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Antioxidant properties of tea investigated by EPR spectroscopy

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Abstract

The antioxidant properties of green, black and mixed (fruit) tea samples of different origin were investigated by means of EPR spectroscopy. A six line EPR spectrum of solid tea samples indicates the presence of Mn(II) ions and it is superimposed with a sharp singlet line attributed to semiquinone radical species ($\Delta H_{pp} = 1$ mT; $g = 2.0022$). Antioxidant properties of aqueous tea extracts in H_2O_2/NaOH/dimethylsulfoxide system generating reactive radicals ($^{\bullet}OH$, $O_2^{\bullet -}$, $^{\bullet}CH_3$) were followed by spin trapping technique. In addition, antioxidant capacity of these samples was assessed using stable radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL). Typically, the highest antioxidant potential to terminate superoxide radicals was found in green teas, followed by black and fruity teas. The pro-oxidant activity of green teas evidenced by spin traps was promoted in samples with higher Mn(II) and ascorbic acid concentrations. Various sources of free radicals used in the antioxidant tests due to their specific action show different termination rates in the presence of the individual tea samples.

Keywords: Tea; Antioxidant; EPR spectroscopy; Free radical; Spin trap; DMPO; POBN; DPPH; TEMPOL; Ascorbic acid

1. Introduction

In the past decade, considerable attention has been focused on the improvement of human health by consumption of functional food [1–4]. Tea, originally prepared from the leaves of *Camellia sinensis*, became one of the most popular beverages all over the world and it has been re-investigated in many studies for its excellent pharmaceutical properties [3–5].

The procedure of tea fermentation substantially affects the tea composition and teas can be generally divided into three basic groups. Green tea (non-fermented) is derived directly from drying and steaming of fresh tea leaves; oolong tea is prepared when fresh tea leaves are subjected to partial fermentation before drying; and black tea undergoes full fermentation before drying and steaming [6]. The fermentation is an oxidative process, in which catechins, major polyphenolic components of green tea leaves, are oxidized or condensed to larger polyphenolic molecules such as theaflavins and thearubigins [6]. Another special category of produced teas represents mixed fruit teas — i.e. mixtures of various medical herbs and fruits [7,8].

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A chemical composition, concentration and mutual relation of major components in tea leaves primary influence the properties of tea [6]. Green tea contains mainly polyphenolic catechins such as (−)-epicatechin (EC); (+)-gallocatechin (GC); (+)-catechin (C); (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3-gallate (EGCG), as well as gallic acid and caffeine, to which some of the beneficial effects of tea have been attributed [3,4,6,9]. However, some researchers assumed that there is no direct correlation between the antioxidant properties of tea and the content of catechins and they suggest that the non-phenolic fraction of green tea plays an important role [10,11]. A special attention was focused on the role of ascorbic acid present in the tea samples, which has excellent antioxidant properties [12–15] and which can also influence the long-term stability of green tea catechins [16]. It should be noted here that catechins can exhibit both antioxidant and pro-oxidant behaviour as attributed by Oshima et al. [17].

Concentration of polyphenols in different tea samples was previously determined by Ohe et al., who found that non-fermented tea contain much more catechins than semi-fermented and fermented ones [5]. The composition and content of major constituents in various sorts of tea may be affected by inter-species variations, cultivation conditions, age of leaves, climatic conditions and by the process of fermentation, influencing the hue, taste and aroma of prepared tea beverages [5].

A lot of experimental methods (HPLC, UV/visible and MS spectroscopy, cyclic voltammetry, analytical methods based on redox processes and other techniques) were previously developed for monitoring the composition of tea samples [6,18–21] and to investigate the behaviour of tea components under various experimental conditions [3,22].

EPR spectroscopy was formerly successfully applied to determination of radical scavenging activity of catechins and their epimers [23–26], for monitoring antioxidant behaviour of selected tea components [27], wines [28–30], cognacs [31] and fruits [15]. In our laboratory, EPR spin trapping techniques were used to investigate beer stability [32,33] and wine antioxidant properties [28].

The main aim of our current investigation was to characterize radical scavenging activity of tea beverages prepared from commercially available teas (green, black, mixed fruit) by means of EPR spectroscopy using as oxidants stable radicals (DPPH and TEMPOL) as well as reactive free radicals generated in system H_2O_2/NaOH/DMSO.

2. Experimental

2.1. Chemicals

The commercially available samples of green, black and mixed (fruit) teas of different origin were supplied by Slovak, Czech and Ukrainian distributors (Table 1). The spin trapping agent α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), stable free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL), as well as L-ascorbic acid (ASC), sodium sulfite (Na_2SO_3) and iodine were purchased from Sigma Chemicals. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO, Aldrich) was freshly distilled before use and stored in a freezer under argon. All solutions were prepared using redistilled water, dimethylsulfoxide (DMSO) and ethanol of spectroscopic grade (Lachema, Czech Republic). Manganese(II) chloride tetrahydrate was obtained from Aldrich.

2.2. Methods and apparatus

Tea extracts were prepared by addition of two grams of solid tea samples in 100 ml of boiling water (black and mixed teas) or 80 °C water (green teas) in accordance with recommended procedure of tea preparation [34]. After three minutes solid tea particles were filtered and fresh tea extracts were used in EPR measurements after cooling to 22 °C.

Reactive radical species for testing of antioxidant activity were generated immediately before EPR measurements by mixing the extracts with NaOH, DMSO and hydrogen peroxide [35]. The formation of short-lived radical species ($^{\bullet}OH$, $^{\bullet}CH_3$, $O_2^{\bullet -}$) was evidenced by addition of DMPO

Table 1
Tea samples characterization (distributor) and abbreviation, along with the determined pH value, Mn(II) and ASC concentrations. (Tea drinks were prepared using 2 g of dry tea and 100 ml of water)

Tea sample	Abbreviation	pH value	Mn(II) ($\mu g\ ml^{-1}$)	Ascorbic acid (mg/1 g of dry tea sample)
China green tea (Sanny Slovakia)	g1	5.46	2.5	18.9
Yunnan green tea (Sanny Slovakia)	g2	5.29	1.3	17.6
Darjeeling SFTGFOP Sungma (Sanny Slovakia)	g3	5.45	1.1	15.2
Melany (Sanny Slovakia)	g4	5.30	2.9	28.6
Green tea (BOP Slovakia)	g5	5.48	2.1	9.5
China Sechwan black tea (Sanny Slovakia)	b1	4.90	2.9	6.7
Golden Nepal (Sanny Slovakia)	b2	5.04	0.8	17.1
Darjeeling FTGFOP I Silverhill (Sanny Slovakia)	b3	5.03	0.8	17.3
Lapsang Souchong (Sanny Slovakia)	b4	4.70	0.1	4.7
Gruzia (Sanny Slovakia)	b5	4.82	3.2	6.0
Orfeus (Sanny Slovakia)	m1	3.01	2.2	0.6
Rooibos orange-spice (Sanny Slovakia)	m2	4.86	2.3	8.9
Rooibos blue star (Sanny Slovakia)	m3	4.58	0.1	6.4
Karkade (hibiscus flowers) (Ukraine)	m4	2.22	7.6	3.0
Fruity Tea (Jemča, Czech Republic)	m5	2.34	5.8	0.8

or POBN spin trapping agents. Rate of DPPH and TEMPOL radical disappearance in the presence of tea extract, measured by EPR spectroscopy, was also used to assess free radical scavenging ability of tea extracts. Reference samples were prepared by replacing the tea extracts by distilled water. Experimental systems used in testing of antioxidant activity of tea extracts are summarized in Table 2. All samples were mixed in an Eppendorf tube and the reaction time was measured from the moment of addition of reactive agents (i.e. hydrogen peroxide, DPPH or TEMPOL). Samples were transferred in a flat EPR cell, tightened with a stopper and inserted into a standard TE_{102} cavity of a Bruker EPR 200D spectrometer working in X-band. Inside the cavity samples were equilibrated at 295 K using a Bruker temperature control unit ER 4111 VT. EPR spectra were recorded using an Aspect 2000 computer connected on line with the EPR spectrometer. All measurements were started precisely four minutes time after last reagents addition and the EPR spectra were accumulated in 3.25 min intervals.

