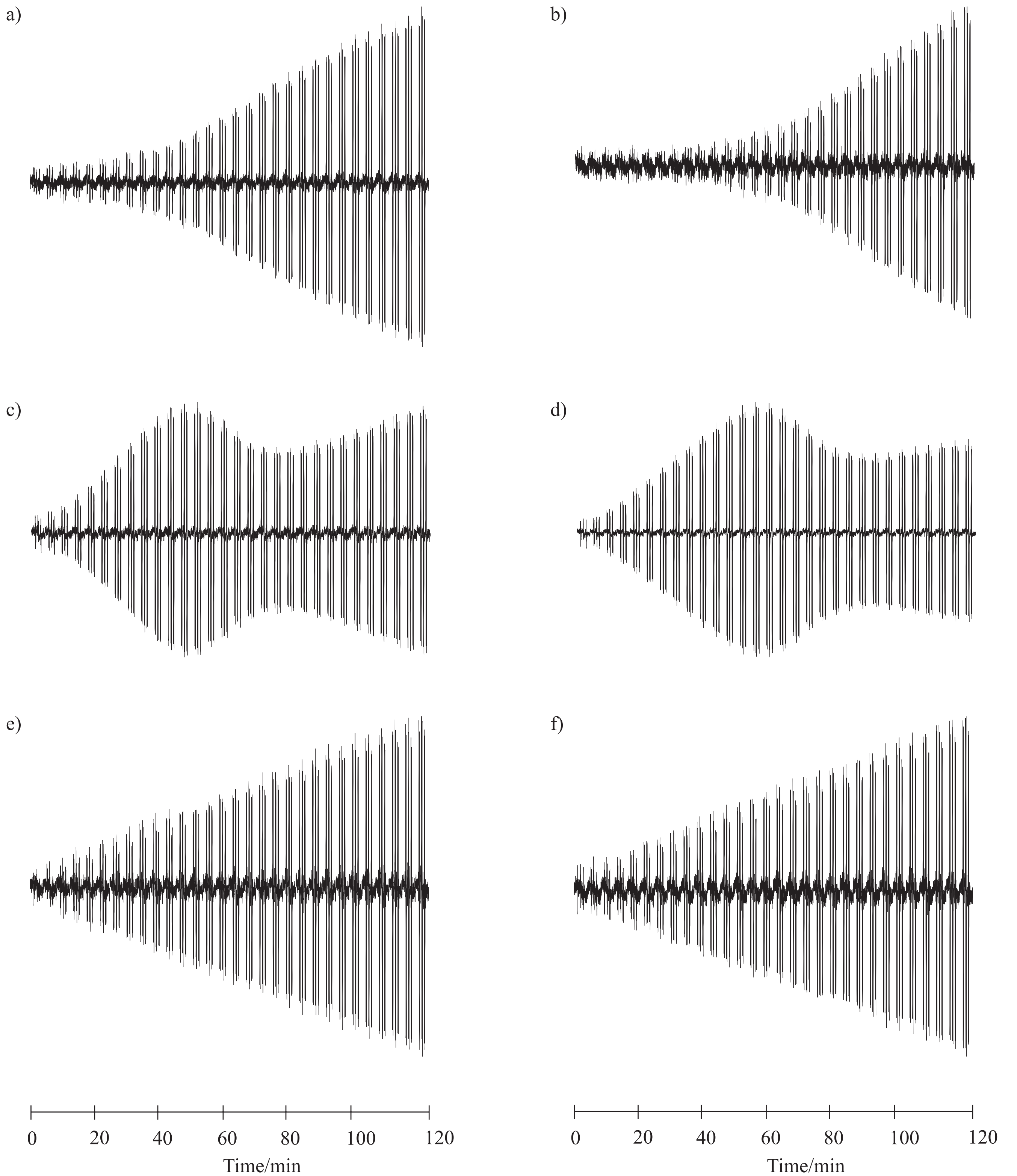

Fig. 1 The time course of EPR spectra monitored over 120 minutes in samples of a), b) lager; c), d) dark; and e), f) non-alcoholic beers at 60 °C in the presence of PBN spin trap. (Sweep width 8 mT)

