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Antioxidant scavenging capacity of Bulgarian red wines by chemiluminescent and stable free radical methods

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The evaluation of the relationship between red wine consumption and its possible beneficial effect has been a subject of scientific interest from decades. Most of the performed investigations comprised evaluation of the influence of factors as wine making technology, used varieties, winery vineyard localization on the antioxidant properties of the product. On the base of these data authors made recommendations associated with the product qualities in response to health concerns.

The aim of the present investigation was to compare the observed antiradical capacity of red wines (variety Merlot and Cabernet) measured by chemiluminescent and spectrophotometric methods. We determined their capability to decrease the concentration of biologically important ROS – superoxide anion radical and hypochlorite. The obtained data were correlated to their scavenging properties against stable free radicals (ABTS and DPPH).

The tested wines have demonstrated antiradical effect in all studied systems. The results from the chemiluminescent assays proved better effectiveness in the hypochlorite elimination compared to the superoxide anion for all wine samples. Highest correlation has been observed between the extent of scavenging activity against both biologically relevant ROS – wine samples possessing better effectiveness against OCl^- had better capability to eliminate $O_2^{\bullet -}$. The correlations concerning comparison of data obtained only using stable free radical systems or ROS and stable free radical were lower.

On this base we suggest that for characterization of the antioxidant potency in response to health concerns for complex samples it is not needless to use also model systems evaluating capability to eliminate different types of biologically important ROS. This is necessary due to the variety of possible antiradical mechanisms of action of the sample components.

Keywords: Chemiluminescence, ROS, wine, antioxidants

INTRODUCTION

In the recent decades has been observed increase in the customers' interest in healthy nutrition habits and life style. This was accompanied by growing requirements to the quality and safety of food and beverages in the European Union Member States and intensification of the studies concerning evaluation of the potential benefits of their main components including antioxidants. One of the most commonly cited in the literature food bio-antioxidant are flavonoids – anthocyanins, catehins, proanthocyanidins, flavanols, phenolic acid and etc. These compounds have been found in highest concentration in the juices and wines made of blueberries, raspberries, black berries and red grape.

The high antioxidant activity of wines, especially of red wines, has been proven by many authors [1, 2]. The reason for this is the high concentration of phenolic compounds – from 2,487 to 4,176 g/dm^3. Red wines are a significant and famous natural source of polyphenols and contribute to the intake of more than 1g per day if moderately consumed [3].

The results from the performed tests evaluating flavonoids reactivity in different model systems show that their antioxidant properties are due to their ability to reduce the concentration of various reactive oxygen species (ROS) as well as to chelate iron [4, 5]. Quercetin and rutin are known to affect the level of lipid peroxidation by scavenging superoxide radicals, suppressing radical generation, iron chelation and by reacting with the produced lipid radicals tackling this way the early stages of lipid peroxidation chain reaction [6]. The anthocyanins have demonstrated capability to suppress the lipid peroxidation processes by inhibiting the auto-oxidation of the linoleic acid and denote themselves as good scavengers of the superoxide radical [7]. There is evidence pointing the cumarin compounds as scavengers of superoxide anions radical and hypochlorite acid [8]. Caffeic acid, gallic acid, ferulic acid etc. have demonstrated capability to decrease the concentration of $O_2^{\bullet -}$ and to inhibit NADPH-oxidase [9].

A quantitative and qualitative analysis of these substances with proven biological effect is made using various modern physical and chemical methods like: spectrophotometric assays, colorimetric methodologies, pH-differential spectrophotometry, HPLC, fluorescent spectroscopy. They have proven themselves as fast, non-destructive and economical methods for qualitative determination and offering opportunity to develop sensor systems on their base for express assessment of the food storage period and evaluation

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of this relationship with various indicators that characterize food quality [10].

The aim of the present investigation was to compare the observed antiradical capacity of red wine samples measured using two groups of analytical methods (chemiluminescent and spectrophotometric) and to compare their effectiveness against stable free radicals and biologically important ROS.