The *g*-values were determined with uncertainty of ± 0.0001 using a manufacturer's supplied internal reference marker containing solid DPPH. EPR

Table 2
The composition of experimental systems used in EPR experiments testing the antioxidant activity of teas

System	Tea drink (μl)	Component (concentration; volume) in experimental system
H_2O_2/NaOH/DMSO/ POBN	100	H_2O_2 (30%; 50 μl)
		NaOH in H_2O (25×10^{-3} mol dm^{-3}; 50 μl)
		DMSO (50 μl)
		POBN in DMSO (0.18 mol dm^{-3}; 50 μl)
		POBN concentration after mixing $c_{POBN}=0.025$ mol dm^{-3}
H_2O_2/NaOH/DMSO/ DMPO	150	H_2O_2 (30%; 50 μl)
		NaOH in H_2O (25×10^{-3} mol dm^{-3}; 50 μl)
		DMSO (50 μl)
		DMPO in DMSO (0.2 mol dm^{-3}; 50 μl)
		DMPO concentration after mixing $c_{DMPO}=0.028$ mol dm^{-3}
DPPH	200	H_2O (100 μl)
		DPPH in ethanol (8.5×10^{-4} mol dm^{-3}; 50 μl)
		DPPH concentration after mixing $c_{DPPH}=1.2\times10^{-4}$ mol dm^{-3}
TEMPOL	200	H_2O (100 μl)
		TEMPOL in ethanol (6.2×10^{-4} mol dm^{-3}; 50 μl)
		TEMPOL concentration after mixing $c_{TEMPOL}=9\times10^{-5}$ mol dm^{-3}

spectra of DMPO and POBN spin adducts were simulated using WinEPR and SimFonia programs (Bruker, Germany). Multi-component experimental EPR spectra were fitted as linear combinations of individual spectra simulations using least-squares minimization procedure with the Scientist Program (MicroMath). Relative concentrations of radical adducts were calculated from contributions of individual spectra to the experimental spectrum after double integration procedure.

The pH of freshly prepared tea drinks was determined at 20 ± 1 °C by means of JENWAY 370 instrument fitted with a combined glass electrode. The concentration of Mn(II) ions in tea solutions was calculated from the double integrated EPR spectra using a calibration curve. A concentration of ascorbic acid in freshly prepared tea extracts was monitored by means of iodometric titration [36].

3. Results and discussions

3.1. EPR spectra of dry tea samples and tea beverages

Transition metal ions are generally paramagnetic by virtue of their partially filled d orbitals. EPR spectra of d^5 ions are characterized by spectral lines with $g_{eff}\approx2.0$, 3.3 and 4.3. Their relative intensities vary with the sample structure and composition. For manganese(II) the resonance at $g_{eff}\approx3.3$, attributed to the rhombic surroundings of Mn(II) ions is usually not observed. The resonances at $g_{eff}\approx2.0$ and $g_{eff}\approx4.3$ arise from transitions between energy levels of the middle Kramer's doublet. The difference between these levels in the case of the resonance at $g_{eff}\approx4.3$ is higher than the transition energy at 9 GHz klystron frequency. Thus, the observed intensity of this line is low. The line at $g_{eff}\approx2.0$ corresponds to the interaction of manganese ion with the weak ligand field of approximately octahedral symmetry. The expected characteristic feature of the Mn(II) EPR spectra is a six-line component centered at $g_{eff}\approx2.0$, flanked by shoulders with a weak feature centered at $g_{eff}\approx4.3$ and a measurable absorption at zero field. The six-line multiplet spectrum results from the hyperfine interaction of the $^6S_{5/2}$ ground state with the ^{55}Mn nucleus ($I=5/2$) [37]. Manganese ions play a significant role in biochemical processes of green plants as cofactors of proteins and enzymes [38]. Mn(II) represents an

essential component in the catalytic splitting of water and in the evolution of oxygen in the photosystem(II) [39].

An EPR spectrum of dry mixed fruit tea (m4) containing predominantly Hibiscus flowers is shown in Fig. 1a. The characteristic feature of this spectrum is a six-line signal centered at $g_{\text{eff}}=1.99$ (inset in Fig. 1a) indicating the presence of Mn(II) ions in a distorted octahedral coordination [37,40]. The sextet lines are not equally spaced, therefore, an average hyperfine coupling $a_{\text{av}}=9.5$ mT was determined, in good agreement with previously published data [40,41]. Furthermore, broad shoulders at $g_{\text{eff}}=2.66$ and 4.32 are evident in the experimental EPR spectrum. These signals have been attributed to the extra-framework Mn(II) ions in a distorted tetrahedral symmetry [37,40]. However, in the EPR spectrum of fermented black tea (b4) shown in Fig. 1b, an additional sharp EPR line was superimposed on the six-line signal of Mn(II) ions (inset in Fig. 1b) characterized by $\Delta H_{\text{pp}}=1$ mT and $g=2.0022$, probably originating from semiquinone structures as described below.

Comparable EPR spectral lines of solid tea samples [42,43], as well as tobacco leaves [44] were recently published by Morsy and Khaled. They attributed the sharp EPR signal to stable radicals of aromatic origin representing semiquinone radicals produced by oxidation of polyphenolic compounds present in plant leaves [42]. Analogously, the sharp signals ($g=2.0073$) superimposed on Mn(II) sextet in EPR spectra of cell wall residue extracted from wheat straw, were also interpreted as semiquinone radical species produced by one-electron oxidation of lignin phenolic groups [45]. Recently, the quantitative determination of semiquinone radical species concentration in the natural polyphenols enabled to decide the degree of transformation of organic matter [46]. Pedersen published a detailed EPR study of semiquinones, which were prepared by chemical redox processes of naturally occurring quinones and quinols and this experimental technique was recommended as an efficient tool for chemotaxonomic studies [47].

The EPR signal of Mn(II) ions observed in the experimental spectra of green (non-fermented) tea samples represents broad line with unresolved splitting characteristic for a relatively undisturbed

