Free radical generation induced by ultrasound in red wine and model wine：An EPR spin-trapping study

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ABSTRACT

Direct evidence for the formation of 1-hydroxylethyl radicals by ultrasound in red wine and air-saturated model wine is presented in this paper. Free radicals are thought to be the key intermediates in the ultrasound processing of wine, but their nature has not been established yet. Electron paramagnetic resonance (EPR) spin trapping with 5,5-dimethyl-l-pyrrolin *N*-oxide (DMPO) was used for the detection of hydroxyl free radicals and 1-hydroxylethyl free radicals. Spin adducts of Hydroxyl free radicals were detected in DMPO aqueous solution after sonication while 1-hydroxylethyl free radical adducts were observed in ultrasound-processed red wine and model wine. The latter radical arose from ethanol oxidation via the hydroxyl radical generated by ultrasound in water, thus providing the first direct evidence of the formation of 1-hydroxylethyl free radical in red wine exposed to ultrasound. Finally, the effects of

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ultrasound frequency, ultrasound power, temperature and ultrasound exposure time were assessed on the intensity of 1-hydroxylethyl radical spin adducts in model wine.

Keywords: Ultrasound, Wine, EPR, Free Radical, Model wine, Spin trapping

1. Introduction

Acoustic cavitation (formation, growth and implosive collapse of bubbles) generated by ultrasonic waves in liquids can induce certain chemical reactions and accelerate reaction rates [1,2]. Because of this, ultrasound is regarded as a promising technique in food technology like processing, preservation and extraction. With regard to winemaking, possible applications of ultrasound to wine microbiology and wine aging have been highlighted [2]. In addition, some researches have been conducted over the last decade on ultrasonic wave treatments for accelerating the aging process of some kinds of wine, such as rice, maize and greengage wines [3-5]. However, to our best knowledge, there is still a lack of papers concerning the application of ultrasound to grape wine and its actual effects. Therefore, further research on the reaction mechanisms and the suitability of ultrasound for winemaking is required.

Generally, free radicals are considered as the important triggering factors to initiate chemical reactions in liquids exposed to ultrasound. Besides the hydroxyl radicals produced by ultrasound in aqueous solution [6], some other free radicals are also generated by ultrasonic waves in some organic liquids [7]. In addition, some species of the above-mentioned free radicals may react with the dissolved substances thus leading to produce secondary radicals [8]. As a consequence, a chain of chemical

reactions could be induced by the free radicals generated by ultrasonic waves. However, whether ultrasonic irradiation has the ability to induce free radicals in red wine, and what kind of free radicals are generated still remains unclear. To the best of our knowledge, the mechanisms of ultrasound's action on modification of some wines are still unclear. It is for these reasons that identification of free radicals generated by ultrasound in red wine and model wine is so valuable. Beyond that, the investigation about effect of ultrasound operational conditions (frequency, power, exposure time and temperature) on formation behaviour of free radical generation is also essential as supplement.

Electron paramagnetic resonance (EPR) spectroscopy is a widely used technique that allows the direct detection of species with unpaired electrons (e.g., free radicals, transition metals) and can often aid in the determination of the radical's identity [9]. A major limitation of this technique is the inability to directly detect some highly reactive radical species with very short life (e.g. superoxide, hydroxyl radicals, sulfur centered radicals and alkoxyl radicals) [10]. Fortunately, this can be overcome through the use of spin traps, which are diamagnetic compounds (often nitrones or nitroso compounds) capable of yielding long-lived radical products upon reaction with free radicals [10]. EPR spin trapping has been successfully used to elucidate many mechanistic questions [11-13]. The assignment of the structure of the radicals, a crucial feature to elucidate the mechanism of the sonochemical reactions, has been successfully approached by EPR spin trapping in numerous studies of aqueous solutions of volatile and non-volatile solutes [11,12,14]. Among the spin traps used in

EPR spectroscopy, the most popular is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), because this spin trap is the most redox inactive, and the ERS spectra of its radical adducts can show more dependence on the structure of the trapped radical than other common nitrone spin traps.

In this paper, we firstly focused on the identification by EPR spin trapping of free radicals induced by low frequency ultrasound in red wine and model wine. Once the free radicals were identified, we assessed the effect of the main operational parameters in ultrasonication, namely ultrasonic power, temperature, ultrasonic frequency and ultrasound exposure time, on the intensity of the free radical spin adducts.

