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Comparison of the total antioxidant status of Bohemian wines during the wine-making process

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Abstract

The total antioxidant status (TAS) of two white and two blue wine varieties from the Žernoseky wine region (North Bohemia, Czech Republic) during the wine-making process was assessed by measurement with different radical scavenging assays: ABTS, DPPH and DMPD. Significant differences in the antioxidant activity between white and red wines were confirmed and changes of TAS during the wine production process were demonstrated. Moreover, differences were ascertained between individual varieties of red wine. No statistically significant relationship between the results provided by the ABTS and DPPH assays was found, obviously due to the fact that each phenolic substance in wine gives a different response to each specific radical used in the assay. The results obtained supported the assumption that variety plays a considerable role in TAS; the blue wine varieties showed a much higher TAS than did the white wines. The ABTS assay showed higher EQA (equivalents of ascorbic acid, mg/ml) values than the DPPH assay.

Keywords: Wine; Wine-making; Total antioxidant status; Free radical cation; DPPH; ABTS; DPMD; Polyphenols

1. Introduction

There is a considerable interest in antioxidants as bioactive components of food with a special role in maintaining health and preventing disease (Kris-Etherton et al., 2002). The consumption of red wine is traditionally appreciated for its positive influence on human health. This influence is still held in high regard nowadays, when more and more people are developing a commitment to healthy nutrition and wine consumption in the Czech Republic is steadily increasing. Wine is seen as a valuable beverage with a tonic and calming effect. Moreover, the polyphenolic substances in wine are, given their capacity to scavenge free radicals, highly powerful antioxidant agents (Fogliano, Verde, Randazzo, & Ritieni, 1999). Rice-Evans and Miller (2005) reported that knowledge of the individual levels of specific antioxidant components may be less useful than the total antioxidant activity determined by the combined reducing activities of its constituents. Several assays have been introduced for measuring the antioxidant ability of single antioxidant compounds and/or complex mixtures (Ozcan, 1997; Roginsky & Lissi, 2005; Wang et al., 2004). Prior and Cao (1999) compared some methods, which have been developed to measure the total antioxidant capacity of biological samples with the goal of adequately assessing oxidative stress in biological systems. Gorinstein et al. (2003) attempted to compare the contents of the main antioxidants and the total antioxidant status (TAS) using different antioxidant assays for some foods with a high antioxidant capacity, such as olive oil.

Because wine, especially red, has a high impact on TAS *in vivo* (Agewall & Doughty, 2000; Perez et al., 2002), and as there is little knowledge about TAS changes during wine-making, a comparison of three TAS assays used for the assessment of the TAS of blue and white wine varieties during the wine-making process was undertaken.

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate); DMPD, N,N-dimethyl-p-phenylenediamine radical cation; EQA, equivalents of ascorbic acid (mg/ml); TAS, total antioxidant status.

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2. Methods and materials

2.1. Material

L-Ascorbic acid was purchased from Sigma–Aldrich, DPPH (2,2-diphenyl-1-picrylhydrazyl) from Sigma Aldridge and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) from Fluka. Ferric chloride, acetic acid and sodium acetate were obtained from Lach-Ner (Czech Republic). All chemicals used were p.a. purity or higher.

Wine samples were obtained from one vineyard and one producer in the Žernoseky wine region (North Bohemia, Czech Republic) during the wine-making process. Two white varieties (Müller-Thurgau ["MT"] and Moravian Muscat ["MM"]), and two blue varieties, (Blauer Portugieser ["BP"] and St. Laurent ["ST"]) were assessed. Samples were collected every week from the beginning of November 2005 until the beginning of April 2006 (21 weeks). In the middle of April the wines were bottled.

Spectrophotometric measurement was performed on a Helios γ instrument (Spectronic Unicam, GB). The results in every assay were obtained from five parallel determinations. The antioxidant activities were expressed in equivalents of ascorbic acid mg/ml (EQA), which could provide the same inactivation as the studied sample volume.