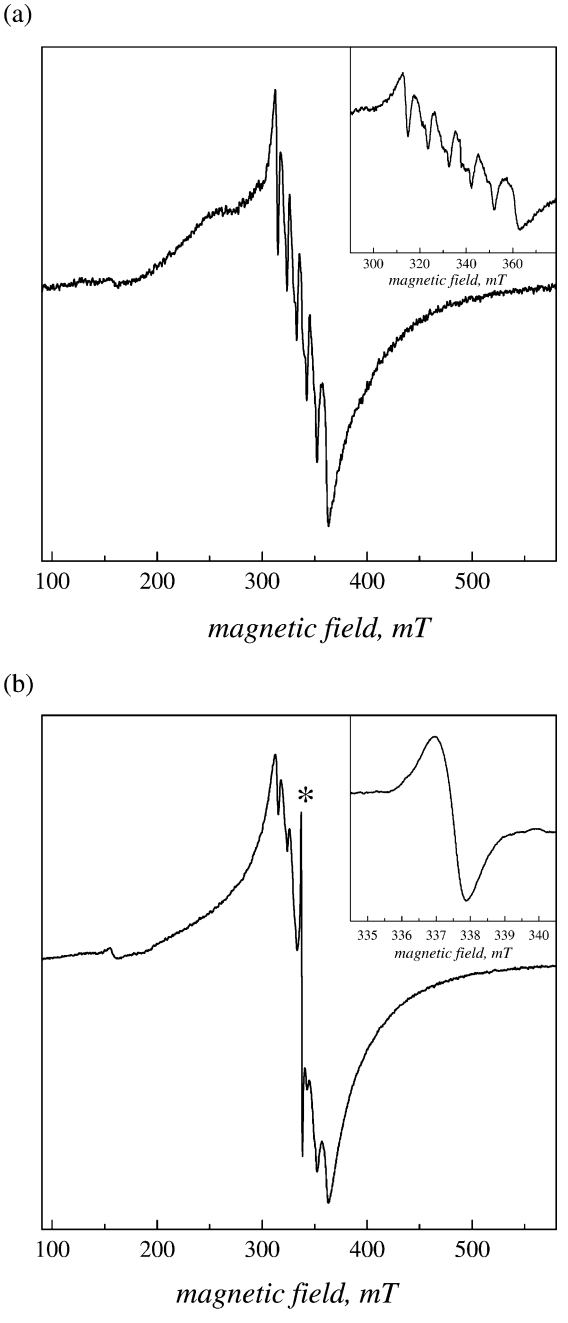


Fig. 1. EPR spectra of dry tea samples measured at 295 K. (a) Mixed fruit tea m4. Inset represents a detailed view at six-line component of Mn(II) ions. (b) Black tea b4. Inset shows a detail of the sharp EPR signal of semiquinone radical (*). Spectrometer settings: centre field, 337 mT; sweep width, 500 mT; gain, 1.25×10^5; modulation, 0.1 mT; scan time, 100 s; microwave power, 20 mW; number of scans, 3.

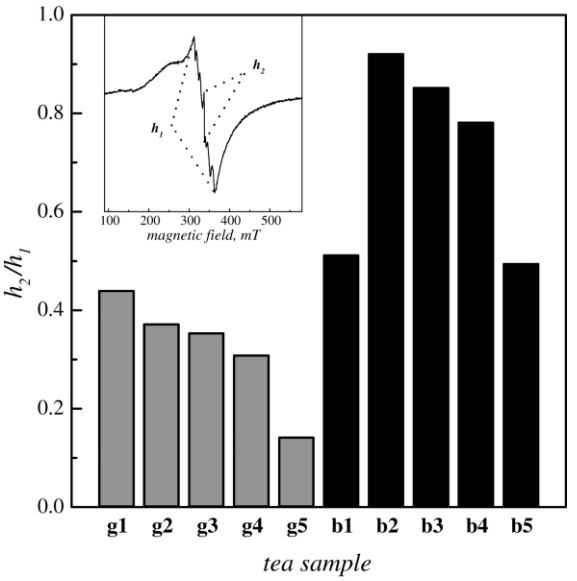


Fig. 2. The values of h_2/h_1 ratio evaluated using EPR spectra of green and black dry tea samples. Inset: EPR spectrum of black tea b5 and demonstration of h_1 and h_2 determination.

bonding of Mn(II) in protein complex [42]. However, the six-line EPR signal of Mn(II) ions is better evident in black (fermented) tea samples (Fig. 1b), which is in a good accord with a damage of the Mn(II)-protein complex of tea leaves during a fermentation procedure. A sharp singlet, previously identified as semiquinone radical structure produced in tea leaves was present in all EPR spectra of green and black teas, but evidently more dominant in black teas. In an attempt to quantify the Mn(II) and semiquinone radical EPR signals in green and black tea samples we evaluated the peak-to-peak height ratio h_2/h_1 (h_1 and h_2 represent the peak-to-peak heights of Mn(II) and semiquinone radical, respectively) as depicted in inset of Fig. 2. The peak-to-peak EPR line widths of both signals in all samples were approximately identical (ΔH_{pp}(Mn) approx. 52 mT and ΔH_{pp}(semiquinone) approx. 1.0 mT). Our data demonstrate that green tea samples (series g1–g5) can be characterized by a value of h_2/h_1 ratio below 0.5, whereas in black tea spectra (series b1–b5) h_2/h_1 ratio is significantly higher (Fig. 2). This is a consequence of the oxidation of natural polyphenols in tea leaves during black tea fermentation producing higher concentration of semiquinone radical species [42,43].

The heterogeneity of mixed (fruit) teas composition is reflected also in the observed EPR spectra of dry tea samples. The tea samples, in which the dominant component represents Hibiscus flowers (Fig. 1a, m4) are characterized by a very well resolved sextet attributable to Mn(II) ions from a damaged Mn(II)-protein complex. However, EPR spectra of Rooibos teas (*Aspalathus linearis*) mixed with dried fruits besides non-resolved Mn(II) broad line and semiquinone sharp signal ($g=2.0022$), contain also additional signals attributed to Fe(III) ions [48,49].

During preparation of tea drinks paramagnetic Mn(II) ions dissolve in water and their concentration can be evaluated using EPR spectroscopy by means of calibration curve. Fig. 3 represents the calibration curve for Mn(II) determination in the experimental tea sample along with the confidence limits for the prediction at 95% confidence level [50,51]. In these experiments no spin traps or other substrates were added to the freshly prepared tea drinks. Since Mn(II) concentrations in aqueous tea extracts are relatively low, higher spectrometer settings (gain, 5×10^5; modulation amplitude, 0.1 mT; microwave power, 20 mW; 10 accumulated scans) had to be used in order to obtain measurable spectra (inset in Fig. 3). The results of Mn(II) concentration measurements in tea drinks are summarized in Table 1. The presence of manganese ions in the tea drinks has to be taken into account since it can influence results of antioxidant activity testing, especially when using experimental systems containing hydrogen peroxide [35,52,53].

3.2. Test of antioxidant and radical scavenging activity of tea beverages

3.2.1. Spin trapping experiments in H_2O_2/NaOH/DMSO/DMPO or POBN systems

The EPR spin trapping method involves trapping of reactive short-lived free radicals by a diamagnetic EPR silent compound (spin trap) via addition to a spin trap double bond to produce a more stable free radical product (spin adduct). Spin adducts are paramagnetic and have EPR spectra

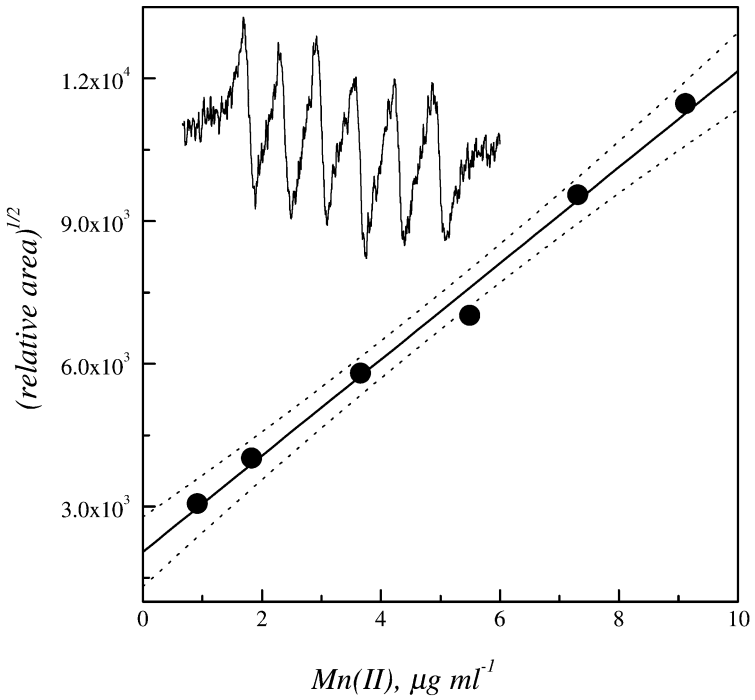


Fig. 3. Linear dependence of square root of Mn(II) EPR signal area on manganese ions concentration utilized for Mn(II) ions determination in tea drinks. The evaluated confidence limits for the prediction were calculated at 95% confidence level. Inset: EPR spectrum (scan 80 mT) of freshly prepared tea drink (sample b1) measured at 295 K. The spectrometer settings are specified in text.

with hyperfine splitting constants and *g*-value characteristic of the type of free radical trapped [54]. Nitrone spin traps (DMPO, POBN) scavenge free radical species via addition to a carbon located in alfa position relative to the nitrogen (Scheme 1) [54].