2. Materials and Methods

2.1. Materials

Ethanol was supplied by Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Catechin of high pressure liquid chromatography grade was purchased from Chinese Food and Drug Inspection Institute. Analytical pure tartaric acid was supplied by Kefeng Chemical Reagent Co. Ltd. (Shanghai, China). Sodium hydroxide was obtained from Tianli Chemical Reagent Co. Ltd. (Tianjin, China). 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA) and was used without further purification. Deionized water was purified by a water system produced by Merck Millipore Scientific Instrument Co. Ltd. (Germany). All the other chemicals and reagents used were of

analytical grade.

2.2. Wine samples

A Cabernet Sauvignon red wine from the vintage 2012 was obtained from Danfeng Winery Ltd. (Shaanxi, China) and used throughout this research. On the other hand, a model wine solution, which contained 12% v/v ethanol, tartaric acid (53 mM) and catechin (12 mM), was used as well. This model wine was adjusted to pH 3.6 with NaOH aqueous solution (5 N) and shaken to achieve air saturation [15].

2.3. Experimental setup

Ultrasonic treatments were carried out in an ultrasonic bath (KQ-300VDE, Kunshan Ultrasonic Equipment Co. Ltd., Jiangsu Province, China) which can work at the frequencies of 45 kHz, 80 kHz and 100 kHz with a variable power output from 120 to 300 W. Ultrasonic energy was delivered from the bottom to the water in the tank by 6 annealed transducers, and the total rated power output was 300 W. For each experimental run, 1 mL sample was loaded into a 1.5 mL centrifuge tube, and then placed in the water bath and fixed at the same position during ultrasound treatment.

2.4. Ultrasonic irradiation experiments

DMPO (500 mM) was directly dissolved into the red wine and the model wine solution, respectively. The following stock solutions were made as well. Firstly, DMPO (500 mM) was dissolved directly into 1 mL of water. Secondly, DMPO (500 mM) was dissolved directly into 1 mL water containing 12% v/v ethanol. Finally, DMPO (500 mM) was dissolved directly into 1 mL water containing 12% v/v ethanol

and tartaric acid (53 mM) with the pH adjusted to 3.6 with NaOH aqueous solution (5 N). All of the stock solutions, model wine and red wine were submitted to 100 kHz ultrasound (300 W power) for 5 min at 20ºC to investigate the formation of free radicals generated by ultrasound.

Afterwards, four sets of experiments were performed with model wine to assess the intensity of free radicals at different ultrasonic conditions. Firstly, the effect of ultrasound frequencies including 45 kHz, 80 kHz and 100 kHz was investigated at a power level of 300 W for 5 min at 20 ºC. Subsequently, the effect of ultrasound power level (120, 180, 240 and 300 W) was assessed, being the ultrasonications performed with 100 kHz ultrasound at 20 ºC for 5 min. In parallel, another set of experiments was carried out at different bath temperatures (20 ºC，30 ºC，40 ºC，50 ºC and 60 ºC, respectively) with the 100 kHz ultrasound frequency at a power level of 120 W for 5 min. Finally, in order to investigate the effects of ultrasound exposure time on the formation of free radicals, ultrasound exposure time (10, 20, 40 and 80 min) was assayed working with 100 kHz, 300 W and 20ºC.

2.5. EPR Spin Trapping

EPR spectra were recorded on a JES-FA200 spectrometer (Japan Electron Optics Laboratory Company, Tokyo, Japan) operating in X-band at room temperature and Spin adducts were quantified. The sweep width was set to 50 G, and the microwave power was set at 37.86 mW. Modulation frequency and modulation amplitude were set at 86.00 kHz and 2.45 G, respectively.

The receiver gain was set to 4.48×10^3. The conversion time and sweep time were set to 20.48 ms and 10.49 s, respectively. The total number of scans was 30 for each sample. 1-hydroxylethyl radical adducts produced a triplet of doublets (hyperfine coupling constants: $a_N = 15.60$ G, $a_H = 2.25$ G) as observed in previous studies [9]. The intensity was quantified by adding the maximum and minimum values of the central doublet.