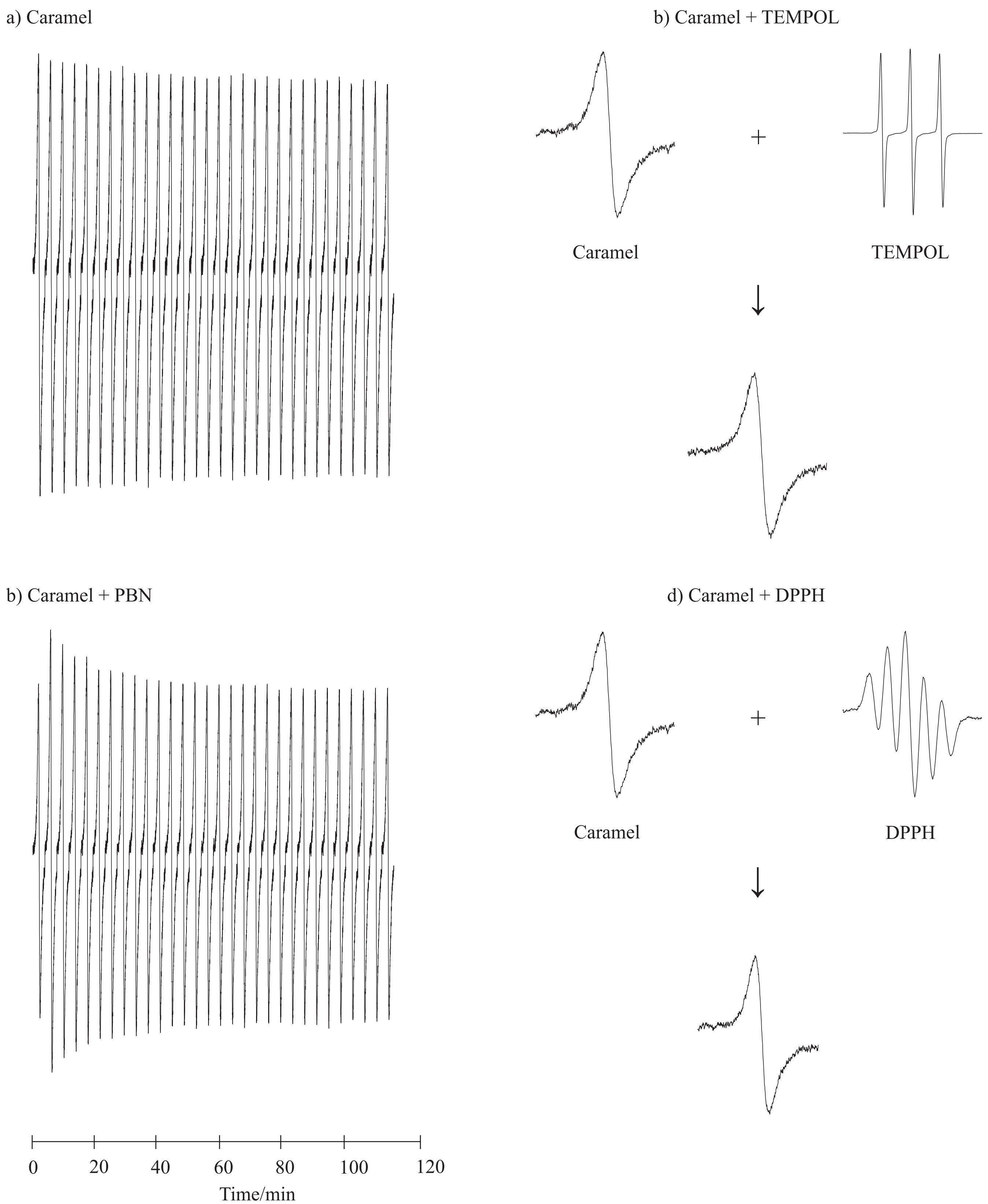

Fig. 2 EPR spectra of caramel solution monitored over 120 minutes at 60 °C: a) without; and b) in the presence of PBN spin trap; and further, EPR spectra of caramel solution and free radical solutions as c) TEMPOL and d) DPPH before and after their mixing. (Sweep width 8 mT)