2.2. DPPH assay

DPPH decolorization was measured after the reaction of sample with the free stable radical DPPH$^{\cdot}$ according to Molyneux (2004). Fresh methanolic solution (2.5 ml) DPPH$^{\cdot}$ were transferred into plastic cuvettes, 10 mm in length, and absorbance at a wavelength of $\lambda = 515$ nm was measured. Absorbance at time t_0 ranged between 0.200 and 1.000 depending on the nature of the sample assayed. Then 5 μl of the sample were added and, after stirring with a hand stirrer in cuvettes, the reaction mixture was left to stand for 5 min. The absorbance was again measured and the percent of inactivation calculated from the decrease of absorbance according to the relationship:

$$\% \text{ of inactivation} = 100 - [(A_{t_5}/A_{t_0}) \times 100] \quad (1)$$

The calibration curves of ascorbic acid (diluted in 13% ethanol v/v) were made for each DPPH absorbance at t_0 (one of the calibration curves, e.g. Fig. 1).

2.3. ABTS assay

The ABTS assay was performed according to the adjusted procedure of Pennycooke, Cox, and Stushnoff (2005): 5 mM water stock solution of ABTS (25 ml) was prepared and 1 g of MnO_2 was added as an oxidizing agent (to produce activated ABTS$^{\cdot+}$ radical). The mixture was stirred and left for 20 min at room temperature. Afterwards, the MnO_2 was removed through centrifugation and syringe-filter filtration (0.25 μm). The activated ABTS^{+} stock solution was diluted in 5 mM phosphate buffer saline (pH 7.4) and adjusted to an absorbance ranging between 0.200 and 1.000, depending on the nature of the assayed sample. The absorbance in a plastic cuvette 10 mm in length at t_0 was recorded and an additional 10 μl of the sample were added, stirred and left to stand for 5 min. A decrease in absorbance after 5 min was measured. The TAS was obtained as mentioned in formula (1) and standardized using the ABTS-ascorbic acid calibration curve for each starting (t_0) absorbance, as mentioned above.

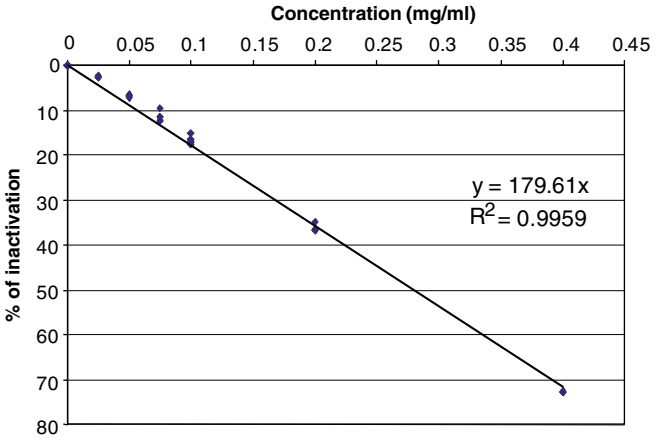

Fig. 1. Example of calibration curve by using ascorbic acid standard (mg/ml) determined by DPPH assay (basic absorbance at t_0=0.350).

2.4. DMPD assay

The DMPD assay was performed strictly according to the method described previously by Fogliano et al. (1999). DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 ml of deionized water. This solution (1 ml) was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the coloured radical cation (DMPD^{+}) was obtained by adding 0.2 ml of a 0.05 M solution of ferric chloride (the final concentration was 0.01 mM). This solution (2 ml) was transferred directly to the plastic cuvette (10 mm in length) and its absorbance at 505 nm was measured (t_0). Sample (100 μl) was added, stirred and left to stand for 10 min. After this time, a decrease in absorbance was measured (t_{10}). Inhibition was calculated according to the Eq. (1) but using absorbancies at t_0 and t_{10}.

2.5. Statistics

Statistical evaluations were performed using the Statistica 7.0 programme at a level of significance of $\alpha = 0.05$.