EXPERIMENTAL

The analysis was carried out using commercially available Bulgarian red wines produced in the time interval 2013 - 2015. All samples were supplied from a winery vineyard situated in the Thracian Plain. The principal used grape variety were Cabernet or Merlot which are famous with their good flavor profile among the customers.

For the evaluation of the antiradical properties of the studied wine samples have been chosen spectrophotometric and chemiluminescent model systems.

Spectrophotometric assays – the spectrophotometric assay comprises the estimation of the studied samples capability to eliminate stable free radicals (ABTS and DPPH). We accomplished the ABTS assay according to Re et al. [11], and the DPPH experiments were performed as described by Groupy et al., [12]. For each of both assays we have prepared fresh radical solution and two groups of samples – control samples where the wine product has been omitted and samples containing different aliquots of the tested wines. The control samples for the ABTS method had absorbance respectively 0.70 ± 0.01 units at 734 nm and for the DPPH absorbance of 0.9 at 518 nm. On the base of the absorbance value for the controls and the wine containing samples was estimated the antioxidant activity by relating the difference in the absorbance measured after 1 h incubation of the control and the wine containing samples to the one of the control as percentage. All the measurements were performed in triplets and the obtained data were presented as means ± SD. Using the calculated results for AOA, the volume wine product reducing the AOA by 50% was calculated – V50. V50 was used as a measure for comparing the antiradical effect of the wine samples - smaller the V50 corresponds to higher antiradical potential of the tested wine.

Chemiluminescent assays – for the chemiluminescent experiments was used LKB 1251 luminometer set at 37°C. The obtained chemiluminescent response (CL) is proportional to the curve area integral of the chemiluminescent curves presented on figure 1a and 2a - it is being calculated on the base of the chemiluminescence signal - time dependences. The presence of substance with radical scavenging properties in the systems will decrease the amount of the radicals and respectively the CL. The ratio of CL in the presence and in the absence of the tested substance was termed CL scavenging index (CL-SI). It was used as indicator for the measured wine samples scavenging properties in both systems. Lower CL-SI corresponds to higher antiradical activity.

For the chemiluminescent methodologies have been chosen assays comprising determination of the wine sample capability to diminish the concentration of biologically important ROS like the superoxide anion radical and the hypochlorite.

Luminol-dependent CL in a system of KO_2 generated superoxide anion radical – the tests were performed using 1 ml samples of phosphate buffer (PB) (50 mM K_2HPO_4/KH_2PO_4, pH 7.4) containing 0.1 mM luminol, and the tested wine samples. In the control samples wine product was omitted. Due to the fast release of superoxide the chemiluminescent response was measured immediately after the addition of 20 μl KO_2 solution dissolved in DMSO. The CL signal was registered for 1 min every 50 milliseconds after the addition of KO_2.

Luminol-dependent CL in a system of NaOCl produced hypochlorite – 1 ml samples PB buffer containing 0.1 mM luminol, 0,06 mM NaOCl and the tested wines (or buffer for the control sample) were prepared. The CL signal was registered for 1 min every 50 milliseconds after the addition of NaOCl.

RESULTS AND DISCUSSION

The evaluation of the antiradical properties of food, wines and beverages in respect to life concerns has always been a subject of interest. In our study have included samples comprising varieties Merlot or Cabernet. They all have demonstrated antiradical effect in all the used systems. The extent of the observed properties depended on both the used method for registration of the scavenging activity and the type of the radical against which their potency were evaluated.

The first step of our experimental work comprised evaluation of the wine potency to decrease the concentration of ROS in chemiluminescent model systems. We chose this method for registration of the antiradical effect of the studied samples due to its' sensitivity, the fact that it has proven itself as reliable, the facts it is being widely used for both pure substances and multicomponent systems and especially because it gives the possibility to estimate the studied properties and effectiveness in the exact moment of the ROS generation. For our experiments we chose enhanced chemiluminescence. The use of various types of luminophores including luminol, isoluminol

or lucigenin in order to enhance the CL activity is necessary due to the fact that the conventional form of the method is characterized by short luminescence tie, low intensity and weak signal. For our experiments we have chosen to use luminol-enhansed chemiluminescence and this gave us the possibility to evaluate the capability of the studied samples to interact even with the very low biologically relevant concentration of the ROS. Due to its lack of selectivity luminol can react with all the generated in the sample ROS, which is important in view of obtaining precise sufficient information for making accurate assumption on the base of the obtained data and also gives us the possibility to compare the results from the different model systems containing different reactive oxygen species [13-15].

