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Tokay wines as scavengers of free radicals (an EPR study)

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Abstract

Wines are increasingly considered to be beneficial to health, so we expanded our previous investigations of red and white wines to Tokay wines, employing electron paramagnetic resonance spectroscopy (EPR). Four sources of free radicals were used in scavenging experiments: thermally decomposed radical initiators, $K_2S_2O_8$ and 2,2′-azo-bis(2-methylpropionamidine) hydrochloride (AAPH), as well as the cation radical of 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) salt (ABTS), and the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The radical-scavenging abilities of 30 samples of Tokay wines from the Slovak region were compared with 10 samples of red and 10 samples of white wines originating from various regions. Tokay wines show a very good scavenging ability, positioned between white and red wines expressed as trolox equivalent antioxidant capacities (TEAC) with 14.8 ± 1.5 for red, 8.1 ± 3.4 Tokay and 3.3 ± 1.6 for white wines in mmol dm^{-3} wine.

Keywords: Tokay wines; Radical scavenger; EPR; Spin trapping technique; DMPO

1. Introduction

An intensive investigation is underway to characterise the beneficial properties of foods (Aruoma, 2003; Kris-Etherton et al., 2004; Park & Surh, 2004; Stanner, Hughes, Kelly, & Buttriss, 2004; Tiwari, 2004; Vitaglione & Fogliano, 2004), since they can serve as a source of antioxidants, able to scavenge and eliminate free radicals, which otherwise may cause oxidative damage to biomolecules, and initiate various illnesses. Consequently the investigations are focussed on foods and drinks containing antioxidants, such as vitamins, flavonoids and other natural antioxidants, which evidently can prevent diseases (Aruoma, 2003; Mira, Silva, Rocha, & Manso, 1999; Rice-Evans, Miller, & Paganga, 1996; Sanchez-Moreno, 2002; Shetty & McCue, 2003; Tiwari, 2004). In this context, wines, as radical scavengers, are also the subject of growing interest (Lopez-Velez, Martinez-Martinez, & Del Valle-Ribes, 2003; Lugasi & Hovari, 2003). Numerous papers have been published on red and white wines (Fernandéz-Pachón, Villaño, Garcia-Parrilla, & Troncoso, 2004; Fogliano, Verde, Randazzo, & Ritieni, 1999; Ho, Hogg, & Silva, 1999; Katalinić, Milos, Modun, Musić, & Boban, 2004; Kilmartin, Zou, & Waterhouse, 2001; Király-Véghely, Katay, Tyihak, & Merillon, 2004; Lopez-Velez et al., 2003; Lugasi & Hovari, 2003; Staško, Liptáková, Malík, & Mišík, 2002; Villaño, Pachón, Troncoso, & Garcia-Parrilla, 2004) but relatively few data are available on Tokay wines. According to published data (Drawert et al., 1976; Kovács & Dinya, 2000; Kovács, Dinya, & Antus, 2004; Miklósy & Kerényi, 2004; Murányi & Kovács, 2000; Schreier, Drawert, Kerényi, & Junker, 1976), the phenolic content of Tokay wines is

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in a range between red and white wines. No data are available on the radical-scavenging ability of Tokay wines.

In previous studies, we described the scavenging abilities of medicines, such as the neuro-protective stobadine (Mišík, Ondriaš, & Staško, 1999), and cardio-protective nifedipine (Ondriaš, Staško, & Gergel', 1997) and also the scavenging properties of beer (Brezová, Polovka, & Staško, 2002), tea (Polovka, Brezová, & Staško, 2003) and glucan derivatives (Kogan et al., 2005), as well as red and white wine samples (Staško et al., 2002) using the EPR spin trapping technique, a method very suitable for describing the radical-scavenging processes. So we used this technique here, and investigated the scavenging ability of Slovak Tokay wines and compared them with those of selected red and white wine samples originating from various sources.

2. Materials and methods

2.1. Materials

As a source of free radicals we used 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), as well as 2,2′-azo-bis(2-methylpropionamidine) hydrochloride (AAPH) from Polysciences, Inc.; and $K_2S_2O_8$ from Merck. 5,5-Dimethylpyrroline-*N*-oxide (DMPO), from Aldrich, served as the spin-trapping agent. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was obtained from Sigma. An overview of the investigated 10 red, 30 Tokay and 10 white wine samples is given in Table 1. A 12% (v/v) aqueous ethanol solution served as a reference allowing evaluation of the contribution of the wine components to the scavenging of free radicals.

2.2. Monitoring of radical-scavenging activity

To monitor the scavenging activity, an EMX EPR spectrometer (Bruker, Germany) was used, equipped with the rectangular (TE_{102}) or cylindrical TM-110 (ER 4103 TM) cavities working at a frequency in X-band region. A 50 μl boron capillary served as sample tube. Its reproducible position in the EPR cavity was maintained by a defined tight fitting with silicon rings in a wide EPR tube. Temperature control was achieved using a Bruker temperature control unit, ER 4111 VT.

The investigations were started by adding or generating reactive free radicals in the reference sample, which consisted of 12% ethanol in water, with a phosphate buffer (pH 7); a spin-trapping agent was added if unstable radicals were thermally generated. Then analogous experiments were carried out, replacing the 12% ethanol with wine samples.

In two series of experiments, free radicals were generated in the thermal decomposition of radical initiators, $K_2S_2O_8$ or the azo compound AAPH. The samples containing radical initiators were placed in the cavity of the EPR spectrometer and tempered at 60 °C. As the generated free radicals ($SO_4^{\bullet -}$ and $^{\bullet}OH$, originating from the $K_2S_2O_8$ initiator and carbon-centred radicals, originating from the azo compound) have a very short life time, their stationary concentrations are very low and cannot be detected directly; consequently the spin trap DMPO was used. Reactive free radicals $R^{\bullet}$ having short life time are added to the DMPO trapping agent, forming longer-lived radical adducts $^{\bullet}$DMPO-R, readily detectable by the EPR technique.

In two another series of experiments DPPH or $ABTS^{\bullet +}$, free radicals stable at room temperature, were used. After the addition of wine samples to the DPPH or $ABTS^{\bullet +}$ solution, the decrease of radical concentration was monitored in the EPR spectrometer as a result of the termination reaction between the free radicals and the antioxidants in the wine samples.