3. Results

The TAS values, measured during the wine-making process with the DPPH assay (Fig. 2) and the ABTS assay (Fig. 3), revealed significant differences between the white and blue wine varieties (Figs. 4–7). The TAS of white wines were significantly lower than those of the blue wine varie-

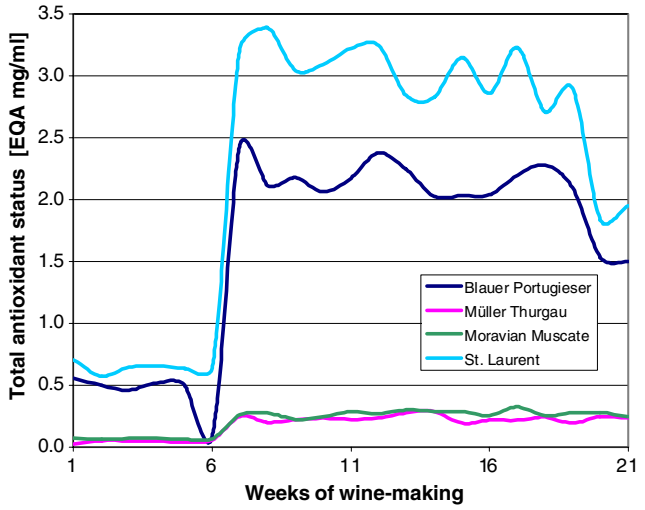

Fig. 2. TAS of wine varieties during wine-making process analysed by DPPH assay.

ties. White wine varieties showed a median value of approximately 0.227 EQA, below the blue wine varieties (2.21 EQA) and had ten times as much TAS when determined by the DPPH assay. In the ABTS assay the EQA values were higher (median 1.45 for white wine varieties and 5.46 EQA for blue wine varieties); the TAS increase was 3.7 times higher in the blue wine varieties than in the white varieties. The difference between the white wine varieties, determined by both methods, was nearly the same (14% by the DPPH assay, 12% by the ABTS assay). The TAS values determined by the ABTS and DPPH assays were strongly affected during the wine-making process. The highest values were determined in the St. Laurent variety (Figs. 2 and 3) with a high increase by week 4 (15 EQA by ABTS assay) and between weeks 6 and 7 (3.4 EQA by DPPH). A similar tendency was found in cv. Blauer Portugieser with an increase during weeks 6–8 (5 EQA by the

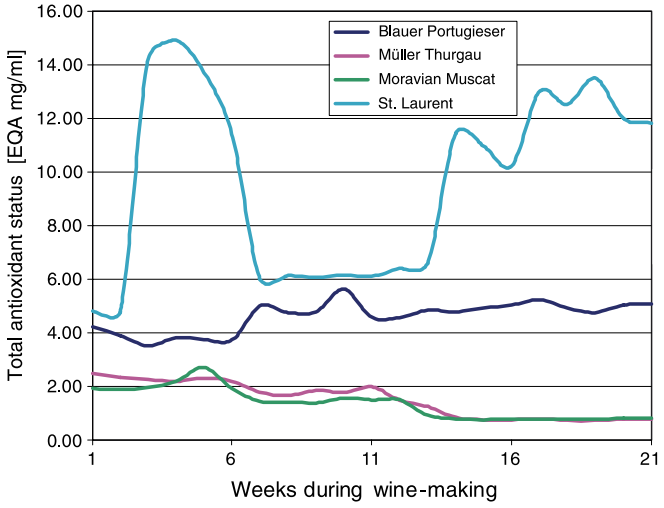

Fig. 3. TAS of wine varieties during wine-making process analysed by ABTS assay.

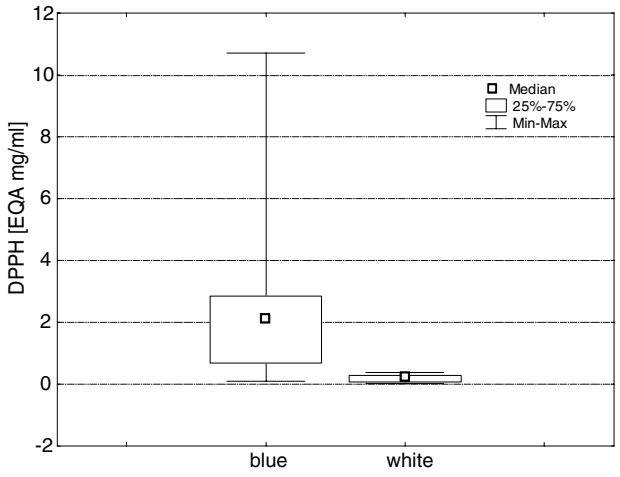

Fig. 4. Comparison of TAS of wine varieties determined by DPPH assay.