In the first system we tested the capability of the samples to influence the CL signal in superoxide anion radical containing system. The scientific interest to the superoxide anion radical despite its low reactivity is due to the fact it is participating in reaction associated with the generation of more harmful types of ROS. It is being generated in relatively high amount by phagocytes (0.5 mM/sec) and in the inner mitochondrial membrane electron transport chain and most importantly given the fact we are determining antiradical properties of wine samples – in several investigations has been claimed that the favorable effect of flavonoids on the myocardium cells are at least partly due to their capability to scavenge $O_2^{\bullet -}$.

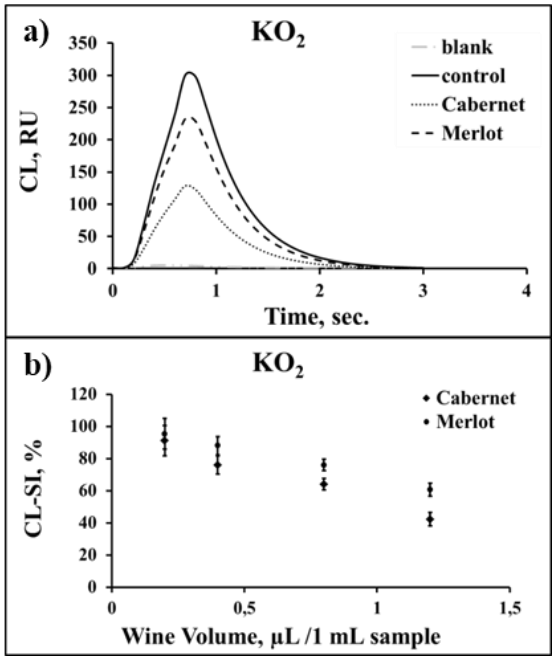

Fig. 1. Reduction of luminol-dependent chemiluminescent response in model system of KO_2 generated superoxide anion radical: (a) typical chemiluminescent curves - X-axis, time of analysis - sec; Y-axis, intensity of chemiluminescence signal - relative units (RU) and (b) dose effect of the radical scavenging activity expressed as chemiluminescent scavenging index (CL-SI%) of wine samples from variety Cabernet and Merlot obtained in the superoxide containing system.

On Figure 1 the results from the evaluation of the capability of the tested wines to decrease the concentration of superoxide anion radical are presented. Figure 1a represents typical chemiluminescent curves after adding equivalent amount representative wine sample from each of both chosen variety and on Figure 1b is being presented the observed linear dependence of the chemiluminescent scavenging index from the volume final wine product added to milliliter sample solution. In order to eliminate the possibility the observed effect to be due to the present ethanol in the final wine product have been performed experiments with samples having equivalent amount of ethanol to the one marked on the wine bottle label. The obtained results didn't denote statistically significant difference between the CL-response of the control sample presented on figure 1a and the ethanol containing one. The obtained results indicate significant decrease of the CL – signal corresponding to lower concentration of the superoxide anion radicals in the samples containing wine product. The observed effect has linear concentration dependence and proves the presence of components possessing $O_2^{\bullet -}$ scavenging activity in the tested samples.

The second part of our chemiluminescent experiments comprises evaluation of the wine samples potency to decrease the samples' hypochlorite concentration. The reason for this is the

found literature data proving the capability of wine to influence and suppress the induced during neutrophil activation oxidative burst. During the activation of this process are being generated superoxide anion radicals, but the observed bactericidal effects is due to the myeloperoxidase catalyzed production of hypochlorite (HOCl), one of the most potent neutrophil ROS, using as substrates hydrogen peroxide (H_2O_2) and chloride (Cl^-). Some authors claimed that the anti-inflammatory effect of wines is due to some of it constituents possessing the capability to eliminate the hypochlorite [7, 8]. In our experiments we determined the wine samples capability to decrease the concentration of hypochlorite at experimental conditions (concentration of this ROS) relevant to the one observed during oxidative burst.