3. Results and discussion

3.1. Identification of free radicals in red wine exposed to ultrasound irradiation

As shown in Figure 1, evidence for the 1-hydroxylethyl free radical formation in ultrasonic-treated red wine was found. This free radical is sufficiently stable to be trapped using nitrone spin traps (e.g., DMPO) and quantified by measuring the intensity of the EPR spectrum corresponding to the spin adduct. The hyperfine coupling constants of the observed spectrum ($a_N = 15.60$ G, $a_H = 2.25$ G) were nearly identical to the values for the DMPO spin adducts formed from the 1-hydroxyethyl radical as mentioned-above [9,16]. Therefore, we can confirm that this kind of free radical does exist in red wine shown in Fig. 1(b), which is in agreement with other authors' reports [9]. 1-hydroxylethyl free radical is considered to be a key radical intermediate in natural oxidation of wine and, as such, has been used to monitor the progress of the oxidation of model wine [15]. As shown in Fig.1(c), the intensity of 1-hydroxylethyl free radical increased after 5 min ultrasound exposure in comparison with that in Fig.1(b), which demonstrated that the ultrasound treatment has

contributed to the formation of the 1-hydroxylethyl free radical directly or indirectly.

3.2. Evidence for the 1-hydroxylethyl free radical formation in stock solutions

In order to investigate the possible influence of some chemical compounds in red wine on the formation behavior of free radicals under ultrasonication, EPR spectra of different stock solutions, model wine and pure water sonicated for 5 min were recorded. As shown in Fig. 2 (a), pure water adding 500 mM DMPO was treated by ultrasound for 5 min, and the typical EPR spectrum of hydroxyl free radical (hyperfine coupling constants: $a_N = a_H = 14.9$ G) was observed, which is in agreement with many previous studies [6,8,11]. By contrast, we did not find EPR spectrum of hydroxyl free radical in other stock solutions. Only 1-hydroxylethyl free radical was observed in other stock solutions containing ethanol. The reason is that ethanol, as a hydroxyl free radical scavenger, has the ability to react with hydroxyl free radicals leading to the formation of the novel 1-hydroxylethyl free radical [8,15], and thus resulted in the disappearance of hydroxyl free radical in solutions with ethanol, i.e. ethanol is a substrate for the hydroxyl free radical and therefore competes with the spin trap [8], which can also be confirmed by the EPR spectra shown in Fig. 2 (c) and Fig. 2 (d). In addition, it seems that tartaric acid did not exert great influence on the free radical generation in water containing 12% v/v ethanol, 53 mM tartaric acid and 500 mM DMPO at pH 3.6 during ultrasonification. Interestingly, the intensity of DMPO/1-hydroxylethyl free radical spin adducts decreased in the model wine solution shown in Fig. 2 (e), which contained 12% (v/v) ethanol, tartaric acid (53 mM)

and catechin (12 mM). It may be attributed to the presence of catechin, which is regarded as a powerful free radical scavenger among different classes of flavonoids, and has a significant scavenging effect on hydroxyl radicals and 1-hydroxylethyl free radicals [17,18].

3.3. Effect of ultrasound frequency on the intensity of DMPO/1-hydroxylethyl free radical spin adducts in a model wine

As shown in Fig. 3, the application of higher ultrasound frequencies led to an increase in the intensity of DMPO / 1-hydroxylethyl free radical spin adducts, which may be attributed to the amounts increasing of the collapsing bubbles of ultrasound caviation as reported by other authors [19-21]. Generally, the effect of ultrasound cavitation could be reduced at higher ultrasonic frequency, since either the rarefaction cycle of the sound wave produces a great negative pressure which is insufficient in its duration and/or intensity to initiate cavitation, or the compression cycle occurs faster than the time for the microbubble to collapse [22-24]. As a consequence, the collapse of bubbles occurs much more rapidly, resulting in the amounts of cavitation bubbles increased and more hydroxyl free radicals released from the bubbles, finally inducing the increase of 1-hydroxylethyl free radical and its spin adducts in model wine.