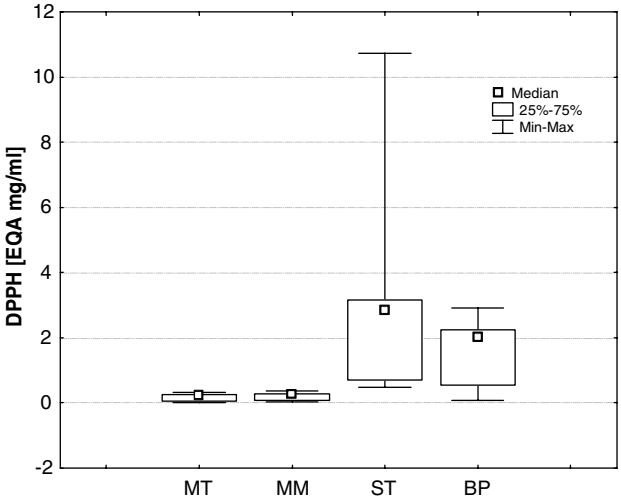

Fig. 5. Comparison of TAS of white and blue wine varieties determined by DPPH assay.

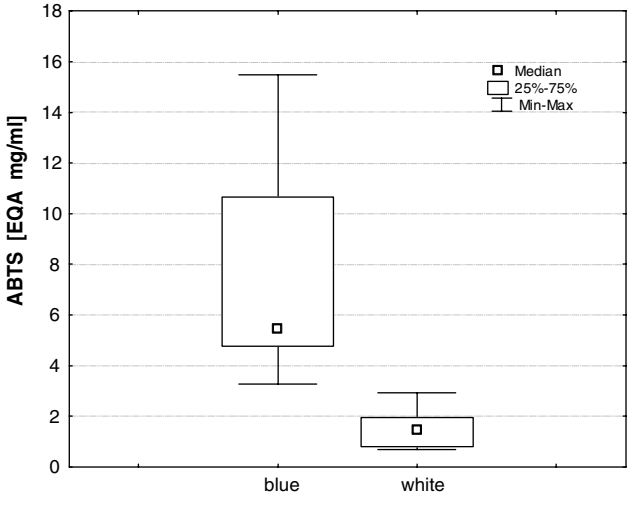

Fig. 6. Comparison of TAS of wine varieties determined by ABTS assay.

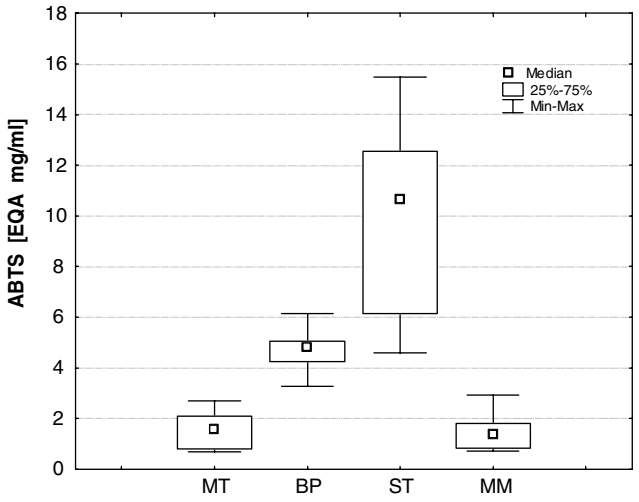

Fig. 7. Comparison of TAS of white and blue wine varieties determined by ABTS assay.

ABTS assay, 3.4 EQA by the DPPH assay). The St. Laurent variety had 40% higher TAS than the Blauer Portugieser when determined by the DPPH assay, and 45% higher when determined by the ABTS assay. A significant difference was found between the blue and white varieties and all varieties except Müller-Thurgau and Moravian Muscat (Table 1). The DMPD assay unfortunately failed in use.