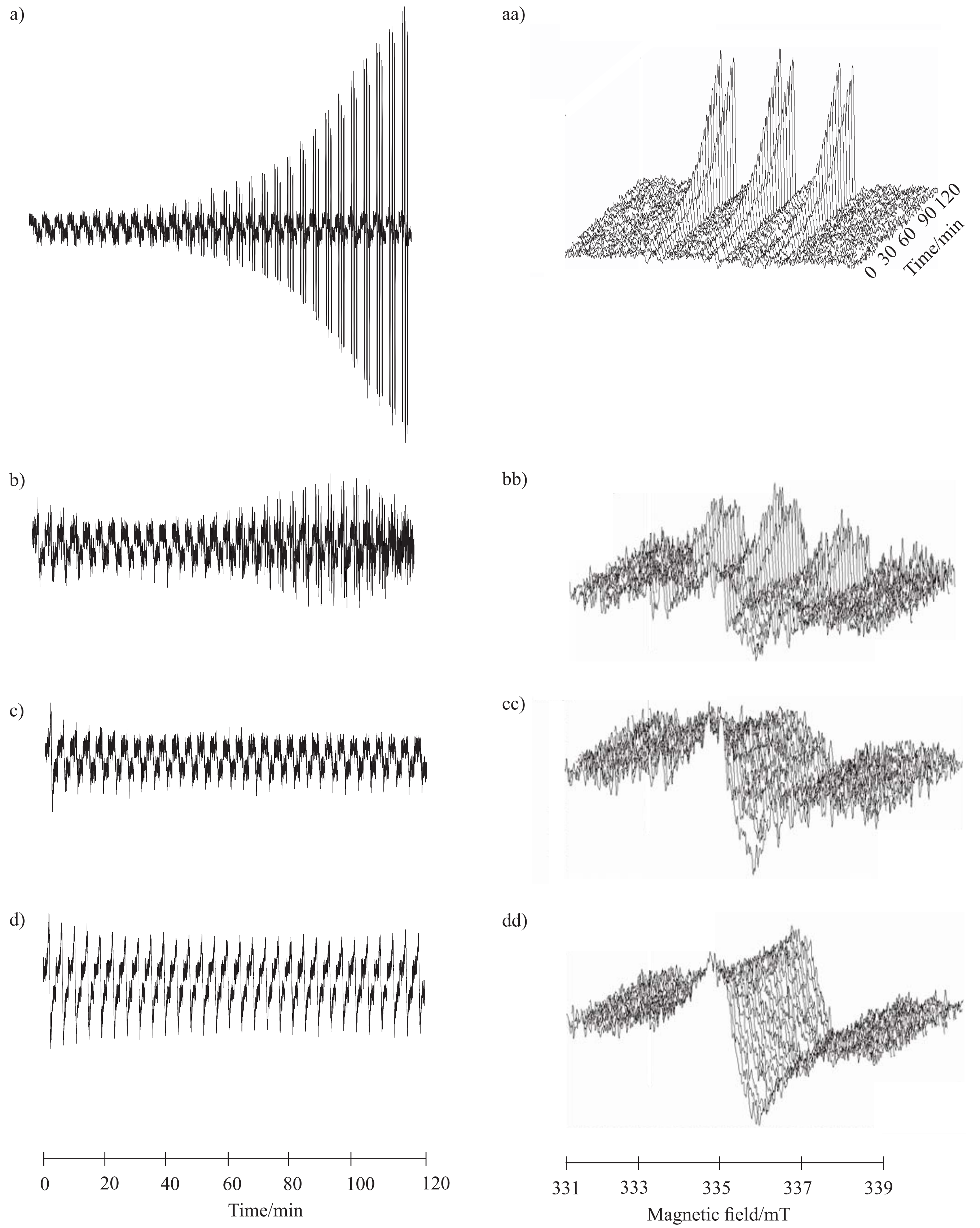

Fig. 3 EPR spectra of 12 % lager beer in two- (a-d) and three- (aa-dd) dimensional presentations monitored over 120 minutes at 60 °C in the presence of spin trapping agent PBN at various volume amounts of caramel solution a) and aa) 0 %; b) and bb) 1 %; c) and cc) 2.5 %; d) and dd) 10 %. (Sweep width 8 mT)