In addition, it is of vital importance to note the choosing of ultrasonic frequencies in experiments to accelerate wines aging. Generally, the higher the ultrasound frequencies, the stronger the intensity of free radicals. Hence, we should take advantage of higher frequency to induce more radicals, but Chang [3] reported that the

20 kHz of ultrasound treatment influenced rice alcoholic beverage aging better than that of 1.6 MHz treatment, which may suggest that too many free radicals induced by high frequency ultrasound would not improve the expectedly quality of wine. Zheng [5] also suggested that low frequency ultrasonic treatment could improve greengage wine well. As a consequence, we choose the lower frequencies (less than 100 kHz) to investigate the ultrasonic effect on the red wine.

3.4. Effect of ultrasound power on the intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine

As a whole, the intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine follows a rising trend with the increase of the ultrasound power (Fig. 4), i.e. ultrasound power has a strong influence on the amounts of free radicals generated, which is in accordance with that reported by other authors [25]. As a rule, Ultrasonic intensity is defined by the power, and it increases at the same reactor area with power [24]. Gogate and pandit (2004)[25] have pointed out that the ultrasonic intensity has a strong effect on the pressures of bubble collapses and local temperatures as well as the number of free radicals generated in the studied solution. As the increase of ultrasonic intensity, the bubble collapses from cavitation will become more violent, and greater sonochemical effects in the collapsing bubbles will happen in consequence. Generally, cavitation is considered as a very dynamic and complicated phenomenon, and higher ultrasonic intensity would create bigger bubbles, consequently the collapse of bubbles would induce higher shear forces. Furthermore, with the increase of ultrasound power,

the pulsation and bubble collapses will occur more rapidly, and amounts of cavitation bubbles will increase, hence producing a higher concentration of free radicals into the model wine solution. Consequently, these free radicals will induce the greater formation of 1-hydroxylethyl free radicals in the ethanol solution, which shows on the increase of the intensity of DMPO / 1-hydroxylethyl free radical spin adducts at higher powers. Except above-mentioned reasons, an increase of ultrasound power also contributes to an increase of acoustic amplitude. The collapsing time, temperature and pressure on the collapses of bubbles are all dependent on the acoustic amplitude, and the collapse of cavitation bubbles is more violent at higher acoustic amplitudes [26]. These are also attributed to the increase of the intensity of DMPO / 1-hydroxylethyl free radical spin adducts. We do need the free radicals, which is easier to be generated by high power ultrasound, to accelerate aging and oxidation of wine [9]. But according to our results [24,27], higher ultrasonic power would lead the degradation of phenolic compounds in red wine, and these compounds are beneficial to health as well known by now. Besides that, Zheng [5] argued that ultrasonic conditions of 300 W could improve the maturation of wine. In order to avoid the unnecessary loss of phenolic compounds and save more energy, we employed the selected ultrasonic power levels to carry out these experiments.

3.5. Effect of ultrasonic temperature on the intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine

As shown in Fig. 5, the intensity of free radical spin adducts in model wine

increased with the increase of ultrasonic temperature from 20°C to 50°C, then followed by a decrease at 60 ℃. Generally, the effect of the ultrasonic temperature on the reactions in solution is a rather complicated phenomenon, since the temperature can affect the gas solubility, surface tension and the vapor pressure of the solutes [24]. For instance, an increase of temperature will cause a decrease of surface tension which can lower the intensity of threshold required to produce cavitation. Besides that, the increase of ultrasonic temperature can also decrease the collapse temperature, since the solution viscosity and/or surface tension decreases with the liquid temperature increasing, and more importantly the vapor pressure increases conversely. In short, the cavitation strength will be reduced at the higher operating temperature, which may be an explanation to the decrease of intensity of free radical spin adducts at the temperature of 60 ℃.

On the other hand, the intensity decrease could also be attributed to the ethanol losses of evaporation at 60 ℃, i.e. the decrease of ethanol concentration in the model wine could lead to a decrease in the 1-hydroxylethyl free radicals. Another explanation could be the chemical instability of DMPO / 1-hydroxylethyl free radical at higher temperature [10]. The higher temperature of 60 ℃ might cause the increase of the degradation rate of DMPO / 1-hydroxylethyl free radical [6], and when its degradation rate is greater than that of its formation rate, a falling trend could be observed.