The H_2O_2/NaOH/DMSO system was established as a non-enzymatic and non-Fenton type system generating reactive radical species ($O_2^{\bullet -}$, $^{\bullet}OH$, $^{\bullet}CH_3$) suitable for evaluation of radical scavenger activity [35]. The generation of reactive radical species is based on the decomposition of hydrogen peroxide in alkaline media [55–58] and can be characterized with Eqs. (1)–(6):

$$H_2O_2 + OH^- \Leftrightarrow HO_2^- + H_2O \quad (1)$$

$$H_2O_2 + HO_2^- \rightarrow O_2^{\bullet -} + {}^{\bullet}OH + H_2O \quad (2)$$

$$^{\bullet}OH + H_2O_2 \rightarrow {}^{\bullet}O_2H + H_2O \quad (3)$$

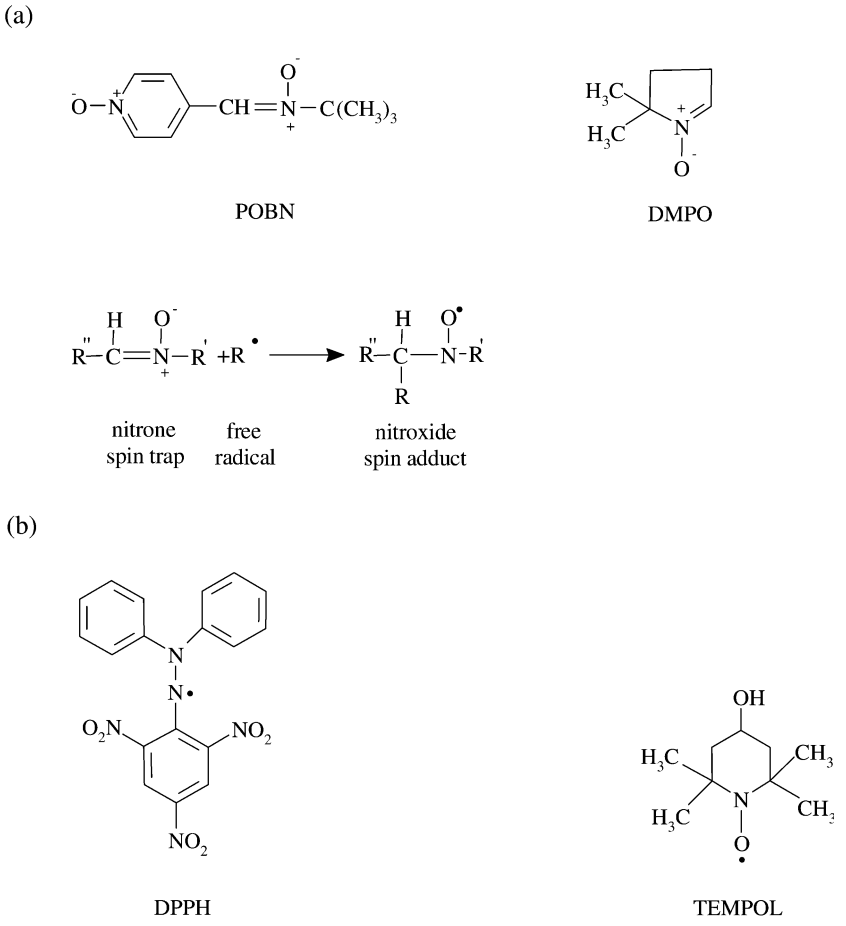
$$^{\bullet}O_2H \leftrightarrow O_2^{-\bullet} + H^+ \quad (4)$$

$$DMSO + {}^{\bullet}OH \rightarrow {}^{\bullet}CH_3 + CH_3SO(OH) \quad (5)$$

$$^{\bullet}CH_3 + H_2O_2 \rightarrow CH_4 + {}^{\bullet}O_2H \quad (6)$$

Spin traps DMPO and POBN were used in our experiments to evaluate the effect of tea solution on the radical formation. The composition of the investigated systems used is summarized in Table 2. In reference samples an active (radical scavenging) tea solution was replaced by distilled water.

DMPO. Fig. 4 shows experimental and simulated EPR spectra obtained in reference sample (water addition instead of tea solution, Fig. 4 reference) and three different tea samples (Fig. 4, g3, b3, m3) using spin trapping agent DMPO in H_2O_2/NaOH/DMSO system 37 min after H_2O_2 addition. The simulation of experimental spectrum in the reference systems was calculated with hyper-



Scheme 1. (a) The structures of spin trapping agents DMPO and POBN and the key reaction of spin trapping technique. (b) The structure of DPPH and TEMPOL free radicals.

fine splittings $a_N = 1.44$ mT, $a_H^\beta = 1.02$ mT, $a_H^\gamma = 0.14$ mT and g-factor $g = 2.0059$, which is attributed to $^{\bullet}$DMPO-O_2^- adduct (Eq. (7)) [52–54].

$$DMPO + O_2^{\bullet -} \rightarrow {}^{\bullet}DMPO\text{-}O_2^- \quad (7)$$

As shown in Fig. 4 in the reference system only formation of superoxide anion-radical adduct $^{\bullet}$DMPO-O_2^- was evidenced, because $^{\bullet}$OH and $^{\bullet}CH_3$ intermediates are under the given reactant concentrations ($c(H_2O_2) = 1.3$ mol dm^{-3}; c(DMSO) = 3.6 mmol dm^{-3}; c(NaOH) = 3.6 mol dm^{-3}) involved in rapid consecutive reactions with hydrogen peroxide leading to superoxide anion-radical formation (Eqs. (4) and (6)). The formation of superoxide adducts is in accord with mechanism proposed by Polyakov for systems containing DMSO and high hydrogen peroxide concentration [59,60].

Significant changes in EPR spectra are found if an analogous radical generation proceeds in the presence of tea solutions. Characteristic spectra of such systems in the case of green or black teas g3, b3 shows Fig. 4, where practically only six-line EPR signal corresponding to carbon-centered adducts was evidenced (Fig. 4, g3, b3). The experimental EPR spectrum measured in the presence of mixed tea m3 is more complex and its