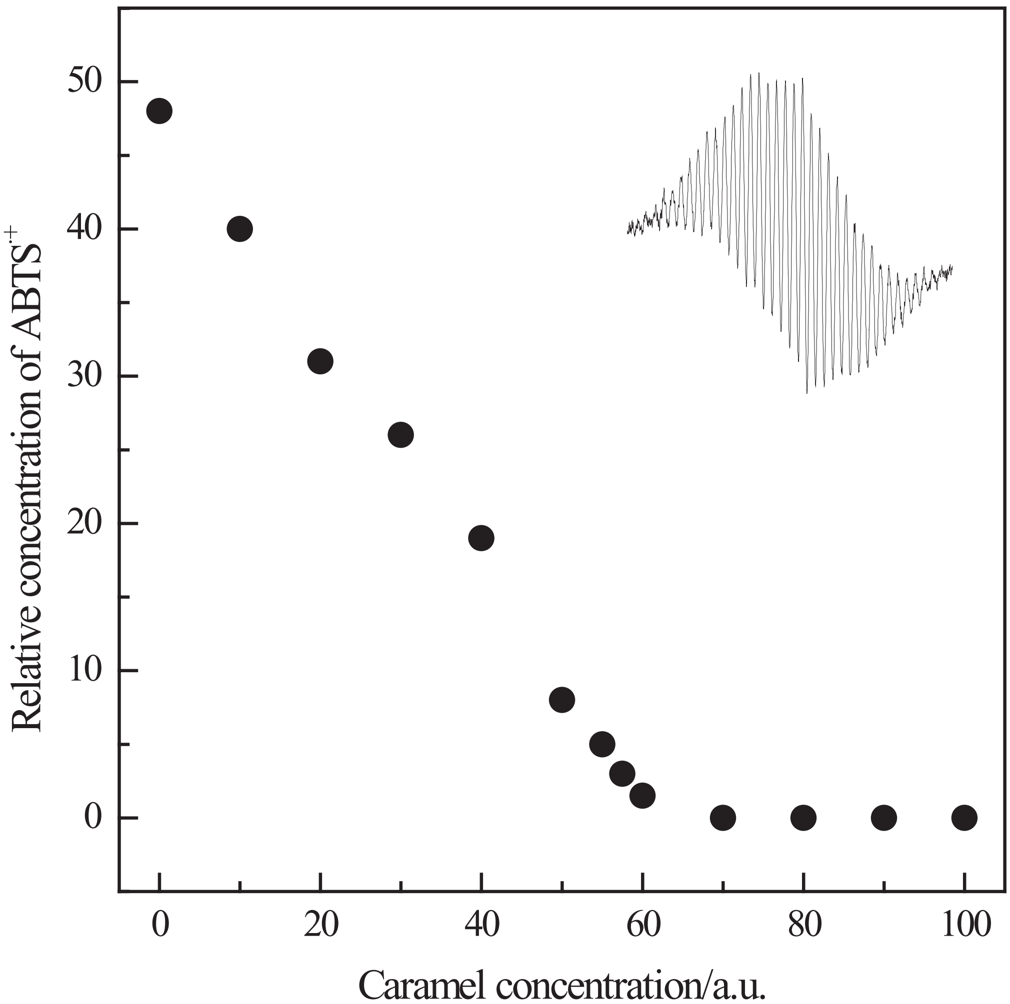

Fig. 4 Decrease of relative $ABTS^{\bullet+}$ concentrations upon the addition of increased caramel concentrations. The inset presents the EPR spectrum of the monitored $ABTS^{\bullet+}$ radical. (Sweep width 3 mT)