2.3. General procedures

Four types of radical sources were used: (i) *$K_2S_2O_8$*. Aqueous solutions of 25 μl 0.2 M DMPO, 12.5 μl 0.1 M phosphate buffer (pH 7), 25 μl 0.1 M $K_2S_2O_8$ and 100 μl of reference or wine sample were mixed, then transferred into a flat (4 mm wide) EPR cell and inserted into the EPR spectrometer. The temperature was raised to 60 °C and, simultaneously, the time course of EPR spectra was monitored for 10 min. (ii) *AAPH*. To aqueous solutions of 25 μl 0.2 M DMPO, 25 μl 0.1 M phosphate buffer (pH 7) and 25 μl 0.01 M AAPH, 25 μl of wine or reference were added, and a 50 μl boron capillary was filled with a corresponding amount of the mixture, placed in the EPR spectrometer at 60 °C, and the time course of the radicals formed was monitored. (iii) *$ABTS^{\bullet +}$*. $ABTS^{\bullet +}$ solution was prepared by mixing equal volumes of the original solution delivered by Sigma with 0.01 M $K_2S_2O_8$ in a buffer with pH 7 and this mixture was left to stand for 16 h in the dark at room temperature according to recommendation published by Re et al. (1999). The concentration of $ABTS^{\bullet +}$ was 50 μmol dm^{-3}, as determined by UV/vis spectroscopy, using the value of molar absorptivity at 735 of nm 1.5×10^4 mol^{-1} dm^3 cm^{-1} (Arts, Haenen, Voss, & Bast, 2004). Then 90 μl of this solution were added to 10 μl of wine probe diluted to 1:100 (v/v) with distilled water. A 50 μl boron capillary was filled with the so-prepared mixture and the time course of $ABTS^{\bullet +}$ cation radical concentration was monitored for 10 min. (iv) *DPPH*. A 10^{-4} M DPPH solution in ethanol was placed in a

Table 1
Investigated wine samples and their characteristics

Nr.	Sample	Year	Category	Region/country
Tokay wines				
1	Tokay, samorodné (szamorodne)	1977	Sweet	Malá Trna/Slovakia
2	Tokay table wine	2001	Dry	Malá Trna/Slovakia
3	Tokay 3 putnové (putyonos)	1979	Sweet	Sl. N. Mesto/Slovakia
4	Tokay samorodné	1999	Dry	Malá Trna/Slovakia
5	Tokay 3 putnové.	1995	Sweet	Velká Trna/Slovakia
6	Tokay samorodné	1989	Dry	Velká Trna/Slovakia
7	Tokay samorodné	1993	Sweet	Velká Trna/Slovakia
8	Tokay samorodné	1993	Sweet	Velká Trna/Slovakia
9	Tokay samorodné	1993	Sweet	Velká Trna/Slovakia
10	Tokay samorodné	1990	Dry	Vinicky/Slovakia
11	Tokay 5 putnové	1990	Sweet	Vinicky/Slovakia
12	Tokay samorodné	1997	Sweet	Malá Trna/Slovakia
13	Furmint BA (Beerenauslese)	2002	Sweet	Velká Trna/Slovakia
14	Muskat zlty BA	2002	Sweet	Velká Trna/Slovakia
15	Tokay samorodné	1997	Sweet	Malá Trna/Slovakia
16	Lipovina (Harslevelu)	1996	Semidry	Malá Trna/Slovakia
17	Tokay samorodné	1996	Sweet	Malá Trna/Slovakia
18	Tokay 2 putnové	1990	Sweet	Malá Trna/Slovakia
19	Tokay 3 putnové	1993	Sweet	MaláTrna/Slovakia
20	Tokay 3 putnové	1993	Sweet	Velká Trna/Slovakia
21	Tokay 3 putnové	1990	Sweet	Vinicky/Slovakia
22	Tokay 3 putnové	1990	Sweet	Malá Trna/Slovakia
23	Tokay 4 putnové	1995	Sweet	Malá Trna/Slovakia
24	Tokay 4 putnové	1990	Sweet	Malá Trna/Slovakia
25	Tokay 5 putnové	1996	Sweet	Malá Trna/Slovakia
26	Tokay 5 putnové	1993	Sweet	Velká Trna/Slovakia
27	Tokay 5 putnové	1990	Sweet	Vinicky/Slovakia
28	Tokay 5 putnové	1990	Sweet	Malá Trna/Slovakia
29	Tokay 5 putnové	1990	Sweet	Malá Trna/Slovakia
30	Tokay 6 putnové	1989	Sweet	Malá Trna/Slovakia
Red wines				
31	Alibernet	2003	Dry	Sv. Jur/Slovakia
32	André	2002	Dry	Vrbové/Slovakia
33	Cuveé (St. Laurent × Blaufränkisch)	2002	Dry	Topolcianky/Slovakia
34	Chevaux des Girondins	2002	Dry	Bordeaux/France
35	St. Laurent	2003	Dry	Pezinok/Slovakia
36	Cabernet Sauvignon	2000	Dry	Velká Trna/Slovakia
37	Cabernet Sauvignon	2002	Dry	Topolcianky/Slovakia
38	Egri Bikáver	2001	Dry	Hungary
39	Bulls Blood	2003	Semidry	Bulgaria
40	Wild cherry wine	2003	Sweet	Bratislava/Slovakia
White wines				
41	Grüner Veltliner	2002	Dry	Modra/Slovakia
42	Irsay Oliver	2001	Dry	Pezinok/Slovakia
43	Chevaux des Girondins	2001	Dry	Bordeaux/France
44	Müller Thurgau	2003	Dry	Sv. Jur/Slovakia
45	Müller Thurgau	2003	Dry	Pezinok/Slovakia
46	Muskat Moravsky	2002	Dry	Modra/Slovakia
47	Pinot Gris	2002	Dry	Modra/Slovakia
48	Mädchentraube	2002	Dry	Vrbové/Slovakia
49	Grüner Veltliner	2001	Dry	Vrable/Slovakia
50	Welschriesling	2003	Dry	Mikulov/Bohemia

1 ml syringe and, in another syringe, 1 part of 12% aqueous ethanol (reference), or 1 part of the wine sample (standard experiment) was diluted with 19 parts of pure ethanol (v/v). Both syringes were attached to a micromixing chamber and connected to a flat cell in the EPR spectrometer. After the simultaneous insertion of both solutions into the flat cell, the time course of DPPH EPR spectra was monitored for 10 min. Generally, the reproducibility of EPR measurements with individual samples was ±5%.