3.6. Effect of ultrasound exposure time on the intensity of DMPO / 1-hydroxylethyl

free radical spin adducts in model wine

Fig. 6 illustrates that the intensity of free radical spin adducts increased with the increase of ultrasound exposure time from 10 min to 80 min, to be specific, the intensity increased rapidly during the initial ultrasonic stage and then the increase slowed down with the further extension of ultrasonic exposure time (for example from 40 min to 80 min), which is in accordance with the results reported by Castellanos [7] and Feng [28], who reported that the yield of hydroxyl radicals in aqueous solution induced by ultrasound increased monotonically at the initial stage, and then tended to stabilize with the further increase of sonication time. The main reason for this interesting phenomenon may be attributed to acoustic cavitation during the first sonication period [29], which was considered as an initial stage of generating and accumulating free radicals, and simultaneously oxidizing the ethanol in model wine. Regarding the slowly increasing stage from 40 to 80 min, the major inducement may be attributed to the limiting amounts of free radicals ultrasonically generated, and the ultrasonic-induced spontaneous degradation of DMPO / free radical spin adducts and DMPO [6,24].

4. Conclusions

EPR spin trapping of free radicals with 5,5-dimethyl-l-pyrrolin *N*-oxide (DMPO) was successfully used to identify the species of free radicals generated by ultrasound irradiation in red wine and model wine. The results show that the 1-hydroxylethyl free radical was captured in red wine and model wine, and its concentration increased after

ultrasonification, which demonstrates that ultrasound does trigger the generation of 1-hydroxylethyl free radicals into wine. Unexpectedly, the hydroxyl free radical was not detected in red wine and model wine, and it is deduced that once the hydroxyl free radical is generated, it will instantaneously attack the ethanol in the red wine and model wine to form the novel 1-hydroxylethyl free radical according to the literature available, i.e. the latter free radical newly formed is more stable than the former one. Furthermore, the presence of catechin in solution exhibited a certain scavenging activity on the 1-hydroxylethyl free radical during ultrasound exposure. With regard to the operational parameters of ultrasound irradiation in model wine, the increase of ultrasound power, frequency and exposure time resulted in an increase in the intensity of DMPO / 1-hydroxylethyl free radical spin adducts. And the increase of temperature (20 to 50 ℃) also promoted the intensity of the DMPO / 1-hydroxylethyl free radical spin adducts, followed by a decrease at the temperature of 60 ℃, which might be attributed to the higher degradation rate of 1-hydroxylethyl free radical spin adducts and DMPO at higher temperature. Nevertheless, the specific forming mechanism of the 1-hydroxylethyl free radical should be further studied in the future. In summary, these results do contribute to understand the mechanism of ultrasound's action on modification of some wines and produce high quality wine with this novel processing technology of ultrasound in winery.

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Figure Captions

Fig. 1 (a) EPR spectrum of red wine without DMPO, (b) EPR spectrum of red wine with 500 mM DMPO and (c) EPR spectrum of red wine with 500 mM DMPO after 5

min ultrasonic irradiation

Fig. 2 EPR spectrum of (a) 500 mM DMPO aqueous solution without ultrasound exposure, (b) 500 mM DMPO aqueous solution after sonication, (c) water containing 12% v/v ethanol and 500 mM DMPO after sonication, (d) water containing 12% v/v ethanol, 53 mM tartaric acid and 500 mM DMPO at pH 3.6 after sonication and (e) model wine after sonication

Fig. 3 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with various ultrasound frequency

Fig. 4 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated at various ultrasound power

Fig. 5 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with different ultrasonic temperature

Fig. 6 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with different ultrasonic time