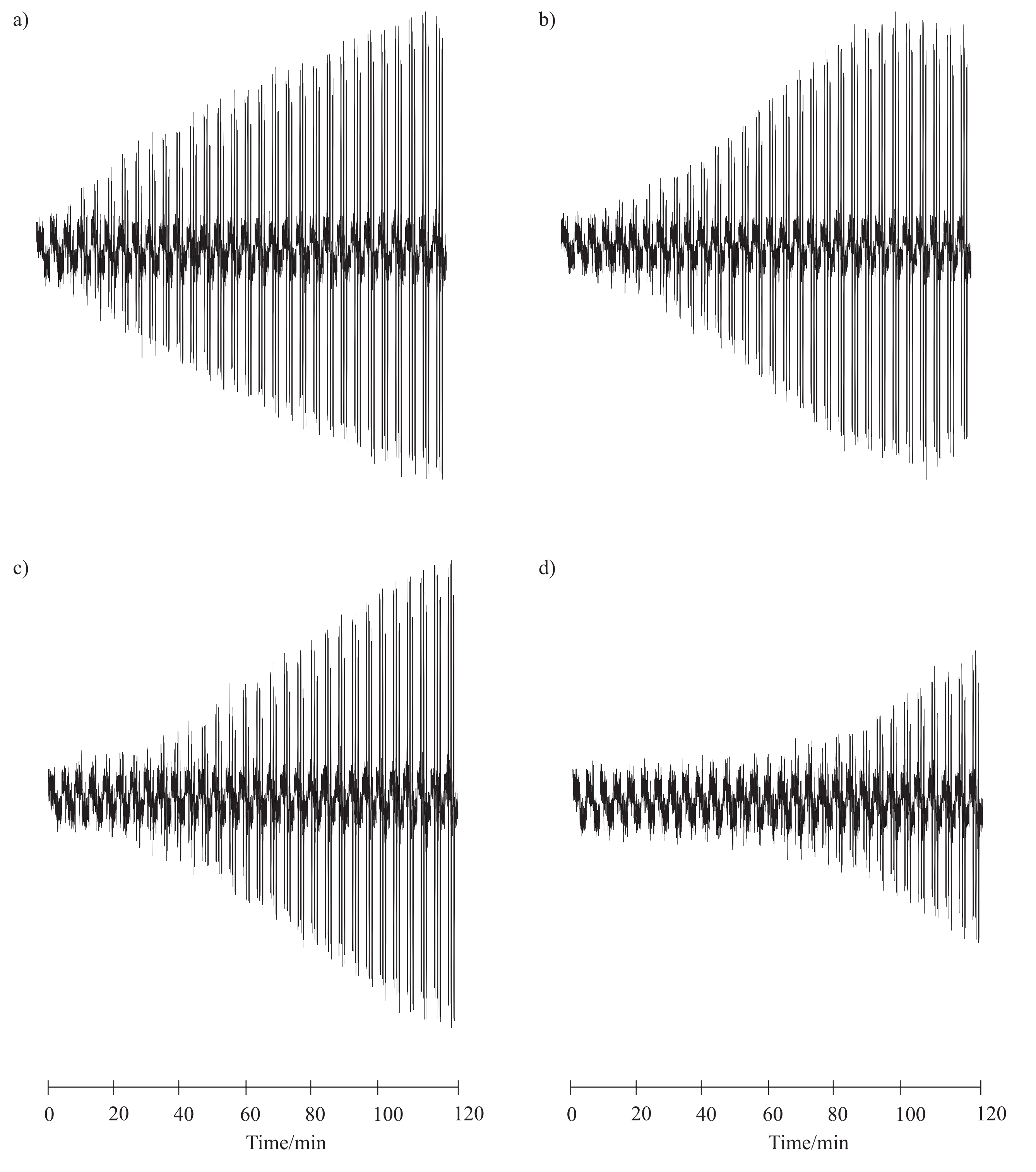

Fig. 5 EPR spectra of a non-alcoholic beer sample monitored over120 minutes at 60 °C in the presence of PBN spin trapping agent at various Na_2SO_3 concentrations: a) 0; b) $2.5x10^{-5}$M; c) $5x10^{-5}$M; d) 10^{-4}M. (Sweep width 8 mT)