4. Discussion

The TAS found by ABTS and DPPH assays in the white and blue wine varieties differed significantly (Figs. 4–7), which is well in accordance with the recent data obtained by de Villiers et al. (2005), suggesting a high polyphenol content in blue wine varieties. Statistically significant differences were also found between individual blue wine varieties, contrary to white varieties (Figs. 4 and 6).

The wine-making process strongly affected the TAS and it was evidently closely related to the content of polyphenolic antioxidant compounds, as Fernández-Pachón, Villaño, Troncoso, and García-Parrilla (2006), in their study, confirmed. This concerns especially gallic, protocatechuic, and caftaric acids and (−)-epigallocatechin gallate. In blue wine varieties, mostly the anthocyanin content is affected by different vinification methods (Gómez-Plaza, Miñano, & López-Roca, 2006). According to our previous results, the stage of maceration, in particular, affects the total polyphenol content in red wines. Differences in the anthocyanin and tannin extractability in grapes during the wine-making process seem to be one of the main factors affecting anthocyanin and polyphenol content and thus the TAS of wines, as indicated in similar results reported by Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, and Gómez-Plaza (2006) and Netzel et al. (2003). Our results are in good accordance with the results obtained by Villaño, Fernández-Pachón, Troncoso, and García-Parrilla (2006) and confirm the impact of oenological practices on the TAS of wines. The maceration and fermentation methods used for red wines have a positive effect on the antioxidant potential. Red wine grapes in our study were cold macerated for 14 days using a standard technology procedure. The significant difference between the blue wine varieties, Blauer Portugieser and St. Laurent, also confirms behavioural differences during vinification, depending on the grape variety.

Another very important factor influencing the TAS results in the study was the addition of SO_2, which acts both as a reducing agent and antibacterial agent. The sharp TAS increase recorded between weeks 6 and 7 (see Fig. 2) is ascribed mainly to the addition of SO_2.

Fifty percent of the determined values in blue wine varieties ranged from 4.5 to 10.5 EQA and 0.5–2 EQA in white wines (determined with the ABTS assay) and from 0.6 to 2.7 EQA in the blue wine varieties determined by the DPPH assay. From this comparison, it is evident that the ABTS assay gives higher values than does the DPPH assay. From the linear correlation and regression analysis of the ABTS and DPPH assays of all wines, a medium correlation between ABTS and DPPH values has been found ($r^2 = 0.7156$ and $r = 0.8459$, $y = 1.6597 + 1.3889x$) and, in blue wine varieties, the results were similar (Fig. 8); in white wine varieties, only a very weak correlation could be found (r^2=0.5512, $r = 0.7424$, y=2.3731 − 4.7292x, Fig. 9). The absolute results obtained by both methods are in good interpretation agreement with the same conclusions (Table 1). However, the lack of strong correlation between these two assays is likely attributable to the fact that every individual phenolic compound contained in wine causes a different response to each specific radical used in the assay. These different phenolic compounds, contained in different concentrations, depend on the selected techno-

Table 1
Comprehensive statistical evaluation of DPPH and ABTS assays [EQA mg/ml]

		DPPH assay			ABTS assay		
	N	Median	Minimum	Maximum	Median	Minimum	Maximum
All varieties	420	0.329	0.012	10.7	3.10	0.693	15.5
Blue varieties	210	2.11	0.082	10.7	5.46	3.26	15.5
White varieties	210	0.227	0.013	0.372	1.45	0.693	2.93
Blauer Portugieser	105	2.00	0.082	2.93	4.79	3.26	6.16
Müller-Thurgau	105	0.215	0.013	0.333	1.55	0.693	2.68
Moravian Muscat	105	0.247	0.040	0.372	1.38	0.704	2.93
St. Laurent	105	2.84	0.479	10.7	10.7	4.61	15.5