2.4. Statistics

The statistical analysis was carried out using an Origin (Microcal) programme. The parameters were evaluated as means and standard deviations at the 0.05 significance level. The evaluated averaged values obtained for red, Tokay and white wines were tested by one-way ANOVA analysis to confirm significant differences in the individual wine groups. Correlation between $DPPH_{eq}$ and TEAC values was calculated by linear regression and ANOVA analysis at the 95% confidence level.

3. Results and discussion

3.1. General

The results obtained are ordered according to the radical sources ($K_2S_2O_8$, AAPH, $ABTS^{\cdot+}$ and DPPH) used in the monitoring of the radical-scavenging activity. Table 2 and Fig. 3 summarise, in the first instance, the relative radical-scavenging activities for the individual groups of wines and initiators with their statistical characteristics, giving standard deviation for a 95% confidence interval. The relatively high deviations from the averaged values found do not represent uncertainties in the measurements, which are relatively low (±5%), but rather the various properties of the individual wine samples. In order to better survey the data obtained, the average scavenging values for the red wines (which are the highest) are set to one (or 100%) and relatively to them are listed the data of Tokay and white wines. Then in the final part the results obtained with $ABTS^{\cdot+}$ are evaluated in trolox equivalent antioxidant capacities (TEAC) and also the results obtained with DPPH are expressed in mmol DPPH equivalents per dm^3 of wine sample.

3.2. $K_2S_2O_8$ radical initiator

A representative time course of EPR spectra from nine selected samples, observed using thermally decomposed $K_2S_2O_8$ radical initiator in the presence of DMPO, is shown in Fig. 1. Experiments were started with the reference sample (Fig. 1(a)) containing 12% ethanol aqueous solution, buffer, DMPO and $K_2S_2O_8$ as specified above. Here, the maximal amplitudes of EPR spectra were found. Replacing the ethanol solution with the white wine samples, the amplitudes of EPR spectra decreased considerably (Fig. 1(b) and (c)). The amplitudes on average were still lower if Tokay wines were used, as illustrated in Fig. 1(d)–(f). The lowest amplitudes, indicating the maximal scavenging activity, were found in the red wine samples, as shown in Fig. 1(g)–(i).

The scavenging activity from the experimental spectra thus obtained was evaluated using the following procedure: the EPR spectra obtained were double-integrated and their time dependence, for four selected representative samples: reference, white, Tokay and red wines, are presented for illustration in Fig. 2. The double integrals of the reference sample (12% ethanol) in Fig. 2 have maximal values, as only ethanol and no further additional natural antioxidants are present in the sample. With the white wine sample (Fig. 2(b)) lower integral values were found, since part of the generated free radicals was scavenged by the antioxidants present in the wine sample. The difference between the double integrals of the reference and the white wine samples characterises the amount of radicals scavenged (RS_{white}) by the antioxidants present in the white wine sample. Applying a similar procedure to Tokay and red wine samples the relative amounts of radical scavenged by Tokay (RS_{Tokay}) and by red wine samples (RS_{red}) were obtained, as shown schematically in Fig. 2. The average value of RS_{red} was set to one (100%) and then the corresponding values of RS_{rel} of individual wine samples were evaluated.

The relative double integrals of the radicals scavenged by the investigated samples, using $K_2S_2O_8$ radical initiator, are summarised in Fig. 3(a). On average, the red wines show the highest value (1.00 ± 0.12) and therewith the highest scavenging activities. Tokay wines have a comparable scavenging activity (0.86 ± 0.15), and on average, the white wine samples show the lowest radical scavenging activities (0.60 ± 0.15).

The inset in Fig. 3(a) shows a characteristic EPR spectrum of DMPO spin adducts, observed using $K_2S_2O_8$ initiator. A quartet (marked with solid circles), characteristic of ·DMPO-OH adduct, dominates there. As a minor by-product, evidenced with relatively low amplitude, there is a sextet characteristic of carbon-centred radicals added to DMPO. Additional details

Table 2
An overview of the average relative values of the scavenging activities found for the red, Tokay and white wines using radical sources DPPH, $ABTS^{\cdot+}$, AAPH, $K_2S_2O_8$, along with their average pH values, relative Mn^{2+} ions and Na_2SO_3 concentrations

Wine	DPPH	$ABTS^{\cdot+}$	AAPH	$K_2S_2O_8$	pH	Mn^{2+}	Na_2SO_3
Red	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.12	3.45 ± 0.20	1.00 ± 0.21	1.00 ± 0.75
Tokay	0.43 ± 0.13	0.55 ± 0.23	0.71 ± 0.16	0.86 ± 0.15	3.21 ± 0.19	0.89 ± 0.50	0.36 ± 0.34
White	0.25 ± 0.07	0.22 ± 0.11	0.37 ± 0.19	0.60 ± 0.15	3.23 ± 0.19	0.95 ± 0.39	1.28 ± 0.92

(The listed deviations do not represent uncertainty in the measurements, but rather disperse properties of the individual wine samples).