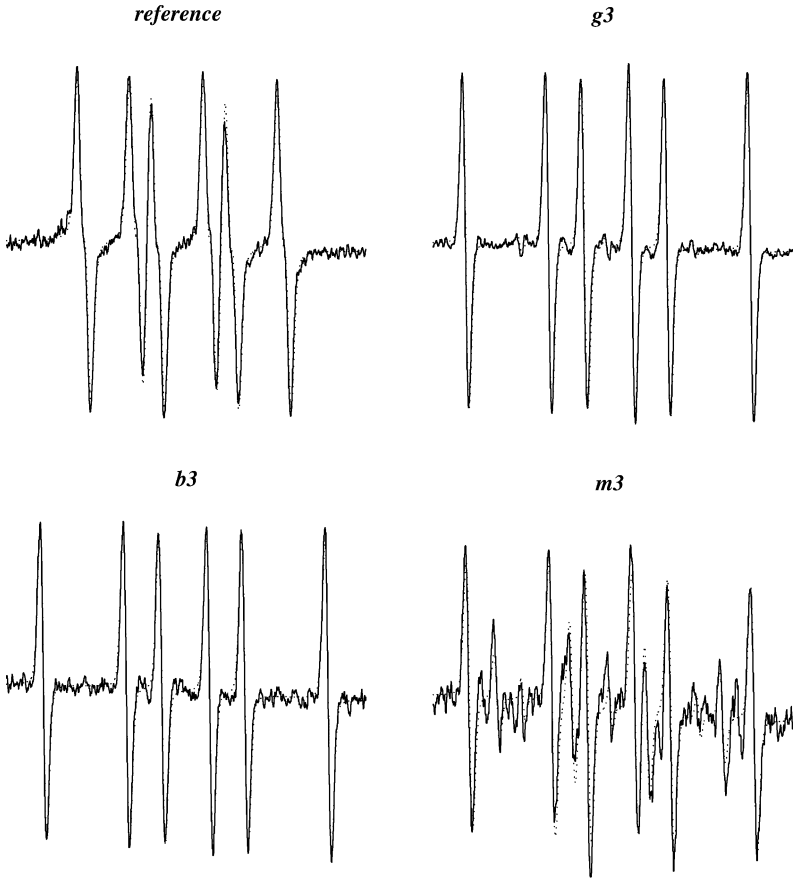


Fig. 4. Experimental (solid line) and simulated (dotted line) EPR spectra of reference (water) and tea samples g3, b3, m3 measured in H_2O_2/NaOH/DMSO solution in the presence of DMPO spin trapping agent. Spectrometer settings: centre field, 337 mT; sweep width, 7 mT; gain, 2.5×10^5; modulation, 0.1 mT; scan time, 50 s; microwave power, 20 mW; number of scans, 3. Experimental spectra were measured 37 min after hydrogen peroxide addition.

simulation revealed the presence of $^{\bullet}$DMPO-CH_3 ($a_N=2.31$ mT, $a_H^{\beta}=1.615$ mT; $g=2.0057$), $^{\bullet}$DMPO-OH ($a_N=1.44$ mT, $a_H^{\beta}=1.48$ mT; $g=2.0059$) and $^{\bullet}$DMPO-CX ($a_N=1.71$ mT; $g=2.0059$) adducts. The later three-line EPR signal of low intensity is evident in the spectra and was attributed to DMPO decomposition product.

The time evolution of EPR spectra measured in H_2O_2/NaOH/DMSO/DMPO system in the presence of Orfeus mixed fruit tea (m1) was different comparing to other tea samples. A simulation of obtained experimental spectra shown in inset of Fig. 5 evidenced the formation of two radical species, namely ascorbyl radical ($a_H=0.18$ mT; $g=2.0053$) and $^{\bullet}$DMPO-CH_3 adduct ($a_N=2.31$ mT, $a_H^{\beta}=1.615$ mT; $g=2.0057$). No EPR signals matching $^{\bullet}$DMPO-OH or $^{\bullet}$DMPO-O_2^- adducts formation were detected. Relative concentrations of ascorbyl radical and $^{\bullet}$DMPO-CH_3 adduct were evaluated from the simulations of the corresponding EPR spectra obtained in various time intervals after H_2O_2 addition. Fig. 5 shows so found relative concentration decrease of ascorbyl radical, coupled with a simultaneous increase of relative $^{\bullet}$DMPO-CH_3 adduct concentration.

Yoshimura et al. [35] previously observed the proportional decrease of $^{\bullet}$DMPO-OH or $^{\bullet}$DMPO-O_2^- signal heights after addition of various con-

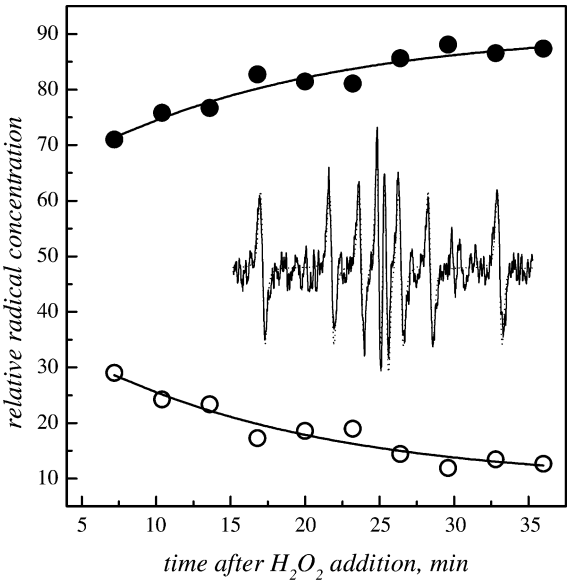


Fig. 5. Time dependence of the relative radical concentration of ascorbyl (○) and •DMPO-CH_3 adduct (●) evaluated from the simulations of EPR spectra measured in tea drink m1. Inset: Experimental (solid line) and simulated (dotted line) EPR spectrum obtained 7 min after hydrogen peroxide addition. Spectrometer settings: centre field, 337 mT; sweep width, 7 mT; gain, 2.5×10^5; modulation, 0.1 mT; scan time, 50 s; microwave power, 20 mW; number of scans, 3.

centration of ascorbic acid to H_2O_2/NaOH/DMSO/DMPO solutions. Conversely, the signal amplitude of •DMPO-CH_3 increased with increasing ascorbic acid concentration. Consequently, they proposed a mechanism of methyl radical generation via reaction with ascorbic acid [35].

POBN. The experimental and simulated EPR spectra obtained in reference samples (water addition instead of tea solution, Fig. 6 reference) and three different tea samples (Fig. 6, g3, b3, m3) using spin trapping agent POBN in H_2O_2/NaOH/DMSO system 37 min after H_2O_2 addition are depicted in Fig. 6. The POBN molecule is a very effective scavenger of radical species produced under the given experimental conditions, as the absolute EPR signal intensities were 2.5 times larger compared to DMPO (Figs. 4 and 6). However, due to limited selectivity of the POBN spin adduct parameters [60], a discussion of the type of radical trapped is not entirely appropriate, and the correct assignment of a radical species generated in system requires supplementary investigations using a more selective spin trap, e.g. DMPO.

The experimental spectrum monitored in reference system (Fig. 6 reference) was simulated as a superposition of two spin adducts, i.e. •POBN-O_2^- ($a_N=1.447$ mT, $a_H^\beta=0.197$ mT, $a_{13C}(6\ ^{13}C)=0.51$ mT; $g=2.0059$; relative concentration 87%) and •POBN-CH_3 ($a_N=1.585$ mT, $a_H^\beta=0.261$ mT, $a_{13C}(6\ ^{13}C)=0.51$ mT; $g=2.0058$; relative concentration 13%). However, the presence of tea solutions caused significant changes in character and intensity of EPR spectra (Fig. 6, g3, b3, m3). The addition of all green and black tea samples fully eliminated the formation of •POBN-O_2^- and •POBN-OH adducts and in EPR spectra only signals corresponding to the formation of •POBN-CH_3 adduct were measured. The reactive oxygen radical scavenging activity of mixed tea m1 and m4 was lower, while the experimental EPR spectra represented mixtures of two individual spectra corresponding to •POBN-CH_3 and •POBN-OH adduct ($a_N=1.448$ mT, $a_H^\beta=0.215$ mT, $a_{13C}(6\ ^{13}C)=0.51$ mT; $g=2.0059$).