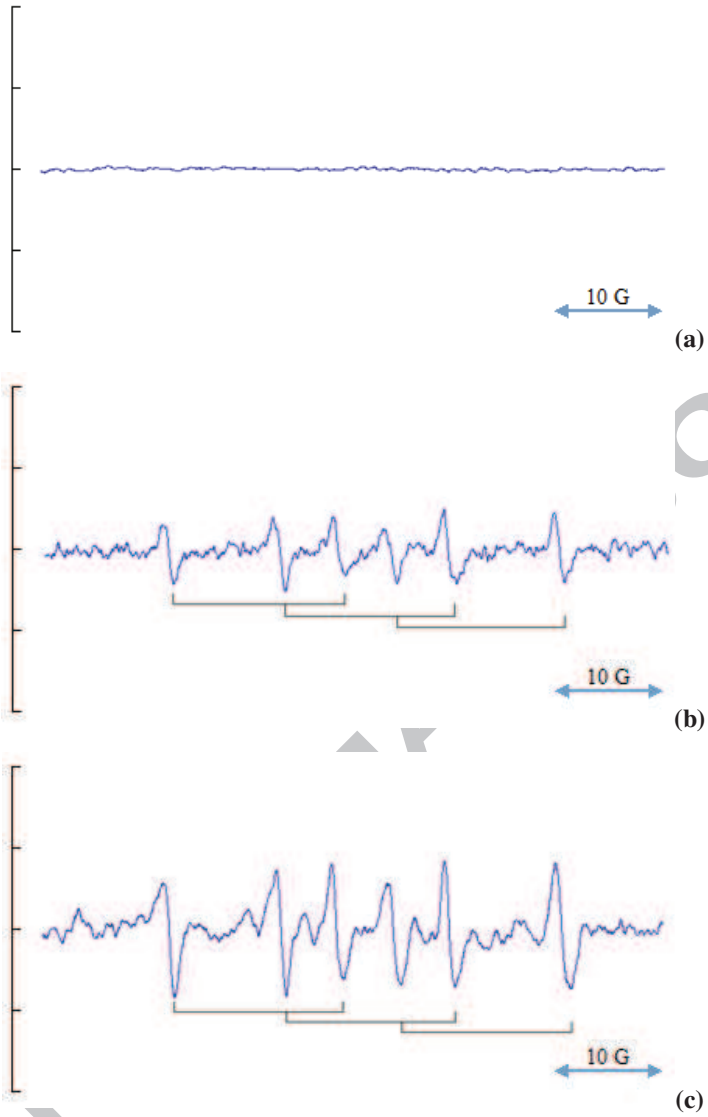
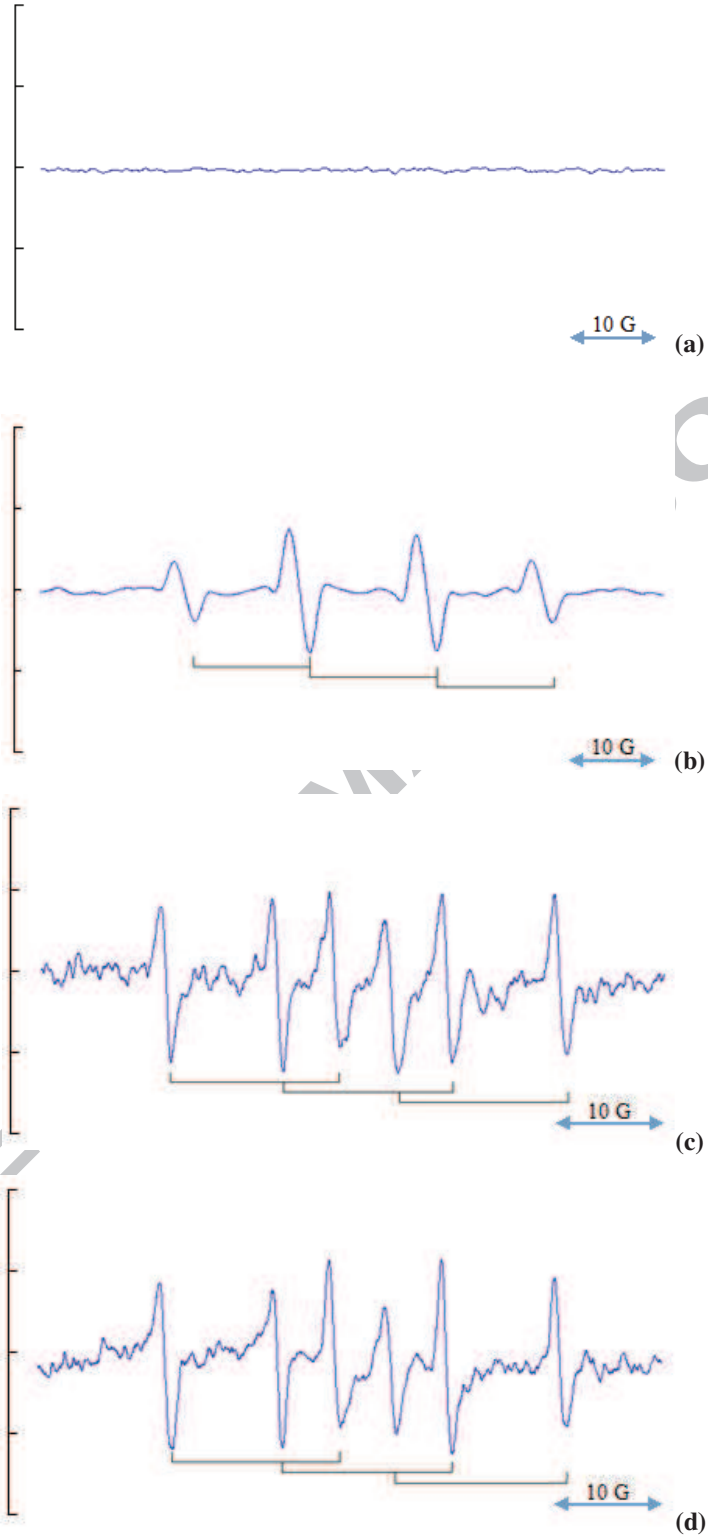
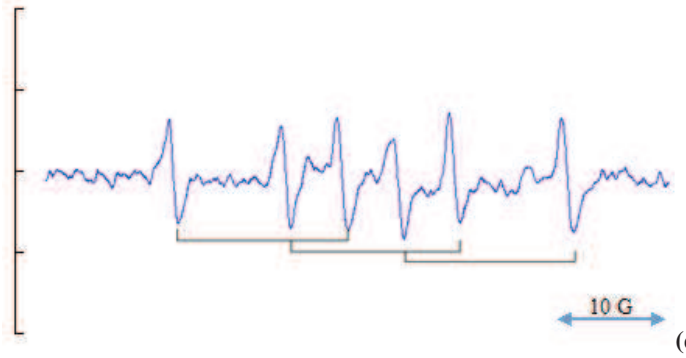