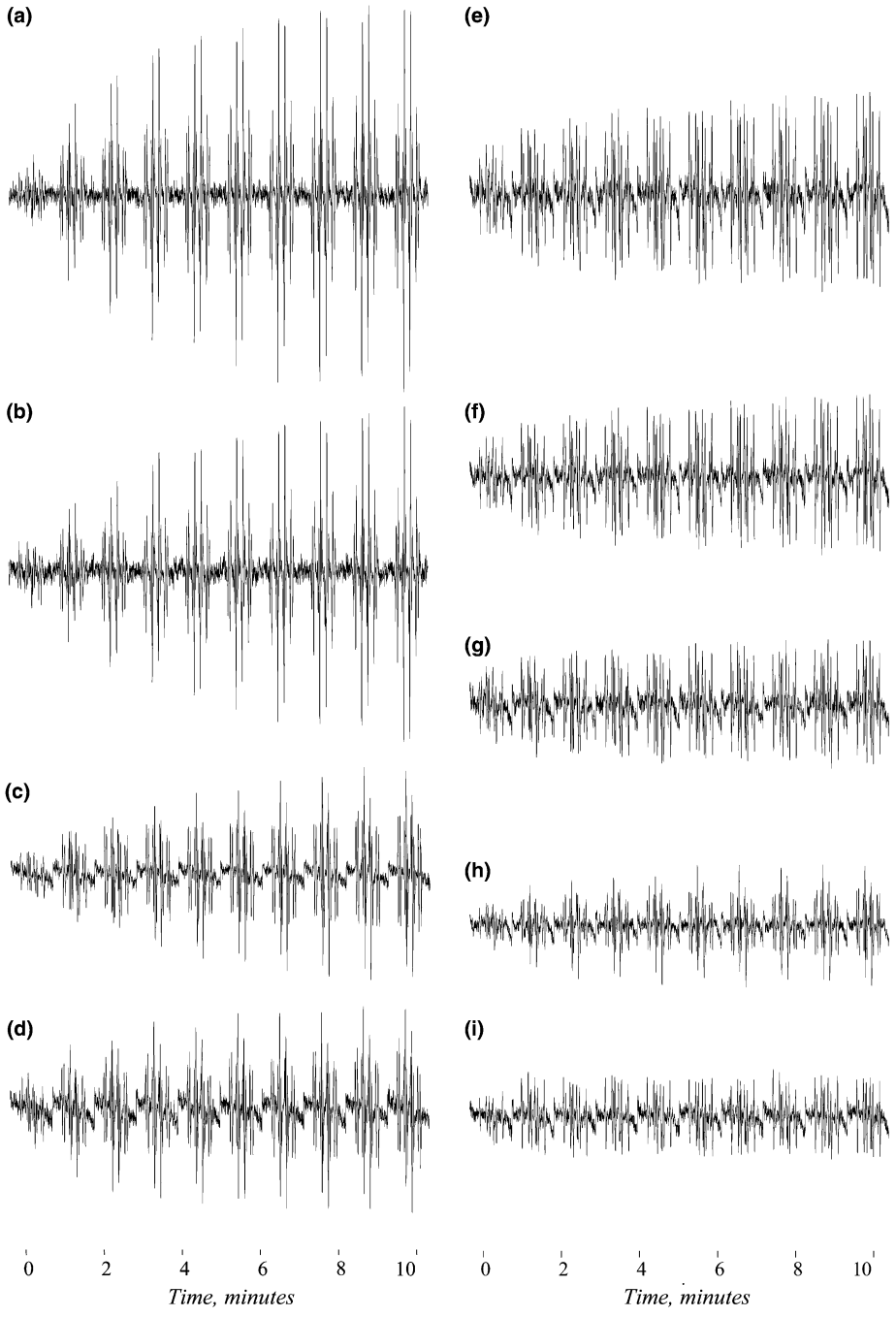

Fig. 1. The time course of EPR spectra from control (a), white (b and c), Tokay (d–f) and red (g–i) wine samples monitored for 10 min at 60 °C in the presence of $K_2S_2O_8$ radical initiator, DMPO spin trap and phosphate buffer (pH 7). The sweep width of the spectra is 10 mT.

on the mechanism of thermal $K_2S_2O_8$ decomposition were described elsewhere (Staško, Brezová, Liptáková, & Šavel, 2000). The DMPO-adduct of the primary $SO_4^{\bullet-}$ radical formed is very unstable (half life of 21 s (Kirino, Ohkuma, & Kwan, 1981)). Additionally, radical $SO_4^{\bullet-}$ is also rapidly terminated with water or wine antioxidants, or, in addition, it can also oxidise DMPO to its cation radical $DMPO^{\bullet+}$ (Chamulitrat, 1999; Eberson, Hartshorn, & Persson, 1997), which hydrolyses in aquatic media, forming ·DMPO-OH adduct (Chignell, Motten, Sik, Parker, & Reszka, 1994) (Eq. (1)). Its EPR spectrum, with characteristic quartet (inset Fig. 3(a)), prevails in all experiments.

$$DMPO^{\bullet+} + H_2O \rightarrow {}^{\bullet}DMPO-OH + H^+ \quad (1)$$

3.3. Free radical generated from azo compound AAPH

Azo compounds (R—N=N—R) easily cleave thermally to form radicals R· (Eq. (2)):

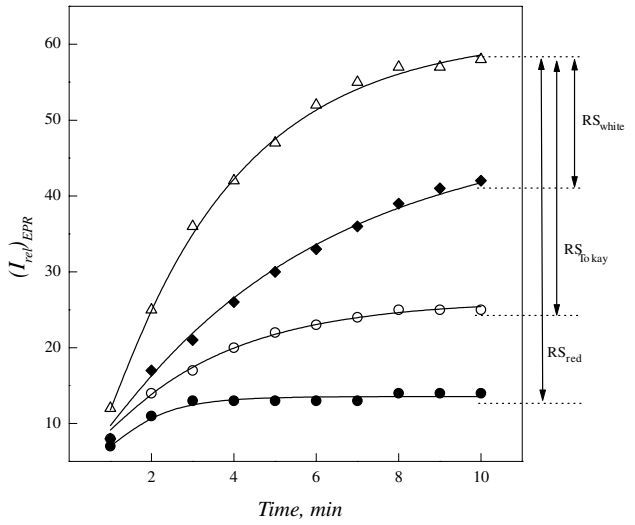

Fig. 2. Scheme employed in the evaluation of relative amounts of radicals scavenged (RS_{rel}). The integral values of EPR spectra of red (●), Tokay (○) and white (◆) wines were subtracted from the integral values of the reference sample (△).

$$R{-}N{=}N{-}R \rightarrow 2R^{\bullet} + N_2 \quad (2)$$

The azo compound AAPH was used in analogous experiments, as described with $K_2S_2O_8$ radical initiator. The generated carbon centred radicals $R^{\bullet}$ are rapidly scavenged by oxygen (Neta, Grodkowski, & Ross, 1996), primary to form peroxyl radicals $ROO^{\bullet}$ (Eq. (3)), or terminated with antioxidants AH present in the wine samples (Eq. (4)), eventually trapped to DMPO (Eq. (5)). A further reaction route of peroxyl radicals is very complex. They may form alkoxy radicals, $RO^{\bullet}$, via tetroxide intermediates, splitting them to $RO^{\bullet}$ and O_2 (Eq. (6)), or terminate to hydroperoxide (Eq. (7)), which can then decompose with transition metals or via other activation to $RO^{\bullet}$ and $^{\bullet}OH$ radicals (Eq. (8)). Various routes are discussed in the literature (Dikalov & Mason, 1999; Dikalov & Mason, 2001; Guo, Qian, & Mason, 2003).