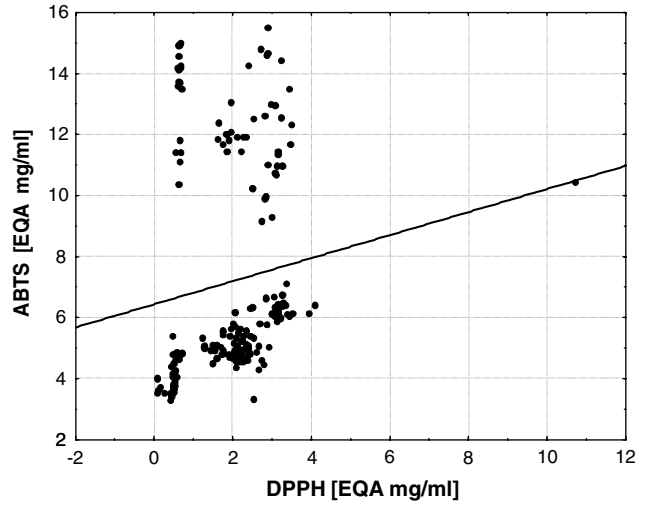

Fig. 8. Comparison of ABTS and DPPH assays of blue wine varieties.

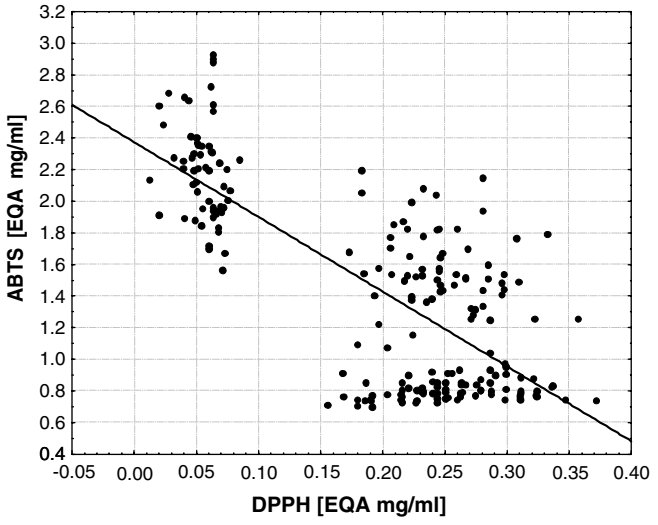

Fig. 9. Comparison of ABTS and DPPH assays of white wine varieties.

logical procedure and duration of individual phases (especially the period of fermentation, period of time in the barrel, storage temperature). A similar lack of correlation between the TAS measured by the TEAC and other assays was reported recently by Wang et al. (2004). Thus, according to these results, different methods should be used in parallel for the estimation of the TAS of biological materials (Prior & Cao, 1999). $ABTS^{+}$ and $DPPH^{\cdot}$ radicals have different stereochemical structures and different paths of genesis and thus they give, after the reaction with the antioxidants, qualitatively different responses to the inactivation of each radical.

The DPMD assay did not acquit itself well. The obtained $DMPD^{\cdot+}$ radical was unstable at laboratory temperatures above 10 °C, even 18 h after its preparation, and its absorbancy changed considerably over time. The measuring cuvettes in this case should be tempered at <10 °C (Rivero-Pérez, Pérez-Magariño, & González-San José, 2002) and a spectrophotometer equipped with a thermostat to maintain a constant low temperature, if needed. Regarding the problem of the genesis of a stable radical with stable absorbancy, this method was not used for further measurements.

5. Conclusions

1. Blue wine varieties have approximately 10 times more TAS (average 2.21 EQA) than have white wine varieties (average 0.227 EQA) in the DPPH test and 3.7 times more in the ABTS test (5.46 EQA and 1.45 EQA, respectively).
2. The very low TAS at the beginning of vinification increased during the wine-making process to maximum values of 0.37 EQA in white wine varieties and 10.7 EQA in blue wine varieties in weeks 6–7, due to the skin maceration of the blue wine grapes.
3. Statistically significant differences between the TAS and the colour of wine varieties, except for Müller Thurgau and Moravian Muscat, were found.
4. Moderate or weak correlations between the DPPH and ABTS assays were found, due to the different responses of individual phenolics to the $ABTS^{+}$ and $DPPH^{\cdot}$ radicals but, in general, the values obtained by the interpretation of results determined by both methods were well comparable. Nevertheless, the correct relationship between results provided by both methods shows only very slight comparability.
5. The DMPD assay proved to be inconvenient, due to the inability to stabilize the produced radical under common laboratory temperature conditions.

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