Fig. 1 (a) EPR spectrum of red wine without DMPO, (b) EPR spectrum of red wine with 500 mM DMPO and (c) EPR spectrum of red wine with 500 mM DMPO after 5 min ultrasonic irradiation





(e)

Fig. 2 EPR spectrum of (a) 500 mM DMPO aqueous solution without ultrasound exposure, (b) 500 mM DMPO aqueous solution after sonication, (c) water containing 12% v/v ethanol and 500 mM DMPO after sonication, (d) water containing 12% v/v ethanol, 53 mM tartaric acid and 500 mM DMPO at pH 3.6 after sonication and (e) model wine after sonication

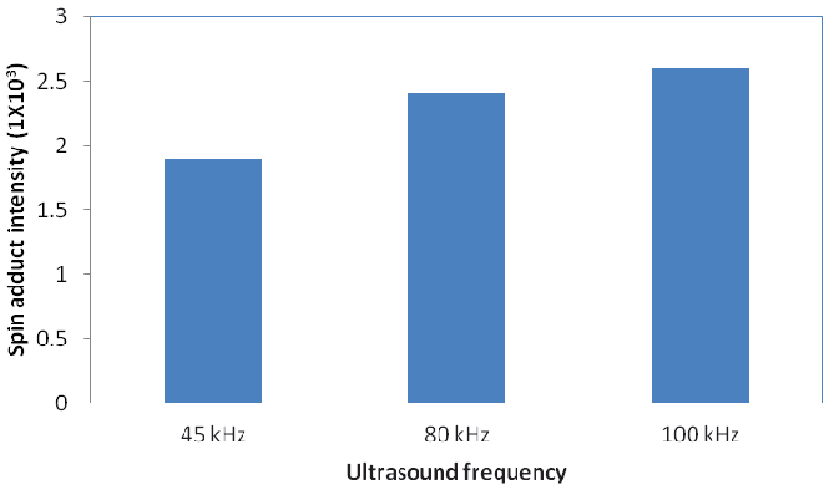


Fig. 3 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with various ultrasound frequency