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (3)$$

$$R^{\bullet} + AH \rightarrow {}^{\bullet}A + RH \quad (4)$$

$$DMPO + R^{\bullet} \rightarrow {}^{\bullet}DMPO{-}R \quad (5)$$

$$2ROO^{\bullet} \rightarrow 2RO^{\bullet} + O_2 \quad (6)$$

$$ROO^{\bullet} + HA \rightarrow ROOH + A^{\bullet} \quad (7)$$

$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH \quad (8)$$

Finally, in our experiments we observed carbon ($R^{\bullet}$) and oxygen-centred ($RO^{\bullet}$, $HO^{\bullet}$) radicals added to DMPO (Eq. (9)):

$$(R^{\bullet}, RO^{\bullet}, HO^{\bullet}) + DMPO \rightarrow {}^{\bullet}DMPO(-R, -OR, -OH) \quad (9)$$

A characteristic EPR spectrum, observed in the experiments with AAPH, is shown in the inset of Fig. 3(b). Two radicals are seen there – carbon-centred (marked with open circles) and oxygen-centred (marked with solid circles). The time course of EPR spectra, monitored during the thermal decomposition of AAPH, was similar to those presented in Fig. 1 using $K_2S_2O_8$ initiator. After evaluating the total double-integrals of the wine samples and subtracting them from the reference (in the same way as described with $K_2S_2O_8$), we obtained the relative scavenging activities (RS_{rel}), as depicted in Fig. 3(b). Here again, the average scavenging ability of red wines is the highest (1.00 ± 0.11), followed by Tokay wines (0.71 ± 0.16), and the white wines showed the lowest relative scavenging capacity (0.37 ± 0.19).

3.4. $ABTS^{\bullet+}$

Whereas in experiments with $K_2S_2O_8$ and AAPH the reactive free radicals were generated through their thermal decomposition, ABTS, after oxidation with persulfate, forms its cation radical stable at room temperature (Re et al., 1999). In the literature, this radical source is increasingly treated as a standard to characterise the antioxidant activity of various systems (Aruoma, 2003; Bartosz, Janaszewska, Ertel, & Bartosz, 1998; Butkovic, Klasinc, & Bors, 2004; Geletii, Balavoine, Efimov, & Kulikova, 2002; Henn & Stehle, 1998; Kefalas, Kallithraka, Parejo, & Makris, 2003; Lee, Kim, Lee, & Lee, 2003; Makris, Psarra, Kallithraka, & Kefalas, 2003; Minussi et al., 2003; Perez, Leighton, Aspee, Aliaga, & Lissi, 2000; Villaño et al., 2004). After mixing $ABTS^{\bullet+}$ solution with 12% aqueous ethanol (serving as reference) or with a wine sample, as described in the experimental part, the time course of EPR spectra was monitored for 10 min in the same way as already demonstrated in Fig. 1. Substracting the spectral integrals of wine samples from that of the reference, the relative value of radicals scavenged (RS_{rel}) was evaluated. Fig. 3(c) shows an overview with the relative activities for red (1.00 ± 0.11), Tokay (0.55 ± 0.23) and white wines (0.22 ± 0.11), confirming the trends demonstrated in Fig. 3(a) and (b).

3.5. DPPH

Analogously to $ABTS^{\bullet+}$, we expanded our investigations of wines, taking a further type of stable free radical, namely DPPH.

For the termination of DPPH radicals, various routes are discussed in the literature (Ancerewicz et al., 1998; Polovka et al., 2003; Ukeda, Adachi, & Sawamura, 2002; Yordanov, 1996). Basically, DPPH is reduced by electron transfer from the antioxidant AH according to reactions (Eqs. (10) and (11)) or terminated according to (Eq. (12)):

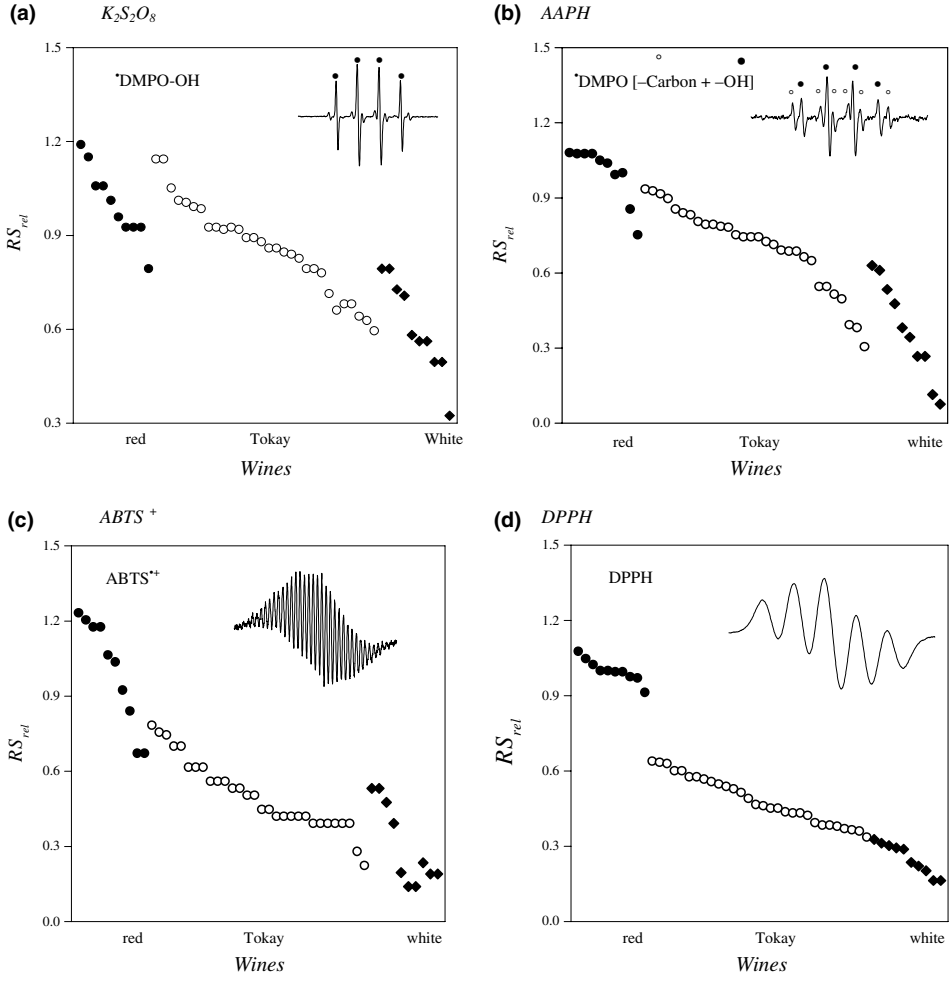

Fig. 3. The relative amounts of radicals scavenged (RS_{rel}) in experiments with (a) $K_2S_2O_8$, (b) AAPH radical initiators and with (c) $ABTS^{\bullet+}$ and (d) DPPH free radicals in the investigations of red (●), Tokay (○) and white (◆) wine samples. Insets represent characteristic EPR spectra of radicals observed.