The ability of green, black and mixed (fruit) tea drinks to decrease the concentration of reactive oxygen radical species was evaluated using both spin traps. We calculated the simulation of experimental EPR spectra matching the signals measured 37 min after hydrogen peroxide addition and then evaluated the EPR signal area of individual simulation component (Figs. 7 and 8). Fig. 7 shows assessment of EPR signal area for radical species identified in H_2O_2/NaOH/DMSO/DMPO systems (•DMPO-O_2^-, •DMPO-CH_3, •DMPO-OH, •DMPO-CX, ascorbyl radical) in the presence of tea drinks of different origin. The addition of green or black tea (g1–g5, b1–b3) solutions to the radical-producing systems fully eliminates formation of •DMPO-OH and •DMPO-O_2^- adducts and consequently, in EPR spectra dominates a six-line EPR signal attributable to •DMPO-CH_3 already shown in Fig. 4. The high values of •DMPO-CH_3 EPR signal area are in good correlation with higher concentration of ascorbic acid in tea beverages [35]. The activity of black teas b4 and b5 to scavenge reactive oxygen radical was lower than that of green teas, since we confirmed the presence of a typical four-line signal matching •DMPO-OH adduct ($a_N=1.485$ mT, $a_H^\beta=1.455$ mT, $g=$

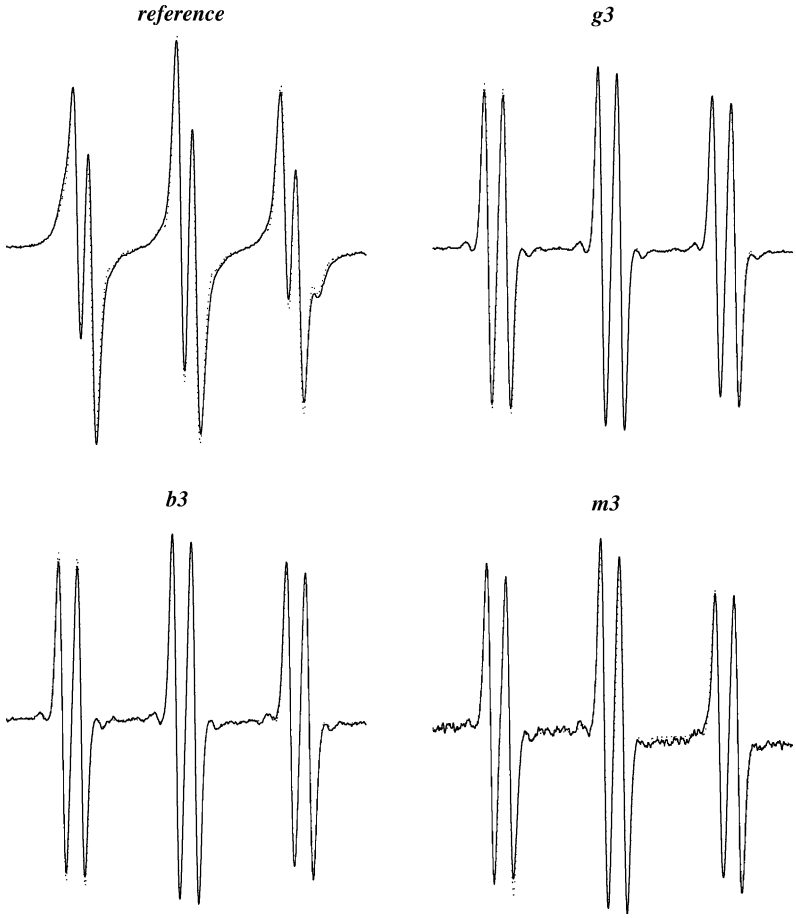


Fig. 6. Experimental (solid line) and simulated (dotted line) EPR spectra of reference (water) and tea samples g3, b3, m3 measured in H_2O_2/NaOH/DMSO solution in the presence of POBN spin trapping agent. Spectrometer settings: centre field, 337 mT; sweep width, 5 mT; gain, 1.0×10^5; modulation, 0.1 mT; scan time, 50 s; microwave power, 20 mW; number of scans, 3. Experimental spectra were measured 37 min after hydrogen peroxide addition.

2.0059). The area of EPR spectra measured in H_2O_2/NaOH/DMSO/DMPO solutions in the presence of mixed fruit teas was significantly lower compared to green or black tea (Fig. 7). Also, here the six-line EPR signal corresponding to the $^{\bullet}$DMPO-CH_3 radical formation predominates in all spectra. But besides the formation of ascorbyl radical found in m1, in mixed teas m2 and m3 we also evidenced the formation of $^{\bullet}$DMPO-OH. This suggests that the efficiency to terminate reactive $^{\bullet}$OH radicals by fruit teas is lower than in the case of green teas.

Results of EPR signal area evaluation for EPR spectra obtained in H_2O_2/NaOH/DMSO/POBN system in the presence of POBN spin trap 37 min after hydrogen peroxide addition are depicted in Fig. 8. The six-line EPR signal dominating in all EPR spectra in the presence of tea solutions was assigned to $^{\bullet}$POBN-CH_3 adduct and only in the presence of m1 and m4 samples the formation of hydroxyl radical adduct was evidenced.

The prepared tea drinks represent complex systems, as they contain various concentrations of polyphenols, phenolic acids, flavonoids, ascorbic acid, as well as traces of metal ions (Mn(II), Fe(III), Cu(II)) [6,42,49]. In an attempt better to understand the results of EPR spin trapping experiments, we determined pH value, Mn(II) and

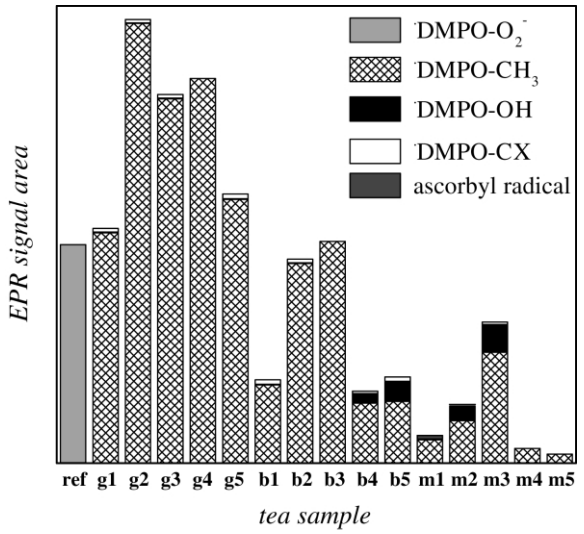


Fig. 7. EPR signal area of individual radical species identified in simulated EPR spectra of reference (water) and tea beverages 37 min after mixing of reactive components in H_2O_2/NaOH/DMSO/DMPO solution. Experimental and simulated EPR spectra of reference, g3, b3 and m3 illustrates Fig. 4.

ascorbic acid concentrations in the investigated tea beverages (Table 1).

The pH-values of all tea drinks in the absence of the reaction system are in acidic region (Table 1). System H_2O_2/NaOH/DMSO producing reactive radicals has pH value of approximately 9. Therefore, the influence of pH changes, resulting from the tea solutions added, on the radical formation is minimal (pH in the region 9–8) [35].

The presence of manganese(II) ions and ascorbic acid in tea samples can considerably influence reaction pathways of superoxide anion-radical and hydroxyl radical in H_2O_2/NaOH/DMSO/spin trap system. Mn(II) ions can be involved in Fenton reaction mechanism with hydrogen peroxide, producing Mn(III) species and hydroxyl radicals (Eq. (8)):

$$Mn(II) + H_2O_2 \rightarrow Mn(III) + {}^{\bullet}OH + OH^- \quad (8)$$

The generated Mn(III) species can react with ascorbic acid/ascorbate forming ascorbyl radicals (Eq. (9)) [61], as well as with superoxide anion-radical (Eq. (10)):

$$Mn(III) + ASC^- \rightarrow Mn(II) + ASC^{\bullet} \quad (9)$$

$$Mn(III) + O_2^{\bullet -} \rightarrow Mn(II) + O_2 \quad (10)$$

As a result, ascorbic acid in the presence of even very small amounts of transition metals can act as a pro-oxidant, initiating additional reactions resulting in production of reactive radical species [35,52]. Under our experimental conditions, such pro-oxidant effect of ascorbate is evidenced as an increase of methyl radical-adduct concentration (Fig. 7). The highest intensities of ·DMPO-CH_3 EPR signals in H_2O_2/NaOH/DMSO solutions were observed using tea beverages with high concentration of ascorbic acid coupled with presence of Mn(II) ions (Fig. 7, g1–g3, b3, Table 1), in line with pro-oxidant mechanism of ascorbic acid promoted with Mn(II) ions.