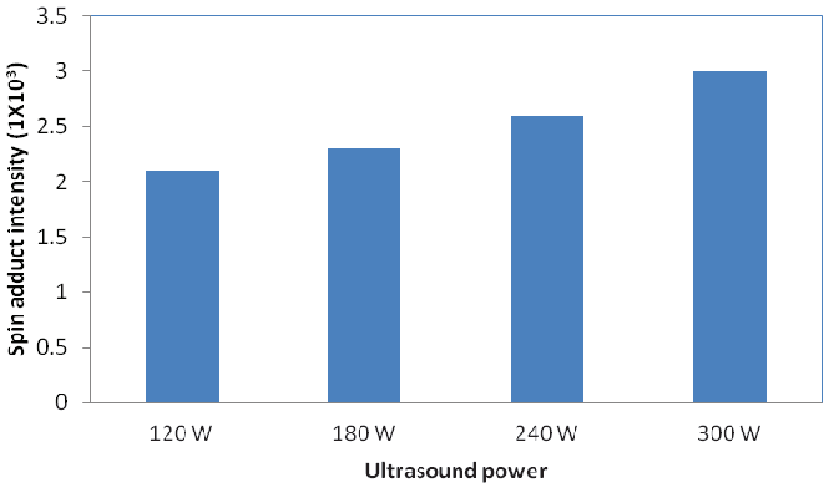


Fig. 4 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated at various ultrasound power

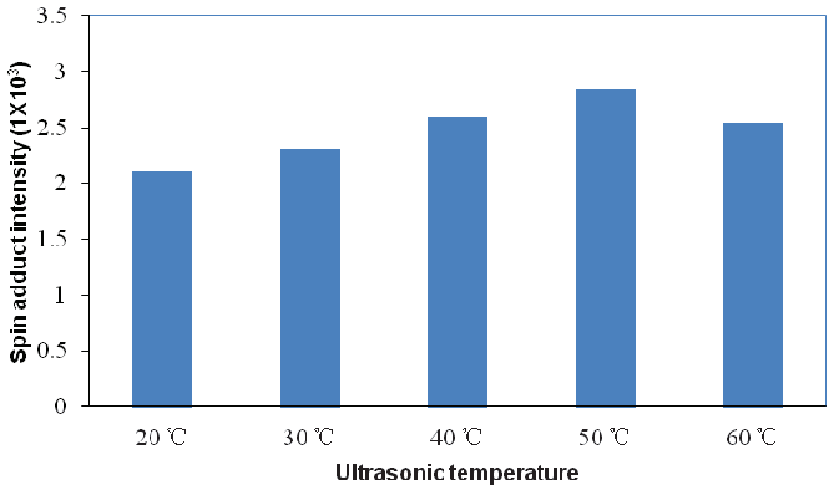


Fig. 5 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with different ultrasonic temperature

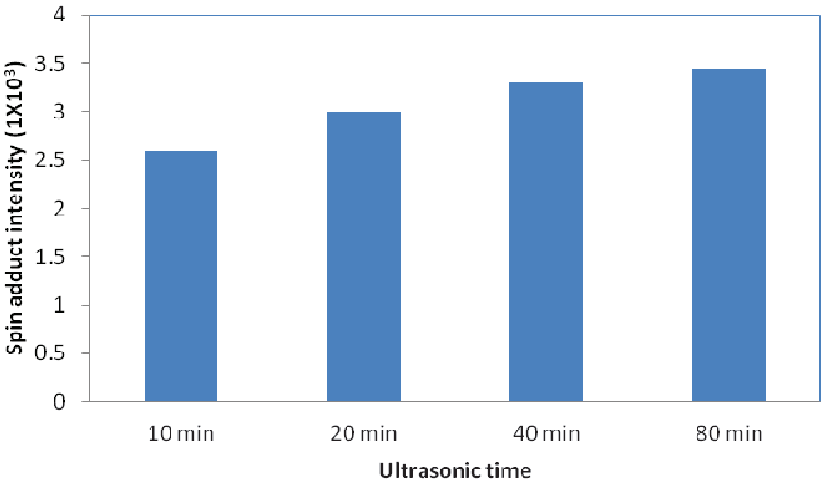


Fig. 6 Intensity of DMPO / 1-hydroxylethyl free radical spin adducts in model wine treated with different ultrasonic time

Highlights

- 1-hydroxylethyl radicals (HER) induced by ultrasound were firstly captured in wine.
- The mechanism of HER formation was discussed in a model wine.
- The effect of ultrasound irradiation on the intensity of HER adducts were investigated.
- The results contribute to understand the modification mechanism of ultrasound on wine.