The presented on figure 2a and 2b data concerning the evaluation of wines potency to decrease the hypochlorite concentration in the chemiluminescent model system of NaOCl generated OCl^- denoted decreased CL-signal and respectively CL-SI index of the containing wine samples compared to the controls. The observed dose-effect response denotes an exponential decay of the CL index with the increase of the wine concentration in the samples. The obtained data prove presence in the studied wine samples of substances possessing the capability to eliminate the OCl^- in the studied system.

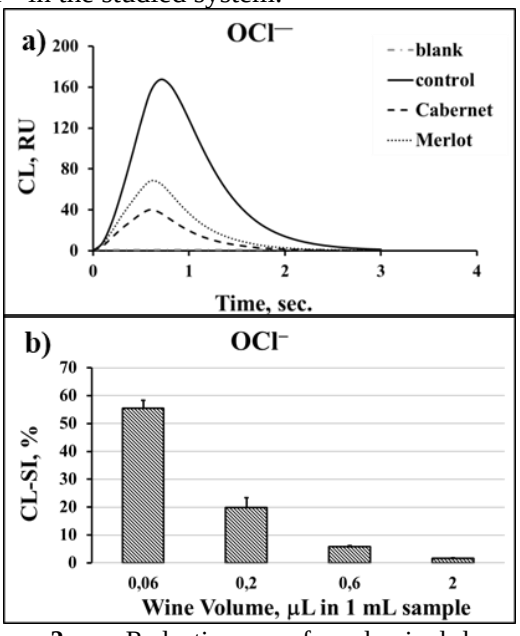

Fig. 2: Reduction of luminol-dependent chemiluminescent response in model system of NaOCl produced hypochlorite: (a) typical chemiluminescent curves - X-axis, time of analysis - sec; Y-axis, intensity of chemiluminescence signal - relative units (RU) and (b) dose effect of the radical scavenging activity expressed as chemiluminescent scavenging index (CL-SI%) of wine samples from variety Cabernet obtained in the hypochlorite anion containing system.

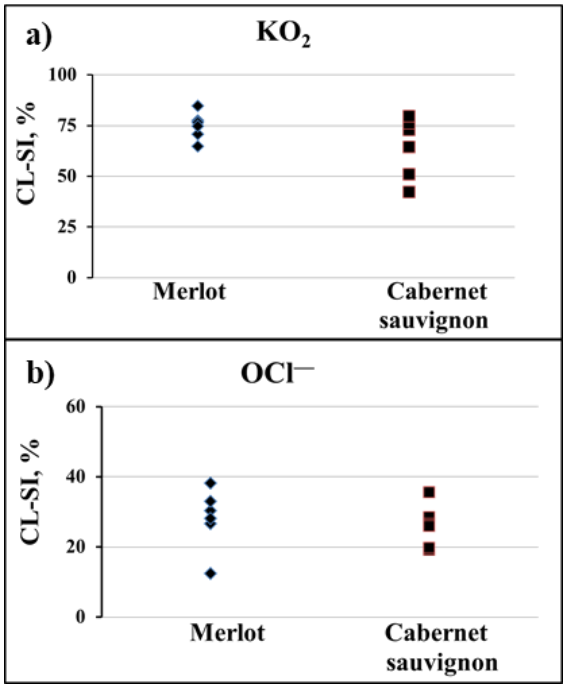

Fig. 3: Scavenging activity against the superoxide anion radical (a) and the hypochlorite (b) of the wine samples (0.2µl/mL) from the variety Cabernet and Merlot.