3.2.2. Radical scavenging activity of tea investigated using DPPH

DPPH is a stable free radical (Scheme 1b), capable to accept electron from reactive radicals,

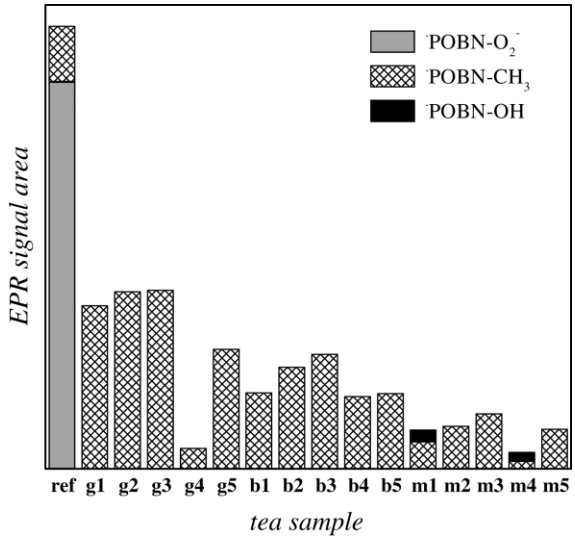


Fig. 8. EPR signal area of individual radical species identified in simulated EPR spectra of reference (water) and tea beverages 37 min after mixing of reactive components in H_2O_2/NaOH/DMSO/POBN solution. Experimental and simulated EPR spectra of reference, g3, b3 and m3 illustrates Fig. 6.

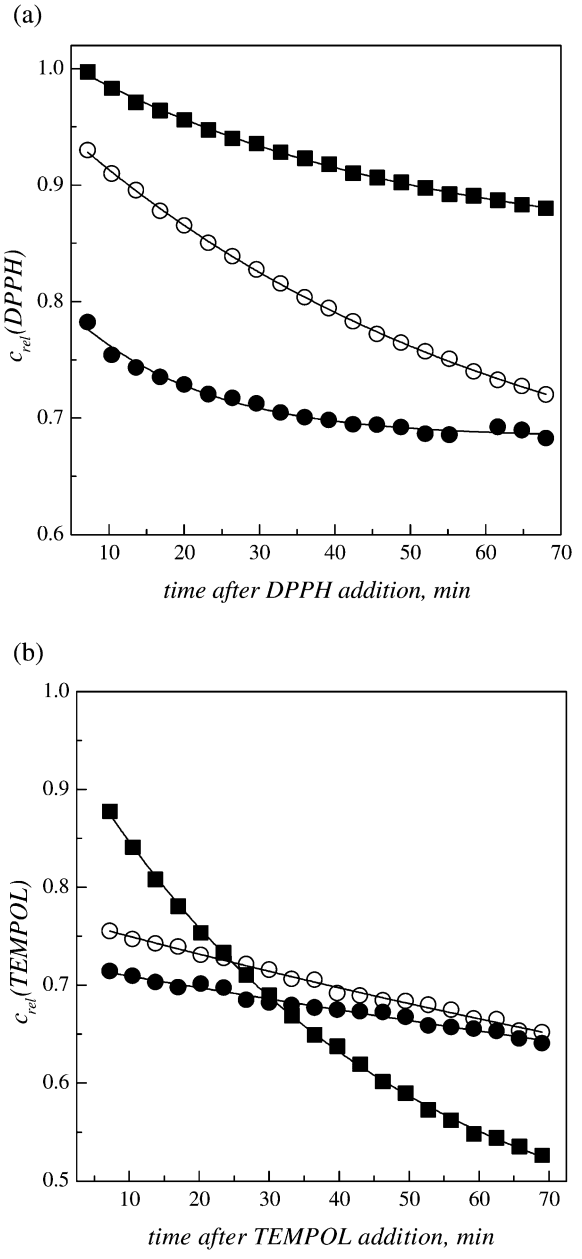


Fig. 9. (a) The time dependence of DPPH relative concentration determined in the solution of green tea g1 (○), black tea b1 (●) and mixed fruit tea m1 (■). (b) The time dependence of TEMPOL relative concentration determined in the solution of green tea g1 (○), black tea b1 (●) and mixed fruit tea m1 (■). The solid lines represent the mathematical simulations of experimental data in accord with the formal first-order kinetics.

thus behaving as a radical scavenger [62]. Additionally, DPPH acts as an electron acceptor from antioxidants (HA) and several electron transfer reactions of DPPH with phenols, amines and other compounds were described in literature [28,62] Eqs. (11)–(13)):

$$^{\bullet}\mathrm{DPPH} + \mathrm{HA} \leftrightarrow \mathrm{DPPH\text{-}H} + {}^{\bullet}\mathrm{A} \quad (11)$$

$$^{\bullet}\mathrm{DPPH} + \mathrm{A}^{-} \leftrightarrow \mathrm{DPPH}^{-} + {}^{\bullet}\mathrm{A} \quad (12)$$

$$^{\bullet}\mathrm{A} + {}^{\bullet}\mathrm{X} \rightarrow \text{non-radical products} \quad (13)$$

The radical $^{\bullet}$A of the antioxidant formed in reaction with DPPH can be sometimes observed directly by EPR (e.g. tocopheroxyl radicals can be generated by this method [14]), but typically the steady-state concentration of $^{\bullet}$A is low, due to the disappearance of these radicals via recombination or disproportionation (Eq. (13)). The depletion of DPPH after addition of antioxidants can be measured by UV/visible spectroscopy ($\lambda_{max} = 520$ nm), EPR spectroscopy, as well as other techniques [28,62–67]. The structural information about the non-radical products produced by the reaction of catechin and epicatechin with DPPH using 1-D and 2-D NMR spectral analysis was investigated by Sang et al., and a possible antioxidant mechanism was proposed [68].

In our experiments ethanolic solutions of DPPH were mixed with solutions of prepared tea drinks (Table 2) and the decline of DPPH signal was monitored by EPR. Under the given experimental conditions the EPR spectrum of DPPH free radical represents a singlet ($\Delta H_{pp} = 0.4$ mT; $g = 2.0036$). The concentration of DPPH was evaluated using the double integrated EPR signal and a calibration curve and the relative DPPH concentration c_{rel}(DPPH) for each reaction time was calculated according to Eq. (14):

$$c_{rel}(\mathrm{DPPH}) = \frac{c_t(\mathrm{DPPH})}{c_0(\mathrm{DPPH})} \quad (14)$$

where c_0(DPPH) represents the initial DPPH concentration and c_t(DPPH) corresponds to the free radical concentration at various times after mixed with tea solution.