$$AH \leftrightarrow A^- + H^+ \quad (10)$$

$$A^- + {}^{\bullet}DPPH \rightarrow A^{\bullet} + {}^{-}DPPH \quad (11)$$

$${}^{\bullet}DPPH + HA \rightarrow A^{\bullet} + H-DPPH \quad (12)$$

In this way an EPR-silent DPPH product is formed. The radicals $A^{\bullet}$ originating from the antioxidant HA are seldom seen in the EPR spectra, as they may be trapped or undergo further consecutive reactions to form EPR-silent products (Ancerewicz et al., 1998).

After mixing DPPH solution with 12% aqueous ethanol (reference) or wine samples, (standard experiment), the EPR spectra were monitored for 10 minutes and the amount of radical scavenged was evaluated as presented in Fig. 3(d) with relative scavenging activity 1.00 ± 0.05 for reds, 0.43 ± 0.13 Tokay and 0.25 ± 0.07 for white wines.

Comparing the relative amounts of radicals scavenged, summarised in Fig. 3, it is evident that, using all four radical sources, the highest relative scavenging activity was found for the red wines, followed by Tokay and then the white wines.

3.6. Mn^{2+} and Na_2SO_3 concentrations

3.6.1. General

From EPR spectra, measured under special conditions, it was also possible to evaluate some further data, such as relative Mn^{2+} and sulphite SO_3^{2-} ion concentrations. Also, the pH values of samples were determined.

3.6.2. Mn^{2+}

The measurements of Mn^{2+} concentrations are presented in Fig. 4. The inset in Fig. 4 shows the characteristic EPR spectrum of Mn^{2+} measured in a wide magnetic field with a sweep width of 100 mT and a central field of 340 mT, with a gain of 10^6 and a modulation amplitude of 0.5 mT. The spectrum shown represents an

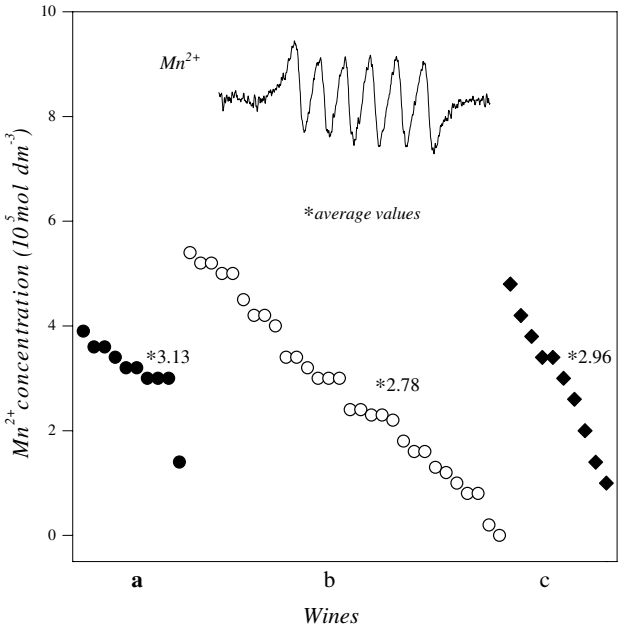

Fig. 4. Concentrations of Mn^{2+} ions evaluated from EPR spectra (inset) observed in red (a, •), Tokay (b, ○) and white wine (c, ♦) samples. Wine groups average values in 10^{-5} mol dm^{-3} $c_{red} = 3.13$, $c_{Tokay} = 2.78$ and $c_{white} = 2.96$.

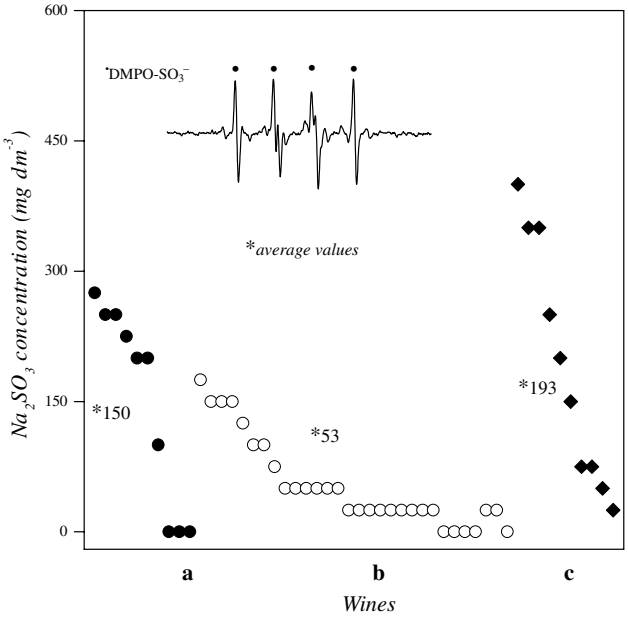

Fig. 5. Concentration of Na_2SO_3 evaluated according to spin-trapping measurements in red (a, •), Tokay (b, ○) and white (c, ♦) wine samples. Wine groups average values in mg dm^{-3} $c_{red} = 150$, $c_{Tokay} = 53$ and $c_{white} = 193$.

accumulation of 3 scans. In our previous paper (Staško et al., 2002), taking a limited number of samples from the Bratislava region, we reported on higher Mn^{2+} concentrations found in the reds and on a lower one in the white wines, correlated with their scavenging activity (a higher one by the reds and a lower one by the white wines). Taking into account here a wide range of samples from various regions, there is no a significant difference in Mn^{2+} concentrations between the red, Tokay and white wines with relative ratio $r{:}T{:}w = (1.00 \pm 0.21){:}(0.89 \pm 0.50){:}(0.95 \pm 0.39)$. The concentration range corresponds to $(0.5\text{–}6) \times 10^{-5}$ mol dm^{-3} Mn^{2+} solutions, as illustrated in Fig. 4.