Comparing the individual results for the different wine samples obtained using both chemiluminescent system (Figure 3a and 3b) we can conclude that all wine samples demonstrated better effectiveness in the hypochlorite elimination compared to the superoxide anion one. The CL-SI values in the superoxide system varies between 42 and 84% and in the hypochlorite one the highest determined CL-SI value is 38.2%

Additional experiments were accomplished in spectrophotometric model systems containing stable free radicals (ABTS and DPPH). Both chosen methods are frequently used in the scientific literature to estimate the antiradical properties of series of complex samples due to the fact that they are relatively cheap, fast and reliable. They are being used by most of the scientists together due to the differences in the chemical reaction associated with the radical elimination in order to obtain possibility for more accurate interpretation of the observed antiradical effect. They are both based on reaction mixture decolorisation proportional to the antiradical properties of the tested samples. The determined from the experiments volumes wine product inducing 50% AOA - V_{50} – are presented on Figure 4. For better interpretation of the results the correlation coefficient from all the model systems are being presented in Table 1.

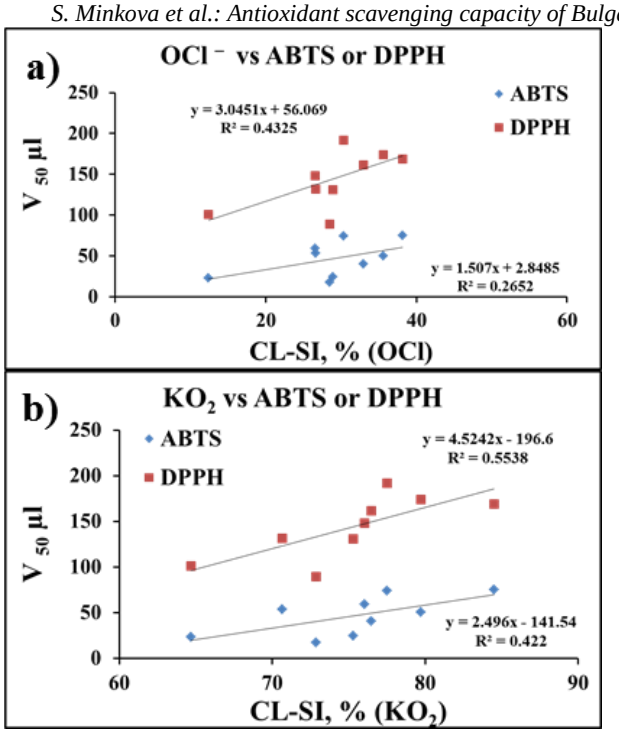

Fig. 4: Correlation between the antiradical effect in the studied chemiluminescent and spectrophotometric model systems.

Table 1: Values of the correlation coefficient between the observed antiradical effect in all used systems.

	ABTS	DPPH	KO_2	OCl^-
ABTS	-	0,6924	0,422	0.2652
DPPH	0,6924	-	0.5538	0.4325
KO_2	0,422	0.5538	-	0.8721
OCl^-	0.2652	0.4325	0.8721	-

On Table 1 the relationship between the CL-SI data from both chemiluminescent model systems containing respectively superoxide anion radical and hypochlorite is presented. A correlation between the extents of scavenging activity against both ROS was observed (R^2= 0,8721). The tendency denote that wine samples possessing better effectiveness against OCl^- had better capability to eliminate $O_2^{\bullet-}$.

On figure 4a and 4b have been compared the data from both spectrophotometric systems with the effectiveness in each of the chemiluminescent. The observed correlation between the results obtained in the stable free radical containing systems and the chemiluminescent was not that high as between the two CL tests.

CONCLUSION

The tested wines have demonstrated antiradical effect in all studied systems. The results from the chemiluminescent assays proved better effectiveness in the hypochlorite elimination compared to the superoxide anion for all wine samples. Higher correlation has been observed between the extent of scavenging activity against both biologically relevant ROS – wine samples possessing better effectiveness against OCl^- had better capability to eliminate $O_2^{\bullet-}$. The correlations comparing data obtained only using stable free radical systems or ROS and stable free radical were lower.

On this base we suggest that for characterization of the antioxidant potency in response to health concerns for complex samples it is not needless to use also model systems evaluating capability to eliminate different types of biologically important ROS. This is necessary due to the variety of possible antiradical mechanisms of action of the sample components – hydrogen atom transfer, single-electron transfer and sequential proton loss-electron transfer mechanisms.

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