Table 3
The formal first-order rate constants, k_{DPPH}, their standard deviations and R-squared values calculated using least square analysis of DPPH relative concentration decrease on time after its addition in solutions containing fresh prepared tea beverages

Tea sample	k_{DPPH}, min^{-1}	Standard deviation of k_{DPPH}, min^{-1}	R-squared
g1	0.00269	7.3×10^{-5}	0.99997
g2	0.0046	1.6×10^{-4}	0.99985
g3	0.0025	1.0×10^{-4}	0.99993
g4	0.00178	1.7×10^{-5}	0.99999
g5	0.00284	2.1×10^{-5}	0.99999
b1	0.0011	1.3×10^{-4}	0.99991
b2	0.0027	1.2×10^{-4}	0.99991
b3	0.00230	7.2×10^{-5}	0.99997
b4	0.00130	9.6×10^{-5}	0.99994
b5	0.00148	8.0×10^{-5}	0.99996
m1	0.00122	5.0×10^{-5}	0.99998
m2	0.00087	3.1×10^{-5}	0.99999
m3	0.00134	6.1×10^{-5}	0.99997
m4	0.00125	6.9×10^{-5}	0.99997
m5	0.00161	6.8×10^{-5}	0.99997

Fig. 9a presents the time dependence of DPPH relative concentration measured in different tea drinks (g1, b1, m1). Various types of tea solutions monitored by DPPH decay show quite different behaviour: in presence of green tea g1 DPPH signal significantly drops during 70 min, but in analogous experiments with black tea b1 a substantial decline is observed only in the first period after DPPH addition. The scavenging activity of mixed fruit tea m1 in DPPH solution is relatively low (Fig. 9a). To compare the DPPH scavenging activity of all tea beverages, the experimental data were fitted by the exponential function (solid lines in Fig. 9a) using least square analysis (program Scientist, MicroMath) and the corresponding values of formal first-order rate constant (k_{DPPH}) were calculated. The statistic parameters of such fitting calculations (sum of square deviations, R-squared, correlation, coefficient of determination) evidenced good agreement of the experimental and calculated data. The calculated values of formal first-order rate constant representing the decrease of DPPH radical concentration, along with their standard deviation and R-squared parameters are summarized in Table 3. The highest values of k_{DPPH} demonstrating the highest DPPH scavenging activity were found for green teas g1, g2, g5 and for black tea b2, whereas in the presence of mixed fruit teas the ability to eliminate DPPH free radical was lower (Table 3).

According to the previous investigations the antioxidant activity of phenolic compounds is determined by their structure and the position and degree of hydroxylation on the ring structure plays a key role [69]. The ability of phenolic compounds to scavenge DPPH radical was suggested to be relative to the redox potential [67]. The elucidation of antioxidant activity of authentic tea drinks is complex problem, as the prepared tea beverages contain various concentrations of phenolic compounds, flavonoids, ascorbic acid, as well as metal ions. From our results it can be concluded that electron and hydrogen donating activity of these compounds determine the DPPH radical scavenging efficiency in tea drinks.

Probably, the lower antioxidative action of mixed fruit teas reflected the presence of different organic acids, previously characterized with lower capability to quench free radical species (e.g. ferulic, vanillic, *p*-hydroxybenzoic and *p*-coumaric acids) [27]. Additionally, a very rapid reaction of ascorbic acid with DPPH free radical [70,71], has to be taken into account by the evaluation of antioxidant activity.

3.2.3. Tea radical scavenging activity investigated using TEMPOL

The antioxidative properties of tea drinks were tested additionally using free radical TEMPOL, which is a stable nitroxide radical, extensively used to investigate properties of biophysical and biochemical systems [72–74]. Generally, TEMPOL can terminate radicals produced in living organisms (Eq. (15)), e.g. it was confirmed that addition of TEMPOL could protect laboratory animals from damage associated with conditions of oxidative and nitrosoactive stress [75].

$$^{\bullet}R + > N\text{-}O^{\bullet} \rightarrow > N\text{-}OR \qquad (15)$$

Furthermore, TEMPOL can be reduced to an EPR silent hydroxylamine and this substance is

able to be re-oxidized again to the paramagnetic nitroxide [32,76]. According to literature, TEMPOL efficiently oxidizes ascorbic acid to dehydroascorbic acid [77–79].

Previously we demonstrated that TEMPOL may be applied as an indicator of radical production during thermally initiated beer ageing, and this degradation process was extensively influenced by the addition of ascorbic acid [32].

The antioxidative potential of tea solutions in the presence of TEMPOL was carried out by an analogous approach as was described for DPPH. We monitored the intensity of nitroxide three-line EPR spectra ($a_N = 1.70$ mT; $g = 2.0060$) after addition of TEMPOL in the tea drinks. Subsequently the experimental spectra were double-integrated and the relative concentration of TEMPOL, c_{rel}(TEMPOL), was determined. Fig. 9b shows the time dependence of TEMPOL relative concentration measured in different tea drinks (g1, b1, m1). The experimental time dependencies of TEMPOL relative concentration here again represent exponential functions according to the formal first-order kinetic model. However, the decline of individual tea samples was different comparing to the DPPH systems (Fig. 9a and b). To evaluate the antioxidative action of all tea samples we calculated, using least square analysis, the corresponding values of formal first-order rate constant k_{TEMPOL}, which are summarized in Table 4. The results obtained expressed once more the dependence of TEMPOL free radical termination reaction on the composition of individual tea beverages. An excellent antioxidant activity was observed for mixed teas m1, m5 and m4 containing Hibiscus flowers, as well as for green tea g2.

It should be noted here, that the role of TEMPOL in our experiments could be affected by the redox potential of the tea sample components in a similar way, as we assumed in the case of DPPH.

Table 4
The formal first-order rate constants, k_{TEMPOL}, their standard deviations and R-squared values calculated using least square analysis of TEMPOL relative concentration decline on time after its addition in solutions containing fresh prepared tea beverages

Tea sample	k_{TEMPOL}, min^{-1}	Standard deviation of k_{TEMPOL}, min^{-1}	R-squared
g1	0.00243	5.3×10^{-5}	0.99998
g2	0.0047	1.4×10^{-4}	0.99992
g3	0.00201	5.6×10^{-5}	0.99998
g4	0.00182	7.9×10^{-5}	0.99996
g5	0.0013	1.4×10^{-4}	0.99988
b1	0.00167	5.6×10^{-5}	0.99998
b2	0.00188	8.1×10^{-5}	0.99996
b3	0.00130	5.6×10^{-5}	0.99998
b4	0.00087	5.2×10^{-5}	0.99998
b5	0.00130	3.5×10^{-5}	0.99999
m1	0.0087	2.5×10^{-4}	0.99965
m2	0.00188	3.7×10^{-5}	0.99999
m3	0.00182	6.9×10^{-5}	0.99997
m4	0.0295	2.5×10^{-4}	0.99979
m5	0.0063	1.3×10^{-4}	0.99975

4. Conclusions

EPR spectroscopy represents a valuable tool to characterize the antioxidant activities of tea samples and authentic tea beverages. The solid state EPR spectra of dry tea leaves evidenced, in addition to Mn(II) ions, a sharp single-line signal attributed to semiquinone radical species. The amount of paramagnetic semiquinone species in black teas is significantly higher than in the dry green tea samples, which is in agreement with semiquinone radical production during the tea fermentation process.

The ability of tea beverages to lower free radical concentrations was determined in the radical producing system ($^{\bullet}OH$, $O_2^{\bullet-}$, $^{\bullet}CH_3$) H_2O_2/NaOH/DMSO using spin trapping EPR technique by means of DMPO and POBN, as well as in experimental systems with added stable free radicals DPPH or TEMPOL. The highest antioxidant potential to terminate superoxide radicals was found in green teas, followed by black and fruity teas. However, the pro-oxidant activity of green teas evidenced by DMPO spin trap was promoted in samples with higher Mn(II) and ascorbic acid concentrations. The simultaneous presence of ascorbic acid, along with Mn(II) ions, additionally generates free radicals, which may formally indicate a lower antioxidant activity of the corresponding samples as a result. Therefore, various radical

sources should be used for antioxidant testing of authentic natural samples to offset these effects.

Acknowledgments

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