3.6.3. Na_2SO_3

In the experiments using $K_2S_2O_8$ radical initiator, already at room temperature the $K_2S_2O_8$ initiator oxidises SO_3^{2-} anions present in wine samples to $SO_3^{\bullet-}$ anion radicals, which are then trapped by DMPO. The EPR spectrum of this adduct, monitored at room temperature is shown in the inset of Fig. 5, along with the corresponding Na_2SO_3 concentrations for the individual samples. They were evaluated by calibrating the spectral integrals of $^{\bullet}DMPO-SO_3^-$ adducts monitored with wine samples with those obtained in analogous experiments with Na_2SO_3 solutions in the presence of $K_2S_2O_8$. According to these results, the red and white wines show, on average, comparable relative concentrations of sulphite radicals added to DMPO: 1.00 ± 0.75 for red, and 1.28 ± 0.92 for white wines. Evidently, the content of sulphite ions in Tokay wines 0.36 ± 0.34 is lower. A significant correlation between the indicated sulphite ion concentration and the scavenging activity is not evident. In further experiments, definite amounts of sulphite were added to the various wine samples. The scavenging activity was not proportional to the growing Na_2SO_3 concentration and varied in different samples. So it was not possible to assign a definite contribution of Na_2SO_3 to wine samples in the scavenging process.

3.7. Relative and absolute evaluation of the scavenging activity

3.7.1. Relative values

As the determination of absolute scavenging activity in $K_2S_2O_8$, and AAPH initiator systems encountered some difficulties and, in order to better survey of the scavenging activity obtained with all four radical initiators, we first chose a relative comparison, setting the scavenging values of red wines (which are the highest) to one and, relatively to them, recording the scavenging activities of Tokay and white wines. The average values so obtained are summarised in Table 2, along with the standard deviations for a 95% confidence interval. The relatively high standard deviations from the averaged values do not result from uncertainty in the measurements, but originate, rather, from the various properties of the individual wine samples. Generally, it is evident that the Tokay wines show a relatively high scavenging ability if compared to the red wines, from 0.43 using DPPH up to 0.86 with $K_2S_2O_8$ radical initiator, and

the white wines a relatively lower one, from 0.25 to 0.60. The one-way ANOVA analysis confirmed, at the 0.05 significance level, the significant differences of averaged values of relative radical-scavenging activities evaluated using DPPH, $ABTS^{\bullet+}$, $K_2S_2O_8$ and AAPH assays for red, Tokay and white wine groups as summarised in Table 2.

The increased scavenging activity from DPPH towards $K_2S_2O_8$ radical sources correlates well with their rising redox potentials, with approximate values vs. NHE: $DPPH^{\bullet}/DPPH^{-} = 0.43$ V (Zhuang, Scholz, & Pragst, 1999), $ABTS^{\bullet+}/ABTS = 0.68$ V (Scott, Chen, Bakac, & Espenson, 1993), AAPH-($ROO^{\bullet}$, $RO^{\bullet}$, $OH^{\bullet}$) = 1.0–2.3 V (Buettner, 1993; Wardman, 1989), $SO_4^{\bullet-}/SO_4^{2-} = 2.5$ to 3.1 V (Neta, Huie, & Ross, 1988; Wardman, 1989). Thus, in the radical source $K_2S_2O_8$, very reactive radicals are generated as $SO_4^{\bullet-}$ and $^{\bullet}OH$ and in AAPH-reactive carbon-centred and secondary $ROO^{\bullet}$, $RO^{\bullet}$ and $^{\bullet}OH$ radicals. Therefore, the relative amounts of radicals scavenged in Tokay and white wines, using $K_2S_2O_8$ and AAPH, are higher than in $ABTS^{\bullet+}$ and DPPH, possessing lower reactive $ABTS^{\bullet+}$ and DPPH radicals. This probably results from the fact that very reactive radicals, originating from $K_2S_2O_8$ and AAPH also oxidise the antioxidants with higher oxidation potentials, which is not the case for $ABTS^{\bullet+}$ and DDPH radical sources representing less-reactive radicals. These trends are also promoted with antioxidants of low oxidation potential present in the wines. The amount is generally highest in red wines, lower in the Tokay and lowest in white wines (Király-Véghely, Tyihák, Albert, Németh, & Kátay, 1998). An analysis and discussion of this phenomenon is beyond the scope of the present investigations and the data obtained, but the mean values found for red, Tokay and white wines can be considered as representative trends.

3.7.2. Absolute scavenging values

A straightforward evaluation of the absolute scavenging capacity was possible in the case of $ABTS^{\bullet+}$ initiator. The absolute $ABTS^{\bullet+}$ concentrations used in EPR experiments were determined from UV/vis measurements (50 μmol dm^{-3}). By monitoring the scavenging in EPR experiments, as described above, from the obtained relative scavenging values, the absolute concentrations changes were evaluated. Considering the equivalent ratio, trolox:$ABTS^{\bullet+}$ = 1:2 (Arts et al., 2004), and a closely similar value was found in our experiment here with $ABTS^{\bullet+}$, the scavenging capacities of the red, Tokay and white wines were expressed in TEAC (mmol/dm^3 wine). The corresponding mean values are summarised in Table 3 along with the standard deviations (14.8 ± 1.5 for red, 8.1 ± 3.4 Tokay and 3.3 ± 1.6 for white wines). Using one-way ANOVA analysis with $\alpha = 0.05$, it was confirmed that the mean values for red, Tokay and white wines were significantly different. The so obtained scavenging capacities correspond well to those published in the literature (Lugasi & Hovari, 2003).

Table 3
An overview of the average values of the radical-scavenging activities found for the red, Tokay and white wine samples evaluated in TEAC and $DPPH_{eq}$ equivalents (mmol dm^{-3} of wine)

Wine	TEAC (mmol dm^{-3})	$DPPH_{eq}$ (mmol dm^{-3})
Red	14.8 ± 1.5	1.80 ± 0.09
Tokay	8.1 ± 3.4	0.77 ± 0.24
White	3.3 ± 1.6	0.45 ± 0.12

Similarly, the evaluation of DPPH equivalent ($DPPH_{eq}$ expressed in mmol/dm^3 wine) was achieved by the known concentrations used in the reference experiment and relative changes monitored with EPR during the scavenging. The values of $DPPH_{eq}$ are 1.8 ± 0.09 for reds, 0.77 ± 0.24 for Tokay and 0.45 ± 0.12 for white wines. They seem to be relatively low. So we sought for evidence of how far they correlated with $ABTS^{\bullet+}$ experiments. Fig. 6 shows the TEAC values found by $ABTS^{\bullet+}$ assay, based the obtained $DPPH_{eq}$ for all 50 wine samples. The output of linear fitting is characterised with the equation $TEAC = 1.2 + 7.9 \times DPPH_{eq}$ (in mmol dm^{-3}) and represents a statistically significant ($P < 0.01$) linear relationship between TEAC and $DPPH_{eq}$ at the 95% confidence level. The correlation coefficient of 0.862 indicates a moderately strong relationship between the variables.