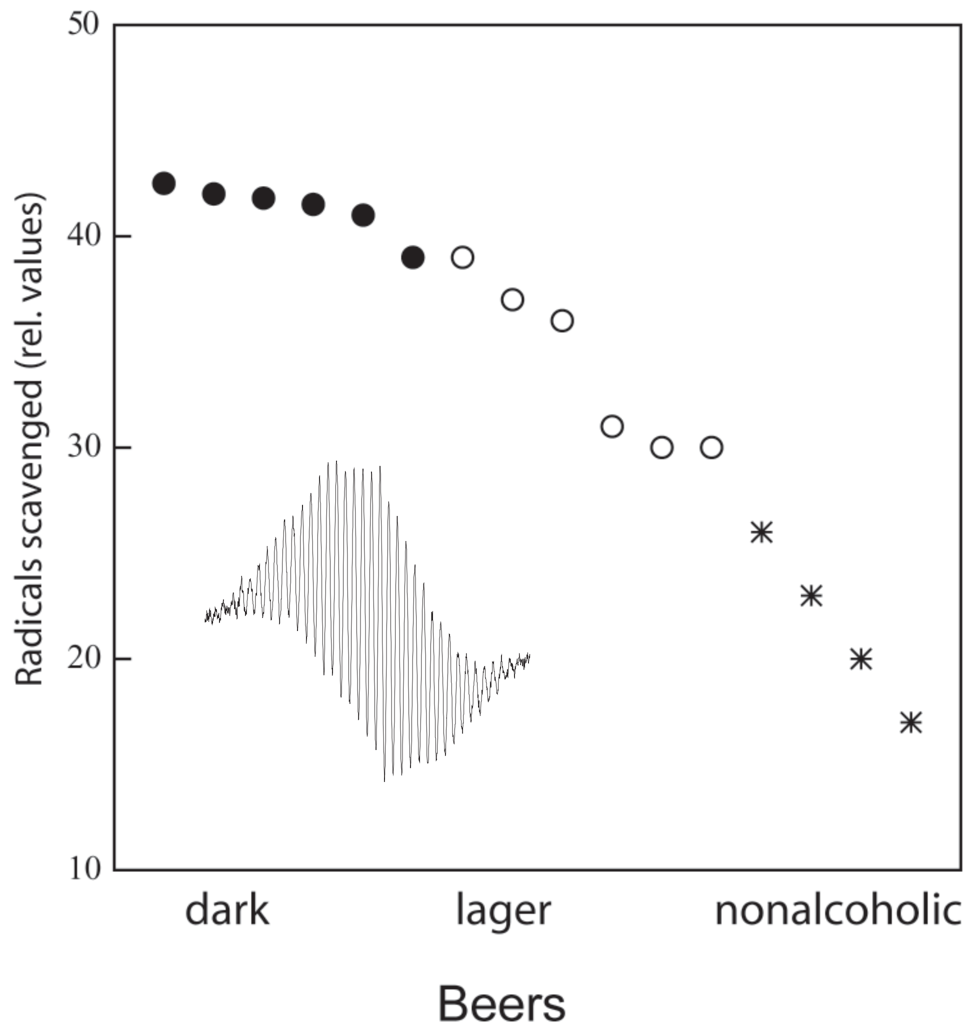

Fig. 6 Relative amounts of ABTS$^{\bullet+}$ radicals scavenged by the dark (●), lager (○) and non-alcoholic (*) beer samples. The inset presents the EPR spectrum of the monitored ABTS$^{\bullet+}$ radical. (Sweep width 3 mT)