To get more specific information on the relationship between TEAC and $DPPH_{eq}$, under analogous

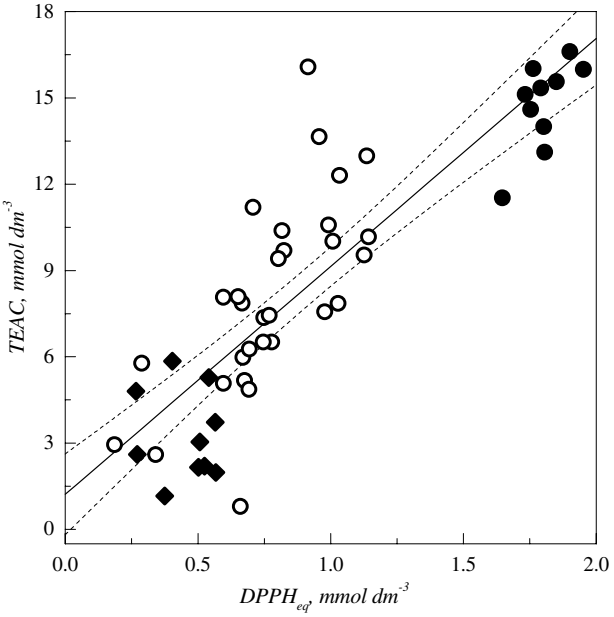

Fig. 6. The linear relationship between TEAC and $DPPH_{eq}$ at 95% confidence level (confidence bands marked with dotted lines) determined for 50 wine samples (10 red (•), 30 Tokay (○) and 10 white (♦)).

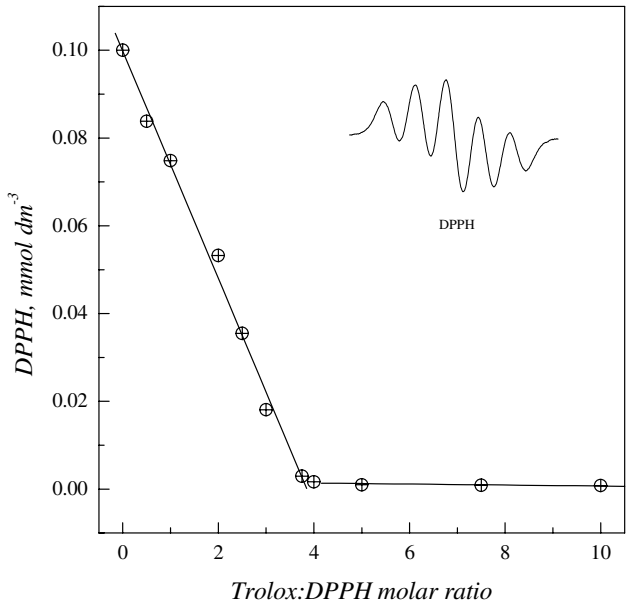

Fig. 7. The concentration decrease of DPPH upon increasing molar ratio trolox:DPPH monitored for 10 min after mixing of DPPH and trolox solutions in ethanol. Inset represents the monitored EPR spectrum of DPPH.

conditions to those described in EPR experiments, we exchanged the wine sample with the variable trolox concentrations and followed the DPPH concentration changes with increasing trolox concentrations. The results are summarised in Fig. 7, where DPPH concentrations measured 10 min after mixing the ethanol solutions of trolox and DPPH, are quoted, based upon the trolox:DPPH molar ratio. The observed concentration changes correspond well to a titration curve with a sharp equivalent point at the molar ratio trolox:DPPH = 4:1. This indicates more complex consecutive reactions of DPPH with antioxidants (phenoxyl radicals) as already considered by Ancerewicz et al. (1998).

To evaluate the absolute scavenging values in the systems with $K_2S_2O_8$ and APPH initiators, we also carried out analogous experiments, as above, with DPPH. To their reference samples, increasing trolox concentrations were added and the corresponding changes were monitored by EPR. The evaluated trolox:$K_2S_2O_8$ or trolox:AAPH ratios were here in the region of about 1:50, indicating that, probably, the absolute values in these initiator systems are strongly modified by the DMPO radical-scavenger necessary in the samples to monitor the scavenging process. But, however, the relative scavenging values obtained with these initiator systems accorded relatively well the trends in the scavenging capacity between the red, Tokay and white wines, analogously as found with $ABTS^{\bullet+}$ and DPPH initiators. The evaluation of the absolute equivalent values, relative to trolox, in $K_2S_2O_8$ and AAPH initiator systems containing the spin trapping agent, remains open.

4. Conclusions

Generally, all scavenging experiments, using various radical initiators, showed relatively high scavenging ability to Tokay wines, with 0.43 ± 0.13 in DPPH up to 0.86 ± 0.15 in $K_2S_2O_8$ initiators (this relatively to the red wine averages). The white wines have the lowest activity with 0.25 ± 0.07 in DPPH and up to to 0.60 ± 0.15 in $K_2S_2O_8$ relative to the red wines. In trolox equivalents (mmol/dm^3 wine) the following TEAC values were found: 14.8 ± 1.5 for red, 8.09 ± 3.4 Tokay and 3.3 ± 1.6 for white wines. Concerning other parameters, the red wines have slightly higher pH values (3.45 ± 0.20) than the Tokay (3.21 ± 0.19) and white (3.23 ± 0.19) wines. There is no significant difference evident in the relative contents of Mn^{2+} ions in the red (1.0 ± 0.21) Tokay (0.89 ± 0.50) and white (0.95 ± 0.39) wines. The relative content of sulphites in the Tokay wines is lower (0.36 ± 0.34) than in the red (1.0 ± 0.75) and white (1.28 ± 0.92) wines.

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